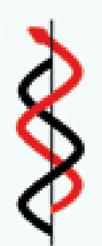

Research

at the

de Duve Institute

and

Brussels Branch of the Ludwig Institute for Cancer Research

August 2011

DE DUVE INSTITUTE

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de Duve Institute

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The de Duve Institute

THE DE DUVE INSTITUTE:
AN INTERNATIONAL BIOMEDICAL RESEARCH INSTITUTE

In 1974, when Christian de Duve founded the Institute of Cellular Pathology (ICP), now renamed the de Duve Institute, he was acutely aware of the constrast between the enormous progress in biological sciences that had occurred in the 20 preceding years and the modesty of the medical advances that had followed. He therefore created a research institute based on the principle that basic research in biology would be pursued by the investigators with complete freedom, but that special attention would be paid to the exploitation of basic advances for medical progress. It was therefore highly appropriate for the Institute to be located on the campus of the Faculty of Medicine of the University of Louvain (Université catholique de Louvain) in Brussels. The University hospital (Cliniques Universitaires St Luc) is located within walking distance of the Institute.

Emile Van Schaftingen

Benoît Van den Eynde

The main commitment of the members of the de Duve Institute is research. Discovery is the endpoint of their efforts and the only element taken into account for their evaluation. The Institute functions in symbiosis with the Faculty of Medicine and many of its senior members hold a Faculty position and have teaching appointments. The influx of doctoral students and postdoctoral fellows from the University is also a key element in the success.

In 1978 the Ludwig Institute for Cancer Research decided to base its Belgian branch within the walls of the de Duve Institute. A fruitful collaboration between the two Institutions has been pursued ever since. Even though the two Institutes are completely independent, the collaboration between the scientists of the de Duve Institute and the Ludwig Institute is extremely close and the sharing of resources is considerable. This strong integration was valued as an asset in a recent evaluation of the Ludwig Institute. The Director of the Brussels Branch of the Ludwig Institute is also a member of the de Duve Institute Directorate.

The de Duve Institute is managed by a directorate of three scientists, presently composed of Emile Van Schaftingen, Benoît Van den Eynde, and Miikka Vikkula. The directorate is appointed by the Board of directors, which comprises eminent members of the Belgian business and finance world, as well as the Rector of the University of Louvain, and three other members of the University.

About 170 researchers work in the de Duve Institute and in the Ludwig Institute, assisted by a technical and administrative staff of about 80 members. Despite this relatively small size, the de Duve Institute has the ambition of pursuing research projects of high quality under conditions that allow original, long-term projects to be pursued. The Institute has an endowment, the strenghtening of which is a goal of the Development and Expansion Council of the de Duve Institute. This endowment is a source of key financing for priority issues, such as the creation of new laboratories for promising young researchers. We expect that the quality of our researchers, supported by sound organisational approaches, will enable the de Duve Institute to stand at the forefront of European Research.

Miikka Vikkula

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ACKNOWLEDGEMENTS

In 2010, the de Duve Institute has attracted major gifts from several foundations, companies and individuals who have been very generous. These sponsors are providing the resources that enable our scientists to better understand and treat diseases that afflict people around the world. Gifts are the lifeblood of new research initiatives and private resources are crucial in underwriting the costs of new laboratories. On an annual basis, fund-raising from private sources has increased during the past decade over levels achieved previously and now supports about 10 % of the Institute's budget.

The appeal for sponsoring postdoctoral fellowships was also widely followed. In 2010 the Institute has been able to allocate the following fellowships, entirely supported by our donors :

The «Haas-Teichen» fellowship was attributed to Reece MARILLIER,

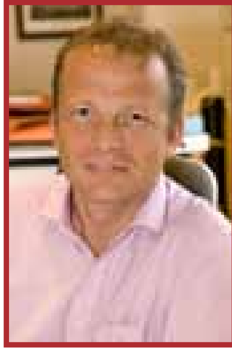
the «Pierre Lacroix» fellowship to Yu-Chiang LAI,

the «Maurange» fellowship to Shreedhara GUPTA,

other fellowships have been awarded by the Institute to Nausicaa ARNOULT and to Seima CHARNI.

We express our gratitude to all who contributed to the financing of post-doctoral fellows and state-of-the art research laboratories at the de Duve Institute, ensuring that this institute will remain at the top of the field in biomedical research.

Luc BERTRAND
President of the Development and Expansion Council



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GENETICS OF HUMAN CARDIOVASCULAR ANOMALIES, CLEFT LIP PALATE AND CEREBRAL TUMORS

The aim of our research is to get insights into the molecular mechanisms underlying a variety of disorders of the cardiovascular and skeletal systems, as well as certain cancers. We are especially interested in evaluating the contribution of genetic variation to human disease. The precise cause of many disorders remains unknown, and current treatments are therefore aimed at alleviating symptoms. Identification of the primary causes as well as modulating factors would allow for the development of treatments that are more "curative" and specific. As this research is based on human DNA extracted from blood and tissue samples from patients, the group works closely with several clinicians and multidiciplinary centers worldwide (e.g. Centre des Malformations Vasculaires, Cliniques universitaires St-Luc; Vascular Anomalies Center, Children's Hospital, Boston, USA; Consultation des Angiomes, Hôpital Lariboisière, Paris, and Centre labiopalatin, Cliniques Universitaires St-Luc).

VENOUS MALFORMATIONS AND GLOMUVENOUS MALFORMATIONS ("GLOMANGIOMAS")

P. Brouillard, N. Limaye, M. Uebelhoer, J. Soblet, M. Amyere, L.M. Boon and M. Vikkula, in collaboration with B.R. Olsen, Harvard Medical School, Boston, USA; J.B. Mulliken and S. Fishman, Children's Hospital, Boston, USA; O. Enjolras, Hôpital Lariboisière, Paris, France; A. Dompmartin, CHU, Caen, France

Venous malformations (VM) are bluish-purple cutaneous and mucosal vascular lesions. They are often congenital, but can appear later in life. They have a tendency to grow slowly with the growth of the child. Glomuvenous malformations (GVM, "glomangiomas") are a special subtype of venous anomalies. They are clinically similar to VMs, yet our clinico-genetic study allowed for their clinical differentiation.

We previously discovered that rare, hereditary venous malformations can be caused by an ac-

tivating mutation in the endothelial cell receptor tyrosine kinase TIE2/TEK (Fig1). The use of high-throughput technologies such as DHPLC and High Resolution Melting, which allow for more efficient and sensitive genetic screens, was instrumental in the identification of several novel mutations amongst affected families [1]. Despite the ubiquitous presence of these germline mutations, the lesions they cause are localized. We therefore hypothesized that lesion-development may require a somatic second hit to locally disrupt the normal allele of the TIE2 gene. We obtained proof for this from one lesion, in which the ligand-binding region of the wild-type allele was deleted somatically, causing a local loss of its ability to function [2]. In addition, we discovered that at least 50% of the far more common sporadic VMs are caused by somatic mutations in TIE2 [2]. On this basis, we suggest somatic changes to be a general genetic mechanism in vascular malformations. All of the TIE2 mutations discovered thus far are intracellular and cause receptor hyperphosphorylation in vitro, although much remains to be learnt as to precisely why this causes lesions. Towards this end, we have begun to carry out functional analyses of the role of TIE2 in VM-pathogenesis, using a variety of in vitro and in vivo methods. These include the generation of mouse models of the anomaly, by "knock-in" substitution of the normal TIE2 allele with the most frequently mutated forms associated with inherited VMCM and sporadic VMs respectively; Affymetrix expression profiling has also been used in order to compare the effects of the wild-type receptor with those of different mutant forms.

In contrast to VMs, glomuvenous malformations (GVM) are mostly, if not always, inherited. We discovered that GVM are caused by loss-of-function mutations in a gene we named glomulin. So far, we have identified 36 different mutations in 140 families. As with VMs, we showed that GVMs appear locally because of the additional alteration of the second allele, likely in vascular smooth muscle cell precursors that populate the lesions. To better understand

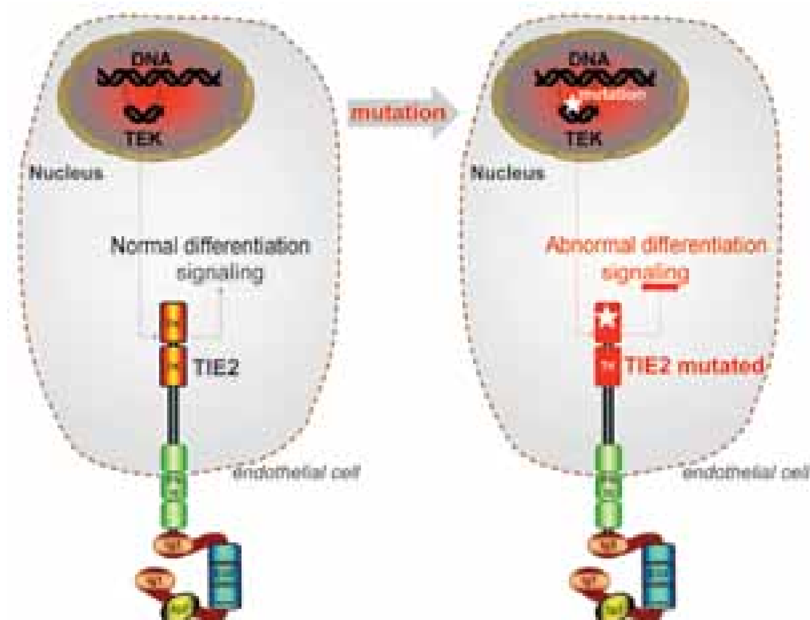

Fig. 1. Mutations in TIE2/TEK cause inherited and sporadic venous malformations. All of the mutations identified cause hyperphosphorylation of this tyrosine kinase receptor, resulting in abnormalities in signaling pathways known to be critical to endothelial cell function (Limaye et al, Nat Genet 2009).

the role of glomulin in normal and pathological conditions, we have created glomulin-deficient mice. While heterozygotes appear normal, homozygous knockouts are embryonic-lethal (Brouillard et al, unpublished). To enable studies beyond this time-point, we generated RNAi conditional knockdown mice, in which glomulin inactivation is cre-inducible (Brouillard et al, unpublished). When triggered during embryonic development, glomulin depletion is likewise lethal. To further study its role specifically in vascular development, we will instead knock it down in a cell-type specific manner. In vitro studies to identify the function as well as any interacting partners of glomulin, a molecule with no known domains or homologs, are also ongoing.

LYMPHEDEMA

A. Mendola, L.M. Boon, M. Vikkula in collaboration with K. Devriendt, KUL; D. Chitayat, Hospital for Sick Children, Toronto, Canada; I. Quere, Montpellier, France

Lymphedema is an external manifestation of lymphatic failure. It may be categorized as primary (idiopathic) or secondary (acquired) lymphedema. Primary hereditary lymphedema can occur at birth (Nonne-Milroy disease) or at puberty (Meige's disease). It is extremely difficult to treat lymphedema. Patients have a lifelong problem with progressive swelling of extremities. We use genetic approaches to unravel the pathophysiology with a view to ameliorating the condition. In some families with Nonne-Milroy disease, missense inactivating mutations in the VEGFR3 gene were identified. Moreover, some sporadic congenital primary lymphedemas are also explained by alterations in VEGFR3. We showed, for the first time, that recessive primary congenital lymphedema can be caused by a particular homozygous VEGFR3 mutation, which has a moderate effect on receptor function and can cause lymphedema only when both alleles are altered [3]. Mutations in the transcription factor gene SOX18 were identified in families with autosomal recessive and dominant hypotrichosis-lymphedema-telangiectasia syndrome, and the forkhead transcription factor FOXC2 is mutated in some families with Meige disease, in association with distichiasis. We recently found that a proportion of sporadic fetal edema of unknown etiology is in fact attributable to mutations in the lymphedema-associated genes VEGFR3 and FOXC2 [4]. In collaboration with a group (RC Hennekam, Dept of Clinical Genetics, Academic Medical Center, The Netherlands) investigating Hennekam syndrome, characterized by lymphedema, lymphangiectasias, mental retardation and unusual facial characteristics, we identified CCBE1 as a gene that causes generalized dysplasia of lymphatic vessels in humans as well as animal models [5].

VASCULAR ANOMALIES AFFECTING CAPILLARIES

N. Revencu, N. Limaye, M. Amyere, L.M. Boon, M. Vikkula in collaboration with J.B. Mulliken, Children's Hospital, Boston, USA; S. Watanabe, Showa University School of Medicine, Tokyo, Japan; A. Dompmartin, CHU de Caen, France; Virginia Sybert, Washington University, Seattle, USA

Capillaries, the smallest blood vessels that connect arterioles to venules, can give rise to various anomalies, two of which are very common: 1) hemangioma, a benign, localized overgrowth of capillary-like vessels, and 2) capillary malformation (CM; commonly known as portwine stain), a localized maldevelopment of capillary like vessels. Hemangiomas have a frequency of up to 12% in 1-year-old children, and typically undergo a period of rapid expansion, followed by spontaneous regression. We have an extensive collection of samples from sporadic as well as rare familial forms of hemangioma, and are using Affymetrix high-density whole genome SNP arrays in order to carry out linkage, loss of heterozygosity and copy number analyses on them in an effort to identify causative genomic variants. Work done with collaborators has demonstrated that per-

turbations of the vascular endothelial growth factor (VEGF) signaling pathway can cause hemangioma pathogenesis [6].

CMs occur in 0.3% of newborns. Unlike hemangiomas, they persist throughout life if not treated. Certain capillary malformations affect other organs, such as the brain in the case of cerebral capillary malformations or CCMs. We discovered that inherited hyperkeratotic cutaneous capillary-venous malformations (HCCVM) associated with CCM are caused by a mutation in the KRIT1 (Krev interaction trapped 1) gene, suggesting it is important not only for cerebral but also for cutaneous vasculature. In addition, genome-wide linkage mapping on families with inherited capillary malformations led us to identify a linked locus CMC1. Screening of positional functional candidate genes resulted in the identification of mutations in the RASA1 gene, a modifier of the Ras signaling pathway. This implies that RAS pathway modulators may serve as a novel therapy for these patients in the future. Ongoing studies have led to the identification of RASA1 mutations in 90 index patients. This has allowed for a more precise clinical description of the clinical signs and symptoms associated with this newly recognized disorder that we have named Capillary malformation-arteriovenous malformation (CM-AVM) [7]. Importantly, capillary lesions can be associated with deeper, more dangerous anomalies about 20% of the time; these include arteriovenous malformations and fistulas (AVM/AVF), Parkes Weber, and Vein-of-Galen aneurysmal malformations, which warrant careful clinical management

CLEFT LIP AND PALATE

M. Ghassibé, L. Desmyter, M. Quentric, N. Revencu, M. Vikkula, in collaboration with Y. Gillerot, B. Bayet, R. Vanwijck, Ch. Verellen-Dumoulin, N. Deggouj, St-Luc, UCL

Cleft lip and palate (CLP) is a congenital anomaly of complex etiology. Predisposition is governed by numerous genetic loci, in combination with environmental factors. Clefts have an incidence of 1/700 births.

We collected DNA samples from a large number of patients affected with popliteal pterygium syndrome, as well as van der Woude syndrome, the most common cleft syndrome. We showed that IRF6 is the major causative gene in our Belgian cohort. This study in turn led to several collaborations that allowed us to carry out a genotype-phenotype correlation on hundreds of patients from different ethnic backgrounds. Results showed that IRF6 is mutated in 69% of VWS patients and 97% of PPS patients. Interestingly, mutation-distribution is non-random: 80% are localized in IRF6 exons 3, 4, 7 and 9 for VWS, and 72% in exon 4 for PPS patients. These findings are of great importance for clinical diagnosis, mutational screens and genetic counseling. We also demonstrated that IRF6 predisposes to non syndromic clefts in Europe and that it is mutated in familial clefts with minor lip anomalies. In parallel, we identified a new gene, FAF1, responsible for cleft palate only and Pierre Robin sequence [8]. This gene is associated with clefts across populations. Zebrafish studies confirmed its role during embryonic development and jaw formation (Fig 2). In parallel, we generated a mouse model where we knocked-out the gene and we are currently phenotyping the mouse in order to understand the mechanism behind craniofacial development and cleft occurrence.

CEREBRAL TUMORS

T. Palm and M. Vikkula, in collaboration with C. Godfraind, Laboratory of Neuropathology, St-Luc, UCL

Morphological characterization and classification of tumors is not always clear. Thus, better (molecular) criteria are needed. We are especially interested in two types of cerebral tumors: oligodendrogliomas and ependymomas. To better understand the molecular alterations

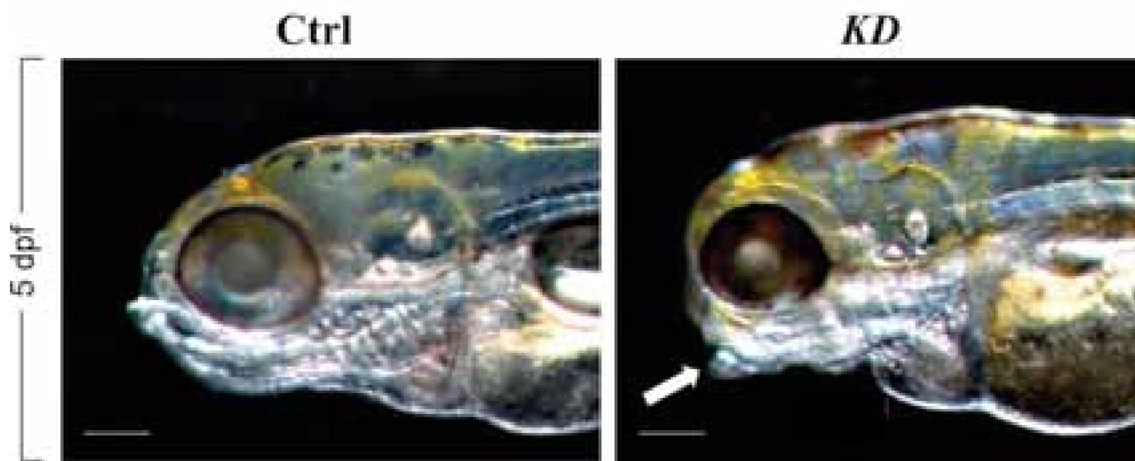

Figure 2 – FAF1 mutations cause abnormalities in jaw development across species. Morpholino-induced knockdown of the protein in zebrafish causes an open-mouthed appearance due to retrognathia, similar to the phenotype in human lacking FAF1. (Ghassibé-Sabbagh et al, Am J Hum Genet 2011)

leading to ependymomal oncogenesis, we performed microarray-based expression profiling on a series of 34 frozen ependymomas. Results of our profiling study are in concordance with the "oncology recapitulates ontology" hypothesis, in which genes implicated in stem cell fate decisions may be important for supporting cancer stem cells as well. Pathways activated in high grade ependymomas were consistent with the histological appearance of a more aggressive tumor phenotype. Using array-CGH, we recognized a subgroup of supratentorial ependymomas affecting young adults, which are characterized by trisomy of chromosome 19.

Within the posterior fossa compartment, ependymomas cluster into three sub-groups. The first corresponds with ependymomas that are histologically of WHO grade II, the second with those of WHO grade III, and the third with a group of ependymomas of a bi-phasic appearance, combining regions of both grades. This sub-group shares gene-sets with tumors of both other groups, and in addition has a glycogen metabolism signature of its own. Whether these groups correspond to three distinct tumoral entities, or demonstrate multifocal tumor progression remains to be investigated.

NEUROENDOCRINE TUMORS

A. Persu, Division of Cardiology, Saint-Luc, UCL; M. Amyere, A. Mendola, A. Vanegeren, M. Vikkula, in collaboration with P. Rustin, INSERM U676, Hôpital Robert Debré, Paris, France

Pheochromocytomas and head and neck paragangliomas are neuroendocrine tumours derived from the neural crest. Paragangliomas are associated with parasympathetic ganglia and are usually non-secreting. By contrast, pheochromocytomas are derived from paraganglia associated with the orthosympathetic system and are characterized by increased secretion of catecholamines and paroxystic hypertension.

The current project aims to look at the nature and frequency of mutations in the known predisposing genes in pheochromocytoma and paraganglioma in Belgium and to detect possible genotype-phenotype correlations. A multicentric collaboration including the main academic centers from Belgium has therefore been established.

The SDH genes code for the subunits of succinate deshydrogenase, at the crossroad of the mitochondrial respiratory chain and Krebs

cycle. Three of the four subunits of succinate deshydrogenase, i.e. SDHD, SDHB, and more rarely SDHC, have been associated with paraganglioma and pheochromocytoma. Furthermore, SDHB mutations have been associated with an increased risk of recurrence and malignancy in several European series. In our study, the prevalence of SDHB was unexpectedly high in head and neck paraganglioma. Surprisingly, tumours associated with such mutations are mainly late-onset unilateral tumours without evidence of recurrence or malignancy. We also described a family with a very rare presentation of severe head and neck paraganglioma with liver and spine localization. No evidence of mutations was found in the known predisposing genes by dHPLC and/or SSCP. An in depth search for the genetic abnormality underlying this unusual form of paraganglioma is currently under way. Last, we are currently involved in an international collaborative effort to look for the clinical spectrum associated with mutations of TMEM127, a recently described pheochromocytoma susceptibility gene

ESSENTIAL HYPERTENSION

A. Persu, Division of Cardiology, Saint-Luc, UCL; N. Limaye, and M. Vikkula.

High blood pressure - commonly called hypertension - is found in almost 20 % of the adult population worldwide and affects 2 million Belgians. Despite the wide range of anti-hypertensive therapies available, blood pressure is adequately controlled in only 30-40% of hypertensive patients. In a large majority of cases, no specific cause is found ("essential hypertension") and high blood pressure is thought to reflect the interplay of lifestyle (obesity, high salt intake) and genetic factors; 30-50 % of blood pressure level is thought to be genetically determined. Despite this, conventional linkage and association studies have failed to establish the role of genetic variants in susceptibility.

In an effort to find genetic variations that account for a significant proportion of blood pressure heritability, and to study the interactions between known variants with mild to moderate effects, we set up a multicentric national genome wide association study (HYPERGEN) with the support of the Belgian Hypertension Committee. We aim to recruit at least 1000 hypertensive patients and 1000 normotensive subjects. Detailed phenotyping including renin and aldosterone dosages are obtained in all patients, and genotyping will be performed using SNP chip technology.

HEMATOLOGICAL MALIGNANCIES AND TUMORS OF SOFT TISSUE AND BONE

H. Antoine-Poirel, V. Havelange, F. Duhoux, J. Bodart, G. Ameye, Human Genetics Center, St.Luc, UCL; with M. Vikkula

The explosion in the identification of genetic biomarkers of hematological malignancies and tumors of soft tissue and bone over the past 20 years has had significant impact on diagnosis, prognosis and treatment, as well as our understanding of the genetic and epigenetic processes that lead to tumorigenesis. Our aim is to characterize genomic alterations in both types of malignancy because their oncogenic mechanisms exhibit significant similarities. Towards this end, we use a variety of techniques including conventional and molecular cytogenetics or FISH, molecular biology, and microarrays to identify partner genes in reciprocal translocations and study their functional consequences. We characterized new partner genes of known multipartner genes MLL/11q23, ABL/9q34, PDGFRβ/5q33, and USP6/17p13, as well as unknown partner genes. We demonstrated that the functional consequences may be more complex than transcriptional dysregulation by promoter-swap of fusion genes.

In an international therapeutic trial of children with mature B-cell lymphomas, we showed the adverse prognostic impact of chromosomal alterations of 13q and 7q, detected by cytogenetics. Using genome-wide SNP array

technology, we found that most 13q alterations lead to an amplification of the microRNA 17-92 cluster, known to interact with the MYC oncogene, a finding confirmed with mature miRNA expression profiling. In addition, we detected numerous cryptic genomic alterations including partial uniparental disomies. Their prognostic value is currently under study in collaborations within different therapeutic trials across Europe

SNP-CHIP PLATFORM AND NEXT GENERATION SEQUENCING PLATFORMS

M. Amyere and M. Vikkula

We host the UCL oligonucleotide microarray platform (Affymetrix), currently used by several groups in the de Duve Institute and UCL for expression profiling as well as genotyping. We also collaborate with several groups from around the world on whole genome mapping studies. In work done with Dr Jüppner from Harvard Medical School, Boston, we genotyped a large family with a new form of hypophosphatemia and mapped this autosomal recessive form (designated ARHP) to chromosome 4q21. This allowed for the identification of causative homozygous mutations in DMP1 (dentin matrix protein 1), which encodes a non-collagenous bone matrix protein expressed in osteoblasts and osteocytes [9]. In collaboration with G. Matthijs from KULeuven, we have used autozygosity mapping along with expression profile analysis to identify a new gene for congenital glycosylation disorder. In collaboration with Dr Vermeesch, also from KULeuven, we genotyped a large family with autosomal-dominant microtia. Copy number analysis led to the identification of five tandem copies of a copy number-variable region at chromosome 4p16, linked to the disease. With the same group, we established that chromosome instability is common during early human embryogenesis in study of 23 pre-implantation embryos from 9 fertile couples. Additionally, rearrangements such as segmental imbalances were observed in 70% of the 23 embryos tested. This explains low human fecundity and identifies post-zygotic chromosome instability as a leading cause of constitutional chromosomal disorders [10]. In an exciting development, this platform will be complemented by a High Throughput Sequencing platform, funded by the Fondation Contre le Cancer. This will further enhance our ability to detect mutations and identify genes that participate in various pathogenic processes.

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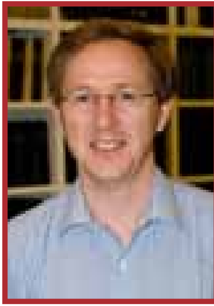

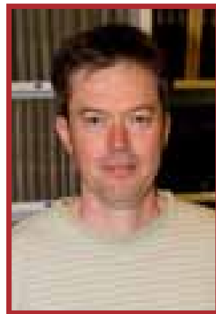

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LIVER AND PANCREAS DEVELOPMENT

The group studies the molecular and cellular mechanisms that govern development of the liver and pancreas, two organs which play essential metabolic roles and which derive from the endoderm (primitive gut of the embryo). The fundamental knowledge gained by this work is essential for improving cell therapy of liver and pancreas diseases (metabolic disease, acute hepatitis, cirrhosis, diabetes), and for understanding the pathophysiology of organ malformations (e.g. polycystic liver diseases). Identifying developmental mechanisms also impacts on understanding abnormal differentiation of liver and pancreatic cancer cells..

LIVER DEVELOPMENT

A. Antoniou, J.-B. Beaudry, R. Carpentier, I. Laudadio, A. Poncy, P. Raynaud

The main cell types of the liver are the hepatocytes, which exert the metabolic functions of the organ, and the biliary cells which delineate the bile ducts. We study how the hepatocytes and biliary cells differentiate and how bile ducts are formed in the embryo. Our preferred model organism to investigate liver development is the mouse, and this includes generation and analysis of transgenic mouse lines. In collaboration, we also use the Zebrafish as a model organism.

The biliary tract consists of intrahepatic bile ducts which collect bile produced by the hepatocytes, and of extrahepatic ducts which drain bile from the liver to the intestine. Biliary cells, also called cholangiocytes, delineate the lumen of the bile ducts and modify the composition of bile. These cells, like hepatocytes, derive from liver progenitor cells called hepatoblasts. Our discovery of the Onecut transcription fac-

tors Onecut-1 (OC-1/HNF-6), OC-2 and OC-3, and the subsequent phenotypic characterization of HNF-6 and OC-2 knockout mice led to the identification of the first transcriptional network regulating bile duct development [1, 2]. Current efforts are devoted to the characterization of the transcription factors and signal transduction pathways that control bile duct development in health and disease.

We have recently identified novel molecular markers that enabled us to revisit the morphogenesis of the intrahepatic bile ducts. We found that biliary morphogenesis occurs according to a new mode of tubulogenesis [3, 4]. Biliary tubulogenesis starts with formation of asymmetrical ductal structures, lined on one side (adjacent to the portal vein) by cholangiocytes and on the other side (adjacent to the liver parenchyma) by hepatoblasts. When the ducts grow from the hilum to the periphery of the liver, the hepatoblasts lining the asymmetrical structures differentiate to cholangiocytes, thereby allowing formation of symmetrical ducts lined only by cholangiocytes. This mode of tubulogenesis is unique as it is to our knowledge the only one characterized by transient asymmetry (Figure 1). We further investigated how this new knowledge impacts on the interpretation of congenital malformations of the bile ducts. To this end we studied several mouse models and samples from human liver fetuses. This work allowed us to propose a new pathogenic classification of biliary malformations (Figure 1) [5].

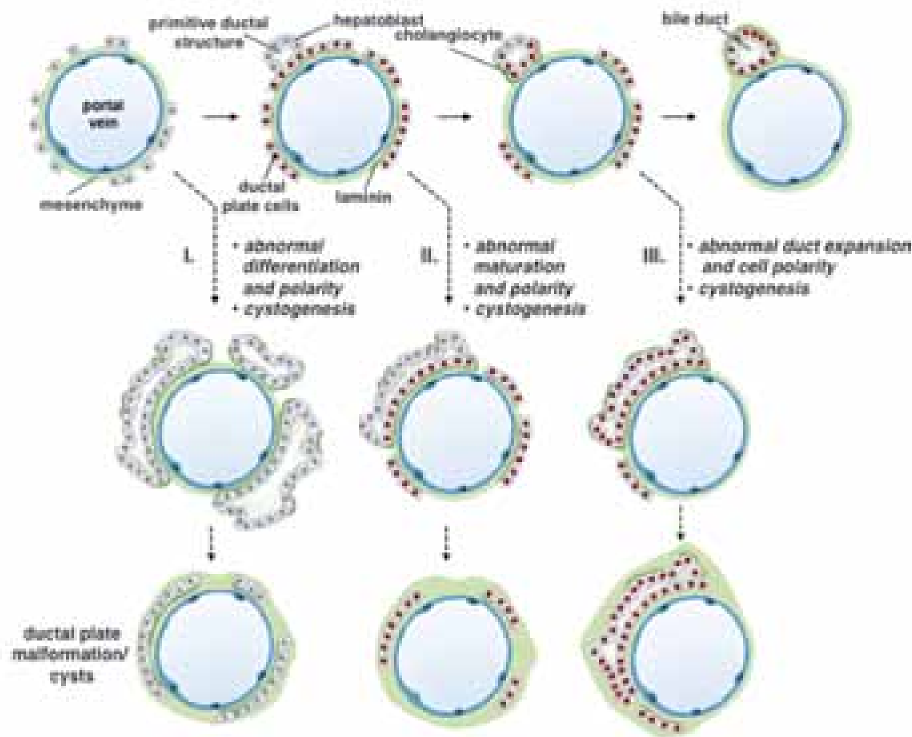

Fig. 1. Classification of biliary malformations based on distinct pathogenic mechanisms. Normal bile duct morphogenesis is illustrated at the top. Malformations can arise by three mechanisms: (I) differentiation of hepatoblasts to ductal plate cells is abnormal and associated with perturbed polarization and cyst formation; (II) asymmetrical ducts are formed but fail to mature to bile ducts; this is associated with formation of cysts and abnormal polarity; (III) differentiation of ductal plate cells and maturation of asymmetrical ducts proceeds but duct expansion is perturbed; this is associated with defects in cell polarity.

The transcription factor network that drives cholangiocyte morphogenesis and bile duct formation has been further investigated. By means of a liver-specific gene inactivation strategy we found that Sox9 controls the timing of bile duct development. Within the biliary transcriptional network Sox9 is located downstream of HNF-6 and upstream of C/EBP-alpha, two factors whose dysfunction is associated with biliary cyst development. In addition, the function of Sox9 was found to be tightly linked with that of the Notch signaling pathway. The latter is deficient in liver of patients affected with Alagille syndrome, a disease characterized by bile duct paucity and severe cholestasis. We pursue this research by evaluating the role of other members of the Sox family.

Our work also addresses the mechanisms of hepatocyte differentiation. HNF-6 and OC-2 are critical for normal differentiation of hepatic precursor cells to hepatocytes or cholangiocytes : in the absence of HNF6 and OC2, the precursor cells generate hybrid hepato-biliary cells instead of distinct hepatocyte and biliary cell populations [2]. Moreover, we found that the hepatic concentration of HNF6 rises during liver development. Specific levels of HNF6 determine recruitment of co-activators at specific stages of development, thereby inducing time-specific expression of HNF6-target genes [6]. Current research focuses on the molecular mechanisms by which HNF-6 and OC-2 fine-tune gene expression at several stages of hepatocyte differentiation.

Since fine-tuning of gene expression is in part exerted by microRNAs (miRNA), we addressed the function of the hepatocyte-specific miR-122 in development. In collaboration with the Katholieke Universiteit Leuven, we found that the expression of enzymes synthesizing ketone bodies rises during development, while that of an enzyme catabolising ketone bodies (2-oxoacid CoA transferase) decreases. We showed that the decrease in 2-oxoacid CoA transferase is in part due to repression mediated by miR-122. Therefore, miR-122 promotes hepatocyte maturation, in part by regulating ketone body metabolism [7].

PANCREAS DEVELOPMENT

M. Colletti, A. Grimont, E. Heinen, P.-P. Prévot, A. Simion

In the embryo, the pancreas develops as an outgrowth of the endoderm, the cell layer that delineates the primitive gut. Pancreatic progenitors derived from the endoderm form two buds (dorsal and ventral) which later fuse to form a single organ. Within these buds the progenitor cells give rise, through a stepwise process, to endocrine, acinar and duct cells. Our group investigates the molecular mechanisms that control development of the various pancreatic cell types.

In collaboration with the CELL research group, we investigated the role of blood vessels in pancreas development, and uncovered the existence of a molecular cross-talk between the blood vessels and the pancreatic epithelium which determines acinar differentiation of epithelial cells. This is illustrated in the report of the CELL research group.

The role of the Onecut transcription factor HNF-6 in pancreas development is being studied since several years. We showed that HNF6 is required for development of endocrine cells and pancreatic ducts [8,9]. When further addressing how HNF6 is controlled and how it exerts its effects, we identified two miRNAs that control HNF6 expression [10], and eighteen miRNAs which are controlled by HNF6. Current research is focussed on the determination of the function of those miRNAs that are downstream of HNF6.

After birth, HNF6 is expressed in the pancreas exclusively in the duct cells where it most likely maintains duct cell identity. Interestingly, there is evidence from other laboratories that pancreatic ductal adenocarcinoma may derive from acinar cells which switch their phenotype from acinar to ductal during progression

to cancer. This process is called acinar-to-ductal metaplasia, and constitutes a preneoplastic state. We hypothesized that the switch in cell identity may depend on the ectopic expression of ductal transcription factors and tested if HNF6 is ectopically induced in acinar cells undergoing metaplasia. This was the case in human pancreas (Figure 2). In addition, we recently collected evidence from mouse models that induction of HNF6 in acinar cells promotes acinar-to-ductal metaplasia, suggesting that HNF6 is a key inducer of preneoplastic lesions.

CONCLUSIONS

Our findings on the role of transcription factors that regulate liver and pancreas development contribute to a better understanding of the diseases affecting these organs. In liver, our work opens perspectives for understanding the pathophysiology of congenital diseases of the liver. Applying our findings to the programmed differentiation of cultured stem cells should also help in developing cell therapy of hepatic deficiencies. In pancreas, our observations on the expression and function of HNF6 in preneoplastic lesions are expected to improve diagnosis and to help preventing progression towards pancreatic ductal adenocarcinoma.

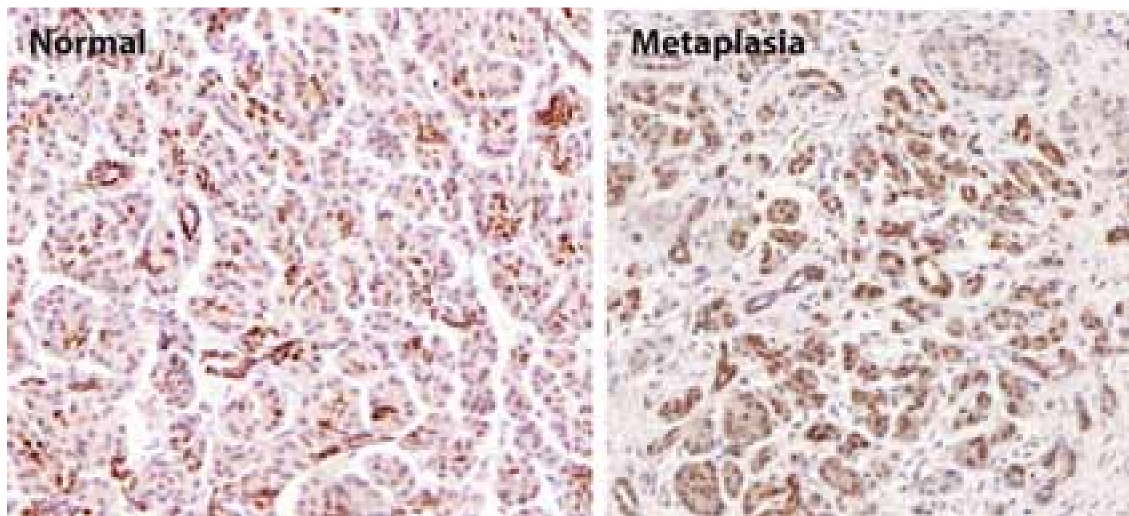

Fig. 2. Sections from human pancreas immunostained to detect HNF6. In normal pancreas, HNF6 is detected in the ducts, whereas in pancreas with acinar-to-ductal metaplasia, HNF6 is detected in ducts as well as in acinar cells acquiring a duct-like morphology.

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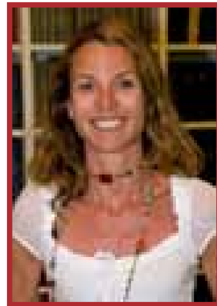

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GENETIC AND EPIGENETIC ALTERATIONS IN GENOMES

Preservation and regulation of genetic information is essential for proper cell function. Consequently, cells have evolved mechanisms of DNA repair, telomere maintenance, and epigenetic regulation of gene expression patterns. Deregulation of these processes contributes to the appearance and progression of cancer cells, which are characterized by genomic rearrangements and dysregulated gene expression patterns. Studies in our group explore the cellular events leading to genomic instability and the mechanisms by which tumour cells maintain their telomeres to acquire immortality. We have demonstrated that epigenetic alterations in tumours, involving loss of DNA methylation marks, can lead to the aberrant activation of a particular group of genes. We are currently investigating how epigenetic marks are established on these genes in embryonic cells and how they become altered in tumour cells.

GENOMIC INSTABILITY IN *SCHIZOSACCHAROMYCES POMBE* FISSION YEAST

S. Lenglez, A. Decottignies

We use *S. pombe* fission yeast to investigate the cellular events driving genomic instability in eukaryotic cells. To get more insight into the nature of chromosomal fragile sites, we characterized *S. pombe* loci that are naturally prone to double-strand break (DSB) formation. From yeast to mammals, different studies reported the insertion of DNA fragments of various sources at experimentally-induced DSBs, including mitochondrial DNA (mtDNA) in budding and fission yeast (1). Interestingly, several studies reported the association of human genetic diseases with *de novo* insertions of mtDNA in the nuclear genome, including a patient exposed to Chernobyl radiations. Moreover, systematic sequencing of eukaryotic nuclear genomes revealed the presence of nuclear sequences of mitochondrial origin (NUMTs) in chromosomes, suggesting that capture of mtDNA fragments at naturally occurring DSBs took place during evolution and remodelled nuclear genomes. By analyzing fission yeast nuclear genome, we found a strong correlation between NUMT localization and chromosomal DNA replication origins (ORIs). Our data suggest that these mtDNA fragments are not part of the ORI but may have been inserted preferentially next to ORIs because these loci are more prone to breakage (2). Using an *in vitro* assay, we further showed that subtelomeric loci associated with a replication origin are highly susceptible to DSB formation in conditions of excessive origin firing (2).

IMPACT OF TELOMERASE ON NF-κB SIGNALING AND CELLULAR RESPONSES TO TNF-α IN HUMAN FIBROBLASTS

M. Mattiussi, G. Tilman, A. Decottignies

Telomeres are specialized protein-DNA structures, which prevent chromosome ends from being recognized as DSBs. Synthesis of telomeric DNA sequences in replicating cells requires telomerase. Cancer cells often show an increased level of telomerase, and this contributes to their unlimited proliferation potential. In some cancers, however, telomeres are maintained in the absence of telomerase activity by one or more mechanisms that are known as alternative lengthening of telomeres (ALT). These two pathways of telomere maintenance are very distinct phenotypically. In telomerase-expressing cells (TEL+), telomere length is very homogenous and telomeres are found at the end of all chromosomes. However, in ALT cells, telomeres are very heterogeneous in length and some chromatids lack telomeres (Fig. 1).

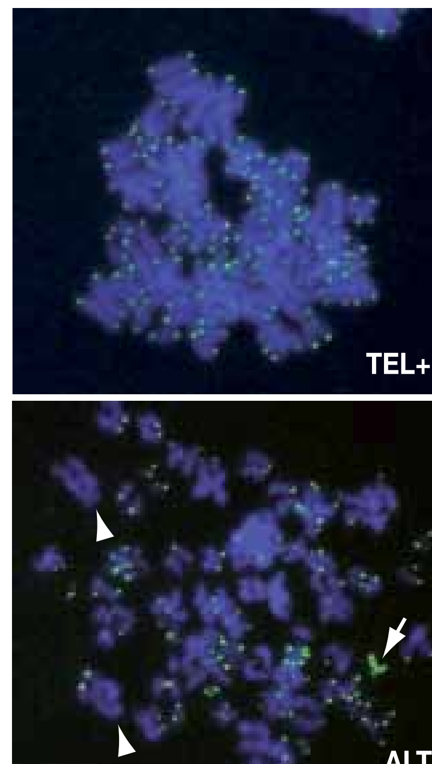

Fig. 1. Telomere-specific fluorescence *in situ* hybridization (FISH) on metaphase chromosomes of telomerase-positive (TEL+) and ALT cancer cells (ALT). Telomeres are hybridized with a fluorescent telomeric probe (green) and DNA is stained with DAPI (blue). In ALT cells, telomeres are very heterogeneous, and even absent at some chromosome ends (arrowheads). ALT cells are further characterized by the presence of extrachromosomal telomeric DNA (arrow).

In addition to its well-established role in telomere synthesis, telomerase exerts non-canonical functions that may promote cancer and stem cell survival, notably as transcriptional cofactor in Wnt-β-catenin signaling pathway. The previously reported physical interaction between telomerase and NF-κBp65 suggested that telomerase may similarly modulate NF-κB pathway, another master regulator of cell proliferation and survival. We investigated telomerase impact on NF-κB signaling in normal human fibroblasts. Strikingly, telomerase overexpression induced constitutive nuclear accumulation of NF-κBp65 that, however, lacked activating Ser-536 phosphorylation. Although NF-κBp65 nuclear accumulation constitutively up-regulated IL-6, basal expression levels of most NF-κB target genes were unaffected, arguing against a general hyperactivation of the pathway. Conversely, prolonged culture of telomerase-expressing fibroblasts down-regulated TNF-α target gene, due to progressive promoter hypermethylation. Telomerase did not either alter NF-κB pathway activation by tumor necrosis factor-α (*TNF*-α). Interestingly however, owing to its ability to reduce reactive oxygen species (ROS), telomerase impaired *SOD2* transcriptional activation as ROS contribute to TNF-α-dependent *SOD2* induction. Accordingly, other ROS-dependent TNF-α-induced processes, including Mitogen Activated Protein Kinase (MAPK) activation and cell death, were reduced by telomerase. Our results demonstrate a new way by which telomerase, through a reduction of ROS, modulates intracellular signaling and gene expression in response to cytokines (3).

DNA HYPOMETHYLATION AND ABERRANT GENE ACTIVATION IN CANCER

A. Loriot, C. De Smet

Genomic DNA in multiple species is modified by the addition of a methyl group to cytosines in CpG dinucleotides. This heritable epigenetic modification is associated with transcriptional repression. Cell-type specific DNA methylation patterns are established during embryonic development, and are usually maintained in adult somatic cells.

DNA methylation patterns often become altered in cancer cells. Alterations include hypermethylation of selected promoters, leading to silencing of critical genes such as tumor suppressor genes, and hypomethylation of numerous other DNA sequences. We have shown that genome hypomethylation in tumors results in the activation of a group of germline-specific genes, which use primarily DNA methylation for repression in somatic tissues (4). These genes, which were originally discovered because their activation in tumors leads to the expression of tumor-specific antigens, were named cancer-germline genes. To date, ~50 cancer-germline genes or gene families have been identified. Several of these were isolated in our group.

The process leading to hypomethylation of DNA sequences in tumors remains obscure. We undertook to address this issue by using MAGEA1, the founding member of the cancer-germline group of genes, as a model. Detailed methylation analyses of the MAGEA1 genomic locus in expressing tumor cells, revealed preferential hypomethylation within the 5' region of the gene (5). Furthermore, transfection experiments with in vitro methylated MAGEA1 constructs, indicated that this site-specific hypomethylation relies on a historical event of DNA demethylation, and on the presence of appropriate transcription factors to protect the region against subsequent remethylation (6). The factors that are responsible for the initial DNA demethylation process and for maintaining cancer-germline gene promoters unmethylated remain to be identified.

DNA METHYLATION CHANGES ASSOCIATED WITH CELL SENESCENCE AND IMMORTALIZATION

G. Tilman, A. Loriot, A. Van Beneden, C. De Smet, A. Decottignies

In human and mouse cells, recent studies have shown that telomeric and subtelomeric chromatin contains histone modifications that are commonly found in heterochromatin. Increasing evidence also indicates that chromatin modifications at chromosome ends are important regulators of mammalian telomeres. In particular, alterations of either histone modifications in telomeric chromatin or of DNA methylation in subtelomeric regions are associated with telomere length deregulation in mouse cells. In addition, a decreased subtelomeric DNA methylation level in mouse cells was reported to be associated with increased homologous recombination between telomeric sequences (T-SCE for Telomeric Sister Chromatid Exchange), a hallmark of human ALT cells.

This prompted us to evaluate the subtelomeric DNA methylation level of human TEL+ and ALT cancer cell lines (7). We detected a significant hypomethylation of subtelomeric DNA in ALT cancer cell lines when compared to TEL+ cell lines. However, subtelomeric DNA was not hypomethylated in ALT cell lines derived from *in vitro* immortalization of human fibroblasts with SV40 T antigen, although T-SCE frequencies in the latter cells were similar to those in ALT cancer cells (7). Strikingly, subtelomeric DNA hypomethylation in ALT cancer cells was also associated with lower global DNA methylation. This observation raised the interesting possibility that DNA demethylation

in tumor cells may be linked to the process that cells use to escape from senescence and/or crisis, two anti-proliferative barriers thought to require bypass during tumorigenesis. Indeed, evidence accumulated during the past decade that senescent and cancer cells share similarly altered global epigenetic profiles that includes changes in DNA methylation, is in agreement with the hypothesis that senescence, whether induced by ageing or by oncogene activation, may be a common step in the tumorigenesis process (8). We are currently investigating whether deep DNA hypomethylation may have favoured the emergence of ALT cells during tumorigenesis.

We also started to address the cellular mechanisms underlying the differences in DNA methylation levels between ALT and TEL+ cancer cells. To this end, we are trying to reproduce, *in vitro*, the demethylation process that operates during tumorigenesis by overexpressing RasV12 oncogene in human dermal fibroblasts. On one hand, RasV12 oncogene is known to induce cellular senescence of primary fibroblasts through activation of the DNA damage response and, on the other hand, this oncogene leads to cellular transformation of p53/pRb-defective cells. As both senescence and transformation may be associated with genomic DNA hypomethylation, we are investigating these two aspects of RasV12 expression.

REGULATION OF TELOMERIC TRANSCRIPTS IN HUMAN CELLS

N. Arnoult, A. Van Beneden, A. Decottignies

Recent studies indicated that telomeres of eukaryotic cells are frequently transcribed, yielding (UUAGGG)n non-coding RNAs in mammalian cells, called "TERRAs". TERRAs have been shown to localize to telomeres where they may impact on diverse aspects of telomere biology, including regulation of telomerase access to telomeres and DNA damage checkpoint. We are interested in understanding how TERRAs are regulated in human cells and what their impact is on telomeres, both in TEL+ and ALT cells.

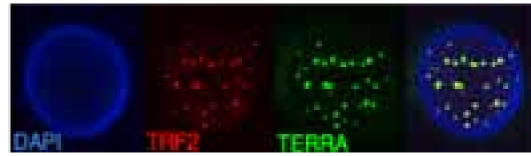

Figure 3. Detection of TERRA telomeric transcripts by RNA-FISH at human telomeres. Telomeres are detected by immunofluorescence against TRF2 telomeric protein (red), TERRAs are hybridized with a fluorescent telomeric probe (green) and DNA is stained with DAPI (blue).

EPIGENETIC REPRESSION OF CANCER-GERMLINE GENES IN HUMAN EMBRYONIC STEM CELLS

G. Parvizi, A. Loriot, C. De Smet

The stage at which cancer-germline genes become methylated during human embryo development has not been determined. We found previously that human cancer-germline genes are repressed and methylated in human blastocyst-derived embryonic stem cells and in comparable embryonal carcinoma cells (9). By performing transfection experiments, we now demonstrated that human embryonal carcinoma cells target active de novo methylation towards MAGEA1, as the gene became methylated and silenced following integration into these cells. Consistently, silencing of MAGEA1 in embryonal carcinoma cells depended on the presence of both DNMT3A and DNMT3B de novo DNA methyltransferases. Other chromatin-related silencing mechanisms, namely histone H3K9 and H3K27 methylation, were not associated with MAGEA1 repression in human embryonal carcinoma cells. This distinguishes the human MAGEA1 gene from its murine counterparts, which rely on H3K9

methylation for repression in embryonic cells. Moreover, by analyzing transcription profiling datasets from human preimplantation embryos, we found that transcription of cancer-germline genes increases up to the morula stage, and then decreases dramatically in blastocysts. Altogether our data indicate that human cancer-germline genes are programmed for repression in the blastocyst, and suggest that de novo DNA methylation is a primary event in this process. They also identify species-specific differences in the underlying epigenetic mechanisms. The disparity between mouse and human MAGEA genes is likely attributable to their poor sequence conservation, especially within regulatory regions, and may explain our previous surprising observation that in vitro methylated human MAGEA1 sequences become demethylated following transfection into mouse embryonic stem cells (10).

UNBIASED SEARCH FOR FACTORS THAT MAINTAIN *MAGEA1* METHYLATION

A. Loriot, C. De Smet

We decided to perform an unbiased search for factors that participate in maintaining methylation within the promoter of *MAGEA1*. To this end we have transduced a lentiviral shRNA library into a human melanoma cell line containing a methylated transgene comprising the *MAGEA1* promoter followed by the sequence encoding the green fluorescent protein (GFP). Transduction of shRNAs directed against factors that contribute to methylation maintenance should lead to de-repression of the transgene and emergence of GFP positive clones. GFP positive cell clones, which emerged at different time points after transduction, have been isolated by cell sorting. The shRNA sequences they contain are currently being identified. Single shRNAs directed against candidate genes will be tested individually to confirm activation and demethylation of the *MAGEA1* transgene.

DEVELOPING PREDICTIVE MARKERS OF RESPONSE TO CHEMOTHERAPY IN BREAST CANCER PATIENTS

V. Rucchin, C. De Smet (BruBreast project: in collaboration with C. Sotiriou and F. Fuks, ULB; J. De Grève, VUB)

Breast cancer is the most frequently encountered type of cancer in women. Although several treatment options are available, one third of the patients eventually die from the disease. The currently used factors for predicting response to therapy are suboptimal and insufficient to explain the differences in survival. The BruBreast project aims to identify markers that would predict the response or resistance to anti-cancer treatment in individual patients with greater accuracy. Practically, the project is accomplished in the context of a multicentric clinical study (coordinated by the Institut Jules Bordet, ULB) aiming at analyzing gene expression profiles associated with response or resistance to epirubicine, one of the most active chemotherapies in breast cancer. We will determine if specific methylation marks are associated with the differentially expressed genes. Our goal is to develop and validate a robust molecular detection kit based on gene expression and methylation markers, which would predict resistance/response to treatment of breast cancer.

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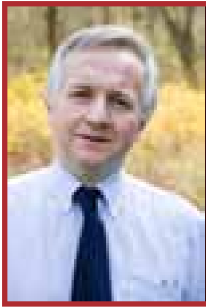

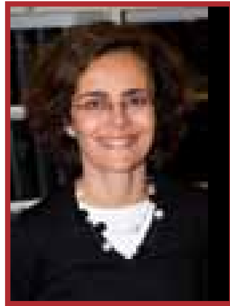

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PROTEIN REPAIR AND INBORN ERRORS OF METABOLISM

The study of the mechanism of formation of an intriguing phosphate ester, fructose 3-phosphate, led us to discover fructosamine 3-kinase, an enzyme serving to remove sugar adducts from proteins. Part of our effort is now devoted to understanding the function of 'fructosamine-3-kinase-related protein', an enzyme that apparently serves to repair ribulosamines. Besides this, our group aims also at elucidating the cause of inborn errors of metabolism. In this respect, a lot of efforts have been made to identify new enzymes. Furthermore, the elucidation of the cause of a metabolic disease, L-2-hydroxyglutaric aciduria, brought us to study another from of repair, 'metabolite repair'.

PROTEIN DEGLYCATION

M. Veiga-da-Cunha, Y. Achouri, E. Van Schaftingen

Free sugars spontaneously react with amino groups of proteins to form Schiff bases, which rearrange to become Amadori products. The Amadori product that is formed when glucose reacts resembles fructose and is therefore named fructosamine. A few years ago, we uncovered an enzyme, fructosamine 3-kinase (FN3K), that phosphorylates the third carbon of fructosamines, making them unstable and causing them to detach from proteins. FN3K is therefore a protein repair enzyme, a conclusion that was supported by the finding that protein-bound fructosamines are significantly more abundant in FN3K$^{-/-}$ mice than in control mice.

We have also identified several other enzymes that are potentially involved in protein deglycation. A first one is fructosamine-3-kinase-related protein (FN3K-RP; ref 2). This enzyme shares about 65 % sequence identity with FN3K and is encoded by a gene that is present next to the FN3K gene on human

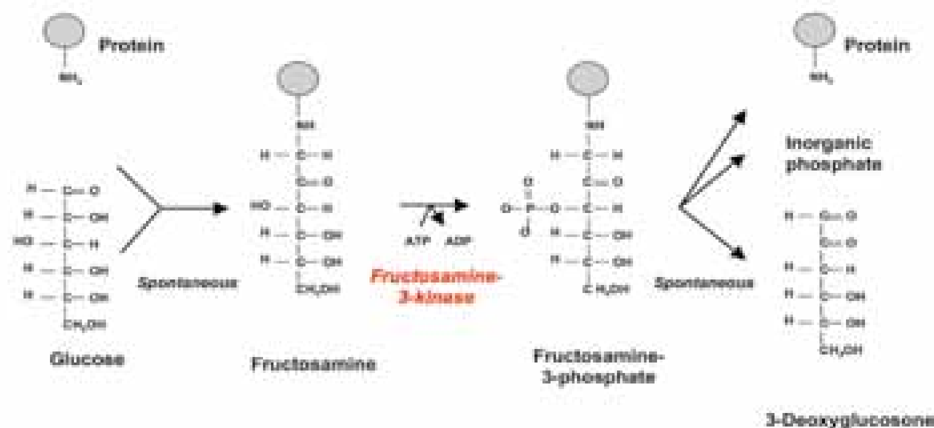

Figure 1. Formation and breakdown of fructosamines

chromosome 17q25. A similar gene arrangement is found in other mammals and in chicken, although not in fishes, indicating that a gene duplication event occurred during or after the fish radiation. FN3K-RP is also a ketoamine 3-kinase, acting best on ribulosamines and erythrulosamines, but not at all on fructosamines. An enzyme with a similar substrate specificity is found in many fishes, in plants and in a significant proportion (≈ 25 %) of bacteria. All ketoamine 3-phosphates are unstable and their spontaneous decomposition regenerates the free amino group, indicating that FN3K-RP is also a protein repair enzyme (7).

It is unlikely that the physiological substrates of FN3K-RP are formed through a reaction of amines with free ribose or erythrose, because these sugars are present at very low concentrations (< 10 μM) in tissues. They are most likely formed through a reaction of proteins with ribose 5-phosphate or erythrose 4-phosphate, two extremely potent glycating agents that react ≈ 80 and 500-fold more rapidly than glucose. The ribulosamine 5-phosphates (Fig. 2) and erythrulosamine 4-phosphates that are formed from phosphorylated intermediates need to be dephosphorylated before being phosphorylated on their third carbon by FN3K-RP, and thereby destabilized and removed from proteins. The phosphatase catalyzing this reaction has recently been identified as LMW-PTP (low-molecular-weight protein-tyrosine-phosphatase). One of our goals is to understand the physiological significance of FN3K-RP-mediated deglycation.

NEUROMETABOLIC DISORDERS

Y. Achouri, G. Noël, R. Rzem, M. Veiga da Cunha, E. Van Schaftingen

D- and L-2-hydroxyglutaric acidurias are distinct neurometabolic diseases characterized by the accumulation of abnormal amounts of either D- or L-2-hydroxyglutarate in cerebrospinal fluid, blood and urine. Work in our lab has led to the elucidation of the metabolism of these compounds (Fig. 2). Both of them are converted to alpha-ketoglutarate by distinct FAD-linked dehydrogenases. The dehydrogenase acting on L-2-hydroxyglutarate is bound to mitochondrial membranes and mutations in its gene are found in virtually all cases of L-2-hydroxyglutaric aciduria (7). The dehydrogenase acting on D-2-hydroxyglutarate is in the mitochondrial matrix and most likely transfers its electrons to the respiratory chain via electron-transfer-flavoprotein (1). It is mutated in about half of the patients with D-2-hydroxyglutaric aciduria.

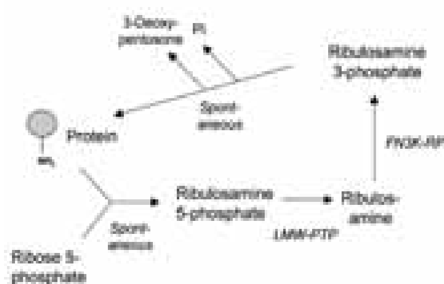

Figure 2. Formation and breakdown of ribulosamines

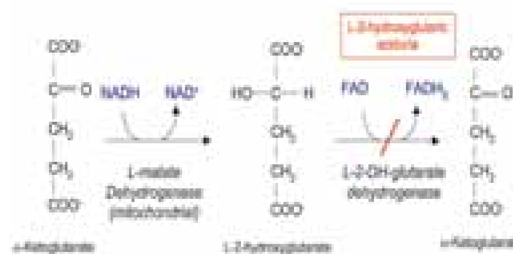

Figure 3. Formation and breakdown of L-2-hydroxyglutarate

The other half of the patients with D-2-hydroxyglutaric aciduria have a peculiar neomutation in isocitrate dehydrogenase 2 (3) (a mitochondrial enzyme). This mutation changes its catalytic activity: the mutated enzyme is no longer able to catalyse the oxidative decarboxylation of isocitrate to alpha-ketoglutarate, but will instead catalyse the NADPH-dependent reduction of alpha-ketoglutarate to L-2-hydroxyglutarate. This production exceeds the capacity of D-2-hydroxyglutarate dehydrogenase, thereby causing accumulation of D-2-hydroxyglutarate in body fluids. The neomutation had initially been found in different types of tumours. Remarkably, D-2-hydroxyglutaric aciduria, unlike L-2-hydroxyglutaric aciduria, does not seem to be associated with an increased cancer risk.

The formation of L-2-hydroxyglutarate is catalyzed by mitochondrial L-malate dehydrogenase (Fig. 3). This enzyme is not completely specific for oxaloacetate : it also reduces, at a very low rate, alpha-ketoglutarate to L-2-hydroxyglutarate. This activity is sufficient to account for the daily formation of L-2-hydroxyglutarate. Since L-2-hydroxyglutarate does not appear to have any role, but to have only toxic effects, L-2-hydroxyglutarate dehydrogenase is a 'repair enzyme' and L-2-hydroxyglutaric aciduria is a disorder of metabolite repair.

We have recently produced a mouse model of L-2-hydroxyglutaric aciduria, which we are analysing in order to confirm the origin of L-2-hydroxyglutarate and the pathophysiological mechanisms of this disease.

IDENTIFICATION OF ENZYMES POTENTIALLY IMPLICATED IN METABOLIC DISEASES

F. Collard, C. Linster, A. Marbaix, G. Tahay, E. Wiame, G. Connerotte, K. Peel, M. Veiga-da-Cunha, E. Van Schaftingen

Synthesis of N-acetyl-aspartate

The brain-specific compound NAA (N-acetylaspartate) is the second most abundant organic molecule in brain. It occurs almost exclusively in neurons, where its concentration reaches ≈ 20 mM. Its abundance is determined in patients by MRS (magnetic resonance spectroscopy) to assess neuronal density and health. The molecular identity of the N-acetyltransferase that catalyses NAA synthesis has remained unknown, because the enzyme is membrane-bound and difficult to purify.

Using a database search approach we have identified its gene. Briefly, we have searched

the human and mouse genome for putative N-acetyltransferases that would be membrane-bound and exclusively expressed in brain. Two candidates were selected in this way (NAT8L and NAT14). They were expressed in HEK cells and NAT8L was shown to be the N-acetylaspartate-producing enzyme (10). A patient deficient in N-acetylaspartate was shown to have a homozygous 19 bp deletion in the coding sequence of the NAT8L gene, further proving that NAT8L is responsible for NAA production. In collaboration with D. Tyteca and P. Courtoy (CELL Unit), we also showed that this enzyme is associated with the endoplasmic reticulum, and not with mitochondria, as often stated previously. The molecular identification of this enzyme will lead to new perspectives in the clarification of the function of N-acetyl aspartate in neurons and for the diagnosis of hypoacetylaspartia in other patients.

Synthesis of N-acetylaspartylglutamate and beta-citrylglutamate

N-acetylaspartylglutamate (NAAG) is the most abundant dipeptide present in vertebrate central nervous system (CNS). β-citrylglutamate, a structural analogue of NAAG is present in testis and immature brain. The role of both compounds is still mysterious. Previous evidence suggests that NAAG is synthesized by an ATP-dependent ligase. As attempts to detect this ligase in brain extracts failed, we searched the mammalian genomes for putative enzymes that could catalyze this type of reaction. We found two putative ligases homologous to *Escherichia coli* RIMK, which ligates glutamates to the C terminus of ribosomal protein S6. One of them, named RIMKLA, is almost exclusively expressed in the CNS, while RIMKLB, which shares 65% sequence identity with RIMKLA, is expressed in the CNS and testis. Both proteins were expressed in bacteria or HEK293T cells and purified. RIMKLA catalyzed the ATP-dependent synthesis of N-acetylaspartylglutamate from N-acetylaspartate and L-glutamate. RIMKLB catalyzed this reaction as well as the synthesis of β-citrylglutamate. The nature of the reaction products was confirmed by mass spectrometry and NMR. RIMKLA was shown to produce stoichiometric amounts of NAAG and ADP, in agreement with its belonging to the ATP-grasp family of ligases. The molecular identification of these two enzymes will facilitate progress in the understanding of the function of NAAG and β-citrylglutamate (1).

Formation of mercapturic acids

NAT8 shares about 30 % identity with NAT8L (aspartate N-acetyltransferase) is predicted to be membrane-bound and to be expressed exclusively in kidney and liver. Based on these features we hypothesized that it would correspond to the acetyltransferase that makes mercapturic acid (N-acetylcysteinyl-S-conjugates), catalysing thereby the last step in one of the major pathways of xenobiotic metabolism (9). This hypothesis was confirmed by expressing this protein in HEK cells and testing its enzymatic activity. In collaboration with D. Tyteca and P. Courtoy, we found that like NAT8L, NAT8 is associated with the endoplasmic reticulum thanks to a non-classical targeting signal that we are now characterizing. The NAT8 gene has recently been shown to be associated with chronic kidney diseases (Chambers et al. Nature Genetics, 2010). Because of the toxicity of non acetylated cysteinyl-S-conjugates, our identification provides a potential explanation for this association.

In relation with the metabolism of xenobiotics, we also carried out for the first time the molecular identification of omega amidase, the enzyme that hydrolyzes alpha-ketoglutaramate, a product made by transaminases using glutamine as an alpha-amino group donor.

Carnosine synthase

Carnosine (beta-alanyl-L-histidine) is a most abundant (concentration ≈ 10 mM) dipeptide in muscle whereas homocarnosine (gamma-aminobutyryl-L-histidine) is the second most abundant dipeptide in brain of most vertebrates and some invertebrates. Their function is still not well established and the enzyme (carnosine synthase) that synthesizes them both was not well characterized and its molecular identity was unknown. To determine this identity, we have purified carnosine synthase from chicken pectoral muscle (2). We found that this enzyme hydrolyses ATP to ADP and inorganic phosphate and not to AMP and pyrophosphate, as previously assumed. Furthermore, by combining a database mining approach with a mass spectrometry analysis of the purified protein, we could show that carnosine synthase corresponds to a protein of unknown function named ATPGD1 in the databases. This was confirmed by expression and purification of human and mouse ATPGD1, which we found to catalyze the synthesis of both carnosine and homocarnosine (2). The identification of the gene encoding carnosine synthase will help getting a better understanding of the biological functions of carnosine and related dipeptides. Furthermore, it opens the perspective of testing if the low homocarnosine level found in the CSF of some patients is due to primary carnosine/homocarnosine synthase deficiency.

A specific pseudouridine 5'-phosphate phosphatase

We previously identified enzymes that metabolize pseudouridine, the fifth-most abundant nucleoside in RNA, and showed that these enzymes are not present in mammals (4). Pseudouridine is indeed not metabolized in mammals, but excreted intact in urine. We have recently identified a specific pseudouridine 5'-phosphatase, which presumably plays an important role in the formation of the free nucleoside from RNA breakdown products. We found indeed that human erythrocyte extracts contain a pseudouridine-5'-phosphatase displaying a Km ≤ 1 µM for its substrate. The activity of the partially purified enzyme was dependent on Mg^{2+}, and was inhibited by Ca^{2+} and vanadate, suggesting that it belonged to the 'haloacid dehalogenase' family of phosphatases. Its low molecular mass (26 kDa) suggested that this phosphatase could correspond to the protein encoded by the HDHD1 gene, present next to the STS (steroid sulfatase) gene on human chromosome Xp22. Purified human recombinant HDHD1 dephosphorylated pseudouridine 5'-phosphate with a catalytic efficiency that was at least 1000-fold higher than that with which it acted on other phosphate esters. The molecular identity of pseudouridine-5'-phosphatase was confirmed by the finding that its activity was negligible (<10% of controls) in extracts of B-cell lymphoblasts or erythrocytes from X-linked ichthyosis patients harbouring a combined deletion of the STS gene (the X-linked ichthyosis gene) and the HDHD1 gene. Furthermore, pseudouridine-5'-phosphatase activity was 1.5-fold higher in erythrocytes from women compared with men, in agreement with the HDHD1 gene undergoing only partial inactivation in females. In conclusion, HDHD1 is a phosphatase specifically involved in dephosphorylation of a modified nucleotide present in RNA (5).

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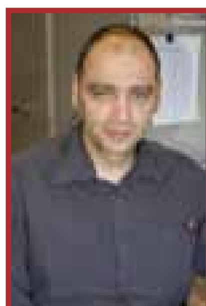

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NUCLEOSIDE ANALOGUES IN LEUKAEMIA

Our group was initially interested in purine metabolism, particularly adenine nucleotide metabolism, and its genetic defects. Thereafter, we expended our investigations on two therapeutic purine nucleoside analogues, 2-chlorodeoxyadenosine and fludarabine, which have revolutionized the treatment of indolent lymphoproliferative disorders. Despite their efficacy, clinical resistance to these drugs is frequently observed. The main objectives of our present studies are to unravel the mechanisms leading to resistance to nucleoside analogues and to find novel therapeutic strategies to counteract them, particularly in chronic lymphocytic leukaemia.

In 1997, a collaborative study of the anti-leukaemic purine nucleoside analogues (PNA), 2-chlorodeoxyadenosine (CdA) and fludarabine (Fig. 1), was started with the Department of Haematology of the University Hospital Saint-Luc. These two deoxyadenosine analogues display remarkable therapeutic properties in indolent lymphoid malignancies including hairy cell leukaemia and chronic lymphocytic leukaemia (CLL). Nevertheless, resistance is also observed, and PNA do not confer a survival advantage when compared to more conventional therapies such as alkylating agents. The aims of our studies are to understand the mechanisms that lead to resistance to nucleoside analogues, and to improve their therapeutic efficacy by searching for synergisms with other compounds.

CdA and fludarabine are prodrugs. To exert their antileukaemic effect, they have to be phosphorylated by deoxycytidine kinase (dCK) into nucleoside analogue monophosphates, followed by conversion into di- and triphosphate derivatives. The latter are the active metabolites. They have been shown to inhibit various processes involved in DNA and RNA synthesis. Moreover, they can be incorporated into newly synthesised DNA, causing chain termination. Together, these actions result in arrest of DNA synthesis (replication and repair) and in the progressive accumulation of DNA strand breaks, leading to apoptosis by mechanisms which are not yet entirely clear (1, for a review).

Fig. 1. Structures of deoxyadenosine and purine nucleoside analogues

MECHANISMS OF ACTION

F. Bontemps, C. Smal, L. Bastin-Coyette, E. de Viron, A. Arts, E. Van Den Neste

To improve our understanding of the mechanisms by which CdA induces apoptosis in CLL cells, we study its effects in EHEB cells, a continuous cell line derived from a patient with CLL. The EHEB cell line was found to be less sensitive (˜10 fold) to CdA than other human lymphoblastic cell lines. This could be partly explained by a lower intracellular accumulation of CdATP, the active metabolite of CdA, due to a reduced dCK activity. In addition, analysis of the cell cycle showed that CdA accelerated the progression from G1 to S phase before inducing cell death (2). This cell response was unexpected because PNA are known to induce accumulation of p53, which typically results in the accumulation of its target p21, inhibition of cyclin-dependent kinase 2 (Cdk2) and G1/S phase arrest. This paradoxical result led us to examine the effects of CdA on the p53-p21 axis in this cell line. We clearly demonstrated that CdA, but also fludarabine and pyrimidine analogues induced p21 depletion, while p53 was upregulated (3). This p21 depletion resulted from an increased proteasomal degradation, which had already been reported after UV-irradiation, but never after nucleoside analogue treatment. In addition, we found that p21 depletion was associated with Cdk2 activation, which could explain the activation of the cell cycle by CdA in this cell line, and by PCNA monoubiquitination, which promotes translesion DNA synthesis and favors DNA repair and cell survival. Further work is needed to determine whether PCNA monoubiquitination could play a role in the clinical resistance to PNA.

In parallel studies, we have analysed the mechanisms of cell death induced by 2-chloroadenosine (2-CAdo) in various types of leukaemic cells. This analogue is not activated by dCK, like CdA, but by adenosine kinase. We found that 2-CAdo was efficiently converted into 2-chloroATP, resulting in ATP depletion, as well as in inhibition of DNA, RNA and protein synthesis. 2-CAdo also caused activation of the intrinsic pathway of apoptosis, which was p53-independent and associated with a decline in the antiapoptotic protein Mcl-1 protein. Prevention of ATP loss by inhibition of AMP deaminase reduced apoptosis, indicating that ATP depletion plays a role in 2-CAdo-induced apoptosis (4).

More recently, we have initiated microarray analyses to identify survival or death pathways that are activated in response to CdA and fludarabine. We intended to compare genes induced or repressed by these PNA in sensitive and refractory CLL patients. We found that, in chemosensitive samples, PNA predominantly increased the expression of p53-dependent genes, among which PLK2 (polo-like kinase 2) was the most highly activated at early time points. Conversely, in chemoresistant samples, p53-dependent and PLK2 responses were abo-

lished. Using qPCR, we confirmed that PNA dose-and time-dependently increased PLK2 expression in chemosensitive but not chemoresistant CLL samples. Analysis of a larger cohort of CLL patients showed that cytotoxicity induced by PNA correlated well with PLK2 mRNA induction. In conclusion, we propose that testing PLK2 activation after a 24-h incubation with PNA could be used to investigate the functional integrity of DNA-damage response pathways in CLL cells, and predict clinical sensitivity to these drugs (5). This study was performed in collaboration with Dr L. Knoops from the Ludwig Institute for Cancer Research (Brussels). The following step was to investigate the role of PLK2 during PNA-induced apoptosis. However, PLK2 could not be detected at the protein level in CLL cells, precluding a role of this kinase in PNA-induced apoptosis. We are currently investigating whether PLK2 expression could be regulated by microRNAs. This study is performed in collaboration with Dr G. Bommer (de Duve Institute).

SEARCH FOR POTENTIATION OF ANTILEUKAEMIC EFFECT OF 2-CHLORODEOXYADENOSINE

E. Van Den Neste, C. Smal, F. Bontemps

Some years ago, we have shown that combination of CdA with DNA-damaging agents, such as cyclophosphamide (CP) derivatives (6) or UV-light, resulted in synergistic cytotoxicity in CLL lymphocytes, due to inhibition of DNA repair. The in vitro synergy between CdA and CP derivatives has provided the rationale for a clinical trial of this combination, which gives encouraging results.

Lately, we have explored the possibility that CdA interacts with the mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) pathway. We have shown that CdA, at concentrations close to the IC50, activated the ERK pathway in EHEB cells. Because activation of this pathway is assumed to exert anti-apoptotic effect, we combined CdA with inhibitors of the ERK pathway. The latter were found to enhance CdA-induced apoptosis. These results suggest that the efficacy of CdA could be strengthened by combination with inhibitors of the ERK pathway (7).

REGULATION OF DEOXYCYTIDINE KINASE ACTIVITY

C. Smal, R. Amsailale, A. Arts, E. Van Den Neste, F. Bontemps

Deoxycytidine kinase (dCK) activates numerous nucleoside analogues used in anticancer and antiviral therapy. Studies of the mechanism(s) that control the activity of this enzyme are thus of particular interest. As literature data suggested that CdA could induce dCK activation through reversible phosphorylation, we decided to investigate this hypothesis. We overexpressed dCK in HEK 293T cells and observed that the enzyme was labelled after incubation with [^{32}P]orthophosphate, confirming that dCK is a phosphoprotein. Tandem mass spectrometry performed by Dr D. Vertommen and Prof. M.H. Rider from the HormPhos unit (de Duve Institute) allowed the identification of four *in vivo* phosphorylation sites, Thr-3, Ser-11, Ser-15 and Ser-74 (Fig. 2). Site-directed mutagenesis demonstrated that Ser-74 phosphorylation was crucial for dCK activity in HEK 293T cells, whereas phosphorylation of other identified sites did not seem essential (8). Phosphorylation of Ser-74 was also observed on endogenous dCK in CCRF-CEM cells and in lymphocytes from CLL patients, in which this phosphorylation was increased by several genotoxic agents (CdA, UV, etoposide…) that enhanced dCK activity, and decreased by sorbitol that diminished dCK activity. Moreover, interindividual variability in dCK activity in CLL lymphocytes could be related to its phosphorylation level on Ser-74 (9). To conclude, our work has demonstrated that dCK activity in leukaemic cells largely depends on the phosphorylation state of Ser-74.

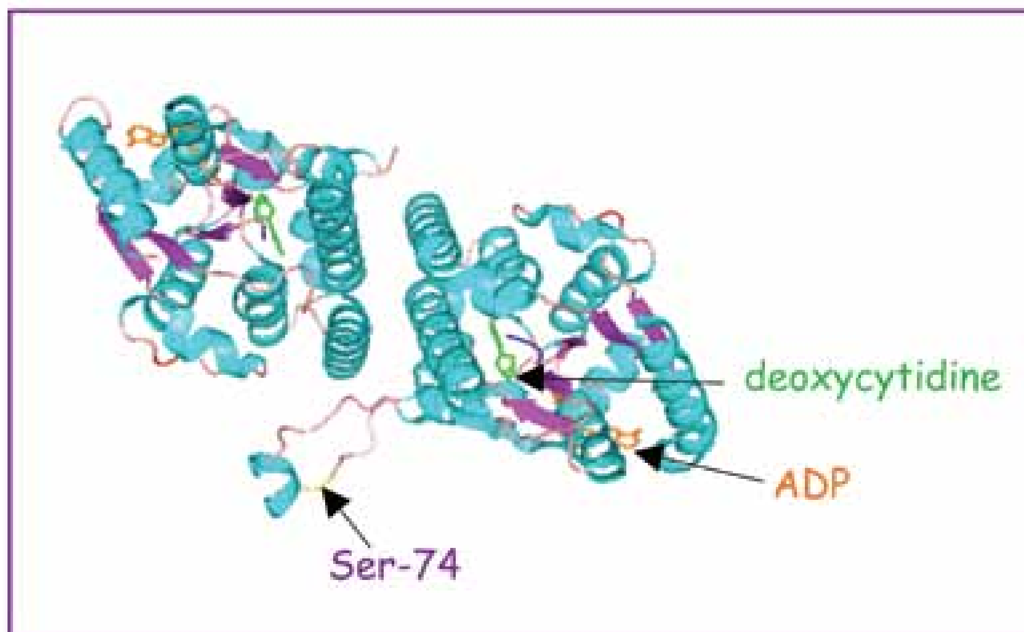

Fig. 2. Three-dimensional structure of deoxycytidine kinase in complex with ADP and deoxycytidine. The Ser-74 phosphorylation site is located at a very flexible 15-residue insert (Ser-63-Asn-77) easily accessible to cellular protein kinases. The N-terminal extremity of dCK (residues 1-19) was flexible and could not be solved. Therefore, the Thr-3, Ser-11 and Ser-15 phosphorylation sites cannot be exactly located.

We are now attempting to identify the protein kinase(s) and the protein phosphatase(s) that control Ser-74 phosphorylation and the signalling pathways that lead to dCK activation following treatment with DNA damaging agents. We showed that casein kinase 1 d (CKI d) could phosphorylate dCK on Ser-74 and increase its activity (10). However, neither CKI d inhibitors nor CKI d siRNA-mediated knock-down modified Ser-74 phosphorylation or dCK activity in cultured cells, precluding a role of CKI d in the regulation of dCK activity *in vivo*. Besides searching for the signalling pathways that control dCK activity, we are investigating whether increase of Ser-74 phosphorylation could enhance efficacy of nucleoside analogues in various types of cancer cells.

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REDOX BIOLOGY

The thiol group of the amino acid cysteine is found in vivo under various oxidation states. This versatility allows cysteine residues to be at the heart of numerous cellular processes by stabilizing protein structures or by fine-tuning protein activity. Some cysteine residues play an essential role in catalysis and in thiol-disulfide exchange reactions by cycling between the thiol state and the disulfide bonded state, such as in ribonucleotide reductase. Another class of cysteine residues is found in many extracellular proteins, which do not benefit from the surveillance of the cellular chaperones and other protection mechanisms. Here, cysteines form disulfide bonds that stabilize protein structure and protect the thiol group from over-oxidation. More recently, some cysteine residues have been shown to play a regulatory role and mediate cellular signaling. For instance, oxidation of cysteine residues to sulfenic acid (-SOH) turns off the activity of protein tyrosine phosphatases. It is now clear that complex enzymatic systems control the oxidation state of cysteine residues, either by reducing or oxidizing them depending on the identity of the protein target, the subcellular compartment and the redox properties of the environment. In our group, we study the mechanisms that control the oxidation state of cysteine residues, mostly using the bacterium Escherichia coli as experimental model. We have two main areas of investigation. First, we study the pathways of disulfide bond formation in the bacterial periplasm. Second, we study the mechanisms that regulate the formation of sulfenic acids by reactive cysteine residues.

DISULFIDE BOND FORMATION IN THE PERIPLASM

In *E. coli*, disulfide bonds are introduced in the periplasm by the Dsb (Disulfide bond) protein family (1, 5).

The primary oxidant is the soluble protein DsbA. DsbA has a CXXC catalytic site motif present within a thioredoxin fold (4). The cysteine residues of this motif are found oxidized in vivo. The disulfide bond of DsbA is very unstable and is rapidly transferred to secreted unfolded proteins. DsbA is then re-oxidized by the inner-membrane protein DsbB that transfers electrons from DsbA to the electron transport chain (Figure 1).

DsbA is a very powerful oxidant that apparently lacks proofreading activity. If the native disulfide bond pattern involves cysteine residues that are non-consecutive in the amino-acid sequence, DsbA can form incorrect

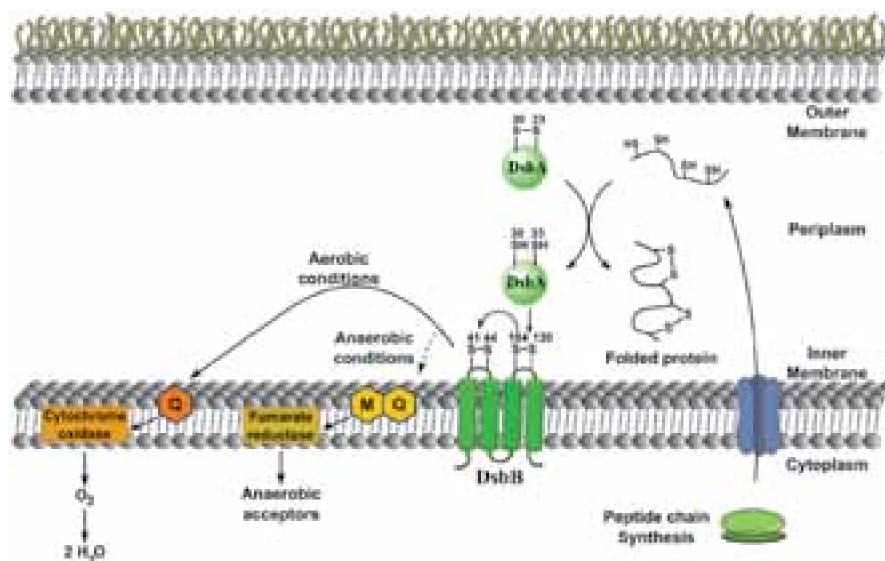

Fig. 1. Disulfide bond formation in the *E. coli* periplasm. Disulfides are introduced into folding proteins by DsbA. DsbA is re-oxidized by DsbB. Electrons flow from the reduced proteins to the CXXC motif of DsbA and then to DsbB. DsbB transfers electrons to the respiratory chain (adapted from (1)).

disulfides. The correction of these non-native disulfides is the role of a disulfide isomerization system. This system involves a soluble periplasmic protein disulfide isomerase, DsbC (Figure 2). Like DsbA, DsbC presents a thioredoxin fold and a CXXC catalytic site motif. In contrast to DsbA, the CXXC motif of DsbC is kept reduced in the periplasm. This allows DsbC to attack non-native disulfides, a necessary step in the isomerization reaction. The protein that keeps DsbC reduced is the membrane protein DsbD. DsbD transfers reducing equivalents from the cytoplasmic thioredoxin system to the periplasm via a succession of disulfide exchange reactions.

In our lab, we are studying the Dsb proteins of E. coli. Over the past few years, we have characterized the disulfide cascade within DsbD, we have identified four proline residues that play an important role in DsbD mechanism and we have identified the proteins that depend on DsbA and DsbC for folding. Moreover, we have engineered a new periplasmic oxidizing system (10) and have characterized the sensitivity of dsbC mutant to copper stress. We have also proposed a revised model for the pathways of disulfide bond formation in the periplasm by showing that DsbC may be acting as a stand-alone protein folding catalyst that is able to cycle from the reduced to the oxidized state upon substrate oxidation and substrate reduction, respectively (9).

Recently, we have identified two new substrates of the protein disulfide isomerase DsbC. Interestingly, these two substrates, LptD and RcsF, are located in the outer membrane and play important roles in the maintain of envelope integrity. LptD is an essential ß-barrel protein that inserts lipopolysacharrides in the outer membrane and whose folding depends on the periplasmic chaperone SurA. We found that deletion of dsbC in strains lacking surA increases E. coli sensitivity to hydrophobic antibiotics due to an impaired assembly of LptD. The definitive evidence that DsbC plays a role in the folding of LptD came from the identification of a LptD-DsbC mixed-disulfide complex (3). RcsF is an outer membrane lipoprotein sensor that detects defects in envelope integrity and transduces the signal to the Rcs phosphorelay. The Rcs phosphorelay is a signaling system that allows bacteria to react to a range of envelope stresses by modulating the expression of specific genes. The Rcs system is required for normal biofilm formation, contributes to antibiotic resistance and regu-

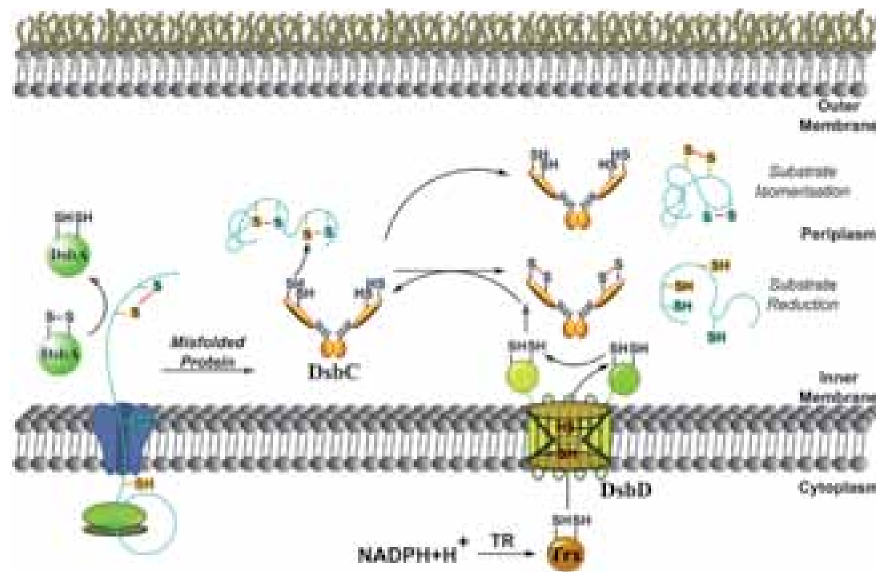

Fig. 2. Disulfide bond reduction/isomerization in the periplasm. DsbA can introduce non-native disulfides when they need to be formed beween non-consecutive cysteines. Incorrect disulfides are either isomerized or reduced by DsbC. Oxidized DsbC is regenerated by the membrane protein DsbD that transfers electrons from the cytoplasmic thioredoxin reductase (TR) / thioredoxin (Trx) system to the periplasm (from reference 1).

lates virulence-associated structures involved in motility and host recognition. In collaboration with Prof. Jean-Paul Declercq (UCL), we solved the structure of RcsF (Figure 3). The fold of the protein is characterized by the presence of a central 4-stranded β sheet, which is conserved in several other proteins, including the copper-binding domain of the Amyloid Precursor Protein (APP). RcsF, which contains four conserved cysteine residues, present two non-consecutive disulfides. We showed that formation of the non-consecutive disulfides of RcsF depends on DsbC: we trapped RcsF in a mixed-disulfide complex with DsbC and we showed that deletion of dsbC prevents the activation of the Rcs phosphorelay by signals that function through RcsF (2).

We have also characterized the DsbA proteins present in the pathogenic bacteria Neisseria meningitides (8), an invasive bacterial pathogen causing life-threatening infection in children worldwide. Host-pathogen interactions, and therefore virulence, depend on the correct folding of many surface-exposed proteins, which often requires disulfide bond formation.

CONTROL OF CYSTEINE SULFENYLATION IN THE PERIPLASM

The periplasm possesses another soluble Dsb protein, DsbG, but the function of this protein has remained elusive. We sought to find the function of DsbG in the periplasm by trapping it with its substrates. We produced a mutant protein in which the second cysteine residue of the CXXC motif is replaced by a serine. This version of the protein is still able to attack a substrate protein to form a mixed-disulfide intermediate. However, due to the absence of the second cysteine residue of the CXXC motif, this mixed disulfide bond cannot be resolved, allowing the purification of the complexes formed between DsbG and its substrates. We identified three periplasmic proteins, in complex with DsbG. The interaction between DsbG and those proteins was confirmed in vitro and in vivo (6).

The three periplasmic proteins (YbiS, ErfK and YnhG) are homologous proteins and belong to the same family of L,-D transpeptidases. Unexpectedly, all three enzymes contain only a single cysteine residue. An intriguing

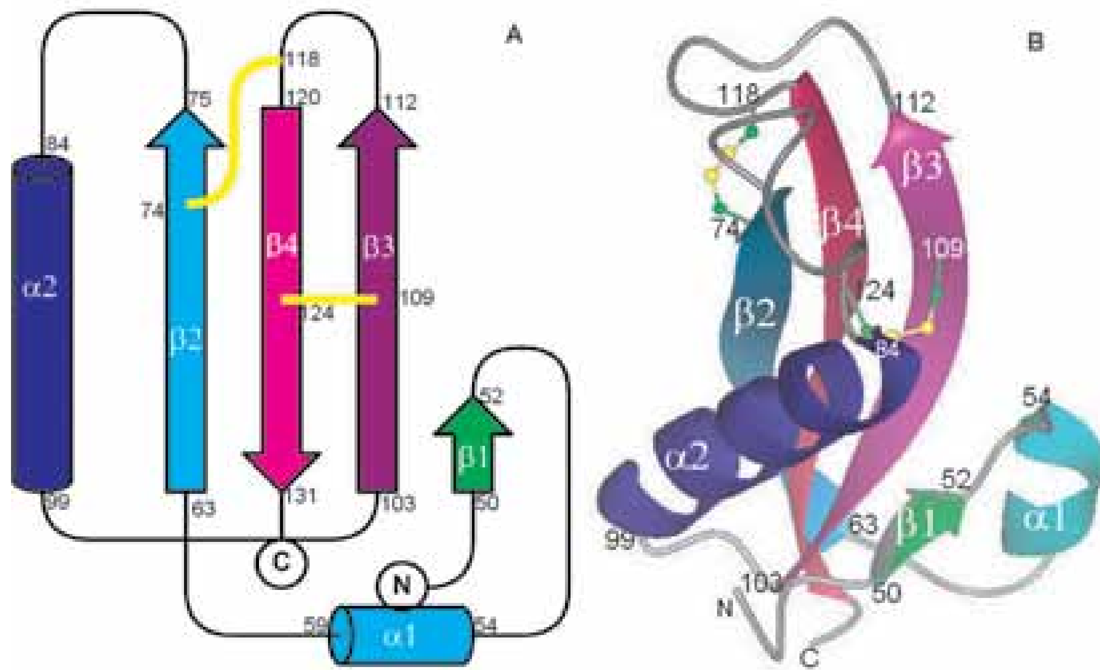

Fig. 3. Overall fold of the RcsF molecule. The protein is colored from green (N-terminal) to pink (C-terminal). A. Topological diagram on which the disulfide bonds are represented by a yellow line. The limits of the secondary structure elements are indicated. B. Ribbon diagram in a more or less similar orientation. The side chains of the cysteine residues taking part in the disulfide bonds are drawn as balls and sticks (from ref. 2).

question pertained therefore to the nature of the oxidation that affects the single cysteine residue of DsbG substrates. We considered the possibility that the cysteine residue might be oxidized to a sulfenic acid (Cys-SOH) by biological oxidants present in the periplasm. Sulfenic acids are highly reactive groups that tend to either rapidly react with other cysteine residues present in the vicinity to form a disulfide bond or to be further oxidized by reactive oxygen species (ROS) to irreversible sulfinic or sulfonic acids.

To test whether the cysteine residue of one of those proteins, YbiS, is indeed able to form a stable sulfenic acid, we used the dimedone-based DAz-1 probe (in collaboration with K. Carroll's lab, The Scripps Institute), which is chemically selective for sulfenic acids. We found that the cysteine residue of YbiS can form a sulfenic acid in vitro and in vivo and that the reduction of this cysteine depends on the presence of DsbG. In the course of our experiments, we observed that, in addition to YbiS, several other periplasmic proteins were also labeled by the probe and that the level of sulfenylation in this compartment is controlled by DsbG and DsbC. We are currently working on the identification of the proteins that were labeled by the DAz probe. On the basis of these results, we proposed the following model. In the oxidizing periplasm, most proteins contain an even number of cysteine residues. These residues form disulfide bonds and are therefore protected from further cysteine oxidation. However, there is a significant number of proteins that contain a single cysteine residue. Because they are not involved in disulfide bonds, these cysteines are vulnerable to oxidation and form sulfenic acids which are susceptible to reaction with small molecule thiols present in the periplasm to form mixed disulfides or to further oxidation to sulfinic and sulfonic acids. DsbG appears to be a key player in a reducing system that protects those single cysteine residues from oxidation. DsbC

could serve as a backup for DsbG and could even have its own subset of favorite sulfenic acid modified substrates to reduce. Both DsbC and DsbG are kept reduced in the periplasm by DsbD, which transfers reducing equivalents from the thioredoxin system across the inner membrane. Thus, the electron flux originating from the cytoplasmic pool of NADPH provides the reducing equivalents required for both the correction of incorrect disulfides and the rescue of sulfenylated orphan cysteines.

Proteins from the thioredoxin superfamily are very widespread and have been identified in the majority of the genomes sequenced so far, making it tempting to speculate that some of these thioredoxin superfamily members, particularly those that are closely related to DsbC and DsbG, play similar roles in controlling the global sulfenic acid content of eukaryotic cellular compartments (6).

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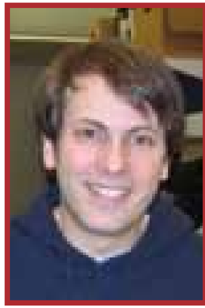

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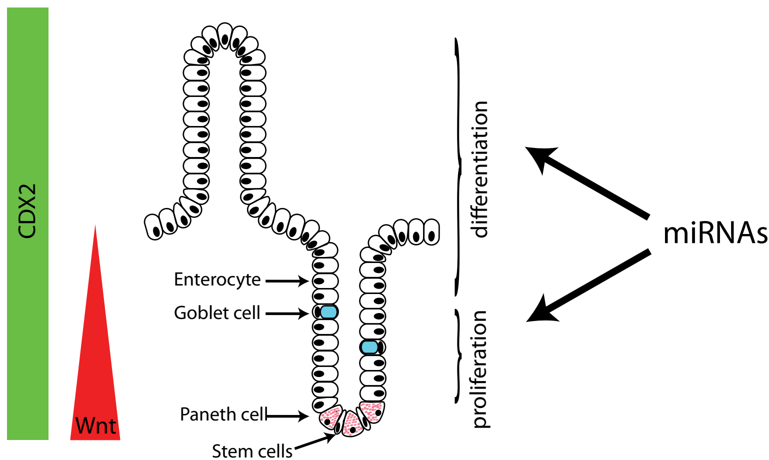
miRNAs IN PHYSIOLOGY AND DISEASE

miRNAs are small non-protein-coding RNAs that can bind to mRNA transcripts of protein coding genes. Upon binding to these mRNAs, they inhibit their translation into proteins. However, each miRNA does not only recognize one target transcript, but rather numerous – in some cases several hundreds – of target transcripts. In addition, for many miRNAs, multiple different genes exist, that encode highly similar or identical mature miRNAs. The potential for combinatorial complexity and functional redundancy is therefore enormous.
We have recently started to work in the unit BCHM focusing on two main topics.

ROLE OF miRNAs IN INTESTINAL DIFFERENTIATION

The intestine is required for the digestion and absorption of essential nutrients and water. In this process, its surface epithelium is exposed to one of the most toxic milieus of the whole body. It has to resist aggressive digestive juices, large pH changes, anaerobic bacteria and numerous toxic compounds. To resist this, its surface epithelium is completely renewed in less than 2 weeks. An intricate network of signaling pathways controls the proliferation and differentiation from intestinal stem cells



Intestinal architecture is maintained by the interplay of many signaling pathways. The intestinal architecture is maintained by the interplay of numerous signaling patways that ensure continuous renewal of intestinal surface epithelia. New cells are generated from a stem cell compartment at the base of the crypts and successively migrate up, where they are eventually shed in the lumen. We are focusing our interest on miRNAs that regulate this process.

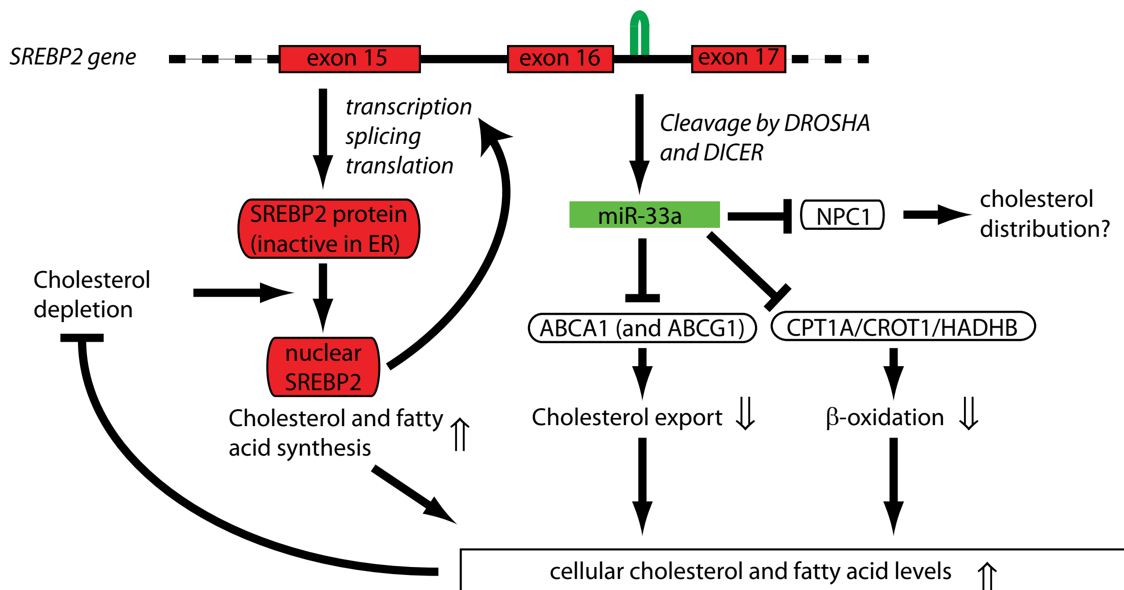
to the mature cell types. We are studying the role of miRNAs in this differentiation process and how they contribute to intestinal differentiation as well as the development of colorectal cancer.

REGULATION OF LIPID METABOLISM BY THE BIFUNCTIONAL LOCUS SREBF2-MIR33

Fatty acids, cholesterol, and their lipid derivatives play essential roles in normal cellular function and serve as structural components, signaling molecules, and/or as storage forms of energy. In multicellular organisms, cellular lipid metabolism is regulated to match the needs both of individual cells and of the entire organism.

The sterol regulatory element-binding factor-2 (SREBF2) gene is a bifunctional locus encoding SREBP-2, a well-known transcriptional regulator of genes involved in cholesterol biosynthesis, and microRNA-33a. We and others have recently shown that miR-33 can reduce the expression of several proteins involved in the cellular export of cholesterol and β-oxidation of fatty acids, thus adding an unexpected layer of complexity and fine-tuning to regulation of lipid homeostasis. In fact, work of other groups has demonstrated that this mechanism might represent a therapeutic target in the treatment of hypercholesterolemia.

We are continuing to investigate the physiological role of miR-33 in different experimental systems.



The bifunctional locus of SREBF2-miR33 regulates cholesterol and fatty acid metabolism. After processing from an intron of SREBF2, miR-33a reduces cellular cholesterol export by inhibiting expression of ABCA1 (and in the mouse ABCG1). In addition, miR-33a reduces mitochondrial fatty acid β-oxidation via inhibition of HADHB, CROT, and CPT1A to increase intracellular lipid levels. Thus the SREBF2 locus uses two distinct mechanisms to maintain lipid homeostasis: regulated transcriptional activity of SREBP-2 and translational repression by miR-33a.

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SIGNAL TRANSDUCTION AND PROTEIN PHOSPHORYLATION

Research in our group focuses on the role of protein phosphorylation in the control of cell function, with special emphasis on the control of metabolism by nutrients, hormones and various stresses. As a model system, we studied 6-phosphofructo-2-kianse (PFK-2)/fructose-2,6-bisphosphatase (FBPase-2) and the control of its activity by various protein kinases. This bifunctional enzyme catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, a potent stimulator of glycolysis that was discovered in this Institute by Van Schaftingen, Hue and Hers in 1980. Our previous work on the regulation of heart PFK-2 activity by phosphorylation led to the study of the insulin and AMP-activated protein kinase (AMPK) signalling cascades, which are now our main research interests.

INSULIN SIGNALLING

L. Toussaint, N. Hussain, D. Vertommen, L. Maisin, M.-A. Gueuning, L. Hue, M.H. Rider, in collaboration with B. Hemmings, Basel

Stimulation of heart glycolysis and adipose tissue lipogenesis by insulin

Insulin stimulates heart glycolysis by increasing glucose transport and by activating PFK-2. This in turn leads to a rise in fructose 2,6-bisphosphate (ref. 1). The recombinant heart PFK-2 isozyme is a substrate of protein kinases of the insulin signalling pathways, such as protein kinase B (PKB), also known as Akt, which is believed to mediate most metabolic effects of insulin. Our previous studies suggested that PFK-2 activation by insulin was dependent on PDK1, a protein kinase upstream of insulin-stimulated protein kinases (Fig. 1). We examined the role of SGK3 (serum- and glucocorticoid-regulated protein kinase-3) in insulin-stimulated PFK-2 activation. Co-transfection of HEK 293T cells with SGK3 siRNA did not affect PFK-2 activation, suggesting that this protein kinase is not required for PFK-2 activation by insulin. Therefore, we re-evaluated the role of PKB. In HEK293 cells co-transfected with heart PFK-2 and total PKB siRNA, insulin-induced PFK-2 activation

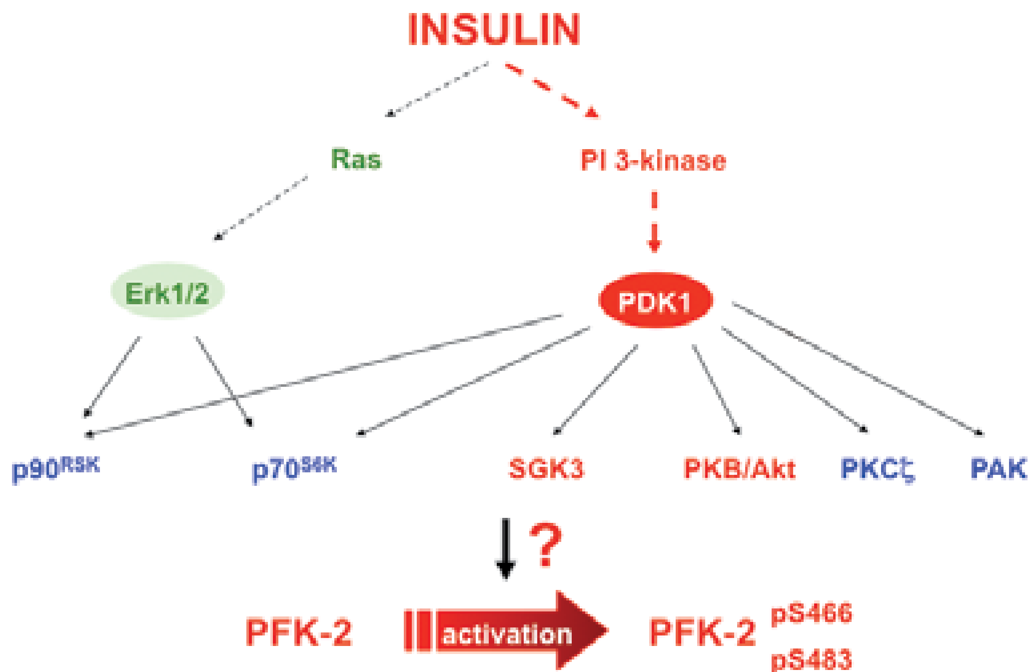

Fig. 1. Protein kinases of signalling pathways that converge on heart PFK-2.

was abrogated. Also in cardiomyocytes treated with the Akti-1/2 PKBα/β-selective inhibitor, PFK-2 activation by insulin was prevented. Our results with PKBβ-knockout mice indicated that this isoform is not required for heart PFK-2 activation by insulin. Moreover, PKBα silencing using the siRNA approach indicated that this PKB isoform is likely to be responsible for heart PFK-2 activation by insulin (ref. 2).

A well-known effect of insulin is the stimulation of adipose tissue lipogenesis, mediated by pyruvate dehydrogenase (PDH) activation via dephosphorylation, and acetyl-CoA carboxylase (ACC) activation. In rat adipocytes incubated with fructose as carbohydrate substrate (to circumvent effects of insulin on glucose uptake), insulin induced lipogenesis was completely abolished by the Akti-1/2 inhibitor suggesting a key role for PKBα/β in the control of this pathway by insulin. Insulin-induced PDH dephosphorylation/activation persisted in the presence of Akti-1/2, indicating that PKB isoforms are not involved in this effect of the hormone. However, ACC Ser79 dephosphorylation by insulin was completely reversed by Akti-1/2. Therefore, a key point of control of the lipogenic pathway by insulin is the dephosphorylation of ACC mediated by PKB, which we showed previously to antagonize AMPK (ref. 3) responsible for ACC Ser79 phosphorylation (see below).

AMP-ACTIVATED PROTEIN KINASE

B. Sid, L. Miranda, L. Bultot, Y. Liu, C. Plaideau, Y.-C. Lai, L, D. Vertommen, N. Hussain, R. Jacobs, Hue, M.H. Rider, in collaboration with S. Carpentier and P. Courtoy, de Duve Institute, S. Horman, UCL, Brussels, K. Sakamoto, Dundee, J. Jenssen, Oslo, K. Storey, Ottawa and B. Viollet, Paris

AMP-activated protein kinase (AMPK) acts as a sensor of cellular energy status. AMPK is activated by an increase in the AMP/ATP ratio as occurs during hypoxia or after ATP depletion with oligomycin. In certain cells, AMPK can also be activated by 5-aminoimidazole-4-carboxamide (AICA)-riboside, which enters cells to be phosphorylated to ZMP, an analogue of AMP, or by the A769662 Abbott compound. Full AMPK activation requires phosphorylation of the α-catalytic subunits at Thr172 by upstream kinases, either LKB1 (the Peutz-Jeghers protein) or calmodulin-dependent protein kinase kinase-β (CaMKKβ). The role of AMPK in the cell is to maintain ATP by stimulating ATP-producing pathways and inhibiting energy-consuming biosynthetic pathways. For example phosphorylation of ACC, the best-known substrate of AMPK, inhibits energy consuming lipogenesis. We contributed to the field by discovering new substrates of AMPK and mechanisms of upstream regulation. We demonstrated that the activation of PFK-2 by AMPK participates in the stimulation of heart glycolysis by ischaemia. We also showed that phosphorylation-induced inactivation of elongation factor 2 (eEF2) by AMPK partly explains the inhibition of protein synthesis by anoxia (ref. 4). Lastly, we demonstrated that PKB-induced phosphorylation of the AMPK catalytic α/β subunits at Ser485/491 in response to insulin antagonizes AMPK activation by LKB1 (ref. 3).

AMPK activation in diapausing insects as a means for surviving energy stress

Winter survival for many insects depends on cold hardiness adaptations as well as entry into a hypometabolic diapause state that minimizes energy expenditure. We investigated whether AMPK could be involved in this adaptation in larvae of two cold hardy insects, *Eurosta*

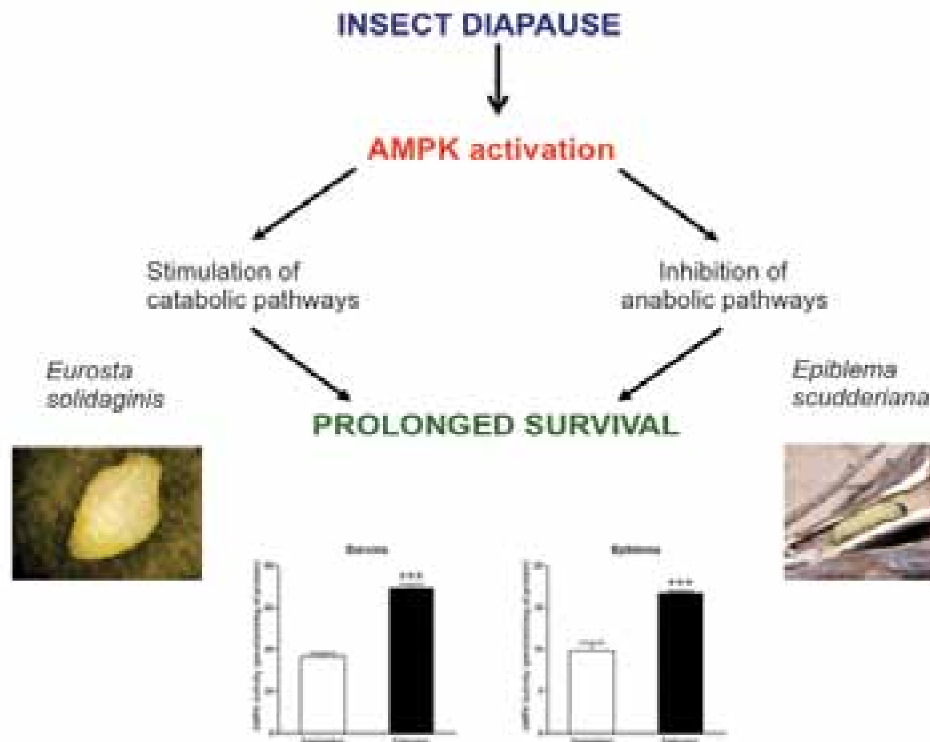

Fig. 2. Role of AMPK to prolong survival during insect diapause.

solidaginis that is freeze tolerant and *Epiblema scudderiana* that uses a freeze avoidance strategy. AMPK activity was almost 2-fold higher in midwinter larvae (February) compared with animals collected in September and accordingly phosphorylation of ACC increased more than 3-fold in both species during midwinter which would strongly suppress lipogenesis. Overall, our study suggests a role for AMPK in minimizing anabolic processes to decrease energy expenditure during insect diapause (Fig. 2; ref. 5).

Control of ion transport by AMPK

We studied whether the mechanism of increased Na+-K+-2Cl- (NKCC1) co-transporter activity by osmotic shrinkage involved AMPK activation. AMPK was found to phosphorylate a recombinant GST-dogfish (1-260) NKCC1 fragment at Ser38 and Ser214, corresponding to Ser77 and Ser242 in human NKCC1, respectively. Incubation of human erythrocytes with A769662 AMPK activator increased Ser242 NKCC1 phosphorylation but did not stimulate $^{86}Rb^+$ uptake. Under hypertonic conditions in human red blood cells (RBCs) incubated with 0.3 M sucrose, NKCC1 activity increased as measured by bumetanide-sensitive $^{86}Rb^+$ uptake and AMPK was activated. However, there was no effect of AMPKα1 deletion in mouse RBCs on the increased rate of $^{86}Rb^+$ uptake induced by hyperosmolarity. AMPK activation by osmotic shrinkage of mouse RBCs was abrogated by the STO-609 CaMKKβ inhibitor, but incubation with STO-609 did not affect the increase in $^{86}Rb^+$ uptake induced by hyperosmolarity. Osmotic shrinkage of human and mouse RBCs led to activation loop phosphorylation of the STE20/SPS1-related proline/alanine-rich kinase (SPAK) at Thr233, which was accompanied by phosphorylation of NKCC1 at Thr203/207/212, one of which (Thr207) is responsible for co-transporter activation. Therefore, phosphorylation-induced activation of NKCC1 by osmotic shrinkage does not involve AMPK and is likely due to SPAK activation (Fig. 3, ref. 6).

Role of AMPK in the control of cytoskeletal actin organization

AMPK function is now recognized to extend beyond metabolic regulation to control cell division, cell polarity and cell migration, all of which depend on the actin cytoskeleton. We studied the effects of A769662 on cytoskeletal organization and signalling in epithelial Madin-Darby canine kidney (MDCK) cells. AMPK activation induced disassembly of stress fibers and accumulation of cortical F-actin. In parallel, Rho-kinase downstream targets, namely myosin regulatory light chain (MLC) and cofilin, were phosphorylated. These effects resembled the morphological changes in MDCK cells exposed to hyperosmotic shock, which led to Ca^{2+}-dependent AMPK activation via CaMKKβ. Indeed, hypertonicity-induced AMPK activation was markedly reduced by the STO-609 CaMKKβ inhibitor, as was the increase in MLC and cofilin phosphorylation. We propose that AMPK links osmotic stress to the reorganization of the actin cytoskeleton (ref. 7).

MASS SPECTROMETRY

D. Vertommen, G. Herinckx, M. Rider in collaboration with C. Sindic, UCL and J.-F. Collet, UCL

The development of mass spectrometry facilities within our laboratory has been an enormous asset to our group and institution. Since the acquisition of our first electrospray mass spectrometer in 1997, the application of mass spectrometry techniques to protein identification, identification of sites of covalent modification and quantification of changes in protein expression has led to well over 30 joint publications. In our own research, it has been paramount in identifying new phosphorylation sites. In 2009 we updated our electrospray machine to the Finnigan LTQ linear ion trap

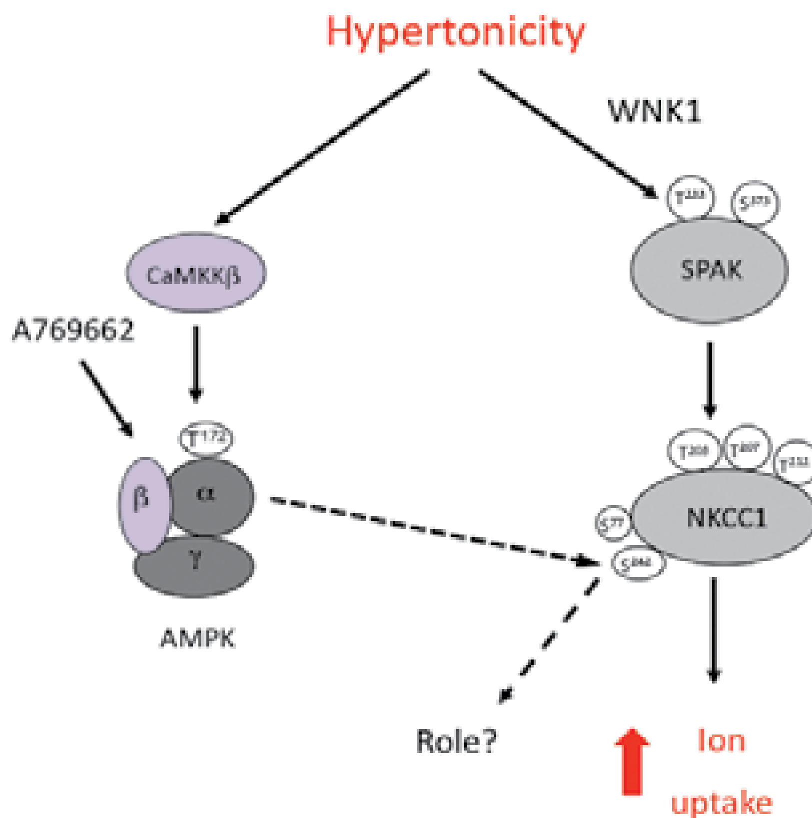

Fig. 3. Cell shrinkage by sucrose treatment in RBCs leads to activation of a1-AMPK and SPAK via the CaMKKβ and WNK (with-no-lysine kinase)-1 pathways, respectively. SPAK activation correlates with NKCC1 Thr203/207/212 phosphorylation and is likely responsible for its activation and increased $^{86}Rb^{+}$ uptake induced by hyperosmolarity. AMPK activation by hyperosmolarity does not affect NKCC1 activity. Treatment with A769662 activates AMPK in RBCs and increases Ser242 NKCC1 phosphorylation, whose function is at present unknown.

equipped with electron-induced transfer dissociation (ETD) fragmentation and we are now part of the UCL proteomics platform MASSPROT, open to the scientific community of the de Duve Institute and UCL and for outside collaborations.

We are continuing our efforts to develop new techniques for the mass spectrometric analysis of proteins. These include a 2D-LC/MS label-free proteomics approach for differential protein expression studies and phosphoproteomics to identify new AMPK substrates, involving hydrophilic interaction chromatography (HILIC) followed by enrichment of phosphopeptides by immobilized metal affinity chromatography (IMAC) and analysis by LC-MS (reviewed in ref. 8). We are also using 14-3-3 pull-downs of extracts from electrically stimulated skeletal muscles to identify new AMPK targets.

We pursue our collaboration with other laboratories within our university and with groups outside and abroad. For example with C. Sindic, the screening of glycoproteins from sera and cerebro-spinal fluid of patients with neurodegenerative diseases is under study to discover new biomarkers. In collaboration with the group of J.-F. Collet, we investigated the mechanisms of outer membrane biogenesis in *E. coli* (refs. 9, 10)

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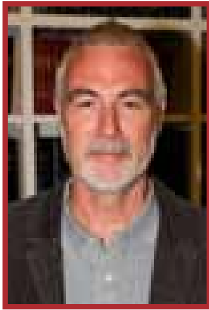

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Nathalie CHEVALIER, Technician
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METABOLISM OF TRYPANOSOMATID PARASITES AND DRUG DISCOVERY

Trypanosomatidae are parasitic protists that cause sleeping sickness in Africa, Chagas' disease in Latin America and various manifestations of leismaniasis in man, in many tropical and subtropical parts of the world. These diseases threaten a few hundreds of millions of people worldwide, affect millions and cause many thousands of deaths each year. The parasites responsible for these diseases are transmitted between humans by insects: tsetse flies, triatomine bugs and sand flies, respectively. There is an urgent need for new, adequate, affordable drugs to treat these diseases, because those currently in use are often toxic and not efficacious, sometimes difficult to administer or too expensive and increasing resistance has been reported against some of them. By a molecular and cell biological investigation of these parasites we intend to provide a basis for the development of such new drugs. African trypanosomes rely entirely on glycolysis for their ATP supply. Interestingly, trypanosomatids are characterized by a unique form of glycolytic compartmentalization by which the majority of the enzymes of this pathway are sequestered inside peroxisome-like organelles called glycosomes. In the past we have validated many of the glycolytic enzymes as potential drug targets. Their structural information is used for the discovery of potent and selective inhibitors. Glycosome assembly and degradation, taking place during cell differentiation, are being studied as well. Many so-called peroxins, proteins involved in glycosome biogenesis, have been identified and also validated as excellent drug targets. Moreover, the availability of the genome sequences of different species of the trypanosomatid parasites causing the three groups of diseases, in combination with various proteomic approaches, has allowed to make a comprehensive inventory of the metabolic capacities of these organisms and to identify other essential differences between the respective parasites and their human host. Other potential drug targets that have been identified are the pentose-phosphate pathway, lipid metabolism and the biosynthesis of biopterin and reduced folate.

METABOLIC PATHWAYS, ENZYMES AND DRUG DISCOVERY

Glycolytic enzymes

V. Hannaert, M. Mazet, P. Michels, in collaboration with L. Gilmore and M. Walkinshaw (University of Edinburgh, Scotland), A. Cavalli and M.-L. Bolognesi (Università di Bologna), O. Thiemann and A. Cordeiro (Universidade de São Paulo, Brazil) and W. Quiñones, L. Avilán and J.-L. Concepción (Universidad de los Andes, Mérida, Venezuela)

Glycolytic enzymes are very important for all trypanosomatids. For *Trypanosoma brucei*, the parasite that causes sleeping sickness when infecting people in sub-Saharan Africa, glycolysis is even the sole free-energy source when the parasite resides in the human blood. Therefore the glycolytic enzymes of these parasites are considered as promising drug targets. Over the years, we have expressed and kinetically characterized trypanosomatid enzymes for all 10 successive steps of the pathway for breakdown of glucose into pyruvate and two additional enzymes present in the glycosome, glycerol-3-phosphate dehydrogenase and glycerol kinase, which also play a crucial role in this process. Structures of most of these enzymes have become available through our collaboration with protein crystallographers elsewhere. All these analyses have revealed important differences between the enzymes of the parasites and the corresponding ones from the human host, offering prospects for developing parasite-enzyme specific potent inhibitors that may be used as lead drugs.

Recently, most of our work has focused on a few selected glycolytic enzymes: phosphofructokinase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and pyruvate kinase from different trypanosomatid parasites. The structures of these enzymes have been determined both without and with various ligands. This has provided insight into mechanisms of catalysis and in the conformational changes required for catalysis by these enzymes. Inhibitors for these enzymes are searched by using them in high-throughput screens of available large libraries of drug-like compounds –mainly at the NIH Chemical Genomics Center, Rockville, USA– and specifically designed libraries and designed by structure-based approaches followed by their synthesis. To date, a series of hit compounds have already been obtained. Some of them inhibit growth of cultured bloodstream-form trypanosomes at concentrations in the micromolar range with no or less effect on cultured human fibroblasts. Detailed analysis of the structure-activity relationship of these compounds is now being used for improving the inhibitors by following medicinal chemistry principles to arrive at leads for anti-parasite drugs. Moreover, our Venezuelan collaborators identified enolase also at the surface of *Trypanosoma cruzi*, the parasite that causes Chagas' disease in Latin-America and *Leishmania* species. In these parasites, which live mainly intracellularly in the human host, this isoform of enolase seems to function as plasminogen receptor, playing a role in the parasites' invasiveness and virulence. This additional location and probable function of enolase offer perspectives to use the enzyme not only as a drug target, but also for vaccination.

For the human pathogenic form of *Leishmania* species not only glucose uptake is important, but also the reverse process of glycolysis, gluconeogenesis; both processes share most of their enzymes. We have also identified and characterized the key enzyme of the gluconeogenic pathway, fructose-1,6-bisphosphatase, of both *T. brucei* and *L. mexicana*. It is present in glycosomes and its activity in *T. brucei* was shown to be silenced by a still unknown mechanism when glycolysis is active.

A small focused library of naphtoquinone-carrying compounds that showed anti-parasite effects in in vitro growth assays with different parasites was synthesized by colleagues at the University of Bologna. Several compounds exhibited potency in the nanomolar range, with up to 80-fold less activity on human cells. By means of a chemical proteomics approach several *T.*

brucei molecular targets of the most promising compound, 2-phenoxy-1,4-naphtoquinone, were identified. Amongst these targets were the glycosomal enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glycerol kinase. Subsequently, these two targets were expressed in bacteria and purified, and then used for inhibition assays. The selected compound was able to inhibit both enzymes with IC_{50} values in the low micromolar range. Supporting the notion that GAPDH is indeed an in situ target was the observation that glucose-grown procyclic trypanosomes, for which the GAPDH activity is crucial, were 28-fold more susceptible to the inhibitor than cells relying much less on GAPDH when they use proline as energy and carbon source. Moreover, it was observed that the compound was able to generate oxygen radicals in mitochondrial fractions of bloodstream-form trypanosomes, but much less so in human mitochondrial preparations, probably related to the highly different respiratory systems of both organisms. This mechanism of inducing the production of oxygen radicals could be additionally responsible for the compound's trypanocidal activity. Indeed, we could show that growth of trypanosomes in which glucose-6-phosphate dehydrogenase (G6PDH) was depleted by RNA interference (RNAi), causing them to be susceptible to oxidative stress, was inhibited at about 100-fold lower concentration of the compound than growth of wild-type trypanosomes. Overall, the molecule showed a multitarget mechanism of action, likely forming the basis of its promising anti-trypanosomatid profile.

Pentose-phosphate pathway enzymes

S. Gupta, M. Alves-Ferreira, P. Michels, in collaboration with A. Cordeiro (Universidade de São Paulo, Brazil)

The pentose-phosphate pathway (PPP) supplies the cells with precursors of nucleotides for DNA and RNA synthesis and the reduced cofactor NADPH for biosynthetic processes and protection against oxidative stress. By knocking down the expression of the first enzyme of the pathway, G6PDH, by RNAi growth of bloodstream-form *T. brucei* is slowed down and eventually the parasites die. Moreover, cells in which the G6PDH has been partially depleted by RNAi are more susceptible to oxidative stress caused by H_2O_2.

We have shown that G6PDH of *T. brucei* and *T. cruzi* are inhibited in an uncompetitive way by the human steroids dihydroepiandrosterone and epiandrosterone and derivatives, with K_i values in the lower micromolar range. In contrast, *L. mexicana* G6PDH is not inhibited. Viability assays demonstrated that the steroids stunt growth of cultured bloodstream-form *T. brucei* and insect-form *T. cruzi,* but not *Leishmania* cells. Importantly, trypanosomes became unsusceptible to the inhibitors when transfected with the *L. mexicana* G6PDH gene and expressing it. Together these findings identified G6PDH as a drug target in trypanosomes and provide prospects for using the steroids to develop leads for a new class of trypanocidal compounds.

The PPP enzyme ribose-5-phosphate isomerase (RPI) catalyses the isomerization of ribulose 5-phosphate and ribose 5-phosphate, to provide the precursors for nucleotide synthesis. Two non-homologous types of RPI exist in nature: RPI-A, that is broadly distributed among most eukaryotic organisms, including humans and some prokaryotes, and RPI-B that is found in most prokaryotic organisms. Also trypanosomatids possess the isoform RPI-B, making it an interesting potential drug target. The *T. brucei* enzyme was expressed in *Escherichia coli*, purified and functionally characterized. The active form of the enzyme is a homodimer and has a $K_m = 1.26 \pm 0.49$ for ribose 5-phosphate, similar to the affinity of the corresponding enzyme from *T. cruzi* and some bacteria. Site-directed mutagenesis was performed to confirm the predicted catalytic role of residue Cys69. Knockdown of the expression of the enzyme by RNAi showed a slight reduction in growth rate of both the bloodstream and procyclic forms of the parasite under usual culture conditions.

Folate metabolism

S. Gupta, V. Hannaert, D. Guerrieri, P. Michels, in collaboration with S. Ferrari and M.-P. Costi (Università di Modena e Reggio Emilia, Italy) and S. Henrich and R. Wade (Heidelberg Institute for Theoretical Studies, Germany)

Enzymes involved in the provision of reduced folate cofactors, such as dihydrofolate reductase (DHFR) and enzymes that use these cofactors, like thymidylate synthase (TS) have been validated as drug targets for cancer and certain bacterial infections and parasitic diseases such as malaria. When DHFR is inhibited, DNA replication is impaired, resulting in cell death. Trypanosomes are auxotrophic for folates and pterins, and inhibition of the enzymes involved in the salvage pathways like the bifunctional DHFR-TS used by trypanosomatids should provide effective treatment. However, antifolates are currently not employed in therapy of trypanosomatid infections, mainly because of the presence of a pteridine reductase (PTR1) –absent from human cells– that is able to carry out successive reductions of both conjugated (folate) and unconjugated (biopterin) pterins and therefore might provide a bypass for DHFR. While DHFR can only reduce folic acid, PTR1 can act on a broader range of substrates. Under physiological conditions, PTR1 is responsible for 10% of the folic acid required by the cell, but inhibition of DHFR often leads to overexpression of PTR1. It was anticipated that folate analogue inhibitors of *Leishmania* PTR1 are potential drugs for combined therapy with DHFR inhibitors. Based on a virtual screening of the Available Chemicals Directory (ACD) database and the known crystal structure of *L. major* PTR1, followed by two rounds of structure-based design to optimize the compounds, 18 drug-like molecules were identified that displayed low micromolar affinities for *L. major* PTR1 and high in vitro specificity when compared to *Leishmania* and human DHFR. The compounds were tested as growth inhibitors of cultured *L. mexicana* and *L. major* promastigotes and human fibroblasts, without and in combination with the DHFR inhibitor pyrimethamine (PYR). Six compounds showed efficacy in combination with PYR, one was active alone and several compounds showed low toxicity for human cells. Interestingly, one compound, Riluzole, a known drug approved for pathologies of the central nervous system, was active in combination and is suitable for early preclinical evaluation of its potential as an antiparasitic drug. Riluzole is notably effective on proliferating parasites. Moreover, the compound was shown to also increase the parasite's susceptibility to oxidative stress.

Thiolase

M. Mazet, P. Michels, in collaboration with R. Wierenga (University of Oulu, Finland) and F. Bringaud (Université Bordeaux Segalen, France)

Thiolases are enzymes that remove an acetyl-CoA group from acyl-CoA in the catabolic β-oxidation of fatty acids, or catalyze the reverse condensation reaction for anabolic processes such as the biosynthesis of sterols and ketone bodies. In humans, six homologous isoforms of thiolase have been described, differing from each other in sequence, oligomeric state, substrate specificity and subcellular localization. Using the sequences of these six isoforms as queries in searches of databases of African trypanosomes, *T. cruzi* and *Leishmania* species, one of these isoforms (called SCP2-thiolase) was found in each of them, another one (TFE-thiolase) in *T. cruzi* and *Leishmania* and a third one (AB-thiolase) only in *T. cruzi*. The role of the different isoforms in the specific metabolism of each the distinct parasites is a topic of study in the TROP unit. The single *T. brucei* SCP2-thiolase has been expressed as recombinant enzyme in *E. coli*, purified and its activity determined. Interestingly, it shows activity in both the degradative and synthetic reaction, with the latter one being 3-fold higher, whereas the synthetic activity of SCP2 from other organisms studied is negligible. mRNA of the thiolase was detected in both bloodstream form and procyclic trypanosomes, but translation of the transcript into

protein only occurred in procyclics as revealed by western blot analysis. The encoded protein has both a predicted N-terminal signal peptide for routing to the mitochondrion and a C-terminal candidate type 1 peroxisome-targeting signal for sorting it to glycosomes. However, both fluorescence analysis of the thiolase with the Green Fluorescent Protein (GFP) fused to either its N- or C-terminus and cell fractionation by differential centrifugation followed by western blot analysis showed only a mitochondrial localization for procyclic cells, irrespective whether the cells were grown with glucose or amino acids as carbon and energy source. When the expression of the thiolase in procyclic cells was knocked down by RNAi, no significant change in growth rate occurred, whether the cells were grown with or without glucose. This absence of a growth phenotype indicates that the metabolic pathway involving this enzyme is not essential for the parasite under either of these growth conditions. Further research is currently being performed to determine the role of this enzyme in the metabolism of the trypanosomes.

Translocation of solutes across the glycosomal membrane

M. Mazet, M. Gualdrón-López, P. Michels, in collaboration with P. Wallemacq (LCBM, UCL) and V. Antonenkov (University of Oulu, Finland)

The glycosomal membrane of *T. brucei* possesses three half-size ABC transporters, designated GAT1-3. GAT1 and GAT3 are expressed in both bloodstream and procyclic-form trypanosomes, whereas GAT2 is only present in bloodstream-form cells. Expression knockdown of GAT1 and GAT3 by RNAi resulted in a growth phenotype that is dependent on the nutritional conditions of the trypanosomes. In the presence of glucose, growth is not affected. When, however, for the GAT1 RNAi cell line, no glucose is available and proline forms the predominant source of free energy, the growth rate is reduced and eventually the trypanosomes die. Glucose-grown cells depleted of GAT1 show a modification of the total cellular fatty-acid composition; no or only minor changes were observed in the levels of most fatty acids, including oleate (C18:1), but the linoleate (C18:2) abundance was significantly increased. We hypothesized that GAT1 is a fatty-acid transporter, like some of its homologues in the peroxisomal membrane of yeasts and mammalian cells. Fatty-acid uptake into glycosomes may be important either for the synthesis of ether-lipids, a process that is crucial for cells, and/or for β-oxidation. Cell fractionation in conjunction with enzyme activity assays, indeed confirmed the association of enzymes of both processes with glycosomes. Glycosomes purified from procyclic wild-type trypanosomes incorporate the activated fatty acid oleoyl-CoA in a temperature-,

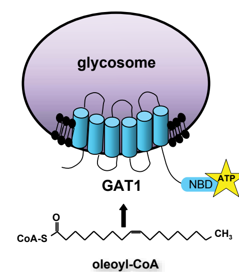

Fig. 1. Model of the half-size glycosomal ABC transporter GAT1 that binds ATP to its nucleotide-binding domain (NBD) to mediate the uptake of oleoyl-CoA into the organelle.

concentration- and ATP-dependent manner, whilst this incorporation was severely reduced in glycosomes from cells in which GAT1 levels had been decreased. This result strongly suggested that GAT1 is an oleate transporter. The increase in linoleate rather than oleate levels upon GAT1 depletion is probably due to the conversion of the latter into the former fatty acid by the high activity of oleate desaturase found in trypanosomes, as described in the report of 2010.

Previously, channel forming activities have been detected for proteins from membranes of mammalian and yeast peroxisomes. We

have now shown similar activities also for glycosomal membrane proteins. When such proteins were solubilized from membranes of highly purified glycosomes and subsequently incorporated into planar lipid bilayers, channels were produced as could be detected by electrophysiological approaches. Preliminary experiments showed the formation of different conductance channels. We hypothesize, by analogy to results obtained for peroxisomes of other organisms, that these channels allow the permeation of small solutes, but restrict the exchange of more bulky compounds such as NAD(H), NADP(H), ADP, ATP, CoA and acetyl/acyl derivatives, etc. Future experiments should lead to the molecular characterization of the channel components and provide information about the assembly and selectivity of the channels.

The metabolic proteome of *Crithidia fasciculata*

F. Opperdoes, in collaboration with S. Beverley (Washington University, St. Louis, USA)

Crithidia fasciculata is a member of the family of Trypanosomatidae and is related to the genera *Leishmania* and *Trypanosoma*. It has a single host life cycle within the mosquito. A limited number of biochemical studies of *Crithidia*, as model organism for the study of the pathogenic trypanosomatids, have been restricted to the cultured choanomastigote stage of the life cycle, which can easily be grown in large quantities in semi-defined culture medium. From these studies it has emerged that *C. fasciculata* has a fully developed mitochondrion with a tricarboxylic acid (TCA) cycle, a complete respiratory chain and it seems to contain essentially the same set of metabolic enzymes as all other trypanosomatids analyzed. Recently its genome has been sequenced and sequence information was placed in the public domain. We have analyzed 406131 translated sequences from open-reading frames and compared them with an in-house database of protein sequences representing 431 metabolic enzymes from previously sequenced trypanosomatid genomes.

The genome analysis of *C. fasciculata* confirms the presence of a fully functional mitochondrion and respiratory chain, as well as the presence of functional glycosomes and a set of peroxins involved in its biogenesis. A classical glycolytic pathway, which partly takes place inside glycosomes, is responsible for the metabolism of exogenous sugars. Carbohydrate metabolism is characterized by an incomplete aerobic oxidation because one of the mitochondrial TCA cycle enzymes is absent and therefore the cycle cannot function as a true cycle. However, the TCA-cycle enzymes present can be used for the interconversion of metabolic building blocks for biosynthetic purposes. Fatty acids are oxidized in the mitochondrion by β-oxidation, while cytosolic fatty-acid synthesis occurs by a number of cytosolic elongases, rather than by Type-I fatty-acid synthetase. Ether-lipid synthesis occurs in part inside glycosomes. Although *C. fasciculata* is able to synthesize its own pyrimidines, it depends on the presence of external purines. It lacks the capacity to oxidize aromatic amino acids and requires an external supply of most of the essential amino acids and vitamins for its growth.

Some trypanosomatids (e.g. *T. brucei*) have an RNAi pathway, while others, such as *T. cruzi and L. major*, have not. The Argonaut protein AGO1 and the Dicer proteins DCL1 and DCL2 are responsible for this activity. In the genome of *C. fasciculata* AGO1 and one of the dicer proteins, DCL2, were detected. A homologue of DCL1 was detectable only at low probability (E = 7e-13). Thus it is likely, but not certain, that *Crithidia* has RNAi capacity.

GLYCOSOME TURNOVER

Glycosome biogenesis in Trypanosoma brucei

M. Gualdrón-López, A. Brennand, P. Michels in collaboration with D. Rigden (University of Liverpool, UK) and R. Erdmann and W. Schliebs (Ruhruniversität Bochum, Germany)

So far, 12 candidate peroxins (acronym PEX), proteins involved in glycosome biogenesis in *T. brucei* have been identified; for the majority of them, their role in glycosome biogenesis has been experimentally confirmed and, using RNAi, their essentiality for the viability of both cultured bloodstream- and procyclic-form trypanosomes has been demonstrated. Most of these peroxins are involved in the transport of newly synthesized glycosomal proteins from the cytosol to the organellar matrix. In recent years, our work has been mostly focused on a detailed investigation of PEX5, 7, 13 and 14. PEX5 and PEX7 are unrelated cytosolic receptors for glycosomal proteins with a C-terminal and a N-terminal peroxisome-targeting signal (PTS1 and PTS2), respectively. PTS-bearing proteins associate with these receptors in the cytosol followed by interaction of the loaded receptor with a membrane-associated docking complex minimally comprising PEX13 and PEX14.

Much of our current research addresses the cycling of PEX5 and the role of PEX4 and PEX22 in that process. In yeast and mammalian peroxisomes it was found that receptor PEX5, after delivery of its cargo into the peroxisomal matrix, is retrieved in a mono- or di-ubiquitination dependent process and cycled back to the cytosol. Non-cycled PEX5 is degraded in proteasomes after its poly-ubiquitination. PEX4, belonging to the family of ubiquitin-conjugating E2 enzymes, is the peroxin that in yeast is responsible for the mono-ubiquitination of PEX5. PEX4 is a cytosolic enzyme that in yeasts and plants is associated with the peroxisomal membrane by binding to the integral membrane protein PEX22. In mammalian cells no PEX4 orthologue and no PEX22 homologue are present, but there another cytosolic E2 enzyme (UbcH5) that is responsible for ubiquitination of the receptor.

T. brucei contains genes coding for 15, highly diverse E2-like enzymes. One of them stands out as a candidate PEX4, it is orthologous to the yeast PEX4, although they only share about 30% sequence identity; it has conserved the characteristic region near the C-terminus containing the cysteine residue that is critical for catalytic activity. Similarly, a trypanosomatid orthologue of yeast and plant PEX22 was identified by homology searches in spite of very low sequence conservation. The residues of yeast PEX4 and PEX22 responsible for their interaction have been conserved in the *T. brucei* candidates.

PEX4 is expressed in bloodstream- and procyclic-form cells as observed by RT-PCR and western blot analysis. By confocal immunofluorescence microscopy a N-terminal GFP-tagged PEX4 was shown to be mainly associated with glycosomes of bloodstream-form trypanosomes. Biochemical analysis showed that it was predominantly localized in the membrane fraction of both life-cycle stages and by protease treatment it appeared to be present on the cytosolic face of the organelles. Only a minor growth phenotype could be observed when its expression was partially knocked down by RNAi or when both alleles of the gene were deleted. Possibly one of the other E2-like proteins could compensate for the PEX4 loss. This is currently under study.

Previously, *T. brucei* PEX13 has been characterized; a glycosomal membrane protein with a Tyr-Gly rich N-terminal region of low sequence complexicity, two transmembrane segments, a SH3 domain in its C-terminal half and, uniquely for the trypanosomatid PEX13, terminating with a PTS1-like sequence. Recently, another candidate PEX13 has been identified in the trypanosomatid databases. Although this new candidate lacks the SH3 domain of

PEX13s, it has a higher overall sequence identity with PEX13s of other organisms and possesses the typical PEX13 N-terminal half with Tyr-Gly motifs and transmembrane segments. It does not contain a PTS1. Indeed, when expressed in procyclic trypanosomes as a GFP-fusion protein, the fluorescence pattern colocalizes with that of glycosomal marker proteins. Current research is devoted to determining its role in glycosome biogenesis. It should be noted that different isoforms of PEX13 have not been found so far in any other organism.

In collaboration with colleagues in Germany, very recently also a PEX16 candidate has been identified with very low sequence identity with its orthologues in other organisms, a peroxisomal membrane protein involved in the integration of other proteins into this membrane. GFP-fusion constructs, expressed in procyclic trypanosomes, colocalized with glycosomal marker proteins. Functional studies of this peroxin are in progress.

Glycosome degradation in *Trypanosoma brucei*

A. Brennand, M. Gualdron-Lopez, P. Michels, in collaboration with E. Pays (Université Libre de Bruxelles), D. Rigden (University of Liverpool, UK) and M. Ginger (Lancaster University, UK)

Trypanosomes encounter highly different environments during the successive stages of their life cycle and have to adapt their metabolism accordingly. Previously we have provided strong indications that, when the trypanosomes develop from the bloodstream into the procyclic form, the adaptation involves a drastic degradation of glycosomes by a selective form of autophagy called pexophagy and the synthesis of new glycosomes with a different repertoire of metabolic enzymes. Furthermore, we performed a bioinformatics analysis that allowed us to identify in the trypanosomatid databases orthologues of about 20 of the 40 known yeast proteins known to be involved in autophagy (ATGs = AuTophaGy-related proteins). Also orthologues specifically required for pexophagy were recognized in trypanosomatids. Recently, this bioinformatics analysis was extended to a taxonomically diverse range of other protists. This analysis, together with similar analyses performed by others for plants, animals and fungi confirmed autophagy as an ancient eukaryotic invention, utilizing a conserved core machinery but also with lineage-specific moderation (specific losses of ATGs) and elaboration (expansion of a paralogous repertoire of some ATGs). This was indeed also observed in trypanosomatids. Some protists seem to have undergone a secondary loss of macroautophagy, the best understood of the autophagy pathways. This is possibly due to adaptation to a very constant niche, two of the three examples found being parasites with a very simple life cycle and one free-living organism adapted to an extreme environment. Furthermore, although pexophagy is a conserved process in all organisms having peroxisomes, it seems to involve at least some proteins that are not widely conserved. With regard to the process in parasitic protists, this may offer opportunities for drug design targeting autophagy proteins in these organisms.

Experimental support for degradation of glycosomes in trypanosomes by autophagy, both under conditions of nutrient deprivation and during differentiation –from long-slender bloodstream forms to short stumpy forms and from short stumpy to procyclic forms– has previously been obtained by immuno-electron and -fluorescence microscopy. Furthermore, several of the *T. brucei* homologues of yeast proteins involved in autophagy, notably that of peroxisomes, have been cloned and sequenced: VPS34, ATG7, ATG8, ATG24 and VAC8. Recombinant forms of ATG8, ATG24 and VAC8, as well as a control protein (actin) have been produced in *E. coli* for production of antisera to be used for immuno-blot and immunofluorescence studies of cells. Also for labeling of organelles (lysosome and autophagosomes) involved in autophagy, procyclic-form trypanosome cell lines expressing an ATG24-myc fusion construct has recently been created, as

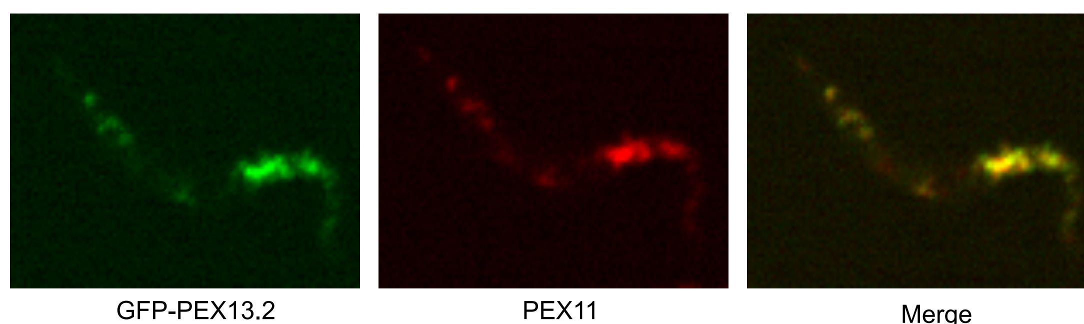

Fig. 2. Subcellular localization of the second isoform of peroxin 13 (PEX13.2) in *T. brucei* by expression of a fusion construct of PEX13.2 with green fluorescent protein (GFP-PEX13.2) in *T. brucei*. The green fluorescence of GFP colocalizes with the red signal of marker protein PEX11, a peroxin present in the glycosomal membrane, as detected by immunofluorescence, giving the yellow merged signal.

well as procyclic cells expressing ATG7-GFP. Using these tools, the localization of the candidate ATG7 and ATG8 has already been analysed. When autophagy was induced in procyclic cells by nutrient deprivation, the putative ATG8 was found in punctuate structures reminiscent of autophagosomes, but only a rather disperse ATG7 signal was recognized. For different pexophagy-related proteins (VAC8, ATG24 and VPS34) bloodstream-form and procyclic RNAi-mutant cell lines have been constructed and are currently being analyzed under standard culturing conditions and under conditions where an increased rate of glycosome turnover is expected. The subcellular localisation of ATG24 and VAC8 is currently being determined as well as the mechanism by which these proteins associate with intracellular membranes.

NATURAL PRODUCTS AS TRYPANOCIDAL DRUGS

V. Hannaert, in collaboration with J. Bero and J. Quetin-Leclercq (Pharmacognosy Research Group, UCL) and I. Ngantchou and B. Nyasse (Université de Yaoundé, Cameroun)

Nature is a potential source of new drugs since it contains a countless quantity of molecules with a great variety of structures and pharmacological activities. The potential of natural products in the treatment of diseases can be seen in traditional medicines. For thousands of years, natural products have played an important role throughout the world in treating and preventing human diseases. Several well established human antiprotist drugs have their origins in nature, such as quinine and artemisinin used to treat malaria. In Africa, plants have been used traditionally for centuries and are still widely employed to treat sleeping sickness. However, no trypanocidal drug molecule from natural origin is currently used against *T. brucei* infections.

In our search for more effective trypanocidal drugs, we investigated plants from different African countries (Benin, Cameroon, Ghana) that are traditionally used as antiparasitics. The selection was based on information obtained from traditional practitioners. Plant extracts were prepared and evaluated in vitro on *T. brucei* bloodstream forms and their selectivity analysed on MRC-5 mammalian fibroblasts. Active extracts were further fractionated to isolate pure compounds. Some of them have been shown to inhibit the growth of trypanosomes in vitro with EC_{50} values in the micromolar range and to have moderate cytotoxicity values towards mammalian cells. Their chemical structures were determined and showed a great diversity. Further investigations have to be made directly on the parasite to study in detail

the action mechanisms of these antitrypanosomal molecules and to determine if they constitute suitable candidates for drug development.

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ENDOCYTOSIS AND EPITHELIAL DIFFERENTIATION

Endocytosis, a central activity of all eukaryotic cells, allows for cell nutrition, regulates the composition of the cell surface and controls transfer of macromolecules across epithelial barriers. This research group has made significant contributions in the dissection of endocytic pathways and in unravelling their contribution to numerous physiopathological processes. Our current investigations focus on endocytosis at the apical membrane of polarized kidney proximal tubular cells (PTC). We surmise that this most active endocytic surface will allow defining rate-limiting components of the different subcellular steps. These should also emerge from the dissection of various genetic defects causing low-molecular weight proteinuria in appropriate mouse models. Recent achievements include: (i), the elucidation of the signalling cascade whereby the paradigmatic oncogene, v-Src, and the master catalyst, Rab5a, impact on the apical actin cytocortex, so as to induce macropinocytosis, like enteropathogens (2); (ii) the elucidation of a deficit of apical endocytosis in ClCn5 KO mice, which mimick Dent's disease, a genetic predisposition to kidney stones (1); (iii) the serendipitous discovery that apical endocytosis of ultrafiltrated lysosomal proteases is a central mechanism for PTC lysosomes biogenesis (3); and (iv) the demonstration that the transcription factor, ZONAB, is a key component for the switch between proliferation and apical differentiation in various epithelia (7). While looking at fluorescent lipid markers to follow "bulk" trafficking and fate of the plasma membrane lipids by live cell imaging, we noticed their propensity to form micrometric domains, distinct from lipid rafts. Multiple approaches indicated a relation with native, endogenous lipid organization. We further provided the first evidence that sphingomyelin forms cytoskeleton-independent but temperature-dependent micrometric domains, which are regulated by cholesterol and membrane tension (8, 10). The group of C. Pierreux studies epithelial tubulogenesis and differentiation, using developing pancreas, salivary and thyroid glands, as complementary models of controlled interconversion between multilayered cell masses and polarized monolayers, with emphasis on paracrine and transcriptional control (6, 9). Two strong assets of our group are a several-decade expertise in structural biology and a versatile cellular and tissular imaging platform that offers conventional and multiphoton microscopy for vital imaging (5), as well as transmission and scanning electron microscopy.

PLASMA MEMBRANE LIPIDS FORM STRUCTURALLY AND KINETICALLY DISTINCT MICROMETRIC DOMAINS, REGULATED BY MEMBRANE TENSION AND AFFECTED IN HAEMATOLOGICAL DISEASES

L. D'Auria, P. Van Der Smissen, P.J. Courtoy and D. Tyteca

Lipids are the most abundant constituents of biological membranes. For several decades, plasma membrane lipids were considered to form a homogenous two-dimensional barrier, acting as solvent for membrane proteins. However, it is now well-accepted that some membrane lipids spontaneously cluster into discrete nanometric "lipid rafts" (liquid-ordered domains), thereby creating small-scale lateral asymmetry. In addition, micrometric domains have been reported using fluorescent lipids incorporated into artificial liposomes, erythrocyte ghosts, and in living cells for non-natural lipids, but the occurrence of micrometric endogenous lipid domains in the plasma membrane of living cells is still questioned.

Our group is addressing whether micrometric lipid domains naturally occur at the plasma membrane, using fluorescent analogs (BODIPY) of sphingomyelin (SM), an abundant outer plasma membrane sphingolipid. Both in erythrocytes and CHO cells, BODIPY-SM insertion labelled (sub)micrometric fluorescent plasma membrane domains. BODIPY-SM domains were randomly distributed in control erythrocytes at 37°C (Fig 1a) and coalesced upon hypotonic shock, excluding structural surface features and endocytosis, and indicating control by surface tension. In CHO cells, direct plasma membrane insertion of BODIPY-SM and intracellular enzymatic conversion of BODIPY-ceramide into BODIPY-SM produced similar surface BODIPY-SM domains. Depletion of endogenous SM by sphingomyelinase cleavage or biosynthetic inhibition impaired (sub)micrometric domains, strongly suggesting that fluorescent domains of exogenous BODIPY-SM reflected endogenous SM compartmentation. Thus, BODIPY-SM spontaneously clusters at the outer leaflet of the plasmalemma of living cells into ordered micrometric domains, defined in size by membrane tension and cholesterol (ref. 8).

Using fluorescent analogs of other outer leaflet plasma membrane lipids, we next show the co-existence of three segregated micrometric phases. Indeed, BODIPY-SM differed from BODIPY-glycosphingolipids and -phosphatidylcholine (PC) domains in temperature dependence, propensity to excimer formation, association with a glycosylphosphatidylinositol (GPI)-anchored fluorescent protein reporter, and lateral diffusion by FRAP, thus demonstrating different lipid phases and boundaries. This was further supported by double labelling experiments and was confirmed by additive occupancy, up to 70% cell surface coverage. Specific alterations of BODIPY-analogs domains by manipulation of corresponding endogenous sphingolipids suggested that distinct fluorescent lipid partition might reflect differential intrinsic propensity of endogenous membrane lipids to form large assemblies (ref. 10).

We have further dissected the mechanism accounting for the formation and maintenance of micrometric lipid plasma membrane domains in living erythrocytes as the most simple model. Experimental or genetic modulation of erythrocyte stretching, cholesterol content (by methyl-beta-cyclodextrin; Fig. 1b) and suppression of membrane-cytoskeleton anchorage via 4.1R complexes (hyperphosphorylation by PKC; Fig. 1c) and band3 complexes (splenectomyzed patient with spherocytosis, a genetic disease of red blood cells characterized by anemia due to erythrocyte fragility; Fig. 1d) differentially affected BODIPY-SM, -glycosphingolipids and -PC micrometric domains. Altogether, these results confirm that different phases coexist at the plasma membrane and point to a key role of membrane tension in micrometric lipid domain maintenance and formation.

Whether the lipid domains play a role in cell physiology and whether endogenous membrane lipids can also form large assemblies are some of our other challenges.

In collaboration with the Cliniques universitaires Saint-Luc, we are now addressing whether and how the organization of fluorescent lipids in micrometric domains is affected in patients with spherocytosis. To find a way to re-establish the erythrocyte membrane of the patients is one of our future goals.

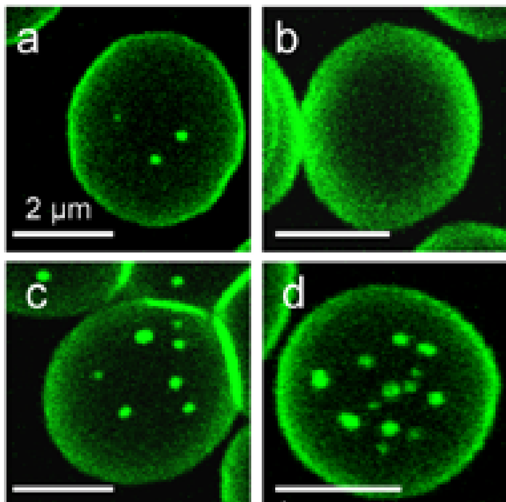

Fig. 1. Modulation of fluorescent sphingomyelin micrometric domains on erythrocytes by pharmacological (b,c) or genetic (d) perturbation of membrane architecture. (a) control erythrocyte at 37°C; (b) + methyl-beta-cyclodextrin (-25% cholesterol); (c) + calyculin A + PMA (hyperphosphorylation of 4.1R complex); (d) untreated erythrocyte from a spherocytotic patient.

DIFFERENTIAL MEMBRANE RECRUITMENT OF SRC MAY SPECIFY ITS DOWNSTREAM SIGNALLING TO MAPK AND PI3K PATHWAYS

Ph. de Diesbach, T. Medts, S. Carpentier, L. D'Auria, P. Van Der Smissen, A. Platek, M. Mettlen, D. Tyteca and P.J. Courtoy

Most Src family members are diacylated and constitutively associate with membrane "lipid rafts" that coordinate signalling. Whether the monoacylated Src also localizes at "rafts" remains controversial. Using polarized MDCK cells expressing the thermosensitive v-Src/tsLA31 variant, we here addressed how Src tyrosine-kinase activation may impact on its (i) membrane recruitment, in particular to "lipid rafts"; (ii) subcellular localization; and (iii) signalling. The kinetics of Src-kinase thermoactivation correlated with its recruitment from the cytosol to sedimentable membranes where Src largely resisted solubilisation by non-ionic detergents at 4 °C, and floated into sucrose density gradients like "lipid rafts". By immunofluorescence, activated Src showed a dual localization, at the apical plasma membrane and at adjacent endocytic vesicles. The plasma membrane Src pool did not colocalize with caveolin-1 and flotillin-2, but extensively overlapped with GM1 labelling by cholera toxin. Severe (~70%) cholesterol extraction with methyl-β-cyclodextrin (MβCD) did not abolish "rafts" floatation, but strongly decreased Src association with floating "rafts" and abolished its localization at the apical plasma membrane.

Src activation independently activated first the MAP-kinase-ERK1/2 pathway, then the PI3-kinase-Akt pathway. MAP-kinase-ERK1/2 activation was insensitive to Mβ CD, which suppressed Akt phosphorylation and apical endocytosis induced by Src, both depending on the PI3-kinase pathway. We therefore suggest that activated Src is recruited at two distinct membrane compartments allowing differential topological signalling, first via ERK1/2 at "non-raft" domains (possibly endosomes), then via PI3-kinase-Akt on a distinct set of "rafts" at the apical plasma membrane. Whether this model is applicable to c-Src remains to be examined (4). Current investigations are addressing which PI3K isoform regulates apical membrane recycling in differentiated PTC and how this works.

CONTRIBUTION OF APICAL ENDOCYTOSIS TO THE BIOGENESIS OF LYSOSOMES AND PATHOGENESIS OF CYSTINOSIS IN KIDNEY PROXIMAL TUBULAR CELLS

P.J. Courtoy, W.R. Lima and H.P. Gaide Chevronnay, in collaboration with C. Antignac (Paris, F), S. Cherqui (Scripps, CA, USA), E.I. Christensen (Aarhus, DK), O. Devuyst (NEFR, UCL)

Recruitment of acid hydrolases to lysosomes generally occurs by intracellular sorting based on recognition of a common mannose 6-phosphate signal in the trans-Golgi network and selective transport to late endosomes/lysosomes. We have obtained direct evidence for an alternative, efficient secretion-recapture pathway mediated by megalin, exemplified by cathepsin B in kidney proximal tubular cells (PTC). We found that in mouse kidneys with defective megalin expression [megalin knockout (KO)] or apical PTC trafficking (ClC-5 KO, see below), the (pro)cathepsin B mRNA level was essentially preserved, but the protein content was greatly decreased and the enzyme was excreted in the urine as mannose 6-phosphate-devoid species.

In polarized PTC monolayers, purified cathepsin B was avidly and selectively taken up at the apical membrane; uptake was abolished by the "receptor-associated protein", a megalin competitor. Direct interaction of cathepsin B with megalin was demonstrated by surface plasmon resonance. Circulating procathepsin B was detected in normal mouse serum. Purified cathepsin B injected into mice was preferentially taken up by kidneys and targeted to lysosomes where it remained active, as shown by autoradiography and subcellular fractionation. A single cathepsin B injection into cathepsin B KO mice could reconstitute full lysosomal enzyme activity in the kidneys. These findings demonstrate a pathway whereby circulating lysosomal enzymes are continuously filtered in glomeruli, reabsorbed by megalin-mediated endocytosis, and transferred into lysosomes to exert their function, providing a major source of enzymes to PTC. These results also extend the significance of megalin in PTC and have several physiopathological and clinical implications (3). Current investigations are addressing the pathophysiology of cystinosis, a multisytemic lysosomal disease due to a defective lysosomal membrane cystine/H+ antiporter. This disease first manifests itself in kidney as a generalized PTC dysfunction, referred to as kidney Fanconi syndrome. Endocytosis of ultrafiltrated plasma proteins rich in disulfide bridges rich must be the main source of lysosomal cystine in PTC. We expect that analysis of Ctns-/- mice (Nevo et al, 2009) will help understanding how cystine accumulation causes apical PTC atrophy and how cystinosis can be corrected by grafting of hematopoetic stem cells (Yeagy et al, 2011).

THE TRANSCRIPTION FACTOR, ZONAB, IS CONTROLLED DURING EPITHELIAL POLARIZATION AND IS A KEY REGULATOR IN THE PROLIFERATION/ DIFFERENTIATION SWITCH

W.R. Lima, S. Dupasquier, C.E. Pierreux and P.J. Courtoy, in collaboration with K. Parreira and O. Devuyst (NEFR)

Epithelial polarization depends, and impacts on, gene expression. The transcription factor, ZONAB, can shuttle between tight junctions and the nucleus to promote expression of cyclin D, and thus participate in the control of proliferation. We have examined whether ZONAB simultaneously represses differentiation, using renal proximal tubular cells (PTC) as a model. During mouse kidney ontogeny and polarization of PTC monolayers in vitro, decreasing ZONAB level inversely correlated with differentiation of the apical endocytic receptors, megalin/cubilin, brush border and primary cilium markers. Conversely, ZONAB was reexpressed in dedifferentiated renal carcinomas.

Sparsely plated PTC formed small islands: peripheral cells, necessarily lacking external tight junctions, strongly expressed nuclear ZONAB, proliferated and failed to differentiate; central cells, able to form continuous junctional belts, lost nuclear ZONAB, stopped proliferating and engaged in apical differentiation (Fig. 2). In confluent PTC monolayers, stable ZONAB transfection repressed expression and function of the endocytic receptors and impaired brush border and primary cilium maturation. Reporter and chromatin immunoprecipitation assays demonstrated that megalin and cubilin are ZONAB target genes. ZONAB expression was regulated by polarity at pre- and posttranscriptional levels (proteasomal degradation). In PTC islands, proteasome inhibition extended nuclear ZONAB to central cells, which reversed their choice from differentiation to proliferation.

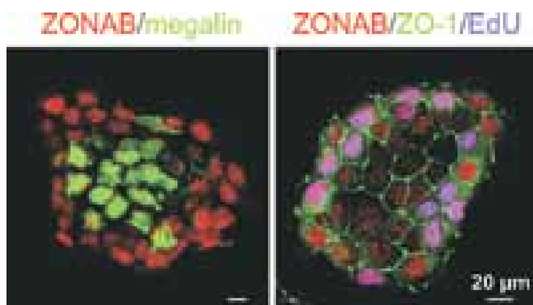

Figure 2. ZONAB is a key regulator in the switch between epithelial proliferation and differentiation. In colonies of kidney proximal tubular cells, ZONAB (immunolabelled in red) is absent in central differentiated cells (expressing megalin, in green, left) but is selectively detected in nuclei of peripheral proliferating cells (simultaneous DNA synthesis, labeled in blue, yields a pink color at right, from ref. 7).

Thus, ZONAB is down-regulated by epithelial polarity at both mRNA and protein levels and acts simultaneously to promote proliferation and repress differentiation. In vitro and in vivo data suggest that ZONAB is a sensor of epithelial density, involved in their switch from proliferation to differentiation (7).

EPITHELIAL DIFFERENTIATION

A.-C. Hick, S. Dupasquier, P.J. Courtoy and C.E. Pierreux, in collaboration with F.P. Lemaigre (LPAD)

Several endoderm-derived organs, such as the pancreas, salivary and thyroid glands, are composed of polarized epithelial monolayers. The pancreas and salivary glands are made of open branched tubules specialized in external secretion (exocrine), while the thyroid glands is organized in closed follicles for internal secretion (endocrine). During embryogenesis, the epithelial cells of these developing organs first form a proliferating mass before reorganizing in specialized monolayers (Fig. 3). Once fully differentiated, epithelial monolayers possess three distinct membrane domains, each able to transmit signals to the nucleus. First, the basal domain ensures adhesion to the extracellular matrix. Next, the lateral domain allows direct interactions with adjacent cells via junctional complexes. Finally, the free apical domain is now facing a lumen and bears a mecano-sensory cilium.

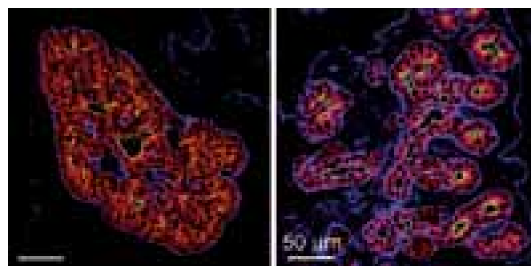

Fig. 3. Branching morphogenesis in exocrine glands. Reorganization in the early pancreatic bud of the multicellular mass of epithelial cells labelled for E-cadherin (left, red) into polarized monolayers with distinct apical domains (mucin, green) and their merging to create tubules (right). Laminin (blue) delineates basement membranes (From Ref 6).

Formation of epithelial monolayers requires a coordinate and dynamic interaction with their environment, composed of mesenchymal and endothelial cells. This process is gland-auto-

nomous, as it is faithfully reproduced in explant cultures. We have shown that embryonic salivary and pancreatic epithelial cells highly express receptors for SDF-1 (Stromal cell-Derived Factor-1), itself abundantly produced by immediately adjacent mesenchymal cells. Pharmacological inhibition of SDF-1:receptor interaction in explants of the pancreas or salivary glands fully abrogated the reorganization of the epithelial masses into monolayers (6). By three-dimensional analysis of the developing pancreas, we also uncovered a dense and close association of the epithelium with the endothelium (Fig. 4). Our in vivo and in vitro data show that endothelial cell recruitment is dependent on VEGF production by the pancreatic epithelium and that endothelial cells, in turn, limit acinar differentiation (9). These data demonstrate that paracrine epithelial:mesenchyme and epithelial:endothelial interactions are crucial for organ differentiation and bring a proof-of-principle that molecular components of these interactions can be readily dissected in explant cultures (6, 9).

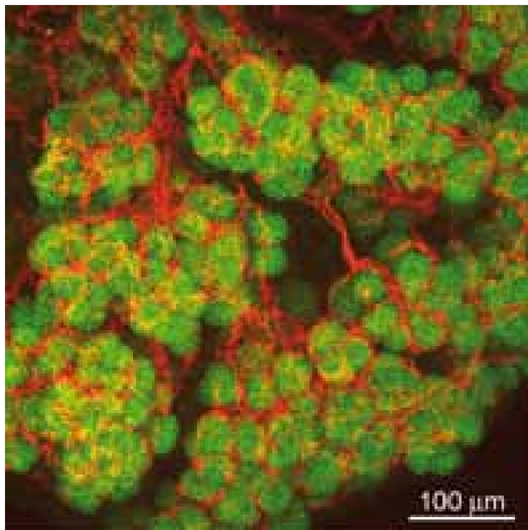

Fig. 4. Epithelial:endothelial interactions in the pancreas. Projections of 40 confocal images of an embryonic pancreas showing the dense and close association of pancreatic epithelial cells, labelled for E-cadherin (green), with endothelial cells, labelled for PECAM (red).

COLLABORATIONS ON MEMBRANE TRAFFICKING AND BRIEF REPORT ON THE CELL AND TISSUE IMAGING PLATFORM

P. Van Der Smissen, D. Tyteca, T. Lac, P.J. Courtoy

Besides sharing the same laboratory and continuing a two-decade fruitfull collaboration with the group of E. Marbaix and P. Henriet (see their report p 74), we have pursued our long-term commitment to promote collaborations by sharing expertise in endocytosis and cellular imaging. For the recent years, see our contribution to the study of the endocytic trafficking of syndecans and its role in signalling by its heparin-sulfate ligands (Zimmermann et al., Dev Cell 2005;9:377-88); of the subcellular trafficking of the thrombopoietin receptor (Royer et al., J Biol Chem. 2005;280:27251-61; see report by S. Constantinescu, p 127) and the amyloid precursor protein, APP (Feyt et al., J Biol Chem 2005;280:33220-7); elucidation of the disputed subcellular localization of aspartate N-acetyltransferase (NAT8L) and its congener, NAT8 (Wiame et al., Biochem J 2010;425:127-36; Veiga da Cunha et al., J Biol Chem J Biol Chem 2010;285:18888-98 see report by E. van Schaftingen, p 32); first evidence for dispersion of the actin cytoskeleton in epithelial cells by AMP-activated kinase (Miranda et al, Biochem Biophys Res Comm 2010;396:656-661; see report by M. Rider, p 51); ultrastructural analysis of differentiating hepatoblasts (Clotman et al., Genes Dev 2005;19:1849-54; see report by F. Lemaigre, p 21) and the biogenesis of glycosomes in Trypanosoma brucei (Galland et al., Biochim Biophys Acta Mol Cell Res 2007;1773:521-35; see report by P. Michels, p 57), or the morphological evidence by FRET of tight interaction between key players of CTL, that is interrupted during their anergy in cancer but can be reversed by galectins (Demotte et al., Immunity 2008;28:414-24; Cancer Res 2010;70:7476-88 see report by P. Van der Bruggen, p 107).

TEN SELECTED PUBLICATIONS OF THE PAST TEN YEARS

1. Christensen EI, Devuyst O, Dom G, Nielsen R, Van Der Smissen P, Verroust P, Leruth M, Guggino WB, Courtoy PJ. *Loss of chloride channel ClC-5 impairs endocytosis by defective trafficking of megalin and cubilin in kidney proximal tubules.* **Proc Natl Acad Sci USA** 2003;100:8472-7.

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Pascale LEMOINE, Technician (part-time)
Yves MARCHAND, Secretary (part-time)

EXTRACELLULAR MATRIX REMODELING

The extracellular matrix (ECM) plays a central role in the structural and functional organization of tissues and organs. ECM constituents, in particular fibrillar collagens, are the most abundant proteins of the human body. Physiological and pathological breakdown of ECM is predominantly achieved by a family of enzymes called matrix metalloproteinases (MMPs; see Fig. 1). Our laboratory was the first to demonstrate that menstrual tissue breakdown is due to a dramatic change in the focal expression and/or activation of MMPs (1). This seminal observation led us to : (i) use this system as a human model to study the regulation of MMPs, in particular cellular interactions that integrate overall hormonal impregnation with local environmental changes; and (ii) explore whether this basic knowledge can lead to a better understanding and a rational treatment of abnormal uterine bleeding, a major health problem (2). We also investigate the control by individual cells of local MMP activity, which can be either increased by recruitment and retention to the plasma membrane (3), or down-regulated by receptor-mediated endocytosis and degradation

MECHANISMS OF MENSTRUAL BREAKDOWN AND REGENERATION: IDENTIFICATION OF NEW CANDIDATE GENES BY TRANSCRIPTOMIC COMPARISON OF MICRODISSECTED TISSUE AREAS

H. Gaide Chevronnay, P.J. Courtoy, E. Marbaix, P. Henriet

The general aim of this study was to further elucidate the mechanisms ensuring the spatio-temporal control of menstrual endometrial remodeling in response to the global regulation by estradiol and progesterone. The experimental strategy relied on two advanced methodologies : (i) to separate, by laser capture microdissection, stromal and glandular cells from degraded or preserved areas of the human endometrium after ultrafast immunolabelling and (ii) to compare their global transcriptome by non-supervised microarray analysis.

First, we compared the transcriptomes of stromal and glandular cells microdissected from (i) the *basalis* as well as from (ii) degraded and (iii) preserved areas of the *functionalis* in

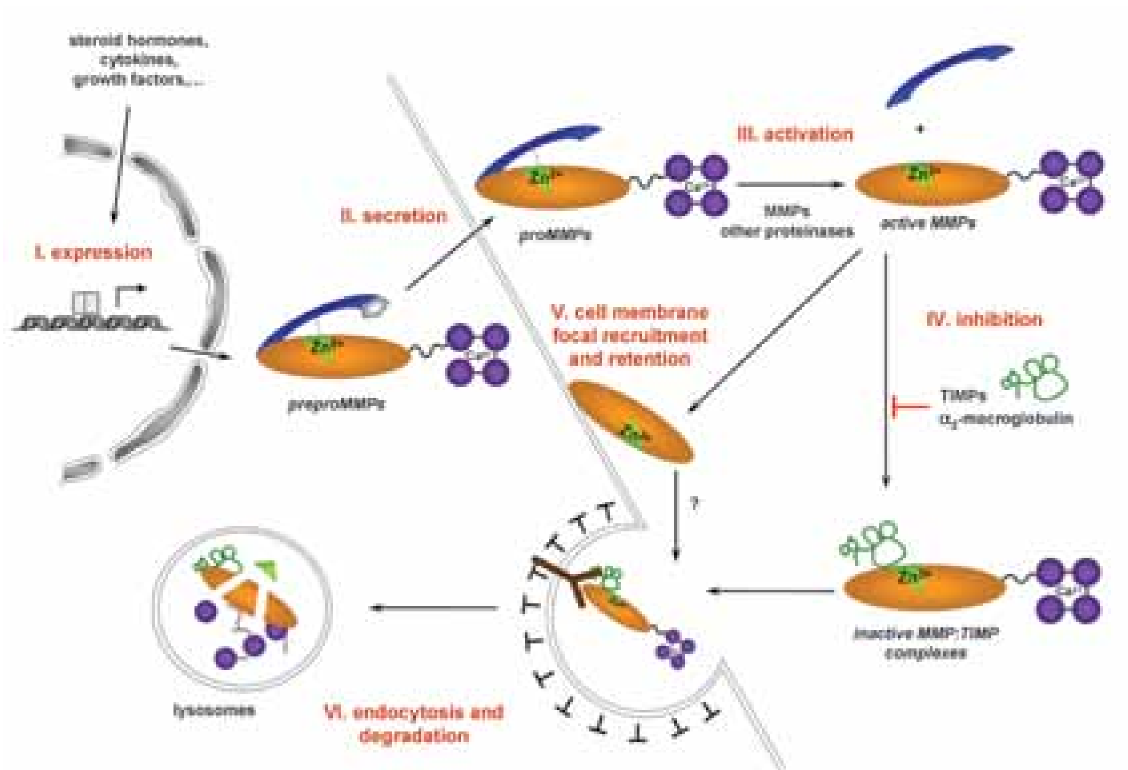

Fig. 1. Regulation of soluble MMP activity in the human endometrium: current model.

MMPs, the major actors of extracellular proteolysis, share a common intramolecular control due to masking by a N-terminal prodomain (blue, here shown with the signal peptide in grey) of the Zn2+-dependent catalytic site (green) within the catalytic domain (orange). All but MMP-7 and -26 (matrilysins, the "mini-MMPs") are linked by a hinge domain (black) to a variable C-terminal hemopexin-like domain stabilized by calcium (mauve), responsible for substrate specificity,). The overall activity of MMPs can be controlled at six different levels: (I) expression; (II) secretion (regulated in a limited number of cell types such as neutrophils); (III) zymogen activation upon prodomain excision; (IV) inhibition of active forms by physiological inhibitors such as TIMPs (represented with their tertiary structure) and α2-macroglobulin; (V) secondary membrane recruitment increasing pericellular activity; and (VI) down-regulation by endocytosis. *In the cycling human endometrium*, MMPs activity is tightly regulated to remodel the extracellular matrix both for blastocyst implantation and, in its absence, for menstrual breakdown of an irreversibly specialized tissue. At menses, the rise of active MMP-1 in the *functionalis* can exceed one-million-fold as compared with mid-phase tissue (1). Several levels of regulation can be evidenced : (I) ovarian steroids and their intracellular receptors as well as cytokines, growth factors and downstream signaling pathways interact to form an integrated system that differentially controls the focal expression of endometrial MMPs and TIMPs. (II) Neutrophils are numerous at menstruation and could contribute to an abundant secretion of MMPs. (III) MMPs can be activated by other MMPs, by plasmin, itself activated during menstruation, or by as yet unidentified proteinases. (IV) TIMPs are particularly abundant in the human endometrium; like MMPs, the level of TIMPs is regulated by ovarian steroids and cytokines. (V) MMP-7 binds to membrane receptors in cholesterol-rich domains, a mechanism which enhances pericellular MMP activity. (VI) Endometrial LRP-1 (brown) binds and internalizes MMP-2 and MMP-2: TIMP-2 complexes, leading to lysosomal degradation. Our research has unraveled (and is focused on) levels (I), (III), (IV), (V) and (VI).

menstrual endometria (8). Algorithms for sample clustering (PCA) segregated biological samples according to cell type and tissue depth, indicating distinct gene expression profiles (Fig. 2). Strikingly, in addition to genes products associated with tissue degradation (MMP and plasmin systems) and apoptosis, lysed areas in the superficial stroma were enriched in gene products associated with ECM biosynthesis (collagens and their processing enzymes). The presence of new synthesized collagens and increased integrin production was confirmed at the protein level. Overexpression of ECM components and adhesion molecules by lysed menstrual fragments could participate in post-menses endometrial reconstruction but also facilitate implantation of endometriotic lesions

In the second part of the study, stromal and glandular areas were microdissected from explants cultured without or with estradiol and progesterone (9). The microarray datasets were also compared to other published endometrial transcriptomes. Moreover, the contribution of proteolysis, hypoxia and mitogen-activated protein kinases (MAPKs) to the regulation of selected genes was further investigated in explant culture. Like in the menstrual endometrium, this analysis identified distinct gene expression profiles in stroma and glands but functional clustering underlined convergence in biological processes, further supporting cooperative interactions between cell types. Only partial overlaps were observed between lists of genes involved in different occurrences of endometrial remodeling, pointing to a limited number of potentially crucial regulators but also to the requirement for additional mechanisms controlling tissue remodeling. This feature was illustrated by a group of genes differentially regulated by ovarian steroids in stroma and glands and sensitive to MAPKs.

In conclusion, we have generated a reliable

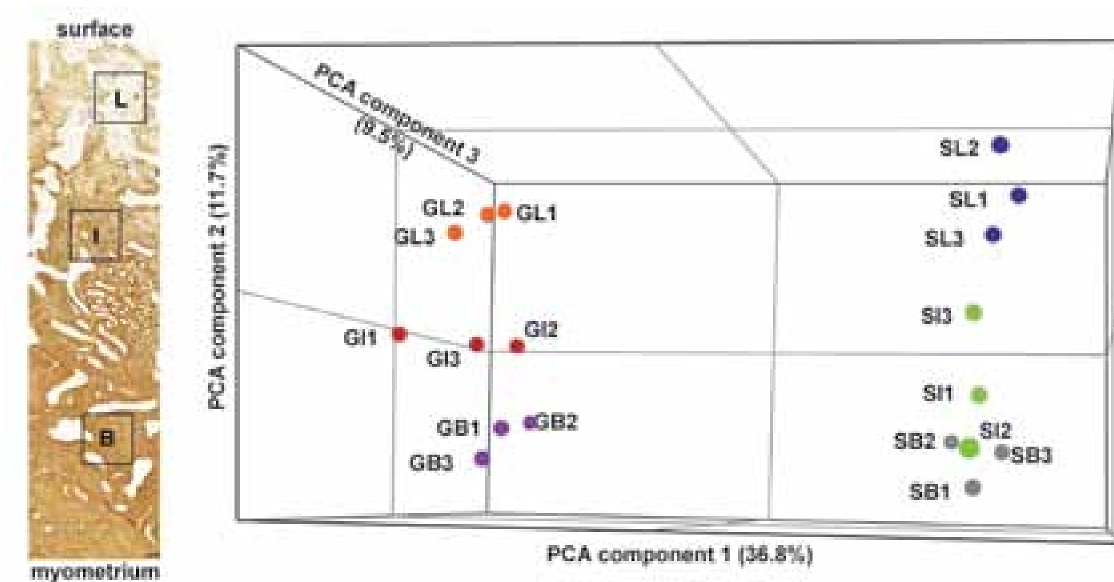

Fig. 2. Comparison of transcriptomes from microdissected areas identifies remarkable cell type- and layer-specific signatures. Tissue samples containing either stroma or glands were microdissected from the three layers of 3 menstrual endometria : lysed superficial *functionalis* (L), preserved intermediate *functionalis* (I) and *basalis* (B). The transcriptomes of the 18 samples were determined using whole genome microarrays. Principal component analysis of the datasets (PCA, at right) clearly indicates (i) the remarkable reproducibility of the biological triplicates (numbers and symbols colors); (ii) a major segregation between stromal (S) and glandular (G) gene expression profiles along axis 1; and (iii) a noticeable segregation between layers along axis 2. For details, see (8).

and useful database of genes differentially regulated in the human endometrium in the context of tissue remodeling. Their comparison suggests that fragments of the functionalis participate in endometrial regeneration during late menstruation, arguing against the classical straightforward model of regeneration from the basalis only. This study also indicates that MAPKs act in concert with hormone withdrawal to locally and specifically control expression of menstrual genes in the superficial layer of the human endometrium.

CELL CHOLESTEROL MODULATES LRP-1 ECTODOMAIN SHEDDING AS A MECHANISM TO REGULATE MMP-2 AND -9 ENDOCYTIC CLEARANCE

C. Selvais, P.J. Courtoy, P. Henriet, E. Marbaix, H. Emonard (in collaboration with S. Dedieu at CNRS, Reims, France)

We have previously shown that the efficient LRP-1-mediated clearance of MMP-2 and -9 activity in non-bleeding endometrium was abrogated upon hormone withdrawal, due to shedding of LRP-1 ectodomain by a metalloproteinase, presumably ADAM-12, itself regulated by estradiol and progesterone (7). Using human fibrosarcoma HT1080 cells, we recently identified two membrane-associated metalloproteinases, ADAM-12 and MT1-MMP that shed LRP-1 ectodomain (10). We compared the shedding potential of classical fibroblastoid HT1080 cells with a spontaneous epithelioid variant, enriched ~2-fold in cholesterol. Although both fibroblastoid and epithelioid HT1080 cells expressed similar levels of LRP-1, ADAM-12, MT1-MMP and of their specific inhibitor TIMP-2, LRP-1 ectodomain shedding from epithelioid cells was ~4-fold lower than from fibroblastoid cells. Release of the ectodomain was triggered by cholesterol depletion in epithelioid cells and impaired by cholesterol overload in fibroblastoid cells. Modulation of LRP-1 shedding on clearance was reflected by accumulation of gelatinases (MMP-2 and -9) in the medium. We conclude that cholesterol exerts an important control on LRP-1 level and function at the plasma membrane by modulating shedding of its ectodomain, and therefore represents a novel regulator of extracellular proteolytic activities (Fig. 3).

ENDOMETRIAL XENOGRAFTS

C. Galant, H. Gaide Chevronnay, P.J. Courtoy, P. Henriet, E. Marbaix (in collaboration with J.M. Foidart, M. Nisolle and A. Béliard at the University of Liège, Belgium)

MMPs are thought to induce menstruation as well as dysfunctional endometrial bleeding, a benign pathology characterized by spontaneous and irregular bleeding associated with menstrual-like stromal breakdown (2). Because menstruation only occurs in few species, in vivo exploration of the physiopathological regulation and role of MMPs is limited. In collaboration with the laboratory of Dr. J.M. Foidart (ULg), we have developed a new experimental model of endometrial xenografts in immunodeficient mice (7). The model allowed us to investigate the alterations of endometrial ECM remodelling upon levonorgestrel treatment and will be used to directly address the role of MMPs in physiological and abnormal endometrial bleeding, endometrial angiogenesis and vessel maturation, as well as in tissue regeneration after menstrual shedding.

Menstrual-like tissue degradation was shown to occur after progesterone withdrawal in a deciduoma induced in the mouse uterus, but involvement of MMPs in this model was not clear. We therefore investigated by immunohistochemistry and quantitative RT-PCR the expression of MMPs in human endometrium xenografted subcutaneously for 3 weeks to immunodeficient mice treated with estradiol- and progesterone-releasing pellets, and compared

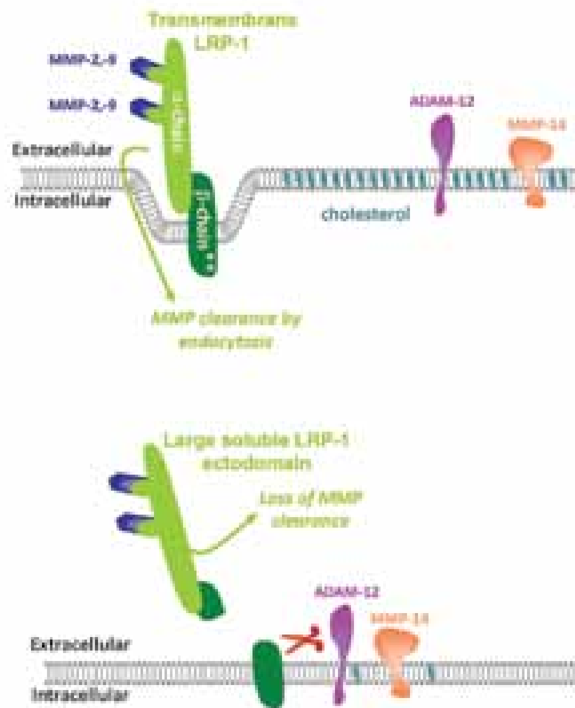

Fig. 3. A model for regulation of gelatinase activity by LRP-1.

Upper panel: Binding of gelatinases (MMP-2 and -9) to LRP-1 triggers avid receptor-mediated endocytosis thanks to its two NPxY motifs (indicated by *). Sheddase activity of ADAM-12 and MT1-MMP is prevented by cholesterol-induced membrane rigidity. Lower panel: Shedding of LRP-1 ectodomain is enhanced by membrane fluidity due to cholesterol depletion. For details, see (7 and 10).

them to the mouse menstruation model and the uterus of the recipient mice.

The decidualized xenografted endometrium showed focal tissue breakdown and bleeding 3 to 4 days after hormonal withdrawal. Human MMP-1, -3, -8 and -9 expression and MMP-2 immunostaining were strongly increased and TIMP-3 expression decreased. MMP-7 immunostaining was increased but not consistently its mRNA level. In the mouse menstruation model, most murine Mmps had high mRNA level in both the deciduoma and the control horn, essentially not affected by hormones withdrawal, whereas increased expression of Mmp-2, -3 and -10 and decreased expression of Mmp-7 were observed in the uterus of the grafted mice.

In conclusion, hormonal withdrawal induces a menstrual-like pattern of expression of most MMPs and TIMPs in human endometrial xenografts but not in the mouse uterus. The xenograft model seems thus appropriate to study the induction of menstruation, in particular changes in the vasculature and infiltration by leukocytes, as well as of its related pathologies.

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4. Gaide Chevronnay HP, Cornet PB, Delvaux D, Lemoine P, Courtoy PJ, Henriet P, Marbaix E. *Opposite regulation of transforming growth factors-beta2 and -beta3 expression in the human endometrium.* **Endocrinology** 2008; 149:1015-25.

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10. Selvais C, D’Auria L, Tyteca D, Perrot G, Lemoine P, Troeberg L, Dedieu S, Noël A, Nagase H, Henriet P, Courtoy PJ, Marbaix E, Emonard H. *Cell cholesterol modulates metalloproteinase-dependent shedding of LRP-1 (low-density lipoprotein receptor-related protein-1) and clearance function.* **FASEB J** 2011; In press.

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GROWTH FACTOR RECEPTORS

From signal transduction to human diseases

Growth factors such as platelet-derived growth factors (PDGF) are secreted proteins that stimulate cell proliferation via transmembrane receptors. PDGF binds to a receptor-tyrosine kinase which signals by phosphorylating various intracellular proteins on tyrosine residues, leading to the regulation of multiple transcription factors and profound changes in genes expression. Understanding how this network of signaling cascades and transcriptional regulations controls cell growth is the first goal of our team.
The uncontrolled activation of PDGF receptors has been linked to several diseases, such as cancer, leukemia and fibrosis. Our second objective is to clarify the role of PDGF receptors in these diseases and to identify the patients who could benefit from a therapy based on the potent, well-tolerated inhibitors of PDGF receptors.

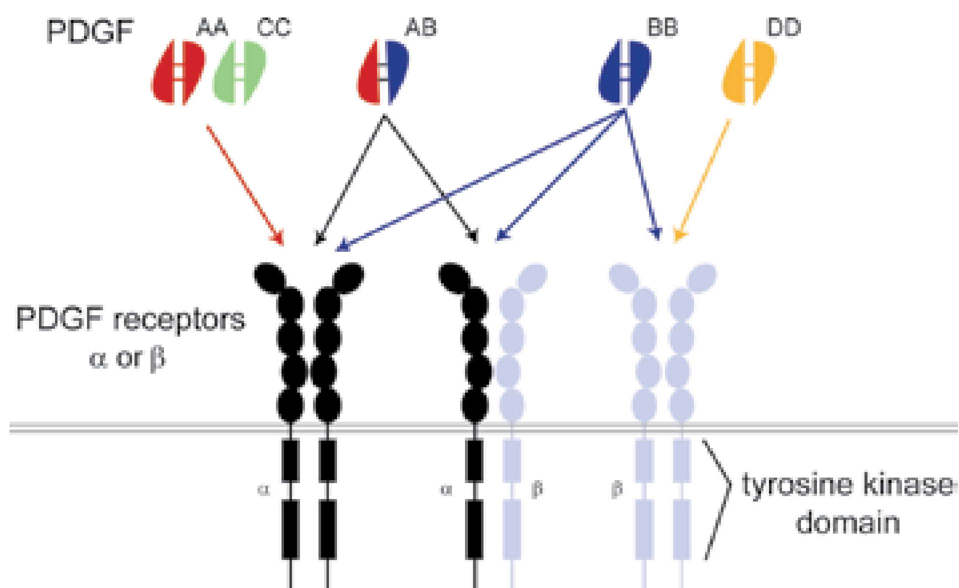

Fig. 1. PDGF receptors and ligands

SIGNAL TRANSDUCTION AND GENE REGULATION BY GROWTH FACTORS: ROLE OF THE TRANSCRIPTION FACTORS FOXO, STAT AND SREBP

A. Coomans de Brachène, A. Essaghir, J.B. Demoulin.

Most of the cellular effects of growth factors are mediated by reprogramming gene expression within the cell nucleus. Each signal transduction cascade controls a number a transcription factors, which activate or repress the expression of many genes. We analyzed the transcriptional program elicited by stimulation of normal human fibroblasts with growth factors (PDGF or FGF) using microarrays. In several successive analyses, we identified hundreds of regulated transcripts that had not previously been linked to PDGF signaling (1-5). We also analyzed gene expression in neural stem cells, glioma, carcinoid tumors and leukemic cells.

One key transcription factor group that is regulated by growth factors is FOXO. These factors induce a cell cycle arrest, increase resistance toward oxidative stress and regulate metabolism. They are inactivated by growth factors via AKT, which phosphorylates three conserved sites within FOXO proteins. Phosphorylated FOXO is excluded from the nucleus and targeted for degradation by proteasomes (Fig. 2). We observed that FOXO mRNA expression is also decreased upon stimulation with growth factors (2). We showed that the promoter of FOXO genes is stimulated by FOXO themselves, a process that is disrupted by growth factors, most likely via AKT, and regulates cell growth. We are now analyzing whether this mechanism could play a role in the proliferation of tumor cells. We also identified several mediators of the effects of FOXO on the cell cycle.

In our microarray analysis, a cluster of genes involved in fatty acid and cholesterol

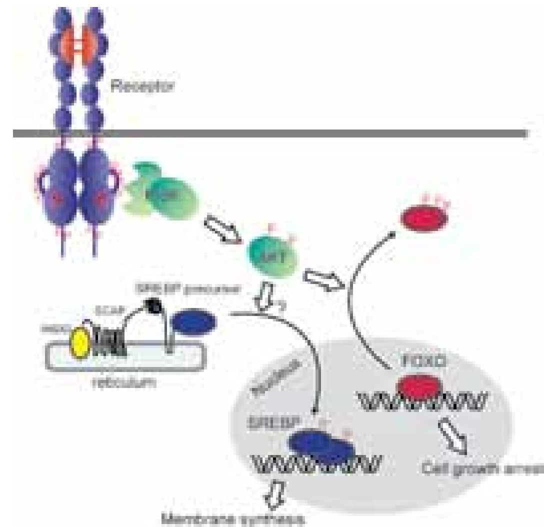

Figure 2. Activation of SREBP and inactivation of FOXO by PDGF

biosynthesis, including stearoyl-CoA desaturase (SCD), fatty acid synthase and hydroxymethylglutaryl-CoA synthase (HMGCS), was up-regulated by PDGF after 24 h of treatment. Their expression correlated with an increase in membrane lipid biosynthesis. All these genes are known to be controlled by sterol regulatory element-binding proteins (SREBP). PDGF increased the amount of mature SREBP-1, and regulated the promoters of SCD and HMGCS in a SREBP-dependent manner. In line with these results, blocking SREBP processing by addition of 25-hydroxycholesterol blunted the effects of PDGF on lipogenic enzymes and PDGF-driven proliferation. SREBP activation was dependent on the phosphatidylinositol 3-kinase (PI3K) pathway, as judged from the effects of the inhibitor LY294002 and mutation of the PDGF receptor β tyrosine residues that bind the regulatory PI3K subunit p85. In conclusion, our results suggest that PDGF induces membrane lipid synthesis via PI3K and the activation of SREBP (Fig 2. and reference 4). The role of SREBP in PDGF and tumor development will be further analyzed. We went on by identifying novel target genes for the SREBP transcription factors. We found that these transcription factors regulate p55γ, a subunit of the PI3K complex and heme oxygenase, which plays an important role in stress

responses (3). These results expand the list of genes regulated by SREBP to targets that are not directly involved in lipid metabolism. We are now trying to understand more precisely the role of the SREBP target genes in growth factor responses.

TFactS: A BIOINFORMATICS TOOL TO PREDICT TRANSCRIPTION FACTOR REGULATION FROM MICROARRAY DATA

A. Essaghir, J.B. Demoulin, in collaboration with J. van Helden (Université Libre de Bruxelles).

Deciphering transcription factor networks from microarray data remains difficult. We have developed a simple method to infer the regulation of transcription factors from microarray data based on well-characterized target genes (1). We generated a catalogue containing 352 transcription factors associated with 2,721 target genes and 6,422 regulations. When it was available, a distinction between transcriptional activation and inhibition was included for each regulation. Next, we built a tool (www.TFactS.org) that compares new submitted gene lists with target genes in the catalogue to detect regulated transcription factors. We validated TFactS with our own microarray experiments and with published lists of regulated genes in various models and compared it to tools based on in silico promoter analysis. We also analyzed the NCI60 cancer microarray dataset and showed the regulation of SOX10, MITF and JUN in melanomas. Our results show that the expression level of transcription factor target genes constitute a robust signature for transcription factor regulation, and can be efficiently used for microarray data mining. We are now introducing new features into this tool and we are using it to analyse cancer genome data.

REARRANGEMENTS OF RECEPTOR TYROSINE KINASE GENES ASSOCIATED WITH LEUKEMIA

C. Montano, L. Noël, A. Velghe, J.B. Demoulin, in collaboration with F. Duhoux, V. Havelange and H. Antoine-Poirel (Cliniques Universitaires Saint-Luc, UCL).

Although PDGF receptors are expressed on platelets and macrophages, PDGF receptor-deficient mice show no primary hematopoietic or immune defect. In vitro, PDGF is a poor mitogen for hematopoietic cells. However, alterations of PDGF receptor genes, as a result of chromosomal translocation or deletion, are found in chronic myeloid neoplasms associated with hypereosinophilia, a disease which affects mostly male patients. In most cases, the rearranged gene produces a hybrid protein comprising the PDGF receptor tyrosine kinase domain and an oligomerisation domain. They also retain the receptor transmembrane domain, which plays a particular role in the activation of these oncoproteins (6, 8). Similar hybrid oncogenes derive from FGF receptors.

TEL-PDGFRβ (TPβ, also called ETV6-PDGFRB) is a hybrid protein produced by the t(5;12) translocation. FIP1L1-PDGFRα (FPα) results from a deletion on chromosome 4q12 (6). These oncogenes were studied in Ba/F3 cells, a mouse hematopoietic cell line that is easy to culture and transfect. In contrast to wild-type PDGF receptors, which are quickly degraded upon activation, we observed that TPβ and FPα escaped down-regulation resulting in the accumulation of these oncoproteins in cells. This was confirmed in leukocytes from patients. Similar data were obtained in cells expressing ZNF198-FGFR1, another fusion protein associated with the 8p11 myeloproliferative syndrome. Ubiquitination of TPβ and FPα was much reduced compared to wild-type receptors. We showed that the accumulation of TPβ is required to activate STAT5 efficiently and transform Ba/F3 cells. Thus, chimeric receptor tyrosine kinases escape efficient ubi-

quitination and degradation through lysosomes and proteasomes (9). This is a new mechanism that contributes to cell transformation by fusion kinase.

TPβ and FPα do not induce eosinophilia in mice. In order to develop a model that is more relevant for the human disease, we introduced TPβ and FPα in human CD34+ cells, which were purified from cord blood and are enriched in hematopoietic stem cells. These cells are able to differentiate normally in vitro into various blood cell types, depending on the cytokine cocktail that is added in the culture medium. We observed that TPβ and FPα induce the proliferation of these cells and their differentiation into eosinophils in the absence of cytokine. We are now analyzing this process in detail.

It is particularly important to identify PDGF receptor alterations in cancer patients, as they can benefit from tyrosine kinase inhibitor therapy. Imatinib mesylate, for instance, is very efficient in patients with leukemia that present a PDGF receptor fusion. In collaboration with the hematology unit of the Saint-Luc university hospital, we identified a novel fusion of the PDGF receptor β with the KANK1 gene in a leukemia patient harboring a t(5;9) translocation (Fig. 3 and reference 7). We are now looking for other mutations in tyrosine kinase genes.

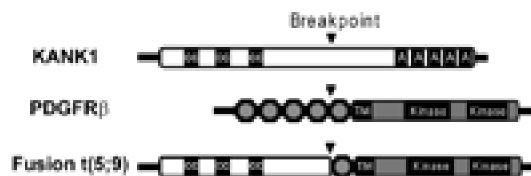

Fig. 3. Structure of the KANK1-PDGFRβ fusion protein created by the t(5;9) translocation.

CC: coiled-coil domain; A: ankyrin repeat; TM: transmembrane domain

ACTIVATION OF PDGF RECEPTORS IN SYSTEMIC SCLEROSIS

S. Charni, J.B. Demoulin, in collaboration with B. Lauwerys and F. Houssiau (Cliniques Universitaires Saint-Luc, UCL).

Systemic sclerosis (also called scleroderma) is a severe connective tissue disease of unknown etiology characterized by vascular alterations, autoimmunity and fibrosis of the skin and multiple internal organs, which is potentially fatal. A recent report suggested that the disease is driven by stimulatory autoantibodies to the platelet-derived growth factor receptors (PDGFR), which stimulate the production of reactive oxygen species (ROS) and collagen by fibroblasts (Baroni et al, New Engl. J. Med 2006; 254:2667). These results opened novel research avenues for the diagnosis and treatment of systemic sclerosis. Several clinical trials using imatinib mesylate, a drug that inhibits PDGF receptors, were promptly initiated following this publication. In order to confirm this important observation, we purified immunoglobulins from 37 patients with systemic sclerosis by protein A/G chromatography. PDGFR activation was tested using four different sensitive bioassays, namely cell proliferation, ROS production, signal transduction and receptor phosphorylation. Purified IgG from patients with scleroderma comprised a panel of antinuclear autoantibodies, but did not specifically activate the PDGFR receptors in any of our tests, compared to controls. As positive control, cell stimulation with PDGF itself consistently produced a strong signal. Our results question the existence of agonistic autoantibodies to PDGFR in scleroderma (10). Four independent research centers have reported similar negative results. We are now trying to identify other factors that activate PDGF receptors in systemic sclerosis and other related fibrotic conditions, such as extensive chronic graft-versus-host disease.

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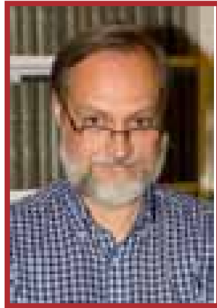

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VIRAL IMMUNITY AND PATHOGENESIS

The possibility for evoluted organisms to survive viral infections depends on the ability of their immune system to eliminate the infectious agent. Therefore, numerous mechanisms, involving different types of immune cells such as cytolytic lymphocytes, T helper and B lymphocytes and macrophages, the molecules that allow those cells to communicate, namely the lymphokines, and the products of those interactions, including antibodies, have been elaborated. On the other hand, viruses have developed strategies to escape the immune system of their hosts, such as large frequencies of mutations or latency, or even to impair this system, which often leads to diseases such as autoimmunity or immunodeficiencies. Our project is to analyse, in murine models, some aspects of these relations between viruses and the immune system.

VIRAL INFECTIONS RESULT IN A DRAMATIC INCREASE IN THE PROPORTION OF IgG2A

Of particular interest is the fact that all antibody responses are not equal. Indeed, depending on their isotype, immunoglobulins display various properties. During the last years, we found that the isotype of antibody responses was influenced by concomitant viral infections. The effect of the virus resulted in a dramatic increase in the proportion of IgG2a, not only in antiviral antibodies, but also in immunoglobulins with an antigenic target unrelated to viral proteins. A dual regulation of antibody responses by gamma-interferon (IFN-γ) and interleukin-6 explains this isotypic bias. Using a model of infection with lactate dehydrogenase-elevating virus (LDV) (1), we could demonstrate that IgG2a antiviral antibodies are more efficient than other isotypes to protect mice against a fatal polioencephalomyelitis induced by the virus (2). However, the modification of the isotype of antibodies reacting with self antigens could potentially lead to more deleterious autoimmune reactions. This property of viruses to enhance selectively the production of one immunoglobulin isotype could depend on the preferential activation of a subset of T helper lymphocytes. Indeed, different subpopulations of those cells, called Th1 and Th2, respectively, are distinguished in particular by their capability of producing selectively IFN-γ or interleukin-4, which can selectively trigger B lymphocytes to produce IgG2a or IgG1, respectively.

ACTIVATION OF NATURAL KILLER CELLS

Many of the influences that viruses may have on diverse immune responses can be explained by the production of pro-inflammatory cytokines, including IFN-γ. Therefore, our analysis of the relationship between viruses and the immune system has focused on the activation, by LDV, of cells from the innate immune system that are able to secrete this cytokine, namely the natural killer (NK) cells. Within a few days after infection, a strong and transient NK cell activation, characterized

by accumulation of this cell population in the spleen, by enhanced IFN-γ message expression and production, as well as by cytolysis of target cell lines was observed. Two pathways of IFN-γ production have been observed that both involve NK cells. The first pathway, found in normal mice, is independent from type I IFN and from interleukin-12. The second pathway involves interleukin-12, but is suppressed by type I IFN (3). Because NK cells and IFN-γ may participate in the defense against viral infection, we analyzed their possible role in the control of LDV titers, with a new agglutination assay. Our results indicate that neither the cytolytic activity of NK cells nor the IFN-γ secretion affect the early and rapid viral replication that follows LDV inoculation.

Similarly, infection with mouse hepatitis virus (MHV) is followed by NK cell activation and leads to IFN-γ production by those activated cells. In contrast to LDV, MHV replication is controlled by this cytokine and animals unresponsive to this molecule quickly die after infection. The protective effect of IFN-γ appears to target infected cells rather than lymphocytes.

Interestingly, NK cell activation results in an increased expression of CD66a (CEACAM-1), an adhesion molecule that serves also as a receptor for MHV. However, this enhanced expression, that is also found on immature NK cells, results from NK cell stimulation with IL-12 and IL-18, but not with LDV (4). Therefore, some, but not all subpopulations of activated NK cells might be susceptible to MHV infection.

ACTIVATION OF MACROPHAGES AND ENHANCED SUSCEPTIBILITY TO ENDOTOXIN SHOCK

Activation of cells of the innate immune system by LDV includes also macrophages and leads to an enhanced response to lipopolysaccharide (LPS), and to an exacerbate susceptibility to endotoxin shock (5). A synergistic effect of LDV and LPS triggered dramatic production of tumor necrosis factor (TNF) and IFN-γ. Susceptibility to LPS shock was completely mediated by TNF, and partially by IFN-γ. Therefore, our model of infection with LDV illustrates how a virus can modulate an ongoing disease initially independent from the infection through modification of the immune microenvironment of its host. In this context, the production of type I IFNs may protect the host against exacerbated pathology by controling the production of IFN-γ.

BLOOD AUTOIMMUNE DISEASES

Virally-induced macrophage activation leads also to an enhanced phagocytic activity, with potential detrimental consequences for ongoing autoimmune diseases. Our analysis has been focused on autoantibody-mediated blood autoimmune diseases. A new experimental model of anti- platelet response was developed in the mouse (6). Immunization of CBA/Ht mice with rat platelets was followed by a transient thrombocytopenia and production of autoantibodies that react with epitope(s)

shared by rat and mouse platelets. Two IgM anti-platelet monoclonal autoantibodies were further analyzed. They recognized mouse platelet antigens and could induce both platelet destruction and impairment of their function (7). This response was found to depend on CD4+ T helper lymphocytes reacting with rat, but not with mouse platelets. These anti-rat platelet T helper cells were mainly of the Th1 phenotype. When transferred into naive mice, they enhanced the anti-mouse platelet antibody response induced by subsequent immunization with rat platelets. In addition, depletion of CD25+ cells enhanced the thrombocytopenia induced by immunization with rat platelets whereas adoptive transfer of CD4+CD25+ cells from immunized mice suppressed it (8). Our results suggest therefore that activation of anti-rat platelet T helper cells can bypass the mechanism of tolerance and result in the secretion of autoreactive antibodies, but this response is still controlled by regulatory T cells that progressively develop after immunization.

We have analysed whether a viral infection could modulate such an autoantibody-mediated autoimmune disease. In mice treated with anti-platelet antibodies at a dose insufficient to induce clinical disease by themselves, infection with LDV or mouse hepatitis virus was followed by severe thrombocytopenia (9). Similarly, administration of anti-erythrocyte monoclonal autoantibody to mice resulted in the development of a transient hemolytic anemia that was dramatically enhanced by a simultaneous infection with LDV, leading to the death of most animals. This viral infection induced an increase in the ability of macrophages to phagocytose in vitro autoantibody-coated red cells, and an enhancement of erythrophagocytosis in the liver (10).

Treatment of thrombopenic or anemic mice with clodronate-containing liposomes and with total IgG indicated that opsonized platelets and erythrocytes were cleared by macrophages. Administration of clodronate-containing liposomes decreased also the in vitro phagocytosis of autoantibody-coated red cells by macrophages from LDV-infected animals. The increase of thrombocytopenia triggered by LDV after administration of anti-platelet antibodies was largely suppressed in animals deficient for IFN-γ receptor. Together, these results suggest that viruses may exacerbate autoantibody-mediated thrombocytopenia and anemia by activating macrophages through IFN-γ production, a mechanism that may account for the pathogenic similarities of multiple infectious agents. Regulation of macrophage activation results in modulation of autoantibody-mediated cell destruction and may be considered as a possible treatment for autoimmune diseases that involve phagocytosis as a pathogenic mechanism.

Together, these two models may correspond to the development of some auto-immune diseases : a first stimulus triggers the production of autoantibodies, through molecular mimicry. A second stimulus, such as a viral infection, leads to the activation of macrophages and results in the destruction of opsonized target cells.

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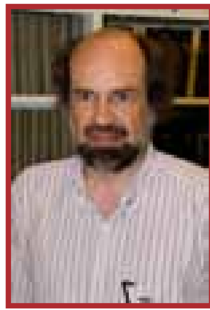

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VIRAL PERSISTENCE AND INTERFERON RESPONSE

Our work focuses on the interplay between viral infections and the immune response of the host. On one hand, we analyze the model infection of the central nervous system by Theiler's virus. This virus can escape the immune defenses of the host and provoke a persistent infection of the central nervous system. Theiler's virus infection and the resulting inflammatory response can lead to a chronic demyelinating disease considered as a model of multiple sclerosis. On the other hand, we analyze the innate immune response against viral pathogens. We focus on the analysis of the type I (IFN-α/β) and type III (IFN-λ) interferon responses which are critically important to control viral infections and to modulate the acquired immune responses.

THEILER'S VIRUS

Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) is a murine picornavirus showing a striking ability to persist in the central nervous system of the host in spite of a specific cellular and humoral immune response. Persistence of the virus is associated with a strong inflammatory response and with lesions of primary demyelination reminiscent of those found in human multiple sclerosis. Our work aims at understanding how a virus can persist in the central nervous system (CNS)

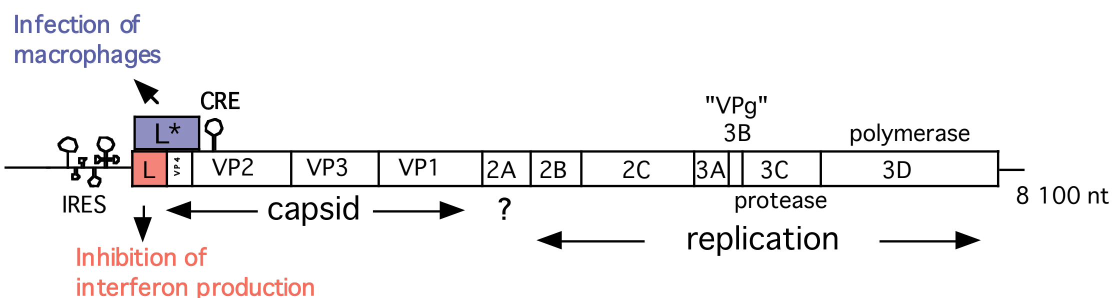

Fig. 1. Genome of Theiler's virus. A large open reading frame encodes a 2000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, to yield the viral mature proteins. Our analysis focuses on 2 proteins, L and L*, that interfere with the host innate immune responses and therefore facilitate the establishment of a persistent infection.

of an immuno-competent host, thus evading the immune response (4, 8).

Recently, a novel human virus closely related to Theiler's virus was discovered and named Saffold virus. This virus is highly prevalent in the human population. Further work is needed to evaluate its virulence potential and its impact on global health.

Analysis of viral proteins involved in Theiler's virus escape of the host immune response.

F. Sorgeloos, F. Borghese, A. De Cock, T. Michiels

Two viral proteins, namely L and L* were found to be dispensable for viral replication in cell culture but to be crucial for persistence of the virus in the central nervous system. Hence, these proteins are believed to interact with host factors in vivo and to counteract the host immune defenses.

INHIBITION OF TYPE-I IFN PRODUCTION AND ALTERATION OF NUCLEO-CYTOPLASMIC TRAFFICKING BY THE L PROTEIN

The leader (L) protein encoded by Theiler's virus is a 76 amino acid-long peptide containing a zinc-binding motif. We observed that this protein exerts pleiotropic activities in infected cells. The first activity that was detected for the L protein is its ability to inhibit the transcription of type I IFN and of chemokine genes (4, 7). This activity likely results from the fact that the L protein inhibits the dimerization of IRF-3, the main transcriptional activator of these genes.

Infection of mice deficient for the type-I interferon receptor (IFNAR-/-) indicates that IFN production inhibition by the L protein also occurs in vivo. However, the inhibition of IFN production is not absolute. If it were, the DA virus would cause a fatal encephalomyelitis in wild-type mice analogous to the disease it causes in IFNAR-/- mice.

The L protein also interferes with nucleo-cytoplasmic trafficking of host proteins and mRNA. It promotes the subcellular redistribution of host nuclear and cytoplasmic proteins (2). It also represses the exportation of mRNA from the nucleus to the cytoplasm, thereby shutting-off the expression of host cell proteins. This might explain the highly toxic nature of this protein. These traffick perturbating activities of the L protein correlate with L-mediated hyperphosphorylation of the Nup proteins that make up the nuclear pore complex (7).

In order to test whether the various activities (IFN transcriptional inhibition versus nucleo-cytoplasmic trafficking perturbation) of the L protein are linked, we subjected the L coding region to random mutagenesis and selected L mutants that lost toxicity. This study revealed that two domains of the L protein are critical for activity: the N-terminal zinc finger and a C-terminal domain that was named «Theilo-domain» since it is conserved in the L protein of Theiloviruses (i.e. Theiler's virus and Saffold virus) but not in the L protein of encephalomyocarditis virus (EMCV) (7).

Mutations of either the Zn-finger or of the Theilodomain abolished all reported activities of the L protein, suggesting that the various activities of the protein are linked. Current efforts are devoted to finding the master L interactor in infected cells.

The L protein encoded by Encephalomyocarditis (EMCV), a related Cardiovirus, shares about 35% amino acid identity with that of Theiler's virus. It differs from the latter by lacking the C-terminal Theilodomain. Yet, we showed, in collaboration with the group of F. van Kuppeveld (NCLMS, Nijmegen, The Netherlands), that the EMCV L protein shares, with the TMEV L protein, the ability to antagonize IFN and chemokine production and to affect nucleocytoplasmic trafficking. Thus, in

this case, the absence of Theilodomain does not prevent the activities of the protein. Solving the structures of these proteins will be of interest to unravel the mechanistic base of their activities.

INFLUENCE OF THE L* PROTEIN ON MACROPHAGE INFECTION AND VIRAL PERSISTENCE

Persistent strains of Theiler's virus produce an 18 KDal protein called L*, which is encoded by an open reading frame (ORF) overlapping the ORF coding the viral polyprotein (see Fig. 1). This protein was shown to enhance the infection of macrophage cell lines and to be critical for persistence of the virus in the central nervous system of the mouse (2).

We showed recently that L* protein is partitioned between the cytoplasm and the mitochondria (Fig. 2). In mitochondria, L* is anchored in the outer membrane, facing the cytosol (9). Current efforts aim at characterizing the function of the L* protein in the infection of macrophages. Macrophages are indeed key players in the demyelinating disease induced by Theiler's virus, being simultaneously effectors of the immune response and targets of viral infection. Our recent data suggest that the L* protein antagonizes an important effector pathway of the interferon response. Thus Theiler's virus would interfere both with the production of IFN by infected cells and with the response of cells to this cytokine. This outlines the major importance of the IFN system in the defense of the host against viral infections.

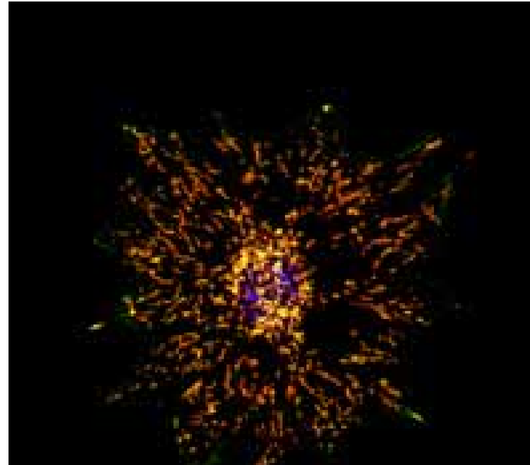

Fig. 2. Mitochondrial localization of L* Mouse embryonic fibroblast expressing L* from a lentiviral vector. L* was immunolabeled in green and mitochondria were labeled in red with mitotracker. Yellow indicates co-localization

TYPE-I AND TYPE-III INTERFERONS

Interferons were the first cytokines to be identified. They were discovered more than 50 years ago by Isaacs and Lindemann who observed that chick chorio-allantoic membranes developped resistance to viral infection after exposure to heat-inactivated influenza virus. Interferons are typically secreted by cells that are infected by a virus. They alert neighbouring cells about the presence of a viral infection and allow these cells to develop an anti-viral state. The interferon system represents a critical protection mechanism of the body against viral infections. In addition, interferons have anticancer properties and modulate the acquired immune response of the host.

Type I IFNs

P. Hermant, F. Sorgeloos, T. Michiels

Mouse and human genomes carry more than a dozen of genes coding for closely related interferon-alpha (IFN-α) subtypes. These interferons and other interferons, like IFN-β, IFN-κ, IFN-ε, IFN-ω, and limitin, form the type-I IFN family. In spite of important sequence divergences, all these IFNs bind the same receptor, raising the question of whether they possess specific functions.

We previously characterized the murine

IFN-α family. 14 IFN-α genes were detected in the mouse genome, two of which, IFN-α13 and IFN-α14, were previously undescribed. In addition the mouse genome contains three IFN-α pseudogenes (3).

Ongoing work aims at understanding the specificities of the various type-I IFN subtypes.

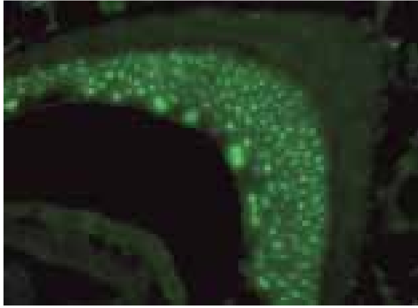

Fig. 3. Reponse to IFN-λ. Section of the urinary epithelium showing a strong response of epithelial cells to IFN-λ. Shown is a fluorescent labelling of the nuclear Mx1 protein, the expression of which is typically induced in response to IFN.

Type-III Interferons (IFN-λ)

M. Minet, F. Sorgeloos, T. Michiels

Recently, another IFN family was described and called «type-III IFN» or «IFN-λ». Although type III IFNs bind a receptor distinct from the type-I IFN receptor, they activate the same signal transduction pathway and upregulate the same set of genes. Thus, they are expected to exert very similar biological activities as type I IFNs. Our work addresses the question of the redundancy of these two IFN systems in vivo.

We observed that the relative expression of IFN-λ over that of IFN-α/β exhibited some extent of tissue specificity and was low in the brain (6). We also used a strategy based on in vivo expression of cloned IFN genes to compare the responses of different tissues to IFN-α and IFN-λ. Interestingly, response to IFN-λ appeared to be **specific to epithelial cells** (Fig. 3), unlike response to IFN-α which occurred in most cell types and was particularly prominent in endothelial cells. Accordingly, tissues with a high epithelial content such as intestine, skin or lungs were the most responsive to IFN-λ and expressed the higher amounts of IFN-λ receptor (9). Work performed in collaboration with the groups of P. Stäheli (Univ. of Freiburg, Germany) and M. Hornef (Univ. of Hannover, Germany) confirmed that IFN-λ participates to the protection of lung and intestine epitheliums (where the response to IFN-λ is prominent) against infection with several viruses such as influenza virus, respiratory syncytial virus or SARS coronavirus. Interestingly, IFN-λ turned out to be the major player in the defense against rotaviruses, common enteric pathogens causing diarrhea (10).

Our data suggest that the IFN-λ system evolved as a specific protection of epithelia and that it might contribute to prevent viral invasion through skin and mucosal surfaces.

Production of IFN-α/β by neurons

M. Kreit, T. Michiels

In collaboration with the teams of Peter Stäheli and Friedemann Weber (Univ. Freiburg, Germany) we analyzed the cells that are responsible for type I IFN production in the central nervous system, during acute encephalitis caused by two neurotropic viruses: Theiler's virus (picornavirus) or La Crosse virus (bunyavirus).

IFN-α/β turned out to be produced by many scattered parenchymal cells and less by cells of the inflammatory foci. Interestingly, besides some macrophages and ependymal cells, neurons turned out to be important producers of both IFN-α and IFN-β. However, not more than 3% of infected neurons expressed IFN, suggesting that some restriction to IFN production might occur in these cells (7).

All CNS cell types analyzed, including neurons, were able to respond to type I IFN by producing Mx or IRF-7. Our data show that, in vivo, in spite of the relative immune privilege of the CNS parenchyma, neurons take an active part to the antiviral defense by being both IFN producers and responders (5).

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HUMAN TUMOR IMMUNOLOGY

Tumor-specific antigens, such as those encoded by the MAGE genes, have been used to vaccinate melanoma patients with detectable disease. About 20 % of the vaccinated patients displayed a tumor regression, a frequency that appears well above the level reported for spontaneous melanoma regressions. Nevertheless, the treatment fails in most patients, and this can probably only be improved by a better understanding of the anti-tumor immune responses of the patients and of the mechanisms of tumor resistance to immune attack.

A first objective is to understand the mechanism of the tumor regressions that occur in a few of the vaccinated patients. Detailed analyses indicated that, surprisingly, the anti-vaccine T lymphocytes are widely outnumbered by other tumor anti-T cells, which recognize tumor-specific antigens different from the vaccine antigens. These anti-tumor T cells represent most of the T cells present in a regressing tumor, and they probably play a major role in the rejection process. We wish to understand why these anti-tumor T cells become activated following vaccination.

A second objective is linked to the observation that the anti-tumor T cells mentioned above are often present in tumors already prior to vaccination. But they appear to be quiescent, as tumor cells co-exist with them without clear signs of immune attack. We are studying various aspects of this co-existence. One is the analysis of lymphocytes infiltrating human melanoma metastases, another is a direct lymphocyte inhibition by melanoma cells in culture, and the last is the analysis of the suppressive or so-called regulatory T cells, which are important attenuators of immune responses.

IMMUNE RESPONSES TO CANCER VACCINE ANTIGENS

G. Hames, P.G. Coulie, in collaboration with J.-F. Baurain, Department of Medical Oncology, Cliniques Universitaires St Luc, and N. van Baren, Brussels branch of the Ludwig Institute for Cancer Research.

To understand why only a few cancer patients vaccinated with defined tumor antigens display an objective tumor regression while most of them do not, it is essential to know whether the anti-tumor T lymphocytes of the patients were amplified by the vaccinations, and whether these amplifications show a correlation with tumor regression. We therefore developed a sensitive approach to detect T cells recognizing known antigens, based on *in vitro* restimulation of blood T lymphocytes with antigenic peptides over two weeks, followed by labeling with tetramers. To evaluate precursor frequencies, these mixed lymphocyte-peptide cultures were conducted under limiting dilution conditions. Cells that were labeled with the tetramer were cloned, the lytic specificity of the clones was verified, and their diversity was analyzed by T-cell receptor sequencing (1, 2).

Focusing on CD8 T cell responses to antigenic peptides presented by HLA-A1 or A2 molecules, we observed surprisingly low levels of anti-vaccine T cells in several of the patients who displayed tumor regression after vaccination. Moreover we did not observe the anticipated correlation between the intensities or breadth (proportions of peptides against which a response is observed) of the immune responses and the clinical impact of the vaccinations (3, and unpublished observations). These results suggest that the main limitation to the clinical efficacy of our therapeutic anticancer vaccines is not the intensity of the induced anti-vaccine T cell responses.

TUMOR REGRESSIONS OBSERVED AFTER VACCINATION: A ROLE FOR TUMOR-SPECIFIC CYTOLYTIC T LYMPHOCYTES THAT DO NOT RECOGNIZE THE VACCINE ANTIGENS

V. Corbière, C. Muller, P.G. Coulie, in collaboration with C. Lurquin and B. Lethé, Brussels branch of the Ludwig Institute for Cancer Research.

In previous work we identified a melanoma patient who displayed a low-level anti-vaccine T-cell response in blood with tumor regression after vaccination with melanoma antigens. Using a genetic approach including T-cell receptor (TCR) cDNA libraries, we found very few anti-vaccine T cells in regressing metastases. However, a far greater number of TCR sequences were found with several of these corresponding to T cell clones specific for non-vaccine tumor antigens (4), suggesting that antigen spreading was occurring in regressing metastases. We also found another TCR belonging to tumor-specific T cells enriched in regressing metastases and detectable in blood only after vaccination. We used the TCR sequence to detect and clone the desired T cells from tumor-infiltrating lymphocytes isolated from the patient. This T cell clone specifically lysed autologous melanoma cells, and its target antigen was identified as the mitochondrial enzyme caseinolytic protease (5). The antigen gene was mutated in the tumor, resulting in production of a neoantigen. Melanoma cell lysis by the T cells was increased by IFN-γ treatment due to preferential processing of the antigenic peptide by the immunoproteasome. These results argue that tumor rejection effectors in the patient were indeed T cells responding to non-vaccine tumor-specific antigens, further supporting our hypothesis (6). We propose that antigen spreading of an antitumor T-cell response to truly tumor-specific antigens contributes decisively to tumor regression.

IN SITU ANALYSIS OF TUMOR-INFILTRATING LYMPHOCYTES

A. Cipponi, N. Remy, P.G. Coulie, in collaboration with N. van Baren, Brussels branch of the Ludwig Institute for Cancer Research.

Our detailed analyses of melanoma patients vaccinated with tumor-specific antigens clearly indicated that anti-tumor T lymphocytes were already present prior to vaccination, both in blood and in some tumors. It is obvious that there is a seemingly pacific coexistence between tumor cells and tumor-specific T lymphocytes that occurs in many of these cancer patients (7). The reasons for this coexistence may well be the key towards improving the clinical efficacy of cancer vaccines. We are gaining information about human tumor-infiltrating or tumor-associated T cells through an *in situ* analysis. Human tumor samples are processed simultaneously for histological analysis including immunochemical detection of immune cells, for complete gene profiling on a fragment of the tumor, and for laser microdissection on frozen material. Whenever possible a small piece is put into culture to derive a melanoma cell line.

We compared the gene expression profiles of pre-vaccine cutaneous metastases from melanoma patients who showed either complete tumor regression or no regression following vaccination with tumor antigens. We observed no relevant difference between the two groups. But we noticed the presence of a specific inflammatory signature, quite variable between samples, and independent of the clinical evolution of the patients. It comprises T cell and macrophage markers. The T cell signature includes activation markers, IFNg target genes, and the *IFNG* transcript itself. Using immunohistology on adjacent tumor sections, we established that this inflammatory signature correlates with the degree of immune cell infiltration in these tumors. Thus melanoma metastases host various degrees of active Th1 inflammation, and we conclude that the immunosuppressive environment in these tumors does not result in a complete inhibition of T cell activation.

HUMAN REGULATORY T CELLS AND TGFβ

S. Lucas, J. Stockis, C. Huygens, E. Gauthy, J. Cuende, N. Remy, M. Panagiotakopoulos, P.G. Coulie.

Regulatory T cells, or Tregs, are a subset of $CD4^+$ lymphocytes specialized in the suppression of immune responses. They are required to prevent the development of auto-immune diseases, but in mice they were also shown to contribute to cancer progression by inhibiting anti-tumor immune responses. Tregs could play a negative role in cancer patients, but this has remained difficult to verify due to the lack of a Treg-specific marker in humans, as well as to an incomplete understanding of the mechanisms underlying their suppressive function.

Our objective is twofold: develop tools to quantify Tregs in human tissues, and identify mechanisms important for their suppressive function which could be specifically targeted to improve the efficiency of cancer vaccines.

Our previous work lead to the obtention of stable human Treg clones, representing long-term cultures of pure lymphocyte populations available for repeated analysis (8). A stable epigenetic mark unambiguously distinguished human Treg clones from non regulatory $CD4^+$ (Thelper) or $CD8^+$ (cytolytic) clones: a conserved region in intron 1 of gene *FOXP3*, encoding a transcription factor indispensable for the development and function of Tregs, was found demethylated in Treg clones only. We set up a methylation-specific real-time PCR assay to quantify demethylated *FOXP3* sequences, indicative of the presence of Treg cells. In collaboration with laboratories from Italy, The Netherlands and Germany, we used this assay to measure Treg frequencies in the blood of

patients who received tumor vaccines in combination with different potentially Treg depleting strategies (9). None of the strategies tested up to now (i.e. low dose Cyclophosphamide, Ontak or Daclizumab) induced a significant decrease in Treg frequencies in a majority of patients. We attempted to use our assay to measure Treg frequencies directly inside tumor samples. However, we observed that melanoma cells themselves could harbor demethylated *FOXP3* sequences, probably as a consequence of aberrant methylation patterns that frequently occur in human tumors. This observation precludes the use of *FOXP3* demethylation as a marker of Treg cells in tumors, unless tumor-infiltrating T cells are separated from tumor prior to analysis (submitted manuscript).

T cell receptor (TCR) stimulation is required for the suppressive function of Tregs. We used expression microarrays to identify functional features that are unique to stimulated Treg clones, by comparison to stimulated Thelper clones. This analysis revealed that a hallmark of stimulated human Treg clones is to produce the active form of TGF-β, a cytokine with well-known immunosuppressive actions. We are currently attempting to identify the mechanisms by which human Tregs can produce active TGF-β.

Many cell types, including Treg and Thelper clones, produce the latent, inactive form of TGF-β. In latent TGF-β, the mature TGF-β protein is bound to the Latency Associated Peptide, LAP, and is thereby prevented from binding to the TGF-β receptor. We recently showed that latent TGF-β, i.e. both LAP and mature TGF-β, binds to GARP, a transmembrane protein containing leucine rich repeats which is present on the surface of stimulated Treg clones but not on Th clones (10). Membrane localization of latent TGF-β mediated by binding to GARP may be necessary for the ability of Tregs to activate TGF-β upon TCR stimulation. As illustrated in the figure below, a model by which activated Tregs would accumulate latent TGF-β on their surface and release its active form in close proximity to their target represents an interesting intermediate between the release of a soluble active TGF-β in the environment, and that of a Treg acting by direct contact with its target. If this model proves to be relevant, it will be important to elucidate the precise mechanism which produces active TGF-β at the surface of Tregs. Our results imply that binding to the GARP receptor is not sufficient, as lentiviral mediated expression of GARP in human Th cells induces binding of latent TGF-β to the cell surface, but does not result in the production of active TGF-

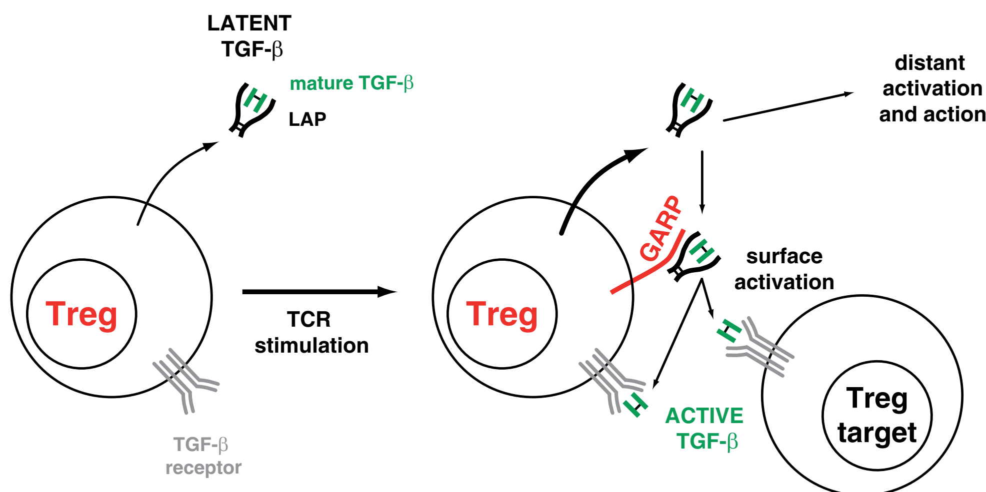

Figure 1. Possible model for TGF-ß production by human Treg clones.

β upon stimulation of these Th cells. We are currently trying to identify additional proteins that interact with GARP, and could represent the missing link for the activation of TGF-β by human Tregs.

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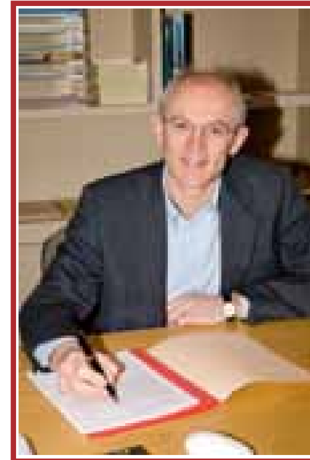
LUDWIG INSTITUTE FOR CANCER RESEARCH

BRUSSELS BRANCH

Cancer is a major concern in human health. The prospects for bringing cancer under control require linked innovative basic and clinical research. In this view, Daniel K. Ludwig created in 1971 the Ludwig Institute for Cancer Research, an international organization bringing together scientists and clinicians from around the world. Many Ludwig investigators are leaders in many areas of science, involving genetics, bioinformatics, immunology, virology, cell biology and signal transduction.



Faithful to the organizing principles laid down by Mr. Ludwig, the Institute conducts its research through ten Branches, located in seven countries. The Branch structure allows the Institute to interact with a number of different research and clinical environments. Each Branch is focused on a research program defined by the Branch Director in relation with the overall objectives of the Institute. The Branches are established in association with University Hospitals, to stimulate close collaborations between research laboratories and the clinic. By organizing and controlling its own clinical trials programs, the Institute has indeed created a continuum that integrates laboratory and clinical research.

The biological properties of any given cancer cell constantly change, allowing tumors to spread and become more aggressive. To overcome these obstacles, the Ludwig Institute has developed a broad-based discovery program that seeks to understand the full complexity of cancer. Research is organized according to the four major programmatic themes that define the Institute: genetics, cell biology, cell signalling and immunology.

Branch staffs vary in size from 30 to over 90, and internationally the Institute employs some 800 scientists, clinicians and support personnel. The quality of the research is monitored on an ongoing basis by the Institute's Scientific Committee and by an external peer review process.

The Brussels Branch of the Institute was created in 1978. It is composed of 93 members and was headed by Thierry Boon until 2009. The Branch is now headed by Benoît Van den Eynde, the current Branch Director.

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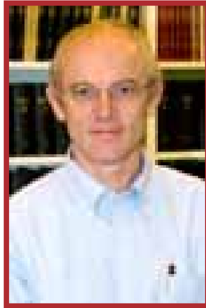

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TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

The group follows three main lines of research. The first focuses on the processing of tumor antigens, studying the role of the proteasome and other proteases in the production of tumor antigenic peptides. The second studies mechanisms whereby tumors resist immune rejection. The third develops new preclinical models for cancer immunotherapy. The long-term goal of these projects is to better understand the interaction of tumors with the immune system and devise strategies to improve the efficacy of cancer vaccines.

PEPTIDE SPLICING BY THE PROTEASOME

A. Dalet, V. Stroobant, N. Vigneron

Tumor antigens relevant for cancer immunotherapy consist of peptides presented by MHC class I molecules and derived from intracellular tumor proteins. They result from the degradation of these proteins, which is mainly exerted by the proteasome. We have described a new mode of production of antigenic peptides by the proteasome, which involves the splicing of peptide fragments, either in the normal or the reverse order (1, 2). We showed that splicing occurs in the proteasome catalytic chamber through a reaction of transpeptidation involving an acyl-enzyme intermediate (Figure 1). We have now described four spliced peptides, two of which are spliced in the reverse order (3). One of these peptides also contains two additional post-translational modifications, resulting in the conversion of asparagines into aspartic acids, through a process a N-glycosylation/deglycosylation. We also compared the efficiency of peptide splicing by

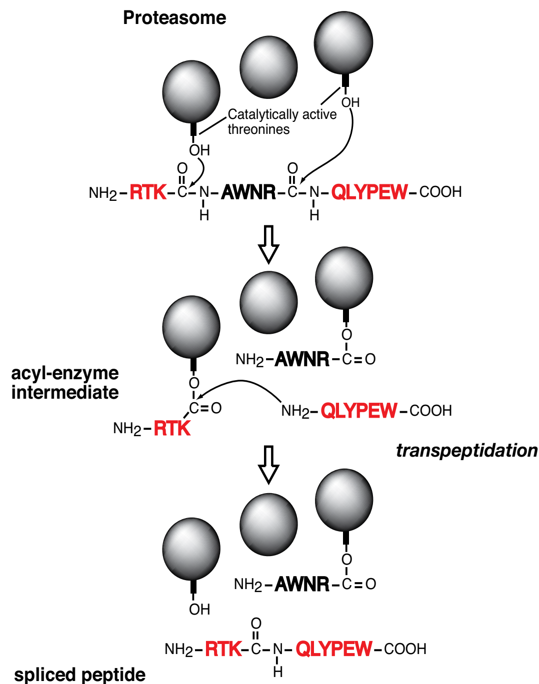

Figure 1. Model of the peptide-splicing reaction in the proteasome. The active site of the catalytic subunits of the proteasome is made up of the side-chain of a threonine residue, which initiates proteolysis by performing a nucleophilic attack on the carbonyl group of the peptide bond. An acyl-enzyme intermediate is formed, which is then liberated by hydrolysis. In the peptide-splicing reaction, a second peptide fragment appears to compete with water molecules for performing a nucleophilic attack on the acyl-enzyme intermediate, resulting in a transpeptidation reaction producing the spliced peptide. Experimental support for this model of reverse proteolysis includes evidence that the energy required to create the new peptide bond is recovered from the peptide bond that is cleaved at the amino-terminus of the excised fragment, and that the amino-terminus of the other fragment needs to be free for transpeptidation to occur.

the standard proteasome and the immunoproteasome, which is found in antigen-presenting cells and cells exposed to interferon-gamma, and contains three inducible catalytic subunits ß1i, ß2i and ß5i instead of the standard catalytic subunits ß1, ß2 and ß5. We found that both proteasomes are able to splice peptides. However, their ability to produce a given spliced peptide varies according to their ability to perform the relevant cleavages to liberate the fragments to splice.

NEW PROTEASOME TYPES THAT ARE INTERMEDIATE BETWEEN THE STANDARD PROTEASOME AND THE IMMUNOPROTEASOME

B. Guillaume, V. Stroobant, A. Busse, E. De Plaen

Using a series of novel antibodies recognizing catalytic subunits of the human proteasome in their native conformation, we identified proteasomes that are intermediate between the standard proteasome and the immunoproteasome (4). They contain only one (ß5i) or two (ß1i and ß5i) of the three inducible catalytic subunits of the immunoproteasome. These intermediate proteasomes represent 30-54% of the proteasome content of human liver, colon, small intestine and kidney. They are also present in human tumor cells and dendritic cells. We studied the processing of a series of antigenic peptides by these intermediate proteasomes, and identified two tumor antigens that are processed exclusively either by intermediate proteasomes ß5i or by intermediate proteasomes ß1i-ß5i. Other functional aspects of these intermediate proteasomes are currently evaluated.

ANTIGENIC PEPTIDE PRODUCTION BY INSULIN-DEGRADING ENZYME

N. Parmentier, V. Stroobant

We studied a proteasome-independent peptide derived form tumor protein MAGE-A3, and we identified insulin-degrading enzyme as the protease producing both the C-terminus and the N-terminus of this peptide (5). This peptide, with sequence EVDPIGHLY, is presented by HLA-A1 and has been widely used in clinical trials of cancer vaccines. Insulin-degrading enzyme (IDE) is a cytosolic metallopeptidase not previously known to play a role in the class I processing pathway.

Cytotoxic T lymphocyte recognition of tumor cells was reduced after metallopeptidase inhibition or IDE silencing. Separate inhibition of the metallopeptidase and the proteasome impaired degradation of MAGE-A3 proteins, and simultaneous inhibition of both further stabilized MAGE-A3 proteins. These results suggest that MAGE-A3 proteins are degraded along two parallel pathways that involve either the proteasome or IDE and produce different sets of antigenic peptides presented by MHC class I molecules.

MODULATION OF TUMOR ANTIGEN EXPRESSION BY INFLAMMATORY CYTOKINES

E. De Plaen, O. Kholmanskikh

We observed that treating some melanoma cell lines with the inflammatory cytokine IL-1ß leads to a 4- to 10-fold decrease in the level of Microphtalmia-associated transcription factor (MITF-M) (6). This effect is NF-kB and JNK-dependent. MITF-M regulates the expression of melanocyte differentiation genes such as Melan-A, tyrosinase and gp100, which encode antigens recognized on melanoma cells by autologous cytolytic T lymphocytes (CTL). Accordingly, treating some melanoma cells with IL-1ß reduced by 40-100% their ability to activate such anti-melanoma CTL.

TUMORAL IMMUNE RESISTANCE THROUGH TRYPTOPHAN DEGRADATION

L. Pilotte, P. Larrieu, V. Stroobant

An important factor limiting the efficacy of immunotherapy is the development of mechanisms allowing tumors to resist or escape immune rejection. Immune resistance mechanisms often involve modulation of the tumoral microenvironment resulting in local immunosuppression. We described one such mechanism, based on the expression by tumor cells of Indoleamine 2,3-dioxygenase (IDO), a tryptophan-degrading enzyme inducing a local tryptophan depletion that severely affects T lymphocyte proliferation (7). Our data in a preclinical model indicate that the efficacy of therapeutic vaccination of cancer patients could be improved by concomitant administration of an IDO inhibitor. In collaboration with the group of Olivier Michielin in Lausanne, we described new compounds able to inhibit IDO in the micromolar range, not only in enzymatic assays but also in cellular assays (8). These compounds will be further optimized with the goal of developing drug candidates. In parallel, a large effort was launched in collaboration with academic and industrial partners to identify IDO inhibitors by high-throughput screening of a chemical library and by structure-base drug design. Two promising families were identified.

We have produced a monoclonal antibody against human IDO, which we used to characterize IDO expression in normal and tumoral tissues. Although others reported high expression of IDO in dendritic cells of murine tumor-draining lymph nodes, our results in humans indicate that a subset of mature human dendritic cells express IDO but these cells are present in normal lymph nodes and not enriched in tumor-draining lymph nodes. However, we observed expression of IDO in a high proportion of human tumors, confirming our initial observation.

NEW PRECLINICAL MODELS FOR CANCER IMMUNOTHERAPY

C. Powis de Tenbossche, (in collaboration with C. Uyttenhove, de Duve Institute and A.-M. Schmitt-Verhulst, CIML, Marseille)

We made transgenic mice, in which we can

induce melanoma in 70% of mice injected with tamoxifen (9). These tumors express the tumor antigen encoded by cancer-germline gene P1A. These tumors can be either highly pigmented and indolent, or unpigmented and highly aggressive. We observed a correlation between aggressive tumor progression and the occurrence of exacerbated systemic inflammation, involving disruption of secondary lymphoid organs, extramedullary hematopoiesis and accumulation of immature myeloid cells, which may contribute to tumoral immune resistance. Current efforts aim to devise therapeutic vaccination approaches able to induce tumor rejection despite this abnormal inflammation.

Cancer-germline genes, which encode tumor antigens of the MAGE-type, are expressed at a low level in the thymus, possibly inducing some level of central immune tolerance that may explain the poor immunogenicity of many of the antigens encoded by these genes. To address this issue, we produced mice that are knockout for cancer-germline gene P1A. These mice are normal and fertile. Their ability to develop an immune response against the P1A-encoded antigen is slightly higher than the wild-type mice, resulting in a better ability to reject P1A-expressing tumors. Analysis of the repertoire of TCR genes revealed some differences in Vß gene usage. This result is consistent with the deletion of high affinity T cells recognizing P1A-encoded antigens in wild-type mice. We conclude that there is a limited central tolerance towards antigens encoded by cancer-germline genes.

BIOMARKER STUDIES IN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) AND RHEUMATOID ARTHRITIS (RA)

B. Lauwerys, J. Ducreux, I. Gutierrez-Roelens, V. Badot, A.-L. Maudoux (in collaboration with F. Houssiau, Unité de Rhumatologie)

In our previous work, we performed high-density transcriptomic studies in synovial biopsies from patients with RA, prior to initiation of therapy with TNF inhibitors. We identified, and confirmed by immunohistochemistry on synovial tissue, several markers of resistance to therapy. Strikingly, we demonstrated that the genes associated with poor response to TNF blockade are induced in synovial fibroblasts by the addition of pro-inflammatory cytokines such as TNF-alpha, IL-1beta or combinations of these cytokines with IL-17, thereby suggesting that these molecules play a role in the mechanisms of resistance to TNF blockade in RA.

IL-7R is one of the genes associated with poor-response to TNF inhibitors in RA. We demonstrated that exposure of RA synovial fibroblasts to pro-inflammatory cytokines induce the production of a soluble form of IL-7R (sIL-7R). sIL-7R can be detected in the sera of patients with RA, and we demonstrated that serum titers are strongly associated with resistance to TNF blockade. We are currently extending these observations in additional cohorts of RA, but also SLE patients. We hypothesize that sIL-7R is a marker of end-organ inflammation (joint, kidney,…), and exposure to pro-inflammatory cytokines.

Finally, we performed high-density transcriptomic studies in synovial biopsies of RA patients prior to and after rituximab (depleting anti-CD20 antibody) therapy. We found that rituximab inhibits IL-17 production in the synovium and induces genes involved in repair mechanisms (10). We also identified synovial markers of response to rituximab therapy, which are different from those predicting response to TNF blockers, thereby demonstrating the specificity of our findings, and their relevance for future clinical applications.

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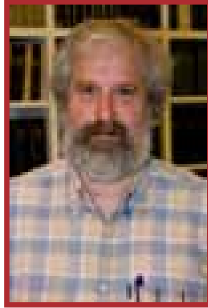

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REGULATION OF T LYMPHOCYTE FUNCTION IN TUMORS

The identification of tumor-specific antigens recognized by T lymphocytes on human cancer cells has elicited numerous vaccination trials of cancer patients with defined tumor antigens. These treatments have induced T cell responses but have shown a low clinical efficacy in tumor-bearing melanoma patients. We believe that progress depends on unraveling the different blockages for efficient tumor destruction. The analysis of the T cell responses of melanoma patients vaccinated against tumor antigens has led us to consider the possibility that the limiting factor for therapeutic success is not the intensity of the anti-vaccine response but the degree of anergy presented by intratumoral lymphocytes. We aim at a better understanding of dysfunctions of the immune system in tumors and more precisely T lymphocyte dysfunctions.

PREVIOUS WORK IN OUR GROUP: IDENTIFICATION OF TUMOR ANTIGENS RECOGNIZED BY T CELLS

In the 1970s it became clear that T lymphocytes, a subset of the white blood cells, were the major effectors of tumor rejection in mice. In the 1980s, human anti-tumor cytolytic T lymphocytes (CTL) were isolated in vitro from the blood lymphocytes of cancer patients, mainly those who had melanoma. Most of these CTL were specific, i.e. they did not kill non-tumor cells. This suggested that they target a marker, or antigen, which is expressed exclusively on tumor cells. We started to study the anti-tumor CTL response of a metastatic melanoma patient and contributed to the definition of several distinct tumor antigens recognized by autologous CTL. In the early 1990s, we identified the gene coding for one of these antigens, and defined the antigenic peptide (1). This was the first description of a gene, MAGE-A1, coding for a human tumor antigen recognized by T lymphocytes.

Genes such as those of the MAGE family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They are therefore referred to as "cancer-germline genes". They encode tumor specific antigens, which have been used in therapeutic vaccination trials of cancer patients (2). A large set

of additional cancer-germline genes have now been identified by different approaches, including purely genetic approaches. As a result, a vast number of sequences are known that can code for tumor-specific shared antigens. The identification of a larger set of antigenic peptides, which are presented by HLA class I and class II molecules and recognized on tumors by T lymphocytes, could be important for therapeutic vaccination trials of cancer patients and serve as tools for a reliable monitoring of the immune response of vaccinated patients (3-4). To that purpose, we have used various approaches that we have loosely named "reverse immunology", because they use gene sequences as starting point (5).

Human tumor antigens recognized by $CD4^+$ or $CD8^+$ T cells are being defined at a regular pace worldwide. Together with colleagues at the de Duve Institute, we read the new publications and incorporate the newly defined antigens in a database accessible at <http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm>.

A MECHANISM CAUSING ANERGY OF CD8 AND CD4 T LYMPHOCYTES

The identification of specific tumor antigens recognized by T lymphocytes on human cancer cells has elicited numerous clinical trials involving vaccination of tumor-bearing cancer patients with defined tumor antigens. These treatments have shown a low clinical efficacy. Among metastatic melanoma patients, about 5% show a complete or partial clinical response following vaccination, whereas an additional 10% show some evidence of tumor regression without clear clinical benefit. We believe that progress depends on unraveling the different blockages for efficient tumor destruction.

The tumors of the patients about to receive the vaccine, already contain T cells directed against tumor antigens. Presumably these T cells are exhausted and this impaired function is maintained by immunosuppressive factors present in the tumor. The T cell response observed in some vaccinated patients reinforce an hypothesis proposed by Thierry Boon and Pierre Coulie: anti-vaccine CTL are not the effectors that kill the tumor cells but their arrival at the tumor site containing exhausted anti-tumor CTL, generates conditions allowing the reawakening of the exhausted CTL and/or activation of new anti-tumor CTL clones, some of them contributing directly to tumor destruction (2, 6). Accordingly, the difference between the responding and the non-responding vaccinated patients is not the intensity of their direct T cell response to the vaccine but the intensity of the immunosuppression inside the tumor. It is therefore important to know which immunosuppressive mechanisms operate in human tumors.

Human tumor-infiltrating T lymphocytes show impaired IFN-γ secretion

Both human CD8 and CD4 tumor-infiltrating T lymphocytes (TIL) were isolated from tumor ascites or solid tumors and compared with T lymphocytes from blood donors. TIL secrete low levels of INF-γ and other cytokines upon non-specific stimulation with anti-CD3 and anti-CD28 antibodies (7-10). TCR were observed to be distant from the co-receptors on the cell surface of TIL, either CD8 or CD4, whereas TCR and the co-receptors colocalized on blood T lymphocytes (Figure 1).

Reversing the anergy of tumor-infiltrating T lymphocytes with galectin ligands

We have attributed the decreased IFN-γ secretion to a reduced mobility of T cell receptors upon trapping in a lattice of glycoproteins clustered by extracellular galectin-3. Indeed, we have shown that treatment of TIL with N-

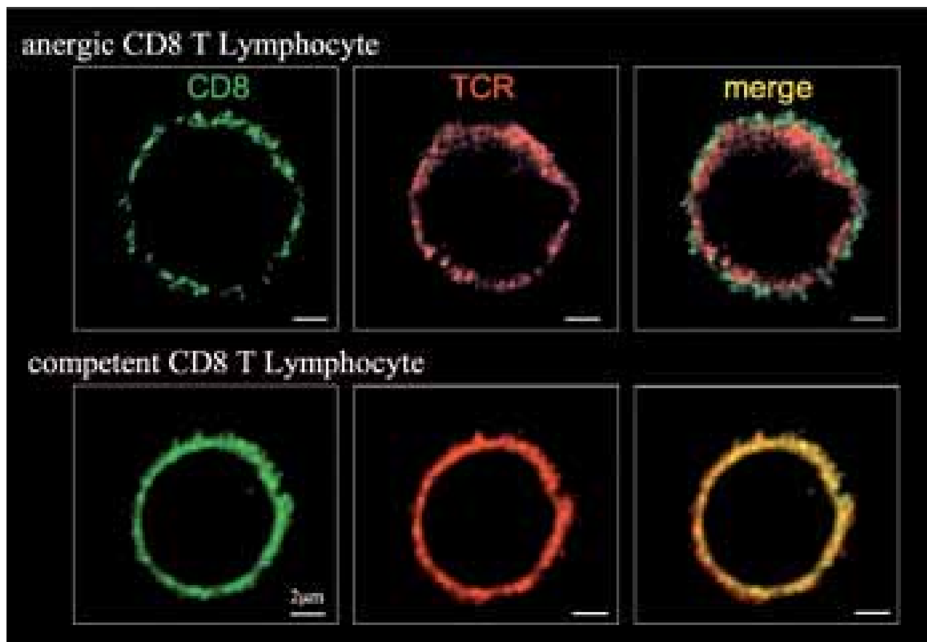

Figure 1. TCR and CD8 do not co-localize on CD8 T cells with impaired functions.

acetyllactosamine (LacNAc), a galectin-competitor ligand, restored this secretion (Figure 2). Our working hypothesis is that TIL have been stimulated by antigen chronically, and that the resulting activation of T cells could modify the expression of enzymes of the N-glycosylation pathway, as shown for murine T cells. The chronically activated TIL, compared to resting T cells, could thus express surface glycoproteins decorated with a set of glycans that are either more numerous or better ligands for galectin-3. Galectin-3 is an abundant lectin in many solid tumors and carcinomatous ascites, and can thus bind to surface glycoproteins of TIL and form lattices that would thereby reduce TCR mobility. This could explain the impaired function of TIL. The release of galectin-3 by soluble competitor ligands would

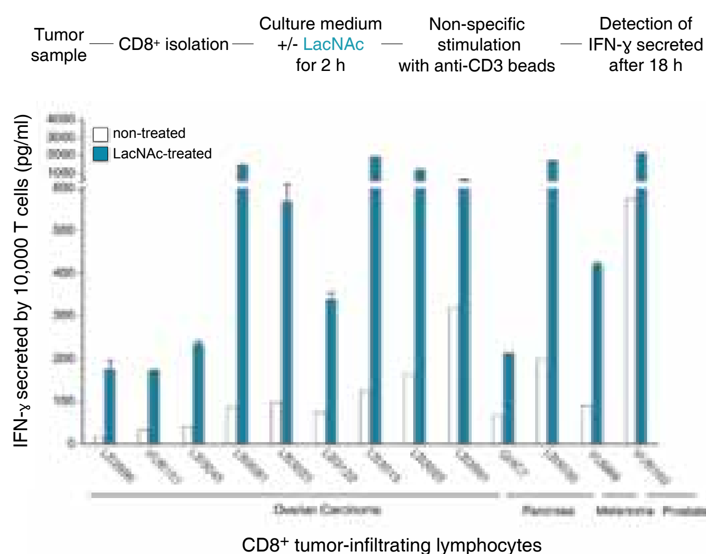

Figure 2. Treatment of tumor-infitrating lymphocytes with a galectin ligand reverses anergy.

restore TCR mobility and boost IFN-γ secretion by TIL. We recently strengthened this hypothesis by showing that both CD4 and CD8 TIL that were treated with an anti-galectin-3 antibody, which could disorganize lattice formation, had an increased IFN-γ secretion compared to untreated cells.

Towards a clinical trial combining vaccination and galectin-binding polysaccharides

Galectin competitor ligands, *e.g.* disaccharides LacNAc, are rapidly eliminated in urine, preventing their use *in vivo*. We recently found that a plant-derived polysaccharide, currently in clinical development, detached galectin-3 from TIL and boosted their IFN-γ secretion. Importantly, we observed that not only $CD8^+$ TIL but also $CD4^+$ TIL that were treated with this polysaccharide secreted more IFN-γ upon ex vivo re-stimulation. In tumor-bearing mice vaccinated with a tumor antigen, injections of this polysaccharide led to tumor rejection in half of the mice, whereas all control mice died. In non-vaccinated mice, the polysaccharide had no effect by itself. These results suggest that a combination of galectin-3 ligands and therapeutic vaccination may induce more tumor regressions in cancer patients than vaccination alone. Translation of these results to the clinic was unfortunately impossible because the company producing this polysaccharide got bankrupted. We recently identified another plant-derived polysaccharide that binds to galectins and was already used in combination with chemotherapy in phase II clinical trials in colorectal cancer patients. This compound was as effective as LacNAc in boosting the secretion of IFN-γ by treated TIL. A clinical trial with this new compound, in combination with anti-tumoral vaccination, will hopefully be launched in 2011 in clinical centers close to the Brussels Branch. We are currently trying to understand the very early activation events that are defective in TIL.

IS THE SPONTANEOUS ANTI-TUMOR T CELL RESPONSE OF BREAST CARCINOMA PATIENTS A CLINICAL PROGNOSTIC FACTOR ?

Several retrospective studies suggest a correlation between the survival of patients with ovarian or colorectal carcinoma and infiltration of their tumors by immune cells. So far, prospective data validating these observations do not exist. In collaboration with Dr Javier Carrasco (Grand Hôpital de Charleroi) and Dr Jean-Pascal Machiels (Cliniques Universitaires St-Luc), we set out a prospective study aimed at looking for a correlation between the clinical outcome of patients with non-metastatic breast carcinoma and their spontaneous anti-tumor T cell response. Considering our experience in quantitative approaches to detect very weak T cell responses in the blood of melanoma patients, Danièle Godelaine set out the evaluation of the frequencies of anti-tumor CD8 T lymphocytes in the blood of breast cancer patients recruited in the two clinical centers. Frequencies are evaluated by mixed lymphocyte-peptide cultures, carried out with HLA-A2 and A3-restricted peptides derived from HER2/neu and hTERT, two proteins usually overexpressed in breast tumors, followed by detection of specific cells with HLA-peptide tetramers. Blood samples are collected before and after surgery. Tumors removed at surgery are analyzed by immunohistology for infiltration by immune cells, and fragments are frozen for further analysis. This prospective study foresees to include 172 patients whose clinical evolution will be followed up over a 5-year period. So far, 25 patients have been included. Eight of them have a frequency against the targeted antigens ranging from 10^{-5} and 10^{-6} among blood CD8 T cells, whereas the frequency in healthy donors blood was estimated to be lower than $5x10^{-7}$. We hope to identify the patients with a better prognosis in order to offer them an adapted care avoiding unnecessary heavy treatments.

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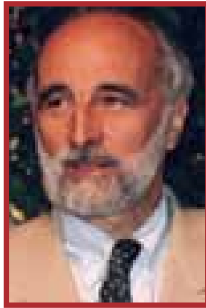

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The identification in the early 1990's of human tumor-specific antigens that are recognized by T cells led to widespread attempts at vaccinating cancer patients with these antigens to induce tumor regression (1). Vaccination of metastatic melanoma patients with MAGE peptides resulted in evidence of tumor regression in about 15% of the patients, with complete and partial clinical responses in only 7% of the patients (2). Why did most patients fail to respond? A plausible hypothesis was that the anti-MAGE T cell response was too weak. However, none of the numerous attempts to boost the efficacy of the vaccines, for instance with adjuvants or by the use of dendritic cells, resulted in improvement of the clinical efficacy.
Our analysis of a few responding patients led us to a different hypothesis. Several groups reported a long time ago that human tumors contain tumor-infiltrating lymphocytes (TILs). These T lymphocytes could be extracted from the tumors and were capable of destroying tumor cells in vitro after short-term cultivation in the presence of IL-2. However, inside the tumor, they must have become inactive ("anergic") at one point, since the tumor is progressing. We made the paradoxical observation that, when vaccination causes complete tumor regression, the T lymphocytes directed against the vaccine antigen are present in the tumor in very small numbers, clearly insufficient to cause rejection. But they reactivate the "anergic" tumor infiltrating T lymphocytes that are present in the tumor in large numbers as a result of a past spontaneous immune response of the patient. It is these reactivated TILs which are capable of destroying the bulk of the tumor cells (3, 4). Our new hypothesis is that what differentiates the non-regressing and the regressing patients is not their direct response to the vaccine but the severity of the anergy of their TILs.
Accordingly, our new strategy to improve anti-tumoral vaccination is to supplement it with a local treatment of the tumor with various cytokines and Toll receptor agonists effectors, as well as antibodies directed against inhibitory cytokines such as TGFß, to reduce the immunosuppression in the tumor. This should facilitate the action of the anti-vaccine T lymphocytes which provide the "spark" firing the regression response. We are exploring this approach in a mouse model of skin grafts and we hope that positive results will lead to new small-scale clinical trials.

INDUCING REJECTION OF NORMALLY TOLERATED GRAFTS IN THE H-Y MOUSE MODEL.

C. Bourdeaux, F. Brasseur, I. Jacquemart, B. Lethé, C. Lurquin, C. Uyttenhove, T. Boon

Female CBA mice do not reject male skin grafts, even though they are able to mount a specific anti-H-Y cytolytic T cell response. Repeated immunizations with male lymphoblasts did not induce rejection of established skin grafts. We tested local approaches to break this tolerance. Repeated local injections of a low dose of IL-12, combined with IFNα, caused graft rejection in all mice. This was also the case when IL-12 was combined with ligands against Toll-like receptors 3, 7 or 9 (TLR). Like IFNα, IL-1α, IL-18 and IL-2 were incapable of inducing rejection on their own, but synergized effectively with IL-12.

We tested combinations of agents that are approved for clinical use. Repeated local injections of a combination of low doses of IL-2, GM-CSF and IFNα with TLR7 ligands gardiquimod or imiquimod caused 100% rejection. The crucial components appear to be IL-2 and gardiquimod. A clinical trial has been launched in which a small number of patients with superficial lesions of metastatic melanoma will receive vaccinations with tumor antigens combined with a local treatment composed of the three cytokines available as registered medicines and Aldara, a cream containing imiquimod.

For this treatment to be therapeutically effective, it would be necessary that the rejection of the locally treated metastasis would result into an antitumoral T-cell expansion that would also eliminate other metastases. In CBA females carrying two male skin grafts, the local treatment of one graft which caused its complete rejection, induced, in a fraction of the mice, a systemic immune response sufficient to cause partial or complete rejection of a distant graft. We are presently exploring additional local and systemic treatments that may improve this distant rejection.

To improve our understanding of the action of the local agents, we follow the fate of naïve CD8 T-lymphocytes endowed with an anti-H-Y-Kk T-cell receptor, collected from transgenic mice and transferred into the grafted mice. With a PCR specific for the α chain gene of this receptor, we were able to assess that these lymphocytes concentrate in the graft as soon as 7 days following the onset of the local treatment with IL-2, GM-CSF, IFNα and imiquimod. In the absence of this treatment, these lymphocytes do not accumulate in the graft.

AMINE-REACTIVE OVA MULTIMERS FOR AUTO-VACCINATION AGAINST CYTOKINES AND OTHER IMMUNE MEDIATORS.

C. Uyttenhove (in collaboration with R. Marillier and J. Van Snick)

We have made significant progress in our efforts to develop auto-vaccines against cytokines as a tool for studying their functions in vivo and develop a panel of monoclonal antibodies of mouse origin against mouse and human cytokines with therapeutic perspectives. Self cytokines linked chemically to a non-self protein or genetically associated to a defined foreign sequence become immunogenic. The proposed rationale underlying this process is that the self-reactive B cell that has captured the complex or fusion protein will present foreign peptides on its MHC Class II membrane proteins and thus attract help from T cells reactive with the non-self structure.

Many gaps still exist in our understanding of the precise mechanisms involved in this auto-vaccination but we recently noted a positive correlation between immunogenicity and immunogen size in a series of anti-IL-12 vaccines that were fractionated according to size. This raised a dilemma, since the larger the complex,

also the greater the risk for structural alterations of the antigen.

To circumvent this problem, we tried a two-step procedure. We first made large ovalbumin (OVA) multimers by treating OVA with glutaraldehyde and, after purifying the polymerized products by size exclusion chromatography, reacted these with the target cytokine before saturating remaining glutaraldehyde sites with a pan-DR epitope peptide (PADRE) to maximize immunogenicity.

Using this procedure, mice were successfully immunized against the chemokine GCP-2/CXCL6, the cytokines GM-CSF, IL-17F, IL-17E/IL-25, IL-27, TGFß-1 and the matrix metalloproteinase-9 MMP-9/gelatinase B. Monoclonal Abs derived from such immunized mice would have obvious advantages for the study and control of the in vivo functions of these proteins. We therefore attempted to generate such reagents from our auto-vaccinated mice (5). Currently, we obtained a mAb specific for mouse and human TFGß-1, MTGFß-1.13A1, that is a potent inhibitor of TGFß-1 bioactivity; an anti- IL-17F mAb, MM17F-8F5, that abrogates the neutrophil chemotactic activity triggered by IL-17F in vitro; a mAb against GCP-2, that provided the first demonstration of the essential role played by this chemokine in rapid neutrophil mobilization after Leishmania major infection; and, finally, a mAb against mouse IL-27, MM27.7B1, that potently inhibited the bioactivity of this cytokine in vitro and is currently used to evaluate the contribution of IL-27 in anti-tumor activities.

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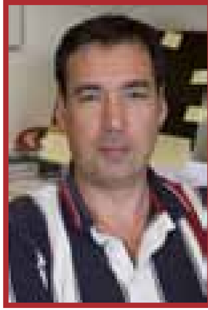

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THERAPEUTIC VACCINATION AND TUMOR EXPRESSION PROFILING GROUP

Cancer cells express tumor-specific antigens that can be targeted by cytolytic T lymphocytes (CTL). These antigens are small peptides derived from endogenous proteins presented at the surface of tumor cells by HLA molecules. In vitro, cytolytic T lymphocytes (CTL) lyse selectively tumor cell lines that express their cognate antigen. Our group has developed small scale clinical immunotherapy trials in which patients with advanced cancer, often metastatic melanoma, have been treated repeatedly with a vaccine containing one or several defined tumor antigens that are expressed by their tumor (Fig. 1). Different immunization modalities, such as vaccination with peptides like MAGE-3.A1 and NA17.A2, or with the MAGE-3 recombinant protein, both with or without adjuvant, or with an ALVAC recombinant viral vector, have already been tested. They are all devoid of severe toxicity. A minority of vaccinated melanoma patients (about 10 to 20%) showed regression of metastatic lesions (Fig. 2). This frequency is far beyond the reported incidence of spontaneous regressions of melanoma metastases, estimated at 0.2-0.3%, indicating that these regressions are linked to the vaccinations. However, only 5% of the patients experience a true clinical benefit. Some of the remissions have lasted for several years. There is no evidence that one of the vaccines tested is more effective against the tumors than the others. The most likely explanation for the poor effectiveness of cancer vaccines is the fact that tumors have acquired the ability to resist destruction by anti-tumoral T cells, following repetitive in vivo challenge with spontaneously occurring immune responses. The molecular mechanisms of tumor resistance remain largely unknown, despite the many candidates that have been proposed. Importantly, we have observed that tumor-infiltrating lymphocytes (TIL) purified from melanoma metastases can rapidly recognize and kill autologous tumor cells in vitro, indicating that tumor resistance is a local effect in the tumor environment. We are following two different approaches to try to improve these results: find more immunogenic vaccines, and combine vaccines with treatments that modify the tumor environment in favor of effective tumor rejection.

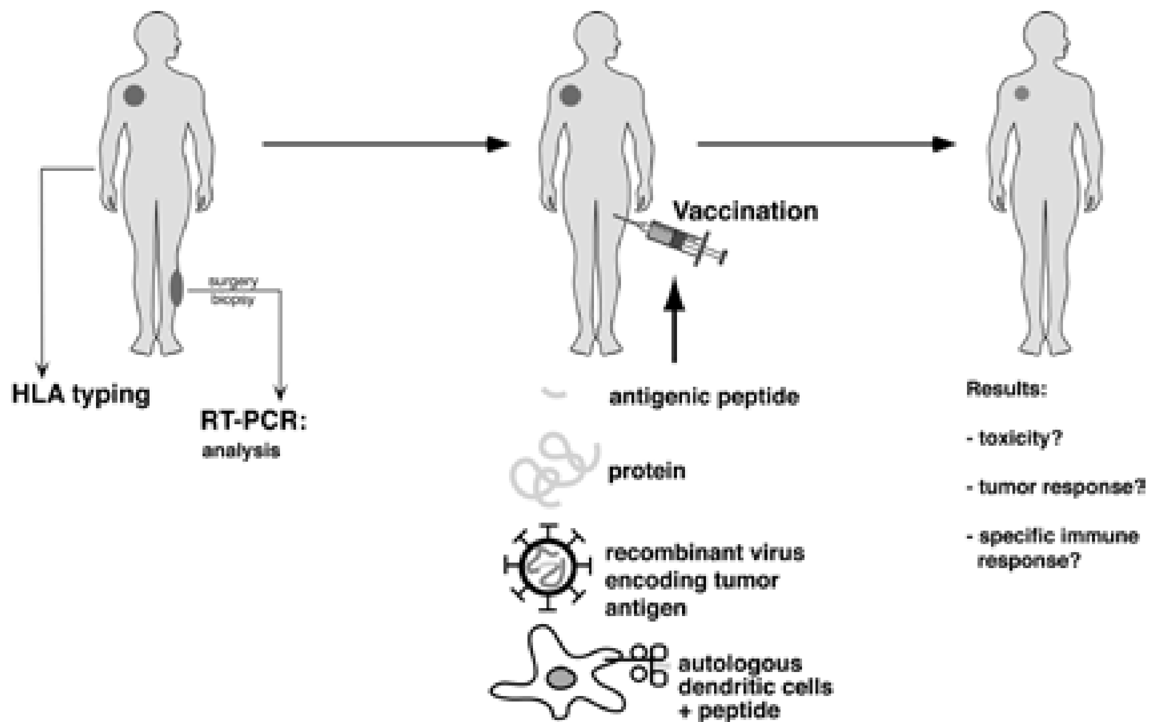

Fig. 1. Principle of anti-tumor vaccination with a defined antigen : The first step is to determine if the patient's tumor cells express the tumor antigen. This can be determined by HLA typing, and by RT-PCR analysis of a tumor sample. Selected patients receive repeated injections of a vaccine with the antigen. Usually this vaccine is a synthetic peptide, a recombinant protein, a recombinant virus coding for the antigen, or dendritic cells derived from the patient's blood and forced to express this antigen. The effect of vaccinations on tumor progression is then assessed. Their immunogenicity is analyzed by comparing the frequency of anti-vaccine CTL in the pre and post-immune blood..

VACCINATION OF MELANOMA PATIENTS WITH THERAVAC, A NEW VACCINE CONCEPT

In collaboration with the groups of J.F. Baurain (Centre du Cancer, Cliniques Universitaires St Luc), P. Coulie, B. Van den Eynde, and Cl. Leclerc (Institut Pasteur, Paris France).

In a recently started phase I clinical trial, we are testing the safety, immunogenicity and anti-tumoral effect of a new promising vaccine called Theravac, developed at Institut Pasteur. Theravac is a recombinant chimeric protein vaccine aimed at targeting dendritic cells (DC) in vivo, and force them to express a Tyrosinase.A2 antigen, a peptide derived from the melanocyte and melanoma-specific tyrosinase protein. Theravac is derived from CyaA, a bacterial toxin that binds specifically to CD11b, an adhesion molecule expressed by dendritic cells and macrophages. Upon binding, a portion of the toxin is internalized and neutralizes its target cell in order to turn off innate immunity at the infectious site. In the recombinant vaccine protein, the toxin activity has been inactivated by insertional mutagenesis, and coupled to the Tyrosinase.A2 peptide. Thus, the unique advantage of this vaccine is its ability to target dendritic cells in vivo, with a putative higher immunogenicity as a consequence. Preclinical experiments have shown that Theravac has a very potent capacity to activate Tyrosinase.A2-specific CTL. In our clinical trial, patients with tyrosinase-expressing metastatic melanoma are immunized with repeated injections of Theravac, at increasing doses, in a classical phase I

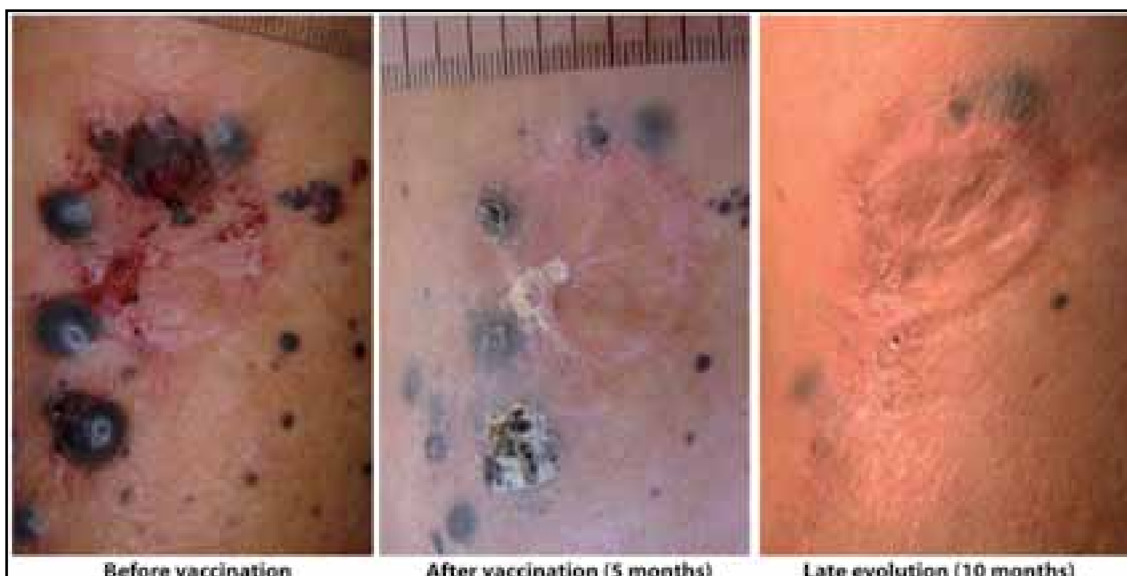

Fig. 2. Example of a complete regression of cutaneous metastases in a melanoma patient after 4 priming vaccinations with an ALVAC recombinant virus expressing the MAGE-3.A1 and MAGE-1.A1 epitopes following by 3 booster vaccinations with the corresponding peptides.

clinical trial design. During the treatment, clinical signs of side effects of the vaccine, including depigmentation occurring as a consequence of anti-melanocyte immune activity, will be recorded, and the size of the metastases will be followed to detect anti-tumoral effect of the vaccine. Blood lymphocytes will be collected before and after the vaccinations to measure the anti-Tyrosinase.A2 immune response. If successful, this new vaccine modality could have a much broader application than in melanoma vaccines.

VACCINATION OF MELANOMA PATIENTS WITH PEPTIDES ASSOCIATED WITH IMMUNOMODULATION OF THE TUMOR ENVIRONMENT

In collaboration with the groups of J.F. Baurain (Centre du Cancer, Cliniques Universitaires St Luc), P. Coulie and T. Boon

In another ongoing clinical trial, melanoma patients with superficial metastases are being vaccinated with a peptide vaccine, either MAGE-3.A1 or NA17.A2, matching the antigenic profile of their tumor. Each of these peptides was previously tested in clinical vaccine trials, and was shown to be well tolerated and associated with tumor regression in some patients. In addition to the vaccine, the patients receive repeated peritumoral injections of a cocktail of pro-inflammatory cytokines and a TLR ligand, in one or two superficial metastases. This local treatment is aimed at inducing a "spark" effect in the tumor environment that could modify it in favor of effective tumor rejection. The same cocktail has been tested in the H-Y mouse model of skin graft rejection, in which it is able to induce effective tissue rejection (see the contribution of T. Boon in this report). As with the other clinical trials run by the group, great attention is given to the collection of biological material (tumor and blood samples), which will allow to study the effect of the treatment on the anti-tumoral immune responses.

VACCINATION OF MELANOMA PATIENTS WITH PEPTIDES ASSOCIATED WITH A GALECTIN-3 INHIBITOR

In collaboration with the groups of J.F. Baurain (Centre du Cancer, Cliniques Universitaires St Luc), P. Coulie and P. van der Bruggen

Recent work in the laboratory has shown that the state of anergy that characterizes tumor-associated T cells can be reversed pharmacologically (see the contribution of P. van der Bruggen in this report). Inhibitors of galectin-3, a protein produced by cancer cells that is able to interfere with effective T cell activation, have been able to reactivate anergic T cells in vitro. In a new clinical trial, in preparation, melanoma patients will receive the same peptide vaccine as in the previous study, in association with repeated infusions of an experimental drug called Davanat®, a plant-extracted oligosaccharide that binds to and inhibits galectins. Galectin-3 is a protein produced by cancer cells that is able to inhibit T cell activation. The group of Pierre van der Bruggen has shown that the anergy that characterizes tumor-associated T cells can be reversed with galectin-3 inhibitors including Davanat®. We hope that this combined treatment will result in the induction of anti-tumoral CTL responses by the vaccine, in synergy with the inhibition of tumor resistance by the galectin-3 inhibitor.

STUDY OF THE INFLAMMATORY ENVIRONMENT IN MELANOMA METASTASES

In collaboration with the groups of P. Coulie (Cellular Genetics Unit, de Duve Institute)

Using the microarray technology, we have established the gene expression profile of a series of tumor samples, mainly cutaneous metastases, obtained from melanoma patients. This approach is combined with systematic immunohistological or immunofluorescence analysis of adjacent cryosections, using antibodies directed against tumor cells, T and B cells, macrophages, blood vessels, and various molecules involved in inflammatory reactions (Fig. 3). In addition, adjacent cryosections are analyzed by performing laser capture microdissection of selected areas, e.g. T cell rich areas, followed by RT-qPCR analysis of T

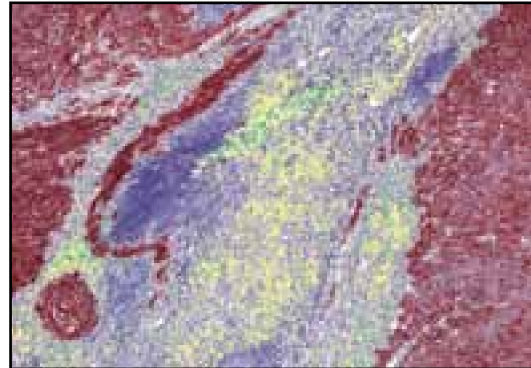

Fig. 3. Example of immune cell infiltration in a melanoma metastasis. The T lymphocytes surround the tumor mass without infiltrating it. This image was reconstructed by superposition of digital microscope images obtained after staining with an antibody directed against melanoma cells (in red) and counterstaining the nuclei with hematoxylin (in blue), followed by elution of antibody and dyes, and by immunofluorescence staining with antibodies directed at CD8+ T lymphocytes (green) and CD4+ T lymphocytes (yellow).

cell, macrophage, melanoma cell and inflammation associated genes. These complementary approaches help us to characterize the inflammatory events that take place inside the metastases, and to understand the interaction between the tumor cells and the inflammatory cells at the tumor site. We are currently characterizing an inflammatory signature that is detected in most tumor samples, and that is associated with T cell activation. We also analyze lymphoid structures present in tumors in which B cell responses seem to occur. The informations gathered from these analyses help us to understand the immune pathways that are active or silent in the tumor environment.

ANALYSIS OF MELANOCYTE-DERIVED TUMORS BY NON-LINEAR OPTICS TECHNIQUES.

Our group collaborates with several other European groups in a project aimed at developing innovative imaging microscopy and endoscopy approaches that might improve cancer diagnosis. These approaches are based on spectroscopical analysis of tissue sections or samples illuminated with one or several laser beams of selected frequencies, using so-called Raman and Coherent Anti-Stokes Raman Spectroscopy (CARS) microscopes. The Raman and CARS effects involve light reflection that depends on the molecular bonds present in the illuminated sample. The objective is to identify spectral signatures associated with tumor cells, which would allow to detect and quantify these cells in conventional microscope preparations without staining. Eventually, this technique coupled to an endoscope might allow to detect the presence of cancer cells in vivo. The current project is focused on melanoma and benign naevus samples, and is at an early, proof-of-feasibility stage of development.

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CYTOKINES IN IMMUNITY AND INFLAMMATION

The cytokine group studies the biological activities of cytokines in inflammatory and tumoral processes, as well as the molecular mechanisms underlying these activities. Our work focuses on Interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is produced by a particular T lymphocyte population, called TH9, and plays a role in immune responses against intestinal parasites and asthma. Dysregulation of IL-9 signalling is also implicated in tumoral transformation and this process has been studied in an in vitro tumorigenesis model, leading to the identification of oncogenic mutations of the JAK1 gene. IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, upregulates the production of acute phase reagents and antibacterial proteins in the liver, the lung and intestinal mucosae, and in the skin. IL-22 appears to play a key role in wound healing and skin inflammation processes such as psoriasis. The role of these cytokines in inflammation is currently being investigated using transgenic and gene-targeted mice for these cytokines and their receptors, and by using an original strategy of anti-cytokine vaccination.

INTERLEUKIN 9

Interleukin-9 (IL-9) was discovered in our group in 1989, through its ability to sustain antigen-independent growth of certain murine T helper clones. Although IL-9 did not turn out to be a T cell growth factor for freshly isolated T cells, it was found particularly potent on T cell lymphomas, as an anti-apoptotic agent. To determine the biological activities of this factor, we generated transgenic mice overexpressing this cytokine. Analysis of these animals disclosed two essential properties of IL-9: its activity on mast cells and eosinophils with consecutive implications in asthma, and its tumorigenic potential in T lymphocytes.

IL-9-transgenic mice : parasite infections and asthma

Although IL-9 overproduction is viable and IL-9 transgenic mice did not show any major abnormality at the first look, they were found to harbor increased numbers of mast cells in the intestinal and respiratory epithelia, and were also characterized by a general hypereosinophilia. This phenotypic characteristic was found to increase the capacity of these animals to expel nematodes like Trichinella spiralis or Trichuris muris., suggesting that IL-9 administration could protect susceptible hosts against these parasites. Conversely, blocking IL-9 activity resulted in a failure to expel T. muris para-

sites and in decreased eosinophilic responses against the parasite (1).

The other side of the coin was the discovery that IL-9 overexpression, such as that characterizing the IL-9 transgenic animals, resulted in bronchial hyperresponsiveness upon exposure to various allergens. Our observations showed that IL-9 promotes asthma through both IL-13-dependent and IL-13-independent pathways (2), as illustrated in figure 1. The potential aggravating role of IL-9 in asthma was confirmed by genetic analyses performed by others and pointing to both IL-9 and the IL-9 receptor genes as major candidate genes for human asthma. In addition, we found that asthma patients produce increased amounts of IL-9. Phase II clinical trials using anti-IL-9 antibodies produced in our laboratory have been initiated in collaboration with Medimmune.

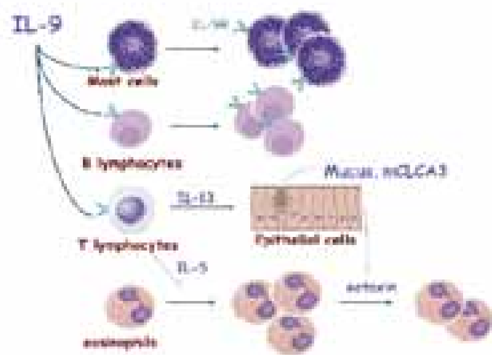

Figure 1. Direct and indirect activities of IL-9 in asthma. IL-9 acts directly on mast cells and B lymphocytes to induce an expansion of these cells and IgE production. IL-9 promotes the proliferation eosinophils indirectly, by upregulating IL-5 production by T cells. Upregulation of IL-13 production by T cells meadiates IL-9 activities on lung epithelial cells, including mucus production and secretion of eotaxin, which is required to recruit eosinophils into the lungs (2).

IL-9-transgenic mice : T cell lymphomas

IL-9 transgenic animals showed normal T cell development and T cell numbers but spontaneously developed thymic lymphomas at low frequency (5%) when maintained in a conventional environment. Two lines of evidence indicate that IL-9 is not a conventional oncogene but rather favors tumor development in response to exogenous stimuli. First, the tumor incidence was significantly lower when mice were maintained under pathogen-free conditions. Secondly, all IL-9 transgenic mice developed T cell lymphomas when exposed to subliminal doses of a chemical carcinogen or to irradiation, that were innocuous in wild type mice (3). The above mentioned anti-apoptotic activity of IL-9 provides an attractive explanation for these observations, namely that IL-9 could lead to increased survival of abnormal cells generated by exposure to minimal doses of oncogenic stimuli. The potential implication of IL-9 in oncology was also confirmed in human systems by its constitutive expression in Hodgkin lymphomas.

IL-9 RECEPTOR AND SIGNAL TRANSDUCTION

Analysis of the mode of action of IL-9 at the molecular level was initiated by the cloning of the murine and human IL-9 receptor (IL-9R) cDNAs (4). By further dissecting the signal transduction cascade triggered by IL-9, we showed that, upon IL-9 binding, the IL-9R associates with a co-receptor protein called γc. This induces the phosphorylation of the JAK1 and JAK3 tyrosine kinases, which are associated with IL-9R and γc, respectively. A single tyrosine residue of the IL-9R is then phosphorylated and acts as a docking site for 3 transcription factors of the STAT family, STAT-1, -3 and -5, which become phosphorylated and migrate to the nucleus, where they activate the transcription of a number of genes. This

pathway is common to many cytokines but is often dispensable for their biological activities. For IL-9, our group demonstrated that activation of the STAT transcription factors is crucial for all the effects of IL-9 studied on various cell lines, including positive and negative regulation of cell proliferation, as well as inhibition of corticoid-induced apoptosis in T cell lymphomas. Further analysis demonstrated that STAT-1, -3 and -5 play specific, redundant and synergistic roles in the different activities of IL-9 in vitro. The pathways responsible for IL-9-induced proliferation were studied in details, and this process was found to depend mainly on the activation of STAT-5, on the recruitment of the IRS-1 adaptor, and on the activation of the Erk MAP-Kinase pathway.

ROLE OF JAK OVEREXPRESSION AND MUTATIONS IN TUMOR CELL TRANSFORMATION

Constitutive activation of the JAK-STAT pathway is frequent in cancer and contributes to oncogenesis. Some of our recent data indicate that JAK overexpression plays a role in such processes. Using a murine proB cell line that strictly depends on IL-3 for growth in vitro, cytokine-independent and tumorigenic clones were derived from a two-step selection process. Cells transfected with a defective IL-9 receptor acquired IL-9 responsiveness during a first step of selection, and progressed after a second selection step to autonomously growing tumorigenic cells. Microarray analysis pointed to JAK1 overexpression as a key genetic event in this transformation. Overexpression of JAK1 not only increased the sensitivity to IL-9 but most importantly allowed a second selection step towards cytokine-independent growth with constitutive STAT activation. This progression was dependent on a functional FERM and kinase JAK1 domain. Similar results were observed after JAK2, JAK3 and TYK2 overexpression. All autonomous cell lines showed an activation of STAT5, ERK1-2 and AKT. Thus, JAK overexpression can be considered as one of the oncogenic events leading to the constitutive activation of the JAK-STAT pathway (5).

Recently, we elucidated the mechanism responsible for the second step of this tumoral transformation process, as we found that the majority of the cytokine-independent tumorigenic clones acquired an activating mutation in the kinase or in the pseudokinase domain of JAK1 (Figure 2).

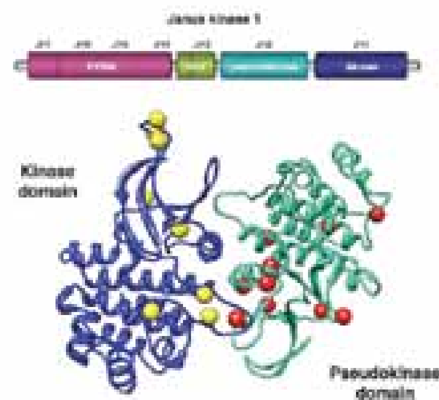

Figure 2. Localization of JAK1 activating mutations in the kinase and pseudokinase domains.

In parallel to these observations, in collaboration with Prof. Marco Tartaglia (University of Rome), we identified activating mutations in JAK1 in 20% of T-cell acute lymphoblastic leukemia (T-ALL) and in 3% of B-ALL patients, confirming the relevance of our in vitro model-derived JAK1 mutations for human malignancies. Further analysis of human ALL samples showed that JAK1-mutated leukemias share a type I IFN transcriptional signature, suggesting that these mutants do not only activate growth-promoting pathways, but also antiviral pathways. Expression of these activating JAK1 mutants in murine hematopoietic cell lines recapitulated this signature in the absence of IFN, but also strongly potentiated

the in vitro response to IFN. Finally, we also showed in an in vivo leukemia model that cells expressing mutants such as JAK1(A634D) are hypersensitive to the anti-proliferative and anti-tumorigenic effect of type I IFN, suggesting that type I IFNs should be considered as a potential therapy for ALL with JAK1 activating mutations (6).

IL-TIF/IL-22 : A NEW CYTOKINE STRUCTURALLY RELATED TO IL-10

Searching for genes specifically regulated by IL-9 in lymphomas, we identified a new gene that turned out to encode a 179 amino acid long protein, including a potential signal peptide, and showing a weak but significant sequence homology with IL-10. This protein, originally designated IL-TIF for IL-10-related T-cell derived Inducible Factor, was later renamed IL-22. Despite its structural homology with IL-10, IL-22 fails to recapitulate any of IL-10 biological activities. Biological activities of IL- 22 include the induction of acute phase proteins in liver (7) and protection against experimental hepatitis and colitis (L. Dumoutier, unpublished results). Among the different T cell subset, IL-22 was found to be preferentialy produced by TH17 cells raising some speculations about its potential role in autoimmune processes (8). Experiments are in progress to establish the role of this cytokine in skin inflammatory processes such as psoriasis, by using mice deficient either in IL-22 or in its receptor.

Although IL-22 does not share any biological activity with IL-10, these 2 cytokines share a common component of their respective receptor complex, IL-10Rß. Anti-IL-10Rß antibodies indeed block the IL-22-induced acute phase response in HepG2 cells (7). All receptor complexes for IL-10-related cytokines include a long chain and a short chain, based on the length of the cytoplasmic domain of these transmembrane proteins. IL-10Rß is a typical short chain component, with only 76 amino acids in the cytoplasmic domain, whose main function seems to consist in recruiting the Tyk2 tyrosine kinase. In addition to IL-10R ß, IL-22 signalling requires the expression of a long chain protein, called IL-22R and comprising a 319 amino acid long cytoplasmic domain. This chain associates with JAK1, and is responsible for the activation of cytoplasmic signalling cascades such as the JAK/STAT, ERK, JNK and p38 MAP kinase pathways. An unexpected featur of the IL-22R chain is the fact that the C-terminal domain of this receptor is constitutively associated with STAT3, and that STAT3 activation by this receptor does not require the phosphorylation of the receptor, in contrast to the mechanism of STAT activation by most other cytokine receptors (9).

Beside this cell membrane IL-22 receptor complex composed of IL-22R and IL-10Rß, we identified a protein of 231 amino acid, showing 33 % amino acid identity with the extracellular domains of IL-22R, respectively, but without any cytoplasmic or transmembrane domain. This soluble receptor has been named IL-22 binding protein (IL-22BP), because it binds IL-22 and blocks its activities in vitro, demonstrating that this protein can act as an IL-22 antagonist.

The crystal structure of IL-22, alone and bound to its cellular receptor IL-22R or to its soluble receptor IL-22BP has been characterized in collaboration with Prof. Igor Polikarpov (University of Sao Paulo) and is illustrated in Figure. 3.

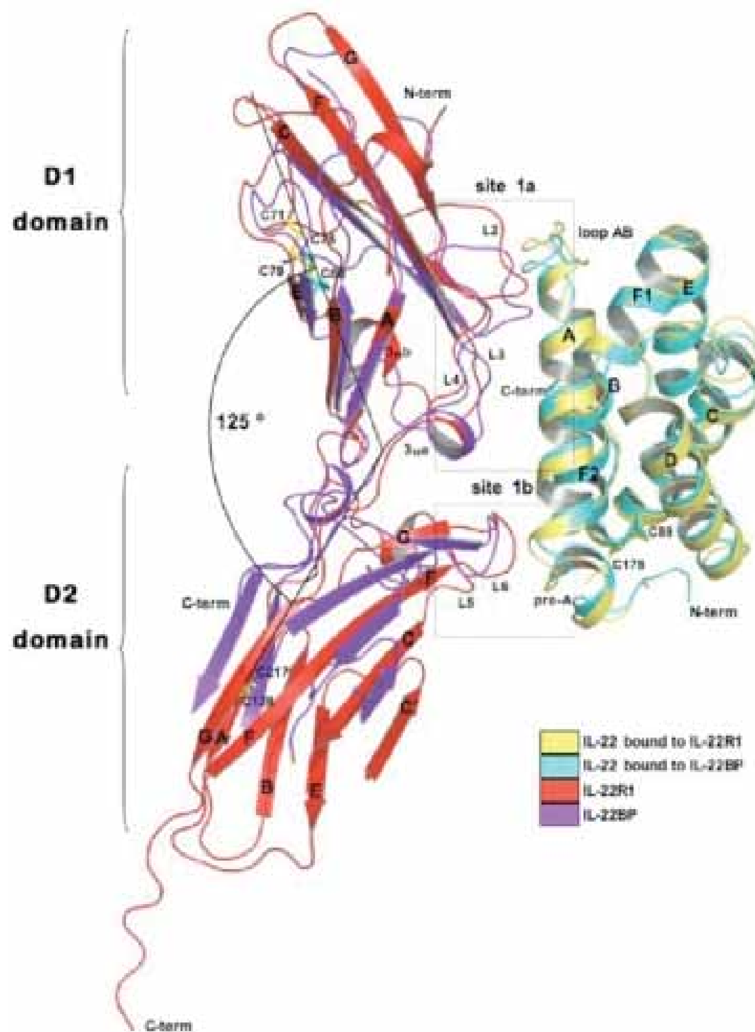

Figure 3. Comparison of IL-22/IL-22BP and IL-22/IL-22R1 binding interfaces. Superposition of IL-22/IL-22BP (cyan/purple blue) and IL-22/IL-22R1 (yellow/red) crystal structures shows their binding interfaces outlined by boxes.

ANTI-CYTOKINE VACCINATION

Beside conventional gene targeting strategies, that were used in our lab to generate mice deficient in the IL-9R, in IL-22 or in IL-22R, we developed a new strategy of anti-cytokine vaccination leading to the production in vaccinated mice of anti-cytokine autoantibody that block the biological activities of endogenous cytokines. Neutralizing auto-antibodies against cytokines such as IL-9, IL-12 and IL-17 have been induced upon vaccination with the autologous cytokines chemically coupled with OVA (IL-9, IL-17) or with the Pan DR T helper epitope PADRE (IL-12). This strategy contributed to demonstrate the role of IL-9 in an intestinal helminth infection (1), of IL-12 in atherosclerosis and of IL-17 in experimental autoimmune encephalomyelitis. More recently, we developed a new procedure of anti-cytokine vaccination by taking advantage of tumor cells as a vaccine against peptides presented at their surface in fusion with a human transmembrane protein. These vaccination methods represent simple and convenient approaches to knock down the in vivo activity of soluble regulatory proteins, including cytokines and their receptors, and are currently validated with additional targets in inflammatory models.

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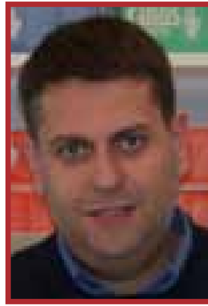

SIGNAL TRANSDUCTION AND MOLECULAR HEMATOLOGY GROUP
STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

Cytokines and their receptors are critical for the formation of mature blood cells and for the function of the immune system. We study the structure and function of several cytokine receptors, such as those for erythropoietin (Epo), thrombopoietin (Tpo), Granulocyte Colony Stimulating Factor (G-CSF), which function as homomeric complexes and of heteromeric receptors such as receptors for interleukins (IL) 2 and 9. Activation of these receptors is triggered by cytokine-induced changes in receptor dimerization/oligomerization, which is transmitted via juxtamembrane and transmembrane domains to the cytosolic region and ultimately to members of the Janus family of tyrosine kinases (JAKs).
Our key interests are: i) the structural basis for transmembrane signaling, especially how transmembrane and juxtamembrane sequences switch-on or -off cytokine receptor signaling; and ii) the mechanisms of JAK attachment to receptors, JAK activation and signaling. The laboratory identified constitutively active oncogenic mutants of JAK2, JAK1 and TYK2 and of cytokine receptors, with some being involved in human blood cancers. Specifically the mechanisms by which JAK2 V617F and TpoR W515 mutants induce, in humans, Myeloproliferative Neoplasms (MPNs), such as Polycythemia Vera, Essential Thrombocythemia or Primary Myelofibrosis are actively pursued. Furthermore, a microRNA was recently identified (miR-28) that appears to be a biomarker for a fraction of MPNs without known molecular cause, and that targets the mRNA for TpoR, inhibiting its translation. A major effort will be dedicated to the identification of molecular defects associated with the latter group of MPNs and with acute myeloid leukemia secondary to MPNs. A close collaborative structure has been created with clinicians and clinical biologists at St Luc Hospital for in-depth study of patient-derived cell

THE MECHANISMS BY WHICH THE MUTANT JAK2 V617F INDUCES POLYCYTHEMIA VERA AND OTHER MYELOPROLIFERATIVE NEOPLASMS IN HUMANS

A. Dusa, C. Pecquet, J.-P.Defour

The JAK-STAT pathway mediates signaling by more than 25 cytokine receptors and is constitutively activated in many cancers. Several mutations in genes coding for JAKs have been identified in the past five years (1). Janus kinases possess two kinase domains, one active and the other, denoted as the pseudo-kinase domain, predicted to be inactive. Four Janus kinases are coded by the human genome JAK1, JAK2, JAK3 and TYK2. JAK2 is crucial for signaling by EpoR, TpoR, the G-CSFR, the interleukin 3 receptor and several other receptors. JAKs are appended to the cytoplasmic juxtamembrane domains of receptors and are switched-on upon ligand binding to the receptors' extracellular domains.

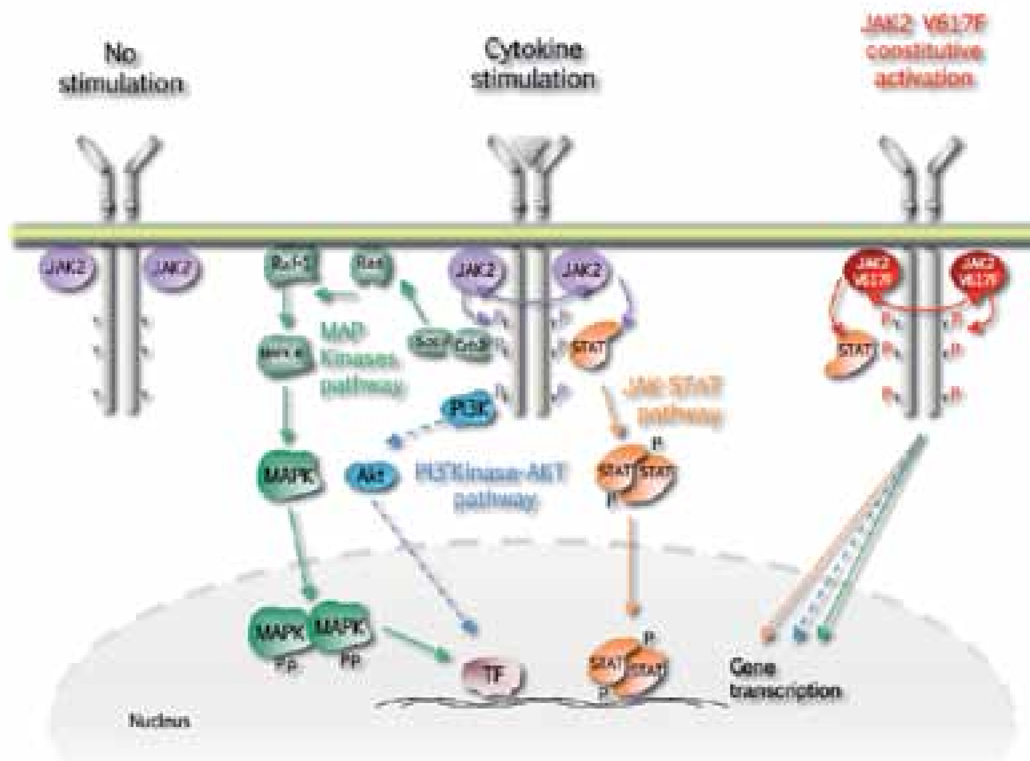

Figure 1. In the absence of cytokine ligands, cytokine receptors (left complex) are preassembled with tyrosine kinases JAK (Janus kinases) in inactive complexes. Cytokine binding to the extracellular domains of receptors (middle complex) induces a conformational change which allows the appended JAKs to cross-phosphorylate and activate each other. In turn, JAKs phosphorylate tyrosine residues (Py) on the cytosolic regions of receptors, which attract SH2- and PTB-containing signaling proteins. These proteins become themselves phosphorylated and either translocate to the nucleus to regulate gene expression (such as STATs, Signal Transducers and Activators of Transcription) or initiate kinase signaling cascades (such as Mitogen Activated Protein-Kinases, MAPK, phosphatydylinositol-3-kinase, PI3K, and Akt). The mutant JAK2 V617F binds to the cytosolic domains of receptors and can trigger signaling in the absence of any cytokine binding to the extracellular receptor domain (right complex). As a consequence, signaling is induced permanently and myeloid progenitors survive, proliferate and differentiate in an uncontrolled manner. (Jean-Michel Heine).

Polycythemia Vera (PV), or the Vaquez-Osler disease, is characterized by excessive production of mature red cells and sometimes of platelets and granulocytes. Two other related diseases, Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) are associated with excessive platelet production and fibrosis (scaring) of the marrow due to excessive myeloid cell proliferation, enzyme release and collagen secretion by marrow fibroblasts.

We postulated that JAK2 is involved in the pathogenesis of these diseases because we showed that JAK2 strongly promotes the maturation and cell-surface localization of TpoR (2), a process that was known to be highly defective in PV.

In collaboration with William Vainchenker at the Institut Gustave Roussy in Paris, we have been involved in the discovery of the unique acquired somatic JAK2 V617F mutation, that is responsible for >98% of Polycythemia Vera and for >50% of Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) cases (3, 4). The mutation in the pseudokinase domain alters a physiologic inhibition exerted by the pseudokinase domain on the kinase domain and allows the mutated JAK2 to bind and activate EpoR, TpoR and G-CSFR in the absence of cytokines (Figure 1). Saturation mutagenesis at position V617 showed that not only Phe, but also Trp, Leu, Ile and Met can activate JAK2, although Trp is the only mutation that exhibits comparable activity with V617F (5). The homologous V617F mutations in JAK1 and TYK2 also enable these kinases to be activated without ligand-binding to cytokine receptors (4). Similarly, at least Trp, Leu and Ile can also activate JAK1, besides Phe, at the homologous V658 position. These results suggested that point mutations in JAK proteins might be involved in different forms of cancers (1). An example of such involvement is the identification of mutants in the pseudokinase domain of JAK1 in approximatively 20% of adult T-lymphoblastic leukemia.

A major effort in the group is geared towards the understanding of how a pseudokinase domain mutation can induce kinase domain activation. The aim is to be able to specifically inhibit mutated JAK2 but not the wild type JAK2, which is crucial for several physiologic processes. Towards that end, we identified pseudokinase residue F595 as absolutely required for constitutive activation by V617F, but not for cytokine-induced activation of JAK2/JAK2 V617F (6). A region around F617 and F595, involving the middle of helix C of the JAK2 pseudokinae domain might be a target for specific JAK2 V617F inhibition (Figure 2).

INVOLVEMENT OF TPOR IN MYELOPROLIFERATIVE DISEASES

C. Pecquet, J.-P. Defour, M. Girardot, E. Leroy

TpoR appears to be central to MPNs: i) most MPN patients strongly down-modulate TpoR levels in megakaryocytes and platelets; ii) mutations in the TpoR intracellular juxtamembrane motif W515 lead to constitutive activation of the receptor, and severe in vivo MPN with myelofibrosis; iii) asparagine mutations in the transmembrane domain of TpoR also activate TpoR and one such mutation has been shown to be associated with ET; iv) alterations of traffic of TpoR to the cell surface can induce thrombocytosis due to insufficient clearance of Tpo and high sensitivity of early megakaryocytes to high Tpo.

We have identified the mechanisms behind the down-modulation of TpoR in MPNs, and showed that JAK2 V617F induces ubiquitinylation, inhibition of recycling and degradation of TpoR (Pecquet et al., submitted). In addition we discovered that Tpo can induce a strong antiproliferative effect in cells that express high JAK2 levels (Pecquet et al., in preparation). This effect can be detected in post-mitotic megakaryocytes (7). We are exploring the precise signaling mediators of this effect and showed that selection against the antiproliferative effect of Tpo occurs in JAK2 V617F

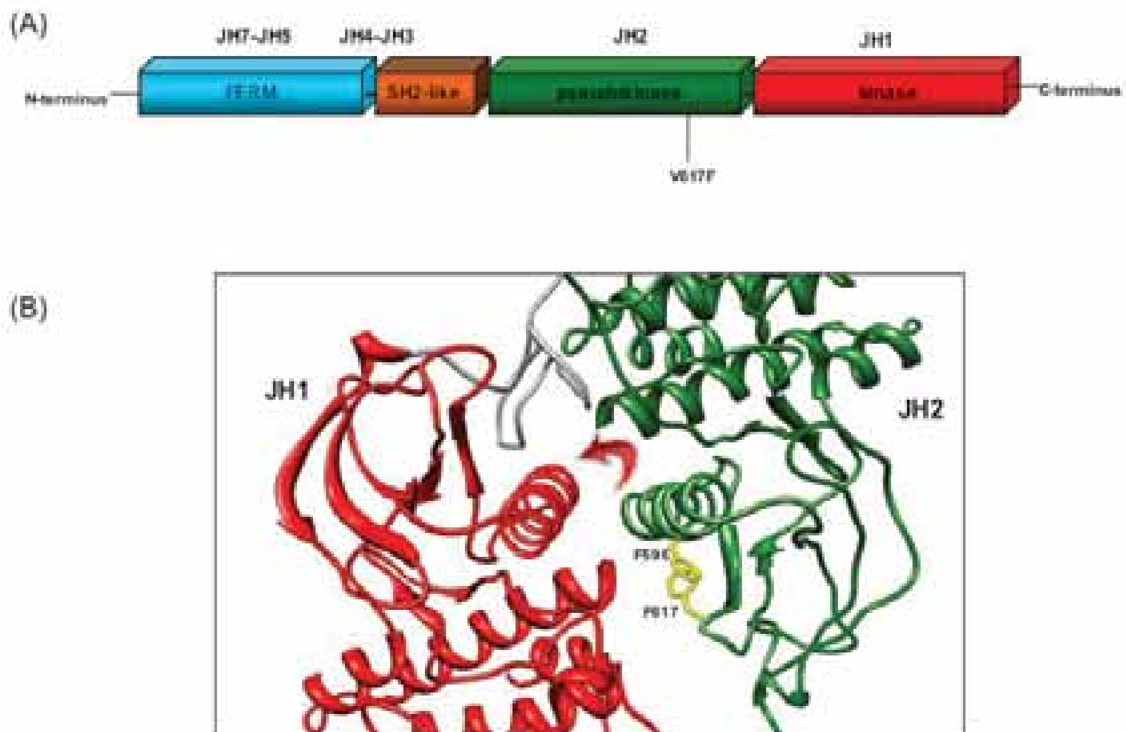

Figure 2. (A) Janus kinase 2 contains several JAK homology domains, JH1, the kinase domain; JH2 the pseudokinase domain; JH3-JH4 the SH2-like domain and JH4-JH7, the FERM (band four point 1, ezrin, radixin, moesin)-like domain. The pesudokinase domain plays a major role in cytokine-dependent activation of the kinase domain, and was implicated in inhibiting the basal activity of the JH1 domain. The V617F mutation is activating the kinase activity of JH1, presumably by preventing the inhibition exerted by JH2 on JH1. The V617F mutation is detected in 98% of PV and approximately 50% of ET and PMF patients. (B) The pseudokinase (JH2) and kinase (JH1) domains of JAK2 are modeled as adopting classical tyrosine kinase structures, interacting with each other and leading to JH1 inhibition. Residue F595 of the helix C of JH2 is required for constitutive activation of JAK2 V617F and of other mutated JAKs proteins, but not for cytokine activation of wild type JAK2. F595 plays a pivotal role in transmitting the conformational change in JH2 to JH1 (red arrow) and eventually in activating the kinase activity of JH1. The region around V617F and the middle of JH2 helix C surrounding F595 could become the target of inhibitors that might specifically decrease constitutive activation of JAK mutants (Alexandra Dusa).

cells, and is partially responsible for TpoR down-modulation in MPN cells, which then continue to proliferate in the presence of Tpo, unlike normal cells.

When the protein sequences of TpoR and the closely related EpoR were aligned, we realized that the TpoR contains a unique amphipathic motif (RWQFP) at the junction between the transmembrane and cytosolic domains. Deletion of this motif (delta5TpoR) results in constitutive activation of the receptor (6), suggesting that these residues maintain the receptor inactive in the absence of TpoR. Mutagenesis of the RWQFP motif showed that W515 (W508 in the murine) is the key residue maintaining human TpoR normally inactive (6). In vivo, in bone marrow reconstituted mice, the delta5TpoR and TpoR W515A induce massive expansion of platelets, neutrophils and immature erythroid progenitors and myelofibrosis by day 45 (Figure 3) (7). Why the

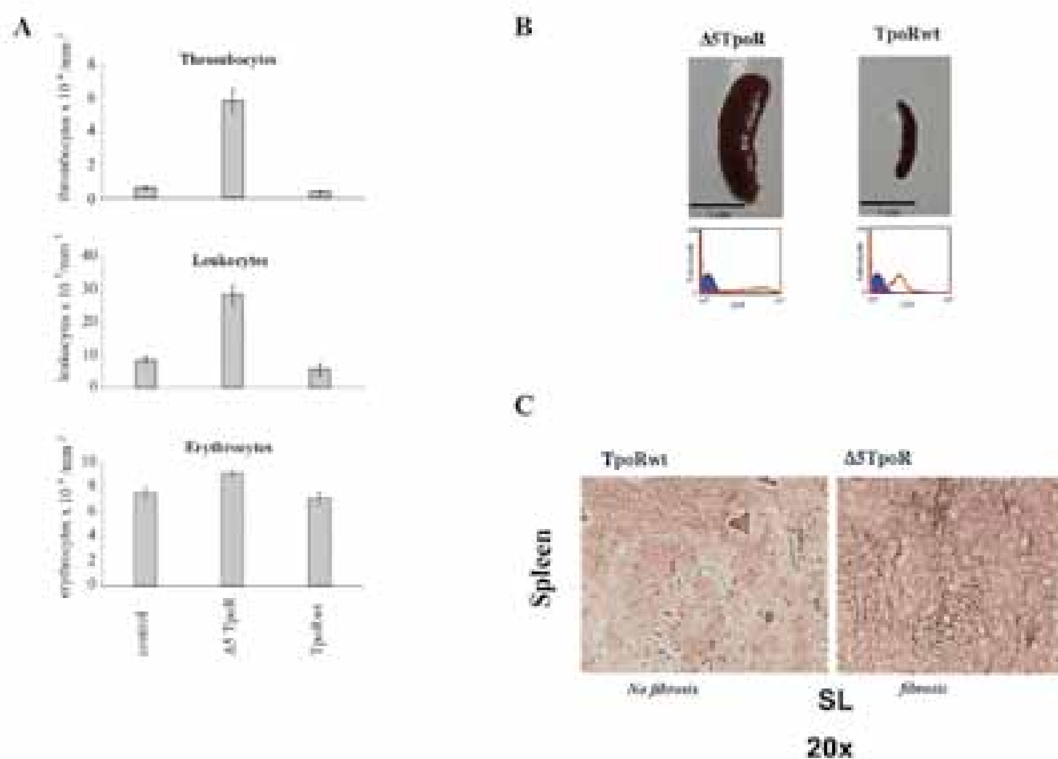

Figure 3. Bone marrow adoptive transfer in lethally-irradiated mice with hematopoietic stem cells expressing the constitutively active Δ5TpoR induces severe myeloproliferative disorder, splenomegaly and fibrosis of the spleen. Δ5TpoR is a mutant where the amphipathic RW[515]QFP motif is deleted, which results in constitutive activation of receptor signaling. (A) Peripheral cell counts recorder 45 days after reconstitution indicates leukocytosis and thrombocytosis induced by Δ5TpoR. (B) Splenomegaly was induced by Δ5TpoR at day 45 post reconstitution. The spleen size in TpoRwt mice was equivalent to that in control healthy mice. Green fluorescence protein (GFP) levels were equal after transduction, but enhanced migration to the spleen and proliferation explain the high GFP levels in Δ5TpoR spleens. (C) Histology of spleen sections of mice reconstituted with the indicated constructs. Silver staining (SL) for reticulin indicates fibrosis of the spleen in the Δ5TpoR mice (Christian Pecquet and Judith Staerk).

phenotype induced by TpoR W515 mutants is much more severe than that of JAK2 V617F is under investigation in our group. We recently established that the myelofibrosis phenotype induced by TpoR W515 mutants depends on cytosolic Y112 (Y626) of TpoR, and appears to involve excessive STAT3 and MAP-kinase signaling (7). Thus, small molecules targeting phosphorylated Y112 (Y626) might be useful in the treatment of myelofibrosis.

CONSTITUTIVE ACTIVATION OF JAK-STAT SIGNALING AND GENES TARGETED BY STAT5 IN TRANSFORMED HEMATOPOIETIC AND PATIENT-DERIVED LEUKEMIA CELLS

M. Girardot, J. Van Hees

Cytokine stimulation of cytokine receptors induces transient activation of the JAK-STAT pathway. In contrast, oncogenic forms of receptors or of JAKs (JAK2 V617F) transmit a

continuous signal which results in constitutive activation of STAT proteins. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors or JAKs in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously (1, 9). A similar picture has been noted in patient-derived leukemia cells. The critical question is which genes are specifically regulated by constitutively active STAT proteins in leukemic cells. Using chromatin immunoprecipitation (Chip) and sequencing of native promoters bound by STAT5 we noted that, in transformed cells, STAT5, and mainly STAT5B, can also bind to low affinity N4 (TTCNNNNGAA) DNA sites, not only to the high affinity N3 sites, which are characteristic of ligand-activated STAT5. We are attempting to identify the promoters actually bound by STAT proteins in living cells in physiologic and pathologic situations. We identified one specific target gene of constitutive active STAT5B signaling in megakaryocytes of MPN patients, namely Lipoma Prefered Partner (LPP) (10), a gene found ot be translocated in rare leukemias. LPP is the host gene for miR-28, which we found to down-modulate TpoR translation, impair megakaryocyte differentiation (10). miR-28 is pathologically overexpressed in 30% of MPNs (10). Targets of miR-28, such as E2F6, are critical cell cycle regulators that might influence the phenotype of myeloproliferative disorders. Furthermore, miR-28 is specifically associated with megakaryocyte proliferation and induces a block in differentiation (10). We are studying the mechanisms of pathologic induction of LPP/miR-28 via constitutively active STAT5.

INTERACTION WITH ST LUC HOSPITAL CLINICIANS AND CLINICAL BIOLOGISTS: IDENTIFICATION OF THE MOLECULAR BASES OF MPNS WITHOUT KNOWN MOLECULAR CAUSE

Under the auspices of an ARC grant (Action de Recherche Concertée of the Université catholique de Louvain) with the St Luc Hospital departments of Hematology (Prof. Cédric Hermans, Prof. Augustin Ferrant, Dr. Laurent Knoops), Clinical Biology (Prof. Dominique Latinne, Dr Hélène Antoine-Poirel) and groups of de Duve Institute (Prof. Mark Rider, Prof. Jean-Baptiste Demoulin) our laboratory is performing a large study on the presence and signaling of JAK2, TpoR, and growth factor receptor mutations in patients with myeloproliferative neoplasms. Next generation sequencing will be employed for well-investigated patients, using primary cells that are characterized for functional defects and that do not harbor known mutations in order to unravel novel molecular defects in MPNs and leukemias.

DETERMINATION OF THE INTERFACE AND ORIENTATION OF THE ACTIVATED EpoR, TpoR AND G-CSFR DIMERS

J.-P. Defour, C. Pecquet, E. Leroy

Epo binding to the erythropoietin receptor (EpoR) results in survival, proliferation and differentiation of erythroid progenitors into mature red blood cells. In the absence of Epo, the cell-surface EpoR is dimerized in an inactive conformation, which is stabilized by interactions between the TM sequences. Epo binding to the extracellular EpoR domain induces a conformational change, which results in the activation of cytosolic JAK2 proteins (8).

To identify the residues that form the interface between the receptor monomers in the activated EpoR dimer we have replaced the EpoR extracellular domain with a coiled-coil dimer of α-helices (9). Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (one), d (four), the register of the coiled-coil α-helices is imposed on the downstream TM α-helix and

intracellular domain.

When each of the seven possible dimeric orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR, only two fusion proteins stimulated the proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells (9). Since the predicted dimeric interfaces of the two active fusion proteins are very close, a unique dimeric EpoR conformation appears to be required for activation of signaling. In this active conformation TM residues L241 and L244 and JM residue W258 are predicted to be in the interface.

Similar studies are undertaken for the related TpoR and G-CSFR. Like the EpoR, the TpoR is thought to signal by activation of JAK2, of several STATs (STAT1, 3 and 5) as well as of MAP-kinase, PI-3-kinase and AktB. However, TpoR and EpoR signal quite differently since only TpoR can induce hematopoietic differentiation of embryonic stem cells or stimulate the earliest stages of hematopoiesis in immature hematopoietic cells. We have shown that TpoR can signal from several distinct dimeric interfaces, and that besides the normal dimeric interface that leads to formation of platelets, other interfaces promote signaling that leads to myeloproliferative and myelodysplastic disorders (Staerk et al., submitted).

STRUCTURE AND FUNCTION OF JUXTA- AND TRANS-MEMBRANE SEQUENCES OF CYTOKINE RECEPTORS

R.-I. Albu, A. Dusa, J.-P. Defour, J. Van Hees, C. Mouton

We have previously shown that the EpoR as well as a fraction of IL2/IL9 receptors exist on the cell surface as a preformed ligand-independent inactive dimers (homomeric and heteromeric in the case of IL2/IL9 receptor complexes). For the EpoR, transmembrane domain interactions stabilize the inactive dimer at the surface and the EpoR TM sequence is an example of TM dimer based on purely hydrophobic sequences (Proc. Natl Acad. Sci USA 2001, 98, 4379-84; EMBO J., 1999, 18, 3334-47). We study potential transmembrane interactions in the context of other transmembrane proteins, such as TpoR, G-CSFR. We use cell surface immunofluorecence co-patching of differentially epitope tagged receptors and protein ligation assays in order to determine the ligand-independent state of cell surface complexes. Preformed cytokine receptor oligomers might be important for supporting signaling by mutated JAKs in the absence of ligand. In addition to cytokine receptors, we study the role of transmembrane dimerization in the amyloidogenic processing of Amyloid Precursor Protein (APP) in collaboration with the group of Prof. Jean-Noel Octave. We identified three Gly-X-X-X-Gly motifs in the juxtamembrane and transmembrane domain of APP and showed that these motifs promote amyloidogenic processing of APP (J. Biol. Chem. 2008 283, 7733)

TRAFFIC OF CYTOKINE RECEPTORS TO THE CELL-SURFACE

R.-I. Albu, C. Pecquet

We have observed that, in hematopoietic cells, over-expression of JAK proteins leads to enhanced cell-surface localization of cytokine receptors (i.e. EpoR TpoR, IL9R, IL2R, γ_c). For some receptors, the effect of the cognate JAK is to promote traffic from the endoplasmic reticulum (ER) to the Golgi apparatus, while for others, such as the TpoR, JAK2 and TYK2 also protect the mature form of the receptor from degradation by the proteasome, and thus JAKs enhance the total amount of cellular receptor (2). In collaboration with Pierre Courtoy, we are employing confocal microscopy of epitope

tagged receptors in order to define the precise intracellular compartments where receptors and JAKs interact. Our working hypothesis is that the N-terminus FERM domain of JAK proteins exerts a generic pro-folding effect on cytosolic domains of cytokine receptors. Furthermore, the extracellular fibronectin type III modules of TpoR (D1, D2, D3 or D4) appear to be critical for efficient cell surface localization of TpoR, which in turn is essential for clearance of Tpo by circulating platelets. We have identified critical determinants in the extracellular domain of TpoR traffic. While a receptor lacking D1D2 (D3D4-TpoR) is not efficiently localized at the cell surface, introducing the juxtamembrane W515K activating mutation and selection for cytokine-independent growth leads to enhanced expression and detectable cell surface localization of the N-terminally truncated D3D4-TpoR. These results support the notion that cell surface localization is a prerequisite for constitutive signaling by TpoR W515 mutants. The roles of precise extracellular domain glycosylation motifs in TpoR traffic and signaling are also determined.

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LINKS

Group:
Ludwig Institute for Cancer Research Ltd.
NewsLink Sept 2005 of our group (http://www.licr.org/12124501528/newslink/0509/)

European Commission Marie Curie Research Training Network ReceptEUR (www.recepteur.org)

RESEARCH

Whitehead Institute for Biomedical Research, MIT, Lodish Lab (http://www.wi.mit.edu/lodish/)

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