Research

at the

de Duve Institute

and

Brussels Branch of the Ludwig Institute for Cancer Research

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DE DUVE INSTITUTE

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de Duve Institute

LICR

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 institution 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 (UCL). This campus is located in Brussels. The University hospital (Clinique 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 since that time. 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.

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 the Rector of the University of Louvain, one of the Pro-rectors, the General Administrator of the University and the Dean of the Faculty of Medicine. Also present in the Board of directors are eminent members of the business community.

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 a limited endowment, which 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.

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In 2009, 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 2009 the Institute has been able to allocate the following fellowships, entirely supported by our donors :

The «Haas-Teichen» fellowship was attributed to Jhansi KOTA,

the «Pierre Lacroix» fellowship to Shreedhara GUPTA,

the «Maurange» fellowship to Sandrine MEDVES,

other fellowships have been awarded by the Institute to Reece MARILLIER and to Nicolas DIF.

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

The basic aim of our research is to get insights into the molecular mechanisms underlying a variety of disorders of cardiovascular and skeletal systems, as well as certain cancers. We are especially interested in evaluating the importance of genetic variation in human disease development. 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 obtained 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, V. Wouters, N. Limaye, M. Uebelhoer, J. Soblet, V. Aerts, 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. We employed the DHPLC system, which allows for more efficient and sensitive screening for such mutations, and identified several novel activating mutations amongst affected families (Fig. 1). We hypothesized that as the lesions are localized, a somatic second hit might be needed in the normal allele of the TIE2 gene, for lesions to develop. 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 [1]. In addition, we discovered that at least 50% of the far more common sporadic VMs are caused by somatic mutations in TIE2 [1]. On this basis, we suggest somatic changes to be a general genetic mechanism in vascular malformations [2]. 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 is also being 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 34 different mutations in 128 families. As in VMs, we showed that GVM lesions appear locally because of the additional alteration of the second allele, likely in vascular smooth muscle cell precursors. To better understand the role of glomulin in normal and pathological conditions, we have created glomulin-deficient mice. While heterozygotes appear normal, homozygous

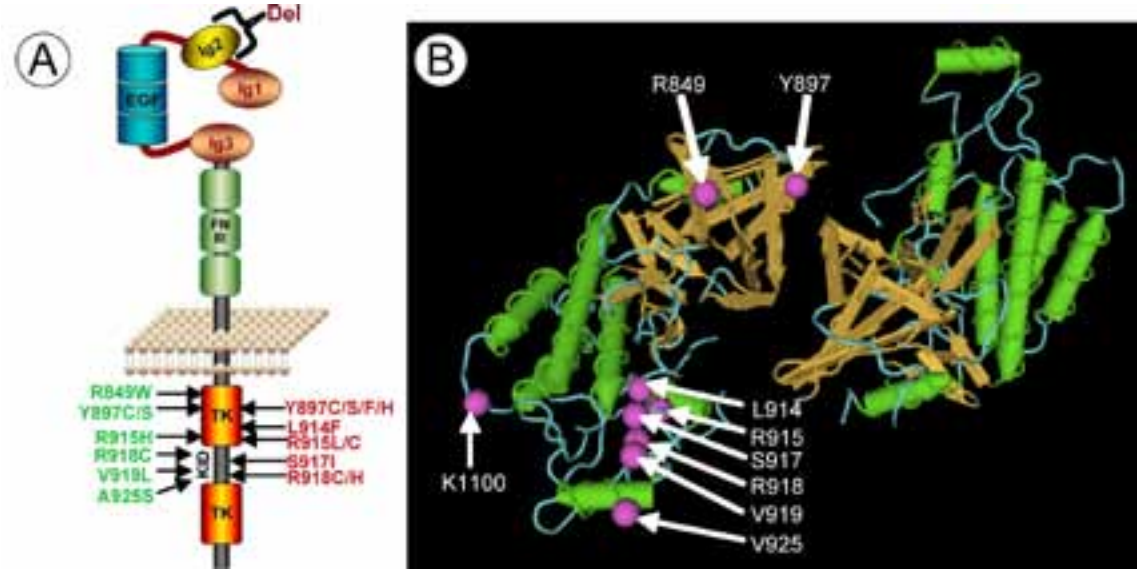

Fig 1. Intracellular TIE2 mutations identified in VM. (A) Inherited changes in VMCM blood samples (left, in green) and somatic changes in sporadic VM lesions (right, in red). A somatic 2nd-hit deletion of the Ig2 ligand-binding domain ("Del") was identified in a lesion from a VMCM patient carrying R849W. (B) The positions of the mutated residues in the intracellular region of TIE2, shown as a dimer.

knockouts are embryonic-lethal. To enable studies beyond this time-point, we generated RNAi conditional knockdown mice, in which glomlin inactivation is cre-inducible. When triggered during embryonic development, glomulin depletion is likewise lethal. We are currently phenotyping these animals further. These mice provide new tools to continue the in vitro and in vivo characterization of glomulin expression and function.

LYMPHEDEMA

A. Ghalamkarpour, A. Mendola, L.M. Boon and M. Vikkula in collaboration with K. Devriendt, KUL; D. Chitayat, Hospital for Sick Children, Toronto, Canada; K. Alitalo, Haartman Institute and Helsinki University, Finland.

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 lifetime problem with progressive swelling of extremities. We use genetic approaches to unravel the pathophysiology. In some families with Nonne-Milroy disease, missense inactivating mutations in the VEGFR3 gene were identified. We also recently found that VEGFR3 mutation can cause hydrops fetalis in Nonne-Milroy transmitting families. Moreover, some sporadic congenital primary lymphedemas are also explained by a VEGFR3 mutation. 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 and 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 have begun to use 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 perturbations of the vascular endothelial growth factor (VEGF) signaling pathway can cause hemangioma pathogenesis [6].

CMs occur in 0.3% of newborns. Unlike

hemangiomas, they stay 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, a 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 54 additional families with RASA1 mutation, accounting for about 30% of those affected. 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 30% 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 responsible for cleft palate only and Pierre Robin sequence [Ghassibe et al, In Prep]. This gene is associated with clefts across populations. Zebrafish studies confirmed its role during embryonic development and jaw formation. 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 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" hy-

pothesis, 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 [8]. 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 (Palm et al, In Prep).

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 [9]. We also described a family with a very rare presentation of severe head and neck paraganglioma with liver and spine localization [10]. 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 antihypertensive 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

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

The genetic nature of hematological malignancies (mainly leukemias, myelodysplasias, chronic myeloproliferative disorders, lymphomas, myelomas) has been clearly established. Despite this, we lack biomarkers for diagnosis, prognosis and treatment, for clinical management as well as for a better understanding of the genetic and epigenetic processes leading to tumorigenesis. Towards this end, we use a variety of techniques including conventional and molecular cytogenetics or FISH, molecular biology, and microarray approaches.

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. Furthermore, 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

M. Amyere and M. Vikkula

We host the UCL DNA-oligonucleotide microarray platform (Affymetrix). This platform is currently used by several groups in the de Duve Institute and UCL for expression profiling as well as genotyping. In collaboration 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. 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 [Foulquier et al, In Prep]. In collaboration with Dr Vermeesch 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 establishes 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].

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LIVER AND PANCREAS DEVELOPMENT

The group studies the molecular and cellular mechanisms that govern differentiation and morphogenesis of the liver and pancreas, two organs which derive from the endoderm (primitive gut of the embryo). The fundamental knowledge gained by this work is essential for understanding the pathophysiology of organ malformations, such as polycystic diseases, and for improving cell therapy of liver and pancreatic diseases, such as hepatic deficiencies (e.g. metabolic deficiencies and cirrhosis) and diabetes.

LIVER DEVELOPMENT

A. Antoniou, R. Carpentier, I. Laudadio, S. Margagliotti, 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.

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 factors 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.

We have recently identified the transcription factor Sox9 as the earliest and most specific biliary cell marker in liver development. Using Sox9 in combination with hepatoblast markers, we analyzed the morphogenesis of the bile ducts and found that it 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 are currently investigating how this new knowledge impacts on the interpretation of congenital malformations of the bile ducts.

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 [5]. 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. We found that the Onecut factors HNF-6 and OC-2 are required for liver expansion at the onset of liver development [6]. They are also critical for normal differentiation of hepatic precursor cells to hepatocytes or cholangiocytes [2], and their

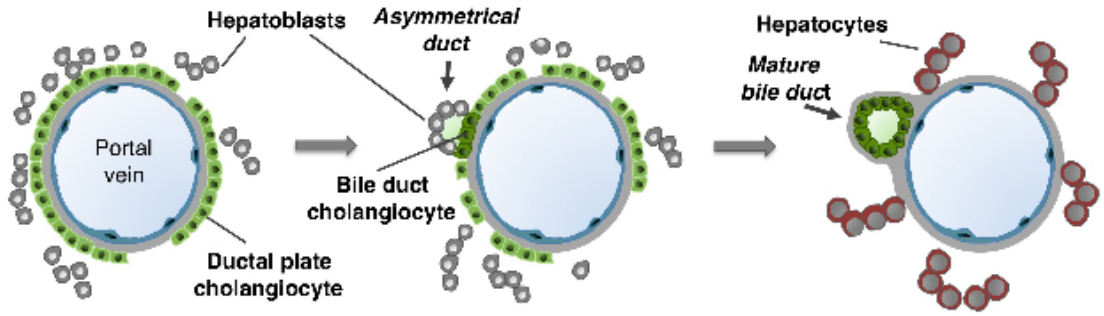

Fig. 1. Development of bile ducts. Left panel: In embryos, hepatoblasts (grey) differentiate to ductal plate cholangiocytes (green) which form a ring of cells around the branches of the portal vein (blue). Middle panel: hepatoblasts become apposed to the ductal plate cells and delineate a developing duct. The latter is asymmetrical as it is lined on the portal side by cholangiocytes (dark green) and on the parenchymal side by hepatoblasts. Right panel: when the ducts have matured, they are entirely lined by cholangiocytes, while parenchymal hepatoblasts have differentiated to hepatocytes (brown) which line up as cords (from Si-Tayeb K, Lemaigre FP, Duncan SA. Organogenesis and development of the liver. Dev. Cell, 18, 175-189, 2010).

level of expression during hepatocyte differentiation determines time-specific gene activation in the liver [7]. Current work focusses on the molecular mechanisms by which HNF-6 and OC-2 fine-tune gene expression at several stages of hepatocyte differentiation. This involves work on the function of microRNAs that repress or are transcriptionally regulated by HNF-6 and OC-2 [8].

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 which is 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.

The Onecut transcription factor HNF-6 is required for endocrine cell development [9]. In HNF-6 knockout mice, endocrine cells fail to develop in the embryo and this results from the lack of HNF6 in pancreatic progenitor cells. In the latter, HNF-6 is required to activate the gene coding for Ngn3, a pro-endocrine transcription factor. We have now looked for microRNAs that are downstream of HNF-6 in pancreatic progenitors cells. We identified several whose expression is stimulated by HNF-6, and we are in the process of characterizing their function. We also found that miR-495 and miR-218 repress translation of HNF-6 and OC-2, respectively, and we characterized the microRNA population that is expressed at the earliest stage of pancreas development [8].

Interestingly, in mice deficient in HNF-6, the endocrine cells and islets of Langerhans have not developed at birth. However, five weeks later, islets of Langerhans have formed. We investigated the source of these neogenic endocrine cells and obtained evidence, including from genetic lineage tracing studies, that Hnf6-/- duct cells can give rise to the endocrine cells (Figure 2). This suggests that in normal conditions, HNF-6 maintains the identity of duct cells, but that its absence in these cells favours their transdifferentiation towards endocrine cells. We are currently investigating how HNF-6 maintains pancreatic duct cell identity.

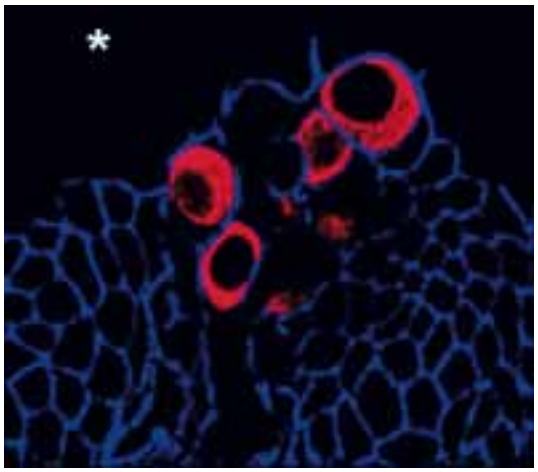

Fig. 2. Endocrine cells differentiate from duct cells in postnatal Hnf6-/- pancreas. Endocrine cells expressing insulin (red) become detectable in the epithelium (e-cadherin staining, blue) lining the lumen (star) of the pancreatic ducts.

CONCLUSIONS

Our work on the signaling pathways and transcription factors in developing liver and pancreas opens perspectives for understanding the pathophysiology of liver and pancreatic congenital diseases. The application of our findings to the programmed differentiation of cultured stem cells should help developing cell therapy of hepatic deficiencies and of pancreatic diseases such as diabetes.

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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 examine how certain DNA repair processes lead to the insertion of mitochondrial DNA sequences into the nuclear genome. We are also exploring the mechanisms by which tumor cells maintain their telomeres to acquire immortality. We have demonstrated that epigenetic alterations in tumors, 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 tumor cells.

DNA DAMAGE REPAIR IN FISSION YEAST *SCHIZOSACCHAROMYCES POMBE*

S. Lenglez, A. Decottignies

DNA repair processes have been well conserved throughout evolution, and yeast has proven to be a good model for their study. We use *S. pombe* to dissect the mechanisms of DNA double-strand break (DSB) repair, a type of genetic lesion arising after exposure to genotoxic agents or during DNA replication. Chromosomal and extrachromosomal DSBs can be induced experimentally in virtually any kind of cell. Such systems led to the dissection of the two major mechanisms of DNA repair: homologous recombination (HR) and non-homologous end-joining (NHEJ). In the lab, fission yeast was used to investigate genetic requirements for microhomology-mediated end-joining (MMEJ), a third DNA repair process that was shown to be related to HR (1). 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 (2) 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, remodeling

the nuclear genome. 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 (Lenglez et al., Genome Research, in revision).

IMPACT OF TELOMERASE ON TGF-β AND NF-κB SIGNALING 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).

In addition to its role in telomere length maintenance, hTERT has been reported to play non-canonical roles in the cell, including modulation of expression of genes implicated in tumorigenesis, through mechanisms that are still largely unknown. Accordingly, it was suggested that, for a given type of cancer, patients with TEL+ tumors may have a poorer pronostic than patients suffering from ALT tumors. We are interested in understanding how telomerase may impact on gene expression in fibroblasts and, more specifically, how telomerase may modulate TGF-β and/or NF-κB signaling, two pathways frequently deregulated in tumors. Our data suggest that hTERT may impact on these two signaling patrhways through both reduced production of reactive oxygen species and nuclear accumulation of RelA/p65 subunit of NF-κB.

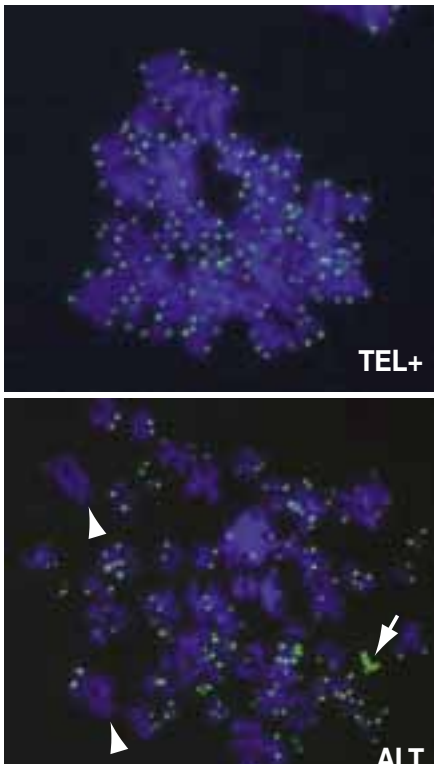

Fig. 1. Telomere-specific fluorescence in situ hybridization (FISH) on metaphase chromosomes of telomerase-positive (*TEL+*) and ALT cancer cells (*ALT*). Telomeres are hybridized with a fluorescent telomeric probe (*green*) and DNA is stained with DAPI (*blue*). In ALT cells, telomeres are very heterogeneous,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

DNA HYPOMETHYLATION AND ABERRANT GENE ACTIVATION IN CANCER

A. Loriot, G. Parvizi, 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 (5).

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 (6). 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 (6-8). The factors that are responsible for the initial DNA demethylation process and for maintaining cancer-germline gene promoters unmethylated remain to be identified. Genetic screenings with a lentiviral human shRNA library, directed against ~20,000 genes, are currently being performed to identify such factors.

DNA METHYLATION CHANGES ASSOCIATED WITH CELL SENESCENCE AND IMMORTALIZATION

G. Tilman, A. Loriot, 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 (9). 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 (9).

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. We are currently analysing the histone modifications at telomeres of ALT and TEL+ cells.

We 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. We also wish to compare the DNA methylation profiles of ALT and TEL+ cells that we expect to arise from SV40 T and RasV12-expressing fibroblasts. The experiments are also performed in other cell types, including melanocytes and mammary epithelial cells.

IMPACT OF TELOMERIC TRANSCRIPTS ON HUMAN TELOMERE REPLICATION

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 telomere replication. Because of the reduced methylation level of subtelomeric DNA in ALT tumor cell lines (9), these cells produce high levels of TERRAs. We are interested in testing whether (and how) TERRAs may affect telomere replication in human cells. During the past few months, we developped the tools to start investigating this.

DNA METHYLATION OF CANCER-GERMLINE GENES IN HUMAN EMBRYONIC STEM CELLS

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

As new methylation patterns are established during early embryo development, embryonic stem (ES) cells provide a suitable experimental system for investigating the molecular mechanisms underlying this epigenetic reprogramming process. ES cells possess both DNA demethylation and de novo methylation activities. Each of these opposing activities appears to be targeted to selected DNA sequences. The mechanisms underlying this targeting are still unclear, but likely involve sequence-specific DNA binding proteins and chromatin modifying enzymes. We recently initiated studies on the epigenetic regulation of cancer-germline genes in human ES cells.

We found cancer-germline genes to be repressed and methylated in human ES cells (obtained from Dr. D. Melton, Harvard University, MA), as well as in human embryonal carcinoma (EC) cells, the malignant counterparts of ES cells (10). This indicates that cancer-germline genes are programmed for methylation-mediated silencing in human ES cells. Our data suggest that the repression of cancer-germline genes in EC cells involves the action of the two de novo DNA methyltransferases DNMT3A and DNMT3B, and that local recruitment of these enzymes is associated with specific histone modifications, including underacetylated

histone H3 and unmethylated lysine 4 of the same histone (H3K4). We are currently searching to identify the factors that mediate these histone modifications. Loss of function of such factors may be a prerequisite for demethylation and activation of cancer-germline genes in tumors. Interestingly, we observed that demethylation and activation of MAGEA1 in tumor cells is associated with a marked gain of H3 acetylation and H3K4 methylation.

DEVELOPING PREDICTIVE MARKERS OF RESPONSE TO CHEMOTHERAPY IN BREAST CANCER PATIENTS

F. Fontaine, 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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PROTEIN REPAIR AND INBORN ERRORS OF METABOLISM

Our laboratory has a longstanding interest in the metabolism of carbohydrates and related compounds. The study of the mechanism of formation of an intriguing phosphate ester, fructose 3-phosphate, led us to identify fructosamine 3-kinase, an enzyme serving to remove sugar adducts from proteins. Other enzymes are potentially implicated in protein deglycation and we try to understand their role. Our group aims also at identifying enzymes that are potentially implicated in inborn errors of metabolism.

PROTEIN DEGLYCATION

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

Chronic elevation of the blood glucose concentration in diabetes appears to be responsible for the long-term complications of this disease. The link between the elevated concentration of glucose and the development of these complications is not clear. One of the theories on this link emphasizes the role of fructosamines. These are formed through a spontaneous reaction (known as 'glycation') of glucose with primary amines, followed by an Amadori rearrangement. Fructosamine 3-kinase (FN3K) is a recently identified enzyme that phosphorylates both low-molecular-weight and protein-bound fructosamines (3). Fructosamine 3-phosphates are unstable, breaking down spontaneously to 3-deoxyglucosone, inorganic phosphate and the amino compound that originally reacted with glucose (Fig. 1).

That FN3K indeed acts as a 'deglycating' enzyme was first indicated by experiments in which erythrocytes were incubated ex vivo with an elevated concentration of glucose, with or without a competitive inhibitor of FN3K. These studies showed also that only part of

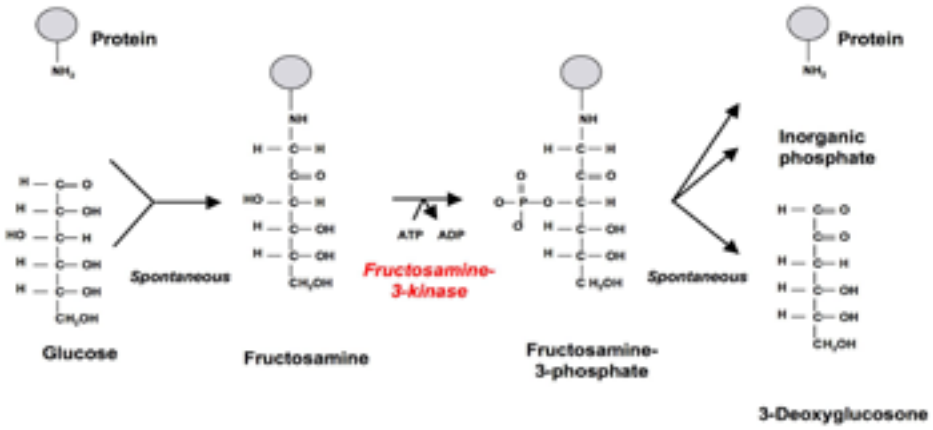

Figure 1. Formation and breakdown of fructosamines

the fructosamines—those that are accessible to FN3K— are cleared in this way. The role of FN3K in deglycation was confirmed and extended by analysis of a mouse FN3K knockout model that we created (8). Protein-bound fructosamines are increased by a factor of about 2 in FN3K-deficient mice. This applies only to intracellular proteins, consistent with the fact that FN3K is a cytosolic enzyme.

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 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.

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, M. Veiga da Cunha, E. Wiame, 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 40 % of the patients with D-2-hydroxyglutaric aciduria.

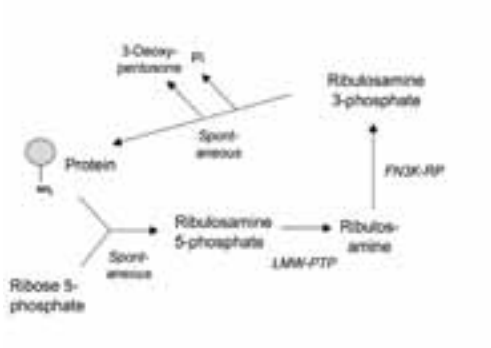

Figure 2. Formation and breakdown of ribulosamines

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. One of our aims is to produce a mouse model of L-2-hydroxyglutaric aciduria in order to confirm the origin of L-2-hydroxyglutarate and understand the physiopathological mechanisms of this disease.

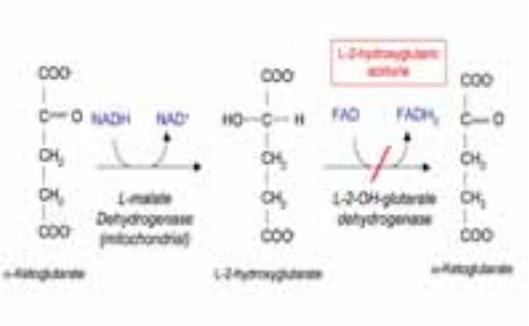

Figure 3. Formation and breakdown of L-2-hydroxyglutarate

FRUCTOSAMINES, MANNOSE BINDING LECTIN AND DIABETES COMPLICATIONS

J. Fortpied, E. Van Schaftingen, in collaboration with Didier Vertommen, Horm Unit

Complement activation via the mannose-binding lectin (MBL) pathway has been proposed to play a role in the pathogenesis of diabetic vascular complications. Since protein glycation is increased in diabetes, we tested the possibility that the glycation product fructoselysine is a ligand for MBL and that its interaction with this protein may initiate complement activation.

We investigated the binding of MBL to fructoselysine by chromatography of human serum on fructoselysine-Sepharose, followed by Western blot and mass spectrometry analysis. We also performed ELISA assays using purified MBL and fructoselysine-derivatized (binding assay) or mannan-coated plates (inhibition assay). Complement activation was determined by the fixation of C3d following incubation of fructoselysine-derivatized plates with serum from subjects producing different levels of MBL.

MBL and its associated proteases were selectively purified from serum by chromatogra-

phy on fructoselysine-Sepharose. Competition experiments indicated that MBL had a similar affinity for mannose, fructose and fructoselysine. MBL bound, in a highly cooperative manner, to fructoselysine-derivatized plates. This binding was associated with complement activation and was much lower with serum from subjects with low MBL genotypes. From this we could conclude that MBL binding to fructoselysine and the ensuing complement activation may provide a physiopathological link between enhanced glycation and complement activation in diabetes. The cooperative character of this binding may explain the high sensitivity of diabetic complications to hyperglycemia (5).

IDENTIFICATION OF ENZYMES POTENTIALLY IMPLICATED IN METABOLIC DISEASES

F. Collard, J. Drozak, S. Jaisson, G. Tahay, E. Wiame, G. Connerotte, K. Peel, E. Van Schaftingen, M. Veiga-da-Cunha

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 P. Courtoy, 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 this most abundant amino acid derivative in neurons and for the diagnosis of hypoacetylaspartia in other patients.

Formation of mercapturic acids

NAT8 shares about 30 % identity with NAT8L (aspartate N-acetyltransferase). It is expressed in kidney and in liver. We could show that this enzyme corresponds 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). In collaboration with Donatienne Tyteca and Pierre Courtoy (Cell Unit), we found that like NAT8L, NAT8 is associated with the endoplasmic reticulum thanks to a non-classical targeting signal that we are now characterizing. NAT8 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 xenobiotic, 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 (4). 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 (4). 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.

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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 this activity, 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 antileukaemic 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 analogues

MECHANISMS OF ACTION

F. Bontemps, C. Smal, L. Bastin-Coyette, E. de Viron, A. Aerts, 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- to 100-fold) to CdA than other human lymphoblastic cell lines. This could be explained by a lower intracellular accumulation of CdATP, the active metabolite of CdA, due to a reduced dCK activity. Unexpectedly, DNA synthesis, measured by thymidine incorporation into DNA, was found to be increased in these cells, up to 2-fold, after a 24 h-incubation with CdA at concentrations close to the IC50 (5 μM) (2). Analysis by flow cytometry, using double labelling with propidium iodide and bromodeoxyuridine, has also shown that CdA provoked an increase in the proportion of cells in S phase, synthesising actively DNA. These results contrasted with those reported in other leukaemic cell lines, like CCRF-CEM cells, in which CdA inhibits DNA synthesis. Kinetics and synchronisation experiments have shown that the increase in the proportion of cells in S-phase induced by CdA in EHEB cells was due to a stimulation of the progression of cells from G1 to S phase. This unexpected result has led us to examine the effects of CdA on proteins involved in the regulation of cell cycle, particularly the cyclin-dependent kinases and p21, and to investigate whether this activation of the transition G1-S contributes to CdA-induced apoptosis in EHEB cells.

In parallel, we have analysed the mechanisms of cell death induced by 2-chloroadenosine (2-CAdo) in various types of leukaemic B-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 Mcl-1 protein level. Prevention of ATP loss by inhibition of AMP deaminase reduced apoptosis, indicating that ATP depletion plays an important role in 2-CAdo-induced apoptosis (3).

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 abolished. 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 (4). This study was performed in collaboration with Dr Knoops from the Ludwig Institute for Cancer Research (Brussels). The following step is to investigate the role of PLK2 during PNA-induced apoptosis.

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 (5) or UV-light, resulted in synergistic cytotoxicity in CLL lymphocytes, due to inhibition of DNA repair (6). 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. Aerts, 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. Our study of dCK was initiated by the discovery, by others and by us, that dCK activity can be increased by various genotoxic agents, including CdA, aphidicolin, etoposide, and UV-C irradiation, without an increase of dCK protein level. Therefore, a post-translational activation of dCK by intracellular signalling pathways was suggested. To investigate this hypothesis, we first analyzed the effect of a variety of activators and inhibitors of protein kinases on dCK activity. We found that some of them could modify dCK activity in several types of leukaemic cells, strengthening the hypothesis of a phosphorylation/dephosphorylation process as a mechanism of regulation of dCK activity. Most particularly, our results indicated that dCK could be down-regulated by the JAK/MAPK pathway and that it might be an in vivo target of protein phosphatase 2A (8).

We then overexpressed dCK in HEK 293T cells and observed that the enzyme was labelled after incubation with [32P]orthophosphate, confirming that dCK is a phosphoprotein. Tandem mass spectrometry performed by Dr D. Vertommen and Prof. M.H. Rider from the Horm-Phos 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 demonstra-

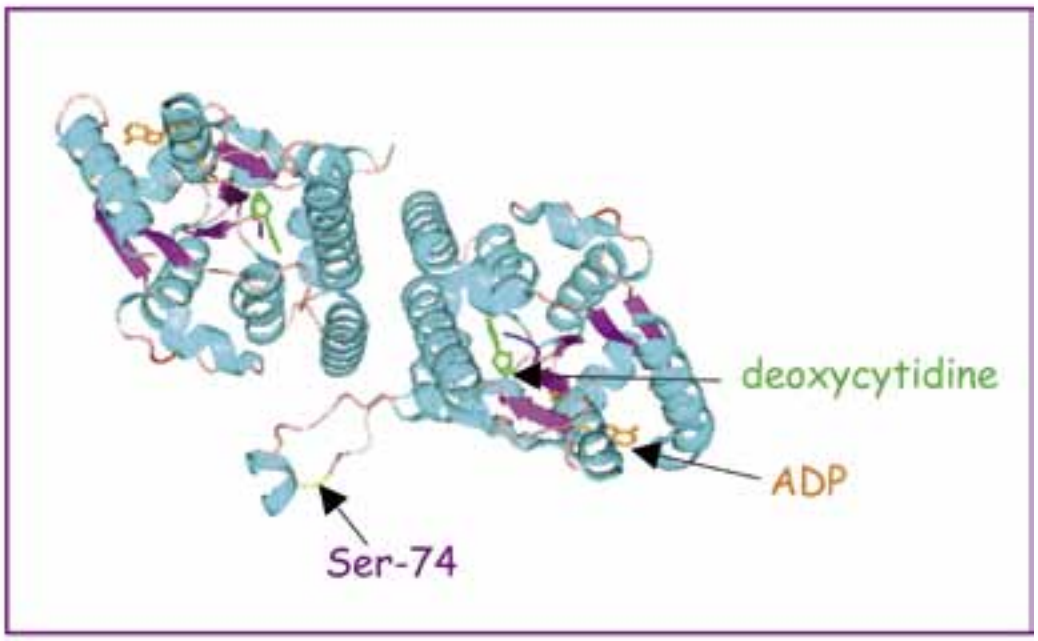

Figure 2. Three-dimensional structure of deoxycytidine kinase in complex with ADP and deoxycytidine. The Ser-74 phosphorylation site is located at a very flexible 15-residue insert (Ser-63-Asn-77) easily accessible to cellular protein kinases. The N-terminal extremity of dCK (residues 1-19) was flexible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

ted that Ser-74 phosphorylation was crucial for dCK activity in HEK 293T cells, whereas phosphorylation of other identified sites did not seem essential (9). Phosphorylation of Ser-74 was also observed on endogenous dCK in CCRF-CEM cells and in lymphocytes from CLL patients, in which Ser-74 phosphorylation was increased by several genotoxic agents (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 (10). To conclude, our work has demonstrated that dCK activity in leukaemic cells largely depends on the phosphorylation state of Ser-74. 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. Moreover, 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 *Escherichia coli*, disulfide bonds are introduced in the periplasm by the Dsb (Disulfide bond) protein family (1, 9).

The primary oxidant is the soluble protein DsbA. DsbA has a CXXC catalytic site motif present within a thioredoxin fold. 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

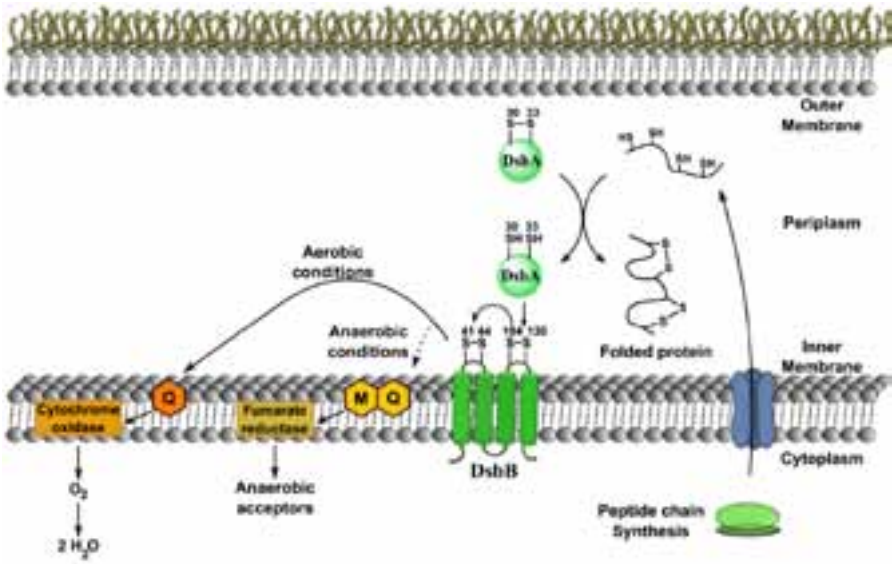

Figure 1. Disulfide bond formation in the *E. coli* periplasm. Disulfides are introduced into folding proteins by DsbA. DsbA is re-oxidized by DsbB. Electrons flow from the reduced proteins to the CXXC motif of DsbA and then to DsbB. DsbB transfers electrons to the respiratory chain (adapted from (1)).

residues that are non-consecutive in the amino-acid sequence, DsbA can form incorrect 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. Recently, we have 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 (7).

We have also characterized the DsbA proteins present in the pathogenic bacteria *Neisseria meningitidis*, 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. Uniquely among bacteria, *N. meningitidis* possesses three genes encoding active DsbAs: NmDsbA1, NmDsbA2 and NmDsbA3. In collaboration with Laurence Serre and Céline Lafaye (Grenoble, France), we have characterized the neisserial enzymes biochemically and structurally. We found that the most striking feature shared by all three is their exceptional oxidizing power. With a redox potential of -80 mV, they are by far the most oxidizing thioredoxin-like enzymes known to date (6).

One of our long-term goal is to understand how disulfide bond formation is coordi-

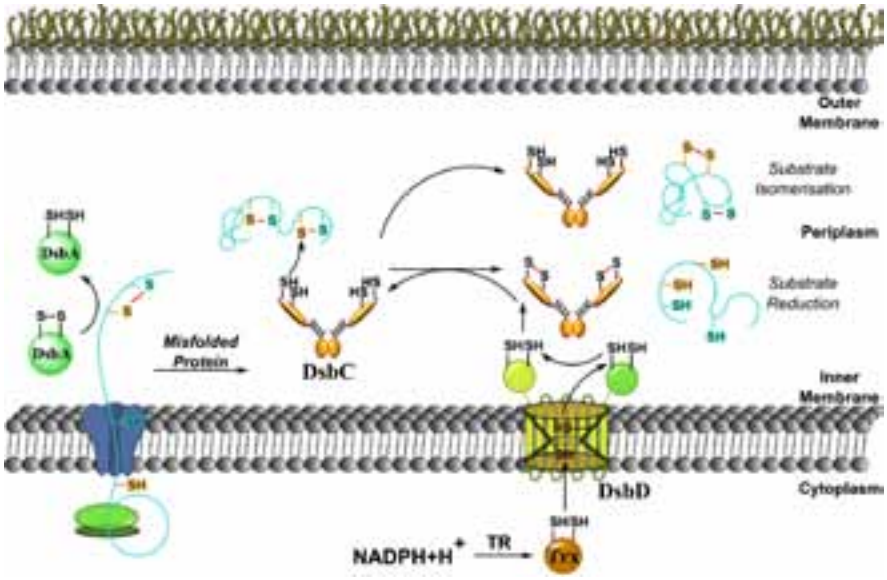

Figure 2. Disulfide bond reduction/isomerization in the periplasm. DsbA can introduce non-native disulfides when they need to be formed beween non-consecutive cysteines. Incorrect disulfides are either isomerized or reduced by DsbC. Oxidized DsbC is regenerated by the membrane protein DsbD that transfers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

nated with the protein secretion and protein folding processes. In particular, we would like to understand how the Dsb proteins cooperate with the chaperones present in the periplasm. However, our understanding of the global folding process is hampered by the poor characterization of the roles of the periplasmic chaperones. Therefore, we have set out to clearly determine the specific role of the periplasmic chaperones involved in envelope biogenesis. We have developed a proteomic technique based on 2D-LC-MS/MS to better characterize the various periplasmic chaperones (2). In particular, we have characterized the function of the *E. coli* periplasmic chaperone SurA. SurA had been proposed to escort β-barrel proteins en route to the OM. However, our understanding of SurA's function was based on studies performed on a small number of OM proteins and its role on the global OM proteome had never been determined. We compared the relative abundance of 64 OM proteins, including 23 β-barrel proteins, in wild-type and surA strains. We found that the loss of *SurA* affects the abundance of 8 β-barrel proteins. Using qPCR, we showed that for 6 of them, the decreased protein abundance could be attributed, at least in part, to decreased mRNA levels in the *surA* strain. In the case of LptD, an essential protein involved in OM biogenesis, our data support a role for SurA in the assembly of this protein and suggest that LptD is a true SurA substrate. Based on our results, we propose a revised model in which only a subset of OM proteins depends on SurA for proper folding and insertion in the OM (5). We are currently using our powerful proteomic technique to characterize other periplasmic chaperones.

CONTROL OF CYSTEINE SULFENYLATION IN THE PERIPLASM

The periplasm possesses another soluble Dsb protein, DsbG, but the function of this protein has remained elusive (1). 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* (3).

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 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, University of Michigan), 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. On the basis of these results, we proposed the following model (3). 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 (Fig. 3).

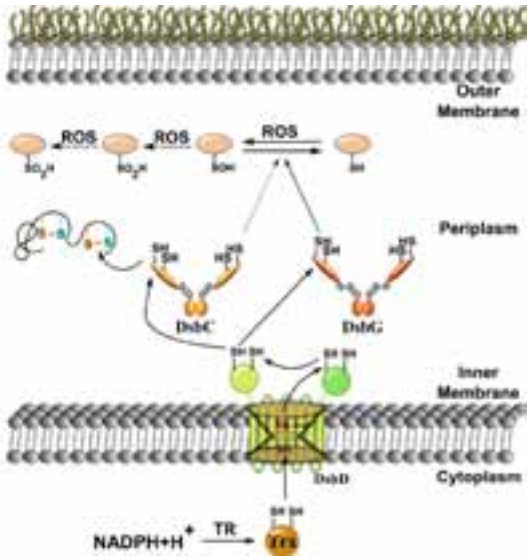

Figure 3. Rescue of sulfenylated cysteine residues in the periplasm. Proteins with single cysteine residues are easy prey for reactive oxygen species present in the periplasm. They form sulfenic acids (-SOH) which are susceptible to irreversible oxidation to sulfinic (-SO2H) and sulfonic acids (-SO3H). DsbG rescues sulfenylated cysteine residues. DsbC could serve as a back up for DsbG. Both DsbC and DsbG are kept reduced by DsbD. (Adapted from (1)).

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 (3).

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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 THE miRNAS DOWNSTREAM OF THE TUMOR SUPPRESSOR p53 IN CANCER

The p53 protein is a transcription factor that acts in the center of a signaling pathway that gets activated by different kinds of cellular stress. Upon exposure to ionizing irradiation, oxidative stress, chemotherapeutic drugs or the activation of oncogenes, a cascade of kinases leads to the stabilization of p53 protein. Depending on the cellular context, this leads to programmed cell death (apoptosis), cell cycle arrest or cellular senescence. Numerous protein-coding transcriptional target genes have been characterized and in a collaborative way contribute to the development of the functional consequences of p53 activation. We have previously characterized the miRNA34 family as direct target genes of the p53 signaling pathway. Presently, we are investigating the contribution of this miRNA family (and others) to the functional consequence of p53 activation *in vivo* and *in vitro*.

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

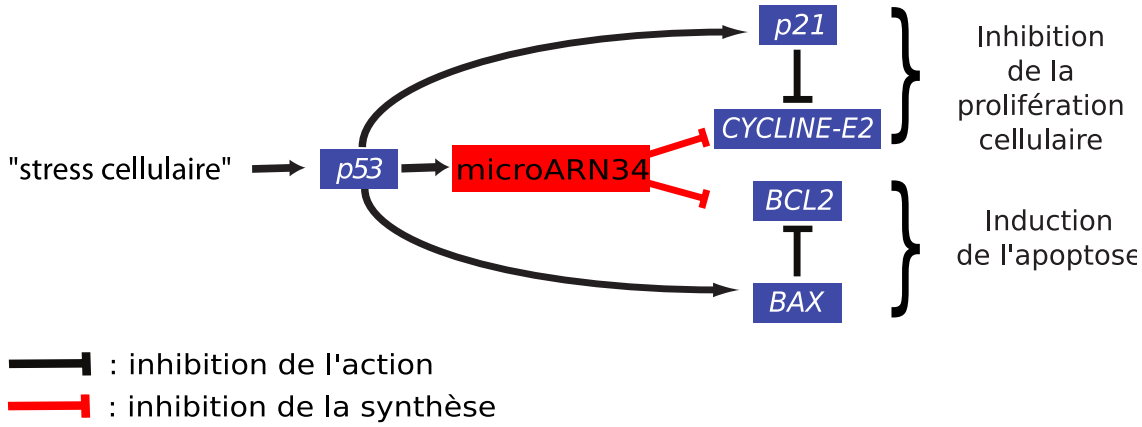

Figure 1. miRNA34 reinforces the phenotypic effect of protein coding p53 target genes. The tumor suppressor protein p53 is stabilized upon different stimuli and can transcriptionally activate numerous protein coding target genes that cooperate to lead to cellular death and cell cycle arrest. Transcription of the miRNA34 family is directly activated by p53 and via downregulation of the translation of the mRNA of antiapoptotic proteins Bcl2 and cell-cycle genes cyclin E2 reinforces the phenotypic effect of the protein coding p53 target genes.

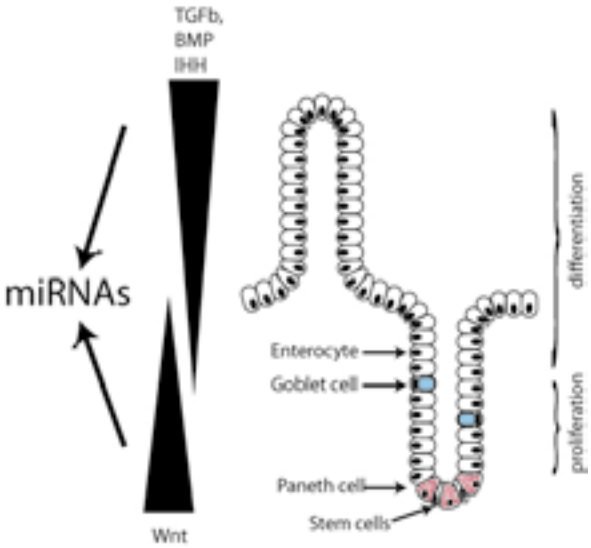

Figure 2. miRNAs play a role in the balance between differentiation and self renewal in the intestine.
The surface epithelium of the small intestine is continuously replenished from stem cells residing at the bottom of the crypt. Several signaling pathways are implicated in the differentiation of intestinal cells and the maintenance of the stem cell pool. We are investigating the role of an intestine specific miRNA that seems to be be involved in these processes.

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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 (PFK2)/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, D. Vertommen, L. Hue, M.H. Rider, in collaboration with B. Hemmings, Basel and D. Alessi, Dundee

Activation of heart PFK-2 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 (1). 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 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 (manuscript under revision).

Figure 1 summarizes the protein kinases from different signalling pathways that converge to phosphorylate heart PFK-2.

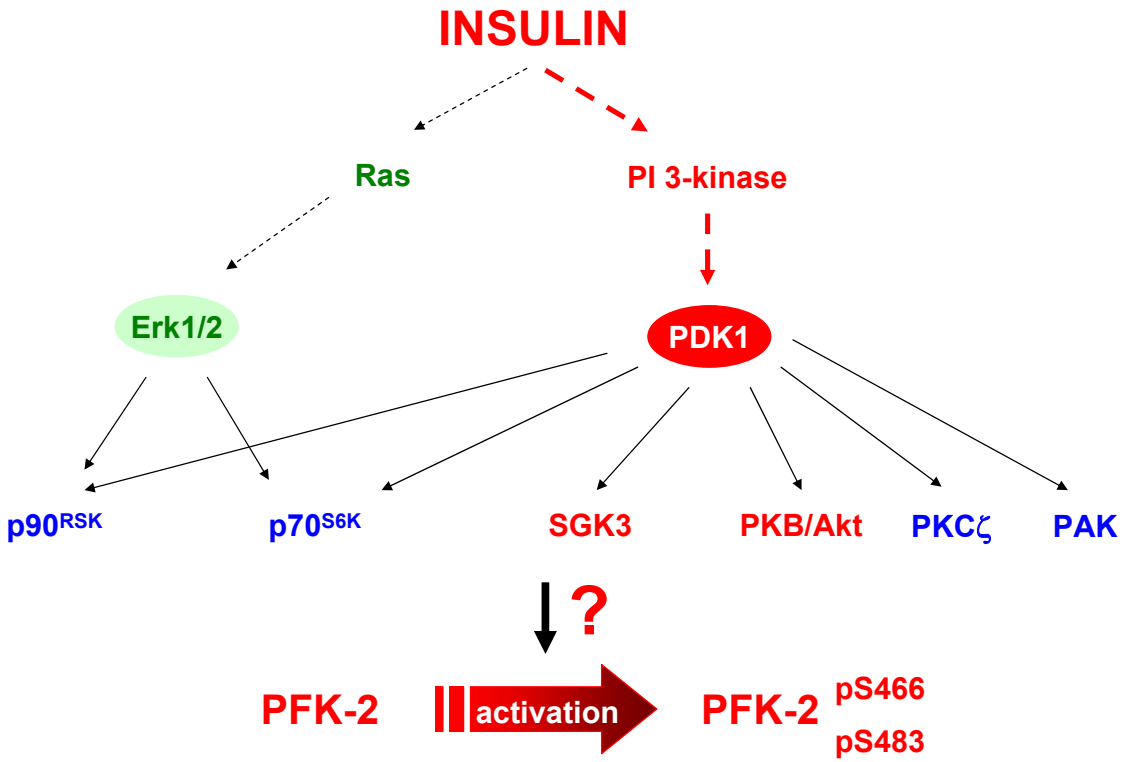

Figure 1. Protein kinases of signalling pathways that converge on heart PFK-2. The numbering of residues refers to the bovine H1 isoform.

AMP-ACTIVATED PROTEIN KINASE

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

The 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 it occurs during hypoxia or after exposure of cells to inhibitors of the mitochondrial respiratory chain, such as 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. For full activation, AMPK α-subunit phosphorylation of its activation oop Thr172 by LKB1 (Peutz-Jegher's protein) or calmodulin-dependent protein kinase kinase-β (CaMKKβ) is required. The role of AMPK in the cell is to maintain ATP by stimulating ATP-producing pathways

and inhibiting energy-consuming biosynthetic pathways. We contributed to the development of this concept by the discovery of new substrates of AMPK. We demonstrated that the activation of PFK-2 by AMPK participates in the stimulation of heart glycolysis by ischaemia (2). Similarly, we showed that phosphorylation-induced inactivation of elongation factor 2 (eEF2) by AMPK explains at least in part the inhibition of protein synthesis by anoxia (ref. 3 and see below).

AMPK activation in animals adapting to extreme energy stress

Long-term survival of oxygen deprivation by animals with well-developed anoxia tolerance depends on multiple biochemical adaptations including strong metabolic rate depression. We investigated whether AMPK could play a regulatory role in the suppression of protein synthesis that occurs when turtles experience anoxic conditions. AMPK activity and the phosphorylation state of ribosomal translation factors (see Fig. 2) were measured in liver, heart, red muscle and white muscle of red-eared slider turtles (*Trachemys scripta elegans*) subjected to 24 h of anoxic submergence. AMPK activity increased 2-fold in white muscle of anoxic turtles compared with aerobic controls but remained unchanged in liver and red muscle, whereas in heart AMPK activity decreased by 40% during anoxia. Eukaryotic elongation factor-2 phosphorylation increased 6- to 8-fold in red and white muscles from anoxic animals but was unchanged in liver and heart. The phosphorylation state of the activating Thr389 site of p70 ribosomal protein S6 kinase was reduced under anoxia in red muscle and heart but was unaffected in liver and white muscle. Exposure to anoxia decreased 40S ri-

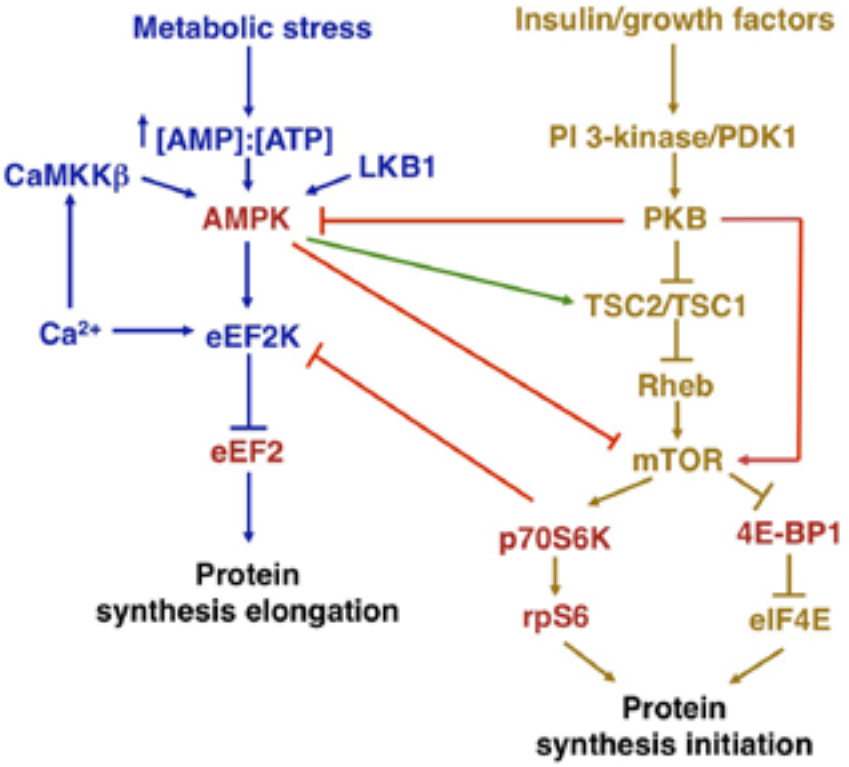

Figure 2. Mechanisms by which AMPK activation inhibits protein synthesis initiation (via a reduction in mTORC1 signalling) and translation elongation (via phosphorylation-induced eEF2K activation).

bosomal protein S6 phosphorylation in heart and promoted eukaryotic initiation factor 4E-binding protein-1 dephosphorylation in red muscle, but surprisingly increased 4E-binding protein-1 phosphorylation in white muscle. The changes in phosphorylation state of translation factors suggest that organ-specific patterns of signalling and response are involved in achieving the anoxia-induced suppression of protein synthesis in turtles (4).

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 5).

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 (6).

We were unable to confirm published data claiming that MLC can be directly phosphorylated by AMPK. Our results indicate that MLC is not a physiological AMPK substrate to explain energy-dependent changes in cell structure and that this published observation was rather due to commercial AMPK contamination by kinases capable of MLC phosphorylation (7).

MASS SPECTROMETRY

D. Vertommen, S. Calberson, 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

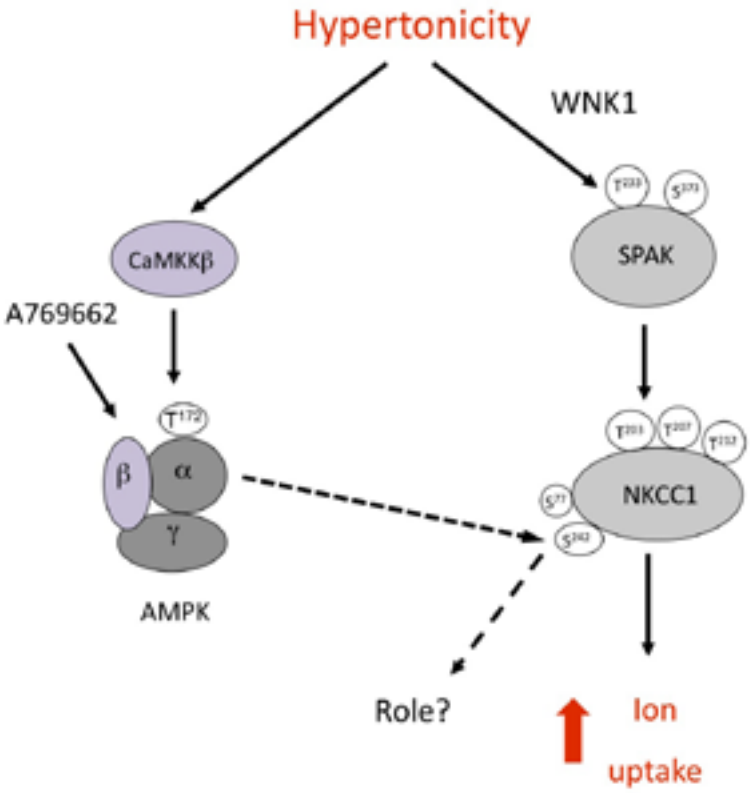

Figure 3. Cell shrinkage by sucrose treatment in RBCs leads to activation of a1-AMPK and SPAK via the CaMKKβ and WNK (with-no-lysine kinase)-1 pathways, respectively. SPAK activation correlates with NKCC1 Thr203/207/212 phosphorylation and is likely responsible for its activation and increased $^{86}Rb^{+}$ 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.

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 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.

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 which has now been validated on membrane proteins (8). For phosphoproteomics to identify new AMPK substrates, a hydrophilic interaction chromatography (HILIC) approach followed by enrichment of phosphopeptides by immobilized metal affinity chromatography (IMAC) and analysis by LC-MS (reviewed in ref. 9) is currently being validated.

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 mechanism that controls sulfenic acid formation in the periplasm of *E. Coli*. Using a diffe-

rential proteomic approach and targeted chemical modification, we discovered that DsbG and DsbC, two thioredoxin-related proteins, control the global sulfenic acid content of the periplasm and protect single cysteine residues from oxidation. DsbG interacts with the YbiS protein and, along with DsbC, regulates oxidation of its catalytic cysteine residue preventing the irreversible modification to sulfinic and sulfonic acids (10).

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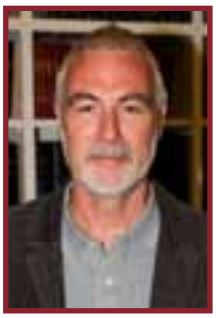

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METABOLIC COMPARTMENTATION IN TRYPANOSOMES

Trypanosomatidae are parasitic protists that cause sleeping sickness, Chagas' disease and leismaniasis in man, diseases that severely affect millions of people in tropical and subtropical parts of the world and cause over a hundred thousand of deaths each year. There is an urgent need for more efficacious and less toxic drugs than those currently in use. By molecular and cell biological investigation of these parasites we intend to provide a basis for the development of such new drugs. Trypanosomes rely on glycolysis for their ATP supply and are characterized by a unique form of glycolytic compartmentation where 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 effective 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 also been identified and validated as excellent drug targets. Moreover, the availability of the genome sequences of three trypanosomatids 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

Systems biology and glycolysis in glycosomes

P. Michels, in collaboration with H. Westerhoff (Vrije Universiteit Amsterdam, The Netherlands) and B. Bakker (Rijksuniversiteit Groningen, The Netherlands

Trypanosoma brucei, the causative agent of sleeping sickness, is transmitted between mammalian hosts by tsetse flies. Glycolysis is the sole free-energy source for the parasite living in the mammalian bloodstream and is therefore a promising drug target. Previously, we demonstrated the importance of a systems biology approach for drug target validation when we investigated the contribution of each enzyme of the pathway to the control of the glycolytic flux. We determined that glucose transport into the cell exerts high control and hence the transporter is a promising target. In general, there is little awareness that the composition of a metabolic network may change in response to drugs. If the response is homeostatic (e.g. through upregulation of the target protein), this may neutralize the initial inhibitory effect. In this scenario the effect on cell growth and survival would be less than anticipated based on the affinity of the drug for its target. By our systems approach we recently showed that inhibition of the glucose transporter at high inhibitor concentrations causes cell death, as expected, but interestingly sublethal concentrations initiate a domino effect in which the metabolic network adapts via respectively (i) partial inhibition of the flux-controlling target protein, (ii) down-regulation of the expression of the target protein and other proteins in the same metabolic pathway and (iii) differentiation of the cells leading to expression of metabolic enzymes and immunogenic coat proteins characteristic for the procyclic life-cycle stage of trypanosomes living in the tsetse fly midgut and not viable in humans. This 'anti-homeostatic' response may offer a possibility for efficient killing of parasites at an acceptable drug dosage.

Glycolytic enzymes

V. Hannaert, P. Michels, in collaboration with L. Gilmore and M. Walkinshaw (University of Edinburgh, Scotland)

Previously, we have expressed and kinetically characterized trypanosomatid enzymes for all steps of the glycolytic pathway. Structures of most of these enzymes have become available through our collaboration with protein crystallographers elsewhere. Notably, coworkers at the University of Edinburgh have determined the structures of *T. brucei* phosphofructokinase, phosphoglycerate kinase and *Leishmania mexicana* phosphoglycerate mutase and pyruvate kinase with and without various ligands. This has provided insight into mechanisms of catalysis and in the conformational changes required for catalysis by these enzymes. Inhibitors are identified by using the enzymes in high-throughput screens of available large libraries of drug-like compounds and specifically designed libraries and by structure-based design and synthesis. To date, a series of hit compounds have already been found. 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.

Pentose-phosphate pathway enzymes

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

The pentose-phosphate pathway 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, glucose-6-phosphate dehydrogenase (G6PDH), by RNA interference (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 for oxidative stress caused by H_2O_2.

We have shown that G6PDH of *T. brucei* and *Trypanosoma cruzi* is uncompetitively inhibited by the human steroids dihydroepiandrosterone and epiandrosterone, with Ki values in the low-micromolar range. In contrast, *L. mexicana* G6PDH is not inhibited. Viability assays demonstrated that both steroids stunt growth of cultured *T. brucei* bloodstream-form cells, but not *Leishmania* cells. Importantly, trypanosomes became unsusceptible to the inhibitors when induced to express a transgene of *L. mexicana* G6PDH. Together these findings identified G6PDH as a drug target in trypanosomes and provide prospects for using the steroids to develop leads for of a new class of anti-trypanosomatid compounds.

Hexokinase (HK), the first enzyme of the glycolytic pathway, of trypanosomatids is located in their glycosomes. In addition, a glucokinase (GlcK) was found in the glycosomes of *T. cruzi* and *Leishmania* species, but not in *T. brucei*. The crystal structure of TcGlcK with bound glucose showed that the sugar was present in its β-anomeric form; this preference was confirmed in activity assays. In contrast, all known HKs have a preference for the α-glucose anomer. The enzymes following the HK in the glycolytic and pentosephosphate pathways, glucose-6-phosphate isomerase and G6PDH, are highly specific for respectively the α- and β-form of the glucose 6-phosphate. The presence of both a HK and GlcK in glycosomes could be related to these different specificities. In *T. brucei*, where GlcK is absent, a glucose-6-phosphate-1-epimerase (G6PE) would be required for the anomerization. Indeed, a homologue of yeast G6PE, with a glycosome-targeting signal, was identified in the trypanosomatid genome databases. The activity was confirmed with recombinant G6PE. Knocking down its expression in bloodstream-form trypanosomes has no effect on their growth in regular HMI-9 medium. However, the cells are susceptible to oxidative stress in a non-reducing medium, very similar to results obtained for trypanosomes in which G6PDH has been depleted. These results suggest a role for G6PE in making glucose 6-phosphate available for the pentosephosphate pathway and NADPH production.

Fatty-acid desaturases

S. Gupta, M. Gualdrón, P. Michels, in collaboration with A. Uttaro (Universidad Nacional de Rosario, Argentina), P. Wallemacq (LCBM, UCL) and J.-P. Dehoux (ANIM, UCL)

Both procyclic and bloodstream-form *T. brucei* are capable of *de novo* synthesis of fatty acids and the process is essential for parasite survival. Polyunsaturated fatty acids (PUFAs) are synthesized by enzymes known as desaturases. Two desaturase enzymes were identified in *T. brucei*: Δ9 desaturase that synthesizes oleate (C18:1Δ12) from stearate (C18) and Δ12 desaturase that converts oleate into linoleate (C18:2Δ9,12). Knocking down the expression of these desaturase enzymes by RNAi, in both procyclic and bloodstream-form *T. brucei*, caused a growth phenotype and also exerted a significant effect on the total fatty-acid composition of the parasite. Isoxyl and 9-thiostearate, known Δ9 desaturase inhibitors, showed an inhibitory effect on the growth of in vitro cultured bloodstream-form trypanosomes with EC_{50} values of 0.1 μM and 1 μM, respectively. Moreover, in a preliminary experiment a significantly reduced parasitaemia was observed by treatment of *T. brucei* mice infected with Isoxyl. Two Δ12 desaturase inhibitors, 12- and 13-thiostearate, totally inhibited parasite growth with EC_{50} of 2 μM and 7 μM, respectively. The results suggest that Δ9 and Δ12 desaturase are essential for both procyclic and bloodstream-form *T. brucei*. The complete

absence of Δ12 enzyme activity in mammalian cells and the significant structural differences between trypanosome and mammalian Δ9 desaturases, highlight these enzymes as promising targets for selective chemotherapeutic intervention against the parasitic disease.

Glycosomal solute transporters

M. Mazet, P. Michels, in collaboration with P Wallemacq (LCBM, UCL)

Previously, three half-size ABC transporters, designated GAT1-3, have been identified in the glycosomal membrane of *T. brucei*. GAT1 and GAT3 are expressed in both bloodstream and procyclic-form trypanosomes, whereas GAT2 is only present in bloodstream-form cells. In order to study the function of the transporters, procyclic RNAi cell lines for depletion of both GAT1 and GAT3 have been created. Expression knockdown of GAT1 and GAT3 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 significant increase in the content of the fatty-acid linoleate (C18:2). We hypothesize that GAT1 is fatty-acid transporter, like some of its homologues in the peroxisomal membrane of yeasts and mammalian cells. The 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. Previous work in TROP, involving cell fractionation in conjunction with enzyme activity assays, indeed showed the probable association of enzymes of both processes with glycosomes.

To address the question if GATs are involved in glycosomal ether-lipid synthesis, the RNAi cell lines were used that we have created for knocking down the expression of the cytosolic enzymes Δ9 desaturase and Δ12 desaturase. Partial depletion of the respective desaturases caused the expected changes in cellular levels of their fatty-acid substrates and products. Interestingly, partial depletion of the Δ9 enzyme resulted in a decrease of GAT1 mRNA levels, whereas partial depletion of Δ12 desaturase caused an increase of the GAT1 transcript. This result strongly suggested that GAT1 is an oleate transporter and that oleate may be incorporated into ether-lipids. Relevant in this respect is that Δ9 desaturase depletion led also to a slight decrease of the ether-lipid biosynthetic enzyme dihydroxyacetone-phosphate acyltransferase. The notion that GAT1 is an oleate transporter has been tested. To that end, glycosomes purified from procyclic trypanosomes were incubated with radiolabelled oleate. Indeed, a time-, temperature-, oleate concentration- and ATP-dependent uptake of the fatty-acid was measured.

GLYCOSOME TURNOVER

Glycosome biogenesis in Trypanosoma brucei

M. Gualdrón, N. Galland, E. Verplaetse, P. Michels

So far, we have identified 11 proteins called peroxins (acronym PEX) involved in glycosome biogenesis in *T. brucei*; 10 of them are involved in the import of organellar matrix proteins. Using RNAi, we have shown the essentiality of most of the currently known peroxins for the viability of both cultured bloodstream and procyclic-form trypanosomes. In previous years, our work has been mostly focused on 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 charged recep-

tor with a membrane-bound docking complex minimally comprising PEX13 and PEX14.

Much of our current research is on the role of PEX4 and PEX22. In yeast and mammalian peroxisomes it was found that receptor PEX5, after delivery of their cargo in the peroxisomal matrix, are recovered in a mono- or di-ubiquitination dependent process and cycled back to the cytosol. Non-recycled 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 monoubiquitination 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 and PEX22 homologues are present, but there a cytosolic E2 enzyme (UbcH5) is responsible for receptor ubiquitination.

The *T. brucei* candidate PEX4 displays only low overall sequence identity (30%) with yeast PEX4, but has a conserved region near the C-terminus that contains the cysteine residue that is critical for catalytic activity. It is expressed in bloodstream and procyclic-form cells as observed by RT-PCR and western blot analysis. By confocal immunofluorescence microscopy

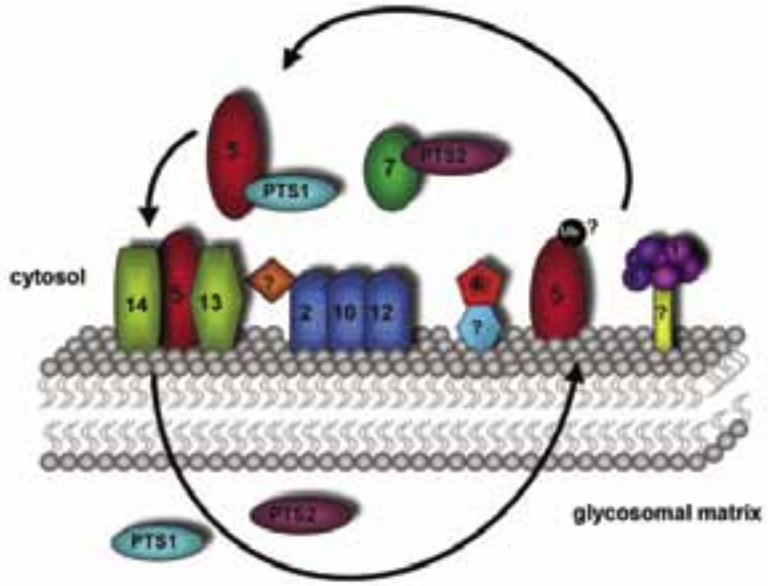

Fig. 1. Model of the import of proteins into the matrix of trypanosomatid glycosomes. Peroxins which have been shown to mediate import of proteins with a type-1 or -2 peroxisome-targeting signal (PTS) into yeast peroxisomes are indicated. The numbered peroxins are the corresponding *Trypanosoma brucei* peroxins identified and currently being studied in the Research Unit for Tropical Diseases. Newly synthesized PTS1 and PTS2 containing proteins are recognized in the cytosol by receptors PEX5 and PEX7, respectively. The cargo-loaded receptors interact with a docking complex (PEX13 and PEX14) at the glycosomal membrane, resulting in the release of the PTS proteins in the organelle's matrix. Receptor PEX5 is cycled back to the cytosol through a ubiquitination process involving E2 ubiquitin-conjugating enzyme PEX4 and E3 ubiquitin-ligase enzymes PEX2, PEX10 and PEX12, as well as AAA-ATPases PEX1 and PEX6.

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. No significant growth phenotype was observed when its expression was partially knocked down by RNAi. However, this partial depletion appeared to affect the abundance of PEX5.

A trypanosomatid orthologue of yeast and plant PEX22 was identified by homology searches in spite of very low sequence conservation. Its partial depletion by RNAi caused some growth retardation in procyclic trypanosomes but not in bloodstream forms. Currently we are developing trypanosome cell lines in which PEX4 and PEX22 can be depleted more severely, for further characterization of their role in glycosome biogenesis. Moreover, additional studies to confirm the glycosomal localization of PEX22 are in progress.

Glycosome degradation in Trypanosoma brucei

A. Brennand, P. Michels, in collaboration with E. Pays (Université Libre de Bruxelles) and 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 form into the procyclic insect 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. A previously performed bioinformatics analysis enabled us to identify in the trypanosomatid databases orthologues of about 20 of the approximately 40 ATGs (= AuTophaGy-related proteins) known to be involved in autophagy in yeast, suggesting a functional but highly streamlined version of the process in these parasites. Also orthologues specifically required for pexophagy were recognized in trypanosomatids. This bioinformatics analysis was very recently extended to a taxonomically diverse range of other unicellular eukaryotes. This analysis 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

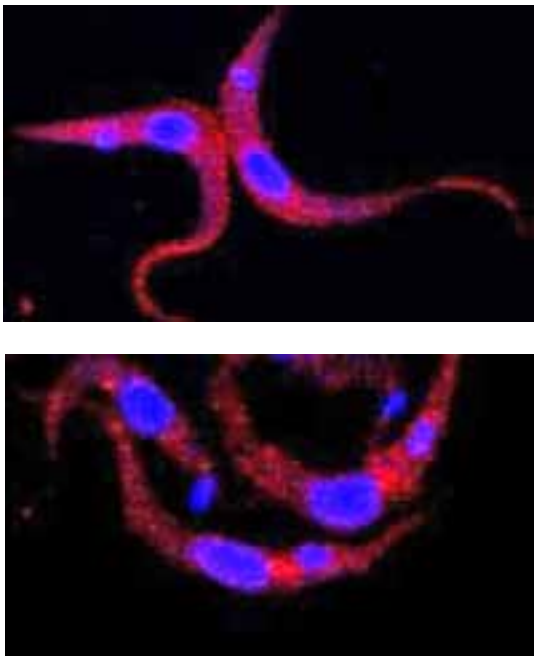

Figure 2. Subcellular localisation of autophagy-related proteins (detected by immunofluorescence; stained in red) in *T. brucei*. (a) cytosolic distribution of TbATG8 in procyclic cells. (b) cytosolic and puctuate distribution of TbATG24 in procyclic cells. The puncta are mainly located between the nucleus and kinetoplast (the large and small structures, respectively, stained in blue by TOPRO) and possibly co-localize with components of the endosomal system.

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.

Several of the *T. brucei* homologues of yeast proteins involved in autophagy, notably that of peroxisomes, were cloned and analyzed: VPS34, ATG7, ATG8, ATG24 and VAC8. 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 (ATG8, VAC8, ATG24 and VPS34) bloodstream-form and procyclic RNAi-mutant cell lines have been constructed and analyzed under standard culturing conditions and under conditions where an increased rate of glycosome turnover is expected. RT-PCR experiments confirmed in each case a decrease of the respective mRNA level upon induction of RNAi. However only a partial depletion was achieved and under those conditions no important growth phenotype was observed. We are currently repeating the experiments with cell lines transfected with RNAi-constructs prepared with a different vector in which we expect a more efficient decrease of the targeted mRNAs.

GENOME ANALYSIS

F. Opperdoes, in collaboration with J. de Jonckheere and M. Ouelette, Quebec, Canada

The amoeboflagellate *Naegleria gruberi* is a free-living protist which belongs to the phylum Heterolobosea, to which also the Euglenozoa (with the euglenoids and the Trypanosomatidae) belong. Early in eukaryotic evolution the Heterolobosea separated from all other eukaryotes . While the Trypanosomatidae lost many of their metabolic capacities as the result of an adaptation of the parasite to its host, *N. gruberi* is free living and was expected to provide a more complete blueprint of the metabolic capacities present in the ancestral eukaryotic cell.

The *N. gruberi* proteome, predicted from 15753 gene models was analyzed and the results are presented here.

Protein sequence analysis revealed that N. gruberi has all the necessary enzymes to feed on oligosaccharides as well as extracellular peptidoglycans from bacterial origin. The resulting monosaccharides are metabolized via a slightly modified glycolytic pathway where hexokinase has been replaced by fructokinase and glucokinase and the classical ATP-dependent phosphofructokinase (PFK) by a PPi-dependent PFK. In addition to a pyruvate kinase, *N. gruberi* has a PPi-dependent pyruvate-phosphate dikinase (PPDK), an enzyme that catalyzes the conversion of PEP into pyruvate similarly as pyruvate kinase except that this enzyme utilizes PPi rather than ATP as phosphoryl donor. Due to the absence of fructose-1,6-bisphosphatase the reversed pathway, called gluconeogenesis, utilizes the enzymes PPDK and a reversed PPi-dependent PFK. *N. gruberi* also has a homologue of the glucokinase regulatory protein which in bacteria has been shown to have etherase activity and catalyzes the delactoylation of bacterial N-acetylmureate 6-phosphate. The products are D-lactate and N-acetylglucose. Together with D-lactate dehydrogenase this allows the protist to degrade bacterial mureine to N-acetylglucoseamine and pyruvate.

Although there are no reports in the literature suggesting the presence of peroxisomes in *Naegleria*, 287 protein sequences with a C-terminal peroxisome-targeting signal were identified. Therefore, the presence of peroxisomes, involved in the detoxification of ROS and the degradation of fatty acids is more than likely.

In addition to a full set of enzymes involved in aerobic mitochondrial metabolism, *Naegleria* is predicted to be able to survive under microaerophylic or anaerobic conditions. Electrons may not only be passed on to oxygen but also nitrate and nitrite, as electron ac-

ceptors. Moreover, the organism is capable of malate dismutation and contains a gene coding for a hydrogenase, an enzyme typically found in the hydrogenosomes of anaerobic protists and present in many anaerobic bacteria. The *Naegleria* mitochondrion thus seems to be the long sought intermediate in mitochondrial evolution that unites biochemical properties of both aerobic and anaerobic mitochondria and hydrogenosomes Although it is not known whether *N. gruberi* is able to thrive under anaerobic conditions, its genome predicts that it should be able to adapt to a great variety of life styles.

A start has been made with the genome project of the lizard parasite *Leishmania tarentolae*.

In order to get a first impression of the metabolic capacities of *L. tarentolae*, over 700 sequences, all of metabolic enzymes from the *Leishmania major* genome database, which were previously analyzed in detail were selected and used in a BlastP search against the *L. tarentolae* predicted proteome. All *L. major* sequences were found to have orthologues in *L. tarentolae*. Also the sequences that previously were found to be specific for *Leishmania* and being absent from e.g. *T. brucei*, are all present in the *L. tarentolae* genome. These include enzymes of sugar metabolism, the urea cycle, folate metabolism and haem synthesis. Therefore, it can be concluded that the metabolic capacities of *L. tarentolae* are essentially identical to that of *L. major*, despite the the fact that the natural host of *L. tarentolae* is the lizard rather than the human. *L. tarentolae* therefore may be a good model organism for the study of *Leishmania* metabolism and drug screening.

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ENDOCYTOSIS AND EPITHELIAL DIFFERENTIATION

Endocytosis, a central activity of all eukaryotic cells, allows for nutrition, regulates plasma membrane and controls macromolecules transport across epithelial barriers. This research group has made significant contributions in the dynamic structure of endocytosis, delineation of pathways and physiopathology. Our current investigations focus on endocytosis at the apical membrane of polarized kidney proximal tubular cells (PTC). We surmise that this most active apical endocytic surface will allow to identify rate-limiting actors and elucidate diseases when these become defective. These molecular actors should also emerge from the study of genetic or acquired mouse models causing low-molecular weight proteinuria. Recent achievements include: (i) delineation 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 (1, 6); (ii) elucidation of defective apical endocytosis in ClC5-KO mice mimicking Dent's disease, a genetic predisposition to kidney stones (2) and CFTR-KO mice, a model of cystic fibrosis in kidneys (5); (iii) serendipitous discovery that apical endocytosis of ultrafiltrated lysosomal proteases is a central mechanism for PTC lysosomes biogenesis (4); and (iv) identification of the transcription factor, ZONAB, as a key component for the switch between proliferation and apical differentiation in various epithelia (9). In view of the distinct composition of apical plasma membrane lipids, we have also undertaken to address whether their micrometric organization impacts on distinct endocytic rate and fate. We thus provided the first evidence that sphingomyelin forms temperature-dependent micrometric domains in living cells (10). Biogenesis of epithelia is the second line of investigations. We focus on epithelial tubulogenesis and differentiation, using developing pancreas and salivary glands in vivo and explants, as complementary models of controlled interconversion between multilayered cell masses and polarized monolayers, with emphasis on paracrine and transcriptional control (8). Our group offers expertise in structural biology and cellular and tissular imaging with a versatile platform, from confocal and multiphoton microscopy (7), to transmission and scanning electron microscopy.

REGULATION OF APICAL ENDOCYTOSIS BY v-SRC IN POLARIZED EPITHELIAL CELLS

M. Mettlen, Ph de Diesbach, T. Medts, S. Carpentier, P. Van Der Smissen, D. Tyteca, P.J. Courtoy

Oncogenic transformation is well known to accelerate the endocytic activity, but the underlying mechanisms remained poorly understood. We originally reported that v-Src causes a profound remodelling of actin cytoskeleton in Rat-1 fibroblasts, resulting in stress fiber disappearance, cortical actin polymerisation, ruffling and macropinocytosis (Amyere et al., Mol Biol Cell 2000;11:3453-67). Since most cancers (i) are of epithelial origin, (ii) and frequently overexpress or overactivate Src; and since (iii) apical endocytosis depends on actin, (iv) which is a major target of Src, we examined whether v-Src would similarly trigger apical endocytosis in MDCK cells. Because stable cell transformation abolishes epithelial polarity due to epithelio-mesenchymatous transition, we resorted to MDCK cells bearing a thermosensitive (ts) v-Src kinase.

When MDCK/tsLA31 cells were plated at high density on a permeable support and cultured at the non-permissive temperature (40°C), a polarized epithelial monolayer could be established, with ~2-fold faster fluid-phase endocytosis at the basolateral than at the apical surface. Shifting to the permissive temperature (34°C) rapidly activated v-Src kinase but preserved a tight monolayer for at least 6 h, allowing to study the effect of Src on polarized endocytosis. During this interval, Src kinase induced apical circular ruffling (Fig. 1) and selectively accelerated apical fluid-phase endocytosis (up to 6-fold). This was accompanied by the induction of macropinosomes, merging into a huge (> 5 μm) apical endocytic vacuole, generated by overflow into the apical recycling compartment (ARE). Preservation of ARE tubulation and of apical polarity indicated that the overall function of this essential compartment was

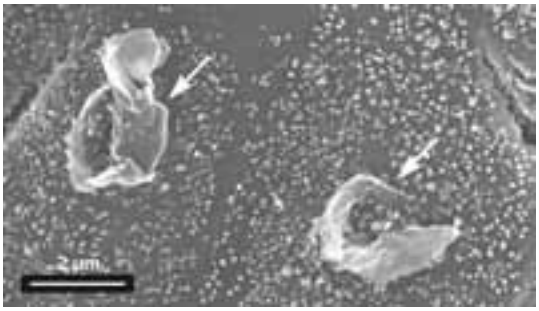

Figure 1. Src causes circular apical ruffling (arrows; scanning electron microscopy, from Ref 3).

however not affected. Macropinosomes and the ARE were labelled for v-Src, Rab11, and rabankyrin-5, but not for early endosome antigen-1, thus distinguishing two separate Rab5-dependent apical endocytic pathways. Ruffling and macropinocytosis depended on an amplification cascade involving PI3K, PLC and PLD, as shown by inhibition by wortmannin, NCDC and 1-butanol, respectively.

These data demonstrate that v-Src selectively affects the dynamics of the apical plasma membrane, where microdomains known as "lipid rafts" are abundant. The mechanisms of Src-induced apical ruffling and macropinocytosis may provide important insights for the apical entry into enterocytes triggered by enteroinvasive pathogens and on the apical differentiation of osteoclasts (3).

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, D. Tyteca, 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" was controversial. Using polarized MDCK cells expressing the thermosensitive v-Src/tsLA31 variant, we 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 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. ERK1/2 activation was insensitive to MβCD, which suppressed Akt phosphorylation and apical endocytosis induced by Src, both depending on the PI3K/Akt 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, then via PI3-kinase-Akt on a distinct set of apical plasma membrane "rafts". Whether this model is applicable to c-Src remains to be examined (6).

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

P.J. Courtoy, M. Leruth, F. N'Kuli and W.R. Lima, in collaboration with E.I. Christensen and his colleagues (Aarhus, DK), M. Jadot (FUNDP, BE) and 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 pro-cathepsin 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 (4).

MOLECULAR MEDICINE: THE CAUSE OF ENDOCYTIC DEFECTS IN KIDNEY FANCONI SYNDROMES

S. Carpentier, W.R. Lima, P. Van Der Smissen, B. Marien, P.J. Courtoy, in collaboration with O. Devuyst (NEFR) and E.I. Christensen (Aarhus, DK)

The highest apical endocytic activity in the body by kidney proximal tubular cells (PTC) allows to recapture all ultrafiltrated low-molecular weight (LMW) proteins. Conversely, defective PTC function in inherited or acquired diseases, collectively referred to as Fanconi syndromes, leads to LMW proteinuria. Dent's disease, also known as X-linked nephrolithiasis, is a paradigm of Fanconi syndromes, due to inactivating mutations of ClC-5. ClC-5 KO mice, which faithfully mimick Dent's disease, showed a severe deficit in PTC endocytosis of a variety of ligands of megalin and cubilin, acting as high-capacity tandem receptors at the apical membrane (brush border) (Fig. 2). Surprisingly, the total megalin pool was not appreciably affected. However, the combination of analytical subcellular fractionation and quantitative ultrastructural immunogold labelling revealed instead that the endocytic receptors were sequestered in apical endosomes and failed to reach the apical membrane, likely as part of a general membrane trafficking defect (2).

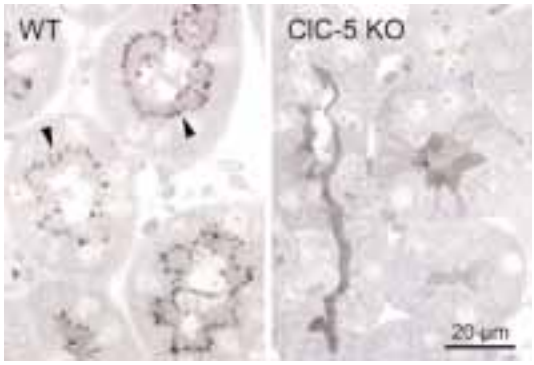

Figure 2. Evidence for a deficit of apical endocytosis of low-molecular weight proteins in kidney proximal tubular cells of ClC-5 KO mice. Arrows indicate cytochemical detection of injected, ultrafiltrated peroxidase. Note the strong labelling by peroxidase of multiple apical PTC endosomes in WT mice, contrasting with poor apical uptake and luminal retention in ClC-5 KO mice.

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

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

Apical endocytosis is a hallmark of epithelial differentiation. 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 both 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 mar-

kers. 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 (see Fig. 3). In confluent PTC monolayers, stable ZONAB overexpression inhibited 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 were ZONAB target genes. ZONAB expression was regulated by polarity at pre- and posttranslational levels (mRNA expression or stability, proteasomal degradation). In PTC islands, proteasome inhibition extended nuclear

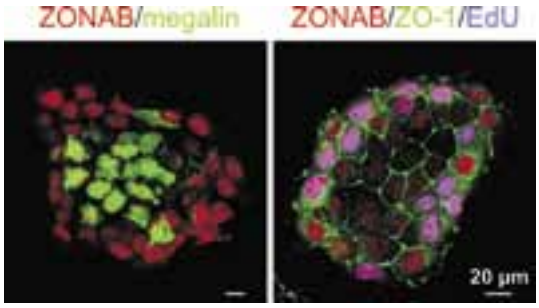

Figure 3. ZONAB is a key regulator in the switch between epithelial proliferation and differentiation. In colonies of kidney proximal tubular cells, ZONAB (immunolabelled in red) is absent in central differentiated cells (expressing megalin, in green, left) but is selectively detected in nuclei of peripheral proliferating cells (DNA synthesis is shown in blue at right).

ZONAB to central cells, which reversed their choice from differentiation to proliferation.

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 (9).

PLASMA MEMBRANE LIPID FLUORESCENT ANALOGS FORM STRUCTURALLY AND KINETICALLY DISTINCT MICROMETRIC DOMAINS

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

Lipids are the most abundant biological membranes constituents. 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 natural occurrence of micrometric lipid domains in the plasma membrane of living cells is questioned. We found that insertion of BODIPY-SM into the outer membrane leaflet of living, featureless erythrocytes labelled (sub) micrometric fluorescent plasma membrane

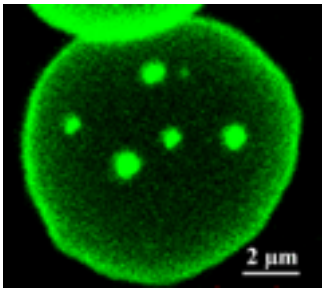

Figure 4. Fluorescent sphingomyelin analogs reveal (sub)micrometric domains on erythrocytes (see Ref 10).

domains (Fig 4). BODIPY-SM domains were randomly distributed in control erythrocytes at

37°C and coalesced upon hypotonic shock, excluding structural surface features and endocytosis and indicating control by surface tension. In CHO cells, either direct plasma membrane insertion of BODIPY-SM or intracellular enzymatic conversion of BODIPY-ceramide into BODIPY-SM produced similar surface BODIPY-SM domains. Domains were extracted by surface back-exchange, resisted endocytosis block (K+-depletion) and actin depolymerization (latrunculin B), and clustered upon cholesterol deprivation. BODIPY-SM excimers demonstrated clustering in ordered 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. Consistent with domain boundaries, fluorescence recovery after photobleaching revealed restriction of BODIPY-SM lateral diffusion over long-range, but not short-range, and various perturbations affected its mobile fraction as predicted from effects on fluorescent domains viewed by confocal microscopy. Taken together, these data reveal that 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, which may reveal similar domains of endogenous SM.

EPITHELIAL DIFFERENTIATION

A.-C. Hick, P.J. Courtoy, C.E. Pierreux, in collaboration with F.P. Lemaigre (HORM)

Several organs, such as the exocrine pancreas and salivary glands, are made of polarized epithelial monolayers with a branched tubular organization. In contrast to the iterative bifidal division of the bronchial tree, their tubular organization requires the integration of three developmental processes : (i) formation of blunt apical cavities within a single compact multicellular mass by epithelial polarization; (ii) confluence of these cavities to create tubules; combined with (iii) branching expansion of tubules and glands (see Fig. 5). 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.

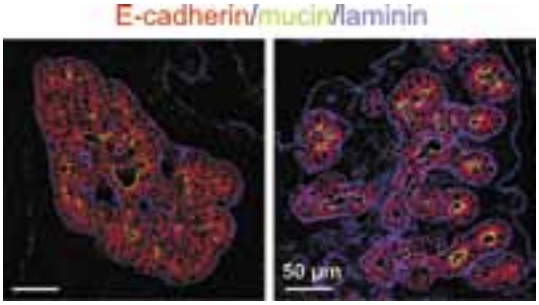

Fig. 5. Branching morphogenesis in exocrine glands. Reorganization in the early pancreatic bud of the multicellular mass of epithelial cells labelled for E-cadherin (left) into polarized monolayers with distinct apical domains (mucin) and their merging to create tubules (right). Laminin delineates basement membranes.

Epithelial monolayers formation requires a coordinate and dynamic interaction with their environment, composed of mesenchymal and endothelial cells. This process is gland-autonomous, 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), which was highly expressed by mesenchymal cells immediately adjacent to the epithelial buds. 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 These data demonstrate that paracrine epithelio-mesenchyme interaction is crucial for branched glandular morphogenesis

and bring a proof-of-principle that molecular components of this interaction can be readily dissected in explant cultures (8).

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

We have pursued our long-term commitment to promote collaborations by sharing expertise in endocytosis and cellular imaging. For recent years, we may cite contribution to the study of endocytic trafficking of syndecans and its role in signalling by its heparin-sulfate ligands (Zimmermann et al., Dev Cell 2005;9:377-88); of biogenesis of vacuolar H^+-ATPase and the role of CFTR in kidney (Jouret et al., J Am Soc Nephrol 2005;16:3235-46); subcellular trafficking of the thrombopoietin receptor (Royer et al., J Biol Chem 2005;280:27251-61; see report by S. Constantinescu) 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 "in press"; see report by E. Van Schaftingen); first evidence for dispersion of the actin cytoskeleton in epithelial cells by AMP-activated kinase (Horman et al, Biochem Biophys. Res. Commun "in press" see report by M. Rider); ultrastructural analysis of differentiating hepatoblasts (Clotman et al., Genes Dev 2005;19:1849-54; see report by F. Lemaigre) 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), or the morphological evidence by FRET of the interaction between key players of CTL that is interrupted during their anergy in cancer (Demotte et al., Immunity 2008;28:414-24; see report by P. van der Bruggen).

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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 innovations: separate, by laser capture microdissection, stromal and glandular cells from degraded or preserved areas of the human endometrium and compare their transcriptome by 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 menstrual endometria (9). Algorithms for sample clustering (PCA) segregated biological

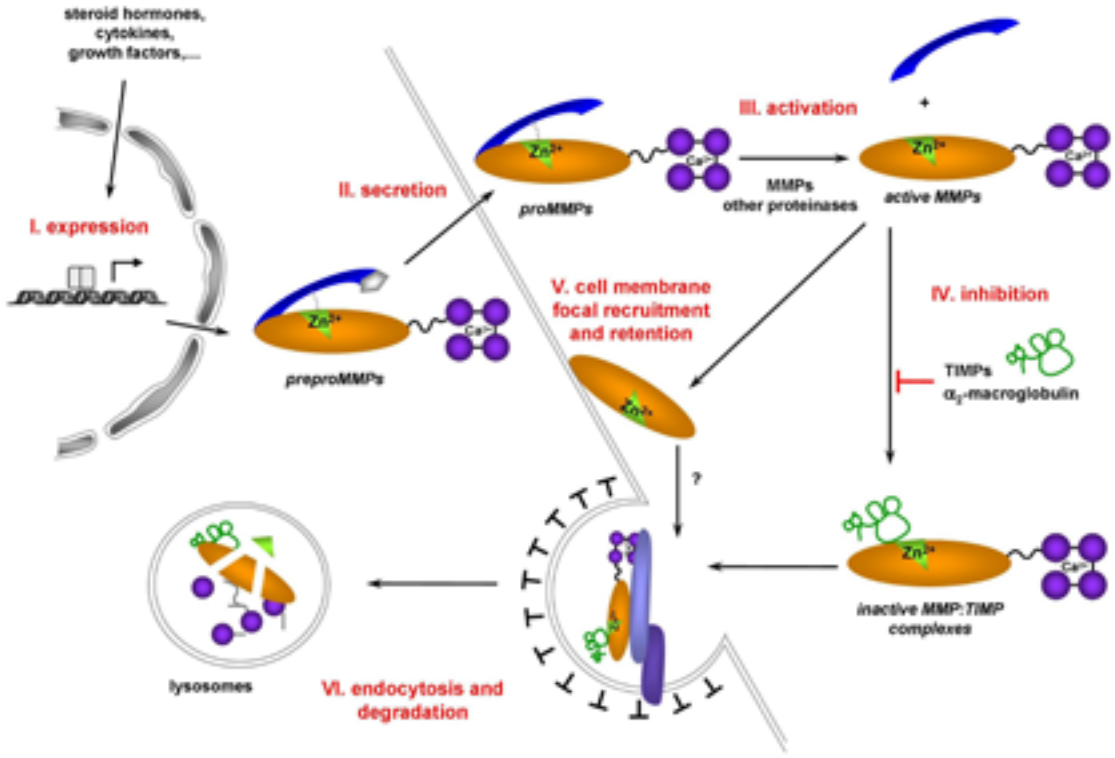

Figure 1. Regulation of soluble MMPs activity in the human endometrium: current model.

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"), contain an additional, variable C-terminal hemopexin-like domain stabilized by calcium (mauve) responsible for substrate specificity, linked by a hinge domain (black). The overall activity of MMPs can be controlled at 6 different levels: (1) expression, (2) secretion (regulated in a limited number of cell types such as neutrophils), (3) zymogen activation upon prodomain excision, (4) inhibition of active forms by physiological inhibitors such as TIMPs (represented with their tertiary structure) and α2-macroglobulin, (5) secondary membrane recruitment increasing pericellular activity, and (6) down-regulation by endocytosis.

In the 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. In particular : (1) 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. (2) Neutrophils are numerous at menstruation and could contribute to an abundant secretion of MMPs. (3) MMPs can be activated by other MMPs, by plasmin, itself activated during menstruation, or by as yet unidentified proteinases. (4) TIMPs are particularly abundant in the human endometrium; like MMPs, the level of TIMPs is regulated by ovarian steroids and cytokines. (5) MMP-7 binds to membrane receptors in cholesterol-rich domains, a mechanism which enhances pericellular MMP activity. (6) 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 (1), (3), (4), (5) and (6).

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.

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

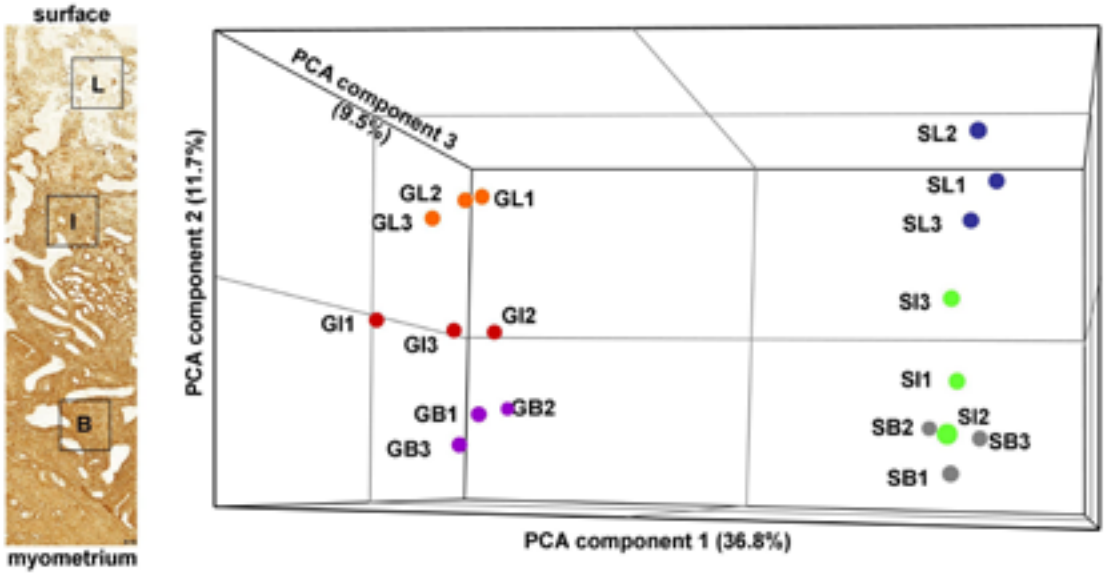

Figure 2. Comparison of transcriptomes from microdissected areas identifies remarkable cell type- and layer-specific signatures. Tissue samples containing either stroma or glands were microdissected from three successive layers of three menstrual endometria : lysed superficial (L), yet preserved intermediate (I) or basal (B). The transcriptomes of the 18 samples were determined using whole genome microarrays. Principal component analysis (PCA, at right) of the datasets 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) an noticeable segregation between layers along axis 2. For details, see (9).

In the second part of the study, stromal and glandular areas were microdissected from explants cultured without or with estradiol and progesterone (manuscript in revision). The microarray datasets were also compared to

remodeling. This feature was nicely illustrated by a group of genes differentially regulated by ovarian steroids in stroma and glands and sensitive to MAPKs.

In conclusion, we have generated useful and reliable lists of gene 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 and 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.

METALLOPROTEINASE-DEPENDENT SHEDDING OF LRP-1 ECTODOMAIN DECREASES ENDOCYTIC CLEARANCE OF ENDOMETRIAL MMP-2 AND -9 AT MENSTRUATION

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

In contrast to menstrual-restricted MMPs, e.g. interstitial collagenase (MMP-1), gelatinases A (MMP-2) and B (MMP-9) mRNAs are abundant throughout the cycle without detectable tissue degradation at proliferative and secretory phases, implying a tight post-translational control of both gelatinases. The receptor-associated protein (RAP), used as LRP antagonist, strongly increased (pro)gelatinases accumulation in medium conditioned by endometrial explants, indicating a role for LRP-1 in their clearance. Although LRP-1 mRNA remained constant throughout the cycle, the protein ectodomain vanished at menses. LRP-1 immunolabeling selectively disappeared in areas of extracellular matrix breakdown in menstrual samples. It also disappeared from explants cultured without estrogen and progesterone due to ectodomain shedding in the medium. The shedding was inhibited by metalloproteinase inhibitors, including a disintegrin and metalloproteinase (ADAM) inhibitor, and by tissue inhibitors of MMPs (TIMP)-3 and -2, but barely by TIMP-1, pointing to ADAM-12 as the putative sheddase. In good agreement, ADAM-12 mRNA expression was repressed by estradiol and progesterone. In conclusion, the efficient LRP-1-mediated clearance of gelatinases activity in non-bleeding endometrium is abrogated upon hormone withdrawal, due to shedding of LRP-1 ectodomain by a metalloproteinase, presumably ADAM-12 (Fig. 3), itself regulated by estradiol and progesterone (8).

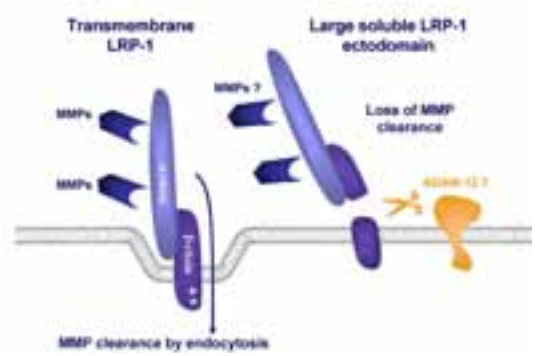

Figure 3. Proposed model for regulation of gelatinase activity by LRP-1. At left, binding of gelatinases (MMP-2 and -9) to LRP-1 triggers avid receptor-mediated endocytosis thanks to its two NPxY motifs (indicated by *). At right, shedding of the large ectodomain, presumably via ADAM-12, prevents its endocytic clearance and may stabilize extracellular gelatinases. For details, see (8).

ENDOMETRIAL XENOGRAFTS

C. Galant, H. Gaide Chevronnay, P.J. Courtoy, P. Henriet, E. Marbaix (in collaboration with 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 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 more appropriate to study the induction of menstruation and of its related pathologies.

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GROWTH FACTOR RECEPTORS

From signal transduction to human diseases

Growth factors are secreted proteins that stimulate cell proliferation via transmembrane receptors. Our research interests are focused on the mechanisms of activation of these receptors. We are particularly interested in receptor-tyrosine kinases that are binding to platelet-derived growth factors (PDGF) and fibroblasts growth factors (FGF) (Fig. 1). These proteins play an important role in the development of the embryo and in wound healing, as well as in cancer and in fibrosis.

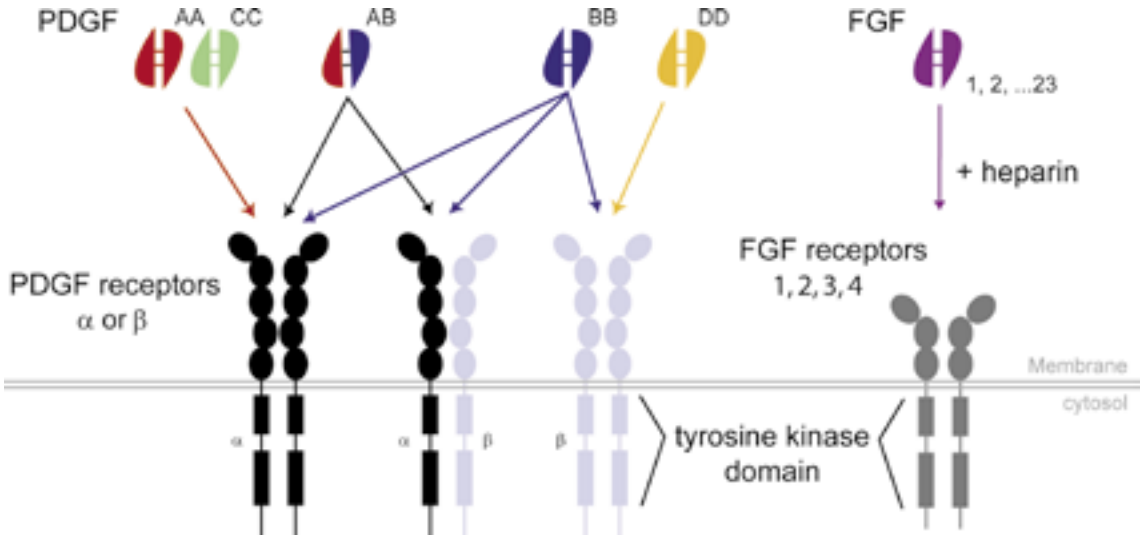

Figure 1. PDGF and FGF receptors and ligands

REARRANGEMENTS OF THE RECEPTOR TYROSINE KINASE GENES ASSOCIATED WITH LEUKEMIA

F. Toffalini, S. Medves, C. Montano, A. Velghe, J.B. Demoulin

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, modifications of PDGF receptor genes, as a result of chromosomal translocation or deletion, are found in chronic myeloid neoplasms associated with eosinophilia (formerly classified as chronic eosinophilic leukemia, atypical chronic myeloid leukemia or chronic myelomonocytic leukemia). In all cases, the rearranged gene produces a hybrid protein comprising the PDGF receptor tyrosine kinase domain and an oligomerisation domain. In most cases, they also retain the receptor transmembrane domain, which plays a particular role in the activation of these oncoproteins (3). 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 in patients with chronic eosinophilic leukemia. 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 α and β, which are quickly degraded upon activation, we observed that TPβ and FPα escaped down-regulation resulting in stabilization of the proteins. High stability of these hybrid oncogenes was confirmed in leukocytes from two patients. Similar data were obtained in cells expressing ZNF198-FGFR1, another fusion protein associated with the 8p11 myeloproliferative syndrome. Cbl-mediated monoubiquitination of receptor lysines targets them for lysosomal degradation. Ubiquitination of TPβ and FPα was much reduced compared to wild-type receptors, despite marked Cbl phosphorylation in cells expressing hybrid receptors. Deletion of the pointed (PNT) domain, impairing TPβ polymerization, strongly destabilized the protein. In conclusion, chimeric receptor tyrosine kinases escape efficient ubiquitination and down-regulation through lysosomes and proteasomes (4).

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 *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 and differentiation of cytokine-independent cells. 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 translocation. 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. 2 and reference 2). We are now looking for other mutations in tyrosine kinase genes.

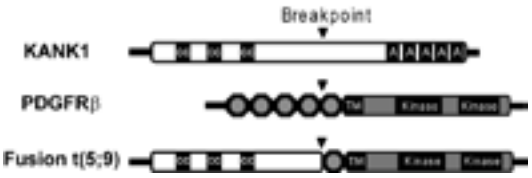

Figure 2. Structure of the KANK1-PDGFRβ fusion protein created by the t(5;9) translocation.

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

ACTIVATION OF PDGF RECEPTORS IN SYSTEMIC SCLEROSIS

S. Charni, J.-F. Classen, J.B. Demoulin

Systemic sclerosis (also called scleroderma) is a severe connective tissue disease of unknown etiology, characterized by fibrosis of the skin and multiple internal organs. 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 α or β 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 (5). Two 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.

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

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

Most cellular effects of growth factor occur through reprogramming gene expression within the cell nucleus. Each signal transduction cascade controls a number a transcription factors that will 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 (6, 10). We also analyzed gene expression in neural stem cells, glioma, carcinoid tumors and leukemic cells (1, 7-10).

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. 3). We observed that FOXO mRNA expression is also decreased upon stimulation with growth factors (6). 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.

In our microarray analysis, a cluster of genes involved in fatty acid and cholesterol biosynthesis, including stearoyl-CoA desaturase (SCD), fatty acid synthase and hydroxy-methylglutaryl-CoA synthase (HMGCS), was up-regulated by

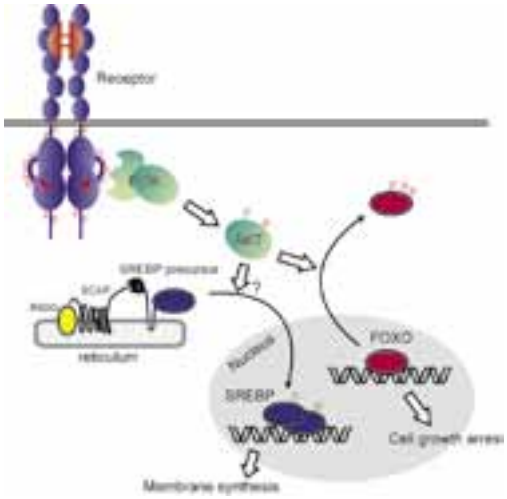

Figure 3. Activation of SREBP and inactivation of FOXO by PDGF

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 b 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 3. and reference 10). 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 (8). 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 these 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 Jacques van Helden (Université Libre de Bruxelles)

Deciphering transcription factor networks from microarray data remains difficult. We have developped 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 using this tool to analyse cancer genome data.

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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. For example, IgG1, one of the major IgG subclass in mice, cannot fix the complement, contrary to IgG2a, another major component of murine immunoglobulins. Such a difference may lead to dramatic variations in the functional effect of antibodies, as their ability to lyse cells they have bound. During the last few 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 (1). In the case of antiviral antibodies, a possible explanation for this phenomenon could be the selection by the infected host of the most appropriate response against the virus. Using a model of infection with lactate dehydrogenase-elevating virus (LDV) (2), we could demonstrate that IgG2a antiviral antibodies are indeed more efficient than other isotypes to protect mice against a fatal polioencephalomyelitis induced by the virus (3). The advantage for the host to select IgG2a in non-antiviral responses is more difficult to understand. In addition, 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-g 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 4). 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. 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 (5). Therefore, some, but not all subpopulations of activated NK cells might be susceptible to MHV infection.

ACTIVATION OF MACROPHAGES AND AUTOIMMUNE DISEASES

Activation of cells of the innate immune system includes also macrophages and leads 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. In contrast, BALB/C mice similarly immunized with rat platelets did not develop thrombocytopenia. 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 (7). 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 (8). 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 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 (9).

Macrophage activation by LDV led also to an enhanced response to lipopolysaccharide (LPS), and to an exacerbate susceptibility to endotoxin shock (10). 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.

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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.

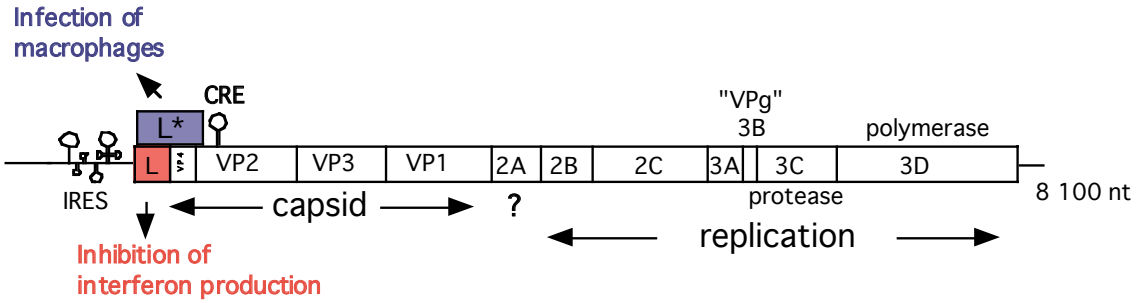

Fig. 1. Genome of Theiler's virus. A large open reading frame encodes a 2000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, to yield the viral mature proteins. Our analysis focuses on 2 proteins, L and L*, that interfere with the host innate immune responses and therefore facilitate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

Our work aims at understanding how a virus can persist in the central nervous system (CNS) of an immuno-competent host, thus evading the immune response.

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, C. Ricour, T. Michiels

Two viral proteins, namely L and L* were found to be dispensible 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 (2, 9). 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 (6).

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 (4). 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 (9).

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) (10).

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.

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 (3).

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.

TYPE-I AND TYPE-III INTERFERONS

Interferons were the first cytokines to be identified. They were discovered about 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 anti-cancer 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 (5).

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

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 (9). 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, 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 M. Mordstein and P. Stäheli (Univ. of Freiburg, 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.

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.

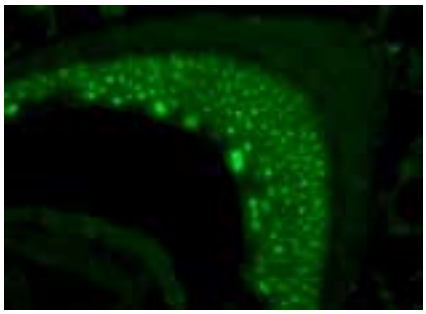

Fig. 2. Reponse to IFN-λ. Section of the urinary epithelium showing a strong response of epithelial cells to IFN-λ. Shown is a fluorescent labelling of the nuclear Mx1 protein, the expression of which is typically induced in response to IFN.

Production of IFN-α/β by neurons

M. Kreit, T. Michiels

In collaboration with the teams of Peter Staeheli 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 (7, 8).

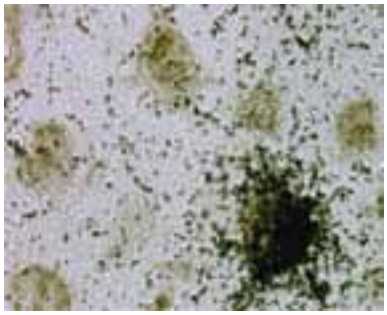

Fig. 3. Neurons can produce IFNα/β. Neuron (immunostained in brown for NeuN) producing IFN-β (detected by in situ hybridization: black dots) after infection with La Crosse virus.

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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 a minority of cancer patients vaccinated with defined tumor antigens display an objective tumor regression while most of them do not, it is essential to know whether and how the anti-tumor immune responses of the patients are either primed, amplified, or modified by the vaccinations. We nevertheless felt that it was crucial to know whether or not low-level responses had occurred and whether such cytolytic T lymphocytes (CTL) responses showed a correlation with tumor regression, in order to understand why most patients failed to show any evidence of regression. We therefore developed a sensitive approach based on in vitro restimulation of blood lymphocytes with the antigenic peptide 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 (TCR) sequencing (1, 2).

Focusing on CD8 T cell responses to antigenic peptides presented by HLA-A1 or A2 molecules, we observed a surprisingly low levels of anti-vaccine T cell responses 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 reponses and the clinical impact of the vaccinations (3, and unpublished observations). All these results suggest that the main limitation to the clinical efficacy of these therapeutic anti-cancer 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.

We described previously a low level anti-vaccine CTL response in the blood of a melanoma patient who displayed tumor regression after vaccination with MAGE antigens. With a genetic approach including T-cell receptor (TCR) cDNA libraries, we also found very few anti-vaccine CTL in the regressing metastases. But we found up to 1,000 times more of other TCR sequences, several of which corresponding to CTL clones specific for non-vaccine tumor antigens (4). We then observed another TCR that was enriched in regressing metastases, and that became detectable in blood only after vaccination. We wished to identify the antigen recognized by the T cells bearing this receptor. We used the TCR sequence to detect and clone the desired T cells out of tumor-infiltrating lymphocytes. The resulting CD8+ clone specifically lysed the autologous melanoma cells and displayed HLA-A2 restriction. Its target antigen was encoded by the gene coding for the mitochondrial enzyme caseinolytic protease. The gene bears a mutation in the tumor, resulting in a new antigenic peptide. These results reinforce our hypothesis that T lymphocytes enriched in tumors as compared to blood (5) do recognize tumor-specific antigens, and that the main effectors of tumor rejection in this patient were CTL to non-vaccine tumor-specific antigens. Of these, the mutated antigen described above is the only one against which no T cells could be detected before vaccination. Following vaccination, the spreading of the anti-tumor T cell response to this truly tumor-specific antigen may have contributed 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 (6). 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 TGFB

S. Lucas, J. Stockis, C. Huygens, 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 (7). 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. We are currently using this assay to monitor frequencies of Tregs in peripheral blood. In collaboration with laboratories from Italy, The Netherlands and Germany, we measured

circulating Treg frequencies in patients who received tumor vaccines in combination with different potentially Treg depleting strategies. 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 will attempt to develop our assay to measure Treg frequencies in tumor samples.

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-ß, a cytokine with well-known immunosuppressive actions. We are currently attempting to identify the mechanisms by which human Tregs can produce active TGF-ß.

mature TGF-ß, 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 (8). Membrane localization of latent TGF-ß mediated by binding to GARP may be necessary for the ability of Tregs to activate TGF-ß upon TCR stimulation. As illustrated in the figure below, a model by which activated Tregs would accumulate latent TGF-ß 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-ß 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-ß 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-ß to the cell surface, but does not

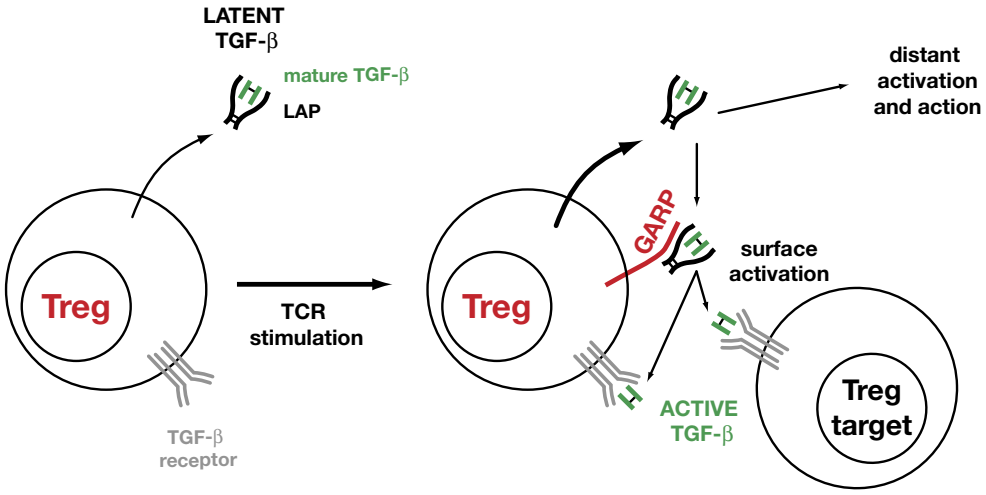

Figure 1. Possible model for TGF-ß production by human Treg clones.

Many cell types, including Treg and Thelper clones, produce the latent, inactive form of TGF-ß. In latent TGF-ß, the mature TGF-ß protein is bound to the Latency Associated Peptide, LAP, and is thereby prevented from binding to the TGF-ß receptor. We recently showed that latent TGF-ß, i.e. both LAP and result in the production of active TGF-ß 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-ß 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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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). In the two cases we initially described, 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 demonstrated that the same mechanism accounts for the splicing of a third spliced peptide, derived from FGF5, despite the fact that the fragments to splice are distant from each other by 40 amino acids (3). We also compared the efficiency of splicing by

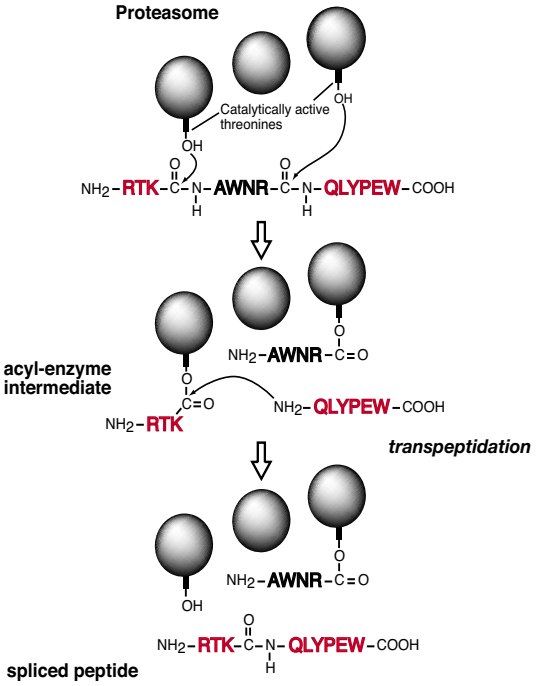

Figure 1. Model of the peptide-splicing reaction in the proteasome. The active site of the catalytic subunits of the proteasome is made up of the side-chain of a threonine residue, which initiates proteolysis by performing a nucleophilic attack on the carbonyl group of the peptide bond. An acyl-enzyme 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 were able to splice peptides, but their relative efficiency was different for each peptide, depending on the major cleavage sites. This is consistent with the transpeptidation model of splicing.

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

B. Guillaume, V. Stroobant, A. Busse

Using a series of novel antibodies recognizing catalytic subunits of the human proteasome in their native conformation, we also identified proteasomes that are intermediate between the standard proteasome and the immunoproteasome. 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.

PRODUCTION OF AN ANTIGENIC PEPTIDE 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 (4). This peptide, with sequence EVDPIGHLY, is presented by HLA-A1 and has been widely used in clinical trials of cancer vaccines. Insulin-degrading enzyme 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 recently 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) (5). 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 (6). 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 (7). 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-based drug design.

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 have devised a mouse melanoma model, in which we can induce melanoma in 70% of mice injected with tamoxifen (8). These tumors express the tumor antigen encoded by cancer-germline gene P1A. They 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 (9).

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 spontaneously. 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.

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

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

SLE is a systemic autoimmune disorder of unknown etiology, characterized by the activation of autoreactive CD4 T and B cells directed against constituents of the chromatin and the production of pathogenic antinuclear antibodies. Recently, several groups identified a characteristic interferon signature in PBMC from SLE patients, i.e. the over-expression of genes induced by type I interferons. We compared SLE synovitis and the synovitis of osteoarthritis (OA) and RA using high-density oligonucleotide spotted microarrays. Our results indicate that SLE arthritis is characterized by a very specific molecular signature that is distinct from that of OA and RA, with up-regulation of interferon (IFN)–inducible genes and down-regulation of genes involved in extracellular matrix (ECM) homeostasis. The latter observation is probably associated with the less destructive character of SLE compared to RA and OA. These results have immediate clinical applications for the differential diagnosis of arthritis.

We also performed global gene expression studies on synovial biopsies from RA patients treated with TNF blockers. We identified gene signatures in pre-treatment synovial tissue that predict the absence of response to TNF blockade. Not surprisingly, these genes can be induced in synovial cells by other inflammatory cytokines (such as IL-1b or IL-17), alone or in combination with TNF-a (10). These observations can be useful to guide therapeutic decisions.

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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 $CD8^+$ 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 CD8 tumor-infiltrating T lymphocytes show impaired IFN-γ secretion

Human CD8 tumor-infiltrating T lymphocytes (TIL) were isolated from tumor ascites or solid tumors and compared with T lymphocytes from blood donors. They were unable to secrete INF-γ or other cytokines after non-specific stimulation with anti-CD3 and anti-CD28 antibodies (7). TCR were observed to be distant from CD8 on the cell surface of TIL, whereas TCR and CD8 co-localized on blood T lymphocytes (Figure 1).

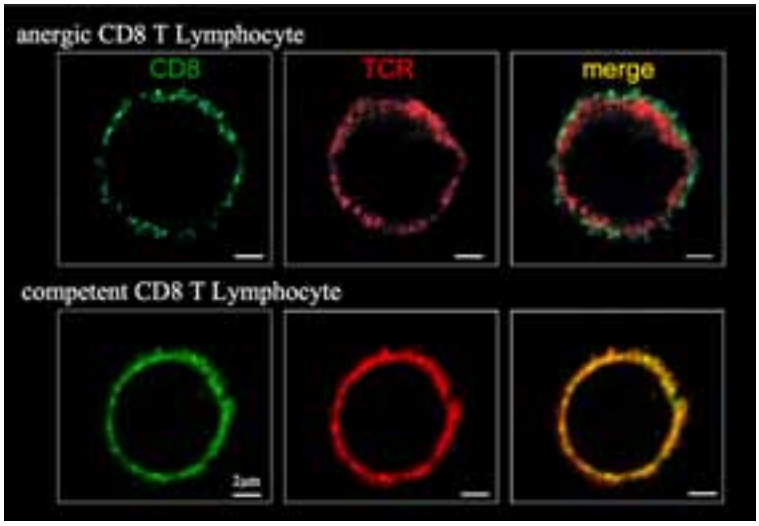


Figure 1. TCR and CD8 do not co-localize on recently stimulated CTL without effector functions.

Why do galectin-3 ligands improve human TIL function?

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-acetyllactosamine (LacNAc), a galectin-competitor ligand, restored this secretion (Figure 2). Our working hypothesis is that TIL have been stimulated by antigen recently, 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 recently 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 restore TCR mobility and boost IFN-g secretion by TIL. We recently strengthened this hypothesis by showing that $CD8^+$ TIL treated with an anti-galectin-3 antibody, which could disorganize lattice formation, had an increased IFN-γ secretion.

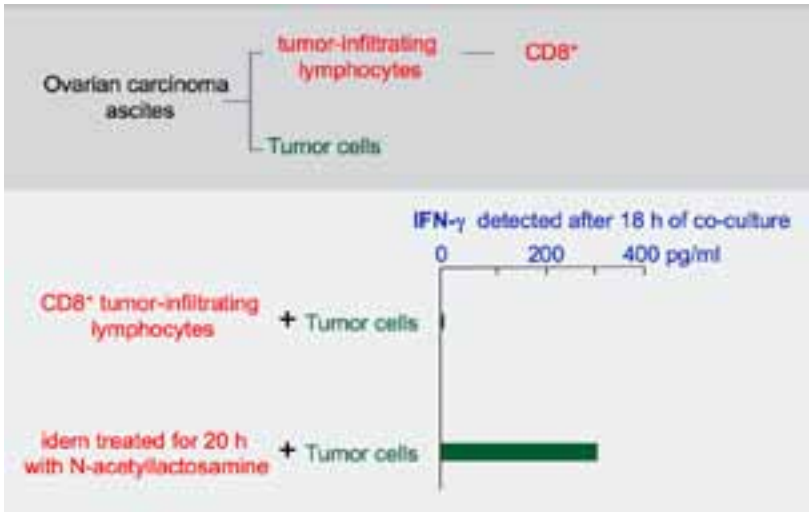


Figure 2. Treatment of tumor-infiltrating lymphocytes with a galectin ligand reverses anergy.

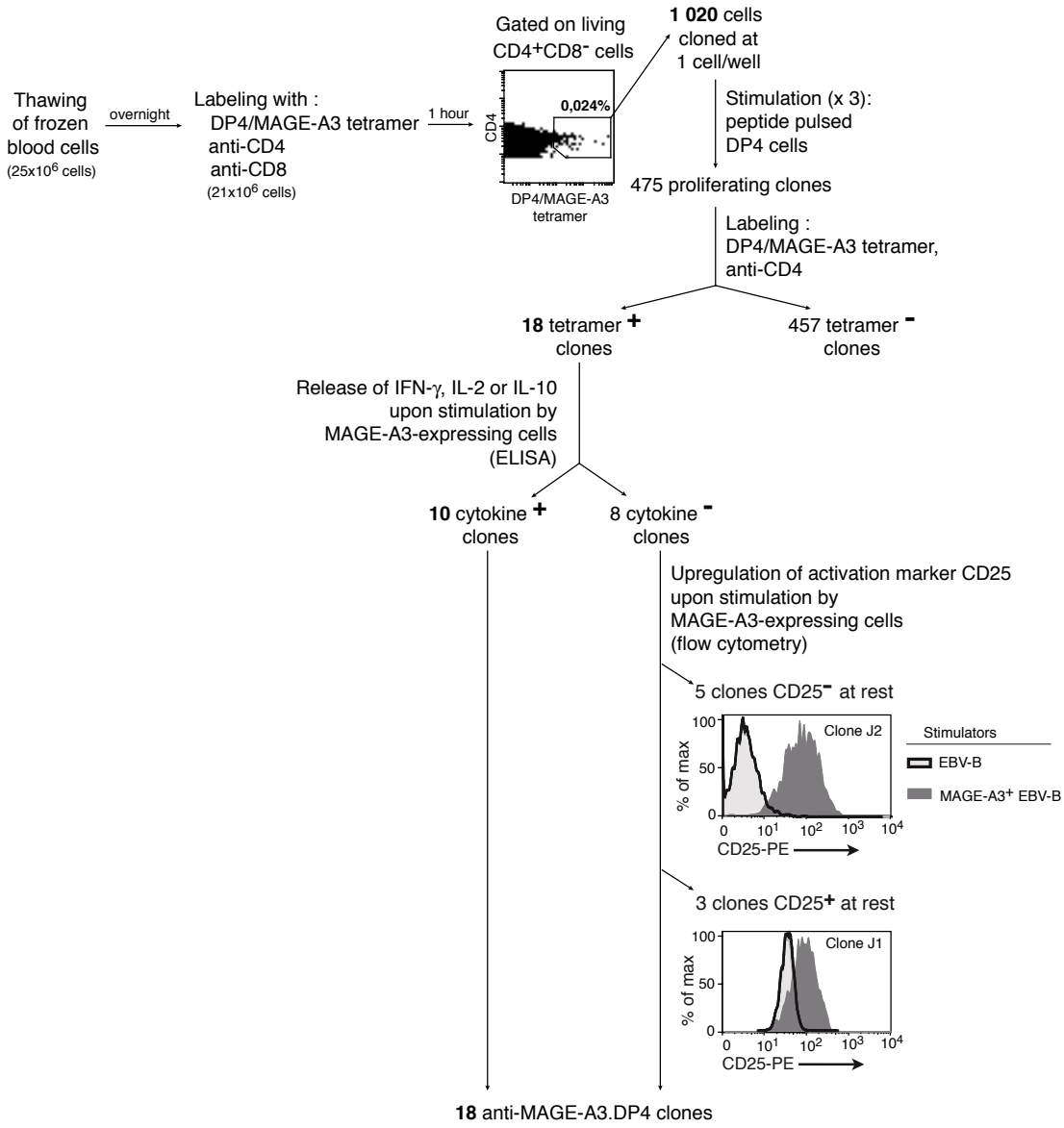
Towards a clinical trial combining vaccination and galectin-binding polysaccharides

These observations indicate that ex vivo human tumor-infiltrating lymphocytes can recover their effector functions with galectin-competitor ligands and suggest that treatment of cancer patients with galectin-competitor ligands could correct the anergy of TIL. It is possible that peptide vaccination combined with local injection of a galectin-competitor ligand will be more effective at producing tumor regression than vaccination alone. Galectin competitor ligands, e.g. disaccharides lactose and LacNAc, are rapidly eliminated in urine, preventing their use in vivo. Other compounds that could block interactions between galectin-3 and glycoproteins are under development by several groups. We found that a plant-derived polysaccharide, which is 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. We therefore intend to pursue clinical trials involving the use of these polysaccharides in combination with anti-tumoral vaccination.

DETECTION OF ANTI-MAGE-A3 REGULATORY T CELLS IN VACCINATED MELANOMA PATIENTS

The presence of regulatory T cells, which have the ability to dampen immune responses, could participate in an immunosuppressive environment in tumors. We have analyzed the blood T cells from 14 vaccinated melanoma patients who carried the HLA-DP4 allele and whose tumor expressed MAGE-A3 (8). The vaccines involved various antigens present on melanoma cells and all contained the MAGE-$A3_{243-258}$ peptide presented to T cells by HLA-DP4. The vaccines were either a mix of peptides without adjuvant, autologous mature dendritic cells loaded with peptides, or a MAGE-A3 protein mixed with adjuvant and combined with peptides. Our approach, outlined in Figure 3, involved the ex vivo selection of $CD4^+$ T cells that were labeled by DP4 MAGE-A3 tetramer and amplified under clonal conditions. A total of 197 $tetramer^+$ stable clones were isolated from 10 out of the 14 patients. Each of the 197 $tetramer^+$ $CD4^+$ T cell clones recognized the MAGE-A3.DP4 antigen. Anti-MAGE-A3.DP4 cells were found in 1 out of 2 patients injected with a mix of peptides without adjuvant, in 3 out of 6 patients injected with peptide-loaded dendritic cells, and in each of the 6 patients injected with a MAGE-A3 protein mixed with adjuvant and peptides. The frequencies in the blood samples collected after at least four vaccines ranged from $2x10^{-6}$ to $2x10^{-3}$ among the $CD4^+$ blood T lymphocytes. We found no correlation between the frequencies of anti-MAGE-A3.DP4 T cells and the clinical evolution of the patients, but the very small number of patients and the diversity of the vaccines preclude any conclusion.

Interestingly, 12 out of 197 clones expressed CD25 in resting state. This $CD4^+CD25^+$ phenotype was evocative of T cells with suppressive activity, known as regulatory T cells. Because the antigen recognized by our $CD4^+$ T cell clones was known, we designed a suppression assay where the potential regulatory T cell clone and an indicator T cell clone are each stimulated with their specific antigen presented by irradiated EBV-B cells. These twelve $CD25^+$ clones had a high capacity to suppress in vitro the proliferation of another T cell clone. Eleven of them had an high FOXP3 expression at rest and an unmethylated *FOXP3* gene. They secrete upon stimulation no IFN-γ, IL-2, IL-4, IL-5 or IL-10, but they produce active TGF-β. Their suppressive activity in vitro seems partly attributable to their secretion of active TGF-β. These regulatory T cell clones represent about 5% of the anti-MAGE-A3.DP4 T



Frequency =
0,024% of tetramer-labeled cells among the CD4+CD8- cells x (**18** anti-MAGE-A3.DP4 clones / **1 020** cloned cells)
= 4 x 10^{-6} among the CD4+ T lymphocytes

Figure 3. Example of the procedure used to obtain anti-MAGE-A3.DP4 T cell clones from blood cells of vaccinated patients. The numbers indicated correspond to an experiment performed with blood cells of patient EB97 collected after the sixth vaccination.

cell clones that we have isolated. In addition to these clones with an unmethylated *FOXP3* gene, several CD25 clones had a significant but lower suppressive activity, expressed *FOXP3* in the resting state but *FOXP3* demethylation was not observed.

This work is the first to describe the presence of anti-vaccine regulatory T cells not only on the basis of markers such as FOXP3, but also on the basis of their suppressive activity in vitro. FOXP3 seems of doubtful value as unique marker for regulatory T cells, due to its transient expression in some activated non-

regulatory T cells and also in some of our resting T cells without suppressive activity. Exclusive to T cells with a suppressive activity and a stable expression of FOXP3 is the demethylation of an intronic sequence of FOXP3. Thus, a quantitative DNA methylation analysis of FOXP3 based on RT-PCR could become a routine technique to identify what, in our opinion, are the best regulatory T cell candidates.

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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, 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 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, I. Jacquemart, B. Lethé, C. Lurquin, C. Uyttenhove, A. Van Pel, 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. We tested several approaches to break this tolerance. Immunization with irradiated male lymphoblasts did not induce rejection of established skin grafts, but repeated local injections of a low dose of IL-12, combined with IFN_, caused rejection in all mice. This was also the case when IL-12 was combined with ligands of Toll-Like Receptors 3, 7 or 9. IL-1_, IL-18 and IL-2 were incapable of inducing rejection on their own, but synergized effectively with IL-12.

To follow lymphocyte infiltration into the graft after cytokine injections, we used adoptive transfer of transgenic T cells bearing a receptor specific for a H-Y antigen. When transferred to a female CBA before grafting, naïve transgenic CD8 T cells did not infiltrate or reject the graft. But a low dose of IL-12 injected locally stimulated infiltration of the graft, whereas IFN_ had no effect. Our results suggest that local delivery of cytokine combinations may enhance the efficacy of anti-tumoral vaccination.

Contrary to naïve transgenic anti-HY CD8 T cells, adoptively transferred activated transgenic CD8 T cells infiltrated the grafts. Interestingly, skin grafts were rejected when the transferred T cells had been stimulated in vitro with H-Y peptide pulsed B-blasts, but were not rejected when the CD8 T cells had been stimulated in vitro with antibodies against CD3 and CD28. We tried to identify the critical difference between the CD8 T cells stimulated with the specific antigen and those stimulated with anti-CD3 antibodies. Comparison of the expression levels of cell surface markers by FACS analysis revealed that a CD8 T cell population stimulated with the H-Y peptide is composed of a larger proportion of CD62L-negative effector memory T lymphocytes, known to circulate in the periphery, whereas the population stimulated with anti-CD3 is composed of more CD62L-positive T lymphocytes, known to home to secondary lymphoid tissues. The functional avidity of the two activated CD8 T cell populations was tested by their capacity to secrete IFNg in response to various concentrations of H-Y peptide. No apparent avidity difference between the two populations was observed.

TGF-SS2 IN MELANOMA CELLS.

B. Lethé, C. Lurquin, in collaboration with J. Stockis

To understand the anergy of tumor-infiltrating lymphocytes in many patients, we have analyzed tumor samples of a series of metastatic melanoma patients, who were vaccinated with the MAGE-3.A1 tumor-specific antigen, for their expression of a series of genes coding for tumor-specific antigens, differentiation antigens and genes putatively associated with immunosuppression. Tumor samples for 26 patients who did not show any tumor regression were compared with 14 patients who showed significant tumor regression. For the gene coding for transforming growth factor beta 1 (TGF-b1) the ratio of non-regressors to regressors was 1.6, for TGF-b2 it was 2.4 and for TGF-b3 it was 1.1. One tumor cell line that was analyzed similarly produced a high amount of the TGF-b2 polypeptide (latent form) and showed SMAD2 phosphorylation, indicating that TGF-b2 is active. Strikingly, this cell line was exceptional being unable to support proliferation of autologous anti-tumoral cytolytic T cells. These results suggest that TGF-b1 and b2 may participate to an immunosuppressive environment protecting melanoma tumors against immune rejec

CONTROL OF IL-9 PRODUCTION BY T HELPER LYMPHOCYTES.

C. Uyttenhove, J. Van Snick

Since its discovery, IL-9 has been considered a TH2 cytokine but recent analyses involving intracellular cytokine staining of naïve CD4 T cells activated in vitro indicated that IL-9 did not fit the TH2 paradigm. In a publication by Marc Veldhoen and Brigitta Stockinger (5), to which we participated by producing the first antibody suitable for intracellular IL-9 detection, it was indeed shown that IL-9 producing cells were selectively induced when CD4 T cells were stimulated in the presence of TGF-b and IL-4, suggesting the existence of a novel T helper subset designated "TH9" (5). We have now provided evidence that these TH9 cells also develop when in vivo primed T cells are restimulated in vitro with the priming antigen in the presence of TGF-b and IL-4, indicating that this cytokine milieu can completely orient an established immune response to selective IL-9 production.

We then started a search for factors other than IL-4 that could stimulate IL-9 production in the presence of TGF-b. We found that TGF-b combination with IL-1a, IL-1b, IL-18, and IL-33 had equivalent IL-9 stimulating activities in all mouse strains tested, including IL-4- and IL-4-R-deficient animals. As IL-9 levels were much lower in TH2 cultures (IL4 + anti-TGF-b) or TH17 cultures (TGF-b + IL-6), these results identify TGF-b/IL-1 and TGF-b/IL-4 as the main control points of IL-9 synthesis.

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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). 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. Whilst these vaccines have shown no toxicity and have been associated with evidence of tumor regression in some patients, their overall anti-tumoral effect is poor. It is thought that this limited effectiveness is a consequence of the acquisition and selection by the tumor of immunosuppressive features, that allow it to resist immune mediated rejection. We are now focusing on new therapeutic approaches that combine a vaccine and an immunomodulatory treatment that is aimed at reverting the immunosuppressive tumor environment.

THERAPEUTIC VACCINATION WITH MAGE TUMOR ANTIGENS

In collaboration with J.F. Baurain (Centre du Cancer, Cliniques Universitaires St Luc) and the group of P. Coulie (Cellular Genetics Unit, de Duve Institute). The clinical trial program was set up and a large part of it was carried out by M. Marchand.

Many if not all human cancers express tumor antigens that can be recognized by T cells. 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 the cognate antigen. MAGE antigens are examples of tumor-specific antigens. They are encoded by MAGE genes, which are expressed in many different tumor types, such as melanoma, non-small cell lung cancer, bladder cancer, head-and-neck cancer and multiple myeloma. These genes are not expressed in normal somatic tissues. They are expressed in germline cells such as spermatogonias, which are devoid of surface HLA class I molecules, and thus can not present MAGE antigens on their surface. Thus, MAGE antigens are good candidates for cancer vaccines, because they are strictly tumor-specific, and are shared by various cancers.

Based on these findings, we have launched phase I/II clinical trials in which patients with advanced cancer, mainly melanoma, were repeatedly immunized with one or more tumor-specific antigens (Figure 1). These trials have two main objectives. First, the effectiveness of various vaccination modalities can be assessed by following the clinical evolution of the tumor, by analyzing whether a specific CTL res-

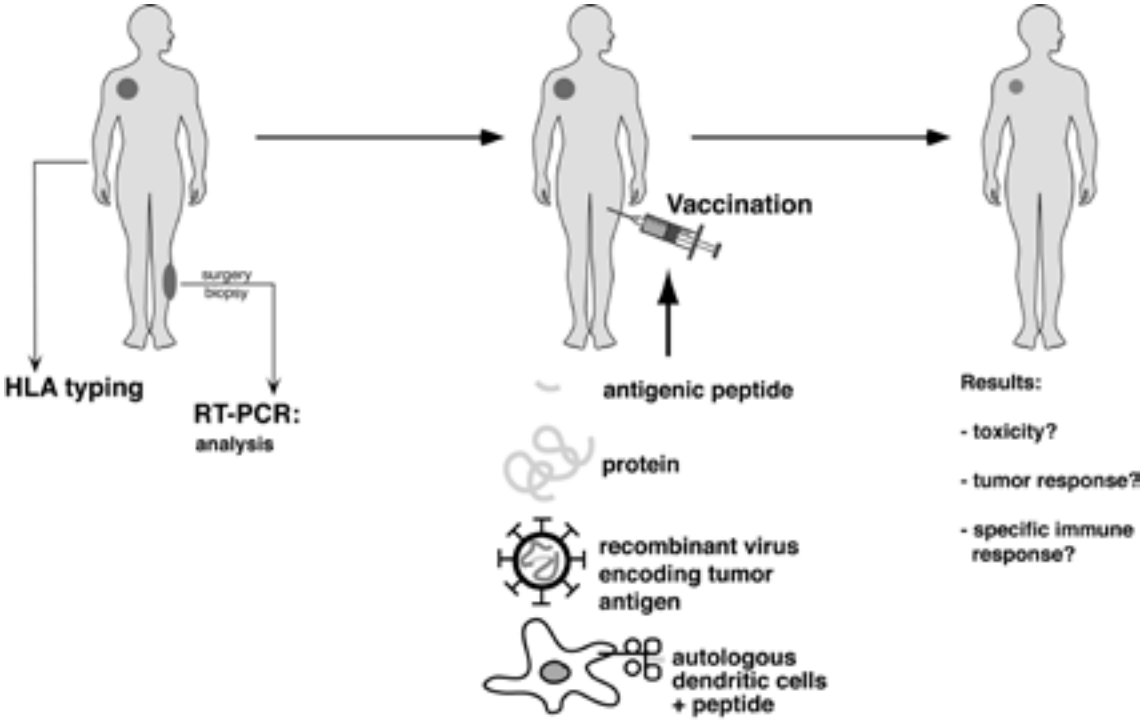

Figure 1. Principle of anti-tumor vaccination with a defined antigen : The first step is to determine if the patient's tumor cells express the tumor antigen. This can be determined by HLA typing, and by RT-PCR analysis of a tumor sample. Selected patients will receive repeated injections of a vaccine 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.

ponse to the vaccine antigen occurred, and by determining whether immunological and clinical responses are correlated. Secondly, these trials allow crucial biological material to be collected from vaccinated patients. Blood samples provide T cells, which allow to analyze the spontaneous and vaccine-induced immune response against tumor antigens. Tumor samples allow to analyze the interaction between cancer cells and immune cells in the tumor environment.

Different immunization modalities, such as immunization with peptides, or with the MAGE-3 recombinant protein, both with or without adjuvant, or with the 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. 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 (Figure 2). 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. CTL responses were detected in a minority of patients vaccinated either with peptides or with the ALVAC virus. These responses were often weak, and, in the case of the MAGE-3.A1 antigen, were observed mostly in patients who had tumor regressions.

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

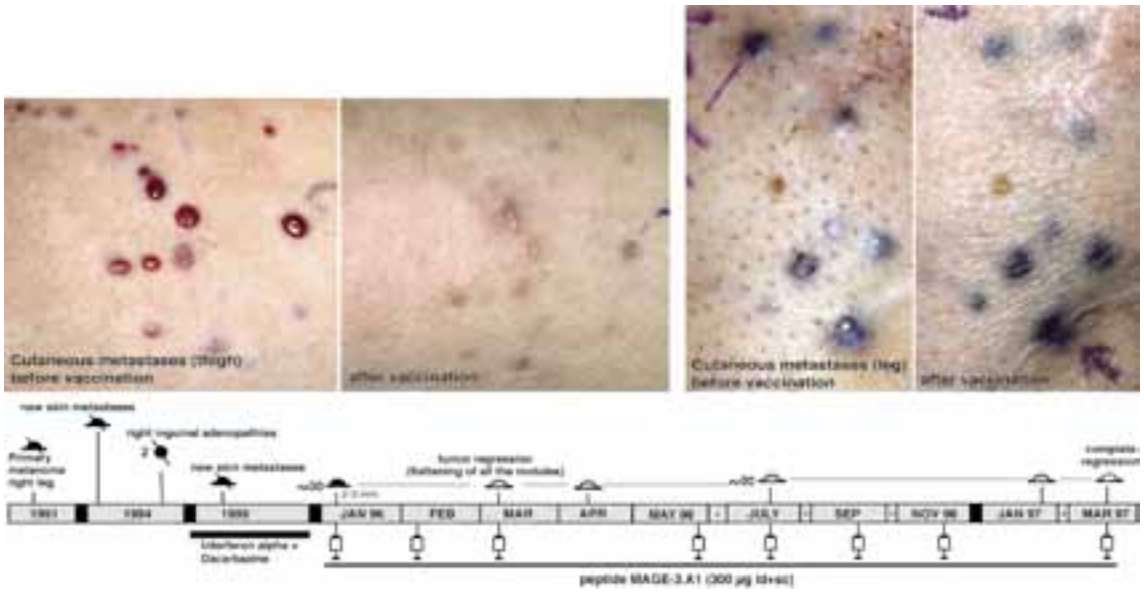

Figure 2. Example of a complete regression of cutaneous metastases in a melanoma patient after repeated vaccinations with the MAGE-3.A1 peptide given without immunological adjuvant.

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 consequence of local immunosuppression in the tumor environment.

We are now setting up a new clinical trial in which patients with advanced melanoma, including superficial metastases, will receive repeated peptide vaccinations, as previously, but now combined with a local immunomodulatory treatment. The latter will associate immunostimulatory cytokines and/or TLR ligands, administered in or close to 1 or 2 superficial metastases. The precise treatment will be chosen on the basis of skin graft rejection experiments performed in a murine model that mimics the situation observed in tumors. The vaccine is aimed at inducing new anti-tumoral T lymphocyte responses, and the local treatment is aimed at modifying the tumor environment in favor of effective tumor rejection.

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 Pierre 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. We are currently developing a new clinical trial, in which patients with advanced melanoma will receive a treatment combining a peptide vaccine and an experimental drug that inhibits galectin-3. 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.

Figure 3. Cryosections obtained from a cutaneous melanoma metastasis were stained with antibodies directed against either melanoma cells (left), T lymphocytes (middle) and macrophages (right), and counterstained with hematoxylin. The corresponding cells appear in red. These images show clearly that the inflammatory cells do not infiltrate, but rather surround the tumor mass.

EXPRESSION PROFILING OF TUMOR SAMPLES FROM VACCINATED PATIENTS

In collaboration with the group 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 analysis of adjacent cryosections, using antibodies directed against tumor cells, T and B cells, macrophages, blood vessels, and various molecules involved in inflammatory reactions (Figure 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 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.

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 parasites 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.

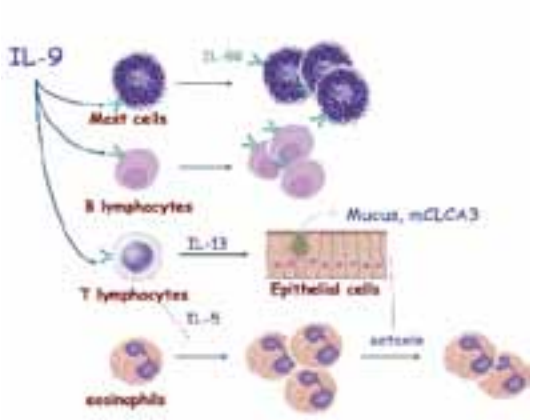

Figure 1. Direct and indirect activities of IL-9 in asthma. IL-9 acts directly on mast cells and B lymphocytes to induce an expansion of these cells and IgE production. IL-9 promotes the proliferation eosinophils indirectly, by upregulating IL-5 production by T cells. Upregulation of IL-13 production 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 in 1992 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 gc. This induces the phosphorylation of the JAK1 and JAK3 tyrosine kinases, which are associated with IL-9R and gc, 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).

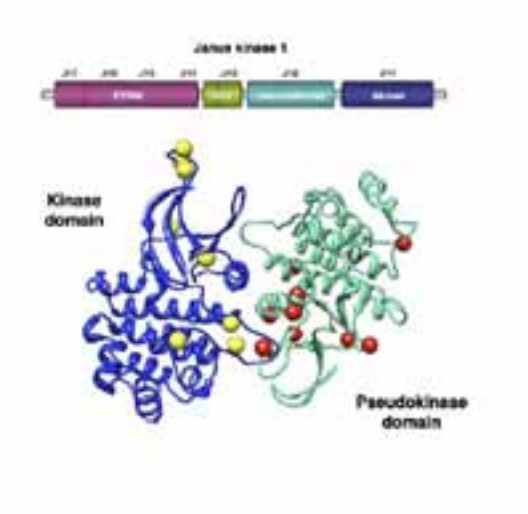

Figure 2. Localization of JAK1 activating mutations in the kinase and pseudokinase domains.

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 defiicent 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.

In addition to its role in IL-22 binding and signalling, the IL-22R chain also forms a functional heterodimeric receptor complex by associating with IL-20Rß, the second short chain member of the IL-10R-related receptor family. This complex mediates STAT-1 and –3 activation by IL-20 and IL-24, but not by IL-22. In addition, IL-20 and IL-24 can also bind to other complexes consisting of IL-20Ra and IL-20Rß (see ref. 10 for a review of this cytokine family).

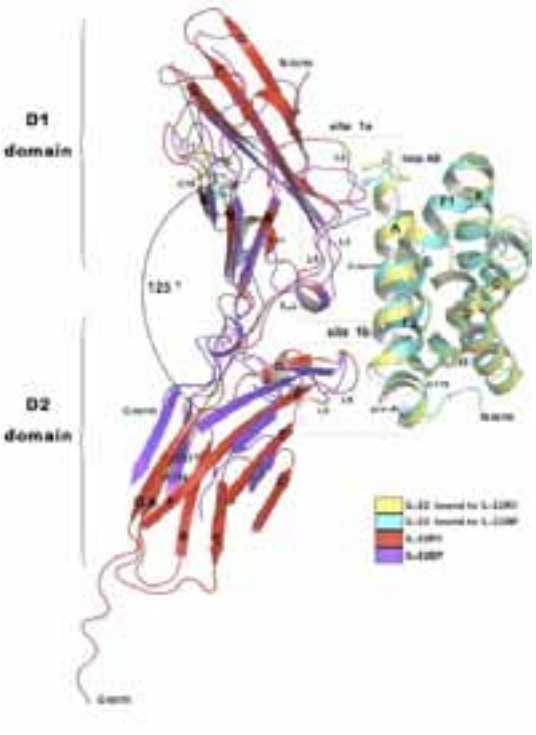

Figure 3. Comparison of IL-22/IL-22BP and IL-22/IL-22R1 binding interfaces. Superposition of IL-22/IL-22BP (cyan/purple blue) and IL-22/IL-22R1 (yellow/red) crystal structures shows their binding interfaces outlined by boxes.

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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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), and interleukins (IL) 2 and 9. Activation of these receptors is triggered by cytokine-induced changes in receptor dimerization/oligomerization, which lead to the activation of cytosolic Janus tyrosine kinases (JAK). Regulation by JAK kinases of receptor traffic, the assembly of cell-surface receptor complexes, the mechanisms of dimerization of transmembrane (TM) and cytosolic juxtamembrane (JM) domains, and mechanisms of JAK catalytic activation are major points of interest. The laboratory identified constitutively active mutants of JAK2, JAK1 and Tyk2 and of thrombopoietin receptor and is actively investigating the mechanisms by which JAK2 V617F and thrombopoietin receptor W515 mutants induce, in humans, Myeloproliferative Neoplasms, such as Polycythemia Vera, Essential Thrombocythemia or Primary Myelofibrosis

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

A. Dusa, C. Pecquet

The JAK-STAT pathway is emerging as a key player in cancer, with several mutations in genes coding for JAKs being identified in the past three years (1). Janus kinases possess two kinase domains, one active and the other, denoted as the pseudokinase domain, inactive. JAK2, one of the four known JAKs (JAK1, JAK2, JAK3 and Tyk2) is crucial for signaling by several cytokine receptors, such as the erythropoietin receptor (EpoR), the thrombopoietin receptor (TpoR), the G-CSF receptor (G-CSFR), the interleukin 3 receptor and the growth hormone receptor. JAKs are appended to the cytoplasmic juxtamembrane domains of receptors and are switched-on upon ligand binding to the receptors' extracellular domains.

Polycythemia Vera (PV), or the Vaquez disease, is characterized by excessive production of mature red cells and sometimes of platelets and granulocytes. Erythroid progenitors in PV are hypersensitive to Epo or independent of

erythropoietin (Epo) for proliferation and differentiation. Strikingly, the traffic of TpoR is defective in myeloid progenitors from PV. A hint that JAK2 or JAK2-binding proteins may be involved in PV came when we showed that the wild type JAK2 strongly promotes the maturation and cell-surface localization of TpoR, the very process that is defective in PV (2).

In collaboration with William Vainchenker at the Institut Gustave Roussy in Paris, we have been involved in the discovery of the 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

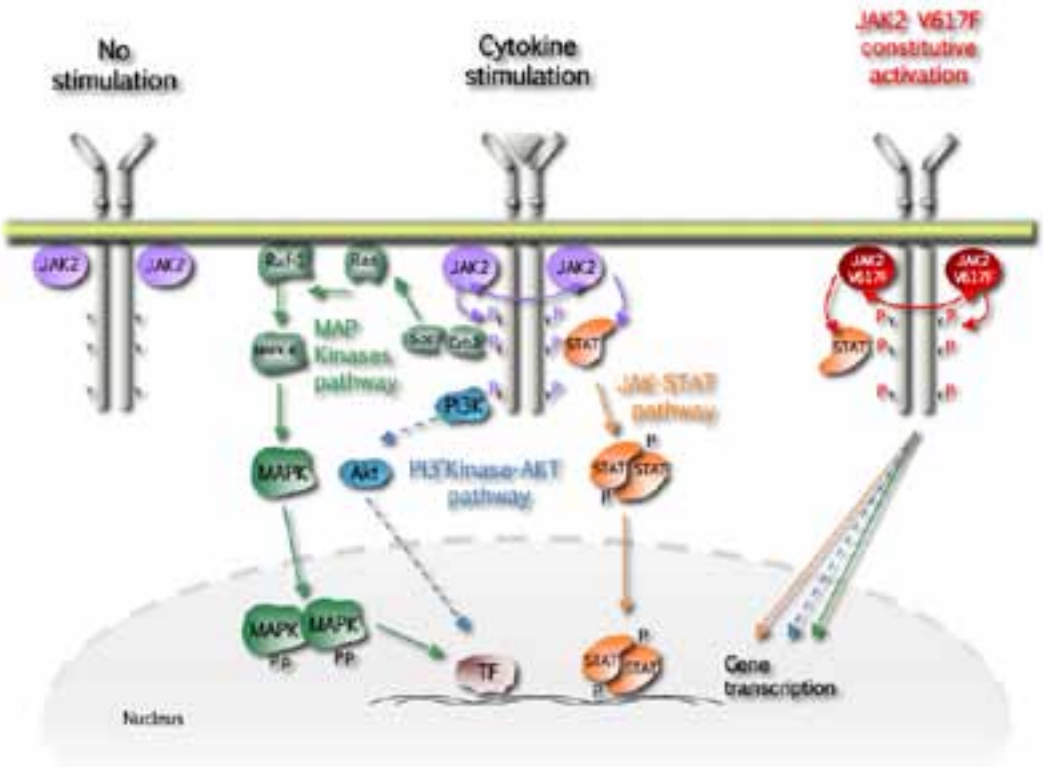

Figure 1. In the absence of cytokine ligands, cytokine receptors (left complex) are preassembled with tyrosine kinases JAK (Janus kinases) in inactive complexes. Cytokine binding to the extracellular domains of receptors (middle complex) induces a conformational change which allows the appended JAKs 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)

to cytokine receptors (4). Similarly, at least Trp, Leu and Ile also can 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 ~ 20% of adult T-lymphoblastic leukemia.

Involvement of TpoR in myeloproliferative diseases

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

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. 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 (7) (Figure 2). We predicted that mutations in the amphipathic motif W515 may exist in patients with myelofibrosis (6). Indeed, residue W515 has been found to be mutated to either leucine or lysine by the groups of D. G. Gilliland and A. Tefferi. Why the 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 of TpoR, and appears to involve excessive MAP-kinase signaling (7). Thus, small molecules targeting phosphorylated Y112 might be useful in the treatment of myelofibrosis.

At present, our laboratory is performing 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), Clinical Biology (Prof. Dominique Latinne) and HORM-PHOS Unit of de Duve Institute (Prof. Mark Rider) a large study on the presence and signaling of JAK2 and TpoR mutations in patients with myeloproliferative neoplasms. Close collaborations with Drs. Laurent Knoops and Jean-Baptiste Demoulin are supported by the ARC project.

DETERMINATION OF THE INTERFACE AND ORIENTATION OF THE ACTIVATED EPOR, TPOR AND G-CSFR DIMERS

N. Caceres, A. Dusa, J.-P. Defour

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 of the receptor, 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 a-helices is imposed on the downstream TM a-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.

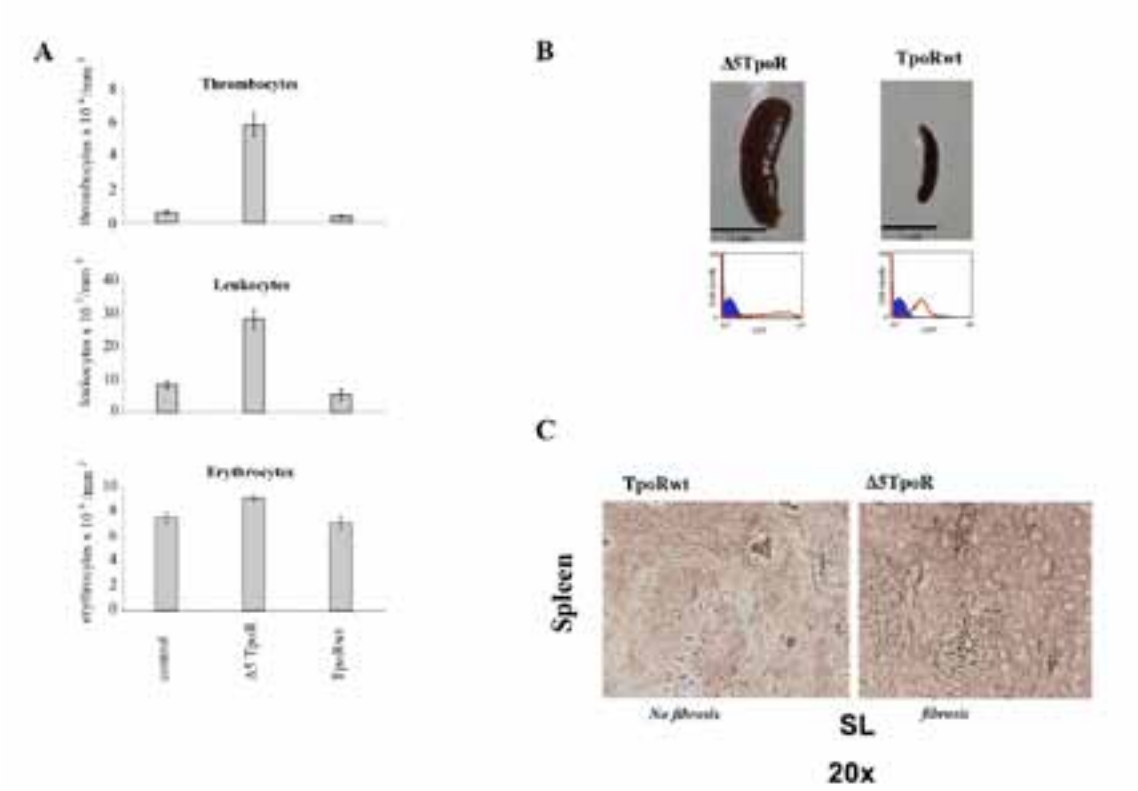

Figure 2. Bone marrow adoptive transfer in lethally-irradiated mice with hematopoietic stem cells expressing the constitutively active Δ5TpoR induces severe myeloproliferative disorder, splenomegaly and fibrosis of the spleen. Δ5TpoR is a mutant where the amphipathic RW515QFP motif is deleted, which 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 (C. Pecquet and J. Staerk).

STRUCTURE AND FUNCTION OF JUXTAMEMBRANE AND TRANSMEMBRANE SEQUENCES OF CYTOKINE RECEPTORS

R.-I. Albu, A. Dusa, 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 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

C. Pecquet, R.-I. Albu

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, gc). 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 the receptor. The W515K activating mutation was introduced in TpoR mutants that lack segments of the extracellular domain; these mutants are normally impaired in their traffic. Selection in the absence of Tpo leads to enhanced cell surface localization of N-terminally truncated TpoR mutants that also posses the activating W515K mutation. Microarray experiments are determining which chaperones or signaling proteins are overexpressed in selected cells, that might stimulate TpoR traffic.

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

M. Girardot

Cytokine stimulation of cytokine receptors, induces transient activation of the JAK-STAT pathway. In contrast, oncogenic forms of re-

ceptors 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 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 to be translocated in rare leukemias. LPP is the host gene for miR-28, which we found to down-modulate TpoR translation, impair megakaryocyte differentiation. miR-28 is pathologically overexpressed in 30% of MPNs (10). Tagets of miR-28, such as E2F6, are critical cell cycle regulators that might influence the phenotype of myeloproliferative disorders (10), thus linking spcific gene induction by constitutive STAT signaling to phenotype of disease.

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