Research

at the

de Duve Institute

and

Brussels Branch of the Ludwig Institute for Cancer Research

August 2009

de Duve Institute

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The de Duve Institute

THE DE DUVE INSTITUTE:
AN INTERNATIONAL BIOMEDICAL RESEARCH INSTITUTE

In 1974, when Christian de Duve founded the Institute of Cellular Pathology (ICP), now renamed the de Duve Institute, he was acutely aware of the constrast between the enormous progress in biological sciences that had occurred in the 20 preceding years and the modesty of the medical advances that had followed. He therefore created a research 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 Prorectors, 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.

Miikka Vikkula

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ACKNOWLEDGMENTS

In 2008, 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 2008 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,

another fellowship was recently awarded by the Institute to Reece MARILLIER.

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

Luc BERTRAND,
President of the Development and Expansion Council

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

The 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 tightly together 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, 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 [1]. 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 activating 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; Wouters et al, in prep). 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 [2]. In addition, we discovered that at least 50% of the far more common sporadic VMs are caused by somatic mutations in TIE2 [2]. All of the 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.

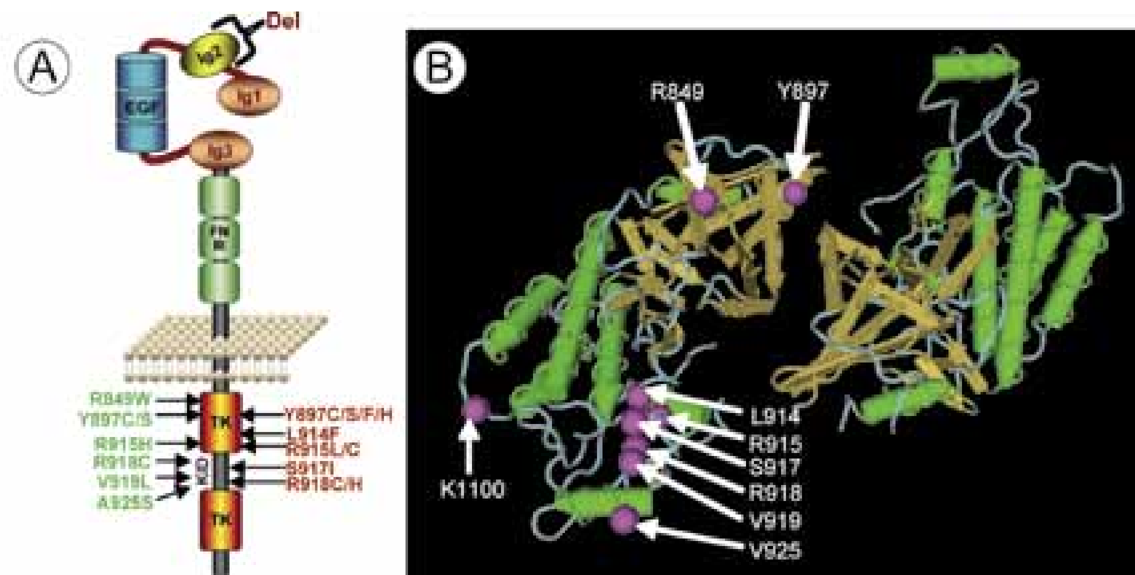

Fig 1. Intracellular TIE2 mutations identified in VM. (A) Inherited changes in VMCM blood samples (left, in green) and somatic changes in sporadic VM lesions (right, in red). A somatic 2nd-hit deletion of the Ig2 ligand-binding domain ("Del") was identified in a lesion from a VMCM patient carrying R849W.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

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 35 different mutations in 133 families. As in VMs, we showed that GVM lesions appear locally be cause of the additional alteration of the second allele, likely in vascular smooth muscle cell pre-

cursors. To better understand the role of glomulin in normal and pathological conditions, we have created glomulin-deficient mice. While heterozygotes appear normal, homozygous knockouts are embryonic-lethal. To enable studies beyond this time-point, we generated RNAi conditional knockdown mice, in which glomulin 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, 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].

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, University of Washington, 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 [5].

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 cuta-

neous capillary-venous malformations (HC-CVM) 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) [6]. 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.

CARDIOPATHIES

M Amyere, I. Gutierrez-Roelens, and M. Vikkula, in collaboration with T. Sluysmans, C. Ovaert, St-Luc, UCL and M. Gewillig and K. Devriendt, KUL

The cardiovascular system can encounter developmental problems affecting the heart. These cardiac defects or cardiopathies vary from physiological septal defects to life-threatening complex malformations. Identification of genetic causes enables tight follow-up and preventive pacemaker implantation. CSX/NKx2.5 gene, an important transcription factor for cardiac development, was mutated in three families, in which atrial septal defect is associated with progressive atrioventricular conduction defect. For families with heterotaxia and situs inversus, whole-genome linkage analysis was performed using 10K Affymetrix SNP-chips, which identified a possible locus for a causative gene.

CLEFT LIP AND PALATE

M. Ghassibé, L. Desmyter, 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 van der Woude syndrome, the most common cleft syndrome, and showed that IRF6 is the major causative gene in our Belgian cohort. Moreover, IRF6 is the gene responsible for popliteal pterygium syndrome. 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. In parallel, we identified a new gene responsible for cleft palate only and Pierre Robin sequence. We are currently generating and phenotyping zebrafish and mouse models to understand the mechanism behind craniofacial development and cleft occurrence across species.

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" hypothesis, in which genes implicated in stem cell fate decisions may be important for supporting cancer stem cells as well. Pathways activated in high grade ependymomas were consistent with the histological appearance of a more aggressive tumor phenotype [7]. 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. 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 [8]. We also described a family with a very rare presentation of severe head and neck paraganglioma with liver and spine localization. No evidence of mutations was found in the known predisposing genes by dHPLC and/or SSCP. An in depth search for the genetic abnormality underlying this unusual form of paraganglioma

is currently under way.

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, M. Herman, 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 Schools, Boston, we genotyped a large family with a new form of hypophosphatemia and mapped this autosomal recessive form (designated ARHP) to chromosome 4q21. This allowed for the identification of causative homozygous mutations in DMP1 (dentin matrix protein 1), which encodes a non-collagenous bone matrix protein expressed in osteoblasts and osteocytes [9]. In collaboration with G. Matthijs from KULeuven, we have been able to combine genotyping and expression profiling in one consanguineous family with a congenital glycosylation disorder. Autozygosity mapping along

with expression profile analysis allowed us to identify a new gene for CDG. 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 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]. 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).

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. This work is currently pursued by the analysis of cyst formation in polycystic diseases, in the light of the new mechanism of tubulogenesis and of the role of Sox9. In addition, the function of Sox9 was found to be tightly linked with that of the Notch signaling pathway [4]. The latter is deficient in liver of patients affected with Alagille syndrome, a disease characterized by bile duct paucity and severe cholestasis.

We have shown that the Transforming Growth Factor-beta (TGF-beta) signaling stimulates differentiation of biliary cells. TGF-beta signaling is detectable in the liver as a gradient, with high signaling activity near the portal vein, where biliary cells differentiate, and lower signaling activity in the parenchyma, where hepatocytes differentiate. The results of this research are now used by collaborating teams who attempt to program in vitro differentiation of stem cells to hepatocytes for cell therapy of liver disease. Our efforts in 2008 were concentrated on the understanding of the role of TGF-beta signaling in the formation of bile ducts. We found that TGF-beta is essential at several steps of biliary development and is a regulator of the transient asymmetry that characterizes bile duct development [3].

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 [5]. They are also critical for normal differentiation of hepatic precursor cells to hepatocytes or cholangiocytes [2], and their level of expression during hepatocyte differentiation determines time-specific gene activation in the liver [6]. Current work focusses on the molecular mechanisms by which HNF-6 and OC-2 fine-tune gene expression at several stages of hepatocyte differentiation.

PANCREAS DEVELOPMENT

M. Colletti, 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 cells that produce insulin, glucagon, somatostatin, pancreatic polypeptide

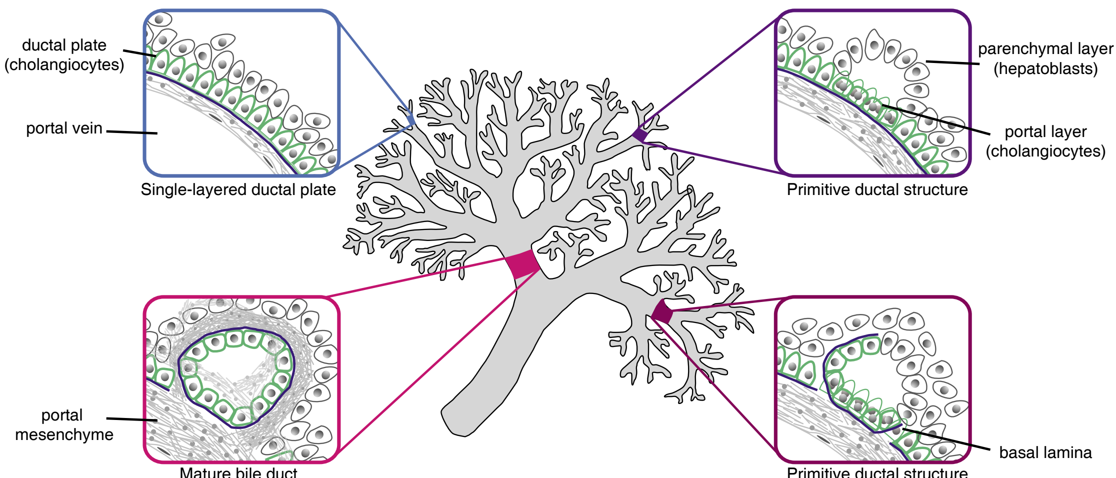


Fig. 1. Bile duct development progresses from the hilum of the liver to the periphery of the lobes. The intrahepatic biliary tree is represented at a stage when ducts have reached maturity near the hilum (lower left), while the most peripheral structures are still in single-layered ductal plate configuration (upper left). Intermediate stages illustrate the progression from asymmetrical to radially symmetrical ducts (upper and lower right).

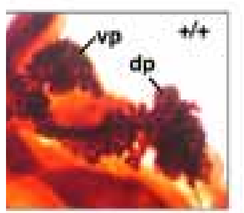
or ghrelin. The endocrine cells associate to form the islets of Langerhans. Our group investigates the transcriptional mechanisms and signal transduction pathways that control how endoderm cells become pancreatic progenitors and how the latter develop into endocrine precursor cells. This research is currently pursued using the mouse, including transgenic mice, as a model organism.

In 2008 we investigated how the transcription factors HNF-6 and OC-2 and FGF signaling control development of pancreatic progenitors from the endoderm, and the subsequent generation of endocrine precursor cells. The Onecut transcription factors HNF-6 and OC-2 exert redundant roles in pancreas morphogenesis and in differentiation of endocrine cells [7,8]. Work has progressed on the molecular mode of action of HNF-6 and OC-2, in particular on the identification of microRNAs that regulate or are regulated by these factors.

FGF-10 is produced by the mesenchyme surrounding the developing pancreas and sustains development of the pancreas [9]. In 2008 we have investigated which receptors mediate FGF-10 signaling in pancreas by analysing mice that are deficient in FGFR1b and/or FGFR2b. The results indicated that FGFR2b is critical for pancreas development and mediates the role of FGF-10 during early pancreas development.

The results of the research on Onecut factors and FGF signaling are being translated in the design of cell therapy of diabetes by collaborating teams who attempt to differentiate stem cells to insulin-producing cells. These collaborations include our participation to the EU-sponsored network BetaCellTherapy.

During pancreas development, the progenitor cells also give rise to exocrine and ductal cells. Our work addresses how pancreatic ducts are generated. These ducts drain the secretions from the pancreatic exocrine cells to the intestine and are delineated by ductal cells. We have shown that the Onecut factor HNF-6 controls a network of genes that is required for the formation of cilia at the apical pole of the ductal cells and for normal development of the ducts. In the absence of HNF-6, the ducts form cysts, much like in human polycystic diseases [10].



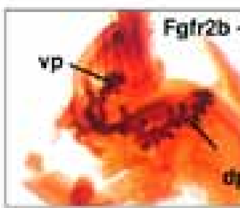


Fig. 2: The pancreas of mouse embryos that are wild-type or knockout for the FGF-10 receptor FGFR2b was studied at embryonic day 14.5 by whole mount immunostaining with an antibody against the pancreatic protein Pdx1. The ventral (vp) and dorsal (dp) parts of the pancreas can be distinguished. In FGFR2b-deficient embryos, the pancreas is severely hypoplastic and shows reduction in its branching pattern. Pancreatic endocrine cells can differentiate in these embryos albeit in reduced numbers (not shown on picture).

Work by our and other teams has provided evidence that ductal cells can transdifferentiate to pancreatic endocrine cells. Current work investigates the transcriptional mechanisms that govern this transdifferentiation process.

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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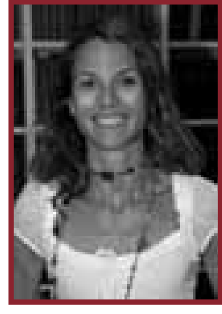

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GENETIC AND EPIGENETIC ALTERATIONS IN GENOMES

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 unlimited proliferation potential. Furthermore, 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*

A. Decottignies, S. Lenglez

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 poorly characterized so far (1).

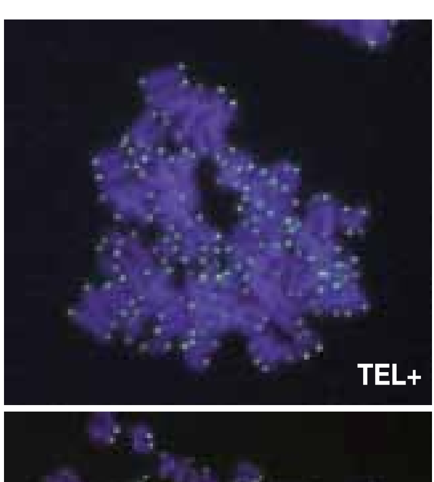
From yeast to mammals, different studies also reported the insertion of DNA fragments of various sources at experimentally-induced DSBs, including mitochondrial DNA (mtDNA) in budding and fission yeast (2). Interestingly, recent studies reported the association of human genetic diseases with *de novo* 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. We are currently investigating the mechanisms responsible for this association.

TELOMERASE AND ALTERNATIVE MECHANISM(S) OF TELOMERE MAINTENANCE IN HUMAN CELLS

M. Mattiussi, G. Tilman and 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). ALT cell lines can also be obtained after *in vitro* immortalization of telomerase-negative human fibroblasts with SV40 T antigen. 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).



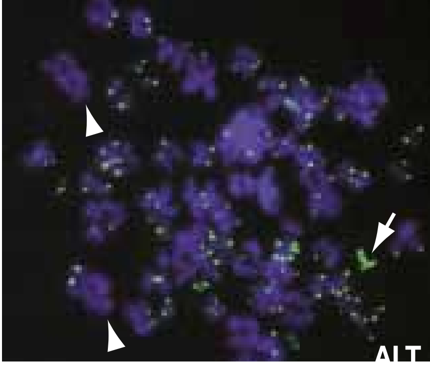


Figure 1. Telomere-specific fluorescence *in situ* hybridization (FISH) on metaphase chromosomes of telomerase-positive (TEL+) and ALT cancer cells (ALT). Telomeres are hybridized with a fluorescent telomeric probe (green) and DNA is stained with DAPI (blue). In ALT cells, telomeres are very heterogeneous,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

In addition to its 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. We are interested in the study of genes that are distinctly regulated in ALT and TEL+ cells. In particular, we are investigating the role of telomerase in the modulation of TGF-β-dependent induction of extracellular matrix protein-encoding gene expression, like periostin (3), collagen or fibronectin in human dermal fibroblasts.

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 generally 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 (6,7). The proposed model of MAGEA1 demethylation and activation during tumor development is illustrated in Figure 2. The factors that are responsible for the initial DNA demethylation process and for maintaining cancer-germline gene promoters unmethylated remain to be identified.

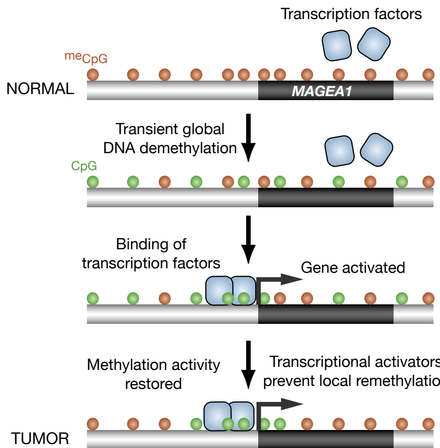


Figure 2. Model for the stable activation of MAGEA1 in tumors.

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 (8). 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 (8). We further showed that reducing T-SCE frequency of ALT cancer cells was not associated with subtelomeric DNA remethylation. Strikingly, subtelomeric DNA hypomethylation in ALT cancer cells was also associated with lower global DNA methylation. This observation raises the interesting possibility that DNA demethylation in tumor cells may be linked to senescence or to the process that cells use to escape from senescence.

We are currently addressing 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.

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 (9). This suggests that cancer-germline genes are programmed for methylation-mediated silencing in human ES cells. Accordingly, transfection experiments indicated that human EC cells direct a potent repression activity towards the promoter of MAGEA1. This was associated with progressive de novo methylation of the transgene. Our group is currently using the chromatin immunoprecipitation (ChIP) technology to define histone modifications associated with the silencing of cancer-germline genes in human ES/EC cells. This should help us to identify the factors that target DNA methylation towards cancer-germline gene promoters in these cells. Loss of function of such factors may be a prerequisite for demethylation and ac-

tivation of cancer-germline genes in tumors.

MOUSE EMBRYONIC STEM CELLS AS A MODEL TO STUDY MAGEA1 DEMETHYLATION

A. Loriot, C. De Smet (In collaboration with O. De Backer, Molecular Physiology Research Unit, FUNDP, Namur)

We found mouse cancer-germline genes to be repressed and methylated in mouse ES cells. Surprisingly however, when *in vitro* methylated human cancer-germline sequences were transfected into mouse ES cells, the integrated transgenes became demethylated (10). It appears therefore that mouse ES cells not only lack appropriate factors to induce *de novo* methylation of human cancer-germline transgenes, but in addition target the demethylation machinery to these sequences. This disparity between mouse and human cancer-germline genes is likely attributable to their poor sequence conservation, especially within regulatory regions. Nevertheless, mouse ES cells offer a valuable experimental model to study the mechanisms of demethylation of human cancer-germline sequences. To this end, we constructed a cDNA library from mouse ES cells, and transfected it into a recipient cell clone containing a selectable human MAGEA1 transgene. In this cellular clone, the transgene is methylated and repressed; its activation should therefore reveal the presence of a cDNA clone capable of inducing DNA demethylation. Transfectants with re-activated MAGEA1 transgenes are being isolated. Mouse ES-derived cDNAs contained in these transfectants will be identified by direct PCR amplification with primers matching vector sequences, and their ability to induce demethylation will be confirmed by repeating the transfection procedure described above.

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, S. Jaisson, M. Veiga-da-Cunha, 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.

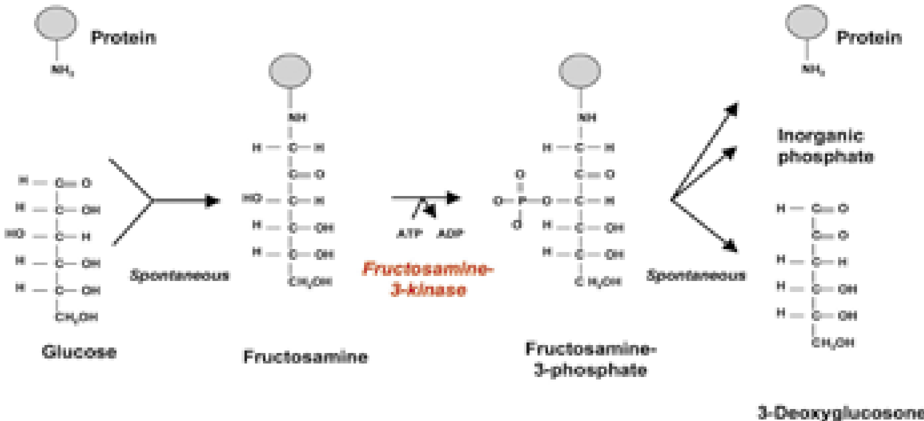

Figure 1. Formation and breakdown of fructosamines

These studies showed also that only part of 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 knock-out model that we created (6). 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 understand the physiological significance of FN3K-RP-mediated deglycation.

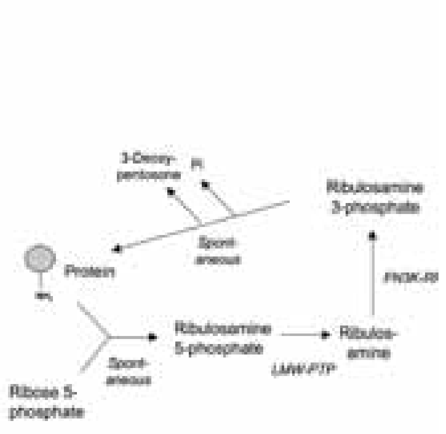

Figure 2. Formation and breakdown of ribulosamines

NEUROMETABOLIC DISORDERS

Y Achouri, F. Collard, J. Drozak, S. Jaisson, G. Noël, K. Peel, G. Tahay, 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 (8). 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.

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.

We are presently studying enzymes involved in the synthesis of brain-specific compounds, such as N-acetylaspartate, with the hope of elucidating as yet unresolved neurometabolic disorders.

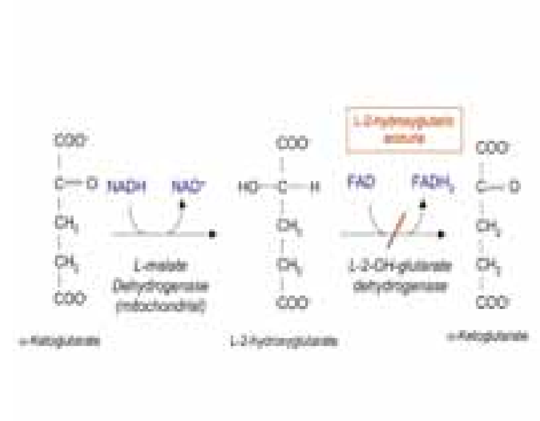

Figure 3. Formation and breakdown of L-2-hydroxyglutarate

METABOLISM OF GLUCOSE 1,6-BISPHOSPHATE

P. Maliekal, M. Veiga-da-Cunha, E. Van Schaftingen

Glucose 1,6-bisphosphate (Glc-1,6-P2), a well known cofactor for phosphoglucomutase and other sugar phosphomutases, is ubiquitously present in tissues. Its concentration is particularly elevated in brain, where it reaches values of >100 µM, i.e. >1000-fold higher than the concentrations required to stimulate phosphoglucomutase. Glc-1,6-P2 has been proposed to be an effector for several enzymes (Fig. 4). Phosphofructokinase and liver pyruvate kinase are both stimulated by this compound, whereas low Km hexokinases, 6-phosphogluconate dehydrogenase, and fructose-1,6-bisphosphatase are inhibited. These effects have been demonstrated in vitro, but under conditions that are not necessarily physiologically relevant. In addition, the occurrence of this regulation in intact cells has not been demonstrated.

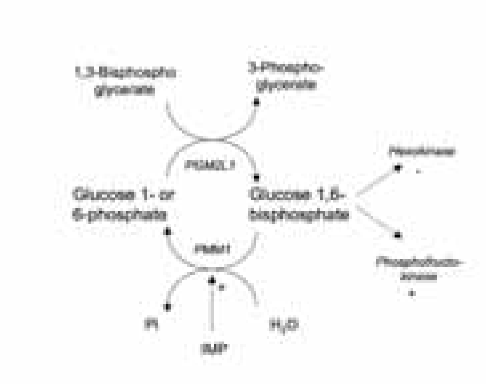

Figure 4. Metabolism and potential regulatory role of glucose 1,6-bisphosphate in brain

We have recently reported the molecular identification of the enzymes that synthesize and degrade Glc-1,6-P2 (6,10). Glc-1,6-P2 is made from 1,3-bisphosphoglycerate and glucose 1-phosphate or glucose 6-phosphate by Glc-1,6-P2 synthase, an enzyme particularly abundant in brain. We have recently identified this enzyme as PGM2L1 (phosphoglucomutase 2-like 1). It belongs to same family as phosphoglucomutase 1, the enzyme that interconverts glucose 6-phosphate and glucose 1-phosphate. Its closest mammalian homologue if PGM2, which serves to interconvert (deoxy)ribose 1-phosphate and (deoxy)ribose 5-phosphate. PGM2 also catalyzes the synthesis of Glc-1,6-P2, although with a lower Vmax than PGM2L1 and a much stronger inhibition by the reaction product Glc-1,6-P2. In comparison with PGM2, PGM2L1 is therefore better suited to provide cells with elevated concentrations of Glc-1,6-P2. This conclusion is supported by the finding that transfection of HEK293T cells with PGM2L1 caused a ≈ 20-fold increase in the concentration of Glc-1,6-P2 whereas transfection with PGM2 did not affect the level of the bisphosphate ester.

Glc-1,6-P2 is degraded by glucose-1,6-bisphosphatase. The brain enzyme, which has been best characterized, is dependent for its activity on the presence of IMP, the concentration of which increases in anoxia. This effect is presumably responsible for the decrease in Glc-1,6-P2 concentration in brain during anoxia. We have recently identified this enzyme to PMM1 (phosphomannomutase 1), an enzyme that was initially thought to be a phosphomannomutase and which we knew to have a modest glucose-1,6-bisphosphatase activity. We have now shown that IMP, but not other nucleotides, stimulated by >100-fold the intrinsic glucose-1,6-bisphosphatase activity of recombinant PMM1 while inhibiting its phosphoglucomutase activity. No such effect is observed with PMM2. Transfection of HEK cells with PMM1 caused a marked decrease (>5-fold) in Glc-1,6-P2 in cells that were or were not cotransfected with PGM2L1. Furthermore, the concentration of Glc-1,6-P2 in wild-type mouse brain decreased with time after ischemia, whereas it did not change in PMM1-deficient mouse brain. Taken together, these data show that PMM1 corresponds to the IMP-stimulated glucose-1,6-bisphosphatase

and that this enzyme is responsible for the degradation of Glc-1,6-P2 in brain. One of our aims is presently to understand the meaning of the regulation of Glc-1,6-P2 concentration by IMP.

METABOLISM OF PSEUDOURIDINE

A. Preumont, E. Van Schaftingen, in collaboration with Jean-François Collet.

Pseudouridine, a non-classical nucleoside present in human urine as a degradation product of RNAs, is one of the few molecules having a glycosidic C-C bond. Through a database mining approach involving transcriptomic data, we have molecularly identified two enzymes that are involved in the metabolism of pseudouridine (Fig. 5) in uropathogenic *Escherichia coli*, the principal agent of urinary tract infections in humans (7). The first enzyme, coded by the gene yeiC, specifically phosphorylates pseudouridine to pseudouridine 5'-phosphate. Accordingly, yeiC(-) mutants are unable to metabolize pseudouridine, in contrast to wild-type *E. coli* UTI89.

The second enzyme, encoded by the gene yeiN belonging to the same operon as yeiC, catalyzes the conversion of pseudouridine 5'-phosphate to uracil and ribose 5-phosphate in a divalent cation-dependent manner. Remarkably, the glycosidic C-C bond of pseudouridine is cleaved in the course of this reaction, indicating that YeiN is the first molecularly identified enzyme able to hydrolyze a glycosidic C-C bond. Though this reaction is easily reversible, the association of YeiN with pseudouridine kinase indicates that it serves physiologically to metabolize pseudouridine 5'-phosphate rather than to form it. YeiN is homologous to *Thermotoga maritima* IndA, a protein with a new fold, which we also showed to act as a pseudouridine-5'-phosphate glycosidase.

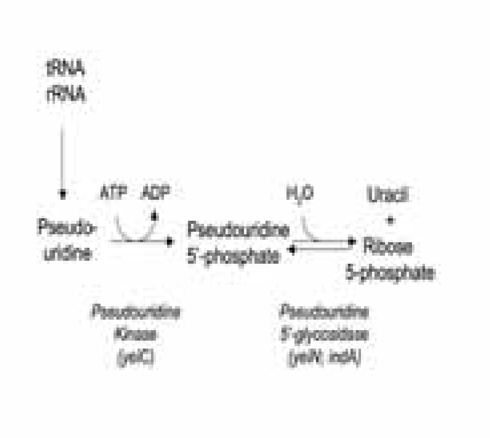

Figure 5. Metabolism of pseudouridine in *Escherichia coli*.

Database mining indicates that most eukaryotes possess homologues of pseudouridine kinase and pseudouridine-5'-phosphate glycosidase and that these are most often associated in a single bifunctional protein. The gene encoding this bifunctional protein is absent from the genomes of man and other mammals, indicating that the capacity for metabolizing pseudouridine has been lost late in evolution. The absence of pseudouridine metabolizing enzymes in man is consistent with the observation that this nucleoside is present in human urine and its urinary excretion is increased in cancer. Our present aim is to identify the mechanism by which pseudouridine is formed in mammalian cells.

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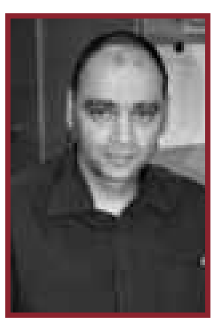

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NUCLEOSIDE ANALOGUES IN LEUKAEMIA

Our group had a long-standing interest in purine metabolism, particularly adenine nucleotide metabolism, and its genetic defects. More recently, 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 B-cell chronic lymphocytic leukaemia.

In 1997, a collaborative study of the antileukaemic nucleoside analogues, 2-chlorodeoxyadenosine (CdA) and fludarabine (Fig. 1), was started with the Department of Haematology of the University Hospital Saint-Luc. These adenosine deaminase-resistant deoxyadenosine analogues display remarkable therapeutic properties in indolent lymphoid malignancies including hairy cell leukaemia and B-cell chronic lymphocytic leukaemia (B-CLL). Nevertheless, resistance is also observed, and nucleoside analogues do not confer a survival advantage when compared to more conventional therapies such as alkylating agents. The aims of the project 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 and in the progressive accumulation of DNA strand breaks, leading to apoptosis by mechanisms which are not yet entirely clear (1, for a review).

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 me-

deoxyadenosine 2-chlorodeoxyadenosine fludarabine

Fig. 1. Structures of deoxyadenosine and analogues

chanisms by which CdA induces apoptosis in B-CLL cells, we study its effects in EHEB cells, a continuous cell line derived from a patient with B-CLL. The EHEB cell line was found to be less sensitive (10- to 100-fold) to the nucleoside analogue CdA than other human lymphoblastic cell lines. This can be explained by a lower intracellular accumulation of CdATP, the cytotoxic metabolite of CdA, due to a reduced dCK activity. Unexpectedly, DNA synthesis, measured by thymidine incorporation into DNA, was found to be increased, up to 2-fold, after a 24 h-incubation with CdA at concentrations close to the IC_{50} (5 µM) (2). Analysis by flow cytometry, using double labelling with propidium iodide and bromodeoxyuridine, has shown that CdA, in EHEB cells, provokes an increase in the proportion of cells in S phase, synthesising actively DNA. These results contrast 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. Accordingly, we found that CdA enhances the activity of cyclin-dependent kinase 2 (Cdk2), a kinase that plays a major role in 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, and to investigate whether Cdk2 activation contributes to CdA-induced apoptosis in EHEB cells. Our further aim will be to elucidate how CdA and possibly other nucleoside analogues activate Cdk2.

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, 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 associated with a decline in Mcl-1 protein level, and p53-independent. 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).

Finally, we have recently initiated microarray analyses to identify survival or death pathways that are activated in response to nucleoside analogue treatment. We intended to compare genes induced or repressed by nucleoside analogues in sensitive and refractory B-CLL patients. We found that some genes were systematically induced in sensitive patients, and not in refractory patients. We are now analysing whether the induction of one or more of these genes play(s) a role in nucleoside analogue-induced cell death. This study is performed in

collaboration with Dr Knoops from the Ludwig Institute at the de Duve Institute.

SEARCH FOR POTENTIATION OF ANTILEUKAEMIC EFFECT OF 2-CHLORODEOXYADENOSINE

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

In recent years, we have shown that combination of CdA with DNA-damaging agents, such as cyclophosphamide (CP) derivatives (4) or UV-light (5), resulted in synergistic cytotoxicity in B-CLL lymphocytes, due to inhibition of DNA repair. The *in vitro* synergy between CdA and CP derivatives has provided the rationale for a clinical trial of this combination, which gives encouraging results (6).

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 IC_{50}, 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

Since deoxycytidine kinase (dCK) activates numerous nucleoside analogues used in anticancer and antiviral therapy, knowledge of its regulation can be expected to allow optimization of the activation of these analogues. Recently, it has been shown by others and by us that dCK activity can be increased by various genotoxic agents, including CdA, aphidicolin, etoposide, and UV-C irradiation. This activation could not be explained by an allosteric effect or by an increase of the protein amount. Therefore, a post-translational activation of dCK by intracellular signalling pathways was suggested. To unravel the mechanism of the activation of dCK, we first investigated 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 [^{32}P]orthophosphate, confirming that dCK is a phosphoprotein. Tandem mass spectrometry performed by Dr D. Vertommen and Prof. M.H. Rider from the Horm unit allowed the identification of four *in vivo* phosphorylation sites, Thr-3, Ser-11, Ser-15 and Ser-74 (Fig. 2). Site-directed mutagenesis demonstrated that Ser-74 phosphorylation was crucial for dCK activity in HEK 293T cells, whereas phosphorylation of other identified sites did not seem essential (9). Phosphorylation of Ser-74 was also observed on endogenous dCK in CCRF-CEM cells and in lymphocytes from B-CLL patients, in which the Ser-74 phosphorylation state 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 B-CLL lymphocytes could be related to its phosphorylation level on Ser-74 (10). To conclude, our work has demonstrated that dCK activity largely depends on the phosphorylation state of Ser-74 in human leukaemic lym-

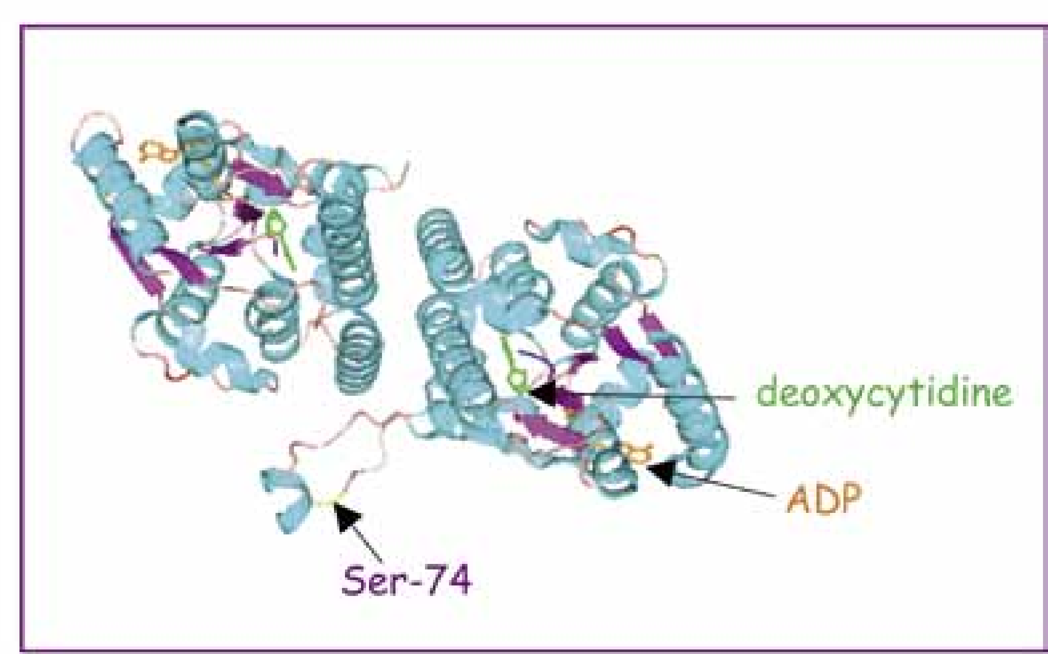

Figure 2. Three-dimensional structure of deoxycytidine kinase in complex with ADP and deoxycytidine. The Ser-74 phosphorylation site is located at a very flexible 15-residue insert (Ser-63-Asn-77) easily accessible to cellular protein kinases. The N-terminal extremity of dCK (residues 1-19) was flexible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

phocytes. We are now attempting to identify the protein kinase(s) and phosphatase(s) that control the phosphorylation of dCK on Ser-74 and the signalling pathways that lead to dCK activation following treatment with DNA damaging agents. In addition, we are analysing the influence of Ser-74 phosphorylation on the kinetic properties of dCK.

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PROTEIN FOLDING IN BACTERIA

To be active and stable, newly synthesized proteins have to fold correctly. However, reaching a proper three-dimensional structure is a real challenge for most proteins. This is especially true for proteins that are secreted to the cell envelope of Gram-negative bacteria. These proteins are assembled in the cytoplasm, are then transported across the inner-membrane and finally fold within the periplasm or the outer membrane, in an environment devoid of any obvious energy source. Moreover, the correct folding of many of these proteins requires the formation of a disulfide bond between two cysteine residues, which is a rate-limiting step of the folding process.

Failure to fold secreted proteins properly affects the integrity of the bacterial cell envelope, which increases the sensitivity of bacteria to antimicrobial drugs.

In our group, we study the pathways that govern the assembly of the cell envelope of Gram-negative bacteria. In particular, we are interested in the mechanisms of disulfide bond formation in the periplasm of Escherichia coli and in the chaperones that assist the folding of outer-membrane proteins.

OXIDATIVE PROTEIN FOLDING IN THE PERIPLASM

G. Connerotte, M. Depuydt, K. Denoncin, P. Leverrier, J.-F. Collet

In the gram-negative bacteria Escherichia coli, disulfide bonds are introduced in the periplasm by the Dsb (Disulfide bond) proteins family [4].

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 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. 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 inner-membrane protein DsbD. DsbD transfers reducing equivalents from the cytoplasmic thioredoxin system to the periplasm via a succession of disulfide exchange reactions. Recently, we showed that DsbC may also 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].

The periplasm possesses another soluble Dsb protein, DsbG, but the function of this protein has remained elusive.

Current efforts in this field are devoted to the characterization of the function of DsbG and the study of DsbA proteins from pathogenic bacteria.

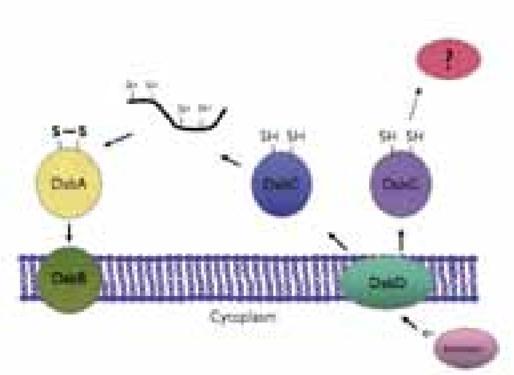

Figure 1. Disulfide bond formation in the E. coli periplasm. Disulfides are introduced into folding proteins by DsbA. DsbA is re-oxidized by DsbB, an inner-membrane protein. Electrons flow from the reduced proteins to the CXXC motif of DsbA and then to DsbB. DsbA can form incorrect disulfides that are corrected by a protein disulfide isomerase DsbC. DsbC is kept reduced by DsbD, a membrane protein that transfers electrons from the cytoplasmic pool of NADPH to the periplasm. DsbD also transfers electrons to DsbG, a protein which resembles DsbC, but whose function is not known.

Identification of the substrates of DsbG

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 proteins in complex with DsbG. The interaction between DsbG and these proteins is currently under investigation.

Structural and biochemical characterization of three DsbA proteins from Neisseria meningitidis

Neisseria meningitidis is 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, the Neisseria meningitidis genome possesses three genes encoding active DsbAs: NmDsbA1, NmDsbA2 and NmDsbA3. NmDsbA1 and NmDsbA2 have been characterized as lipoproteins involved in natural competence and in host interactive biology, while the function of NmDsbA3 remains unknown. In collaboration with Laurence Serre and Céline Lafaye (Grenoble, France), we have characterized the neisserial enzymes biochemicaly and structurally. We found that the crystal structures of the Neisse-

ria DsbA are highly similar and adopt the classical Escherichi coli DsbA fold .

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. Analysis of the crystal structure of the NmDsbAs provided some clues to the extraordinary oxidizing properties of these enzymes. One major difference between the E. coli DsbA and the NmDsbAs is the presence of a threonine residue within the catalytic site (Figure 2). This residue makes a hydrogen bond with the catalytic cysteine that helps stabilizing the reduced form of the enzymes. As the difference in stability between the oxidized and reduced forms of an oxidoreductase is the driving force to oxidize substrate proteins, this threonine residue likely plays a key role in dictating the extraordinary oxidizing power of these enzymes.

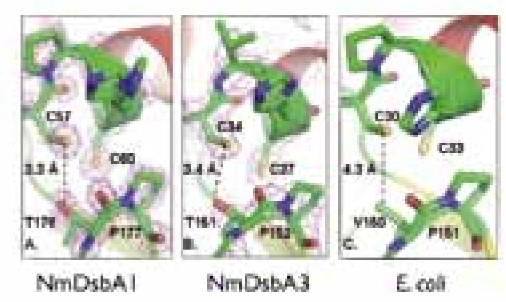

Figure 2. Neisserial DsbA are powerful oxidants. In contrast to E. coli DsbA, NmDsbAs possess a threonine residue in their catalytic site. This residue is hydrogen-bonded to the catalytic cysteine residue, further stabilizing the reduced form of the proteins.

OUTER-MEMBRANE BIOGENESIS

G. Connerotte, H. El Hajjaji, K. Denoncin, P. Leverrier, J.-F. Collet

We recently started to study the mechanisms of outer-membrane biogenesis in E. coli. The outer-membrane (OM) is a permeability barrier that is essential for the viability of Gram-bacteria and protects them against various antibiotics. OM components are synthesized in the cytoplasm or in the inner-membrane. The mechanisms by which unfolded proteins and lipids are then transported through the hydrophilic periplasm and are inserted in the OM are obscure. Our overall goal is to solve the fascinating problem of how such a complex macromolecular structure is assembled in a compartment devoid of energy. The proteins that are involved in OM biogenesis are also attractive targets for the design of new antibiotics and anti-inflammatory drugs.

First, in order to advance our understanding of the mechanisms that govern OM biogenesis, we have developed a proteomic technique based on 2D-LC-MS/MS to study of the OM proteome. In particular, we have characterized the function of the E. coli periplasmic chaperone SurA [10]. SurA had been proposed to escort b-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 b-barrel proteins, in wild-type and surA strains. We found that the loss of SurA affects the abundance of 8 b-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 (Figure 3). We are currently using our powerful proteomic technique to characterize mutants that presents defects in OM integrity.

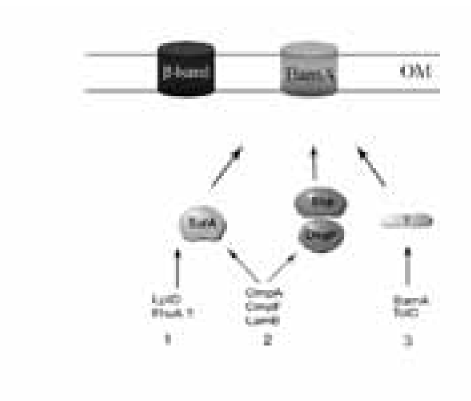

Figure 3. Transport of unfolded b-barrel proteins across the periplasm. Unfolded b-barrel proteins are escorted across the periplasm by periplasmic chaperones before reaching the OM where they are inserted by the Bam complex. There are 3 known periplasmic chaperones that interact with OMPs: SurA, Skp and DegP. Our results indicate that only a subset of OMPs strongly depend on SurA for biogenesis. We propose to divide the OMPs in three groups. Group 1 includes b-barrel proteins, such as LptD, that greatly depend on the presence of SurA for biogenesis. Group 2 includes b-barrel proteins such as the major OMPs OmpA, OmpF and LamB that preferentially interact with SurA for biogenesis but are also able to interact with the other periplasmic chaperones. Group 3 includes b-barrel proteins such as BamA and TolC that do not seem to require the presence of SurA for biogenesis.

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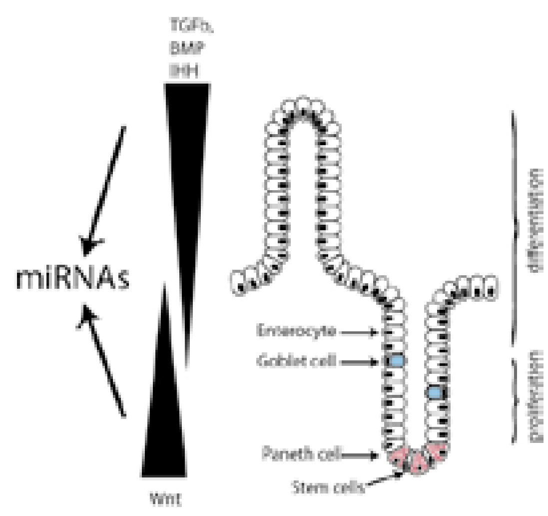
miRNAS IN PHYSIOLOGY AND DISEASE

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

ROLE OF miRNAS IN INTESTINAL DIFFERENTIATION

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



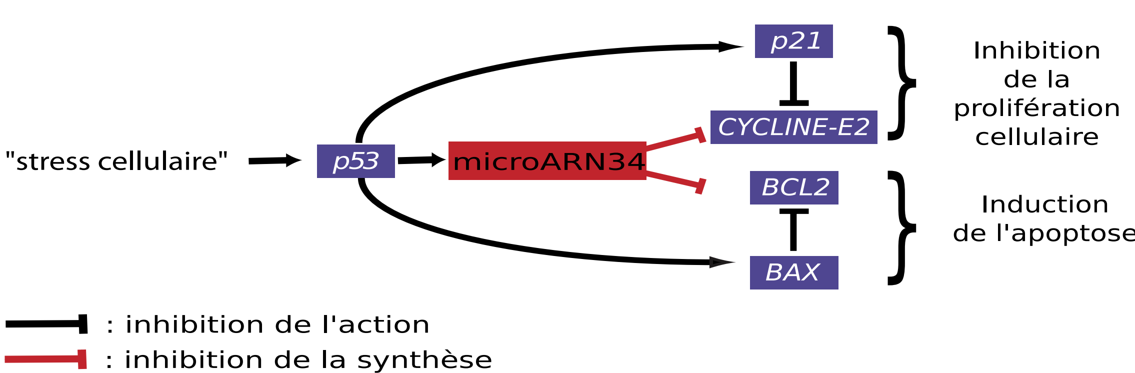
ROLE OF THE miRNAS DOWNSTREAM OF THE TUMOR SUPPRESSOR p53 IN CANCER

The p53 protein is a transcription factor that acts in the center of a signaling pathway that is activated by different forms of cellular stress. Upon exposure to ionizing radiation, oxidative stress, chemotherapeutic drugs or the activation of oncogenes, a cascade of kinases leads to the stabilization of the 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 of p53 have been described and seem to contribute to the observed effects: Upregulation of proteins like p21 induce cell cycle arrest, while proteins like BAX and PUMA lead to an increase in apoptosis. Overall, p53 function is inactivated in the majority of human cancer cases thereby allowing cancer cells to proliferate and survive in an uncontrolled manner.

We have previously identified the miRNA34 family as direct p53 target genes. Notably, this family of miRNAs seems to cooperate with protein-coding p53 target genes (see figure below):

On the one hand, miRNA34 can bind to the 3'UTR and downregulate translation of the transcripts encoding the cell cycle progression genes Cyclin E2 and CDK6, and thereby reinforce cell cycle arrest induced by p21. On the other hand, inhibition of the translation of the anti-apoptotic protein Bcl2 makes cells more susceptible to the pro-apoptotic action of BAX and PUMA.

Presently, we are further investigating the role of miRNA34 family members in tumor development *in vivo* and *in vitro*.



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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 PDK-1, a protein kinase upstream of PKB. Our first results using a dominant-negative PKB in HEK293 transfections suggested that PKB did not participate in heart PFK-2 activation by insulin. Therefore, we examined the role of two other insulin-stimulated protein kinases, namely protein kinase C zeta (PKCzeta) and SGK3 (serum- and glucocorticoid-regulated protein kinase-3) in insulin-stimulated PFK-2 activation. Co-transfection of HEK 293T cells with SGK3 siRNA or with a kinase-inactive PKCzeta construct (2) did not affect PFK-2 activation, suggesting that these protein kinase are 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. Our results with PKB beta-knockout mice indicated that this isoform is not required for heart PFK-2 activation by insulin. Moreover, PKB alpha silencing using the siRNA approach indicated that this PKB isoform is likely to be responsible for heart PFK-2 activation by insulin (manuscript in preparation).

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

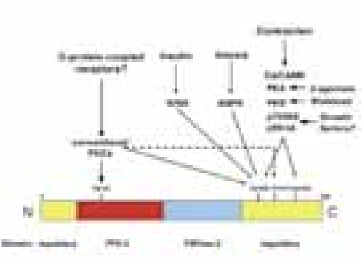

Fig. 1. Protein kinases of signalling pathways that converge on heart PFK-2. The numbering of residues refers to the bovine H1 isoform.

AMP-ACTIVATED PROTEIN KINASE

B. Sid, D. Vertommen, L. Miranda, L. Bultot, Y. Liu, C. Plaideau, L. Hue, M.H. Rider, in collaboration with N. Morel, UCL, Brussels, D.G. Hardie, Dundee, D. Alessi, Dundee, D. Neumann, Zurich, J. Jenssen, Oslo, K. Storey, Ottawa, B. Viollet, Paris and M.P. Walsh, Calgary

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 into ZMP, an analogue of AMP, or by the A769662 Abbott compound. 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 (3). Similarly, we showed that the inactivation of elongation factor 2 (eEF2) by AMPK explains at least in part the inhibition of protein synthesis by anoxia (4 and see below). We are currently engaged in identifying new substrates of AMPK.

AMPK activation inhibits protein synthesis

Protein synthesis, in particular peptide chain elongation, consumes a large proportion of intracellular ATP. We previously showed that in hepatocytes, AMPK activation was associated with protein synthesis inhibition. This was due to eEF2 inactivation via AMPK-induced phosphorylation and activation of eEF2 kinase (eEF2K), the kinase that phosphorylates eEF2 at its inactivating Thr56 site, providing a novel mechanism for the inhibition of protein synthesis (4). In a model of electrical stimulation of rat epitrochlearis skeletal muscles where protein synthesis was inhibited during contraction, Thr56 eEF2 phosphorylation increased rapidly, but this was not related to AMPK activation. Moreover, the inhibition of protein synthesis during contraction could not be explained by inhibition of the mammalian target of rapamycin (mTOR)/p70 ribosomal S6 kinase (p70S6K)/4E-BP1 pathway (Fig. 2). The inhibition of protein synthesis by contraction can be explained by an increase in eEF2 phosphorylation at the onset of contraction, probably secondary to a rise in calcium (5).

AMPK activation in animals adapting to extreme energy stress

Freezing is one of most serious environmental stresses faced by living organisms. We investigated whether AMPK could play a role in metabolic re-sculpting that occurs in animals that adapt to extreme energy stress. AMPK was proposed to play an important role in freeze-tolerance in frogs (6) and anoxia-tolerance in turtles (in press).

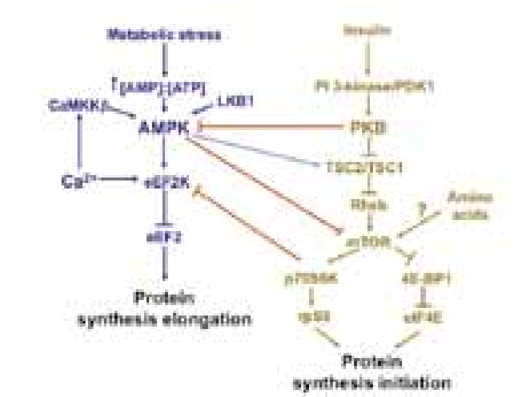

Fig. 2 Cross-talk between the insulin/PKB/mTOR and AMPK signalling pathways with respect to the control of protein synthesisand glucose transport

Control of ion transport by AMPK

We investigated whether AMPK would control the NKCC1 (Na+-K+-2Cl-) co-transporter. NKCC1 was phosphorylated in vitro by AMPK at two sites in a recombinant GST-fusion construct containing the N-terminal cytosolic domain of shark NKCC1. The phosphorylation sites were identified by mass spectrometry and correspond to Ser77 and Ser242 in the human sequence. Incubation of human erythrocytes with the A769662 AMPK activator led to time-dependent AMPK activation, however bumetanide-sensitive 86Rb uptake (to measure NKCC1 activity) was not increased. However, 86Rb uptake was increased by osmotic shock in parallel with AMPK activation. The role of AMPK in hyperosmolarity-induced NKCC1 activation is being studied by measuring 86Rb uptake in erythrocytes from alpha1-AMPK knockout mice.

Role of AMPK in smooth muscle contraction and control of cytoskeletal actin organization

Smooth muscle myosin light chain kinase (smMLCK) was found to be a new AMPK target whose phosphorylation is inhibitory for contraction (7). Phosphorylation at Ser815 by AMPK desensitized smMLCK by increasing the concentration of CaM required for half-maximal activation. In primary cultures of rat aortic smooth muscle cells, vasoconstrictors activated AMPK in a calcium-dependent manner via CaM-dependent protein kinase kinase-beta (CaMKKbeta), a known upstream kinase of AMPK. In aortic rings from mice in which alpha1, the major catalytic subunit isoform of AMPK in arterial smooth muscle, had been deleted, KCl- or phenylephrine-induced contraction was increased. Therefore, AMPK attenuates contraction by phosphorylating and inactivating smMLCK, which could contribute to reduced ATP turnover in the tonic phase of contraction.

We were unable to confirm published data claiming that myosin regulatory light chains (MLC) can be directly phosphorylated by AMPK at the smMLCK Ser19 site. 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 (8).

We studied the effect of pharmacological activators of AMPK (AICA riboside and A762669) on cytoskeletal organization and signalling in epithelial Madin-Darby canine kidney (MDCK) cells. AMPK activation induced peripheral F-actin accumulation and disassem-

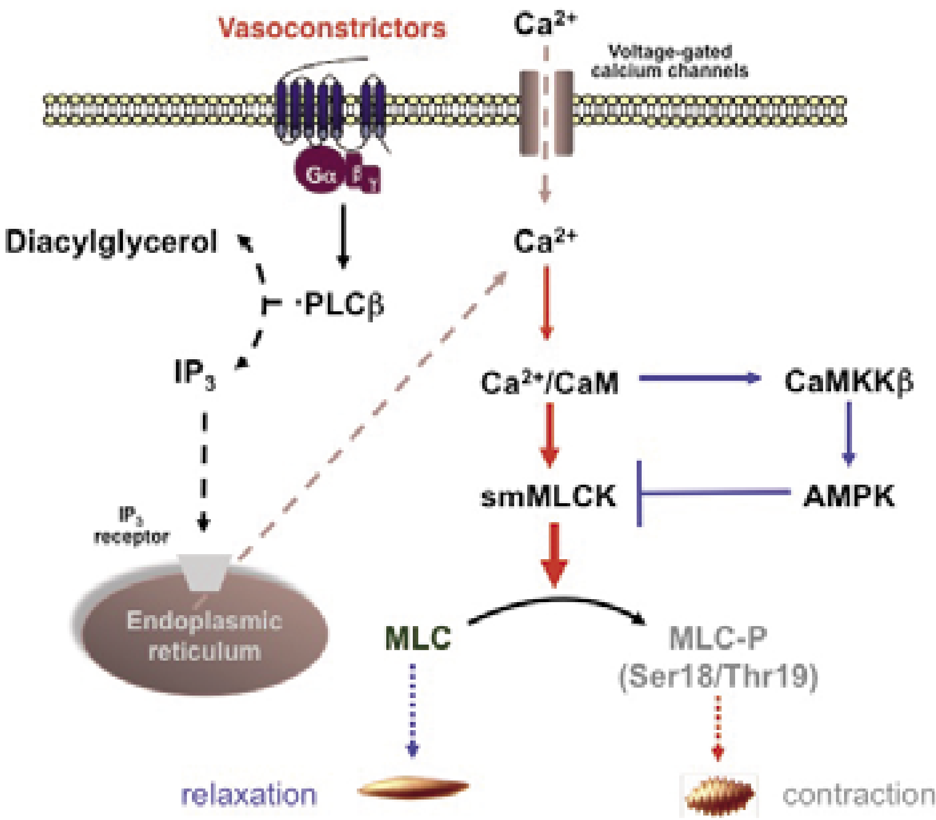

Fig. 3. AMPK activation is induced by vasoconstrictors acting through Gq-coupled receptors, via the calmodulin kinase kinase–β (CaMKKβ) pathway in smooth muscle cells. AMPK attenuates contraction by phosphorylating and desensitizing smooth muscle myosin light chain kinase (smMLCK), thereby contributing to reduced ATP turnover in the tonic phase of contraction.

bly of stress fibers. In parallel, RhoA was activated, associated with an increase in phosphorylation of Rho-kinase (ROK) downstream targets, MLC and cofilin. Accordingly, the A769662-induced increase in MLC and cofilin phosphorylation was prevented by H1152, a ROK inhibitor. These effects resemble the morphological changes in MDCK cells exposed to hyperosmotic shock and ATP-depletion by 2-deoxyglucose treatment where AMPK was activated and MLC phosphorylation increased (manuscript in preparation). We propose that AMPK links osmotic and cellular energy stress to the reorganization of the actin cytoskeleton via the RhoA/ROK pathway.

MASS SPECTROMETRY

D. Vertommen, S. Calberson, M. Rider in collaboration with F. Opperdoes/P. Michels, UCL, C. Sindic, UCL and J.-F. Collet, UCL

The development of mass spectrometry facilities within our laboratory has been an enormous asset to our group and institution. Since the acquisition of our first electrospray mass spectrometer in 1997, the application of mass spectrometry techniques to protein identification, identification of sites of covalent modification and quantification of changes in protein expression has led to well over 30

joint publications. In our own research, it enabled us to identify new phosphorylation sites in several targets of protein kinases and in the AMPK complex. In January 2008, we updated our electrospray machine to the Finnigan LTQ linear ion trap equipped with electron-induced transfer dissociation (ETD) fragmentation.

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. 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 is used. We pursue our collaboration with other laboratories within our university and with other universities. For example with F. Opperdoes and P. Michels, label-free proteomics has been used to study differential protein expression in glycosomes and mitochondria from the bloodstream and insect stages of the Trypanosoma brucei sleeping sickness parasite (9). In collabortaion with C. Sindic, the screening of glycoproteins from sera and cerebrospinal fluid of patients with neurodegenerative diseases is under study to discover new biomarkers. In collaboration with the group of J.-F. Collet, loss of the chaperone SurA in E. coli was studied. Using a differential proteomics approach based on 2-D LC-MS(n), the relative abundance of 64 outer membrane proteins, including 23 beta-barrel proteins, was compared in wild-type and SurA strains. Unexpectedly, the loss of SurA affected the abundance of eight beta-barrel proteins. Based on the results, a revised model was proposed in which only a subset of outer membrane proteins depends on SurA for proper folding and insertion in the outer membrane (10).

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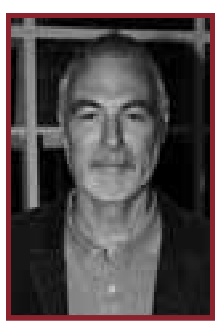

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METABOLIC COMPARTMENTATION IN TRYPANOSOMES

Trypanosomatidae are protozoan parasites which cause sleeping sickness, Chagas' disease and leismaniasis in man and are responsible for the deaths of millions of people each year. For these diseases better drugs are urgently needed. By studying the molecular and cell biology of these tropical parasites we try to provide a basis for the development of new and better 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 essential differences between the respective parasites and their human host.

ENZYMES AND PATHWAYS

Leishmania differentiation and changes in its metabolic repertoire

F. Opperdoes, in collaboration with D. Zilberstein (Technion Institute of Technology, Haifa, Israel)

An iTRAQ proteomics approach was used to compare the proteomes during the differentiation of insect-form *Leishmania donovani* promastigotes to the human intracellular amastigote form. The analysis identified and quantified a large proportion of the total of the parasite's predicted proteome across 7 time points during differentiation. An increase in gluconeogenesis enzymes was accompanied by a decrease in glycolytic capacity. At the same time, enzymes of the β-oxidation of fatty acids, amino-acid catabolism, tricarboxylic acid cycle and the mitochondrial respiratory chain and oxidative

phosphorylation were all up-regulated. The main energy sources of the differentiating parasite shifted from glucose to fatty acids and amino acids. Furthermore, glycerol and amino acids were used as precursors for sugar synthesis, compensating for the lack of exogenous sugars during the intracellular stage.

MITOCHONDRIAL COMPLEX I OF TRYPANOSOMATIDS

F. Opperdoes, P. Michels

The presence of a functional mitochondrial complex I, or NADH dehydrogenase, in Trypanosomatidae is still debated. Several subunits of complex I have been identified by biochemical studies, but the overall composition of the complex has remained elusive. The available literature related to this elusive mitochondrial activity was reviewed and together with a bioinformatic analysis of the trypanosomatid genomes the composition of a putative complex I was predicted. It comprises at least 19 subunits and has minimally a mass of 660 kDa. It is larger than the corresponding bacterial complex but smaller than the typical mitochondrial one of eukaryotes. All subunits known to be involved in electron transport are present, but the complex does not seem to be involved in energy transduction because four membrane subunits, normally encoded by the mitochondrial genome and supposed to be involved in proton extrusion, are missing (Fig. 1).

COMPARTMENTATION OF GLYCOLYSIS IN GLYCOSOMES

P. Michels, in collaboration with B. Bakker (Vrije Universiteit Amsterdam, The Netherlands)

Previously, it has been proposed, based on computer experimentation using a validated mathematical model of glycolysis of trypanosomes, that the compartmentation of the pathway within glycosomes serves to compensate for the lack of feed-back activity regulation of the substrate-phosphorylating enzymes at the beginning of the pathway. Such regulation is considered crucial to prevent uncontrolled accumulation of glycolytic intermediates, but surprisingly was not found for the trypanosomatid enzymes. Indeed, experimental support for this hypothesis has now been obtained by using a *Trypanosoma brucei* cell line in which glycosome biogenesis can be disrupted by inducing the arrest of synthesis of the biogenesis factor PEX14 by RNA interference (RNAi). The depletion of PEX14 prevents import of newly synthesized glycolytic enzymes into glycosomes and, as a consequence, causes intracellular accumulation of glucose 6-phosphate and glycerol 3-phosphate to very high levels, followed by growth arrest and cell death.

TRYPANOSOMATID PHOSPHOFRUCTOKINASE AND PYRUVATE KINASE

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

Our coworkers at the University of Edinburgh have succeeded in solving the crystal structures of *T. brucei* phosphofructokinase (PFK) and *Leishmania mexicana* pyruvate kinase (PYK) as apo-enzymes without substrates and fixed in the low-activity (T) state and as holoenzymes with different ligands in intermediate and high activity (R) states. This has provided insight into the conformational changes required for catalysis by these enzymes. Moreover, the data obtained for PYK show how the allosteric activator fructose 2,6-bisphosphate binds to an effector site that is very different from that in other PYKs, and dramatically stabilizes the enzyme in an active conformation. Our current research is devoted to testing aspects of the model of the allosteric mechanism of PYK, as inferred from the structural analysis, by site-directed mutagenesis and kinetic studies. Moreover, the newly collected information is being used to further optimize the selective inhibitors of these two parasite enzymes

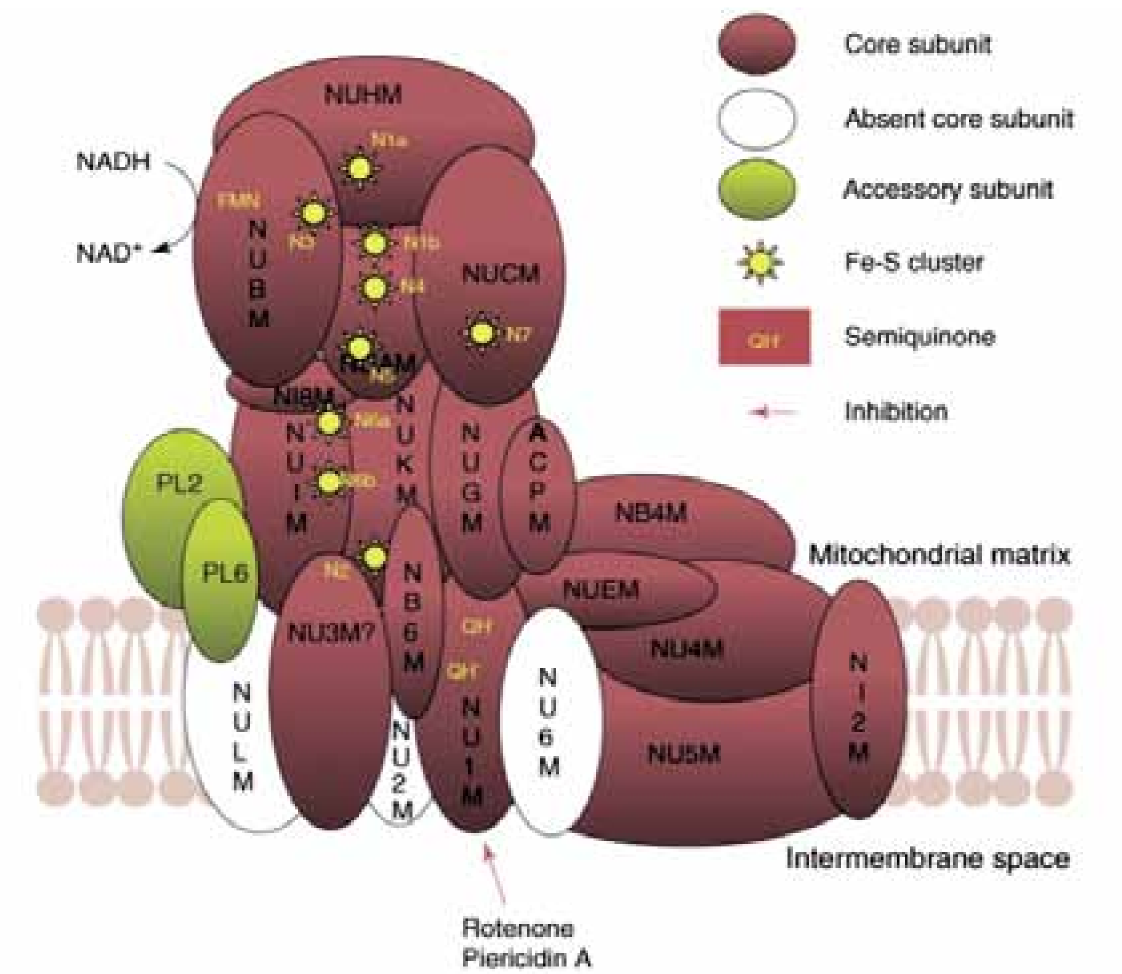

Fig. 1. Schematic structure of *T. brucei* complex I.

described in our previous annual reports and which are designed to bind in the enzymes' active and effector sites.

FATTY ACID DESATURATION BY TRYPANOSOMES

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

Lipids of trypanosomatids contain a relatively high proportion of polyunsaturated fatty acids (PUFAs), notably oleate (C18:1Δ12) and linoleate. (C18:2Δ9,12). We therefore studied the enzymes responsible for de synthesis of these PUFAs in trypanosomes, the Δ12 desaturase and Δ9 desaturase, respectively. Candidate genes for these enzymes have been identified and used to create *T. brucei* cell lines in which the expression of these enzymes can be knocked down by RNAi. Lowering the expression of each of the enzymes in procyclic trypanosomes caused growth retardation. Using these cell lines, the identity of the enzymes was confirmed, because depletion of the putative Δ9 desaturase caused an increase in their stearate (C18) levels and a decrease in their oleate and linoleate content, and depleting the candidate Δ12 enzyme resulted in an increase of the oleate and a decrease of the linoleate content. Addition of oleate to the medium of the Δ9 RNAi cell line could partially restore the growth phenotype. No effect was seen upon addition of

linoleate, probably because trypanosomes are not able to take it up. Using desaturase inhibitors, our Argentine colleagues have chemically validated fatty-acid desaturases as drug targets against Chagas' disease in Latin America caused by *Trypanosoma cruzi*. Currently we are creating bloodstream-form *T. brucei* RNAi cell lines for Δ9 and Δ12 desaturases in order to genetically validate these enzymes also as drug targets against African sleeping sickness.

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. Procyclic cell lines in which RNAi-dependent depletion of GAT1 and GAT3 has been induced continue growing well in the presence of glucose. However when glucose as source for ATP production is replaced by proline, i.e. the regular substrate of insect-stage trypanosomes, growth of the GAT1-RNAi induced cells is affected, followed by death of the trypanosomes, but not the growth of the GAT3-RNAi induced trypanosomes. Knockdown of GAT1 expression in glucose-grown cells caused a change in the lipid fatty-acid composition of the cells, most notably an increase in their linoleate level. Probably GAT1 is a linoleate transporter. Glycosomal uptake of this fatty acid may be important for either β-oxidation inside the organelles, possibly essential in energy metabolism in the absence of glucose, or for ether-lipid biosynthesis, an important process localized inside the glycosomes.

GLYCOSOME TURNOVER

Glycosome biogenesis in *Trypanosoma brucei*

N. Galland, E. Verplaetse, M. Gualdrón, V. Hannaert, P. Michels, in collaboration with W. Hol (Univerity of Washington, Seattle, USA)

Our research during the last few years has shown that biogenesis of glycosomes in trypanosomatids and that of peroxisomes in other organisms occur via homologous processes involving proteins called peroxins, which exert their function through multiple, transient interactions with each other. Yet, considerable variations have been observed between glycosome and peroxisome biogenesis and the 11 *T. brucei* peroxins identified previously show very limited sequence identity with their homologues from human and other organisms. This low sequence identity, together with the essentiality of the peroxins for trypanosome viability, as shown in expression knock-down experiments, render them excellent drug-target candidates. Our recent research has focused on aspects of the import of proteins into the glycosomal matrix: the characterization of PEX5, a cytosolic receptor for proteins with a type 1 peroxisome-targeting signal (PTS1), the binding of the cargo-loaded receptor to the docking complex peroxins PEX13 and PEX14 in the glycosomal membrane, and the retrieval of the discharged receptor. Trypanosomatids have an atypical PEX13 that could only be found by a bespoke bioinformatics approach involving pattern searches and domain analysis. Its interaction with PEX5 and PEX14 has been demonstrated in two-hybrid analysis. Indications have been obtained that retrieval of PEX5 involves its modification by one or a few ubiquitin moieties. New candidate peroxins possibly involved in this ubiquitination have been identified: PEX4, an ubiquitin-conjugating protein, and its glycosomal-membrane anchoring protein, PEX22. These peroxins are currently being analyzed.

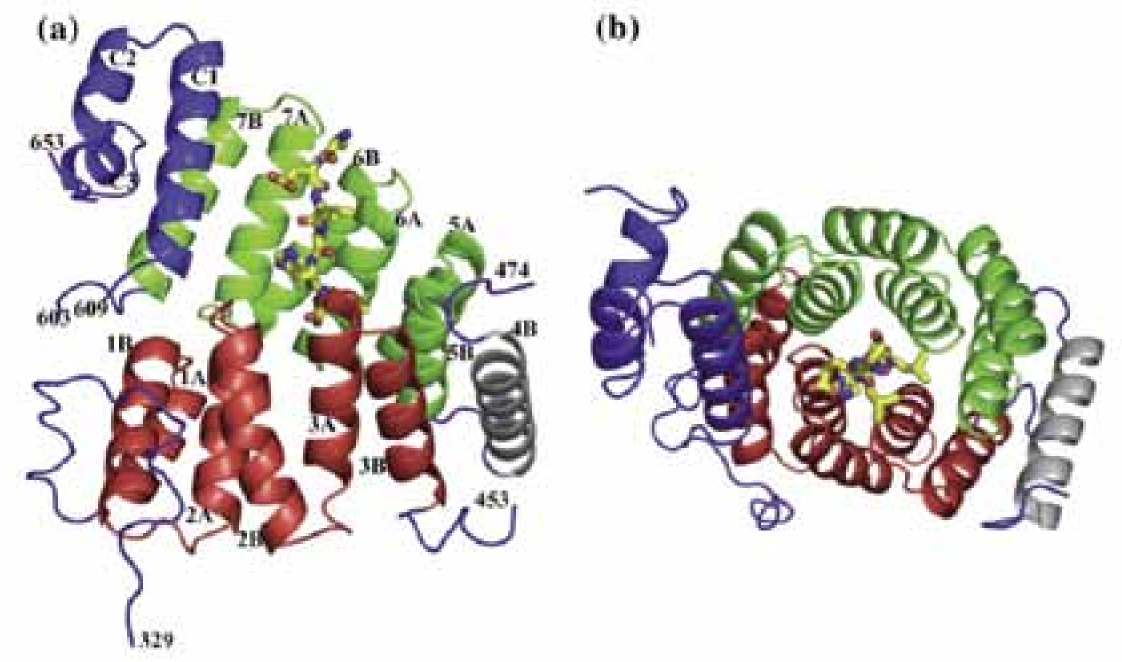

Fig. 2. Structure of the *T.brucei* PEX5 PTS1-binding domain in complex with a PTS1-peptide (a) Structure of the TbPEX5 domain with the PGI PTS1 C-terminal peptide, Ac-FNELSHL, containing the PTS1 sequence of glucose-6-phosphate isomerase. (b) Rotated top view of the structure showing the peptide bound between the two subdomains.

Comparison of crystal structures of the PTS1-binding domain of *T. brucei* PEX5 in complex with a different PTS1-containing peptides (Fig. 2) and that of the human receptor has revealed unique possibilities for binding compounds that will interfere selectively with PTS1 binding or release in trypanosomatids and consequently for disrupting glycosome biogenesis and preventing parasite growth. Computational approaches and compound library screening to discover such compounds have been initiated.

GLYCOSOME DEGRADATION IN *TRYPANOSOMA BRUCEI*

A. Brennand, P. Michels, in collaboration with E. Pays (Université Libre de Bruxelles) and D. Rigden (University of Liverpool, England)

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. In a previously performed bioinformatics analysis othologues of about 20 of the 40 known yeast proteins known to be involved in autophagy (ATGs), or specifically in pexophagy, were identified in the trypanosomatid databases. We have started the analysis of several of them: *T. brucei* candidates for VPS34, ATG7, ATG8, ATG24 and VAC8. The localization of the candidate ATG7 and ATG8 has been determined; upon induction of autophagy in procyclic cells by nutrient deprivation, as expected, the putative ATG8 quickly became concentrated in punctuate structures suggestive of autophagosomes, and a rather disperse ATG7 signal was observed. For different autophagy- and pexophagy-related proteins (VAC8, ATG8, ATG24

and VPS34) bloodstream-form and procyclic RNAi cell lines have been constructed and are currently being analyzed under standard culturing conditions and under conditions where an increased rate of glycosome turnover is expected.

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ENDOCYTOSIS AND EPITHELIAL DIFFERENTIATION

Endocytosis, a central activity of all eukaryotic cells, allows for cell nutrition, regulates the composition of the cell surface and controls transfer of macromolecules across epithelial barriers. The role of endocytosis in signalling, and, conversely, signalling to endocytosis, are fields of intense investigations. This research group has made significant contributions in the dissection of endocytic pathways and in unravelling the contribution of endocytosis to physiopathology (3, 4, 6, 8, 9), parasitology (2) and pharmacology. Our current goal is to help unravelling the molecular machineries governing the endocytic activity at the apical membrane of epithelial cells, with strong emphasis on the kidney proximal tubule, one of the most active endocytic sites in the body, and a fascinating tissue of major clinical importance. Recent achievements include: (i), the elucidation of the signalling cascade whereby the paradigmatic oncogene, v-Src, impacts on the actin cytocortex, so as to induce macropinocytosis specifically at the apical domain of polarized epithelial cells, like enteropathogens (1, 5, 7, 10); (ii) the physiopathological control of rate-limiting endocytic regulators whereby apical endocytosis finely tunes thyroid hormones production (3, 9); (iii) the elucidation of a deficit of apical endocytosis in a genetic form of kidney stones (4); and (iv) the demonstration of a major role of apical endocytosis in the (mannose 6-phosphate-independent) biogenesis of kidney lysosomes (6). In view of the distinct composition of apical plasma membrane lipids, we have also undertaken to address the relation between their organization as micrometric domains, with their distinct endocytic rate and fate. Dr. C. Pierreux has recently moved from HORM to this Unit to develop with us new projects on epithelial differentiation and on tubulogenesis, with emphasis on the developing pancreas and salivary glands as complementary models of controlled interconversion between multilayered cell masses and polarized monolayers. Two strong assets of our group are a several-decade expertise in structural biology and a versatile cellular and tissular imaging platform, including live cell imaging, confocal, multiphoton, 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 have 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 (2). 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 swelling of the apical recycling compartment (ARE). Preservation of ARE tubulation and of apical polarity indicated that function of this essential compartment was not affected. Macropinosomes and the ARE were labelled

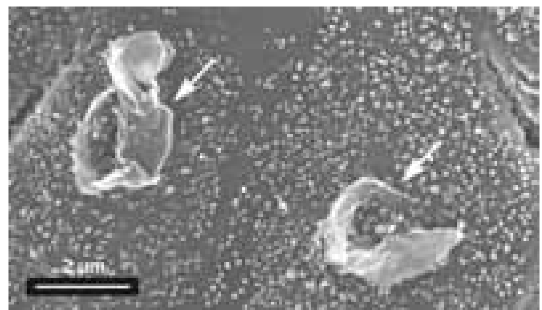

Fig. 1. Src causes circular apical ruffling (arrows; scanning electron microscopy, from Ref 5).

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 (5).

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

Ph. de Diesbach, T. Medts, S. Carpentier, L. D'Auria, P. Van Der Smissen, A. Platek, M. Mettlen, M.F. van den Hove, 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" remains controversial. Using polarized MDCK

cells expressing the thermosensitive v-Src/tsLA31 variant, we here addressed how Src tyrosine-kinase activation may impact on its (i) membrane recruitment, in particular to "lipid rafts"; (ii) subcellular localization; and (iii) signalling. The kinetics of Src-kinase thermoactivation correlated with its recruitment from the cytosol to sedimentable membranes where Src largely resisted solubilisation by non-ionic detergents at 4 °C, and floated into sucrose density gradients like "lipid rafts". By immunofluorescence, activated Src showed a dual localization, at apical endosomes/macropinosomes and at the apical plasma membrane. 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-b-cyclodextrin (MbCD) did not abolish "rafts" floatation, but strongly decreased Src association with floating "rafts" and abolished its localization at the apical plasma membrane.

Src activation independently activated first the MAP-kinase-ERK1/2 pathway, then the PI3-kinase-Akt pathway. MAP-kinase-ERK1/2 activation was insensitive to MβCD, which suppressed Akt phosphorylation and apical endocytosis induced by Src, both depending on the PI3-kinase pathway. We therefore suggest that activated Src is recruited at two distinct membrane compartments allowing differential topological signalling, first via ERK1/2 at "non-raft" domains (possibly endosomes), then via PI3-kinase-Akt on a distinct set of "rafts" at the apical plasma membrane. Whether this model is applicable to c-Src remains to be examined (10).

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

P.J. Courtoy, W.R. Lima and A. Caplanusi, in collaboration with E.I. Christensen and his colleagues (Aarhus, DK), O. Devuyst (NEFR, UCL) and M. Jadot (FUNDP, BE)

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

In polarized PTC monolayers, purified cathepsin B was avidly and selectively taken up at the apical membrane; uptake was abolished by the "receptor-associated protein", a megalin competitor. Direct interaction of cathepsin B with megalin was demonstrated by surface plasmon resonance. Circulating procathepsin B was detected in normal mouse serum. Purified cathepsin B injected into mice was preferentially taken up by kidneys and targeted to lysosomes where it remained active, as shown by autoradiography and subcellular fractionation. A single cathepsin B injection into cathepsin B KO mice could reconstitute full lysosomal enzyme activity in the kidneys. These findings demonstrate a pathway whereby circulating lysosomal enzymes are continuously filtered in glomeruli, reabsorbed by megalin-mediated endocytosis, and transferred into lysosomes to

exert their function, providing a major source of enzymes to PTC. These results also extend the significance of megalin in PTC and have several physiopathological and clinical implications (6).

MOLECULAR MEDICINE: ROLE OF AN ENDOCYTIC TRAFFICKING DEFECT OF KIDNEY PROXIMAL TUBULAR CELLS IN HEREDITARY KIDNEY STONES

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

Kidney proximal tubular cells (PTC) show one of the highest endocytic activities in the body, 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. A paradigm of Fanconi syndromes is Dent's disease, or X-linked nephrolithiasis, due to inactivating mutations of ClC-5. To understand the pathophysiology of Dent's disease, we have studied ClC-5 KO mice. These mice 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, as part of a general membrane trafficking defect (4).

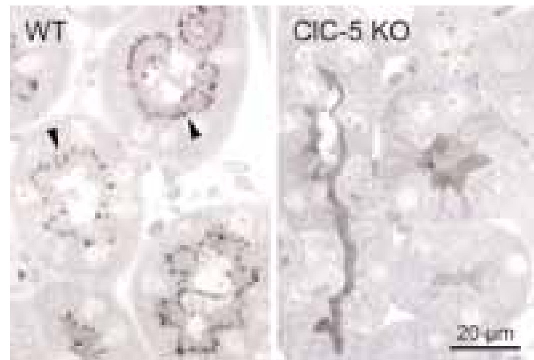

Fig. 2. Evidence for a deficit of apical endocytosis of low-molecular weight proteins in kidney proximal tubular cells of ClC-5 KO mice. Arrows indicate cytochemical detection of injected, ultrafiltrated peroxidase. Note the strong labelling by peroxidase of multiple apical PTC endosomes in WT mice,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

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 branched 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. 3). Once fully differentiated, epithelial monolayers possess three distinct membrane domains, each able to transmit signals to the nucleus. First, the basal domain ensures adhesion with 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.

Epithelial monolayers formation requires a coordinate and dynamic interaction with their

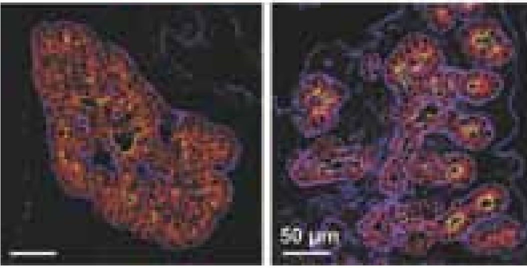

Fig. 3. Branching morphogenesis in exocrine glands. Reorganization in the early pancreatic bud of the multicellular mass of epithelial cells labelled for E-cadherin (left, red) into polarized monolayers with distinct apical domains (mucin, green) and their merging to create tubules (right). Laminin (blue)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

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 is higly 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 (Hick et al., submitted).

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)

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

Sparsely plated PTC formed small islands: peripheral cells, necessarily lacking external tight junctions, strongly expressed nuclear ZONAB, proliferated and failed to differentiate; central cells, able to form continuous junctional belts, lost nuclear ZONAB, stopped proliferating and engaged in apical differentiation (see Fig. 4). In confluent PTC monolayers, stable ZONAB transfection repressed expression and function of the endocytic receptors and impaired brush border and primary cilium maturation. Reporter and chromatin immunoprecipitation assays demonstrated that megalin and cubilin are ZONAB target genes. ZONAB expression was regulated by polarity at pre- and posttranscriptional levels (proteasomal degradation). In PTC islands, proteasome inhibition extended nuclear ZONAB to central cells, which reversed their choice from differentiation to proliferation.

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 (Lima et al., submitted).

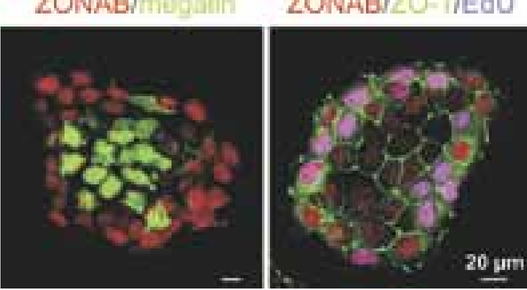

Fig. 4. ZONAB is a key regulator in the switch between epithelial proliferation and differentiation. In colonies of kidney proximal tubular cells, ZONAB (immunolabelled in red) is absent in central differentiated cells (expressing megalin, in green, left) but is selectively detected in nuclei of peripheral proliferating cells (DNA synthesis is shown in blue at right).

PLASMA MEMBRANE LIPIDS FORM STRUCTURALLY AND KINETICALLY DISTINCT MICROMETRIC DOMAINS

D. Tyteca, L. D'Auria, P. Van Der Smissen, T. Medts, S. Carpentier, P.J. Courtoy

Lipids are the most abundant constituents of biological membranes. For several decades, plasma membrane lipids were considered to form an 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 still questioned. Our group is addressing whether micrometric lipid domains naturally occur at the plasma membrane, using fluorescent lipid analogs, and how many distinct micrometric phases may coexist. We found that 10 different lipid analogs spontaneously formed micrometric domains, covering the bulk of the plasma membrane. These domains did not reflect endocytosis nor structural surface features such as microvilli, as they could be readily observed on living erythrocytes as well. Domains with non-overlapping distributions and distinct lateral diffusion (as assayed by fluorescence recovery after photobleaching) could be evidenced. Perturbations of endogenous lipids selectively affected clustering of their fluorescent analogs, indicating that domains did not depend on substitution with the fluorophores tested. The relevance of the micrometric organization of membrane lipids to their endocytic rate and fate and to overall cell polarity is currently being investigated.

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

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

We have pursued our long-term commitment to promote collaborations by sharing expertise in endocytosis and cellular imaging. For the recent years, we may cite our contribution to the study of endocytic trafficking of syndecans and its role in signalling by its heparin-sulfate ligands (Zimmermann et al., 2005, Dev. Cell 9:377-88); of biogenesis of vacuolar H+-ATPase and the role of CFTR in kidney (Jouret et al., 2005, J. Am. Soc. Nephrol. 16:3235-46); of subcellular trafficking of the thrombopoietin receptor (Royer et al., 2005, J. Biol. Chem. 280:27251-61) and the amyloid precursor protein, APP (Feyt et al., 2005, J. Biol. Chem. 280:33220-7; 2007, Biochem. Biophys. Res. Commun. 357:1004-10); the ultrastructural analysis of differentiating hepatoblasts (Clotman et al., 2005, Genes Dev. 19:1849-54) and the biogenesis of glycosomes in Trypanosoma brucei (Galland et al., 2007, Biochim. Biophys. Acta Mol. Cell. Res. 1773:521-35), or the morphological evidence by FRET of the interac-

tion between key players of CTL that is interrupted during anergy (Demotte et al., 2008, Immunity 28:414-24).

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EXTRACELLULAR MATRIX BREAKDOWN

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 bleeding 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 (5), or down-regulated by receptor-mediated endocytosis and degradation.

DIFFERENTIAL CONTROL OF ENDOMETRIAL MMPS AND CYTOKINES BY OVARIAN STEROIDS, IN VIVO AND IN EXPLANTS

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

Both endocrine and paracrine factors participate in controlling the expression and activity of MMPs involved in menstrual breakdown of the human endometrium. Several genes encoding MMPs and cytokines show maximal endometrial mRNA concentrations around menstruation. However, their expression profiles substantially diverge during the other phases of the cycle. Although these variations are in part explained by differential repression of transcription by estradiol and/or progesterone receptors, there is increasing evidence of the major contribution of specific local regulators which finely adjust in time, space and amplitude the effect of ovarian steroids on the expression of genes required for menstrual ECM breakdown (3, 4).

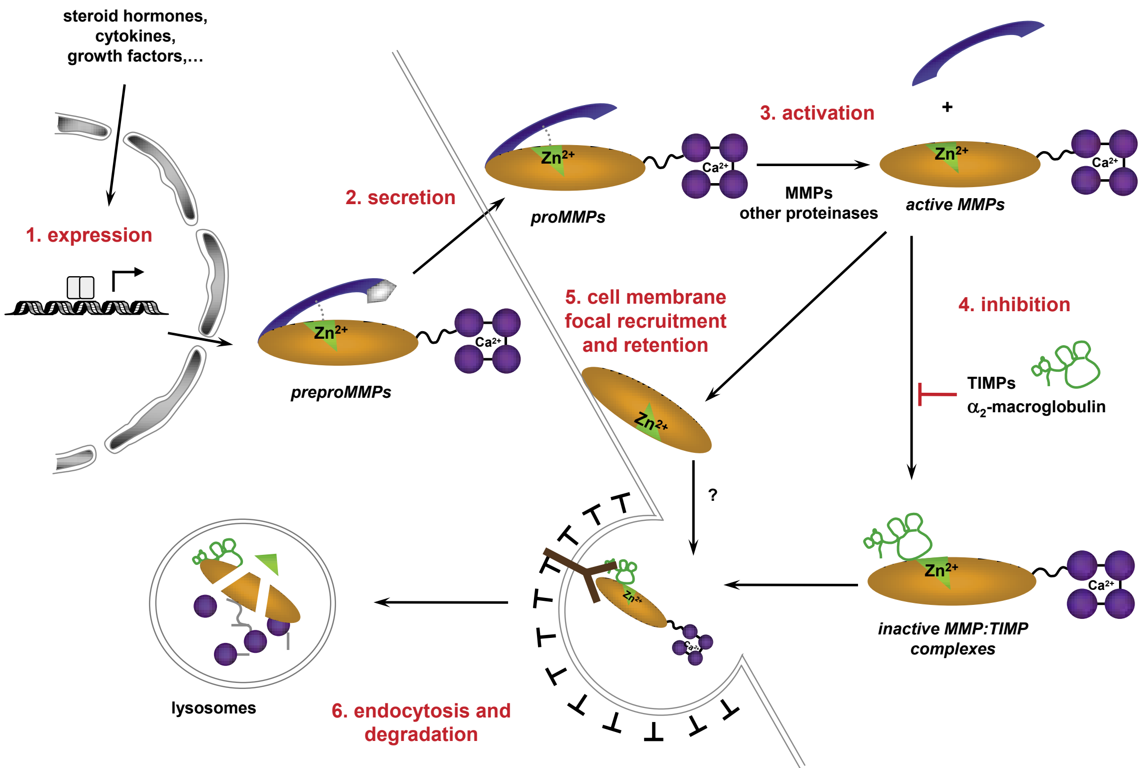


Figure 1. Regulation of soluble MMPs activity in the human endometrium: current model. MMPs, the major actors of extracellular proteolysis, share a common intramolecular control due to masking by a N-terminal prodomain (blue, here shown with the signal peptide in grey) of the Zn2+-dependent catalytic 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).

Among local regulators, transforming growth factors-β (TGF-βs) have been reported to mediate the repression by progesterone of several MMPs in the human endometrium, thereby preventing menstrual breakdown but reports were conflicting on the expression profiles, source and regulation of the TGF-β system in this tissue. We therefore readdressed by real-time RT-PCR and ELISA the expression of the three TGF-βs (total and mature forms) and their two receptors throughout the menstrual cycle, as well as their regulation by ovarian steroids in cultured explants, including in microdissected epithelial and stromal compartments (Fig. 2, adapted from ref. 6). This comprehensive study on a huge collection of endometrial samples evidenced a differential regulation of TGF-β isoforms expression. TGF-β2 and -β3 are differentially expressed during the menstrual cycle and differentially regulated by progesterone in epithelial vs stromal cells. The study also disclosed an opposite regulation of TGF-β2 and -β3 by cAMP and MAPK which could account for their distinct expression in vivo.

We also showed a decade ago that stromal expression of MMP-1 (interstitial collagenase) is induced in the menstruating endometrium by the paracrine action of epithelium-derived interleukin-1α (IL-1α). Recently, we further characterized the mechanisms by which estradiol and progesterone control endometrial IL-1α expression and bioactivity (7). Combining in situ hybridization, explant culture, laser capture microdissection, quantitative PCR, ELISA and immunolabeling, we found that IL-1α is synthesized by epithelial cells throughout the cycle but estradiol and/or progesterone prevent(s) its release. In contrast, endometrial stromal cells produce IL-1α only at menses and during irregular bleeding in areas of tissue breakdown. Stromal expression of IL-1α, like that of MMP-1, is repressed by progesterone (alone or with estradiol) but triggered by epithelium-derived IL-1α released upon withdrawal of both hormones. Altogether, these experiments suggest that IL-1α released by epithelial cells triggers the production of IL-1α by stromal cells in a paracrine amplification loop to induce MMP-1 expression during menstruation and dysfunctional bleeding. All three steps of this amplification cascade are repressed by estradiol and progesterone.

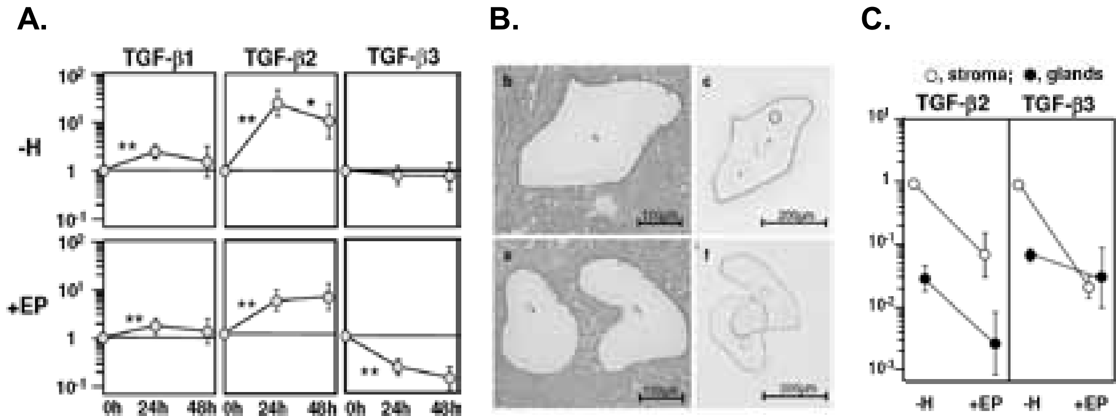


Figure 2. Differential expression of TGF-β2 and -β3 by endometrial explants. A. Explants were cultured in the absence (-H) or presence (+EP) of estradiol and progesterone for 24h or 48h. TGF-β mRNA levels were quantified by RT-PCR and normalized according to β-actin mRNA (geometric means with 95% confidence interval by reference to the non-cultured tissue, 0h). B,C. Laser capture microdissection of stroma (b,c) and glands (e,f) collected after 24h of culture highlights a differential regulation of TGF-β2 and -β3 by EP specifically in glands (mRNA levels in C presented by comparison to levels in stroma -H ; from ref. 6).

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

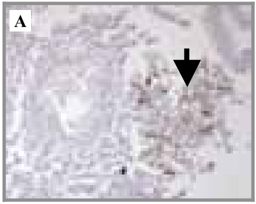
C. Selvais, P.J. Courtoy, P. Henriet, E. Marbaix, H. Emonard (in collaboration with CNRS, Reims, France)

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, itself regulated by estradiol and progesterone (10).

ROLE OF MATRIX METALLOPROTEINASES IN ABNORMAL ENDOMETRIAL BLEEDING

C. Galant, J.-L. Brun, M.-L. Alvarez, A. Béliard, P.J. Courtoy, P. Henriet, E. Marbaix (in collaboration with the Hôpital universitaire Pellegrin, Bordeaux, France, and with the University of Liège, Belgium)

Looking for a role of MMPs in triggering the occurrence of abnormal bleeding in addition to normal menstrual bleeding, we investigated patients showing irregular dysfunctional bleeding, a spontaneous condition that cannot be accounted for by primary organic lesions of the uterus (2). As in patients on long-term progesterone contraception, irregular dysfunctional bleeding is associated with menstrual-like stromal breakdown, particularly in foci containing low levels of ovarian steroids receptors, and correlates with increased expression and activity of several MMPs, together with decreased production of TIMP-1 (Fig. 3). Neutrophils were further recruited to the same foci, released abundant proMMP-8 and -9, and thereby increased ECM proteolysis.



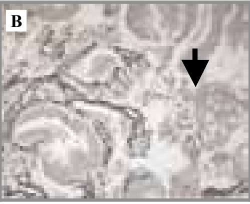


Figure 3. Focal expression of MMPs correlates with ECM breakdown. A. MMP-3 immunostaining. B. Silver staining. Arrows show that MMP-3 expression is restricted to the area showing disappearance of collagen-rich argyrophilic fibers of the endometrial stroma during menstruation

Menorrhagia, or excessive menstrual bleeding, is also a frequent cause of medical consultation and surgical treatment. Thermal ablation of the endometrium is currently largely used

as a less aggressive but efficient surgical procedure, despite rapid regeneration of the mucosa in most patients. We investigated whether the level of MMPs activity could be involved in the amount of menstrual bleeding in such patients and found that menstrual endometria regenerated after thermal ablation showed smaller areas of collagen fibres lysis and contained more TIMP-1 and TIMP-2 compared with endometria from non-treated women with excessive menstrual bleeding, providing a first molecular explanation for the decreased amount of bleeding (8).

In collaboration with the laboratory of Dr. J.M. Foidart (ULg), we have developed a new experimental model of endometrial xenografts in immunodeficient mice (9). 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.

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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). These proteins play an important role in the development of the embryo and in wound healing, as well as in cancer and in fibrosis.

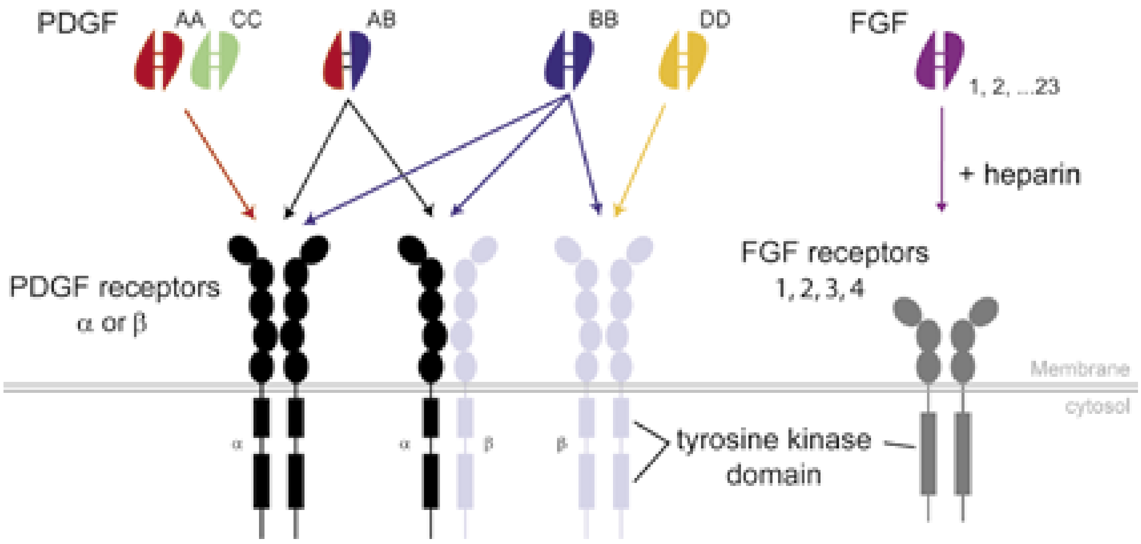

Figure 1. PDGF and FGF receptors and ligands

REARRANGEMENTS OF THE RECEPTOR TYROSINE KINASE GENES ASSOCIATED WITH LEUKEMIA

F. Toffalini, S. Medves, C. Montano, 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. 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 (1).

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 hybrid PDGF receptor in a leukemia patient harboring a t(5;9) translocation. We are now looking for other mutations in tyrosine kinase genes.

ACTIVATION OF PDGF RECEPTORS IN SYSTEMIC SCLEROSIS

J.-F. Classen, S. Lenglez, J.B. Demoulin in collaboration with B. Lauwerys and F. Houssiau (Rheumatology Unit).

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 (Ba-

roni 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 (2). 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 in other related fibrotic conditions, such as extensive chronic graft-versus-host disease.

SIGNAL TRANSDUCTION AND GENE REGULATION BY GROWTH FACTORS

A. Essaghir, N. Dif, 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 (3, 8). We also analyzed gene expression in neural stem cells, glioma, carcinoid tumors and leukemic cells (4, 6).

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

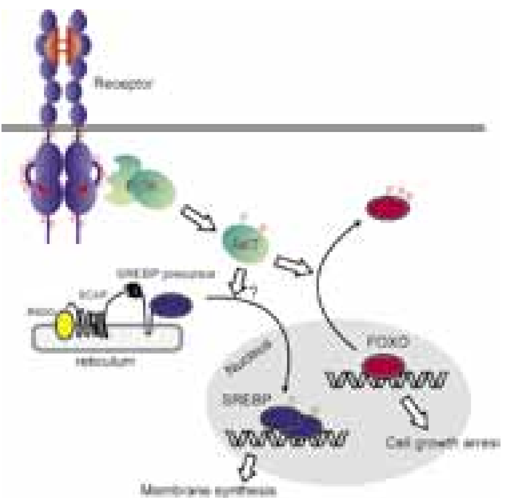

Figure 2. Activation of SREBP and inactivation of FOXO by PDGF

GROWTH FACTORS STIMULATE LIPID SYNTHESIS BY ACTIVATING THE SREBP TRANSCRIPTION FACTORS

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

In our microarray analysis, a cluster of genes involved in fatty acid and cholesterol biosynthesis, including stearoyl-CoA desaturase (SCD), fatty acid synthase and hydroxymethylglutaryl-CoA synthase (HMGCS), was up-regulated by PDGF after 24 h of treatment. Their expression correlated with an increase in membrane lipid biosynthesis. All these genes are known to be controlled by sterol regulatory element-binding proteins (SREBP). PDGF increased the amount of mature SREBP-1, and regulated the promoters of SCD and HMGCS in a SREBP-dependent manner. In line with these results, blocking SREBP processing by addition of 25-hydroxycholesterol blunted the effects of PDGF on lipogenic enzymes and PDGF-driven proliferation. SREBP activation was dependent on the phosphatidylinositol 3-kinase (PI3K) pathway, as judged from the effects of the inhibitor LY294002 and mutation of the PDGF receptor β tyrosine residues that bind the regulatory PI3K subunit p85. In conclusion, our results suggest that PDGF induces membrane lipid synthesis via PI3K and the activation of SREBP (8). 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 (5). 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.

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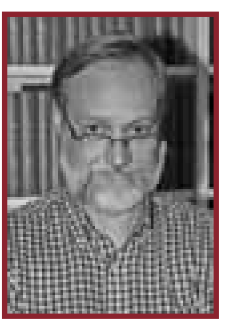

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VIRAL IMMUNITY AND PATHOGENESIS

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

VIRAL INFECTIONS RESULT IN A DRAMATIC INCREASE IN THE PROPORTION OF IGG2A

Of particular interest is the fact that all antibody responses are not equal. Indeed, depending on their isotype, immunoglobulins display various properties. 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-g) 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-g. 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-g message expression and production, as well as by cytolysis of target cell lines was observed 4). Two pathways of IFN-g 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-g 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-g 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-g 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-g 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-g receptor. Together, these results suggest that viruses may exacerbate autoantibody-mediated thrombocytopenia and anemia by activating macrophages through IFN-g 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).

Finally, the Docile strain of lymphocytic choriomeningitis virus (LCMV) induces also anemia in a number of inbred strains of mice, including C3HeB/FeJ and CBA/Ht animals. A difference in the kinetics of anemia and of compensatory reticulocytosis suggested that impaired erythropoiesis was the major pathogenic mechanism involved in CBA/Ht, but not in C3HeB/FeJ mice. In both mouse strains an anti-erythrocyte autoantibody production that depended on the presence of functional CD4+ T lymphocytes was observed. Although depletion of T helper lymphocytes prevented anemia in C3HeB/FeJ mice, this treatment largely failed to inhibit the development of the disease in CBA/Ht animals. This observation indicated that the anti-erythrocyte autoimmune response induced by the infection was at least partly responsible for the anemia of C3HeB/FeJ, but not of CBA/Ht mice. Erythrophagocytosis was enhanced in both mouse strains after LCMV infection, but did not appear to be a major cause of anemia. These data clearly indicate that similar disease profiles induced by the same virus in two different host strains can be the result of distinctly different mechanisms. 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-g. Susceptibility to LPS shock was completely mediated by TNF, and partially by IFN-g.

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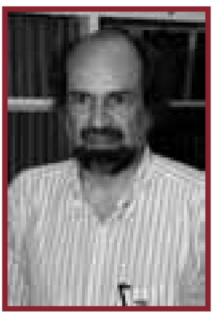

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VIRAL PERSISTENCE AND INTERFERON RESPONSE

Our work focuses on the interplay between viral infections and the immune response of the host. On one hand, we analyze the model infection of the central nervous system by Theiler's virus. This virus can escape the immune defenses of the host and provoke a persistent infection of the central nervous system. Infection by this virus 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 in the particular context of the central nervous system. 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.

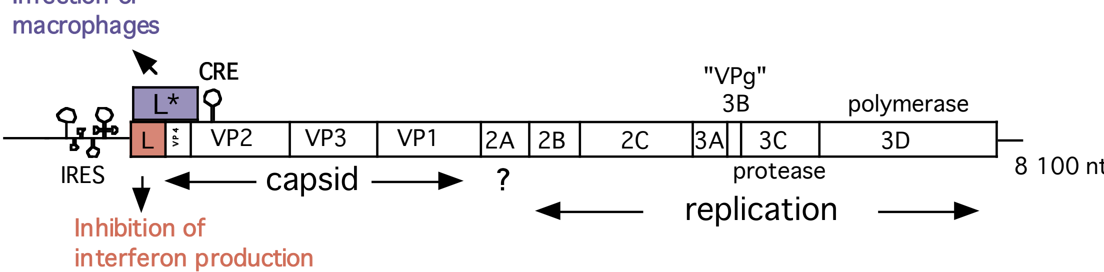

Fig. 1. Genome of Theiler's virus. A large open reading frame encodes a 2000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, to yield the viral mature proteins. Our analysis focuses on 2 proteins, L and L*, that interfere with the host innate immune responses and therefore facilitate 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 showed previously that the L protein could inhibit production of type-I interferons (IFNs) by infected cells (2). Mutation of the zinc-finger is sufficient to abolish the anti-IFN activity of the L protein in vitro and to dramatically impair viral persistence in the central nervous system of mice.

We also showed that the leader protein interferes with nucleo-cytoplasmic trafficking of host cell proteins (4) and with nuclear export of mRNA (10). These activities were associated with hyperphosphorylation of the Nup proteins that make up the nuclear pore complex (10). Perturbation of nucleo-cytoplasmic trafficking can constitute a effective way to inhibit early cell defense mechanisms. Indeed, the induction of many genes involved in early host defense, such as genes coding cytokines and chemokines, depends on the nuclear translocation of transcription factors such as NFκB or IRF-3 into the nucleus.

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, 10).

The L protein encoded by Encephalomyocarditis (EMCV), a related Cardiovirus, shares about 35% amino acid identity with that of Theiler's virus. It differs from the latter by lacking a serine/threonine-rich C-terminal domain and by carrying phosphorylated residues not conserved in Theiler's virus L protein. In spite of these differences, the L protein of EMCV shares, with that of Theiler's virus, the ability to inhibit the transcription of type I interferon, cytokine and chemokine genes and to interfere with nucleo-cytoplasmic trafficking of host-cell proteins. Recent work performed in collaboration with S. Hato and F. van Kuppeveld (NCLMS, Nijmegen, The Netherlands) confirmed the importance of the L protein of EMCV in counteracting the IFN response in vivo.

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 in-

fection 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)

S. Paul, M.Minet, 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 recently 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).

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. Future work will address the physiolgical importance of the IFN-λ system.

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, S. Paul, T. Michiels

In peripheral organs, a specialized population of dendritic cells known as «plasmacytoid» dendritic cells (pDCs) was reported to account for most of the type I IFN production. Constitutive expression of the IRF-7 transcription factor enables pDCs to rapidly synthesize large amounts of IFNs after viral infection. In the central nervous system, however, these cells are reportedly absent or very rare.

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).

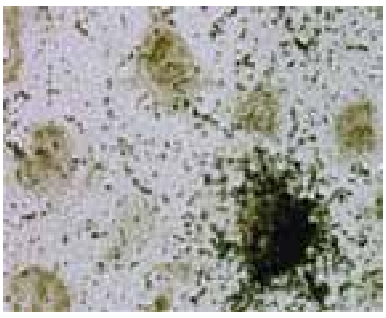

Fig. 3. Neurons can produce IFNα/β. Neuron (immunostained in brown for NeuN) producing IFN-β (detected by in situ hybridization: black dots) after infection with La Crosse virus.

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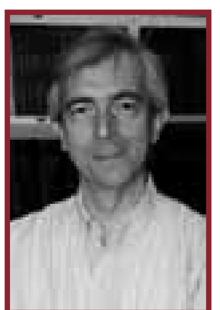

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HUMAN TUMOR IMMUNOLOGY

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 assess the immunogenicity of tumor vaccines and to compare various vaccine modalities. We have developed very sensitive methods for the detection of anti-tumor T lymphocytes, and apply them to patients included in cancer vaccination trials conducted by Dr. Jean-François Baurain at the Cliniques Universitaires St Luc and by the clinical team of the Ludwig Institute.

A second objective is to understand the mechanism of the tumor regressions that occasionally occur in vaccinated patients. Detailed analyses of a few such patients indicated that, surprisingly, the anti-vaccine T lymphocytes are largely 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. Along this line, we are setting up an in situ genetic approach to explore the functional status of T lymphocytes present within melanoma tumors.

That local immunosuppression could be involved in preventing tumor rejection is compatible with the observation that the anti-tumor T cells mentioned above are often present in tumors prior to vaccination. Considering that suppressive or so-called regulatory T cells are important attenuators of immune responses, we analyze their role in vaccinated patients.

IMMUNE RESPONSES TO CANCER VACCINE ANTIGENS

G. Hames, V. Corbière, P.G. Coulie, in collaboration with A.-M. Feyens and 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 responses 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 anticancer vaccines is not the intensity of the induced anti-vaccine T cell responses.

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

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

Because the low levels of anti-vaccine T cells might be insufficient to produce on their own the observed tumor regressions, we examined the possibility that CTL directed against other antigens present on the tumor might contribute to the regressions. For 7 vaccinated melanoma patients, selected because it had been possible to derive a permanent cell line from their tumor cells, we estimated the blood frequencies of CTL directed against any antigen present on the tumor cells. For all these patients, anti-tumor CTL were found at high frequencies in the blood after vaccination. Unexpectedly, they were already present at similar high frequencies before vaccination. The frequency of anti-tumor CTL observed after vaccination was 12 fold to 20,000 fold higher than that of the anti-vaccine CTL (4). Because T cells directed at other tumor antigens than the vaccine antigen could make an important contribution to the tumor regressions, we felt that it was necessary to define the precise nature of their target antigens. We focused our effort on patient EB81, who had shown complete regression of a large number of cutaneous metastases following vaccination with ALVAC-MAGE. A majority of anti-tumor CTL clones recognized antigens encoded by *MAGE-C2*, a cancer-germline gene (4). Others recognized an antigen encoded by *gp100*, a melanocytic differentiation gene. In conclusion we are facing a paradoxical situation where the melanoma patients that are

being vaccinated, have already mounted a high spontaneous response against the types of antigens used in the vaccines. At the time of vaccination this spontaneous T cell response is clearly ineffective in halting tumor progression. The analysis of metastases of vaccinated patients indicated not only the presence of anti-vaccine T cells, with a very modest enrichment compared to the blood, but mainly of much higher proportions of the other anti-tumor T cells, considerably enriched compared to the blood (5). These results suggest that the anti-vaccine CTL may not be the principal effectors that kill the bulk of the tumor cells. They may exert their effect mainly by an interaction with the tumor that creates conditions enabling the stimulation of large numbers of CTL directed against other tumor antigens, which then proceed to destroy the tumor cells (6).

IN SITU ANALYSIS OF TUMOR-INFILTRATING LYMPHOCYTES

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

Our observations that some vaccinated melanoma patients could display a clear tumor regression with a very low or sometimes undetectable anti-vaccine T cell response led us to analyse the other anti-tumor T cells present in these patients, namely the T lymphocytes that recognized tumor-specific antigens different from those of the vaccines. Detailed analyses carried out in two vaccinated patients 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 cancer patients. The reasons for this coexistence may well be the key towards improving the clinical efficacy of cancer vaccines. Our approach is to gain information about human tumor-infiltrating or tumor-associated T cells through an in situ genetic 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. A small piece is systematically put into culture to derive a melanoma cell line. We use laser microdissection to isolate small numbers (1-50) of tumor cells or T lymphocytes infiltrating the tumor. A major difficulty of the approach is to maintain RNA integrity throughout the process: we measured that the RNA recovery in microdissection is usually around 1%, which is too low for the analysis of 1-50 cells. Over the last year we worked out every aspect of these experiments, and improved the recovery by a factor of 10-20, depending on whether or not an histological staining is required. We can now measure with RT-qPCR the expression of 6-8 genes on a microdissected fragment of about 50 cells. We are setting up a global cDNA amplification, in order to carry out complete gene expression profiling on fractions of the samples. We have adapted and are comparing two methods of amplification that can theoretically be used on single cells.

HUMAN REGULATORY T CELLS AND TGFβ

S. Lucas, J. Stockis, 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 mecha-

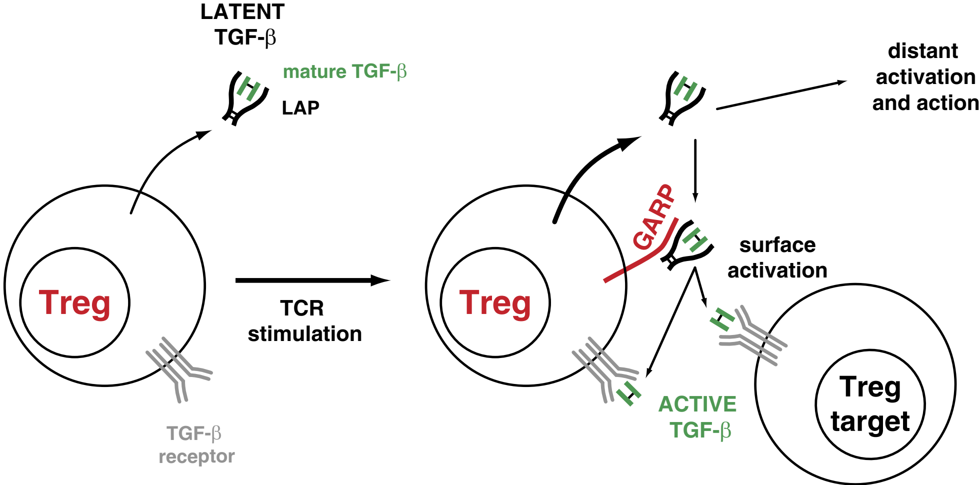

Figure 1. Possible model for TGF-ß production by human Treg clones.

nisms 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. 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 will determine whether this assay can be used to monitor the distribution of Treg cells in human tissue samples such as peripheral blood or tumor fragments. Such monitoring should help to evaluate the role of human Treg cells in the inhibition of spontaneous or vaccine-induced anti-tumor immune responses.

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.

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 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. 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 above, 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 result in the production of active TGF-ß upon stimulation of these Th cells.

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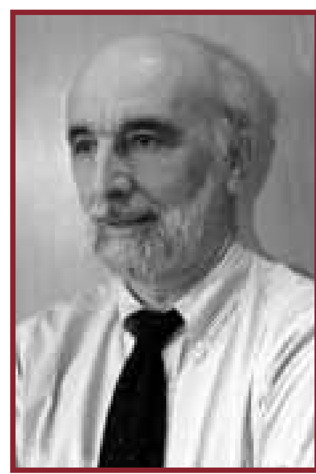
LUDWIG INSTITUTE FOR CANCER RESEARCH

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 nine 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 600 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 90 members and is headed by Thierry Boon, Branch Director.

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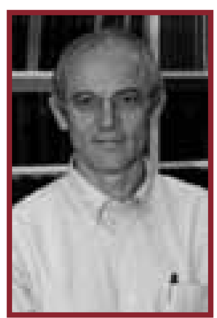

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TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

Building up on the molecular definition of tumor antigens recognized by T cells, our group mainly focuses on two aspects of tumor immunology, namely the processing of tumor antigens and the study of animal models to optimize cancer immunotherapy and evaluate tumor resistance mechanisms.

Tumor antigens recognized by Cytolytic T Lymphocytes (CTL) consist of peptides that are presented by MHC molecules at the cell surface and derive from intracellular proteins that are degraded by the proteasome. The intracellular pathway leading from the protein to the peptide/MHC complex is known as "antigen processing". Our group focuses on the proteasome and recently described a new mode of production of antigenic peptides by the proteasome, based on cutting and pasting peptide fragments to form a new spliced peptide. The first example was a peptide derived from human melanocyte protein gp100. This antigenic peptide is nine-amino acid long and is produced by the splicing of two fragments that were initially non-contiguous in the parental protein. The splicing is made by the proteasome, is tightly coupled to the proteolytic reaction, and appears to occur by transpeptidation involving an acyl-enzyme intermediate. We also described a second example of spliced peptide, which is a minor histocompatibility antigen, and where the two fragments are rearranged before splicing. We are currently working on additional spliced peptides. We are also studying the processing differences between the standard proteasome, which is present in most cells, and the immunoproteasome which is found in dendritic cells and in cells exposed to interferon-gamma. Several tumor antigens - including spliced peptides - were found to be processed differently by the two proteasome types, usually because of a preferential

cleavage made by one or the other proteasome within the antigenic peptide itself. We also study new proteasome types that are intermediate between the standard proteasome and the immunoproteasome.

Translation of knowledge on tumor antigens into efficient cancer immunotherapy requires additional studies on the various strategies that can be used. Some of these studies can be done in preclinical animal models. The study of such a model allowed us to uncover a powerful mechanism of tumor resistance, which is based on tryptophan catabolism by indoleamine-2,3 dioxygenase, an enzyme that we found to be frequently expressed in tumors. The resulting local tryptophan shortage appears to prevent the proliferation of lymphocytes at the tumor site. Inhibitors of indoleamine-2,3 dioxygenase can be used in vivo to counteract this tumor resistance mechanism. We are searching for new IDO inhibitors that could be developed clinically. We also study additional tumor resistance mechanisms.

The currently available murine models are limited by the fact that they are based on transplantation of tumor cells grown in vitro into a healthy animal. This does not recapitulate the long-term host/tumor relationship that occurs in humans when a tumor slowly develops within a normal tissue. To circumvent this limitation and obtain more relevant information from such preclinical models, we have build a new mouse melanoma model where tumors expressing a given antigen can be induced, using a transgenic system based on Cre-lox recombination.

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

B. Guillaume, V. Stroobant, W. Ma

Antigens recognized by cytolytic T lymphocytes (CTL), such as viral or tumor antigens, usually consist of peptides of 8-10 amino acids in length, which are presented by MHC class I molecules at the cell surface. Because such peptides derive from intracellular proteins, a processing step is required before they can be exposed to the cell surface in association with MHC molecules. Firstly, the peptide is produced through degradation of the parental protein by the proteasome. Secondly, it is taken up by a dedicated transporter named TAP and translocated inside the endoplasmic reticulum where it meets and associates with newly synthesized MHC molecules. The first step of cleavage by the proteasome is crucial in that cleavage location determines the precise sequence of the final antigenic peptide. We have observed that this cleavage may occur differently in some cells, depending on their proteasome content. The proteasome comes in two forms: the standard proteasome, which is found in most cells, and the immunoproteasome, which is expressed by mature dendritic cells and by cells exposed to interferon-gamma (IFNg).

We previously reported that a class-I restricted antigenic peptide derived from an ubiquitous human protein was processed efficiently by the standard proteasome but not by the immunoproteasome. As a result, the relevant epitope is not presented efficiently by mature dendritic cells, which contain immunoproteasomes (1). We have now extended those observations to several antigenic peptides of interest for cancer immunotherapy, including HLA-A2-restricted epitopes derived from tyrosinase, Melan-AMART1 and gp100. On the contrary, we showed that other tumor epitopes, which are derived from MAGE-3 and MAGE-C2, are processed by the immunoproteasome but not by the standard proteasome and the-

refore are presented to CTL only by tumor cells pre-treated with IFNg (2). By analyzing the peptidic fragments produced after in vitro digestion with the two proteasome types, we found that the differential processing can result from two mechanisms. In some cases, one of the proteasome types predominantly cleaves within the sequence of the epitope, resulting in its destruction (2). In other cases, the difference lies in the efficiency of cleavage at the C-terminal end of the antigenic peptide. These observations may have major implications for cancer immunotherapy, as they imply that the peptide repertoire presented by tumor cells may differ from the repertoire presented by antigen-presenting cells (3). It is therefore essential to study those processing differences in detail, so as to define the most effective vaccination strategy for each epitope. We have also observed the presence in many tumor lines of proteasome types that are intermediate between the standard proteasome and the immunoproteasome. These intermediate proteasomes contain only some of the three catalytic subunits of the immunoproteasome, i.e. only ß5i or only ß1i and ß5i. In terms of production of antigenic peptides, these intermediate proteasomes produce more or less the same peptides as the immunoproteasome, and therefore explain the recognition of tumor cells by CTL directed against these peptides in the absence of IFNg. However, we have also identified two antigenic peptides that are produced exclusively by intermediate proteasomes.

ANTIGENIC PEPTIDES PRODUCED BY PEPTIDE SPLICING IN THE PROTEASOME

A. Dalet, V. Stroobant (in collaboration with Edus Warren, Fred Hutchinson Cancer Research Center, Seattle, USA)

By studying the antigen recognized by a CTL clone isolated from a melanoma patient, we identified an antigenic peptide composed of two non-contiguous fragments of the same protein, namely the melanocytic protein gp100. The production of this peptide requires the excision of an intervening fragment of 4 amino acids and the splicing of a fragment of 3 residues with a fragment of 6 residues. We have shown that this splicing is exerted by the proteasome and can be reproduced in vitro by incubating a precursor peptide with purified proteasomes. Splicing is coupled directly to peptide bond cleavage by the proteasome and appears to occur by transpeptidation involving an acyl-enzyme intermediate (Fig. 1) (4). The splicing reaction appears not to involve a particular motif, but rather to result from a low-efficiency reversal of the proteolysis reaction. Its occurrence is depending only on the occurrence of peptide cleavage.

We have also identified a second antigenic peptide produced by peptide splicing in the proteasome (5). This peptide is recognized by CTL directed against a minor histocompatibility antigen. The CTL was isolated from a multiple myeloma patient treated with HLA-identical bone marrow transplantation. The peptide is encoded by the polymorphic region of a gene ubiquitously expressed. Again it is made by the joining of two fragments that are initially non-contiguous in the parental protein. In addition, the two fragments are inverted in the spliced peptide, i.e. the fragment that was more N-terminal in the parental protein ends up at the C-terminal side of the spliced peptide, and vice-versa. We showed that splicing and transposition could be reproduced in vitro with purified proteasomes. The splicing mechanism based on transpeptidation immediately after peptide bond cleavage is compatible with a transposition of the fragments prior to splicing. Another spliced peptide was observed previously by Hanada et al. We have now demonstrated that this third spliced peptide, which is derived from protein FGF-5, is also produced by transpeptidation inside the proteasome, despite the presence of a much longer segment between the two spliced fragments. We also showed that both the standard proteasome and the immunoproteasome are able

to splice peptides, but we noted differences in their efficiency to produce individual peptides. These differences are related to differences in the efficiency of cleavage at the relevant positions to produce the fragments to splice. This observation confirms the transpeptidation model, which predicts that the efficiency of splicing of a given peptide is mainly dependent on the efficiency of cleavage of the fragments to splice. Altogether, these results indicate that spliced peptides are not uncommon and may represent a significant part of the peptide repertoire presented by MHC class I molecules.

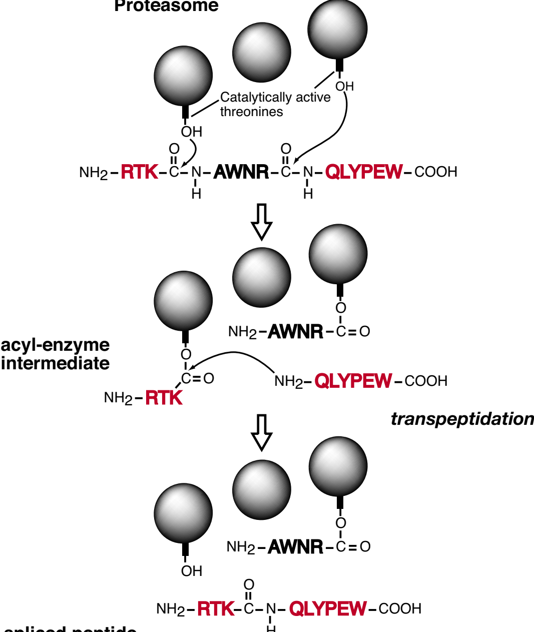

Figure 1. Model of the peptide-splicing reaction in the proteasome. The active site of the catalytic subunits of the proteasome is made up of the side-chain of a threonine residue, which initiates proteolysis by performing a nucleophilic attack on the carbonyl group of the peptide bond. An acyl-enzyme 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.

IDENTIFICATION OF NEW ANTIGENS RECOGNIZED BY AUTOLOGOUS CTL ON HUMAN MELANOMA

W. Ma, N. Vigneron (in collaboration with P. Coulie)

Melanoma patient EB81 was vaccinated with a MAGE-type antigen and showed regression of all cutaneous metastases. Blood lymphocytes collected after the regression were stimulated with autologous tumor cells, and CTL clones were obtained. Surprisingly, none of these clones was directed against the antigen used for vaccination. Using a cDNA expression cloning approach, we identified the antigens recognized by three of them. These antigens correspond to three distinct peptides all derived from MAGE-C2, a gene with a cancer-germline expression pattern, which is expressed in about 40% of melanomas and 30% of bladder carcinomas. Two peptides are presented by HLA-A2, and one by HLA-B57 (6). Because of their strict tumor-specificity and their wide expression in tumors, these new antigens represent promising targets for cancer immunotherapy. The processing of two of these peptides is dependent on the immunoproteasome (2).

We also started to study the intracellular processing pathway responsible for the cross-presentation of long peptides by dendritic cells.

A TUMOR IMMUNE ESCAPE MECHANISM BASED ON TRYPTOPHAN DEGRADATION BY INDOLEAMINE 2,3 DIOXYGENASE

L. Pilotte, I. Théate, C. Uyttenhove, D. Donckers, V. Stroobant, D. Colau, P. Larrieu

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme that catalyses rapid tryptophan degradation. Because tryptophan can freely cross the plasma membrane, IDO expression results in a local depletion of tryptophan in the extracellular medium surrounding the expressing cell. Tryptophan depletion was shown to impair T lymphocyte proliferation, and therefore IDO expression represents of powerful immunosuppressive mechanism that accounts, for example, for maternal tolerance to allogeneic fetuses, where IDO expression by placenta was found to play an essential role. Expression of IDO can be induced by interferon-gamma in many cellular types, including macrophages and dendritic cells, and appears to play a prominent role in immune regulation.

We have observed that many human tumors express IDO in a constitutive manner (7). To determine whether IDO expression provides tumor cells with a survival advantage by allowing their escape from immune rejection in vivo, we used the well-characterized model system of mouse tumor P815, where the antigen encoded by gene P1A is the major target of the rejection response. We observed that expression of IDO by P815 tumor cells prevents their rejection by pre-immunized mice.

This effect can be partly reverted by systemic treatment of mice with an inhibitor of IDO, in the absence of noticeable toxicity (7). These results suggest that the efficacy of therapeutic vaccination of cancer patients could be improved by concomitant administration of an IDO inhibitor. We are currently searching for new IDO inhibitors that could be developed at the clinical level. We have produced a novel monoclonal antibody against human IDO (in collaboration with the group of Jean-Christophe Renauld). Using this antibody we have studied the expression of IDO in normal tissues in the steady state. Besides endothelial cells of the placenta, the only normal cells that express IDO are dendritic cells located in lymphoid organs, including lymph nodes, spleen, gut lamina propria and thymus. We characterized those cells as mature myeloid dendritic cells. Contrary to published reports, we found that plasmacytoid dendritic cells do not express IDO, and that IDO-expressing dendritic cells are not enriched in tumor-draining lymph nodes. Using in vitro derived dendritic cells, we confirmed that immature dendritic cells do not express IDO, while mature dendritic cells express high levels of IDO.

AN INDUCIBLE MOUSE MODEL OF MELANOMA EXPRESSING A DEFINED TUMOR ANTIGEN

C. Powis de Tenbossche, C. Uyttenhove, G. Warnier (in collaboration with A.-M. Schmitt-Verhulst, CIML, Marseille)

Cancer immunotherapy based on vaccination with defined tumor antigens has not yet shown strong clinical efficacy, despite promising results in preclinical models. This discrepancy might result from the fact that available preclinical models rely on transplantable tumors, which do not recapitulate the long-term host-tumor interplay that occurs in patients during progressive tumor development and results in tumor tolerance. To create a faithful preclinical model for cancer immunotherapy, we generated a transgenic mouse strain developing autologous melanomas expressing a defined tumor antigen recognized by T cells (8). We chose the antigen encoded by P1A, a well-characterized murine cancer germline gene. To transform melanocytes, we aimed at simultaneously activating the Ras pathway and inactivating tumor suppressor Ink4a/Arf, thereby reproducing two genetic events frequently observed in hu-

man melanoma. The melanomas are induced by s.c. injection of 4-OH-tamoxifen (OHT). By activating a CreER recombinase expressed from a melanocyte-specific promoter, this treatment induces the loss of the conditional Ink4a/Arf gene in melanocytes. Because the CreER gene itself is also flanked by loxP sites, the activation of CreER also induces the deletion of its own coding sequence and thereby allows melanocyte-specific expression of genes H-ras and P1A, which are located downstream on the same transgene. All melanomas induced in those mice with OHT show activation of the Ras pathway and deletion of gene Ink4a/Arf. In addition, these melanomas express P1A and are recognized by P1A-specific T lymphocytes. This model will allow to characterize the interactions between the immune system and naturally occurring tumors, and thereby to optimize immunotherapy approaches targeting a defined tumor antigen. We have now optimized the induction of melanoma in this model, and we reach an incidence of 70-80% tumors. In many tumor-bearing mice, we observed an accumulation of immature myeloid cells bearing both the CD11b and Gr1 markers. We are characterizing those cells, which might correspond to the myeloid-derived suppressor cells described in other models. In parallel, we have developed a strain of mice transgenic for the P1A-specific T cell receptor, which will be useful for such studies.

IMPACT OF THE THYMIC EXPRESSION OF CANCER-GERMLINE GENE P1A ON THE IMMUNOGENICITY OF P1A-DERIVED TUMOR ANTIGEN P815AB

I. Huijbers, C. Uyttenhove, D. Donckers, (in collaboration with A.-M. Schmitt-Verhulst, CIML, Marseille)

The genes encoding tumor antigens of the MAGE type are encoded by so-called cancer-germline genes, whose expression is restricted to tumor cells and male germline cells, which do not bear MHC class I molecules. Recently, the group of Kyewski described the expression of such genes in a subset of epithelial cells located in the thymic medulla. This raised the question of the induction of a certain level of central tolerance to the antigens encoded by cancer-germline genes, which could explain the relatively poor immunogenicity of these antigens. To address this question, we have created a line of mice deficient for gene P1A, which is the murine prototypic cancer germline gene. Those P1AKO mice were healthy and fertile. When immunized against the P1A-derived antigen, they mounted slightly stronger specific immune responses than wild type mice. Similarly, P1AKO mice rejected P1A-expressing tumors more readily than wild type mice. Those results confirm a certain level of tolerance to antigens encoded by cancer-germline genes. However, this tolerance is limited, since wild type mice can develop responses to P1A-derived antigens, as opposed to P1A-transgenic mice that express P1A in all tissues and are fully tolerant to P1A.

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 os-

teoarthritis (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 (9). These observations can be useful to guide therapeutic decisions.

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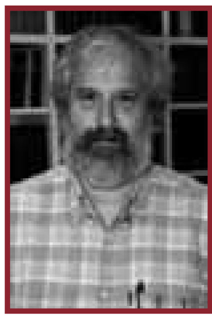

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REGULATION OF T LYMPHOCYTE FUNCTION IN TUMORS

We have recently discovered a new type of anergy of human CD8 T cells, which is observed on tumor-infiltrating lymphocytes. We are analyzing the mechanism of this type of anergy and we look for agents that reverse this anergy. 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 therefore intend to pursue clinical trials involving the use of these agents in combination with anti-tumoral vaccination.

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. To that purpose, we have used various approaches that we have loosely named "reverse immunology", because they use gene sequences as starting point (3).

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

ANALYSIS OF THE T CELL RESPONSES OF CANCER PATIENTS

$CD8^+$ T cells

We analyzed the T cell response in one patient who regressed upon vaccination with autologous dendritic cells pulsed with MAGE-3.A1 and MAGE-3.DP4 peptides. This patient developed a mixed tumor response, with disappearance/reduction of metastases and appearance of new metastases (Figure 1). In many MAGE-vaccinated patients who show tumor regression, the frequency of anti-vaccine T cells is very low. We wondered how such a low number of T cells could provide the main component of the specific effectors destroying the tumor cells. This led us to evaluate the frequency of all the T cells that recognized the autologous tumor cell line. We performed an in-depth longitudinal analysis of the anti-vaccine and anti-tumor responses, in the blood and in tumor sites of this patient (4). Before vaccination, the patient already had high frequencies of anti-tumor T cells in the blood. Skin metastases contained T cells but they were apparently inactive in destroying tumor cells. Upon vaccination, a modest anti-vaccine response was observed and this response lasted over the observation period of three years, with no evident concentration of anti-vaccine T cells

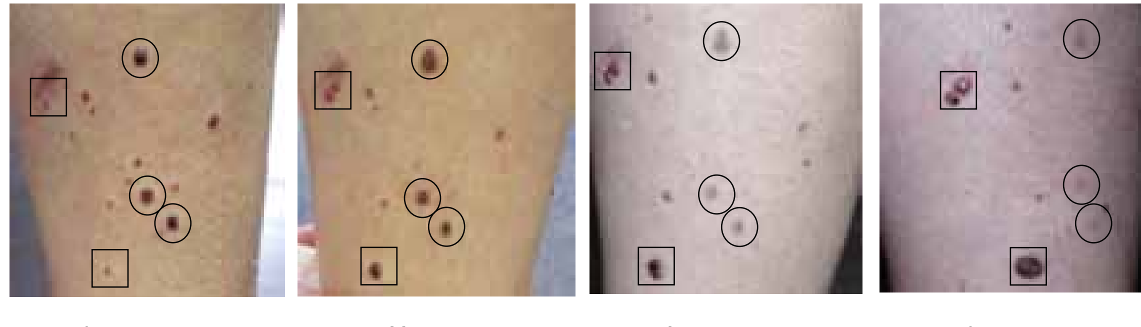

Figure 1. Development of a mixed tumor response in patient LB2586. Regressing lesions on the posterior face of the right leg are encircled. The squares frame lesions that progressed over the same period of time.

at tumor sites. Interestingly, a new anti-tumor CTL clone appeared in the blood after vaccination and was found to be enriched by more than 1,000-fold in the metastases. This anti-tumor CTL was directed against a previously unknown antigen, a MAGE-C2-derived peptide presented by HLA-B44 (Figure 2) (5).

Three other MAGE-C2 antigenic peptide were identified with CTL isolated from melanoma patients who displayed impressive CTL responses against these antigens, reaching frequencies in a tumor metastasis of several percent of CD8 T cells. Whether this makes MAGE-C2 derived antigens particularly useful for anti-tumoral vaccines will have to be determined by clinical experimentation.

The CD8 T cell response observed in this patient reinforce an hypothesis proposed by T. Boon and P. 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.

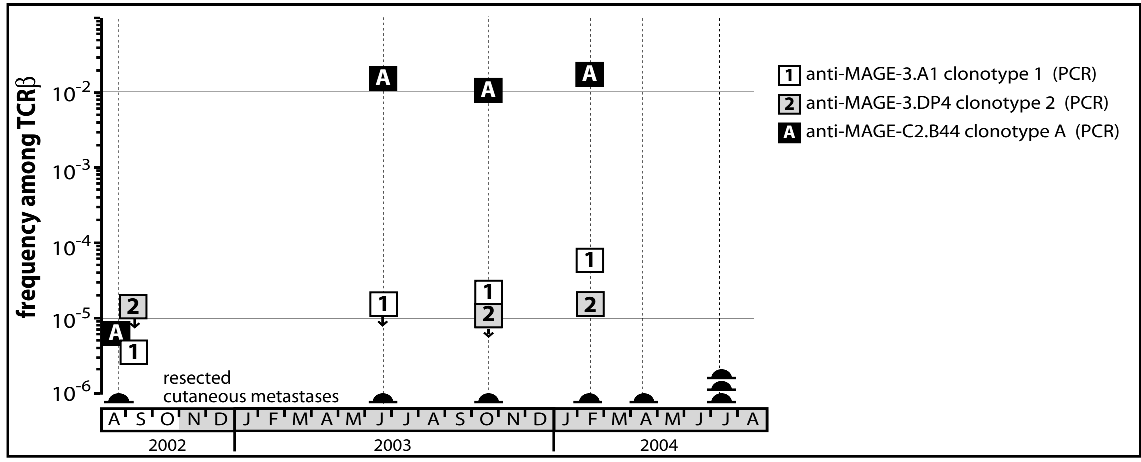

Figure 2. Frequency and diversity of anti-vaccine and anti-tumor T cells in various skin metastases of patient LB2586. The frequencies were estimated by PCR using primers specific for the CD3/TCRβ region of each clonotype.

CD4+ T cells and CD4+ regulatory T cells

We have analyzed the blood T cells from 14 vaccinated melanoma patients who carried the HLA-DP4 allele and whose tumor expressed MAGE-A3 (6). The vaccines involved various antigens present on melanoma cells and all contained the MAGE-A3243-258 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 (7). 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

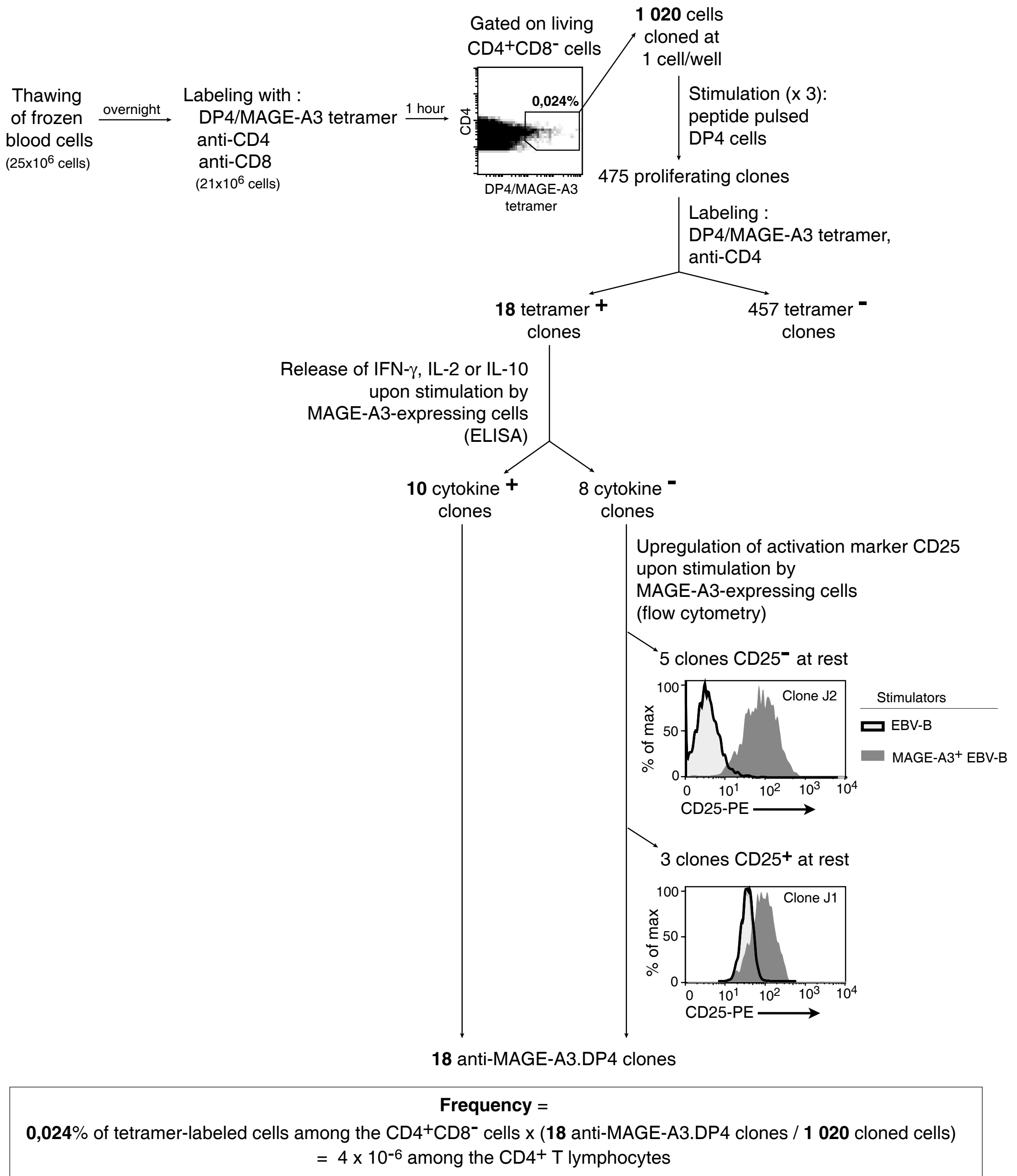

Figure 3. Example of the procedure used to obtain anti-MAGE-A3.DP4 T cell clones from blood cells of vaccinated patients. The numbers indicated correspond to an experiment performed with blood cells of patient EB97 collected after the sixth vaccination.

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, twelve 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 an 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. These regulatory T cell clones represent about 5% of the anti-MAGE-A3.DP4 T 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.

We conclude that MAGE-A3.DP4 vaccination can produce $CD4^+$ T cells that may influence the outcome of the treatment by exerting regulatory T cell function. The anti-MAGE-A3.DP4 potential regulatory T cells were isolated from the blood of four patients, including two patients with a mixed clinical response. This suggests at first that the occurrence of these T cells is not sufficient to prevent tumor regression following vaccination. We have nevertheless no information about the presence of these cells at the tumor site. Quantitative DNA methylation analysis of FOXP3 together with the relative frequencies of regulatory T cells and effector T cells at the tumor site could become critical pieces of information to better establish the impact of regulatory T cells on the clinical outcome of vaccinated cancer patients.

A SCENARIO TO EXPLAIN THE LOW LEVEL OF CLINICAL RESPONSES IN VACCINATED PATIENTS

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 understanding why a small minority of patients shows tumor regression following vaccination, whereas the majority does not.

Recent analyses of the T cell responses of melanoma patients has led us to propose the following scenario (2, 8). Most melanoma patients produce a spontaneous T cell response against melanoma tumor antigens at a relatively early stage of the disease (primary tumor or early metastatic tumor) (Figure 4).

These T cells can eliminate some tumors at an early stage, but often they do not succeed in eliminating the tumor and they become anergic. Thus, the tumors of the patients about to receive the vaccine, already contain anergic T cells directed against tumor antigens. Presumably this anergy is maintained by immunosuppressive factors present in the tumor. A few patients show tumor regression following vaccination because some T cells generated by the vaccine penetrate inside the tumor, attack some tumor cells and succeed in reversing the local immunosuppression, possibly by releasing cytokines or chemokines. This reactivates the anergic T cells located inside the tumor and elicits new anti-tumoral T cells. These T cells then proceed to the elimination of the tumor cells. Most patients do not show tumor regres-

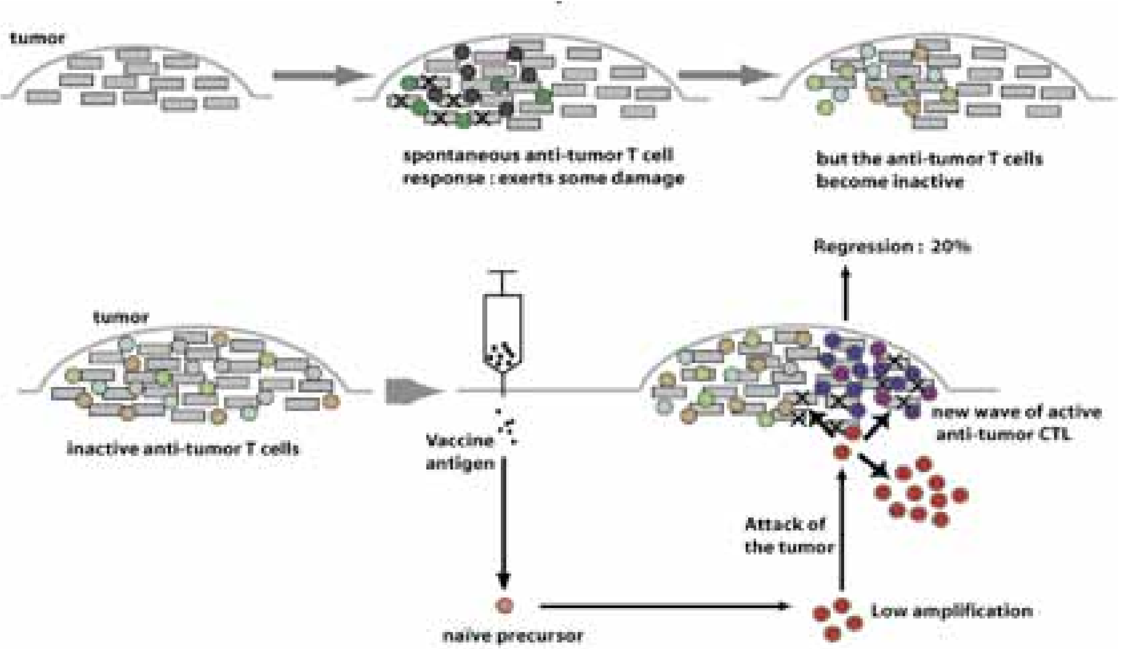

Figure 4. Our working hypothesis: T cells generated by the vaccine succeed in reversing the local immunosuppression.

sion because the few anti-vaccine T cells that reach the tumor are immediately overwhelmed by the immunosuppressive environment.

Accordingly, our working hypothesis is that the crucial difference between the responding and the non-responding 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.

A MECHANISM CAUSING ANERGY OF CD8+ T LYMPHOCYTES

We have identified a novel mechanism causing anergy of human CD8 cytolytic T lymphocytes (CTL), including tumor-infiltrating lymphocytes (9-10). This type of anergy appears to occur transiently during the normal stimulation cycle of T lymphocytes.

We observed that, a few days after antigen stimulation, CTL clones lose the capacity to secrete cytokines and in some case the cytolytic activity. These functions are recovered gradually and are usually completely restored after two weeks. TCRs and CD8 co-receptors were colocalized at the cell surface of functional CTL but, on the contrary, distant at the cell surface of non-functional CTL (Figure 5).

TCR and CD8 do not co-localize on tumor-infiltrating lymphocytes

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

Glycoprotein-galectin lattices restrain

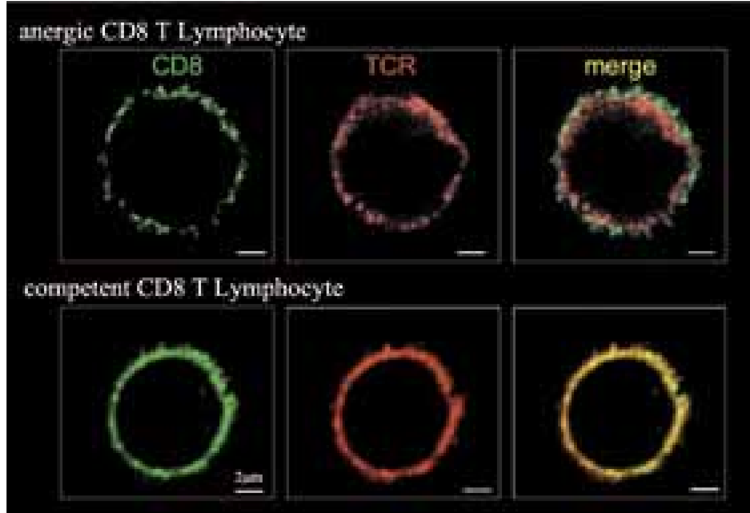

Figure 5. TCR and CD8 do not co-localize on recently stimulated CTL without effector functions.

mobility of TCR

On the basis of the work of other groups, we hypothesized that the absence of TCR-CD8 co-localization at the cell surface of anergic T cells is due to the loss of mobility of the TCR, which is trapped in a lattice of galectin-3. The presence of galectin-3 in ascites and solid tumors has been shown in many studies. To test this hypothesis, tumor-infiltrating lymphocytes were incubated with N-acetyllactosamine, a disaccharide ligand of galectin-3. This treatment restored the TCR-CD8 co-localization and the capacity to secrete IFN-γ and other cytokines after stimulation (Figure 6).

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 ligands and suggest that treatment of cancer patients with galectin ligands could correct the anergy of tumor-infiltrating lymphocytes. It is possible that peptide vaccination combined with local injection of a galectin ligand will be more effective at producing tumor regression than vaccination alone. We have recently identified a polysaccharide already approved for clinical use that was more efficient than

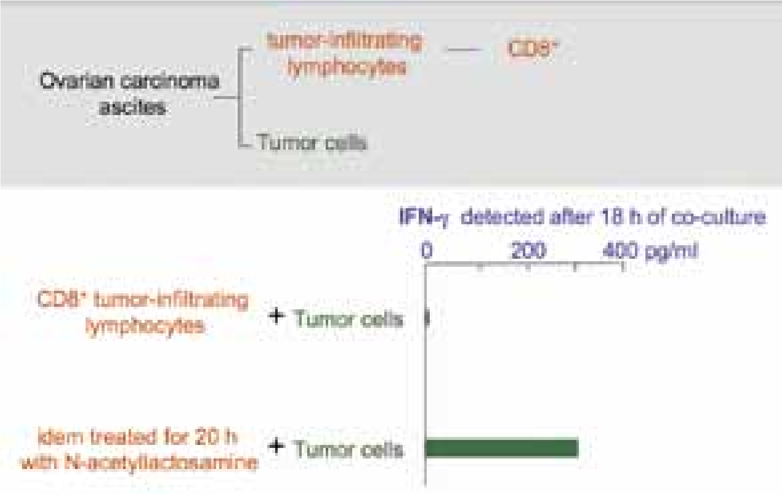

Figure 6. Treatment of tumor-infitrating lymphocytes with a galectin ligand reverses anergy.

N-acetyllactosamine to correct the anergy of CTL clones and human tumor-infiltrating lymphocytes. We therefore intend to launch a clinical trial combining peptide vaccination and injections of galectin-binding polysaccharides in melanoma tumor-bearing metastatic patients.

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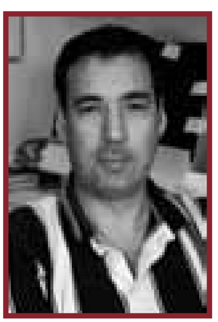

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THERAPEUTIC VACCINATION AND TUMOR EXPRESSION PROFILING GROUP

Tumor cells carry antigens such as MAGE antigens that are absent from normal tissues, and that can be targeted by cytolytic T lymphocytes (CTL). Whilst it is possible to make such CTL recognize and kill autologous tumor cells in vitro, the precise way to induce an effective immune response against tumor antigens in cancer patients remains unknown. In our initial clinical vaccination trials, patients with a MAGE-expressing cancer, often metastatic melanoma, have been treated repeatedly with a MAGE vaccine. We observed evidence of tumor regression in several patients, including a few who experienced a long lasting complete remission. However, the great majority of vaccinated patients had a continuous tumor progression.
These trials allow to collect crucial biological material, which can be analyzed in order to understand what happens in regressing tumors, and why it doesn't occur in progressing tumors. Blood and tumor samples provide anti-tumoral CTL clones, which can be functionally characterized. Tumor samples can be analyzed by expression microarrays and immunohistology, to study the interaction between the tumor environment and the immune cells at the transcriptional level. New treatment modalities can then be defined based on the knowledge acquired from these analyses.

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.

Tumor cells carry antigens such as MAGE antigens that are absent from normal tissues, and that can be targeted by cytolytic T lymphocytes (CTL). Whilst it is possible to make such CTL recognize and kill autologous tumor cells in vitro, it is not known yet how to effectively induce CTL responses against tumor antigens in cancer patients that lead to tumor destruction.

In our clinical vaccination trials, patients with a MAGE-expressing cancer, often melanoma, are treated repeatedly with a MAGE vaccine (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 response to the vaccine antigen occurred, and by determining whether immunological and clinical responses are correlated.

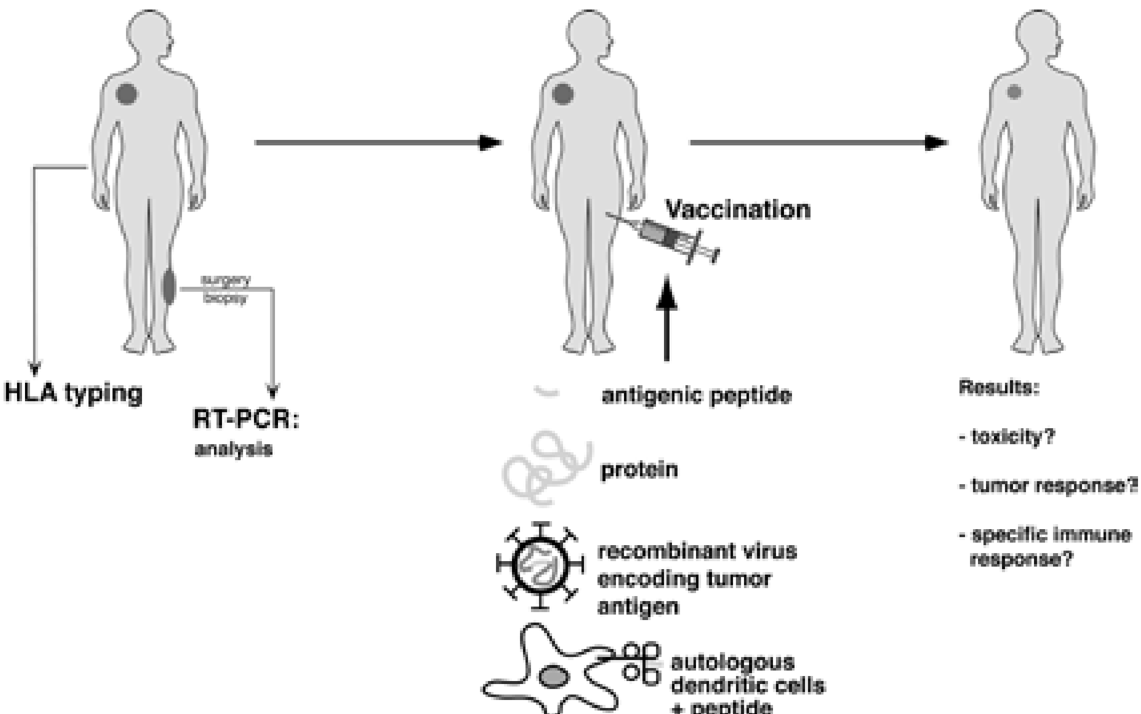

Figure 1. Principle of anti-tumor vaccination with a defined antigen : The first step is to determine if the patient's tumor cells express the tumor antigen. This can be determined by HLA typing, and by RT-PCR analysis of a tumor sample. Selected patients will receive repeated injections of a vaccine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

Secondly, these trials allow crucial biological material to be collected from vaccinated patients. Blood samples provide anti-tumoral CTL clones, which can be functionally characterized.

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 tried, they all are devoid of significant 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 shown until now is the fact that tumors have acquired the ability to resist destruction by antitumoral T cells, following repetitive in vivo challenge with spontaneously occurring immune responses. Even though the molecular mechanisms of tumor resistance remain largely unknown, recent

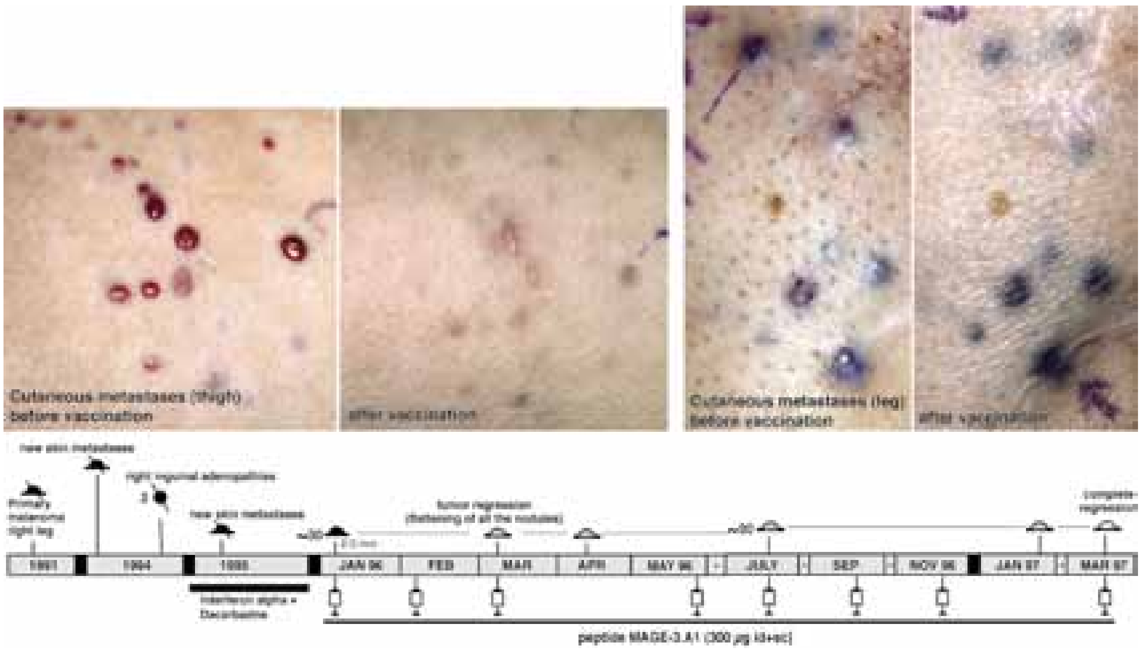

Figure 2

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.

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.

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, usually before the vaccine treatment was started. These data 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. 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).

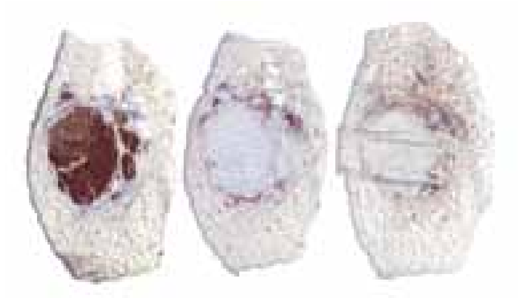

Figure 3. Cryosections obtained from a cutaneous melanoma metastasis were stained with antibodies directed against either melanoma cells (left), T lymphocytes (middle) and macrophages (right), and counterstained with hematoxylin. The corresponding cells appear in red. These images show clearly that the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

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CYTOKINES IN IMMUNITY AND INFLAMMATION

The cytokine group studies the biology of Interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is a TH2 cytokine that plays a role in immune responses against intestinal parasites and asthma. IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, upregulates the production of acute phase reagents in the liver. Its activity in inflammatory responses is modulated by a specific antagonist, the IL-22 binding protein (IL-22BP). The role of IL-9 and IL-22 in inflammation is currently being investigated using transgenic and gene-targeted mice for these cytokines and their receptors.

INTERLEUKIN 9

J.-C. Renauld, J. Van Snick, L. Knoops, V. Steenwinckel, M. Stevens

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. We further identified human IL-9 by cross-hybridization with the mouse gene. 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 three essential properties of IL-9: its tumorigenic potential in T lymphocytes, its stimulatory activity on a particular subset of B lymphocytes, and its activity on mast cells and eosinophils with consecutive implications in asthma.

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. 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-transgenic mice : B1 cell expansion

Further analysis of these IL-9-transgenic mice showed that a particular B lymphocyte population, called B-1 lymphocytes and usually restricted to the peritoneal and pleuropericardial cavities, were dramatically expanded in response to IL-9 overproduction. In addition, such cells were also found in the blood and in the lungs of IL-9 transgenic mice. This observation is reminiscent of mice that are prone to the development of diseases that are characterized by the production of autoantibodies, such as Systemic Lupus Erythematosus, and suggests that IL-9 might play a role in some autoimmune processes.

IL-9-transgenic mice : parasite infections and asthma

In addition, IL-9 transgenic mice 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. This was confirmed by taking advantage of a new strategy of anti-cytokine vaccination: mice vaccinated against their own IL-9 failed to expel T.muris parasites and had a decreased eosinophilic response 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. Recent studies indicated that IL-9 promotes asthma through both IL-13-dependent and IL-13-independent pathways (2). 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 I clinical trials using anti-IL-9 antibodies produced in our laboratory have been initiated in collaboration with Medimmune.

IL-9 RECEPTOR AND SIGNAL TRANSDUCTION

J.-C. Renauld, L. Knoops, T. Hornakova, M. Stevens

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. By further dissecting the signal transduction cascade triggered by IL-9, we showed that, upon IL-9 binding, the IL-9R associates with a co-receptor protein called γc. This induces the phosphorylation of the JAK1 and JAK3 tyrosine kinases, which are associated with IL-9R and γc, respectively. A single tyrosine residue of the IL-9R is then phosphorylated and acts as a docking site for 3 transcription factors of the STAT family, STAT-1, -3 and -5, which become phosphorylated and migrate to the nucleus, where they activate the transcription of a number of genes. This pathway is common to many cytokines but is often dispensable for their biological activities. For IL-9, our group demonstrated that activation of the STAT transcription factors is crucial for all the effects of IL-9 studied on various cell lines, including positive and negative regulation of cell proliferation, as well as inhibition of corticoid-induced apoptosis in T cell lymphomas. Further analysis demonstrated that STAT-1, -3 and -5 play specific, redundant and synergistic roles in the different activities of IL-9 in vitro (3).

The pathways responsible for IL-9-induced proliferation were studied in details, and this process was found to depend mainly on the ac-

tivation of STAT-5, on the recruitment of the IRS-1 adaptor, and on the activation of the Erk MAP-Kinase pathway.

The signal transduction pathway downstream the IL-9 receptor is illustrated in Fig. 1.

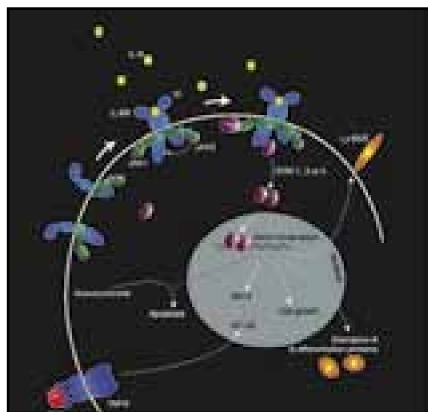

Figure 1. IL-9 receptor signalling.

ANTI-APOPTOTIC ACTIVITY OF I-309RECEPTOR AND SIGNAL TRANSDUCTION

J.-C. Renauld, A. Tounsi, J. Van Snick

Incidentally, our studies of this particular model of the regulation of cell death by cytokines, lead them to purify another protein called I-309, originally described as a human chemotactic factor, and that turned out to exert a significant anti-apoptotic activity for thymic lymphomas (4). However, I-309 and IL-9 trigger completely different pathways and it was shown that the I-309 anti-apoptotic activity was dependent on the activation of G-proteins and the Ras/MAPKinase pathway, whereas the IL-9-mediated effect was not. More recently, we showed that a viral protein related to human chemotactic factors (vMIP-I), and isolated from Herpes viruses that induce T cell tumors, has the same anti-apoptotic activity by binding to the I-309 receptor.

ROLE OF JAK OVEREXPRESSION IN TUMOR CELL TRANSFORMATION

J.-C. Renauld, L. Knoops, T. Hornakova, M. Stevens

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 but only TYK2-overexpressing cell lines showed a constitutive activation of STAT3. Thus, JAK overexpression can be considered as one of the oncogenic events leading to the constitutive activation of the JAK-STAT pathway (5).

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

L. Dumoutier, M. de Heusch, J.-C. Renauld

Searches for genes specifically regulated by IL-9 in lymphomas lead to the cloning of

a 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. Recombinant human IL-22 was produced (with D. Colau, LICR) and its crystallograhic structure solved. 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 (6) 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.

Analysis of genomic databases lead to the identification of a new receptor belonging to the IL-10 receptor family (7). This gene is located in the chromosome 6q24, at 24 kb from the IFNGR1 gene and at 152 kb from the IL-20R. It encodes a protein of 231 amino acid, showing 33 % and 34 % amino acid identity with the extracellular domains of the IL-22R and the IL-20R, respectively, but no cytoplasmic nor transmembrane domains were found. IL-22BP is highly expressed in the placenta, in the breast, in the mammary gland and in the skin. A specific interaction was demonstrated between insolubilized IL-22 and an IL-22BP-Ig fusion protein. Moreover, recombinant IL-22BP could block IL-22 biological activity demonstrating that this protein can act as an IL-22 antagonist.

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 (6). 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 feature 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 (Dumoutier et al., submitted for publication).

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-20R ß, 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 (8). In addition, IL-20 and IL-24 can also bind to other complexes consisting of IL-20Rα and IL-20Rß. This promiscuity in cytokine receptor usage is illustrated in Fig 2 (see also ref. 9 for a review of this cytokine family).

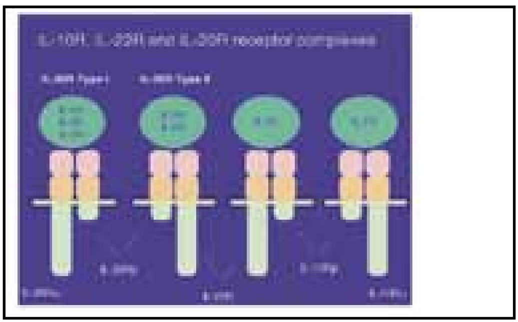

Figure 2. Receptor complexes for IL-10, IL-20 and IL-22

LICR2: A NEW CYTOKINE RECEPTOR MEDIATING ANTIVIRAL ACTIVITIES

J.-C. Renauld, L. Dumoutier

Type II cytokine receptors include receptors for type I and Ii interferons (IFNs) and for IL-10-related cytokines. These transmembrane proteins are almost exclusively related by their extracellular part, which consists of tandem fibronectin type II domains, whereas the cytoplasmic domain is associated with a tyrosine kinase of the Janus Kinase (JAK family). By screening genomic databases for similarity with the extracellular domain of these receptors, we identified a new receptor that we called LICR2 (Likely Interleukin or Cytokine rceptor 2). This receptor binds new cytokines designated IFN-λ1-3, and mediates the same activities as those mediated by the receptors for IFN-α and ß, including antiviral and antiproliferative activities (10), raising the possibility of therapeutic applications in viral infections and cancer.

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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 or Primary Myelofibrosis.

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

A. Dusa, C. Pecquet, J.-M. Heine

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 and become independent of erythropoietin (Epo) for proli-

feration and differentiation. Strikingly, the traffic of TpoR is defective in myeloid progenitors from PV. A hint that JAK2 or a JAK2-binding protein 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

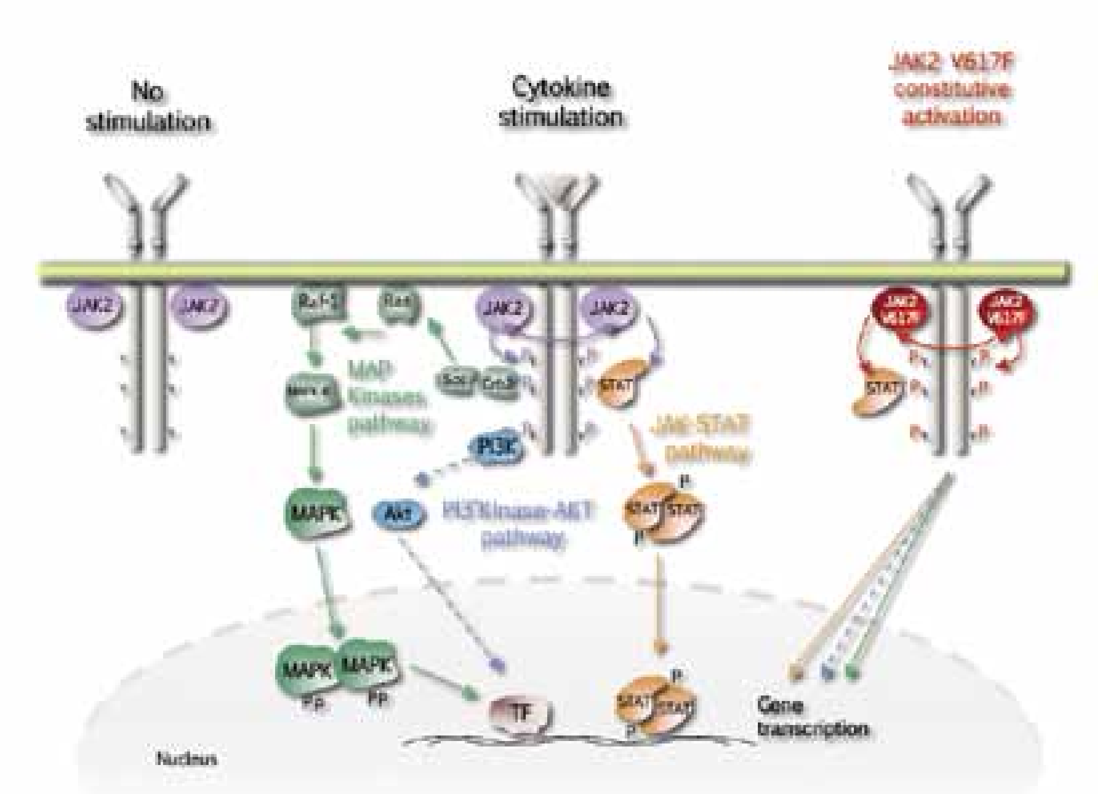

Figure 1. In the absence of cytokine ligands, cytokine receptors (left complex) are preassembled with tyrosine kinases JAK (Janus kinases) in inactive complexes. Cytokine binding to the extracellular domains of receptors (middle complex) induces a conformational change which allows the appended JAKs 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 and Leu can activate JAK1 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.-M. Heine, 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. Further stimulation with Tpo of the delta5TpoR leads to an excess of immature erythroid progenitors at the expense of megakaryocyte differentiation (6). In vivo, in reconstituted mice, the delta5TpoR induces massive expansion of platelets, neutrophils and immature erythroid progenitors and eventually myelofibrosis (Pecquet et al., in preparation). Within the KWQFP motif (RWQFP in the human), the key residues that maintain the receptor inactive are the K/R and W residues; mutation of either of the two residues to alanine activates the receptor. We predicted that such mutations 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.

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. Augustin Ferrant) and Clinical Biology (Prof. Dominique Latinne) a large study on the presence 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

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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 (7).

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 (8). Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (one), d (four), the register of the coiled-coil α-helices is imposed on the downstream TM α-helix and intracellular domain.

When each of the seven possible dimeric orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR, only two fusion proteins stimulated the proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells (8). Since the predicted dimeric interfaces of the two active fusion proteins are very close, a unique dimeric EpoR conforma-

tion 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.

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

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We have previously shown that the EpoR exists on the cell surface as a preformed ligand-independent inactive dimer stabilized by transmembrane domain interactions. To explore the details of inactive and active dimeric interfaces of cytokine receptors we undertake two approaches: (i) cysteine scanning mutagenesis of the extracellular juxtamembrane and TM domains; (ii) cell surface immunofluorecence co-patching. We isolated three constitutively active

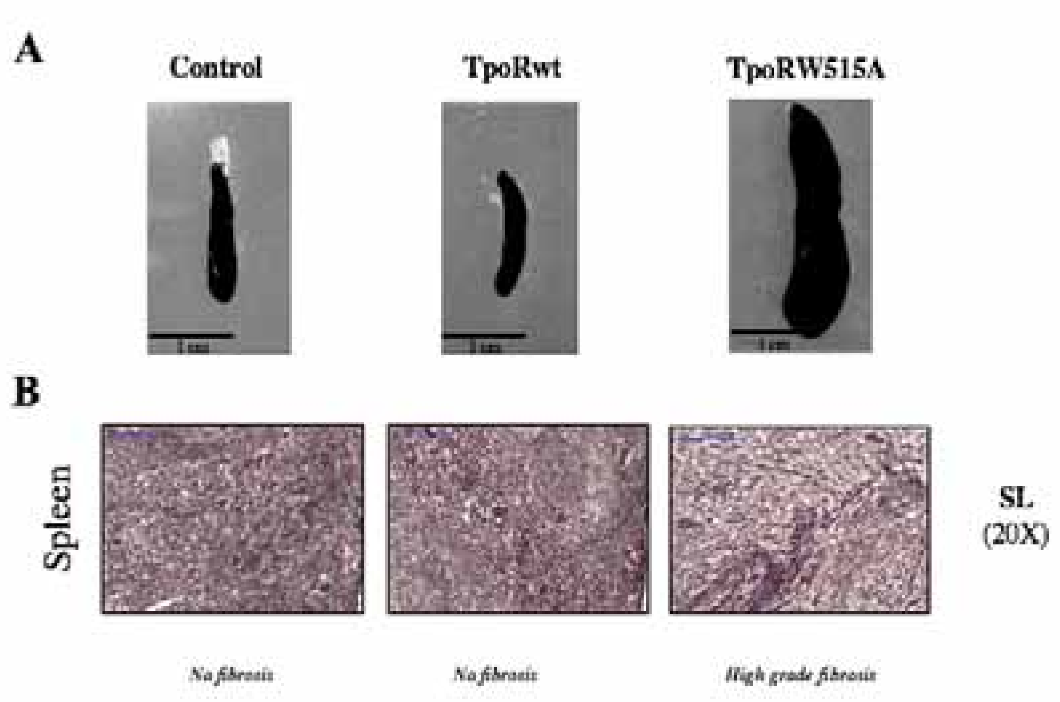

Figure 2. Bone marrow adoptive transfer in irradiated mice with hematopoietic stem cells expressing the constitutively active TpoR W515A induces severe myeloproliferative disorder, splenomegaly and fibrosis of the spleen. (A) Splenomegaly was induced by the TpoR W515A mutant at day 45 after reconstitution in contrast to the wild type TpoR or empty vector control. All mice presented >80% GFP positive cells, indicating expression of the bicistronic retroviral construct. (B) Histology of spleen sections of mice reconstituted with the indicated constructs. Silver staining (SL) for reticulin indicates extensive fibrosis of the spleen in the TpoR W515A mice. (Christian Pecquet)

novel mutants of the EpoR where residues L223, L226 or I227 were mutated to cysteine (9). Many other cysteine mutants formed disulfide bonds, but only EpoR L223C, L226C and I227C dimerized in a conformation compatible with signaling. Cysteine-mediated maleimidyl crosslinking indicated that the first five TM residues are not helical and that the interface of the active EpoR dimer contains residues L241 and L244. These studies led to the notion that sequences flanking the transmembrane domain might play important roles in receptor function as «switch» regions and also may regulate transmembrane protein oligomerization.

More recently, using copatching assays, we have identified preformed homomeric complexes of IL2Rβ, IL9Rα and γ-chain, and heteromeric IL2Rβ- γ-chain and IL9Rα- γ-chain complexes (10). Such ligand-independent complexes might be important for the pre-assembly of signaling complexes at the cell surface. Furthermore, signaling by a constitutive active JAK3 mutant depends on preformed heteromeric complexes that contain gamma chain (10).

TRAFFIC OF CYTOKINE RECEPTORS TO THE CELL-SURFACE

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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. We are testing this hypothesis on several different cytokine receptors and are investigating the link between proper folding in the ER and transport to the cell-surface. Using a fetal liver retroviral cDNA library cloned in pMX-IRES-CD2, we are attempting to clone novel proteins that can regulate traffic and stability of TpoR.

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 receptors or of JAKs (JAK2 V617F) transmit a continuous signal which results in constitutive activation of STAT proteins. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors or JAKs in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously (1). A similar picture has been noted in patient-derived leukemia cells. The critical questions we would like to answer concern: (i) the mechanisms by which the JAK-STAT pathway remains permanently activated in transformed cells; (ii) 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 can also bind to low affinity 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 use a modified version of the chromatin immunoprecipitation assay pioneered by Alex Varshavsky in conjunction with DNA microarray genomic profiling. One specific target gene of constitutive active signaling in megakaryocytes is the host gene for miR-28, a microRNA we identified to be pathologically induced in 30% of MPNs. Tagets of miR-28, such as E2F6, are critical cell cycle regulators that might influence the phenotype of myeloproliferative disorders, thus linking spcific gene induction by constitutive STAT signaling to phenotype of disease.

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