Research at

the de Duve Institute

and at

the Brussels Branch of

the Ludwig Institute for Cancer Research (LICR)

August 2007

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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 of the Institute.

In 1978 the Ludwig Institute for Cancer Research decided to base its Belgian branch within the walls of the de Duve Institute. A fruitful collaboration between the two Institutions has been pursued since that time. Even though the two Institutes are completely independent, the collaboration between the scientists of the de Duve Institute and the Ludwig Institute is extremely close and the sharing of resources is considerable.

The de Duve Institute is managed by a directorate of three scientists, presently composed of Emile Van Schaftingen, Benoît Van den Eynde, and Miikka Vikkula. The directorate is appointed by the Board of directors, which comprises the Rector of the University of Louvain, one of the Pro-rectors, the General Administrator of the University and the Dean of the Faculty of Medicine. Also present in the Board of directors are eminent members of the business community.

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

Miikka Vikkula

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ACKNOWLEDGMENTS

In 2006, 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 nearly tripled during the past decade over levels achieved previously and now supports 6 % of the Institute's budget.

The appeal for sponsoring postdoctoral fellowships was also widely followed. In 2006 the Institute has been able to allocate the following fellowships, entirely supported by our donors :

the «Haas-Teichen» fellowship to Susan COORT

the «Pierre Lacroix» fellowship to Nisha LIMAYE

the «Umicore» fellowship to Artur CORDEIRO

Two fellowships were awarded by the Institute to Mariana IGOILLO and Christian PECQUET

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.

Jean PETERBROECK,
President of the Development and Expansion Council

PROTEIN REPAIR AND INBORN ERRORS OF METABOLISM

Emile VAN SCHAFTINGEN, Member
Maria VEIGA-da-CUNHA, Associate Member
Thierry de BARSY, Emeritus Member

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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 [3], an enzyme which proved to be a repair enzyme permitting the removal of sugar adducts from proteins (deglycation). More recent work has led us to identify additional enzymes that are potentially also involved in deglycation, but if and why protein deglycation is important is still an open question. Our laboratory aims also at identifying the enzymatic defects underlying 'new' inborn errors of metabolism.

Protein deglycation

Y. Achouri, T. Sokolova, J. Fortpied, R. Gemayel, A. Preumont, K. Peel, M. Veiga-da-Cunha, E. Van Schaftingen in collaboration with M.H. Rider and D. Vertommen, Horm Unit

Fructosamine 3-kinase

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 yet 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 [3] that phosphorylates both low-molecular-weight and protein-bound fructosamines. Fructosamine 3-phosphates are unstable, breaking down spontaneously to 3-deoxyglucosone, inorganic phosphate and the amino compound that originally reacted with glucose (Fig. 1).

The role of FN3K as a protein-repair enzyme is studied on a mouse knock-out model obtained by targeted gene inactivation of the FN3K gene. The level of haemoglobin-bound fructosamines is about 2.5-fold higher in FN3K$^{-/-}$ mice than in control (Fn3k$^{+/+}$) or

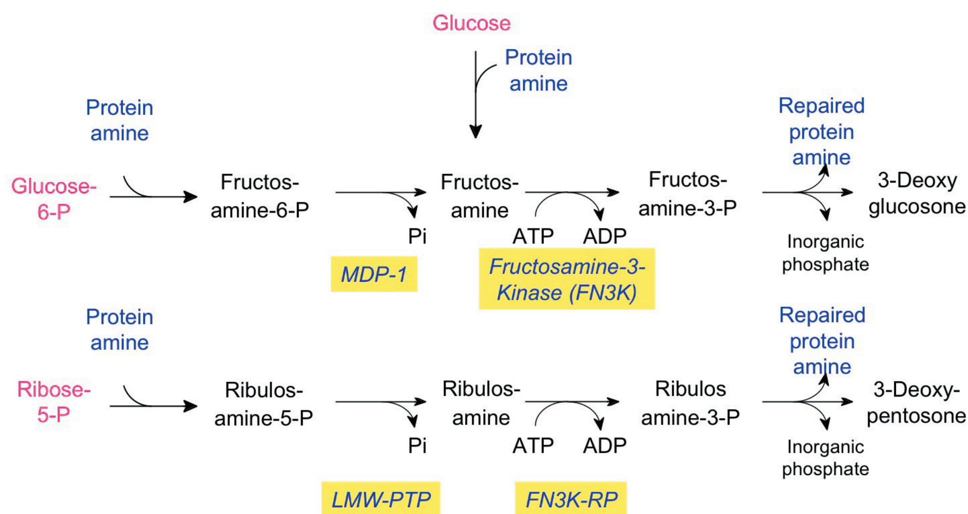

Figure 1. Role of fructosamine 3-kinase and other enzymes in the deglycation of proteins

$Fn3k^{+/-}$ mice [10]. Other cytosolic proteins (FN3K is a cytosolic enzyme) are also significantly more glycated in FN3K-deficient mice than in control mice and this applies to all investigated tissues. These findings indicate that FN3K is indeed able to remove fructosamines from proteins in vivo, though it is unable to remove all of them. In vitro studies on purified proteins indicate that this is due to a lack of accessibility of this enzyme to 'buried' fructosamines. As $FN3K^{-/-}$ mice are apparently healthy, we still need to understand why it is important to repair protein glycation. One of our aims is to identify physiological or biochemical processes that are affected by FN3K deficiency.

An intriguing observation made in these studies is that the glycation level of cytosolic muscle proteins is only slightly lower than that found in liver and erythrocytes despite a 100-fold lower cytosolic concentration of glucose. The explanation for this paradoxical observation is that fructosamines may also be formed from glucose 6-phosphate, which is more abundant than glucose in muscle cytosol and which is intrinsically 5-fold more reactive than glucose. We have identified an enzyme that dephosphorylates fructosamine 6-phosphates to fructosamines [5]. Our hypothesis is that this enzyme, for which protein-bound fructosamine 6-phosphates appear to be the best substrates, teams up with FN3K to free proteins from the glycation products that are formed from glucose 6-phosphate.

Fructosamine-3-kinase related protein

Fructosamine-3-kinase-related protein (FN3K-RP) is an enzyme that shares about 65 % sequence identity with FN3K and is encoded by a gene that neighbours the FN3K gene on human chromosome 17 [2]. Intriguingly, FN3K-RP does not phosphorylate fructosamines, but well ribulosamines and erythrulosamines. The ribulosamines 3-phosphates and erythrulosamine 3-phosphates that are so formed are unstable (even more so than fructosamine 3-phosphates) and their spontaneous breakdown also leads to the regeneration of a free amino group. Remarkably, plant and bacterial homologues of FN3K also phosphorylate ribulosamines and erythrulosamines, but not fructosamines indicating that functional homologues of FN3K-RP are more widely distributed then functional homologues of FN3K.

How are the substrates of FN3K-RP found? It is unlikely that they arise through a

reaction of amines with free ribose or erythrose, because these sugars are present at very low concentration (< 10 μM) in tissues. Our present hypothesis is that substrates of FN3K-RP are 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. This view is consistent with the observation that FN3K-RP, which is rather evenly distributed in mammalian tissues, is poorly active in skeletal muscle, a tissue known for its particularly low pentose phosphate cycle activity.

The ribulosamine 5-phosphates 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. A phosphatase that catalyses the dephosphorylation of ribulosamine 5-phosphates has recently been purified from human erythrocytes and identified as 'low-molecular weight tyrosine phosphatase A' (LMW-PTP-A) [4]. The ribulosamine-5-phosphatase activity of LMW-PTP-A was found to be higher than its protein-tyrosine-phosphatase activity. Furthermore, several bacterial genomes contain an operon encoding both a FN3K homologue and a LMW-PTP homologue, suggesting that this type of phosphatase is involved in the repair of ribulosamine 5-phosphates and/or erythrulosamine 4-phosphates. The mechanism of formation of the substrates for FN3K-RP is one of the main questions we are presently trying to answer.

As a 'side-product' of our studies on the metabolism of fructosamines in eukaryotes, we have also identified several enzymes that metabolise glycation products in bacteria. These enzymes allow the metabolism of glycation products formed from different hexoses, namely D-glucose, D-fructose and D-allose, and from different amino acids. Typically, the glycation products are phosphorylated on the sixth carbon of their sugar moiety by an ATP-dependent kinase or a 'PTS' (phospho-transfer system, which uses phosphoenolpyruvate as phosphoryl donor) and the 6-phosphoderivative is then used by an enzyme catalysing an Amadori rearrangement. As only few bacteria possess the enzymatic arsenal to metabolise these compounds, glycation products offer 'nutrient niches' for selected bacteria.

Disorders of L-serine biosynthesis

Y. Achouri, E. Wiame, E. Van Schaftingen

L-Serine, one of the twenty 'standard' amino acids, is important not only for protein synthesis, but also for the formation of several other biomolecules including phospholipids, sphingolipids, nucleotides, and two other amino acids, glycine and cysteine. L-serine is a 'non-essential' amino acid, which means that humans and other mammals have the enzymatic equipment necessary for its synthesis, i.e., a three step pathway branching from glycolysis at the level of 3-phosphoglycerate (Fig. 2). Although substantial amounts of serine are present in the food or result from the endogenous degradation of proteins, it is now clear that *de novo* serine biosynthesis is essential for the proper development of the brain. This conclusion derives from the identification of serine deficiency disorders.

About ten years ago, our group identified, in collaboration with Prof. J. Jaeken (KULeuven), 3-phosphoglycerate dehydrogenase deficiency in patients with a severe neurological disorder. Patients with this deficiency have been treated with supplemental serine and glycine with varying degrees of success. Phosphoserine phosphatase deficiency has also been identified in one patient.

We have recently identified the first cases of phosphoserine aminotransferase deficiency (in collaboration with colleagues in Newcastle and Leuven) [6]. This new disorder of serine biosynthesis has been discovered in two siblings showing low concentrations of serine and glycine in plasma and CSF. The index case

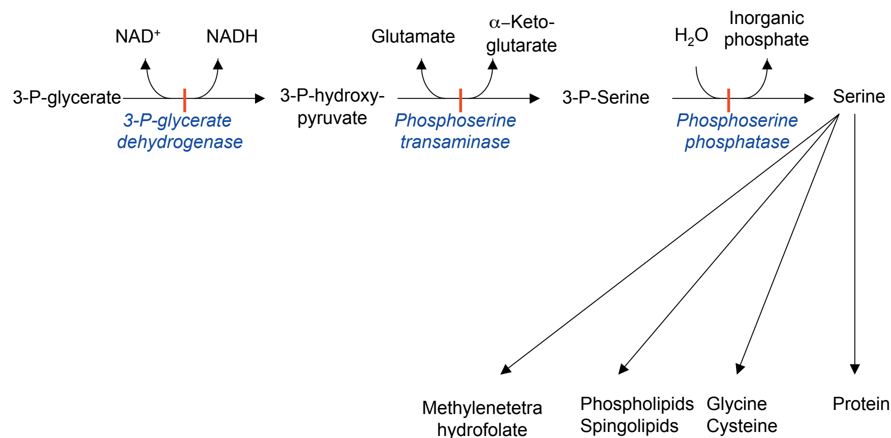

Figure 2. The pathway of serine biosynthesis and its defects.

presented with intractable seizures, acquired microcephaly, hypertonia and psychomotor retardation, and died at seven months of age despite supplementation with serine and glycine from 11 weeks of age. The younger sibling was treated from birth leading to a normal outcome at 3 years of age. Mutational analysis of the three genes encoding the enzymes of serine biosynthesis revealed compound heterozygosity for two mutations in the phosphoserine aminotransferase gene. One mutation is frameshifting whereas the other is a point mutation that result in a marked decrease in the enzymatic activity. These findings indicate that phosphoserine aminotransferase deficiency is a new form of serine deficiency that can be treated successfully if the diagnosis is made soon after birth.

2-Hydroxyglutaric acidurias

Y. Achouri, T. Kardon, R. Rzem, G. Connerotte, G. Noël, Th. De Barsy, M. Veiga-da-Cunha, E. Van Schaftingen

D- and L-2-hydroxyglutaric acidurias are distinct neurometabolic diseases characterized by the accumulation of abnormal amounts of either D- or L-2-hydroxyglutarate in cerebrospinal fluid, blood and urine. Work in our lab has led to the elucidation of the metabolism of these compounds (Fig. 3). 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 responsible for L-2-hydroxyglutaric aciduria [9]. 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. It is mutated in a significant fraction of the patients with D-2-hydroxyglutaric aciduria. One of our aims is to identify the other cause(s) of D-2-hydroxyglutaric aciduria.

Formation of D-2-hydroxyglutarate is catalysed by an enzyme that metabolizes 4-hydroxybutyrate (which is produced endogenously but is also a drug- and an abuse drug). Unlike other alcohol dehydrogenases this enzyme does not tranfer electrons onto free NAD or NADP, but onto alpha-ketoglutarate (Kaufman et al. 1988). We recently cloned this enzyme and showed that it corresponds to a protein designated as 'iron-dependent alcohol dehydrogenase', the enzyme that metabolizes 4-hydroxy butyrate [7]. This enzyme comprises binding sites for NAD (which is presumably tightly bound, serving as an intermediary electron acceptor) and for a divalent cation. The cloning of this enzyme opens perspectives for the study of the mechanism of action of 4-hydroxybutyrate.

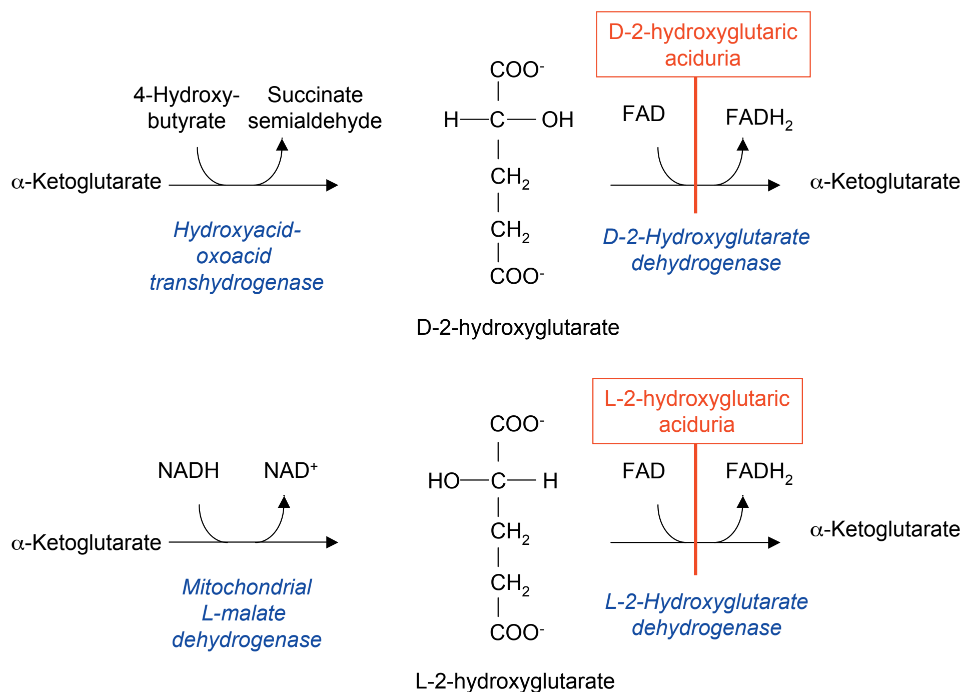

Figure 3. Formation and degradation of D- and L-2-hydroxyglutarate and metabolic defects in 2-hydroxyglutaric acidurias. See Text.

Formation of L-2-hydroxyglutarate appears to be catalysed by mitochondrial L-malate dehydrogenase. This enzyme is not entirely specific for oxaloacetate, since it can also reduce alpha-ketoglutarate with a 10^7 lower catalytic efficiency. Though very low, this activity is sufficient to account for the daily formation of L-2-hydroxyglutarate. Since L-2-hydroxyglutarate does not appear to have any physiological 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.

Other inborn errors of metabolism

P. Maliekal, G. Connerotte, G. Noël, T. de Barsy, E. Van Schaftingen

Our group is also involved in the enzymatic diagnosis of glycogen storage diseases and of congenital disorders of glycosylation (as a member of the Euroglycanet network). In the latter context, one of our aims is to identify enzymes in the glycosylation pathway that have not yet been molecularly identified. This led us to report recently the identification of the gene encoding N-acetylneuraminate-9-phosphate phosphatase.

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PURINE NUCLEOSIDE ANALOGUES IN LEUKAEMIA

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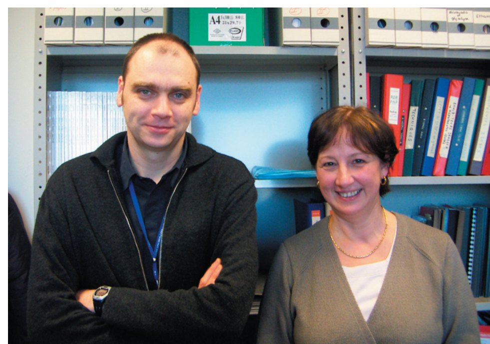

Our group had a long-standing interest in purine metabolism, particularly adenine nucleotide metabolism and its genetic defects (1). In previous work, we demonstrated that, in several tissues, the catabolism of AMP, leading to the production of uric acid and/or allantoin, proceeds only by way of AMP deaminase, a highly regulated enzyme which is normally more than 95 % inhibited. Dephosphorylation of AMP into adenosine also occurs but does not contribute to the formation of purine catabolites, owing to recycling by way of adenosine kinase. Interruption of this recycling plays a crucial role in the elevation of adenosine, a major regulatory compound, under anoxic conditions. We have also shown that a variety of rat and human tissues contain a cytosolic 5'-nucleotidase which hydrolyzes preferentially IMP and GMP, is stimulated by ATP and 2,3-bisphosphoglycerate, and inhibited by Pi. More recently, we expended our investigations on therapeutic purine nucleoside analogues. The main objective of our present studies is to find means to increase their efficacy in hematologic disesases, and particularly in B-cell chronic lymphocytic leukaemia.

In 1997, a collaborative study of the anti-leukaemic nucleoside, 2-chloro-2'-deoxy adenosine (CdA), was started with the Department of Haematology of the University Hospital Saint-Luc. This adenosine deaminase-resistant deoxyadenosine analogue displays 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 CdA does 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 CdA, and to improve its therapeutic efficacy by searching for synergisms with other compounds.

To exert its antileukaemic effect, CdA has to be phosphorylated by deoxycytidine kinase (dCK) into CdAMP, followed by conversion into CdADP and CdATP. The latter, the active metabolite of CdA, has been shown to inhibit a variety of enzymes involved in DNA synthesis, including ribonucleotide reductase and DNA polymerase α. Moreover, CdATP 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 (2, for a review).

Mechanism of action of 2-chloro-2'-deoxyadenosine

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

To improve our understanding of the mechanisms by which CdA induces apoptosis in

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 increased in EHEB cells, up to 2-fold, after a 24 h-incubation with CdA at concentrations close to the IC_{50} (10 μM) (3). 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 and provokes an accumulation of most cells in either early S phase or at the G1-S border. Kinetics and synchronisation experiments have shown that 10 μM CdA stimulates the progression of EHEB cells from G1 to S phase, rather than blocking them in 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. Inhibition of cdk2 by roscovitine prevents the stimulation of S-phase entry by CdA and also significantly reduced the activation of caspase-3 by CdA. In conclusion, we show a new mode of cellular response to CdA, implying activation of cdk2 and acceleration of S-phase entry. These effects could contribute to CdA-induced apoptosis, as suggested by the antagonism between CdA and roscovitine. Our present aims are to elucidate how CdA and possibly other nucleoside analogues activate cdk2, and by which mechanism(s) this activation contributes to apoptosis.

In addition, we have recently initiated microarray analyses to identify survival or death pathways that are activated in response to purine analogue treatment. We intend to compare genes induced or repressed by purine analogues in sensitive and refractory B-CLL patients. This study is performed in collaboration with Dr. Knoops from the Ludwig Institute at the ICP.

Search for potentiation of anti-leukaemic effect of 2-chloro-2'-deoxyadenosine

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. 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 dCK activity

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

Since 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 was not explained by an allosteric effect or by an increase of the

protein amount. 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 labeled 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 ICP allowed the identification of four *in vivo* phosphorylation sites, Thr3, Ser11, Ser15 and Ser74. Site-directed mutagenesis demonstrated that Ser74 phosphorylation was crucial for dCK activity in HEK 293T cells, whereas phosphorylation of other identified sites did not seem essential (9). Phosphorylation of Ser74 was also observed on endogenous dCK in CCRF-CEM cells and in lymphocytes from B-cell chronic lymphocytic leukemia (B-CLL) patients, in which the Ser74 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 Ser74 (10). To conclude, our work has demonstrated that dCK activity largely depends on the phosphorylation state of Ser-74 in human leukaemic lymphocytes. We plan now to identify the protein kinase(s) responsible for the phosphorylation of dCK on Ser74.

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REDOX BIOLOGY IN BACTERIA

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Oxidation of two cysteine residues leads to the formation of a disulfide bond and the concomitant release of two electrons. This oxidation reaction is harmful to most cytoplasmic proteins and may lead to protein misfolding and aggregation. Both eukaryotic and prokaryotic cells possess mechanisms to ensure that cytoplasmic cysteines are kept reduced. These mechanisms involve enzymes like thioredoxins and glutaredoxins. In contrast, formation of disulfide bonds is a required step in the folding pathway of many secreted proteins and takes place in the eukaryotic endoplasmic reticulum or the bacterial periplasm. In our group we study the mechanisms that govern disulfide bond formation and reduction in the Gram⁻ bacteria Escherichia coli.

Oxidative protein folding in the periplasm

G. Connerotte, M. Depuydt, J.-F. Collet

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

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 is composed of two soluble periplasmic proteins, DsbC and DsbG, which function as disulfide isomerase proteins. Like DsbA, DsbC and DsbG possess a thioredoxin fold and a CXXC catalytic site motif. In contrast to DsbA, the CXXC motif of DsbC and DsbG is kept reduced in the periplasm. This allows them to attack non-native disulfides, a necessary step in the isomerization reaction. The protein that keeps DsbC and DsbG 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.

The transmembrane electron transporter DsbD

The mechanism by which DsbD transports electrons across the membrane is unknown. Our goal is to examine this longstanding mystery of how reducing equivalents get across membranes.

We purified to homogeneity 3 polypeptides corresponding to the 3 domains of DsbD. Using these domains, we could reconstitute a DsbD activity and, for the first time, recons-

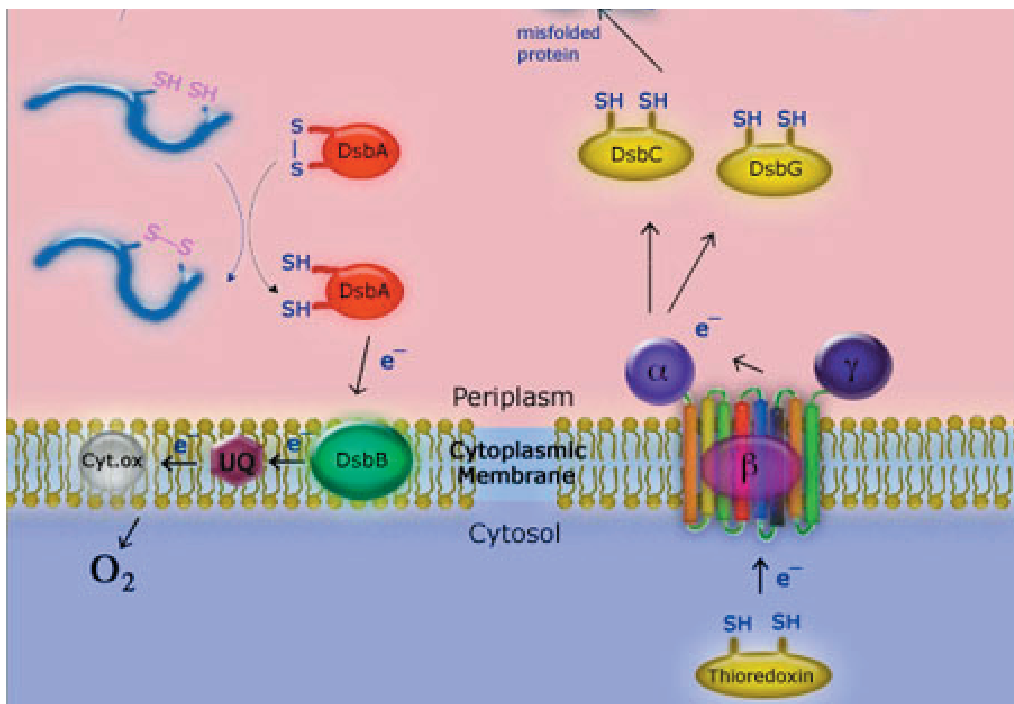

Figure 1. Disulfide bond formation in the E. coli periplasm. Disulfides are introduced into folding proteins by DsbA. DsbA is re-oxidized by DsbB, a inner-membrane protein that generates disulfides *de novo* from quinone reduction. DsbC and DsbG are protein disulfide isomerases that correct incorrect disulfides. They are recycled by DsbD, a transmembrane protein that transfers electrons from the cytoplasmic thioredoxin system to the periplasm.

titute in vitro the electron transport pathway from NADPH and thioredoxin to DsbC and DsbG. We also provided evidence that DsbD's conformation depends on its oxidation state. Using site-directed mutagenesis, we showed that four highly conserved proline residues surrounding DsbD's membrane-embedded catalytic cysteines may have an important functional role, possibly conferring conformational flexibility to DsbD [5].

In collaboration with Todd Lowther's lab (Wake Forest University), we showed that in another bacteria, *Neiserria gonorrheae*, DsbD is involved in the defense mechanisms against oxidative stress by transferring reducing equivalents to the periplasmic methionine sulfoxide reductase PilB [6].

The protein disulfide isomerase DsbC

We characterized the role of DsbC using a combination of microbiological, biochemical and proteomics approach. We found that *E. coli* requires DsbC for growth under certain oxidative stress conditions [3]. We also showed that DsbC is able to cooperate with DsbA in a DsbD independent manner to fold *E. coli* envelope proteins [7]. We found that the simultaneous absence of DsbA and DsbC has severe consequences on *E. coli* viability and outer-membrane integrity. *dsbAdsbC* mutants have a number of phenotypes not exhibited by either *dsbA*, *dsbC* or *dsbAdsbD* mutants: they exhibit an increased permeability of the outer-membrane, are resistant to the lambdoid-phage Φ80, and are unable to assemble the maltoporin LamB. In collaboration with D. Vertommen (HORM unit), we developed a 2D-LC-MS/MS proteomics approach that allowed us to estimate the abundance of ≈ 130 secreted proteins in various *dsb*⁻ strains. We observed that the absence of DsbA and DsbC affects the global protein content of the periplasm and leads to a decreased abundance of several cysteine-containing proteins. These changes are not observed in the periplasm of a *dsbAdsbD* mutant. Our data indicate therefore that DsbC's

function is not limited to the framework of the disulfide isomerization pathway. On the basis of our results, we have proposed a new model for the oxidative protein folding pathways in *E. coli*.

2D-LC-MS/MS data also highlighted dramatic changes that occur in all strains impaired in disulfide formation, including the induction of several proteins known to be induced under periplasmic stress conditions and other uncharacterized proteins. We also identified new DsbA and DsbC substrates. The formation of a disulfide bond in a select group of these substrates was confirmed by combining 2D-LC-MS/MS and thiol-trapping.

Outer-membrane biogenesis

This past year, we 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. 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.

Recently, two protein complexes have been identified in *E. coli*. The Imp complex appears to be involved in lipopolysaccharides insertion whereas the YaeT complex appears to play a role in the insertion of β-barrel proteins. However, we do not know how these complexes work, what are the roles of the various components and whether other proteins are involved. We have started to study the mechanisms of these complexes and the role of their various components using 2D-LC-MS/MS. We have adapted the technique developed to study the periplasmic proteome of *dsb*⁻ strains. The first results are encouraging. They allowed us to identify 73% of the known OM proteins and to determine what are the OM proteins that depend on the YaeT complex for insertion.

Oxidative stress in the *E. coli* cytoplasm

C. Bouillot, J.-F. Collet, H. El Hajjaji

In *E. coli*, numerous proteins are involved in the defense mechanisms against oxidative stress. Proteins like catalases and peroxiredoxins are on the front lines and directly react with harmful reactive oxygen species (ROS) to convert them to innocuous products. Other proteins, like thioredoxins, supply the primary ROS scavengers with the reducing equivalents they need to fight the redox battle and also reduce aminoacids modified by the ROS.

Thioredoxins are small redox proteins present in many eukaryotic and prokaryotic genomes. They share a similar 3-dimensional structure and possess a conserved WCGPC catalytic motif. They reduce disulfide bonds formed between cysteine residues as a result of either a catalytic activity or oxidative stress. In the course of the reaction, the two catalytic cysteine residues of the WCGPC motif are oxidized. They are then converted back to the reduced form by thioredoxin reductase, a NADPH-dependent protein.

Two thioredoxins have been described in *E. coli*. The first one, Trx1, is a 12 kDa protein that has been identified 40 years ago and is well characterized. The second thioredoxin, Trx2, coded by the gene *trxC*, has been discovered more recently. The sequence of this 15 kDa protein presents 28 % of identity with Trx1. Trx2 has two striking characteristics that differentiate it from Trx1 and suggest that it has a specific role to play:-1- It has been reported that the expression of Trx2 is under OxyR control, a transcription factor that induces the expression of several antioxidant genes. -2- Trx2

contains an additional amino-terminal domain of 32 residues in which two CXXC motifs are present. We have shown that these additional cysteine residues tightly bind zinc. Upon oxidation with hydrogen peroxide in vitro, the zinc is released, which triggers a conformational change. Our goal is to find the function of this unique zinc binding thioredoxin. We have characterized Trx2 in vitro. We determined the redox potential of the catalytic CXXC motif and showed that Trx2 is less reducing than Trx1. We also determined the pKa of the catalytic cysteine residues. The zinc domain does not influence the redox properties of the protein. We also measured the stability of the protein. We found that Trx2 is less stable than Trx1. A truncated form of Trx2 lacking the zinc domain is significantly less stable than the wild-type protein, which suggests that the zinc domain is involved in the stability of this protein. Using western blots, we found that Trx2 forms high molecular weight complexes under oxidative stress conditions. The characterization of these complexes is under way.

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TISSUE-SPECIFIC TRANSCRIPTION FACTORS IN DEVELOPMENT

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Vivien O'CONNOR, Secretary (half-time)

A number of human diseases are characterized by malformations or functional deficiencies in the liver or pancreas. The aim of the research in our group is to characterize the molecular mechanisms that control development of the liver and pancreas. This will help to understand such diseases and to treat them by pharmacological or cell therapy-based approaches. The present focus is on the study of the role of the Onecut transcription factors, discovered in our laboratory. They define a class of conserved homeoproteins, with three members in mammals : HNF-6 or OC-1, OC-2 and OC-3 (1). The role of the Onecut factors in development has been addressed by studying knockout mice generated in our laboratory.

Control of liver development by Onecut factors

A. Antoniou, J.-B. Beaudry, F. Clotman, S. Margagliotti, C. Pierreux, P. Raynaud, I. Laudadio.

During liver development, hepatoblasts (the liver precursor cells), differentiate into hepatocytes, which exert the metabolic functions of the liver, and into biliary cells which delineate the bile ducts. We have found that HNF-6 and OC-2 control the segregation of the hepatocytic and biliary lineages in the developing liver (2). Indeed, in mice knockout for both HNF-6 and OC-2, the hepatoblasts fail to differentiate properly into hepatocytes or biliary cells. Instead, the hepatoblasts give rise to hybrid 'hepato-biliary' cells which display characteristics of hepatocytes and biliary cells.

We have shown that the Transforming Growth Factor β (TGFβ) is required in liver for differentiation of biliary cells. TGFβ 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 (2). We have also found that Onecut factors control the gradient of TGFβ signaling (2), and this led to the conclusion that hepatic cell differentiation is controlled by Onecut factors via a modulation of TGFβ signaling.

In mice knockout for Hnf6 and for Oc2, biliary differentiation occurs but the bile ducts are abnormal (Fig. 1). The mice suffer from cholestasis and display a phenotype that resembles human biliary diseases called «ductal plate malformations», which are related to biliary atresia (3). The *Hnf6* knockout mice also lack hepatic artery branches, an anomaly which can aldo be found associated with human biliary diseases (4). Therefore, our findings indicate that HNF-6 is essential for bile duct development.

HNF-6 also controls the maturation of hepatocytes, i.e. it controls the time-specific expression of genes in hepatocytes during development. We found that the concentration of HNF-6 in liver progressively rises during liver development, allowing HNF-6 to reach threshold concentration levels required for interaction with co-activators and for activating gene expression at specific time-points. This work revealed an unexpected biological mechanism whereby the expression level of a transcription factor is critical to determine timely expression of genes during development (5).

We are currently investigating the pathogeny of human congenital malformations of the liver, as well as the role of HNF-6, OC-2, and TGFβ signaling in hepatic development.

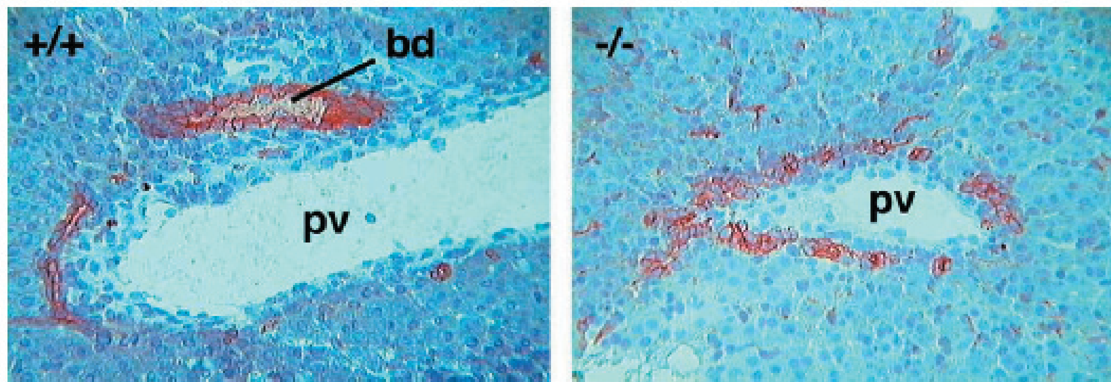

Figure 1. Abnormal development of the intrahepatic bile ducts in HNF-6 knockout mice. Immunohistochemistry of liver sections 10 days after birth shows in normal mice (left panel) a typical bile duct (bd) delineated by biliary cells (stained red) embedded in mesenchyme. In contrast, biliary cells do not form bile ducts in *Hnf6*-/- mice (right panel) and remain dispersed as a layer around a branch of the portal vein (pv).

Control of endocrine pancreas development by Onecut factors

E. Heinen, A. Simion

During pancreas development, cells lining the primitive gut (endoderm) start to express pancreas-specific genes. These pluripotent pancreatic progenitors, then proliferate and differentiate into precursors of the pancreatic endocrine, exocrine or ductal cells, and so contribute to the formation of the pancreas.

We found that HNF-6 is required for differentiation of endocrine precursors, since the pancreas of *Hnf6* knockout mice is devoid of islets of Langerhans (Fig. 2). HNF-6 controls endocrine development by stimulating the expression of the transcription factor Ngn-3, a factor which is essential for development of endocrine cell precursors (6). Moreover, the analysis of mice knockout for both HNF-6 and OC-2 indicates that both factors redundantly control Ngn-3 expression. The Onecut factor OC-3 is not involved in endocrine pancreas development, but is expressed in endocrine cells of the gut (7).

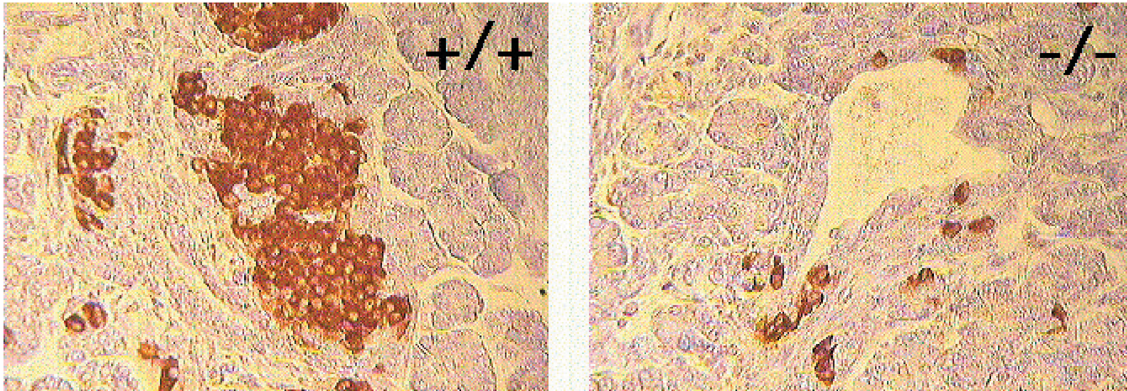

Figure 2. Abnormal development of the endocrine pancreas in HNF-6 knockout mice. Immunohistochemistry of tissue sections four days after birth shows that only a few insulin-producing cells (brown) are found near pancreatic ducts in *Hnf6* knockout mice (-/- ; right panel), instead of organized endocrine islets as in wild-type mice (+/+ ; left panel).

Control of pancreatic duct development by Onecut factors

C. Pierreux

We found that HNF-6 is required for development of the pancreatic ducts (8). Indeed, in *Hnf6* knockout mice the ducts are abnormal and form cysts (Fig. 3). This is associated with an absence of primary cilia at the apical pole of the ductal cells and with reduced expression of fibrocystin and cystin, two proteins known to control the function and the formation of cilia. Patients deficient in these proteins suffer from polycystic kidney disease, a hereditary disease associated with cysts in kidneys, liver and pancreas. This work is the first to identify a transcription factor that controls pancreatic duct development. It also characterizes HNF-6 as a regulator of cilium formation.

We are currently investigating the mesenchymal signals and the signal transduction pathways which regulate the development of pancreatic ducts.

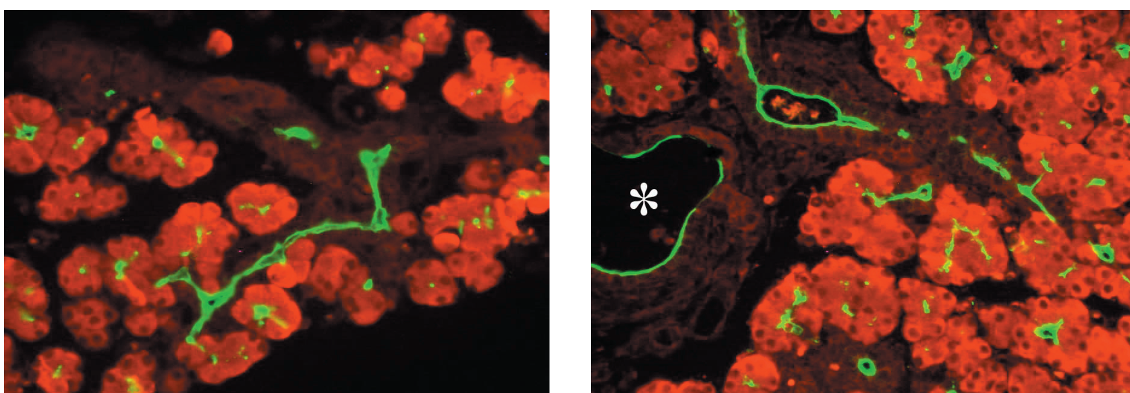

Figure 3. Formation of cysts in the pancreatic ducts in *Hnf6* knockout mice. Immunofluorescence analysis of tissue sections two days before birth shows that specific segments of the pancreatic ducts develop cysts (asterisks). The ducts are stained with an anti-Mucin-1 antibody (green) and the exocrine acini are stained with an anti-carboxypeptidase A antibody (red). Left panel, wild-type pancreas (+/+); right panel, *Hnf6* knockout pancreas (-/-).

Control of endoderm development by Onecut factors

C. Pierreux, A. Simion

The endoderm is an embryonic cell layer that lines the primitive gut in early embryos and gives rise to the liver and pancreas. To understand how liver and pancreas development is initiated, one needs to characterize the transcription factor network involved, and how it is controlled by extracellular signals secreted by neighboring tissues.

The transcription factor network was analyzed by studying the factors that are regulated by HNF-6 in the endoderm, as well as those that control HNF-6 expression. We have investigated the genes regulated by HNF-6 in the endoderm by studying the gene expression profile in *Hnf6* knockout embryos. We showed that HNF-6 controls expression of Pdx-1, a factor critical for pancreas development (8). This project also benefited from a new technique set up in our laboratory, with which a whole embryo is cultured for 24 h after electroporation-mediated gene transfer in the endoderm. This approach allowed us to demonstrate that HNF-6 controls expression of OC-3 in the endoderm.

We have also studied the *Hnf6* gene regulatory sequences that direct expression of HNF-6 in the endoderm (9). We cloned *Hnf6* sequences upstream of the β-gal reporter gene and identified the gene regions that drive expression of HNF-6 in the endoderm and in early liver and pancreas (Fig. 4).

Furthermore, using mouse genetic models that have a defective pancreatic mesenchyme or that lack expression of Fibroblast Growth Factor-10 (FGF-10), we showed that FGF-10 secretion by the mesenchyme is essential for pancreas development and for expression of pancreas-specific transcription factors (10).

Taken together, our data define a transcriptional cascade that regulates the initiation of pancreas development in the endoderm

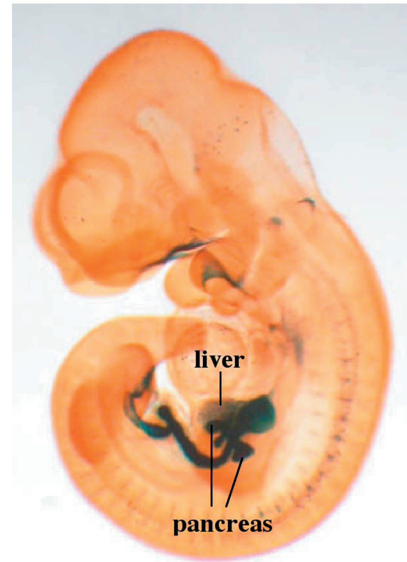

Figure 4. *Hnf6* gene regions drive expression of beta-galactosidase in liver and pancreas of transgenic embryos at embryonic day 10.5.

Conclusions

Our work led to the discovery of the Onecut transcription factors. The characterization of their molecular mode of action, and of their role in development of the endoderm, liver and pancreas, has shed light on how transcription factors control cell differentiation and organ formation. Our findings open perspectives for understanding the pathophysiology of liver and pancreatic congenital diseases. The application of our findings to the programmed differentiation of cultured cells should help developing cell therapy of hepatic deficiencies and of pancreatic diseases such as diabetes.

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SIGNAL TRANSDUCTION AND PROTEIN PHOSPHORYLATION

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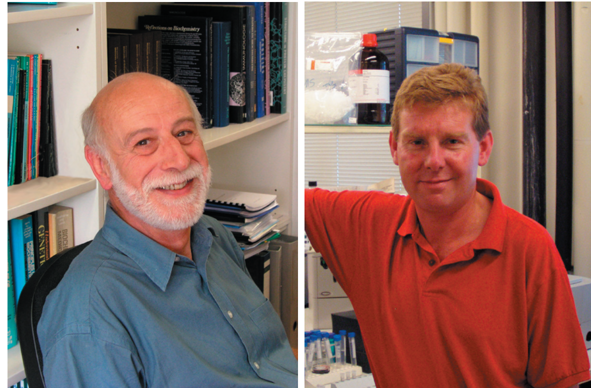
Freddy ABRASSART, Technical Staff (part-time)



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

V. Mouton, D. Vertommen, L. Hue, M.H. Rider, in collaboration with L. Bertrand, UCL, Brussels, 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. The mechanism involved in this insulin-induced activation of heart PFK-2 is being studied both *in vitro* and in intact cells. Recombinant heart PFK-2 isozyme is a substrate of several protein kinases, especially 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 the activation of PFK-2 by insulin was PDK1-dependent but did not require PKB. However, this last result was not confirmed in cells co-transfected with heart PFK-2 and PKB RNAi. Experiments with insulin-perfused hearts from PKB-β isoform knockout mice showed that this isoform was not mediating heart PFK-2 activation by insulin.

We purified a wortmannin-sensitive and insulin-stimulated protein kinase (WISK). WISK phosphorylates heart PFK-2 mainly on Ser466 leading to its activation. Our recent work indicated that WISK contains protein kinase C zeta (PKCz). However, since PKCz is not activated by insulin in heart, it is not required for insulin-induced PFK-z activation in this organ. This conclusion was confirmed by experiments

in cells transfected with a kinase-inactive PKCz construct, which failed to prevent insulin-induced PFK-2 activation (1).

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

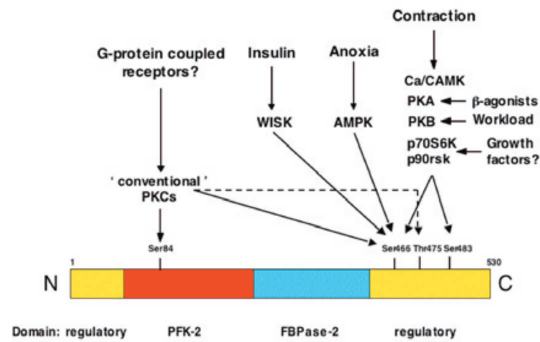


Figure 1. Protein kinases of signalling pathways that converge on heart PFK-2. The numbering of residues refers to the bovine H1 isoform (2).

AMP-activated protein kinase

S. Horman, D. Vertommen, B. Guigas, L. Miranda, L. Hue, M.H. Rider, in collaboration with D. Carling and A. Woods, London, D.G. Hardie, Dundee, P. Ferré and F. Foufelle, Paris, T. Walliman and U. Schlattner, Zurich, J. Jenssen, Oslo, E. Richter and B. Kiens, Copenhagen, K. Storey, Ottawa, X. Leverve and R. Favier, Grenoble and B. Viollet and S. Vaulont, Paris

The AMP-activated protein kinase (AMPK) acts as a sensor of cellular energy status. AMPK is activated by an increase in the AMP/ATP ratio as it occurs during hypoxia or after exposure of cells to inhibitors of the mitochondrial respiratory chain, such as oligomycin. The role of AMPK in the cell is to maintain ATP by stimulating ATP-producing pathways and by 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 anoxic rat hepatocytes or in hepatocytes treated with AICA-riboside, 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 (4,5). In skeletal muscle biopsies from exercising men, Thr56 eEF2 phosphorylation increased by more than 5-fold in < 1 min and the increase was sustained for 90 min of exercise. However, this increase in Thr56 eEF2 phosphorylation could not have been the consequence of AMPK activation, which was only significant after 10 min of exercise. Also in a model of electrical stimulation of rat epitrochlearis skeletal muscles where protein synthesis was inhibited by 75% during contraction, Thr56 eEF2 phosphorylation increased 10-fold in 1 min, but this was not related to AMPK activation. 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 (see Fig. 2). The inhibition of protein synthesis in this muscle system can be explained by an increase in eEF2 phosphorylation at the onset of contraction, probably secondary to a rise in Ca^{2+}, but does not result from a decrease in mTOR signalling.

Stress-induced activation of AMPK in the freeze-tolerant frog Rana sylvatica (6)

Freezing is one of most serious environmental stresses faced by living organisms. An

understanding of mechanisms of freeze-tolerance has obvious medical implications for cryopreservation and organ transplantation. Survival in the frozen state depends on adaptations that, amongst others, include resistance to dehydration due to the exit of cellular water into extracellular ice and long-term anoxia caused by the freezing of blood plasma. We investigated whether AMPK could play a role in metabolic re-sculpting during freezing. AMPK was activated in liver and skeletal muscle of frozen wood frogs (*Rana sylvatica*) and in the liver, this was associated with a rise in eEF2 phosphorylation. Using a panel of phosphospecific antibodies, we investigated the effect of AMPK activation on signalling downstream of mTOR (Fig. 2). In spite of AMPK activation in skeletal muscle from frozen/thawed animals, ribosomal protein S6 and 4E-BP1 phosphorylation increased dramatically, probably to increase translation of a subset of mRNAs necessary for adaptation in this condition. However, the main role of AMPK activation during freezing would be to shut off all energy consuming processes in all organs, to favour massive glycogenolysis and glucose export from the liver and to stimulate glucose uptake into nonhepatic tissues for cryoprotection.

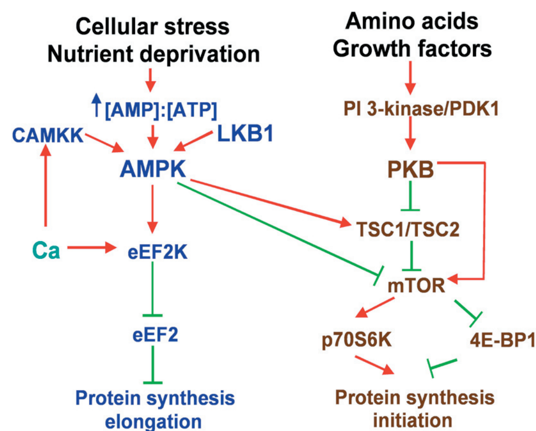


Figure 2. Cross talk between the insulin/PKB/mTOR and AMPK signalling pathways with respect to the control of protein synthesis.

Insulin antagonizes AMPK activation in heart via hierarchical phosphorylation by PKB

Previous studies showed that insulin antagonizes AMPK activation by ischaemia and that PKB might be implicated. We investigated whether the direct phosphorylation of AMPK by PKB might participate in this effect (7). PKB phosphorylated AMPK at Ser485 of the α1-subunits. In perfused rat hearts, phosphorylation of this site was increased by insulin and insulin pre-treatment decreased the phosphorylation of the activating Thr172 site in a subsequent ischaemic episode. Therefore, the effect of insulin to antagonize AMPK activation involves a hierarchical mechanism whereby Ser485 phosphorylation by PKB reduces subsequent phosphorylation of Thr172 by the upstream activating kinase (LKB1).

Mechanism for the inhibition of glucose uptake in hepatocytes by AMPK activators

In most tissues, oxygen deprivation stimulates glycolysis, a phenomenon known as the Pasteur effect. We found that this was not the case in hepatocytes from starved rats. In these cells, anoxia and other agents known to activate AMPK, inhibited glucose uptake, which, in liver, depends mainly on glucokinase activity. Surprisingly the inhibition of glucose uptake by these agents was not mediated by AMPK, because it persisted in hepatocytes from mice deficient in AMPK α1 and α2 isoforms (8). The inhibition of glucose uptake could result from a perturbation of the intracellular distribution of glucokinase. Glucokinase activation results from its translocation from the nucleus, where it is bound and inhibited by the regulatory protein, to the cytosol. This translocation is impaired under hypoxic conditions and is probably related to a decrease in the concentration of ATP, the latter resulting from an inhibition of mitochondrial respiration.

Inhibition of cell respiration by AICAR

AICA riboside, often referred to as AICAR,

has been extensively used in certain cells to activate AMPK, since it enters these cells to be phosphorylated into ZMP, an analogue of AMP. We investigated the effects of AICA riboside on mitochondrial oxidative phosphorylation in hepatocytes. AICA riboside was found to inhibit cell respiration and to decrease inorganic phosphate, ATP, AMP and total adenine nucleotide contents. This effect persisted in hepatocytes from mice lacking both isoforms of the catalytic subunits of AMPK, thus demonstrating that the AICA riboside effects were not mediated by AMPK. In *in vitro* experiments, ZMP directly inhibited the mitochondrial respiratory-chain complex 1. In addition, inhibition of respiration by AICA riboside was potentiated in cells incubated with fructose to deplete their adenine nucleotide and inorganic phosphate contents. We conclude that AICA-riboside inhibits cellular respiration by an AMPK-independent mechanism that likely results from a combination of phosphate depletion and ZMP accumulation (9). Our results also demonstrate that the effects of AICA riboside are not necessarily caused by AMPK activation and that their interpretation should be taken with caution.

Mass spectrometry

D. Vertommen, M.H. Rider in collaboration with J.-F. Collet, E. Van Schaftingen, F. Bontemps, UCL, F. Decquiedt, FUSAGX, Gembloux

We are continuing our efforts to develop new techniques for the mass spectrometric analysis of proteins. These include methods for the enrichment of phosphopeptides with a view to identify new AMPK substrates and establishing a 2D-LC/MS label-free proteomics approach for differential protein expression studies. This is currently been applied to study periplasmic oxidative protein folding and outer membrane biogenesis in *E. coli* in collaboration with Dr J-F Collet (submitted for publication). We pursue our collaboration with other laboratories within the Institute and from other universities. Along with the group of E. Van Schaftingen we identified several new enzymes. With the team of F. Bontemps we identified *in vivo* phosphorylation sites in human deoxycytidine kinase, an enzyme of the deoxyribonucleoside salvage pathway that is necessary for the activation of anti-cancer drugs (J. Biol. Chem., 2006, 281: 4887-4893). Lastly, in collaboration with the group of F. Decquiedt (FUSAGX) we identified new phosphorylation sites in class IIa histone deacetylases (HDAC) for EMK and C-TAK1, two members of the microtubule affinity-regulating kinase (MARK). Phosphorylation alters the subcellular localization and repressive function of HDAC7, and multisite hierarchical phosphorylation by a variety of kinases could allow for sophisticated regulation of class IIa HDACs function (10).

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ENDOCYTOSIS

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Marie-France VAN DEN HOVE, Member

M. Adrian CAPLANUSI, Visiting Scientist
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Francisca N'KULI, Technician
Yves MARCHAND, Secretary

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 (1; see Fig. 1) and in unravelling the contribution of endocytosis to physiopathology (4,6,9,10), parasitology (3) and pharmacology (5). We are currently addressing the molecular machineries controlling the endocytic activity at the apical surface of epithelial cells and upon malignant transformation. Recent achievements include: (i), the elucidation of the signalling cascade whereby the paradigmatic oncogenes, v -Src and K-Ras, control the actin cytocortex (2), so as to perturb motility (7) and to induce macropinocytosis (2), specifically at the apical domain of polarized epithelial cells (8), like enteropathogens; (ii) the fine tuning of apical endocytosis leading to regulated production of thyroid hormones (4,10); and (iii) the elucidation of a deficit of apical endocytosis in a genetic form of kidney stones (6); and (iv) the demonstration of a major role of apical endocytosis in the (mannose 6-phosphate-independent) biogenesis of kidney lysosomes (9).

Regulation of endocytosis by v-Src in polarized cells

M. Mettlen, P. Van Der Smissen, S. Carpentier, Ph. de Diesbach, 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 and K-Ras cause a profound remodelling of actin cytoskeleton in Rat-1 fibroblasts, resulting in stress fibre disappearance, cortical actin polymerisation, ruffling and macropinocytosis (2). Since most cancers are of epithelial origin, and since apical endocytosis depends on actin, we examined whether v-Src would similarly trigger fluid-phase endocytosis in MDCK cells and whether apical endocytosis would be selectively affected. 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 a 2-fold faster rate

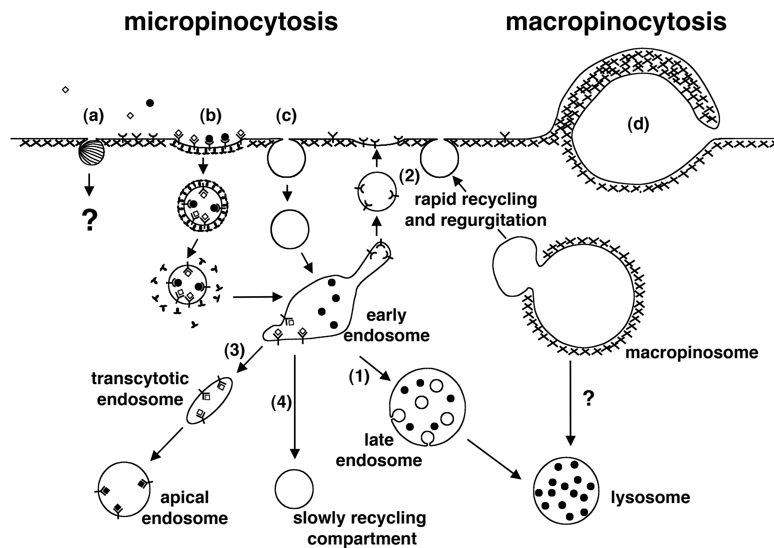

Figure 1. Pathways of endocytosis. This scheme represents four possible modes of vesicular entry of solutes into cells : (a) caveolae; (b) clathrin- and dynamin-associated pits, or «coated pits»; (c) clathrin- and dynamin-independent micropinocytic pits; and (d) macropinocytosis. Crosses represent cortical actin. It further emphasises the endosomes as sorting organelles after micropinocytosis and outlines the four endocytic routes inside the cell : (1) the degradative pathway to lysosomes; (2) the recycling pathway, back to the plasma membrane; (3) transcytosis to the opposite membrane domain (here illustrated from basolateral to apical); and (4) sequestration into slowly recycling endosomes.

of 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 and selectively accelerated apical fluid-phase endocytosis (up to 6-fold) (Fig.2). 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 labeled for v-Src, Rab11, and rabankyrin-5, but not for early endosome antigen-1, thus distinguishing two separate Rab5-dependent 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 show that v-Src selectively affects the dynamics of the apical plasma membrane, where microdomains known as "lipid rafts" are abundant. Current investigations address the interaction between v-Src and "lipid rafts", as well as the effect of v-Src on polarized membrane lipid trafficking. The mechanisms of Src-induced apical ruffling and macropinocytosis could shed light on the apical entry into enterocytes triggered by enteroinvasive pathogens and on the apical differentiation of osteoclasts (8).

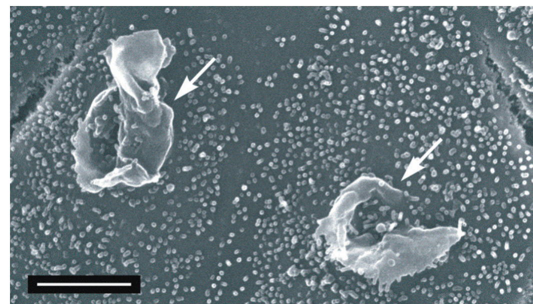

Fig. 2. Src causes circular apical ruffling (scanning electron microscopy, arrows; scale bar : 2 µm).

Relation between endocytosis and cell motility

A. Platek, M. Mettlen, D. Tyteca, Ph. de Diesbach, P.J. Courtoy

Endocytosis may contribute to cell motility by selective removal of plasma membrane constituents from the trailing edge and their recycling to the leading edge. Since v-Src accelerates both motility and endocytosis, we examined whether these two processes are linked and depend on the same regulatory machinery. To this aim, we made use of fibroblasts and epithelial cells harbouring thermosensitive v-Src kinase (Rat-1/tsLA29 and MDCK/tsLA31 cells). In both cell lines, v-Src activation accelerated cell motility by ~ 2-fold, as evidenced by the population-based wound healing assay and by single cell recording in Dunn chambers. Accelerated motility was selectively abrogated by PI3K, PLC and PLD inhibitors. These observations indeed suggest a link between accelerated motility and endocytosis.

In addition, v-Src activation abrogated directionality of cell motility in response to chemotactic growth factor (GF) gradients. v-Src down-regulated GF-receptors by about 2-fold, but directional motility of non-transformed cells was maintained at half receptor occupancy. The loss of chemotactic response to platelet-derived growth factor (PDGF) gradient was further examined in Rat-1/tsLA29 fibroblasts. At non-permissive temperature, PDGF receptor (PDGFR) signalling, assessed by phosphoY^{751}-specific Western blotting (a docking site for PI3K), was not detected without PDGF and was induced by low PDGF concentrations. Both PI3K immunolabeling and a live cell imaging probe of its phosphatidylinositol 3,4,5 tris-phosphate product showed translocation at lamellipodia polarized towards a PDGF gradient, with the concomitant orientation of centrosomes and of PDGFR- and Src-bearing endosomes. Src thermoactivation without PDGF caused Y^{751} phosphorylation, which further increased with PDGF. Src and PDGFR activation were reciprocal and synergistic. As a consequence, PI3K was recruited and activated along the entire plasma membrane without PDGF and did not polarize in response to a PDGF gradient. Src activation also abrogated polarization of centrosomes and of PDGFR-bearing endosomes. Thus, PDGFR signalling persists despite strong Src kinase activity, but diffuse activation of PI3K by Src abrogates cell polarization and chemotaxis: “signalling requires silence” (7).

A strict control of the rate of apical endocytosis regulates thyroid hormone production

P.J. Courtoy, K. Croizet, D. Tyteca, Ph. de Diesbach, M.F. van den Hove

The production of thyroid hormones by thyrocytes is achieved by apical endocytosis of thyroglobulin (Tg) stored in the colloid and intracellular proteolysis, but the cellular mechanisms responsible for its fine regulation are not clear. Since both substrates and hydrolytic enzymes are in vast excess, we hypothesised that the production of thyroid hormones is regulated by their encounter, i.e. depends on rate-limiting endocytic catalysts, such as Rab5 and Rab7. This hypothesis was tested by two approaches. First, we analysed clinical samples of autonomous hyperactive adenomas that result from activating mutations of the TSH receptor/cAMP cascade. The expression of Rab5a and Rab7 was increased up to 6-fold in adenomas by reference to perinodular quiescent tissues. Increased Rab5a and Rab7 expression was selective, coordinate, and correlated with a decrease of the residual Tg content. Second, regulation by TSH of the rate of Tg endocytosis and thyroid hormone production was tested *in vitro*, using primary cultures of normal thyrocytes in polarized monolayers that closely mimic apical Tg secretion then endocytosis, and basolateral release of thyroid hormones. TSH, or forskolin to mimic downstream activation of the cAMP cascade, increased Rab5a and Rab7 expression, apical Tg endocytosis and basolateral thyroid hormone secretion by 2-fold (4).

Since the activity of Rab catalysts depends on their GTP-bound state, we further addressed whether TSH also controls Rab5a activity by promoting GDP/GTP exchange factor(s) (GEF). In hyperactive autonomous adenomas, Rab5a-GEF total activity (availability) and sedimentability (engagement) were both increased by reference to quiescent perinodular tissue. Comparing all *in vivo* samples, GEF activity correlated with Tg depletion and expression of Rab5a and Rabex-5 (the classical Rab5-GEF). In polarized human thyrocyte monolayers, a 2-hours TSH exposure did not affect Rab5a-GEF but promoted its membrane recruitment; after 4 days, TSH increased total and sedimentable Rab5a-GEF, and Rabex-5 expression. Thus, the TSH receptor/cAMP cascade stimulates endocytosis by a two-stage mechanism: (i) rapid membrane recruitment of Rab5a-GEF; then (ii) coordinate increased expression of Rab5a and Rab5a-GEF. Comparing all *in vitro* experiments, sedimentable Rab5a-GEF strongly correlated with apical endocytosis and lysosomal transfer of Tg, and with basolateral secretion of derived hormones (Fig. 3). In conclusion, we provide the first clinical and experimental evidence that control of the expression and activity of a rate-limiting endocytic catalyst finely tunes a normal cellular function, ultimately controlling whole-body metabolism (10).

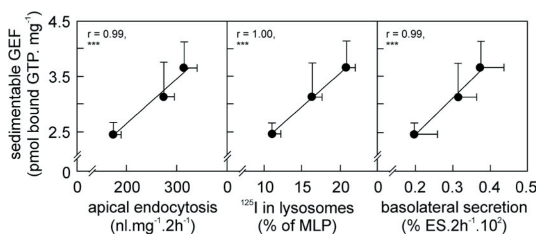

Fig. 3. Fine regulation of Rab5a activity strongly correlates with apical endocytosis and lysosomal transfer of Tg, and with basolateral secretion of derived hormones.

Role of apical endocytosis in the biogenesis of lysosomes in kidney proximal tubular cells

P.J. Courtoy, G. Dom, W. Rezende-Lima, in collaboration with E.I. Christensen and his colleagues (Aarhus University, Denmark), O. Devuyst (NEFR, UCL), M. Jadot (FUNDP, Belgium)

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, and 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), 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, and uptake was abolished by the megalin competitor, receptor-associated protein. Direct interaction of cathepsin B with megalin was demonstrated by surface plasmon resonance. 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 (9).

Molecular medicine: role of an endocytic trafficking defect of kidney proximal tubular cells in hereditary kidney stones

C. Auzanneau, W. Rezende-Lima, S. Carpentier, P.J. Courtoy, in collaboration with O. Devuyst (NEFR)

Kidney proximal tubular cells (PTC) show one of the highest endocytic activity 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, in collaboration with E.I. Christensen (Aarhus, DK), O. Devuyst (NEFR, UCL) and W.B. Guggino (Johns Hopkins, Baltimore, MD, USA). 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. 4). In contrast, fluid-phase endocytosis was better preserved, pointing to a role of megalin and cubilin trafficking. By the combination of analytical subcellular fractionation and ultrastructural immunogold staining, we could demonstrate that both receptors failed to reach the apical membrane, as part of a general membrane trafficking defect (6).

ClC-5, being primary located at PTC endosomes, was proposed to provide the inward chloride conductance necessary to neutralize the membrane potential generated by the electrogenic vacuolar ATPase, in order to support endosomal acidification. We addressed this hypothesis by measuring *in vitro* acidification of early and late endosomes labeled by apical fluid-phase endocytosis of FITC-dextran. ATP-dependent fluorescence quenching depended on chloride, but the acidification rate was undistinguishable between wild-type and ClC-5 KO mice. This indicates that another chloride channel plays a key role in normal PTC endosome acidification, or can compensate for ClC-5 absence. The trafficking defect in ClC-5 KO mice points to a role of ClC-5 in another compartment (ARE ?), or to another function of this channel. We currently address the molecular mechanisms regulating apical receptor-mediated endocytosis in polarized PTC monolayers.

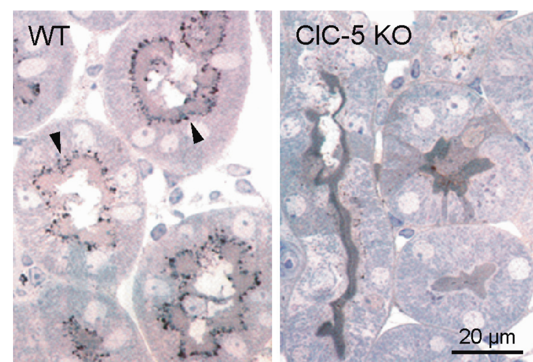

Fig. 4. Evidence for a deficit of apical endocyosis of low-molecular weight proteins in kidney proximal tubular cells of ClC-5 KO mice (cytochemistry of injected, ultrafiltrated peroxidase; note the strong labelling by peroxidase of multiple apical PTC endosomes in WT mice, contrasting with poor apical uptake and luminal retention in ClC-5 KO mice; bars, 10 µm).

Collaborations on endocytosis and cell imaging

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 the endocytic trafficking of syndecans and its role in signalling by its heparin-sulfate ligands (Zimmermann et al., 2005, Dev. Cell 9:377-88); of the biogenesis of vacuolar H+-ATPase and the role of CFTR in kidney (Jouret et al., 2005, 2007, J. Am. Soc. Neph-

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EXTRACELLULAR MATRIX BREAKDOWN

Etienne MARBAIX, Member
Patrick HENRIET, Associate Member

Pierre J. COURTOY, Member
Yves DECLERCK, Visiting scientist
Hervé EMONARD, Guest Investigator
Christine GALANT, Assistant Member
Héloïse GAIDE CHEVRONNAY, Graduate Stud.
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Charlotte SELVAIS, Graduate Student
Denis DELVAUX, Technician
Pascale LEMOINE, Technician (part-time)
Yves MARCHAND, Secretary

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 neutral metalloproteinases, called matrix metalloproteinases (MMPs; see Fig. 1). Our group has demonstrated a decade ago that menstrual bleeding in women is due to a dramatic change in the expression or activation of MMPs (1). This seminal observation led us to : (i) exploit 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 (5). Recently, our group has entered a new field of research, investigating how local MMP activity may be controlled by individual cells, either by local concentration upon plasma membrane binding, or conversely by receptor-mediated endocytosis and degradation.

Differential control of endometrial MMPs and cytokines by ovarian steroids, *in vivo* and in explants

C. Pretto, H. Gaide Chevronnay, 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. They substantially diverge, however, in their expression profile during the other phases of the cycle, indicating differential regulation by estradiol and progesterone.

To directly measure the effect of the ovarian steroids, mRNA amounts of selected genes (MMPs, TIMPs, EBAF, IL-1 and TGF-β) were quantified in a large collection of endometrial samples collected throughout the menstrual cycle and examined without or after culture as explants (3, 6, 8). Differential expression patterns were identified *in vivo* according to the time frame, sharpness and magnitude of the changes (up to 5 logs, see Fig. 2). The differential response to the ovarian steroids was mimicked in explant culture. Altogether, these observations suggest that, *in vivo*, different pathways finely tune in time, space and amplitude the global control by estradiol and progesterone of the expression of genes required for menstrual ECM breakdown.

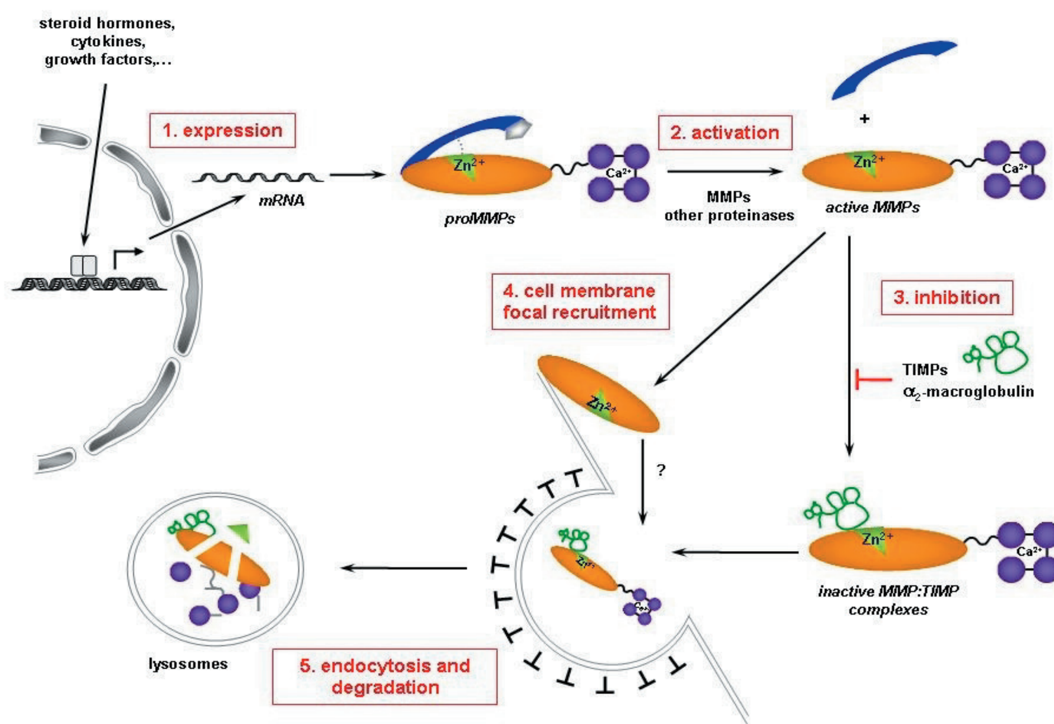

Figure 1. Regulation of MMPs activity in the human endometrium: current model. A. General features on MMPs structure and regulation. MMPs, the major actors of extracellular proteolysis, share a common intramolecular control due to masking by a N-terminal prodomain (blue, here shown with the signal peptide in grey) of the Zn^{2+}-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 (mauve) responsible for substrate specificity, linked by a hinge domain (black). Some MMPs are originally inserted in the plasma membrane by a transmembrane domain or a GPI-anchor (the so-called MT-MMPs, not shown). The activity of MMPs is overall controlled at 5 different levels : (1) expression, (2) zymogen activation by prodomain excision, (3) inhibition of active forms by physiological inhibitors, (4) secondary membrane binding and focalized activity, and (5) endocytosis and degradation. Whereas MMPs secretion is constitutive in almost all cells, it can be additionally regulated in neutrophils. B. Regulation of MMPs action in the human endometrium. Extracellular matrix remodeling in the human endometrium must be tightly controlled, both for blastocyst implantation and, in its absence, for menstrual breakdown of an irreversibly specialized tissue. (1) Expression: ovarian steroids, their intracellular receptors and cytokines interact to form an integrated system that differentially controls the focal expression of endometrial MMPs and TIMPs. (2) Activation: MMPs can be activated by other MMPs, by plasmin, itself activated during menstruation, or by as yet unidentified proteinases. (3) Inhibition: TIMPs are particularly abundant in the human endometrium; like MMPs, the level of TIMPs is regulated by ovarian steroids and cytokines. (4) Secondary plasma membrane concentration: MMP-7 binds to sulphated membrane receptors in cholesterol-rich domain, a mechanism which enhances pericellular MMP activity. (5) Endocytosis and degradation: endometrial LRP-1 binds and internalizes MMP-2 and MMP-2: TIMP-2 complexes, leading to lysosomal degradation. Our research has unravelled and is focused on levels (1), (2), (4) and (5).

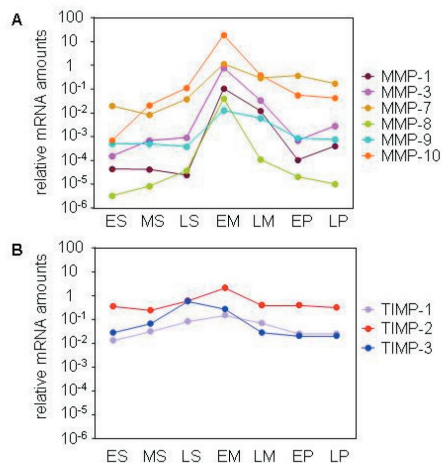

Fig. 2. Differential control of MMPs and TIMPs mRNA expression in human endometrium throughout the menstrual cycle. This representation of the cycle starts after ovulation by the progesterone-dominated secretory phase, is centered on the steroids-deprivated menstrual phase and ends by the estrogen-driven proliferative phase. ES, MS, LS : early, mid, late secretory phase; EM, LM : early, late menstrual phase; EP, LP : early, late proliferative phase. mRNA levels are normalized to β-actin mRNA. Notice the logarithmic scale.

Binding of active MMP-7 to the plasma membrane enhances its pericellular activity and prevents its inhibition by TIMPs

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

Matrix metalloproteinase-7 (MMP-7, matrilysin-1) is specifically expressed by epithelial cells and modulates crucial biological events by processing a variety of epithelial cell surface-associated effectors. We therefore addressed how MMP-7, a "mini MMP" essentially limited to its catalytic domain, can interact with human epithelial cells while keeping its activity. Active MMP-7, but not its latent proform, was preferentially retained in cultured endometrial explants. Immunohistochemical investigations revealed that active MMP-7 was strictly localized at the periphery of glandular epithelial cells, contrasting with the diffuse intracellular pattern displayed by the latent proenzyme. Incubation of recombinant latent and active ^{125}I-MMP-7 with Ishikawa cells (derived from a human endometrial carcinoma) confirmed that only the active species bound to the epithelial cell surface; binding was saturable, specific and of high affinity (K_D ~ 2.5 nM). Prior cholesterol depletion by β-methyl cyclodextrin or competition by heparin each decreased by ~ half MMP-7 binding. Furthermore, a cell surface heparan sulphate proteoglycan, CD44v3, partly colocalized with active MMP-7 at the surface of glandular epithelial cells, as shown by confocal microscopy. Complexing with natural (proteins) or synthetic (small drugs) MMPs inhibitors impeded MMP-7 binding to the cell surface, suggesting interaction via its catalytic domain. However, membrane-bound MMP-7 efficiently degraded a natural substrate, casein, but became resistant towards its physiological inhibitor, TIMP-2. We conclude on the coexistence of at least two types of sulphated binding sites (one of which being CD44v3) at cholesterol-rich domains, allowing for cell-surface focalization of active MMP-7, that not only remains functional but even becomes resistant to natural inhibition (10).

LRP-mediated endocytic clearance of proMMP-2:TIMP-2 complexes and free TIMP-2

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

We found that addition to endometrial explant cultures of RAP, a natural antagonist of the low-density lipoprotein receptor-related protein (LRP-1) considerably increased the accumulation of MMP-2 and -9 in the conditioned medium. This observation suggested that LRP-mediated endocytosis of MMPs could represent a potent additional regulatory mechanism of MMPs activity during the menstrual cycle. We further demonstrated that proMMP-2:TIMP-2 complexes and free TIMP-2 are efficiently cleared by endocytosis via LRP-1 in

the HT1080 human fibrosarcoma cell line, a process requiring prior binding of TIMP-2 to another, non-identified receptor (4). We currently aim at its identification. Beside acting as an endocytic receptor, LRP-1 is involved in transducing various extracellular signals (reviewed in 7). In addition, TIMP-2 is known to abrogate the mitogenic effect of EGF, but the nature of its cell-surface receptor also remains unknown. We found that RAP suppressed inhibition by TIMP-2 of the EGF mitogenic potential, suggesting that LRP-1 could act as a (co)-receptor for cell signalling by TIMP-2.

Role of matrix metalloproteinases in abnormal endometrial bleeding

C. Galant, P. Henriet, P.J. Courtoy, E. Marbaix

Since MMPs play a key role in initiating normal menstrual breakdown, we looked for their contribution in metrorrhagia, a frequent pathological condition characterized by abnormal bleeding. In collaboration with Dr. J. Donnez (UCL), we investigated irregular dysfunctional bleeding, a condition that cannot be accounted for by primary organic lesions, such as uterine fibromas, endometrial polyps or cancer. We found these disorders to be also associated with menstrual-like stromal breakdown, particularly in foci containing low levels of ovarian steroids receptors, and increased expression and activity of several MMPs, together with decreased production of TIMP-1 (5, see Fig. 3). Inflammatory cells, in particular neutrophils, were further recruited to the same sites and found to release their abundant proMMP-8 and -9, which, upon activation, should increase ECM proteolysis. These results confirmed *in vivo* our previous findings obtained with cultured explants from patients on long-term progestinic contraception (2).

In collaboration with Dr. M. Nisolle (Laboratory of Dr. J.M. Foidart, ULg), we are also developing a new experimental model of endometrial xenograft in immunodeficient mice, which should enable us to manipulate *in vivo* the activity of MMPs and thereby better understand their role in physiological and abnormal endometrial bleeding, as well as in endometrial angiogenesis and vessel maturation through pericyte recruitment (8).

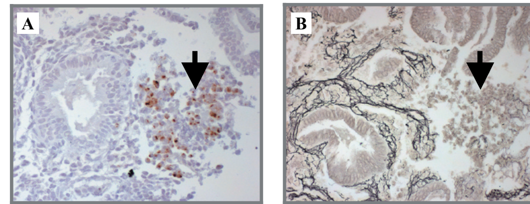

Fig.3. Focal expression of MMPs correlates with ECM breakdown. Arrows show that MMP-3 immunostaining (A) is restricted to the area showing disappearance of collagen-rich argyrophilic fibers of the endometrial stroma during menstruation (B).

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METABOLIC COMPARTMENTATION IN TRYPANOSOMES

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Paul A.M. MICHELS, Member

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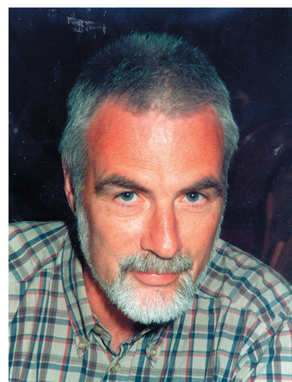
Freddy ABRASSART, Technician
Nathalie CHEVALIER, Technician
Joris VAN ROY (till August 2006), Technician
Françoise MYLLE, Secretary



Fred Opperdoes

Paul Michels

Trypanosomes and Leishmania are haemoflagellated protozoan parasites which cause sleeping sickness, Chagas' disease and leismaniasis. Together these diseases are responsible for the annual deaths of millions of people in the tropics. The arsenal of drugs available for treatment is very limited and most drugs are not adequate and have undesirable side-effects. It is for these reasons that new and better drugs are urgently needed. The Research Unit for Tropical Diseases of the de Duve Institute is trying to better understand the biochemistry of these organisms in order to develop such 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 are sequestered inside peroxisome-like organelles called glycosomes. Since many years, we study the functional and structural properties of the glycolytic enzymes of Trypanosoma brucei and their closely related relatives: T. cruzi and Leishmania mexicana. The collected information is being used for the design of effective and selective inhibitors by structure-based and catalytic mechanism-based approaches. Also the enzymes of another pathway of carbohydrate metabolism, the hexose monophosphate pathway, are associated with the glycosomes and thus are an interesting subject for study. Expression knock-down experiments using RNA interference has allowed us to validate these enzymes as targets for drug development. Glycosome assembly and degradation, processes of utmost importance during cell differentiation, are being studied as well. The «peroxins», proteins involved in glycosome biogenesis, have also our special attention with regard to drug discovery. Most peroxins already described in other organisms have also been identified in T. brucei and validated as excellent drug targets. Finally, the recent completion of the genome sequences of three trypanosomatids has allowed us the make a comprehensive inventory of the metabolic capacities of these organisms and to identify not only metabolic differences between the various representatives of this family, but also to establish essential differences between the parasites and their host.

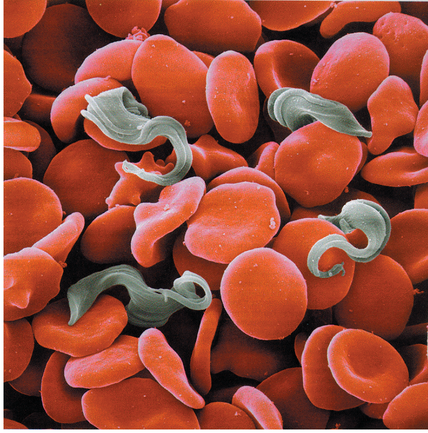


Figure 1. Trypanosomes surrounded by red blood cells.

Pathways and enzymes of carbohydrate metabolism

Energy metabolism

F. Opperdoes, V. Hannaert, P. Michels

The energy metabolism of *T. brucei* differs significantly from that of its hosts and changes drastically during the life cycle. Glycolysis is the major route for the generation of energy in the bloodstream form trypanosome and the glycolytic enzymes have been validated as drug targets. In 2006 we have continued our studies on these enzymes in close collaboration with other laboratories.

Enolase

P. Michels, in collaboration with W. Quinones, Universidad de Los Andes, Venezuela

We have cloned and overexpressed in *Escherichia coli* the enolase from *L. mexicana*. This enolase shares with enolases from other trypanosomatids the presence of three atypical residues, each with a reactive side group, near the active site. Subcellular fractionation and immunofluorescence studies showed that in addition to being a cytosolic enzyme, the leishmania enolase is associated with the external face of the plasma membrane, where it probably functions as a plasminogen receptor. This is currently further investigated.

Phosphofructokinase

P. Michels, in collaboration Linda Forthergill-Gilmore, University of Edinburgh, Scotland

We have also succeeded in resolving the first crystal structure of phosphofructokinase from a eukaryotic organism by using the *T. brucei* enzyme. This ATP-dependent phosphofructokinase (PFK) enables comparisons to be made with the crystal structures of bacterial ATP-dependent and PPi-dependent PFKs. Several features thought to be characteristic of PPi-dependent PFKs are present in the trypanosome ATP-dependent PFK as well. These two types of enzyme are more similar to each other than to the bacterial or mammalian ATP-dependent PFKs. However, there are critical differences at the active site of PPi-dependent PFKs that are sufficient to prevent the binding of ATP. This crystal structure of a eukaryotic PFK has enabled us to propose a detailed model of human muscle PFK. The *T. brucei* enzyme shows unique active site features that offer opportunities for structure-based drug discovery for the treatment of sleeping sickness and other diseases caused by the trypanosomatid family of protozoan parasites.

Fructose-bisphosphate aldolase

P. Michels, in collaboration with C. Blonski, Université Paul Sabatier, Toulouse, France and J. Sygusch, University of Montreal, Canada

An irreversible competitive inhibitor hydroxynaphthaldehyde phosphate was synthesized that is highly selective against the glycolytic enzyme fructose 1,6-bisphosphate aldolase from *T. brucei*. Inhibition involves Schiff base formation by the inhibitor aldehyde with active site Lys116 followed by reaction of the resultant Schiff base with a second residue. Molecular simulations indicate significantly greater molecular geometries conducive for nucleophilic attack in *T. brucei* aldolase than the mamma-

lian isozyme and suggest Ser48 as the Schiff base modifying residue. A pro-drug form of the inhibitor kills cultured trypanosomes at low micromolecular concentrations.

Glycerol-3-phosphate dehydrogenase

P. Michels, Daniel Guerra, in collaboration with A. Decottignies, GECE, and B. Bakker, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

In trypanosomatids NADH produced in the glycolytic pathway is reoxidized by a dihydroxyacetone-phosphate/glycerol-3-phosphate shuttle comprising a glycosomal NAD-linked and a mitochondrial FAD-linked glycerol-3-phosphate dehydrogenase. The genes for the mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase were identified in *T. brucei* and *Leishmania* major genomes. We have expressed the *L. major* gene in the yeast *Saccharomyces cerevisiae* and confirmed the subcellular localization and activity of the produced enzyme. Using cultured *T. brucei* procyclic and *L. mexicana* promastigote cells with a permeabilized plasma membrane and containing intact glycosomes, it was shown that dihydroxyacetone phosphate is converted into pyruvate, and stimulates oxygen consumption, indicating that all components of the glycerol 3-phosphate/dihydoxyacetone phosphate shuttle between glycosomes and mitochondrion are present in these insect stages of both organisms. A computer model allowed to get insight into the metabolic role of the shuttle in these insect-stage parasites.

Glycosome biogenesis

H. Krazy, N. Galland, E. Verplaetse, P. Michels, in collaboration with W. Hol, University of Washington, Seattle, USA

After the characterization of PEX5 and PEX14 (see previous reports) we have analysed in 2006 several additional *T. brucei* orthologues of yeast and mammalian peroxins involved in import of peroxisomal matrix proteins: PEX1, PEX6, PEX7, PEX10, PEX12 and PEX19. Cellular depletion of these peroxins by RNAi severely affected growth of trypanosomes. Both by immunofluorescence studies of intact cells and subcellular fractionation experiments it was shown that RNAi-dependent knockdown of expression of each of these peroxins led to partial mis-localization to the cytosol of several glycosomal enzymes each utilizing a different type of glycosome-targeting signal. These proteins could be divided in the following classes: (i) proteins with the C-terminal type 1 peroxisome-targeting signal PTS1; (ii) the N-terminal signal PTS2 and (iii) a protein for which the sorting information is present in a polypeptide-internal fragment not containing an identifiable consensus sequence. This demonstrates that all glycosomal matrix proteins are imported via this peroxin import system. All peroxins analyzed so far have only little sequence identity with their human counterparts (between 16 and 32%). Currently, we are using different approaches to analyze the interactions occurring between different peroxins when they mediate uptake of proteins into the glycosomal matrix, in order to reveal essential domains or motifs that could be exploited for drug discovery.

Analysis of glycosome solute transporters

C. Yernaux, M. Igoillo-Esteve, P. Michels, in collaboration with M. Fransen, Katholieke Universiteit Leuven

We have identified three ABC transporters (GAT1-3 for Glycosomal ABC Transporters) associated with the glycosomal membrane of *T. brucei*. These polypeptides resemble their homologues from peroxisomes in that they contain only one transmembrane domain and a single nucleotide-binding domain. Their glycosomal localization was confirmed and by expression of fluorescent deletion constructs, the glycosome-targeting determinant of two of the three transporters were identified. We also identified the *T. brucei* homologue of peroxin PEX19, a chaperonin and/or receptor

for cytosolically synthesized proteins destined for insertion into the peroxisomal membrane. We were able to show that PEX19 interacts with glycosomal ABC-transporter fragments containing an organelle-targeting determinant. The GAT transporters are differentially regulated in different stages of the *T. brucei* life cycle. Complementation experiments in yeast mutants deficient for the homologous proteins suggest that GAT2 is involved in the metabolism of fatty acids.

Turnover of glycosomes

M. Herman, P. Michels in collaboration with D. Rigden, University of Liverpool, England, and E. Pays, Université Libre de Bruxelles

The different life-cycle stages of trypanosomes have been shown to vary considerably in their metabolic capacities. We hypothesize that the compartmentation of important parts of its metabolic machinery inside glycosomes allows the trypanosome to adapt efficiently to the changing nutritional conditions encountered during its life-cycle. The necessary adaptation of the metabolic repertoire would then be achieved by degradation of entire glycosomes by autophagy and simultaneous formation of new organelles with a different enzyme content. Autophagy is the process by which cellular components are directed to and degraded in the vacuole or lysosome and has been studied largely in yeasts. Searches in the trypanosomatid genome databases, supplemented with advanced bioinformatic analyses, unambiguously indicated the occurrence of autophagy in these parasites. Trypanosomatid orthologues of yeast proteins involved in different stages of autophagy were identified. However, at most only half of the components characterized in yeasts are present in trypanosomatids suggesting an unexpectedly streamlined version of autophagy occurs in these organisms.

Using an experimental model system developed for studying the differentiation of *T. brucei* long-slender bloodstream forms via short-

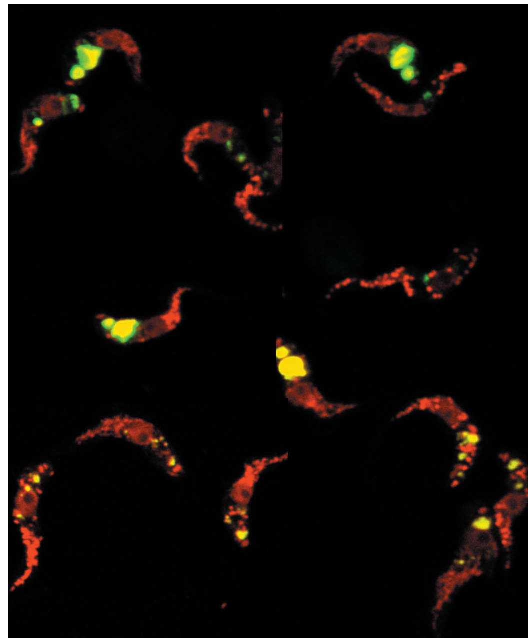


Figure 2. Autophagy of glycosomes inside lysosomes. Immunofluorescence image of trypanosomes in the process of digesting glycosomes during the differentiation from bloodstream to procyclic form *in vitro*. The lysosome (stained in green) is enlarged and colocalizes with some glycosomes (stained with aldolase in red). Co-localization results in a yellow color.

stumpy forms into cultured procyclic forms representative of trypanosomes living in the insect midgut, we performed preliminary studies on the turnover of the organelles. Indeed, strong indications were obtained that glycosome degradation during differentiation involves autophagy.

Metabolic capacities of trypanosomatids

F. Opperdoes, P. Michels, J.-P. Szikora

The completion in 2005 of the genome sequences of three trypanosomatids has allowed us to analyse the metabolic capacities of these organisms and to compare them in detail. Three enzymes of histidine metabolism are only found in *T. cruzi*, one of them, an imidazolonepropionase, seems of bacterial

origin. Specific for the two trypanosome species, because absent from *L. major*, are sedoheptulose-1,7-bisphosphatase and threonine dehydrogenase. An alternative oxidase is only present in *T. brucei*. From a metabolic point of view *L. major* is the most complete of the three organisms, with some 30 metabolic genes not found in the other two trypanosomatids. *T. brucei* is the most crippled of the three. Differences are mainly found at the level of fatty-acid oxidation and amino-acid metabolism. *L. major* glycosomes are capable of degrading many sugars other than glucose, fructose and mannose. This probably represents an adaptation to the sandfly vector, which feeds not only on blood but also on plant nectar and honey dew. *T. brucei* seems to have lost this possibility as an adaptation to the glucose-rich body fluids of the vertebrate host. A bioinformatic analysis of their protein sequences using information about peroxisome-targeting signals has revealed that glycosomes are not only involved in glycolysis and the hexose-monophosphate pathway, but also reactions of gluconeogenesis, purine salvage and pyrimidine biosynthesis. The organelles appear to contain also enzymes of β-oxidation of fatty acids, fatty-acid elongation, the biosynthesis of ether lipids, several steps of isoprenoid synthesis and oxidant stress protection. Several review papers and chapters in books dealing with these database-mining results have been prepared.

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

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Mélanie UEBELHOER, Graduate Student
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Thomas PALM, Graduate Student
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The basic aim of our research is to get insights into the molecular mechanisms behind human disease, and especially to evaluate the importance of genetic variation in disease development. For many disorders, the cause is unknown, and therefore current treatments are aimed at alleviating symptoms. Identification of the primary causes as well as the modulating factors would allow to develop treatments that are more "curative" and more specific. To this end, we use a genetic approach. We are interested in disorders affecting the cardiovascular and the skeletal systems, as well as certain cancers of the nervous system. 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, glomuvenous malformations ("glomangiomas") and Maffucci syndrome

P. Brouillard, M. Amyere, M. Uebelhoer, V. Aerts, V. Wouters, N. Limaye, L.M. Boon, 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 and A. Dompmartin, CHU, Caen, France

Venous malformations (VM) are bluish-purple cutaneous and mucosal vascular lesions. They are often congenital, but can appear later in life. They have a tendency to grow slowly

with the growth of the child. Glomuvenous malformations (GVM, "glomangiomas") are a special subtype of venous anomalies. They are clinically similar to VMs, yet our recent study has allowed clinical differentiation (1).

We have previously identified that hereditary venous malformations can be caused by an activating mutation in the receptor tyrosine kinase TIE2/TEK. In contrast to inherited VMs, inherited glomuvenous malformations are caused by mutations in the gene we named "glomulin". By screening several families in which GVMs are inherited, we have discovered that about 70% of the individuals with inherited GVM show one of four common glomulin mutations (2). This allows efficient genetic diagnosis, although the rest 30%, have a unique mutation. The DHPLC system that we set up for screening this and some other genes of interest allows easier, faster and more sensitive screening for such mutations.

Glomulin does not have sequence identities to known proteins, nor does it contain known functional domains. Thus, its molecular function has stayed unknown. To start to unravel the role of glomulin in angiogenesis, we have studied its expression. Glomulin was found in almost all tissues, but almost exclusively in vascular smooth muscle cells (3).

As most of the identified mutations cause premature STOP codons in the coding sequence of glomulin, loss-of-function is the most likely mechanism causing GVM. Furthermore, we hypothesized that as the lesions are localized, a somatic second hit might be needed in the normal allele of the glomulin gene, for lesions to develop. We have obtained proof for this from one lesion. To further study glomulin function, we have cloned 20kb of the murine glomulin gene to be used to create a construct for inactivating glomulin by homologous recombination in murine embryonic stem cells.

Maffucci syndrome is a rare non-hereditary disorder characterized by venous-like cutaneous lesions associated with enchondromas and increased risk of cancer. With world-wide collaborations we have collected samples from a series of patients with the goal of identifying possible underlying genetic defects.

Lymphedema

A. Ghalamkarpour, L.M. Boon, M. Vikkula in collaboration with K. Devriendt, KUL, D. Chitayat, Hospital for Sick Children, Toronto, Canada

Primary lymphedema can occur at birth (Milroy disease) or at puberty (Meige's disease). It is extremely difficult to treat and the patients have a life-time problem with progressive swelling of extremities. We use genetics to unravel the pathophysiology. A family, in which primary lymphedema was present at birth in several family members, confirmed linkage to 5q33-34 and led to the identification of a mutation in the *VEGFR3* gene. *In vitro* expression studies demonstrated that the mutated receptor has lost its autophosphorylation capacity. More recently, we have shown that a VEGFR3 mutation can also cause a severe intrauterine condition, hydrops fetalis (4).

The continued studies have led to the identification of a transcription factor gene, SOX18, to be mutated in two families with recessively inherited congenital lymphedema, and in another family, with dominantly inherited lymphedema. All individuals with a SOX18 mutation had also hypothrichosis. This study identified the third human gene known to cause lymphedema, a disorder currently without cure.

Vascular anomalies affecting capillaries

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

Capillaries, the smallest blood vessels that

connect arterioles to venules, can give rise to various anomalies, two of which are very common: 1) hemangioma, a benign, localized overgrowth of capillary-like vessels, and 2) capillary malformation (CM; commonly known as portwine stain), a localized maldevelopment of capillary like vessels. Hemangiomas have a frequency of up to 12 % in 1-year-old children, and CMs occur in 0,3% of newborns. Whereas hemangiomas usually disappear spontaneously, capillary malformations stay throughout life, if not treated. Other types of cutaneous capillary anomalies also exist. In addition, some can affect other organs, such as the brain, in case of CCMs, cerebral capillary malformations.

As the molecular mechanisms leading to these localized capillary lesions are unknown, we have collected clinical information and samples from families in which more than two individuals are affected. These studies led to the discovery that inherited hyperkeratotic cutaneous capillary-venous malformations (HCCVM) associated with cerebral capillary malformations are caused by a mutation in the KRIT1 (Krev interaction trapped 1) gene. This suggested that KRIT1, a possible intracellular signaling molecule, is important not only for cerebral but also for cutaneous vasculature (5). In addition, a genome-wide linkage mapping on families with inherited capillary malformations identified a linked locus CMC1. Screening of positional functional candidate genes led to the identification of mutations in the RASA1 gene, a modifier of Ras signaling pathway (6). This implies that RAS signaling pathway modulators may serve as a novel therapy for these patients in the future (7). Ongoing studies have led to the identification of 24 additional families with RASA1 mutation allowing more precise clinical description of the associated clinical signs ans symptoms (Revencu et al, submitted).

Cardiopathies

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

The cardiovascular system may also encounter developmental problems affecting the heart. These cardiac defects, cardiopathies, vary from physiological septal defects to life-threatening complex malformations. To get insight into the molecular mechanisms behind these phenotypes, we collect samples from families with possibly hereditary cardiopathies. In three families, in which atrial septal defect is associated with progressive atrioventricular conduction defect, we identified three novel mutations in the CSX/Nkx2.5 gene, an important transcription factor for cardiac development. Identification of mutation carriers is crucial, as in the few studied families the first "symptom" has sometimes been sudden death. Identification of mutations allows genetic testing in the respective families, enabling tight follow-up and preventive pacemaker implantation.

More recently, we have performed a whole-genome linkage analysis using the 10K Affymetrix SNP-chips and identified a possible locus for a gene causing heterotaxia, situs inversus (Fig. 1) (8).

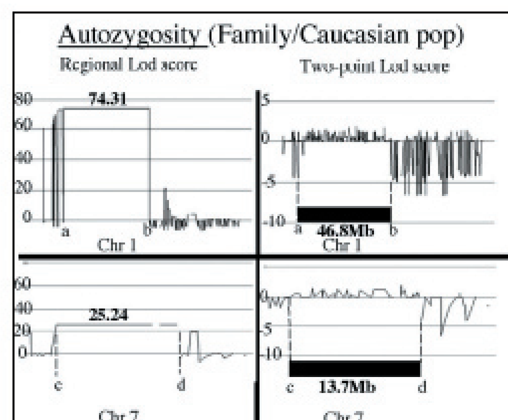

Figure 1. Regional and two-point Lod scores calculated by Genespring GT based on a Caucasian population. The highest regional Lod scores of 74.31 and 25.24, when comparing the family data with a Caucasian control population were obtained, in chromosome 1 and 7 respectively. (adapted from [8]).

Cleft lip and palate

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

Our main project in collaboration with Centre labio-palatin, St Luc, is to unravel the molecular background of syndromic and non-syndromic cleft lip and/or palate.

In addition, collaboration with the cleft lip and palate center of the CHRU Lille (Prof. Ph. Pellerin) has been initiated. These studies have led to the identification of IRF6 mutations causing Van der Woude syndrome in a large cohort of European patients (Ghassibé et al, in preparation). More recently, we also observed association between IRF6 gene polymorphisms and non-syndromic, sporadic cleft lip and palate (Fig. 2)(9)

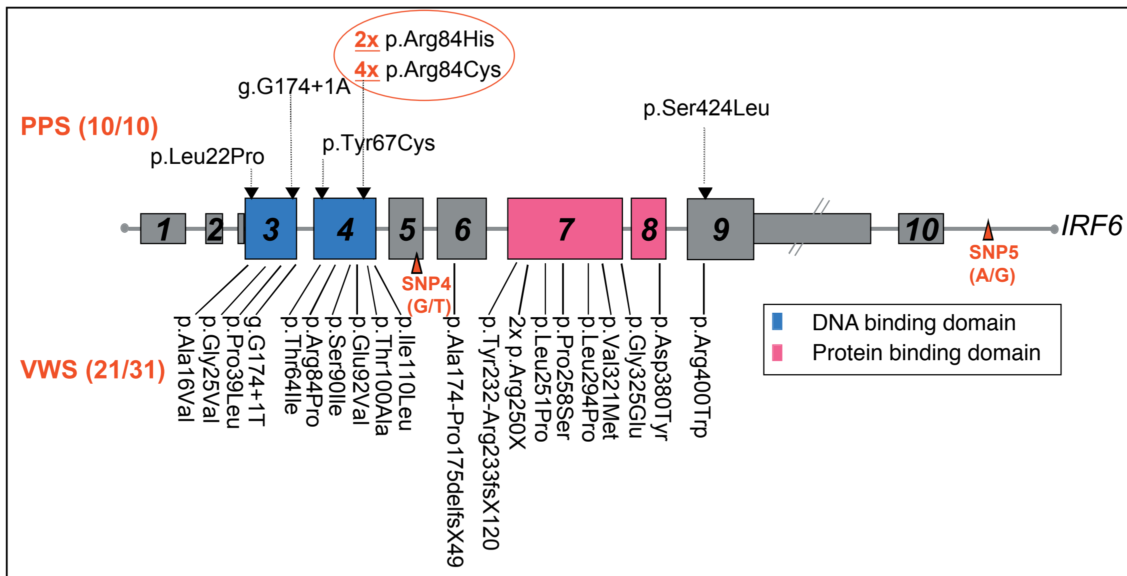

Figure 2. Mutation frequency in the IRF6 gene in Europe.

Other cutaneous disorders

M. Amyere, L.M. Boon, M. Vikkula, in collaboration with B. Olsen, Harvard Medical School and Th. Vogt, University of Regensburg, Germany

A peculiar pigmentation problem in some individuals from the town of Teublitz was identified by Dr Th. Vogt, Germany. As this progressive hereditary hyperpigmentation affects some individuals from the same small village, and is extremely rare, it is plausible that all the individuals carry the same ancestral mutation. Based on this hypothesis, we have performed a whole genome DNA-chip based polymorphic marker analysis and identified a possible locus for the mutated gene (Amyere et al., in preparation). This is the first step towards identification of the causative gene and unravelling the pathway involved in this mirrow-image causing pigmentation disorder.

Cerebral tumors

Th. Palm, 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. In addition, the causative genes are often unknown. We are especially interested in two types of cerebral tumors: oligodendrogliomas and ependymal tumours. Using DNA, extracted from formalin-fixed and paraffin-embedded tissues, we have performed loss-of-heterozygosity tes-

ting. A restricted screening was performed in a number of oligodendroglial tumours as well as in a large series of ependymal tumours. For oligodendrogliomas, this allowed us to identify and define specific histological characteristics for tumors that have lost chromosome 1p and 19q and that are known to have a preferable response to chemotherapy (10). This data had direct clinical relevance. In addition, we identified methylation differences in ependymomas depending on their age and location. More recently, using array CGH, we have identified a subgroup of ependymal tumors with a characteristic chromosomal anomaly (Rousseau et al, submitted).

Conclusions

With the genetic approach described, the GEHU group has unravelled genetic defects behind several human disorders. These discoveries have created international and national collaboration to understand the mechanisms by which these genetic defects alter development and organ function. Already now, this data has, in some cases, made more precise clinical diagnosis possible, thus, directly aiding treatment better. In the more distant future, we hope that this knowledge will help develop novel targeted therapies.

Selected publications

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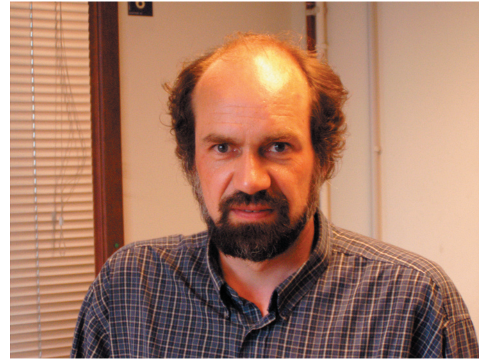
VIRAL PERSISTENCE AND INTERFERON RESPONSE



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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 which 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 interferon (IFN-α/β) response which is critically important to control viral infections and to modulate the acquired immune response.

Theiler's virus

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

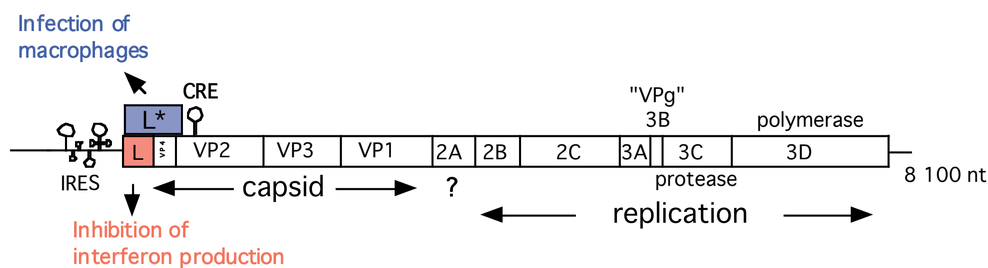

Figure 1. Genome of Theiler's virus. A large open reading frame encodes a 2000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, into 12 mature proteins. An additional protein (L*) is encoded by an alternative open reading frame. Translation of both ORFs is driven by an Internal Ribosome Entry Site (IRES). Protein L* was shown to facilitate the infection of macrophages and viral persistence (3). Protein L inhibits type-I interferon production and affects nucleo-cytoplasmic trafficking of host proteins (2, 4). The role of protein 2A is unknown. VP1, VP2, VP3 and VP4 form the viral capsid. 3B (also called VPg) is covalently linked to the 5' end of the RNA molecule during encapsidation and replication. 3C is the protease responsible for most of the cleavages occurring during polyprotein processing. 3D is the RNA-dependent RNA polymerase. Proteins 2B, 2C, 3A participate in the replication complex. A replication signal has been discovered in the VP2 coding sequence and is denoted CRE for «cis-acting replication element» (1).

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

C. Ricour, F. Sorgeloos, V. and T. Michiels

Two viral proteins, namely L and L* were found to be crucial for persistence of the virus in the central nervous system though they are not required for replication of the virus in cell culture. 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 SJL/J mice.

We also showed that the leader protein interferes with nucleo-cytoplasmic trafficking of host cell proteins (4). 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).

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. Nevertheless, 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 (7). 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.

Interestingly, analysis of viral RNA replication of chimeric viruses constructed between TMEV and EMCV raised the hypothesis that L proteins of TMEV and EMCV diverged during evolution to adapt to the different replication fitness of these viruses (7).

Influence of the L* protein on macrophage infection and viral persistence

Persistent strains of Theiler's virus produce a 18 kDa protein called L*, which is encoded by an open reading frame (ORF) overlapping the ORF coding the viral polyprotein (see Fig. 1). This protein was shown to enhance the infection of macrophage cell lines and to be critical for persistence of the virus in the central nervous system of the mouse (3).

Current efforts aim at characterizing the function of the L* protein in the infection of macrophages. Macrophages are indeed key players in the demyelinating disease induced by Theiler's virus, being simultaneously effectors of the immune response and targets of viral infection. Our recent data suggest that the L* protein antagonizes an important effector pathway of the interferon response. Thus Theiler's virus would interfere both with the production of IFN by infected cells and with the

response of cells to this cytokine. This outlines the major importance of the IFN system in the defense of the host against viral infections.

Type-I Interferons

Mouse and human genomes carry more than a dozen of genes coding for closely related interferon-alpha (IFN-α) subtypes. These interferons as well as other type-I interferons, like IFN-β, IFN-κ, IFN-ε, and limitin, are thought to bind the same receptor, raising the question of whether they possess specific functions.

Taking advantage of the recent availability of the mouse genome sequencing data, 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).

The various IFN-α subtypes displayed a good correlation between their antiviral and antiproliferative potencies, suggesting that IFN-α subtypes did not diverge primarily to acquire specific biological activities, but probably evolved to acquire specific expression patterns.

We are now analyzing the reason for the multiplicity of IFN genes in human and mouse genomes. We currently analyze whether expression of different IFN subtypes differ in a tissue-specific fashion. We also analyze why some IFNs are glycosylated while others are not. Finally, we analyze the IFN response in the particular context of the central nervous system.

Glycosylation of murine IFN-β

C. Sommereyns, T. Michiels

Analysis of available mammalian IFN-β sequences showed that they all carry 1 to 5 predicted N-glycosylation sites. Murine IFN-β contains three predicted N-glycosylation sites (Asn29, Asn69, Asn76). Surprisingly, one of these sites (Asn29) is located in the AB loop of the IFN molecule, in a region predicted to interact with the type-I interferon receptor.

We showed that all three N-glycosylation sites predicted from the sequence, including Asn29, indeed carry N-linked sugars. Mutation of individual N-glycosylation sites had a weak negative influence on IFN antiviral activity. In contrast, the complete loss of glycosylation dramatically decreased activity. Although the 3D structures of different type-I IFNs are closely related, our data suggest that the interaction of murine IFN-β with the IFNAR receptor might differ locally from that of human IFN-α2 and of human IFN-β (8).

We are now analyzing the influence of IFN N-glycosylation on the biological activity of these cytokines in vivo.

Production of IFN-α/β by neurons

S. Paul, S. Delhaye, 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 cel-

ls, 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 (9).

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 (9, 10).

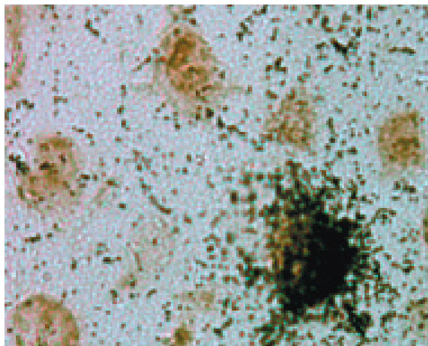

Fig. 2. Neurons can produce IFNα/β: Neuron (immunostained in brown for NeuN) producing IFN-β (detected by in situ hybridization: black dots) after infection with La Crosse virus.

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VIRAL IMMUNITY AND PATHOGENESIS

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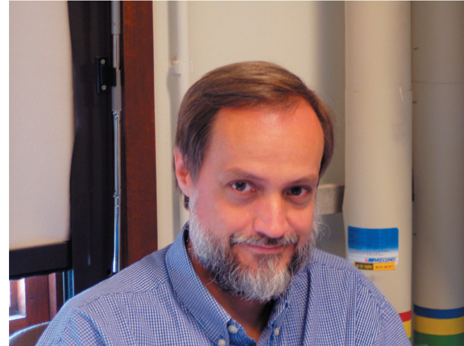

The possibility for evoluted organisms to survive viral infections depends on the ability of their immune system to eliminate the infectious agent. Therefore, numerous mechanisms, involving different types of immune cells such as cytolytic lymphocytes, T helper and B lymphocytes and macrophages, the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

Viral infections result in a dramatic increase in the proportion of IgG2a

Of particular interest is the fact that all antibody responses are not equal. Indeed, depending on their isotype, immunoglobulins display various properties. For example, IgG1, one of the major IgG subclass in mice, cannot fix the complement, contrary to IgG2a, another major component of murine immunoglobulins. Such a difference may lead to dramatic variations in the functional effect of antibodies, as their ability to lyse cells they have bound. During the last few years, we found that the isotype of antibody responses was influenced by concomitant viral infections. The effect of the virus resulted in a dramatic increase in the proportion of IgG2a, not only in antiviral antibodies, but also in immunoglobulins with an antigenic target unrelated to viral proteins. A dual regulation of antibody responses by gamma-interferon (IFN-γ) and interleukin-6 explains this isotypic bias (1, 2). 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) (3), 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 (4, 5). 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-γ or interleukin-4, which can selectively trigger B lymphocytes to produce IgG2a or IgG1, respectively.

Activation of natural killer cells

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

Within a few days after infection, a strong and transient NK cell activation, characterized by accumulation of this cell population in the spleen, by enhanced IFN-γ message expression and production, as well as by cytolysis of target cell lines was observed. Because NK cells and IFN-γ may participate in the defense against viral infection, we analyzed their possible role in the control of LDV titers, with a new agglutination assay. Our results indicate that neither the cytolytic activity of NK cells nor the IFN-γ secretion affect the early and rapid viral replication that follows LDV inoculation (6).

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 (7). 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. The specificity of the antibody response elicited in these two mouse strains differed markedly, with platelet glycoprotein Ib recognized in CBA/Ht, but not in BALB/C animals.

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 (8). 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 (9).

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

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 (10).

Macrophage activation by LDV led also to an enhanced response to lipopolysaccharide (LPS), and to an exacerbate susceptibility to endotoxin shock. A synergistic effect of LDV and LPS triggered dramatic production of tumor necrosis factor (TNF) and IFN-γ. Susceptibility to LPS shock was completely mediated by TNF, and partially by IFN-γ. In contrast, type I IFNs protected mice against the shock by controlling IFN-γ production, suggesting that two distinct pathways may lead to the production of the latter cytokine.

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SIGNAL TRANSDUCTION BY GROWTH FACTOR RECEPTORS

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Growth factors are soluble proteins that stimulate cell proliferation and migration through receptor tyrosine kinases. Our group is mainly interested in Platelet-derived growth factors (PDGF), which play an important role during wound healing and embryonic development. Four PDGF isoforms have been described (A, B, C and D), which bind with different affinities to two specific receptor tyrosine kinases, the PDGF α*- and* β*-receptors. Activated receptors phosphorylate a large number of substrates, including themselves, thereby initiating a complex network of signaling cascades (Fig. 1). Uncontrolled PDGF receptor activation is associated with brain, stomach, skin and hematological cancers. Our goal is to understand in detail (a) how PDGF receptors are activated, and (b) how signal transduction pathways regulate gene expression and, ultimately, cell growth.*

PDGF receptor activation

A. Nzokirantevye, A. Kallin, F. Chiara, J.B. Demoulin, in collaboration with C.H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden.

PDGF receptors have a unique long C-terminal domain. We isolated proteins binding to a peptide corresponding to the PDGF β-receptor C-terminus. Using mass spectrometry, these proteins were identified as the PDZ-domain containing adaptors NHERF and NHERF-2. NHERFs binding did not modify the receptor activity, but regulated the reorganization of the cytoskeleton by PDGF (1).

Using a panel of PDGF β-receptor mutants with progressive C-terminal truncations, we observed that deletion of the last 46 residues, which contain a Pro/Glu-rich motif, increased the activity of the receptor in the absence of ligand, compared to wild-type receptors (2). By contrast, the kinase activity of mutant and wild-type receptors that were pre-activated by treatment with PDGF was comparable. Using

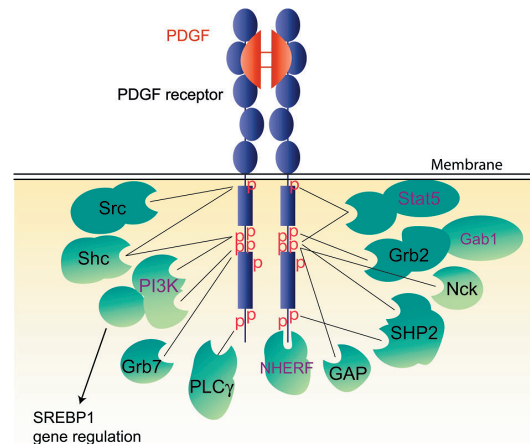

Figure 1. Signal transduction by the PDGF receptors. Signaling molecules of special interest for our group are indicated in purple. The receptor (blue) contains five extracellular immunoglobulin-like domains and an intracellular tyrosine kinase domain (boxes). p, phosphorylated tyrosine residue.

a conformation-sensitive antibody, we found that truncated receptors exhibited an active conformation even in the absence of PDGF. A soluble peptide containing the Pro/Glu-rich motif specifically inhibited the PDGF β-re-

ceptor kinase activity. Whereas deletion of this motif was not enough to confer ligand-independent transforming ability to the receptor, it dramatically enhanced the effect of the weakly activating D850N mutation in a focus formation assay. These findings indicate that allosteric inhibition of the PDGF β-receptor by its C-terminal tail is one of the mechanisms involved in keeping the receptor inactive in the absence of ligand.

In conclusion, we identified two distinct motifs in the PDGF receptor C-terminus: one binding to NHERF and one binding to and inhibiting the kinase domain (Fig. 2).

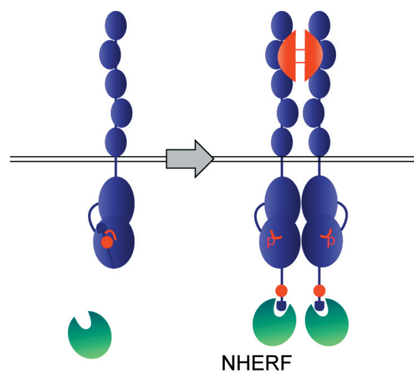

Figure 2. Role of the PDGF receptor C-terminal domain. In the inactive conformation, a Pro/Glu-rich acidic motif (red disk) blocks the kinase domain. Our data suggest that the C-terminal tail is released upon receptor activation and the NHERF binding site (blue disk) becomes accessible.

Gene regulation by PDGF and other growth factors

J.B. Demoulin, N. Dif, A. Kallin, C. Marbehant, A. Essaghir.

We analyzed the transcriptional program elicited by stimulation of normal human fibroblasts with PDGF using cDNA microarrays produced by the Sanger/Ludwig/CRUK consortium. We identified 103 significantly regulated transcripts that had not previously been linked to PDGF signaling. Among them, 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 β-receptor 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 (3). The role of SREBP in PDGF and tumor development will be further analyzed (8). Using Affymetric arrays, we obtained a more complete picture of gene regulation by PDGF. We are now focusing on genes regulated by transcription factors of the STAT, AP1 and forkhead families. We are also comparing gene regulation by PDGF receptors in normal cells and in tumor cells (5).

Gene expression profile of neural stem cells treated with FGF-2 or PDGF-AA

J.B. Demoulin, A. Essaghir, in collaboration with M. Enarsson and K. Forsberg-Nilsson, Biomedicum, and C.-H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden.

Understanding and controlling stem cell differentiation *in vitro* is critical for the development of stem cell-based therapies. The group of Karin Forsberg-Nilsson had previously shown that PDGF-AA stimulates the expansion of neuronal progenitors from neural stem

cells, but is unable to replace fibroblast-growth factor 2 (FGF-2) as a stem cell mitogen. In the present study, we compared gene expression in neural stem cells that were grown in the presence of FGF-2 to that of cells cultured with PDGF-AA or in the absence of growth factor, which induces differentiation. Most of the transcripts enriched in FGF-2-treated cells were connected to cell division (70%) and showed limited overlap with previously described stem cell transcriptional signatures. Interestingly, these regulated transcripts included the core set of genes that are periodically regulated during yeast mitosis, suggesting a conserved mechanism of regulation from yeast to mammals.

PDGF-AA significantly regulated 156 genes, 146 of which were also modified by differentiation in the absence of growth factor. The latter condition affected the expression of 153 additional genes which were regulated less than two fold by PDGF-AA. Among the genes induced by differentiation was the PDGF-A gene itself, suggesting that in the absence of exogenously added growth factors, cells make their own PDGF-AA in an autocrine manner. In addition, we identified several poorly characterized transcripts that were highly regulated and may play a role in stem cell differentiation. Our results suggested that the genetic program elicited by PDGF-AA was not unique, but intermediate between the ones of FGF-2-cultured stem cells and differentiated cells (5). This genomic study will help finding important genes involved in neural stem cell differentiation.

Rearrangements of the PDGF receptor genes associated with myeloproliferative diseases

F. Toffalini, C. Montana, A. Nzokirantevye, J.B. Demoulin, in collaboration with H. Poirel, Department of Hematology, Saint-Luc Hospital, UCL.

Although PDGF receptors are expressed on platelets and macrophages, deficient mice show no obvious 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 causes chronic malignant hemopathies, such as chronic eosinophilic leukemia, atypical chronic myeloid leukemia and chronic myelomonocytic leukemia. In all cases, the rearranged gene produces a hybrid protein comprising the PDGF receptor tyrosine kinase domain and an oligomerisation domain. Transfection of these constructs in Ba/F3 cells stimulated their proliferation, by contrast to wild type receptors. We and others have observed that PDGF receptor hybrids activate STAT5, an important transcription factor for Ba/F3 proliferation (6), whereas the wild-type PDGF receptors do not. Our group will further investigate why the wild-type PDGF receptors are unable to drive hematopoietic cell proliferation (even in the presence of PDGF) as opposed to these hybrid proteins.

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HUMAN TUMOR IMMUNOLOGY*

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*In association with the Ludwig Institute: see **Analysis of T cell responses of vaccinated cancer patients** (research at LICR, Brussels)

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 display 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 part 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 now 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 (1, 3, 4). One objective is to compare immunological adjuvants, another is to compare antigens.

A second part is to understand the mechanism of the tumor regressions that occasionally occur in vaccinated patients. The detailed analysis of one such patient 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 (6, 7). Why these anti-tumor T cells become activated following vaccination with antigens that they do not recognize is not clear.

That local immunosuppression could be involved in preventing tumor rejection is compatible with the observation that the anti-tumor T cells mentioned above are systematically present in tumors already before vaccination. Considering that suppressive or so-called regulatory T cells are recognized as important attenuators of immune responses, we have initiated an analyzis of their role in the vaccinated patients.

Monitoring anti-vaccine T cell responses in melanoma patients vaccinated with defined tumor-specific antigens

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We focused on the analysis of CD8 T cell responses, mostly to antigenic peptides presented by HLA-A1 or A2 molecules.

Several small clinical trials have been performed with the MAGE-A3 antigenic peptide EVDPIGHLY, presented by HLA-A1 (2, 9). Table 1 presents a summary of the results obtained in patients who showed evidence of tumor regression and in patients who did not, after vaccination with ALVAC-MAGE, a recombinant poxvirus containing a minigene encoding the MAGE-3.A1 peptide, with dendritic cells loaded with the peptide (G. Schuler, Erlangen and K. Thielemans, Vrije Universiteit van Brussel), or with the MAGE-3.A1 peptide alone. The observed correlation between CTL responses and tumor regression supports the notion that the rejection is caused by the vaccine (4).

Currently, we are analyzing patients included in vaccination trials involving several peptides presented by HLA-A2 molecules. Two immunological adjuvants are compared: Montanide ISA51, a clinical grade incomplete Freund's adjuvant, and CpG7909, a ligand for Toll-like receptor 9. Four different antigenic peptides are combined with Montanide, and 8 with CpG. For each patient, blood frequencies of T cells against each individual peptide are measured. Preliminary results indicate a hierarchy in the immunogenicity of these peptides.

Table 1. Summary of anti-MAGE-3.A1 CTL responses in vaccinated melanoma patients.

	CTL response in patients with	
Vaccination mode	**evidence of tumor regression**	**no evidence of tumor regression**
ALVAC-MAGE	3/4	1/11
Dendritic cells + peptide MAGE-3.A1	3/3	0/3
Peptide MAGE-3.A1	1/7	0/13
	7/14	1/27

Tumor regressions observed after vaccination: a possible 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.

It is clear from Table 1 that several vaccinated melanoma patients displayed tumor regression in the absence of a detectable anti-vaccine CTL response. In addition, even among those vaccinated patients who showed a CTL response, most had a low frequency of anti-MAGE-3.A1 CTL in the blood, ranging between 10^{-6} and 10^{-5} of CD8 T cells. Because we felt that such a level of CTL might be insufficient to produce on its own the observed tumor regressions, we examined the possibility that CTL directed against other antigens present on the tumor might contribute to the regression. For seven vaccinated melanoma patients, selected because it had been possible to derive a per-

manent cell line from their tumor cells, we estimated the blood frequencies of CTL directed against any antigen present on the tumor cells. For all seven patients, anti-tumor CTL were found at high frequencies, i.e. from 10^{-4} to 3×10^{-3} of the CD8 T cells, 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 considerably higher than that of the anti-vaccine CTL, ranging from 12 fold to 20,000 fold higher (6).

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 (5, 6). 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.

To evaluate the potential contribution of the "anti-tumor" T cells to the tumor rejection that occured following vaccination, we measured the frequency of the anti-vaccine and anti-tumor T cells in metastases of patient EB81. The frequency of anti-MAGE-3.A1 T cells was 2.5×10^{-6} of CD8 T cells in the blood and it was 6-fold higher in a metastasis. An anti-tumor CTL recognizing an antigen encoded by *MAGE-C2* showed a considerably higher enrichment: its blood frequency was 9×10^{-5}, and it was about 1,000 times higher in the tumor. Several other anti-tumor T cell clonotypes also had frequencies above 1% and appeared to constitute the majority of the T cells present in metastases (7). 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.

Specificity of a T cell clone infiltrating a melanoma metastasis that regressed following vaccination.

V. Corbière in collaboration with W. Ma, J. Chapiro, B. Van den Eynde, Brussels branch of the Ludwig Institute for Cancer Research

The repertoire of TCR sequences present in regressing metastases from patient EB81 indicated the presence of several highly repeated clonotypes, with the two most frequent clonotypes representing each 3-9% of all TCRß sequences present (7). One of the two clonotypes belonged to a tumor-specific CTL clone found in post-vaccination blood and recognizing a MAGE-C2 peptide presented by HLA-A2 molecules (5).

The other clonotype, named TCR 101, did not correspond to any of the anti-tumor T cell clones that were derived from the blood. Considering the high proportion of tumor-infiltrating T lymphocytes that expressed this TCR, we surmised that it corresponded to a tumor-specific T cell clone. The identification of the target antigen of these T cells required first to derive a stable T cell clone expressing TCR 101. This was achieved by deriving a collection of T cell clones from tumor-infiltrating lymphocytes that had been frozen immediately after surgery, and screening the clones with a PCR amplification that was specific for TCR 101. A stable T cell clone could eventually be derived that expressed this receptor. This $CD8^+$ clone displayed lytic activity and showed specificity for the autologous melanoma cells. Using a cDNA library prepared with mRNA extracted from the tumor cells, we found that the antigenic

peptide, presented by HLA-A2 molecules, was encoded by a mutated sequence in the gene coding for caseinolytic protease, a mitochondrial enzyme. Lysis of the tumor cells by the CTL was strongly increased by IFN-γ, and this appeared to result from a preferential processing of the antigenic peptide by the immunoproteasome. These results strengthen the notion that tumor-specific CTL present in melanoma metastases that regress following vaccination do not recognize vaccine antigens but other tumor-specific antigens. The local production of IFN-γ may shape the repertoire of these CTL by favoring the presentation of antigenic peptides processed by the immunoproteasome.

Functional analysis of tumor-specific T cell clones

T. Connerotte, P.G. Coulie

As a first step in the analysis of the functional properties of anti-vaccine CTL, we have compared the cytokine secretion profiles of anti-MAGE-3.A1 CTL clones derived from patients vaccinated either with peptide, ALVAC-MAGE, or dendritic cells pulsed with the peptide. Somewhat surprisingly, most CTL clones found after vaccination with dendritic cells produced IL-10, whereas those found after peptide or ALVAC did not. Gene expression profiling of a set of CTL clones indicated that the CTL clones that expressed the *IL-10* gene expressed also the genes coding for prostaglandin D2 synthase and the costimulatory molecule CD40L. The CTL clones that did not express IL-10 expressed the genes coding for granzymes K and H, which are found in the lytic granules of most CTL and whose functions are not clear. These results suggest that different modalities of immunization generate different types of effector cells. We will analyse more patients in order to test whether the presence of anti-vaccine T cells that secrete IL-10 is associated with tumor regression or progression, and try to analyse the production of IL-10 by anti-vaccine T cells *in vivo*.

Clonal analysis of regulatory T cells from cancer patients

S. Lucas, J. Stockis, T. Aerts, P.G. Coulie

Regulatory T cells, or T_{regs}, are a subset of $CD4^+$ lymphocytes specialized in the suppression of immune responses. Their existence was initially revealed by their ability to prevent the development of auto-immune diseases in mice. It has long been proposed that T_{regs} could play a negative role in cancer patients, by inhibiting spontaneous or vaccination-induced anti-tumor immune responses. Our long term objective is to develop tools to test whether anti-tumor T cells in cancer patients are under the negative influence of T_{reg} cells. Because the mechanisms by which T_{reg} cells inhibit immune responses are still poorly understood, we first attempted to identify genes important for the function of human T_{regs}. We derived a panel of T_{reg} clones from lymphocytes infiltrating a melanoma metastasis. A large diversity of T cell receptors was found among these T_{reg} clones, a small minority of which were directed against tumor antigens.

Microarray analysis lead us to the identification of 46 genes differentially regulated in activated T_{reg} clones compared to conventional $CD4^+$ T cell clones. Among these T_{reg} specific genes, *SOX4* and *ID3* encode transcription factors whose overexpression in activated T_{regs} was confirmed by quantitative RT-PCR analysis. Retrovirally-mediated overexpression of *SOX4* and *ID3* in conventional T cell clones did not lead to the acquisition of a suppressive phenotype in vitro. However, it appeared that both *SOX4* and *ID3* expression in conventional T cells is induced by TGFβ signalling. We postulated that overexpression of *SOX4* and *ID3* in activated T_{regs} resulted from autocrine TGFβ signalling in these cells. To test this hypothesis, we used microarrays to analyze gene expression profiles of TGFβ-treated CD4+ T cell clones, and of $CD4^+$ T cell clones cocultured with T_{reg} clones, which we refer to as “suppressed” T cells. This analysis revealed important similarities between the T_{reg}-speci-

fic, TGFβ-specific and suppression-specific gene signatures in CD4+ T cell clones. We are now focusing on the consequences of TGFβ signalling in human T cells. We have observed that some human T cells are resistant to the inhibitory effects of TGFβ measured in vitro. We are currently trying to understand what underlies the resistance or sensitivity to TGFβ in human T cells. Resistance to TGFβ could modulate the efficiency of anti-tumor immune responses.

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DNA DAMAGE REPAIR AND TELOMERE MAINTENANCE

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Cellular DNA is constantly subjected to damage. Genotoxic lesions include DNA double-strand breaks (DSBs) which can be caused either by external agents such as ionizing radiations or by physiological cellular processes such as V(D)J recombination in the immune system or meiosis. In dividing cells, DNA replication provides another major source of DSBs. Cells from all organisms have evolved several mechanisms to re-seal DSBs as proper repair of chromosome breaks is necessary to prevent genomic rearrangements, a hallmark of cancer cells, or cell death. DNA repair mechanisms have been well conserved throughout evolution and yeast has proven to be a good model for their study.

In the absence of any protection, extremities of linear chromosomes, like human chromosomes, would also be recognized as DSBs by the cell and subjected to DNA repair, resulting in chromosomal fusions. To circumvent this problem, chromosomal extremities are protected by specialized DNA-protein complexes, the telomeres. Synthesis of telomeric DNA repeats requires the ribonucleoprotein enzyme telomerase in replicating cells. In most adult cells, telomerase gene expression is switched off as cells have stopped dividing. Hence, proliferation of cancer cells requires the re-establishment of a telomere maintenance mechanism. In about 80 % of the cases, this is achieved by re-activation of the telomerase gene during tumorigenesis while 20 % of cancer cells rely on telomerase-independent mechanism(s) to maintain their telomeres.

DNA damage repair in fission yeast Schizosaccharomyces pombe

A. Decottignies

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). From yeast to mammals, different studies have reported the insertion of DNA fragments of various sources at experimentally-induced DSBs, including mitochondrial DNA (mtDNA) in budding yeast and repetitive DNA in mammalian cells. 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 budding yeast and human nuclear genomes revealed the presence of nuclear sequences of mitochondrial origin (NUMTs) in chromosomes. Similarly, the S. pombe nuclear genome comprises 33 NUMTs (22-358 bp-long) [1], a value close to that reported in budding yeast. For comparison, it has been reported that the human nuclear genome comprises between 211 and 612 NUMTs, depending on the threshold values used for BLAST analysis. Hence,

it appears that capture of mtDNA fragments at naturally occurring DSBs took place during evolution in eukaryotic cells, remodeling the nuclear genome. Careful analysis of NUMT distribution in both budding and fission yeast nuclear genomes revealed a preferential insertion of NUMTs into gene promoters (60-70% of NUMTs). Together with the recent discovery that DSBs are involved in regulated gene transcription through topoisomerase IIb action, this raises the hypothesis that NUMT formation may be coupled to gene transcription. This is currently under investigation.

A new simple extrachromosomal DSB repair assay in fission yeast revealed that DSB repair is associated with the capture of endogenously produced mtDNA fragments in nearly 30 % of the events [1], supporting the hypothesis that DSB repair is a universal mutagenic mechanism responsible for the insertion of linear mtDNA molecules into chromosome breaks, providing a novel mechanism of human inherited disease. A series of yeast mutants revealed that mtDNA insertion at DSBs is dependent on the NHEJ machinery and the evolutionary conserved Mre11 complex [1]. Strikingly, capture of mtDNA fragments is highly increased in yeast cells grown to stationary phase [1], a condition resembling most of the cells of multicellular organisms since: 1) most energy comes from mitochondrial respiration and 2) cells have exited from the cell cycle, i.e., have entered the G0 phase. Carboxypeptidase Y is required for the increased capture of mtDNA at DSBs in stationary phase yeast cells, suggesting that vacuolar turnover of mitochondria may be involved. Moreover, tubular network of mitochondria is lost in stationary phase and mitochondria fluorescence localizes to cytoplasmic spots, a fraction of which being enriched around the nucleus [1], raising the hypothesis that this may help the transfer of nucleic acids from mitochondria to the nucleus.

The same extrachromosomal DSB repair assay in fission yeast was used to investigate genetic requirements for microhomology-mediated end- joining (MMEJ), a DNA repair process poorly characterized so far. Construction of a series of yeast mutants revealed that MMEJ is a DNA repair pathway related to homologous recombination [2]. Specifically, MMEJ was found to require rad22 and exo1, two genes implicated in the single-strand annealing mechanism of HR. Mismatch repair genes are also involved in the process. Finally, the study investigated the critical number of microhomologous nucleotides required for efficient MMEJ as well as the distance between DSB end and the microhomologous region [2].

Alternative mechanism(s) of telomere maintenance

G. Tilman, M. Mattiussi, A. Decottignies

Activation of a telomere maintenance mechanism is indispensable for the immortalization of human cells. Most cancer cells maintain their telomeres via telomerase activation. 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). Hence, successful telomere-targeted anticancer therapy might require the inhibition of both telomerase and ALT mechanisms. ALT cell lines can also be obtained after in vitro immortalization of telomerase-negative human fibroblasts with SV40 T antigen. Such a procedure has indeed been reported to give an average of 40 % ALT and 60 % TEL+ survivors following crisis which occurs after about 80 population doublings, when telomeres have reached a critical length (Fig. 1). These two pathways of telomere maintenance are very distinct phenotypically (Fig. 2). In telomerase-expressing cells (TEL+), telomere length is very homogenous (around 5 kb) and telomeres are found at the end of all chromosomes. However, in ALT cells, telomeres are very heterogeneous, ranging from 0 to 50 kb and some chromatids lack telomeres (Fig. 2). In addition, hTERT has been reported to play non-canonical roles in the cell, including modulation of the expression of ge-

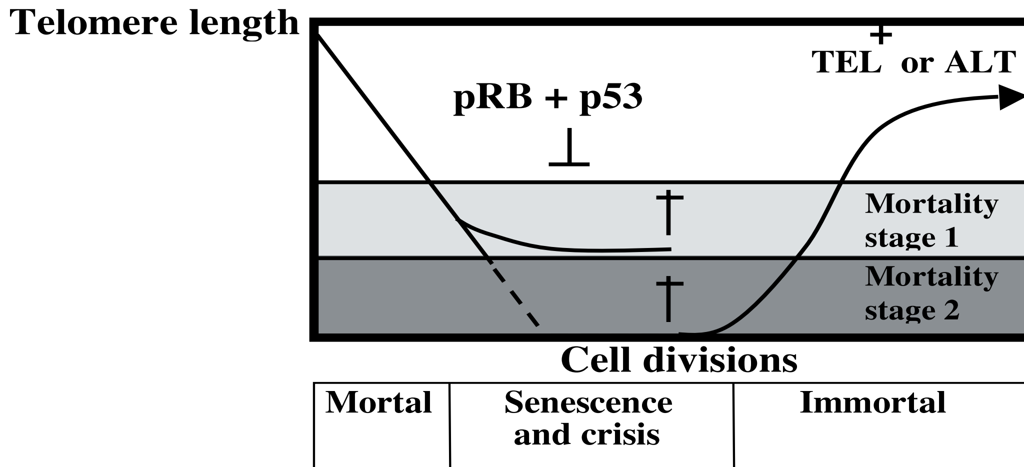

Figure 1. Telomere length in the course of cellular transformation. In telomerase-negative cells, telomere length decreases with continuous replication. At a critical telomere length, cells enter M1 through the action of p53/pRb and eventually die. Upon inactivation of p53/pRb, cells continue to divide and enter M2 characterized by huge genomic instability. A small percentage of cells survive by re-activation of a telomere maintenance mechanism.

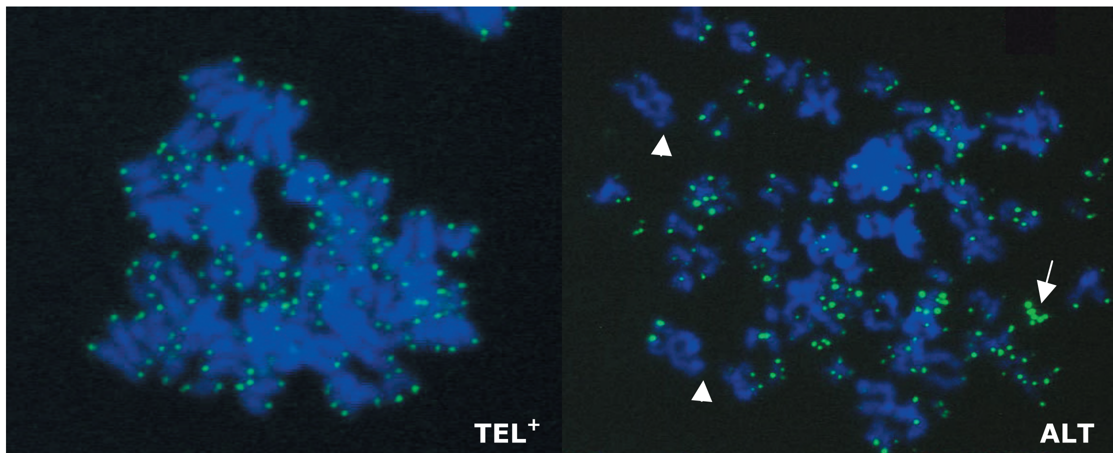

Figure 2. Telomere-specific fluorescence in situ hybridization (FISH) on metaphase chromosomes of telomerase-positive and ALT cancer cells. Telomeres are hybridized with a fluorescent telomeric probe (green) and DNA is stained with DAPI (blue). In ALT cells, telomeres are very heterogeneous and not always present at chromosome ends (arrowheads). ALT cells are further characterized by the presence of extrachromosomal telomeric DNA (arrow).

nes implicated in tumorigenesis, through mechanisms that are still largely unknown.

Our study aims to identify genes that are distinctly regulated in ALT and TEL+ cells. To achieve this goal, we compared the global gene expression profiles of ALT and matching TEL+ human immortalized fibroblasts by RDA (Representational Difference Analysis). We identified a series of genes showing distinct expression levels in ALT and TEL+ cell lines. Genes with higher expression in TEL+ cells

included periostin, COL6A3, fibronectin and ACTG2 characterized by pro-tumorigenic properties. On the opposite, the expression of tumor-suppressor gene DAL1 and IGSF4C was higher in ALT cells. We are currently investigating the effect of telomerase on the expression of these genes by either introducing hTERT/hTR genes in telomerase-negative cells or by reducing hTERT/hTR expression in TEL+ cells. In addition, we are investigating the effect of telomere erosion on gene expression.

Our work also focused on the distribution of POSTN periostin gene expression in both normal and tumoral tissues. POSTN encodes a secreted protein which, through binding to aVb3 or aVb5 integrins, promotes metastatic growth, angiogenesis and cell motility. Our results suggest that periostin expression may be mediated almost exclusively by stromal fibroblasts [3]. We are currently investigating the factors that modulate periostin gene expression in fibroblasts.

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Ludwig Institute for Cancer Research (LICR)

Brussels branch

The Ludwig Institute for Cancer Research

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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TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

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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. In the second example of spliced peptide, which is a minor histocompatibility antigen, the two fragments are rearranged before splicing.

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.

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.*

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.*

Differential processing of tumor antigens by standard proteasomes and the immunoproteasomes and intermediate proteasomes

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 (IFNγ).

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). This could explain how certain potentially autoreactive CTL can escape tolerance induction in the thymus and fail to be activated in the periphery. We have now extended those observations to several antigenic peptides of interest for cancer immunotherapy, including HLA-A2-restricted epitopes derived from tyrosinase, Melan-A^{MART1} 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 therefore are presented to CTL only by tumor cells pre-treated with IFNγ (2). By analysing 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). The peptide repertoire of tumor cells themselves may vary according to the localization of the tumor (e.g. primary tumor versus lymph node metastasis) and its level of exposure to IFNγ. It is therefore essential to study those processing differences in detail, so as to define the most effective vaccination strategy for each epitope and to use the appropriate combination of antigens in order to minimize the risk of tumor escape by proteasome switching.

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. We are currently characterizing these intermediate proteasomes, in terms of function and expression.

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. Together with the previous description of a peptide produced by protein splicing of FGF-5, this is the third example of antige-

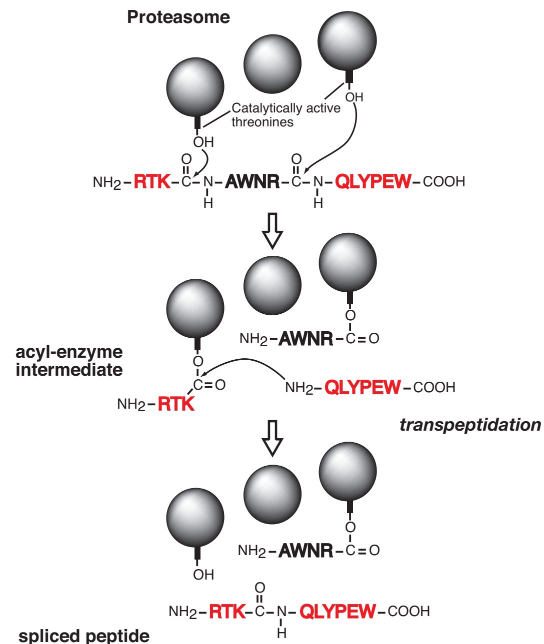

Figure 1. Model of the peptide-splicing reaction in the proteasome. The active site of the catalytic subunits of the proteasome is made up of the side-chain of a threonine residue, which initiates proteolysis by performing a nucleophilic attack on the carbonyl group of the peptide bond. An acyl-enzyme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

nic peptide produced by splicing. 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.

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-germ-line 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).

A novel tumor immune escape mechanism based on tryptophan degradation by indoleamine 2,3 dioxygenase

C. Uyttenhove, L. Pilotte, I. Théate, D. Donckers, N. Parmentier, V. Stroobant, D. Colau

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.

An inducible mouse model of melanoma expressing a defined tumor antigen

C. Hervé (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 germ line 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 human 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. In parallel, we have developed a strain of mice transgenic for the P1A-specific T cell receptor, which will be useful for such studies.

Physiopathology of systemic lupus erythematosus (SLE)

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. From a biological point of view, the disease is characterized by overt polyclonal B cell activation and CD4 T cell-driven production of specific autoantibodies directed against constituents of the chromatin. These antibodies (in particular the double-stranded DNA antibodies) are pathogenic and associated with the most severe manifestations of the disease. In order to better understand the underlying molecular pathways, we performed analyses of global gene expression on sorted CD4 T and B cells from SLE patients as compared to controls and patients with rheumatoid arthritis (RA), using Genechip U133 Plus 2.0 arrays. We also performed similar experiments on synovial tissue from SLE patients with arthritis. We found the presence of a strong type 1 interferon signature in SLE samples, i.e. the presence of numerous interferon-induced genes, as previously observed in SLE PBMC by other groups. We are currently investigating the physiopathological pathways that are dysregulated by the over-expression of these genes, using PBMC from patients and animal models of the disease.

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TUMOR GENETICS GROUP

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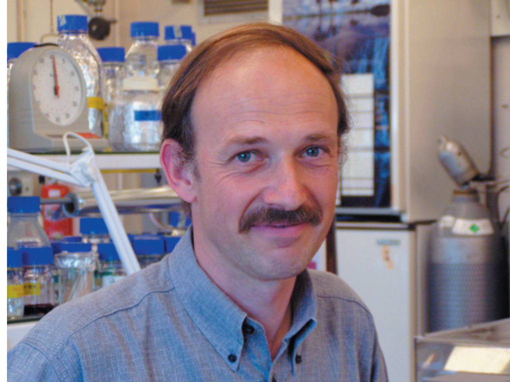

Human tumors express specific antigens arising from the activation of genes, such as MAGE, BAGE, GAGE and LAGE/NY-ESO1, which are normally expressed only in germ cells. As germ cells are not subject to scrutiny by the immune system, antigens encoded by these genes are strictly tumor-specific. The group of Etienne De Plaen and Charles De Smet has identified new genes that are specifically expressed in tumors and in germ cells. Most of these genes have their normal site of expression in spermatogonia, the pre-meiotic stage of sperm development, and are located on the X chromosome. Efforts are now devoted to deciphering the mechanism leading to the activation of "cancer-germline" genes in tumor cells and determining their function.

Charles De Smet and his group are studying the mechanisms leading to the activation of "cancer-germline" genes in tumors. It was previously shown by the group that these genes rely primarily on DNA methylation for their repression in normal somatic tissues, and that their activation in tumors is a consequence of the overall genome demethylation process that often accompanies tumorigenesis. Stable activation of cancer-germline genes in tumors does not require a permanent demethylating activity, but depends on the presence of specific transcription factors that maintain the promoter region unmethylated (1). Antisense-mediated knock-down experiments indicated that DNMT1 is the primary DNA methyltransferase to maintain methylation of cancer-germline genes (2), and that transient down-regulation of this enzyme suffices to induce stable activation of cancer-germline genes. This is supporting the view that hypomethylation of these genes in tumors results from a historical event of demethylation (2). The group is now trying to identify factors that induce DNA demethylation. Embryonic stem cells, which appear to have a demethylating activity, are currently tested as a potential source for the identification of such factors.

To analyze the functions of a MAGE protein, MAGE-A1, Etienne De Plaen and his group searched to identify binding partners of this protein. Using yeast two-hybrid screening, they found interaction between MAGE-A1 and transcriptional regulator SKIP (3). SKIP is an adaptor protein that connects DNA-binding proteins to proteins that activate or repress transcription. Their results suggest that by binding to SKIP and by recruiting histone deacetylase 1, protein MAGE-A1 present in the nucleus represses transcription. In addition, the group has observed an interaction between MAGE-A1 and DNA methyltransferases (Dnmt). Since recruitment of Dnmt3a by the Myc transcription factor has been shown to repress the p21Cip1 promoter (4), the group is now trying to evaluate whether promoters could be repressed by MAGE-A1 in the presence of Dnmt.

Finally, in collaboration with Nicolas Van Baren and Francis Brasseur, the group is presently analyzing the molecular mechanisms by

which IFN-γ, TGF–β and TNF–α inhibits the expression of melanocyte differentiation genes. Data indicate that a soluble autocrine factor is released by the IFN-γ-treated melanoma cells. The group is in the process of isolating and characterizing this factor.

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IDENTIFICATION OF HUMAN TUMOR ANTIGENS

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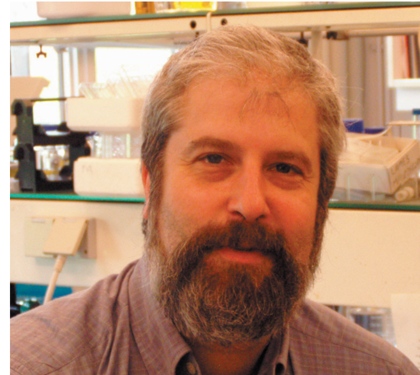

The group led by Pierre van der Bruggen has defined antigenic peptides encoded by genes such as those of the MAGE family. These peptides have been used in therapeutic vaccination trials of cancer patients and have served as tools for a reliable monitoring of the immune response of vaccinated patients. Efforts have been devoted to set up assays that accurately monitor CD4+ T cell responses to cancer vaccines. For patients vaccinated with a protein, we have validated a quantitative approach to isolate anti-vaccine T cells directed at all possible HLA-peptide combinations that could be targeted by the response. For patients vaccinated with a peptide, we have validated the use of the first HLA-DP4 tetramer, which was folded with a MAGE-3 peptide. By screening blood cells of vaccinated patients with this tetramer, we found that injections of the MAGE-3.DP4 peptide resulted in the activation and proliferation of specific T cells with various cytokine profiles, including IL-10-producing T cells and regulatory T cells. The group is currently involved in the study of functional defects of T cells. We have observed that human CTL clones lose their specific cytolytic activity and cytokine production under certain stimulation conditions. These inactive CTL simultaneously lose their labeling by an HLA-peptide tetramer, even though the amount of TCR-CD3 at their surface is not reduced. These results suggest the existence of a new type of functional defect of CTL.

New MAGE antigens recognized by CD8+ and CD4+ T cells

S. Ottaviani, Z. Sun, V. Stroobant

"Cancer germline" 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 encode shared tumor specific antigens, which have been used in therapeutic vaccination trials of cancer patients. The first antigens and genes that code for these antigens were identified with anti-tumor cytolytic T lymphocytes obtained from cancer patients (1). A few HLA class I-restricted antigenic peptides were identified by this "direct approach". A large set of additional cancer-germline genes have now been identified by purely genetic approaches. As a result, a vast number of sequences are known that can code for tumor-specific shared antigens, but most of the encoded antigenic peptides have not been identified yet. The identification of a large number of antigenic peptides presented by HLA class I and class II molecules is likely to be important for the future of clinical trials with defined antigenic peptides. A large set of peptide/HLA combinations will alleviate HLA restriction and widen the set of eligible patients. It will also facilitate the design of concurrent

immunizations against several antigens. Such immunizations could increase the primary antitumor efficacy of the vaccine.

We have used approaches that we have loosely named «reverse immunology» (2). They aim at identifying antigenic peptides recognized by T cells, using gene sequences as starting point. We have focused this search on the cancer-germline genes, which are expected to code for tumor-specific shared antigens on the basis of their pattern of expression.

Search for antigenic peptides recognized by CD8⁺ T cells

In a first approach, we stimulated $CD8^+$ T lymphocytes with dendritic cells transduced with viral vectors containing complete MAGE-coding sequences. As this requires the processing of the antigen by the dendritic cells, we surmised that the peptides that would be identified would also be processed in the tumors expressing the *MAGE* genes. A difficulty of the use of recombinant viruses is the activation of CTL precursors directed against viral antigens. We circumvented this problem by using different vectors for the stimulation of the microcultures, for the lytic assay with the responder T cells, and for the cloning step. This procedure is summarized in Fig. 1. Dendritic cells were infected with either an adenovirus or a canarypoxvirus, and they were used to stimulate microcultures of autologous $CD8^+$ T lymphocytes (3). After three weekly stimulations, the responder cells were tested for lysis on autologous EBV-B cells infected with vaccinia-MAGE. Positive microcultures were cloned. To identify the antigenic peptide, the resulting CTL clone was tested for lysis of autologous EBV-B cells pulsed with a complete set of peptides of 16 amino acids that overlap by 12. When a peptide scored positive, shorter peptides were synthesized to identify the shortest optimal peptide. To identify the HLA presenting molecule, the CTL clones were tested for stimulation by cells transfected with the MAGE cDNA together with cDNAs coding for the possible HLA presenting molecules. Finally, relevant tumor targets were used in a lysis assay to ascertain that the antigen was also processed by tumor cells.

In a second approach, a large number of

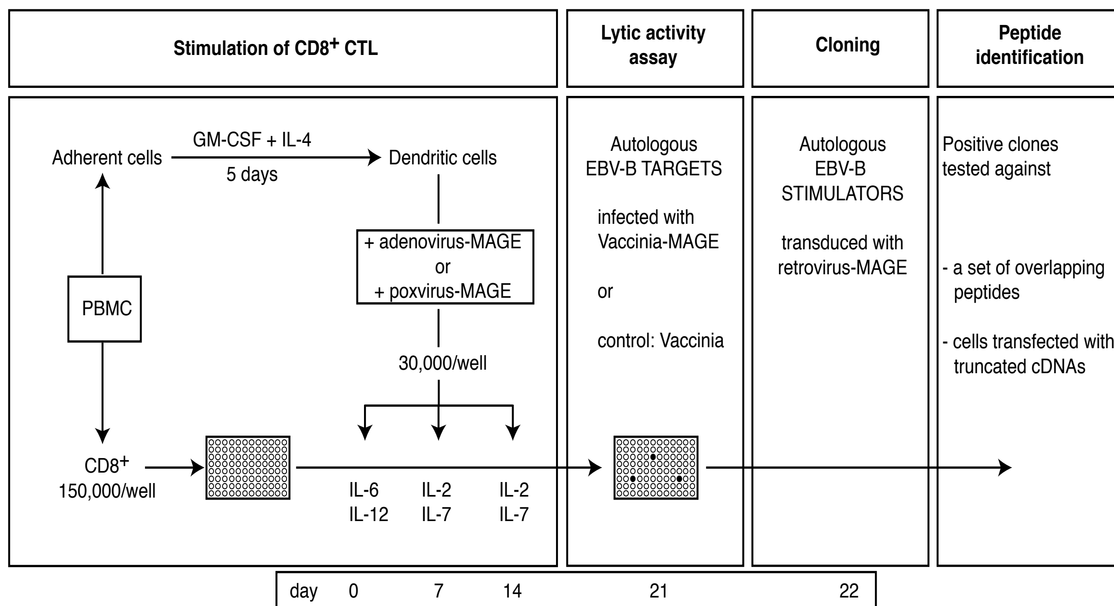

Figure 1. Overview of the procedure to obtain anti-MAGE CD8⁺ CTL clones by stimulation with dendritic cells infected with viral vectors carrying a MAGE coding sequence.

T cells are incubated with HLA/peptide multimers conjugated to phycoerythrin (PE), and with anti-PE antibodies coupled to magnetic beads. The multimer-positive cells are first enriched by magnetic sorting. The selected cells are distributed in microwells and stimulated with peptide-pulsed autologous mature dendritic cells on days 0 and 7. The microcultures are screened on day 19 for the presence of cells specifically labeled with multimers (Fig. 2). Relevant tumor targets were used in a lysis assay to ascertain that the antigen was also processed by tumor cells (4).

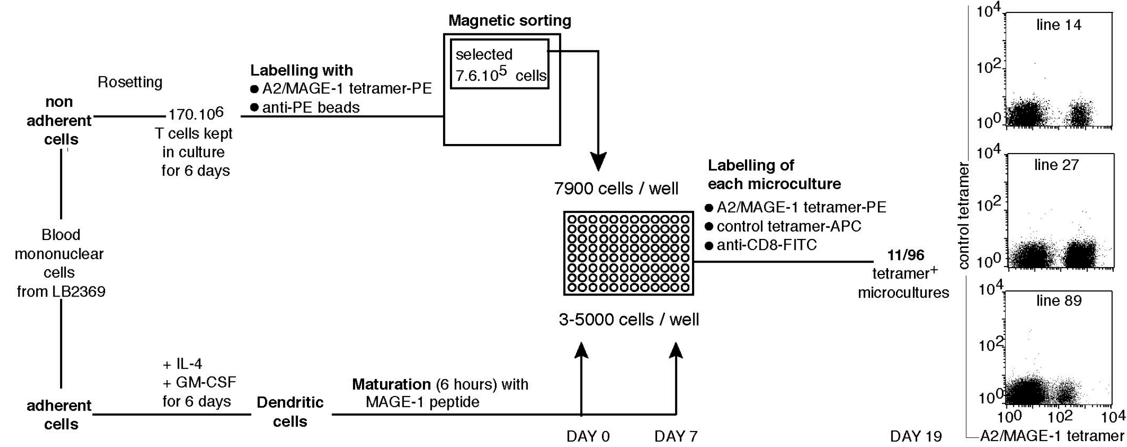

Figure 2. Overview of the procedure using HLA-peptide fluorescent multimers to isolate rare anti-MAGE-1 CD8+ CLTL clones.

Search for antigenic peptides recognized by CD4+ T cells

Studies in several animal models have demonstrated an important role for CD4+ T cells in inducing and maintaining anti-tumor immunity. It is therefore possible that the addition of antigenic peptides presented by class II to those presented by class I will improve the efficacy of therapeutic anti-tumor vaccination. To identify new HLA-peptide combinations, we used dendritic cells loaded with a recombinant MAGE protein to stimulate autologous CD4+ T lymphocytes (5). After four weekly stimulations, the responder cells were tested for their ability to secrete IFN-γ upon stimulation with the antigen, and the positive microcultures were cloned.

To identify the antigenic peptide, the positive clones were stimulated with a set of peptides of 16 amino acids that overlapped by 12 and covered the entire MAGE protein sequence. The positive peptide was then tested for recognition on several Epstein-Barr virus immortalized B cell lines (EBV-B cell lines) to identify the HLA presenting molecule.

MAGE-1 and MAGE-3 antigenic peptides identified by these procedures are listed in a database (http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm).

Study of the significance of the CD45RA expression on memory and effector CD8+ T cells.

P. van der Bruggen (in collaboration with J. Carrasco, D. Godelaine, A. Van Pel)

The expression of CD45RA on CCR7− human CD8+ memory T cells is widely consi-

dered to be a marker of terminal differentiation. We studied the time course of CD45RA and CCR7 expression on human antitumoral CTL clones and blood $CD8^+$ T cells after antigenic stimulation. Our results indicate that $CD45RA^+CCR7^-$ $CD8^+$ T cells are resting memory cells which, upon antigenic stimulation and during the next 10 days, proliferate, lose CD45RA and transiently acquire CCR7. In the absence of further antigenic stimulation, they progressively re-express CD45RA and become $CD45RA^+CCR7^-$ (6). We conclude that the expression of CD45RA on these cells is indicative of the time elapsed since the last antigenic stimulation rather than the incapacity to proliferate or a particularly high lytic potential. This concept leads to a reinterpretation of the significance of the presence of $CD45RA^+$ $CD8^+$ memory cells in patients affected by viral infections or by cancer.

Detection of CD4 T cell response in vaccinated cancer patients

D. Colau, V. François, S. Ottaviani

Patients injected with a protein

Immunizing patients with a MAGE-3 recombinant protein ought to induce T cell responses against several MAGE-3 peptides, including peptides recognized by CD4 T cells, and this might result in a more effective anti-tumor response. Moreover, protein vaccination alleviates the need to select patients according to their HLA, as many peptides presented by various HLA alleles are expected to be presented.

Quantitative evaluation of T cell responses of patients receiving anti-tumoral vaccination with a protein is difficult because of the large number of possible HLA-peptide combinations that could be targeted by the response. To evaluate the response of patients vaccinated with protein MAGE-3, we have developed an approach which involves overnight stimulation of blood T cells with autologous dendritic cells loaded with the protein, sorting by flow cytometry of the T cells that produce IFN-γ, cloning of these cells, and evaluation of the number of T cell clones that secrete IFN-γ upon stimulation with the antigen (8).

Patients injected with class II-restricted peptides

For therapeutic vaccination trials, the vaccine can consist of defined antigenic peptides. This approach greatly facilitates the monitoring of the T cell response, because the presumed target of the T cells is completely defined. It allows the use of HLA-peptide tetramers to detect T cell responses in patients. Although the production of soluble MHC class I multimers has rapidly expanded since their first use in 1996, the development of MHC class II complexes has proved to be more difficult probably due to the intrinsic structural instability of soluble class II molecules. We have developed a very sensitive approach using fluorescent HLA class II/peptide multimers to detect antigen specific CD4 T cells in cancer patients injected with vaccines containing the MAGE-$3_{243-258}$.DP4 peptide.

Production of HLA-DP4 multimers loaded with the MAGE-$3_{243-258}$ peptide

Dimers of soluble DP4.MAGE-3 complexes were produced in Drosophila S2 cells (Fig. 3A). The DPA1*0103 α and DPB1*0401 β chains were truncated to remove their transmembrane and cytosolic regions. Acidic and basic leucine zipper peptides were added at the C-terminus of the α and β chain respectively, to allow pairing of the chains. The MAGE-3 antigenic peptide was covalently linked to the N-terminal end of the β chain. The α chain was further modified by the addition of the murine IgG2a Fc domain to allow the dimerizaton of the DP4.MAGE-3 complex and further purification of the dimer by affinity chromatography. Fluorescent multimeric complexes (Fc Ig multimers) were produ-

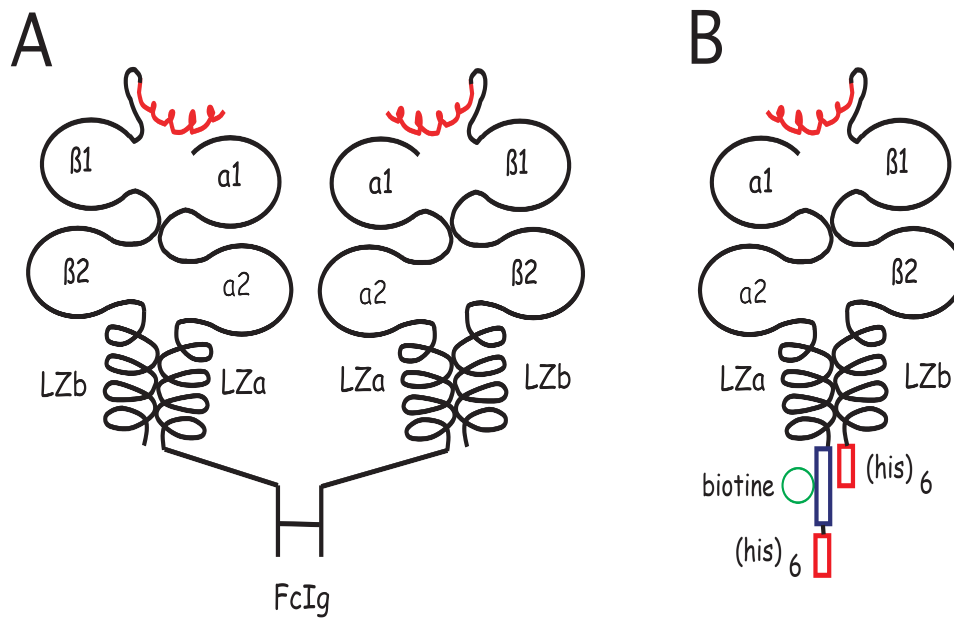

Figure 3. Schematic structures of DP4.MAGE-3 complexes.

ced by the addition of a biotinylated anti-Fc IgG2a antibody and PE labeled streptavidin. We also set up the construction of multimers from biotinylated DP4.MAGE-3 monomers (Fig. 3B). The structure of the DP4.MAGE-3 monomers was similar to the DP4.MAGE-3-FcIg construct with the following differences: the murine FcIg portion of the recombinant α chain, was exchanged for a sequence of six histidines and a tag for biotinylation followed by six histidines was fused to the C-terminus of the recombinant β chain. Monomers were produced in Drosophila S2 cells and purified by metal affinity chromatography. Fluorescent complexes (birA multimers) were obtained by the addition of PE labeled streptavidin. The specificity of the two multimers was demonstrated by staining of anti-MAGE-3.DP4 $CD4^+$ clones. Non-specific binding of the multimers to PBLs was evaluated on lymphocytes from a non cancerous $DP4^+$ patient. Staining with the DP4.MAGE-3 birA multimers showed a 5-fold lower level of non-specific binding (0.01% of the CD4 cells) in comparison to the DP4.MAGE-3-FcIg multimers (0.05%).

Analysis of the anti-MAGE-3.DP4 CD4 T cell responses in vaccinated cancer patients.

DP4.MAGE-3 birA multimers were used to stain *ex vivo* peripheral blood lymphocytes from melanoma patients injected with dendritic cells pulsed with several HLA class I and class II tumor antigenic peptides, including the $MAGE\text{-}3_{243\text{-}258}$ peptide (7). The multimer positive CD4 T cells were sorted and amplified in clonal conditions. Specificity of the clones was assessed by their ability to secrete cytokines or to upregulate activation markers upon contact with the MAGE-3 antigen. Using this approach, low frequencies of about $1x10^{-6}$ anti-MAGE-3.DP4 CD4 cells among CD4 cells could be detected.

Several patients injected with the MAGE-3.DP4 peptide, with or without adjuvant, or pulsed on dendritic cells were analyzed. The immune responses were polyclonal with frequencies ranging from $3x10^{-6}$ to $6x10^{-3}$. We found no correlation between the type of vaccine and the functional phenotype of the anti-vaccine T cells. A total of 200 $multimer^+$ clones were obtained that either upregulate activation marker CD25 upon contact with the MAGE-3.DP4 antigen, or release IFN-γ, IL-

10 or IL-2. Their functional diversity was high. Among these clones, 12 $CD4^+$ $multimer^+$ clones could be considered as regulatory T cell clones (Treg), as the rested cells expressed CD25 and foxp3, released no cytokine upon antigenic stimulation, and were able to suppress the proliferation of $CD4^+CD25^-$ T cells upon stimulation with anti-CD3/CD28 antibodies. This is the first report of human Treg clones with a known specificity that were induced by vaccination. Considering that expression of foxp3, in humans, was reported not to be sufficient to identify Treg cells, we took advantage of our large collection of anti-MAGE-3.DP4 clones to re-examine this question. We have designed an assay to measure the suppressive activity of the different clones after stimulation with antigenic peptides and in the presence of IL-2. In this assay, the proliferation of an indicator $CD4^+$ T cell clone upon stimulation with a MAGE-3.DR1 peptide was estimated by flow cytometry. This assay was performed together with an analysis of (a) foxp3 expression both at the protein and at the mRNA level, (b) the cytokines released upon antigenic stimulation, and (c) the diversity of the TCR repertoire.

A reversible functional defect of $CD8^+$ T lymphocytes involving loss of tetramer labeling

N. Demotte, C. Wildmann, D. Colau

We have observed that human CTL clones lose their specific cytolytic activity and cytokine production under certain stimulation conditions, while retaining an antigen-dependent growth pattern. The labeling of these inactive CTL by an HLA-peptide tetramer was strongly reduced, even though the amount of T cell receptor (TCR) at their surface is similar (9).

These first observations are now confirmed with 13 CD8 T cell clones and 20 CD4 T cell clones. Rested T cells have a $tetramer^{high}$ phenotype whereas all the clones become $tetramer^{low}$ after antigenic stimulation. A complete recovery of tetramer staining is usually observed within 2-3 weeks. The reduced tetramer staining is not the result of a downmodulation of the TCR. $Tetramer^{high}$ and $tetramer^{low}$ T cells are compared for their CD8 and TCR distribution on the membrane by confocal microscopy and electronic microscopy. In addition, microarray analyses are performed to compare the gene expression profiles of $tetramer^{high}$ and $tetramer^{low}$ cells.

Our results indicate that tetramers may fail to reveal some CD8 and CD4 specific T cells bearing the relevant TCR, even when such functionally arrested T cells retain the potential to participate in immune responses. They also suggest the existence of a new type of functional defect of T cells.

Theileria parva candidate vaccine antigens recognized by immune bovine cytotoxic T lymphocytes

A Collaboration between:
The International Livestock Research Institute (ILRI), Nairobi, Kenya,
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East Coast fever, caused by the tick-borne intracellular apicomplexan parasite *Theileria parva*, is a highly fatal lymphoproliferative disease of cattle. The pathogenic schizont-induced lymphocyte transformation is a unique cancer-like condition that is reversible with parasite removal. Schizont-infected cell-directed $CD8^+$ CTL constitute the dominant protective bovine immune response after a single exposure to infection. However, the schizont antigens targeted by *T. parva*-specific CTL are undefined.

Two approaches were pursued to antigen identification, both dependent on screening of transiently transfected antigen-presenting cells with CTL from live vaccine-immunized cattle of diverse bovine leukocyte antigen (BoLA) MHC class I genotypes (10). For the first approach, a cDNA library was constructed in Brussels with RNA extracted from schizont. Pools of cDNA were transfected either in immortalized bovine skin fibroblasts or in monkey COS cells. The first screening of this library was performed in Brussels with CTL imported from Kenya and the other screening were performed at ILRI, Kenya. In a second approach, genes that were predicted by using preliminary sequence data from one of the four *T. parva* chromosomes to contain a secretion signal, were cloned, transiently transfected in antigen-presenting cells and tested for recognition by CTL. The approach was based on the observation that the schizont lies free in the host cell cytoplasm whereby signal peptide-containing parasite proteins would directly access the host cell MHC class I antigen processing and presentation pathway. Five candidate vaccine antigens that are the targets of MHC class I-restricted CTL from immune cattle were identified. $CD8^+$ T cell responses to these antigens were boosted in *T. parva*-immune cattle resolving a challenge infection and, when used to immunize naïve cattle, induced CTL responses that significantly correlated with survival from a lethal parasite challenge. These data provide a basis for developing a CTL-targeted anti-East Coast fever subunit vaccine. In order to have tools to monitor anti-vaccine T cell responses in immunized animals, bovine MHC-peptide multimers have been constructed and produced in Brussels. The specificity of these multimers was validated by staining of relevant and non-relevant CTL clones. Experiments are in progress to define the optimal conditions to detect CTL in PBL from immunized animals.

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THERAPEUTIC VACCINATION AND TUMOR GENE EXPRESSION PROFILING GROUP

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Tumor cells carry antigens such as MAGE antigens that are absent from normal tissues, and that can be targeted by cytolytic T lymphocytes (CTL) (1). Whilst it is possible to make such CTL recognize and kill autologous tumor cells in vitro, the precise way to induce an effective CTL response against a MAGE antigen in cancer patients is not known yet. In clinical vaccination trials, patients with a MAGE-expressing cancer, often melanoma, are treated repeatedly with a MAGE vaccine. 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. 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. Tumor samples can be analyzed by expression microarrays, which allows to study the interaction between the tumor environment and the immune cells at the transcriptional level. New vaccination 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)

We have set up small-scale clinical trials aimed at evaluating the toxicity, the antitumoral effectiveness and the immunological response in cancer patients immunized with MAGE vaccines involving either peptides, a recombinant protein or a recombinant viral vector. A total of about 380 patients have been included in these multicenter trials.

Clinical trials with the MAGE-3.A1 peptide

In a pilot study, the synthetic Mage-3.A1 peptide was administered to 45 HLA-A1 patients with *MAGE-3* expressing melanoma, by subcutaneous (s.c.) and intradermal (i.d.) injections of 100 or 300 µg of peptide on three occasions at monthly intervals. No significant toxicity was reported. Of the 25 melanoma patients with measurable disease who received all 3 immunizations, seven displayed tumor regressions. We observed 3 complete responses (CR), 1 partial response (PR) and 3 mixed responses i.e., a regression of some metastases while others appear, progress, or stabilize (MxR) (2).

Other vaccination modalities involving the same peptide were investigated in melanoma patients with measurable disease (3). This peptide was mixed with the immunological adjuvant MPL + QS21 and injected intramuscularly at 4-week intervals to 5 patients, without any evidence of tumor regression. A combination of the MAGE-3.A1 and MAGE-1.A1 peptides

was administered s.c. and i.d. every 3 weeks to 11 patients. Two of them experienced tumor regression (1 CR, 1 MxR). MAGE-3.A1 was injected s.c. and i.d. every 10-11 days instead of every 3-4 weeks to analyze whether vaccination at higher frequency could improve the clinical response rate. Among 21 patients treated, three had regressions of tumor lesions (3 MxR). The same peptide was associated with the HLA class II-restricted MAGE-3.DP4 peptide, in order to induce both CD8 and CD4 T cell responses, hoping for an improved immunological and antitumoral effectiveness. None of the 7 patients evaluable after 9 i.d. and s.c. vaccinations given every 10-11 days had tumor regression.

Initial assessment of the CTL responses induced by vaccination with the MAGE-3.A1 peptide was hampered by the lack of sensitivity of available CTL monitoring techniques. More recently, a new approach with improved sensitivity, involving lymphocyte-peptide culture and the use of HLA/peptide tetramers, was used to document a significant increase in CTLp frequency in a patient who showed tumor regression following vaccination with this peptide at high frequency. This method also showed that the CTL response was monoclonal. It was extended to 19 other patients who received this peptide without adjuvant. None had a detectable CTL response, indicating that this vaccine is weakly immunogenic (4).

In another study, patients with completely resected primary or regional metastatic melanoma with a high risk of relapse have been vaccinated with the MAGE-3.A1 peptide injected i.d. and s.c. every 2 weeks on 6 occasions. The purpose was to analyze whether vaccination of melanoma patients with less advanced disease in the adjuvant setting would improve the immunological response to a peptide vaccine. No CTL response was detected by our tetramer assay in the 6 patients who have received the complete treatment, including 3 patients with a resected tumor that did not express the appropriate antigen and who are assumed to be immunologically naive.

An ongoing study tests a cocktail of 8 different HLA-A2 restricted peptides mixed with an immunological adjuvant, either CpG7909 or Montanide ISA51. CpG7909 is an immunostimulatory CpG-containing oligonucleotide, which activates antigen presenting cells after binding to Toll-like receptor 9, and is thought to enhance CTL responses. Montanide is similar to incomplete Freund's adjuvant. It forms a water-in-oil emulsion with the peptides in solution, which allows to administer the vaccine mix as a long-lasting depot into the skin. The vaccine is injected on 6 occasions by i.d. and s.c. routes, at 2-week intervals. Fourteen patients are planned in each treatment arm. The purpose is to determine whether the adjuvanted multipeptide vaccine increases the CTL responses, and whether improved tumor response rates will be achieved. Fourteen patients have already received the peptides + CpG7909 vaccine, which was well tolerated. Three of them have shown evidence of tumor regression (all MxR). CTL responses against at least one of the 8 tumor antigens were detected in 6 patients, none of whom had a tumor response. A majority of these CTL responses were directed at the NY-ESO-1.A2 antigen. The second treatment arm in which Montanide is combined with the peptides is ongoing. Four patients have already been included.

Clinical trials with the MAGE-3 protein

In a phase I/II trial, the recombinant Mage-3 protein was tested as a vaccine in patients with MAGE-3 expressing cancer, mainly melanoma. The patients received either 30, 100 or 300 μg of the protein, with or without the immunological adjuvants MPL and QS21, repeatedly by intramuscular injection. No severe toxicity was reported. Among 33 evaluable melanoma patients, four experienced regressions of metastatic lesions, 2 partial and 2 mixed responses. A partial response was also observed in a patient with metastatic bladder cancer (5).

The clinical efficacy of the MAGE-3 protein injected i.d. and s.c. without adjuvant in non-visceral melanoma patients was tested

in another study. Patients received 300 µg of MAGE-3 protein on 6 occasions at 3-week intervals. Five out of 26 evaluable patients have shown regressions, including 1 partial response and 4 mixed responses. Thus this vaccine does not seem to induce more regressions than the MAGE-3.A1 peptide, but it does not require that the patient carries a specific HLA type (6). We then mixed this recombinant protein with adjuvant AS15 containing CpG 7909 in addition to MPL and QS21, and combined these i.m. injections with the administration of selected class I or class II peptides by i.d. and s.c. routes, which may result in the simultaneous activation of both $CD8^+$ and $CD4^+$ specific T lymphocytes. 11 patients were included in that study before its early closure. Three of them had a mixed response.

Clinical trial with an ALVAC-MAGE virus

40 patients with advanced cancer, including 37 with melanoma, were vaccinated with a recombinant canarypox (ALVAC) virus containing a minigene that encodes the MAGE-1.A1 and MAGE-3.A1 antigens, followed by booster vaccinations with the 2 corresponding peptides. The treatment comprised 4 ALVAC injections followed by 3 peptides injections, all i.d. and s.c., separated by 3 weeks each. Local inflammatory reactions at the sites of ALVAC injection were common, but were moderate in intensity and transient in duration. Among the 30 melanoma patients who received at least 4 ALVAC vaccinations, six experienced regression of one or more melanoma metastases. Significant CTL responses were detected in 3 of 4 patients with regressions, and in only one of 11 patients with disease progression, which indicates a significant correlation between immune and antitumor responses.

Summary of relevant observations and perspectives

Immunization with peptides, or with the MAGE-3 recombinant protein, both with or without adjuvant, or with the ALVAC recombinant viral vector, is devoid of significant toxicity. A minority of vaccinated melanoma patients (about 10 to 20%) show 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. 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 (4).

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, through unknown mechanisms (see « Analysis of T cell responses of vaccinated cancer patients » below). Future strategies aimed at improving cancer immunotherapy will undoubtedly rely on the characterization of these resistance mechanisms, which should define new important therapeutic targets. Vaccination at earlier stages, when the patient has no more detectable tumor after surgery but has a high risk of relapse, is another strategy that is being developed.

Gene expression profiling of tumor samples from vaccinated patients

Using the microarray technology, we have established the gene expression profile of a series of tumor samples, mainly cutaneous metastases, obtained from melanoma patients before the vaccine treatment was started. Comparative analysis between samples from patients who experienced either tumor regressions or no regression at all is ongoing. We hope to identify

genes whose expression is predictive of tumor response to cancer vaccines. The identity of such genes might help us to understand what happens in patients with tumor regression, and why this doesn't occur in patients without regression. We also use the microarray data to characterize the inflammatory events that take place inside those metastases, and to understand the interaction between the tumor cells and the inflammatory cells at the tumor site.

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ANALYSIS OF T CELL RESPONSES OF VACCINATED CANCER PATIENTS

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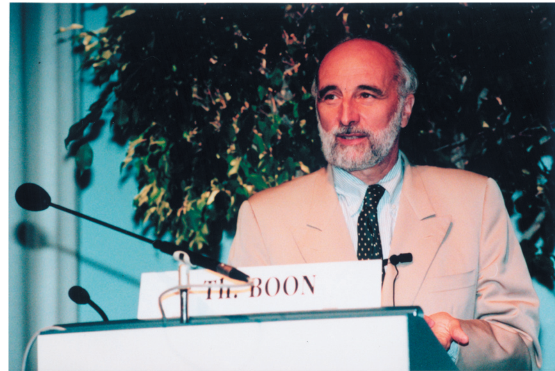

The work of our group is aimed at understanding why some metastatic melanoma patients show tumor regression following vaccination whereas most patients do not. Recent results suggest that a local immunosuppressive environment at the tumor sites may be the main barrier to the efficacy of immunotherapy. Our work is performed in close collaboration with the group of Pierre Coulie.

Study of the anti-vaccine and anti-tumoral T cell responses in melanoma patients vaccinated with an antigen encoded by gene MAGE-3

When we initiated our attempts to vaccinate metastatic melanoma patients with tumor-specific antigens, our belief was that very strong cytolytic T lymphocyte (CTL) responses would be required for tumor regressions to occur. Ten years later, our immunotherapy trials can be summarized as follows. Only a small proportion of patients shows tumor regression : about 20% of the patients show some evidence of regression and about 6% of the patients show a level of tumor regression that can be considered to be clinically beneficial.

Most of the patients who regress do so despite a frequency of anti-vaccine T cells in the blood which is lower than 1/100,000 of CD8 T cells. This frequency is remarkably stable over several months. The anti-vaccine CTL are usually a single T cell clone, except when the patients are vaccinated with tumor antigens presented by dendritic cells. There the response is usually polyclonal, but it is noteworthy that the rate of tumor regression is not higher.

In patients vaccinated with a recombinant ALVAC virus coding for an antigen of gene MAGE-3, we observed that detectable anti-MAGE-3 CTL responses showed correlation with tumor regression. But the paradox remained that tumor regressions were observed in patients who made very low CTL responses against the vaccine.

The analysis of patient EB-81 vaccinated with ALVAC-MAGE-3 indicated that this patient had, in addition to a blood frequency of anti-MAGE-3 CTL of about 1/300,000, a hundredfold higher frequency of CTL directed against other tumor antigens. Moreover, these “anti-tumor” CTL were already present before vaccination. The antigens recognized by the anti-tumor CTL of patient EB-81 were identified. Most of these CTL recognized antigens encoded by gene MAGE-C.2, another gene belonging to the same family as MAGE-3.

To understand better what happened in the

tumor, we used genetic approaches, namely PCR amplification of T cell receptor sequences, to evaluate the presence inside the metastases of patient EB-81 of the anti-vaccine CTL and of the main anti-MAGE-C2 CTL. The results were that the anti-vaccine CTL were barely enriched at the tumor sites relative to the blood whereas the anti-tumor CTL showed more than a hundredfold enrichment.

The results obtained in patient EB-81 have been completely confirmed in another patient treated with dendritic cells pulsed with a MAGE antigen.

These results led to a complete reversal of our views about the processes that lead to tumor regression. We now believe that as a melanoma evolves, there arises a spontaneous T cell response against specific tumor antigens. Thus, an immunosurveillance process occurs and probably results in the complete elimination of some tumors at an early stage. However, many tumors appear to escape this response. They manage to produce an immunosuppressive environment that renders ineffective the large number of T cells present in the tumor. A recent analysis of another patient indicates that the spontaneous T cell response against melanoma can occur at the stage of the primary tumor.

It appears that, in some vaccinated patients, a few anti-vaccine T cells manage to get a foothold in the tumor : they resist the local immunosuppressive conditions long enough to attack some tumor cells, and this results in a focal reversal of the immunosuppressive conditions. This in turn causes the restimulation and the proliferation of other anti-tumor T cells and it is these T cells that carry out the elimination of the bulk of the tumor cells (Fig. 1). To summarize, the anti-vaccine T cells serve only as a “spark” that activates the regression of the tumor.

A result of this process is that the anti-vaccine T cells are also restimulated. Hence the correlation between regression and anti-vaccine T cell responses.

Our results also indicate that a new wave of naïve anti-tumoral T cells may be stimulated and amplified in the course of the tumor regression process.

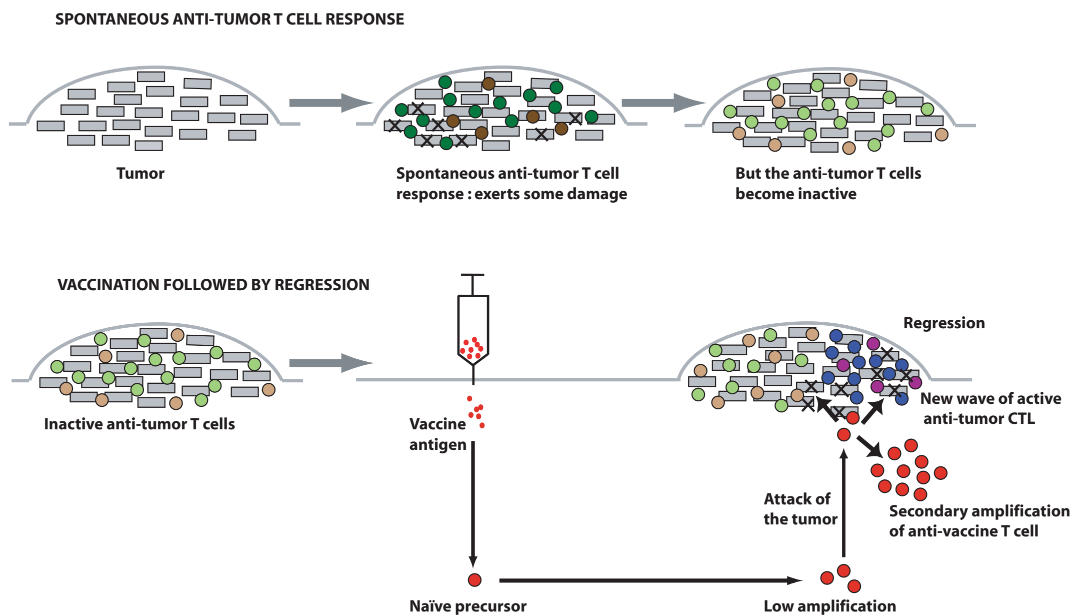

Figure 1

In conclusion, we suspect that the difference between the patients who show tumor regression following vaccination and those who do not, is the "tightness" of the immunosuppressive conditions at the tumor sites. This tightness may vary from one metastatic site to another, explaining why in many vaccinated patients some metastases regress whereas others don't.

In 2006, GlaxoSmithKline Biologicals reported the result of a randomized phase II adjuvant immunotherapy trial performed on non-small cell lung cancer patients. The 244 patients received several vaccinations with Mage-3 protein after complete surgical removal of their tumor. A statistically significant reduction of relapse within 2 years was observed in the vaccinated patients. Interestingly, the benefit was observed only for those patients where lymph node involvement had been observed at the time of surgery, and for the other patients from whom only minimal lymph node removal had been performed. We interpret this as evidence supporting the notion that the effectiveness of the vaccine depends on the presence in the tumor of anti-tumor T-cells resulting from an anterior spontaneous response. In those patients where the local lymph nodes are completely removed before the development of this spontaneous T cell response, the vaccine is ineffective.

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CYTOKINES IN IMMUNITY AND INFLAMMATION

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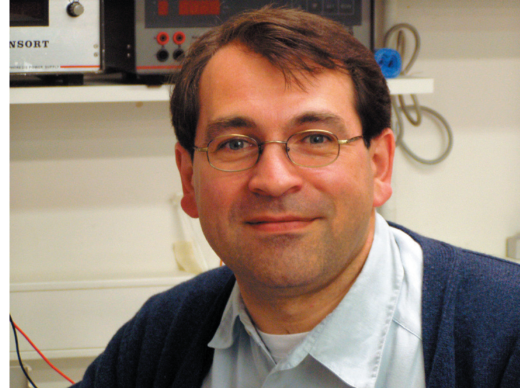

The cytokine group studies the biology of Interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is a TH2 cytokine that plays a role in immune responses against intestinal parasites and asthma. IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, upregulates 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 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 (1).

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 (2).

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. 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 activation 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.

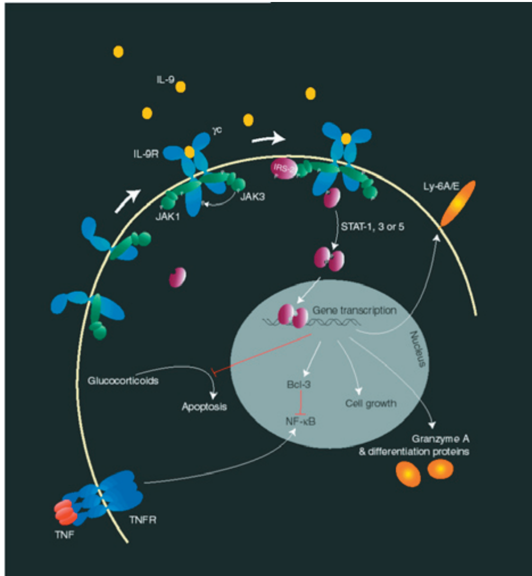

Figure 1.

Anti-apoptotic activity of I-309 receptor 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, led us 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. 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.

IL-9-induced genes

J.-C. Renauld, J. Van Snick, L. Dumoutier, L. Knoops, A. Tounsi, M. Stevens

To further characterize the mechanisms involved in the anti-apoptotic activity of IL-9 in this experimental model, we sought to identify genes induced by IL-9 in T cell lymphomas. Among the genes we identified, three open unexpected perspectives: BCL3, M-Ras and IL-TIF/IL-22.

BCL3 : indirect modulation of NF-κB

BCL3 is a gene originally identified at the breakpoint of translocations found in B cell leukemia, resulting in its transcriptional activation. The BCL3 protein interacts with NF-κB transcription factors and its induction by IL-9 represents a novel mechanism of NF-κB regulation by cytokines, and a new crosstalk between the JAK/STAT and NF-κB signal transduction pathway (4). BCL3 induction might play a role in the antiapoptotic activity of cytokines such as IL-4 and IL-9.

M-Ras : transcriptional regulation of the Ras-MAPKinase pathway

M-Ras is a new member of the Ras oncogene superfamily. The Ras proteins are known to regulate various cellular processes such as proliferation and apoptosis, when they are in their activated form, in association with a GTP molecule. Contrasting with the potent upregulation of M-Ras expression, M-Ras was not activated by IL-9 at the level of GTP binding. However, other cytokines such as IL-3 increased GTP binding to M-Ras, suggesting that M-Ras induction might represent a new mechanism of cooperativity between cytokines. Constitutively activated M-Ras mutants trigger the MAP Kinase pathway and induce proliferation of cytokine-dependent cells (5).

IL-TIF/IL-22 : a new cytokine structurally related to IL-10

IL-TIF is a new gene that turned out to encode a 179 amino acid long protein, including a potential signal peptide, and showing a weak but significant sequence homology with IL-10. This protein, originally designated IL-TIF for

IL-10-related T-cell derived Inducible Factor, was later renamed IL-22. Its expression is induced by IL-9 in thymic lymphomas, T cells and mast cells and by lectins in freshly isolated spleen cells. In addition, constitutive expression of IL-22 was detected by RT-PCR in thymus and brain, suggesting that the role of this new factor is not restricted to the immune system. Preliminary experiments showed that IL-22 induces STAT activation in various cell lines, suggesting that this factor might mediate some of the activities of IL-9. Biological activities of IL- 22 include the induction of acute phase proteins in liver (6) and protection against experimental heatitis (L. Dumoutier, unpublished results). 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.

Analysis of genome databases led 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.

In addition to its role in IL-22 binding and signalling, the IL-22R chain also forms a functional heterodimeric receptor complex by associating with IL-20Rß, the second short chain member of the IL-10R-related receptor family. This complex mediates STAT-1 and –3 activation by IL-20 and IL-24, but not by IL-22 (8). In addition, IL-20 and IL-24 can also bind to

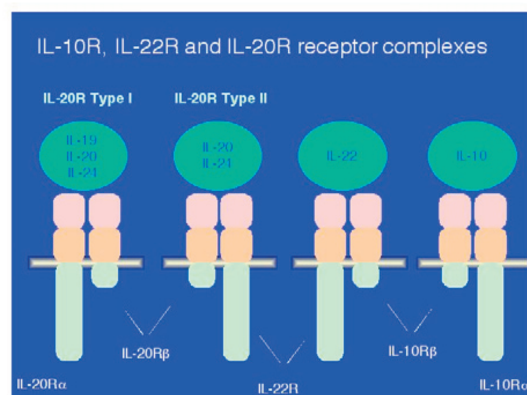

Figure 2.

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 new cytokine family).

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

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Cytokines and their receptors are critical for the formation of mature blood cells and for the function of the immune system. Signaling by cytokine receptors is triggered by ligand-induced changes in receptor dimerization/oligomerization, which induces the activation of cytosolic Janus tyrosine kinases (JAK). We study the signal transduction mechanisms and biologic functions of cytokine receptors such as the receptors for erythropoietin (Epo), thrombopoietin (Tpo), and Granulocyte-Colony-Stimulating Factor (G-CSF). The assembly of cell-surface receptor complexes, the structure and orientation of the transmembrane (TM) and cytosolic juxtamembrane (JM) domains, and the regulation by JAK kinases of receptor traffic are major focuses. The laboratory is actively investigating the mechanisms by which a JAK2 point mutant (V617F) induces Polycythemia Vera and myeloproliferative diseases in humans, as well as the role of TpoR mutants in myelofibrosis.

The mechanisms by which a mutant JAK2 induces Polycythemia Vera and myeloproliferative diseases in humans

J. Staerk, C. Pecquet

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 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. In addition to their role in signaling, JAKs appear to play chaperone roles for promoting traffic of cytokine receptors to the cell surface.

Polycythemia Vera (PV)(known also as the Vaquez disease) is characterized by excessive production of mature red cells and sometimes of platelets and granulocytes. Erythroid progenitors in PV are hypersensitive to Epo or independent of erythropoietin (Epo) for proliferation and differentiation. Strikingly, the traffic of TpoR is defective in myeloid progenitors from PV. A hint that JAK2 or JAK2-binding proteins may be involved in PV came when we showed that the wild type JAK2 strongly promotes the maturation and cell-surface localization of TpoR, the very process that is defective in PV (1).

In collaboration with Prof. William Vainchenker and his INSERM unit at the Institut Gustave Roussy in Paris, we have been involved in the discovery of the JAK2 V617F mutation in a majority of Polycythemia Vera patients (2, 3). The mutation in the pseudokinase domain alters a physiologic inhibition exerted by the pseudokinase domain on the kinase domain (Figure 1B and C). This mutant is found in >80% of PV patients and in 50% of Essential Thrombocythemia and Idiopathic Myelofibrosis (IMF), two other diseases that belong to the myeloproliferative syndromes (Figure 1). Current projects include the determination of downstream signaling proteins activated by the mutant JAK2, and the characterization of

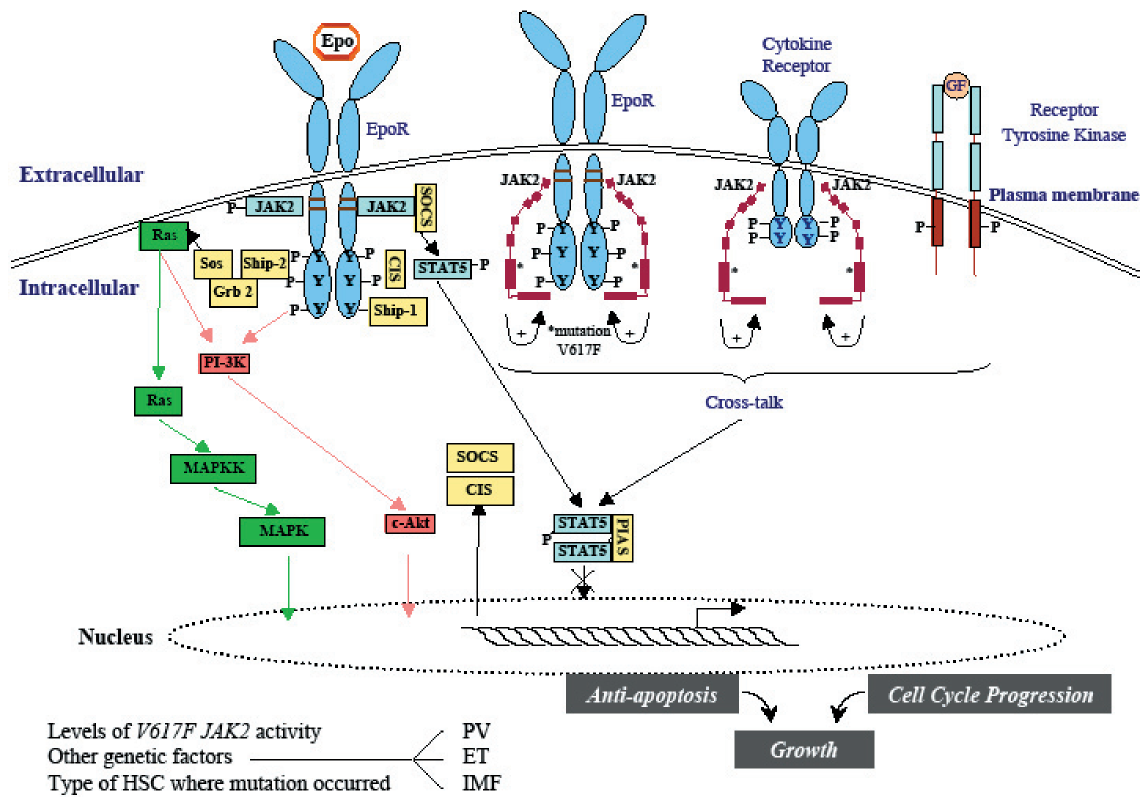

Figure 1. Cytokine receptors such as the erythropoetin receptor (EpoR) arrive at the cell surface after JAK2 had bound to their cytosolic domains early on in the secretory pathway. (A). When ligand (i.e. Epo for the EpoR) binds to the extracellular domain of the EpoR, the prebound JAK2 molecules trans-phosphorylate each other, become active and phosphorylate the receptors themselves and various signaling molecules, which become recruited to phosphorylated tyrosine residues of the receptor and of JAK2. Pathways that become activated are Ras-MAP-kinase, PI-3-kinase-AktB and STAT (STAT5 and STAT3), which upon dimerization are translocated to the nucleus and regulate gene expression. The end result is Epo-dependent activation of cell survival, proliferation and differentiation of erythroid progenitors. Normally JAK2 is inactive in the absence of the conformational change imposed by ligand activation of the receptor. (B,C). When V617 in the JH2 domain of JAK2 is mutated to phenylalanine (V617F) the inhibition exerted by the JH2 (pseudokinase domain) on JH1 is abolished and JAK2 can become active in the absence of ligand-activated receptors. Receptors such as EpoR (B) or others (C) such as thrombopoietin or G-CSF receptors can become phosphorylated and activated in the absence of ligand activation. (D). In cells that express the JAK2 V617F mutant, binding of growth factors such as Insulin-like growth factor 1 (IGF1) to its receptor IGF1R, a tyrosine kinase receptor, leads to the activation of the JAK-STAT pathway, which normally is only activated by cytokines and cell proliferation. This cross-talk between tyrosine kinase and cytokine receptors amplifies the proliferation of myeloid progenitors in patients with myeloproliferative diseases.

cytokine receptor signaling in the presence of the mutant and wild type JAK2 (Figure 1A and D). While the JAK2 wild type is down-modulated by SOCS3 (suppressor of Cytokine Signalling-3), we recently showed that signaling by JAK2V617F is potentiated by SOCS3 (5). Strikingly, the homologous mutations in JAK1 and Tyk2 also enable these kinases to be activated without ligand-binding to cytokine receptors (4). These results suggest that point mutations in JAK proteins may be involved in different forms of cancers and autoimmune diseases.

Involvement of TpoR in myeloproliferative diseases

J. Staerk, M. Girardot, N. Caceres

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, 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 (Staerk et al., in preparation). Within the KWQFP motif (RWQFP in the human), the key residues that maintain the receptor inactive are the K 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 and, indeed, the first reports from other groups and ours on mutations in this motif will soon be published. Thus, it is likely that among the JAK2 V617F-negative myelofibrosis patients some will harbor mutations in the TpoR RWQFP motif.

At present our laborarory 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 Clincal Biology (Prof. Dominique Latinne) a large study on the presence of JAK2 and TpoR mutations in patients with myeloproliferative diseases.

Determination of the interface and orientation of the activated EpoR and TpoR dimers

J. Staerk, A. Dusa, N. Caceres

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, 8).

To identify the residues that form the interface between the receptor monomers in the activated EpoR dimer we have replaced the EpoR extracellular domain with a coiled-coil dimer of α-helices (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 conformation appears to be required for activation of

signaling. In this active conformation TM residues L241 and L244 and JM residue W258 are predicted to be in the interface.

The thrombopoietin receptor (TpoR) is essential for formation of platelets, for renewing hematopoietic stem cells and for expanding myeloid progenitors. Like the EpoR, the TpoR is thought to signal by activation of JAK2, of several STATs (STAT1, 3 and 5) as well as of MAP-kinase, PI-3-kinase and AktB. However, TpoR and EpoR signal quite differently since only TpoR can induce hematopoietic differentiation of embryonic stem cells or stimulate the earliest stages of hematopoiesis in immature hematopoietic cells.

We applied the coiled coil approach to determine the active interface of the TpoR. Unlike the EpoR, where essentially one conformation is productive for signaling, for the TpoR six of seven orientations were inducing cell proliferation. Closer examination showed that different biologic effects were induced by different orientations, i.e. megakaryocyte differentiation, renewal of early hematopoietic progenitors or cell-to-cell adhesion. These differently oriented dimers appear to activate separate pathways besides the common JAK2 molecule. Current experiments aim at using these dimers as baits to purify novel signaling proteins and at determining the in vivo effects, in mice of expressing the different TpoR dimers in hematopoietic stem cells.

Structural studies on the transmembrane and juxtamembrane cytosolic sequences of the EpoR

K. Kubatzky, A. Dusa

To define the interfaces of the active and inactive EpoR dimers we performed cysteine scanning mutagenesis of the extracellular juxtamembrane and TM domains (9). We isolated three constitutively active novel mutants of the EpoR where residues L223, L226 or I227 were mutated to cysteine. These three mutants as well as cysteine mutants of residues 220-230 formed disulfide-bonded dimers. 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.

Traffic of cytokine receptors to the cell-surface: the common γ chain (γc) of the IL2/IL9 receptor complexes and TpoR

C. Diaconu, C. Pecquet

We have observed that, in hematopoietic cells, over-expression of JAK proteins leads to enhanced cell-surface localization of cognate cytokine receptors (i.e. TpoR, IL9R, IL2R, γc). The IL9Rα which requires JAK1 for signaling is expressed at higher levels on the cell-surface when JAK1 but not JAK2 or JAK3 is overexpressed. For the common γc it is uniquely JAK3 that promotes traffic to the cell surface. In collaboration with Pierre Courtoy we are examining the intracellular location of receptors as a function of expression levels of JAKs. In the absence of JAK3 it appears that γc accumulates in an endosome-like vesicle. For the TpoR, both JAK2 and Tyk2 strongly promote traffic, stability and recycling of the mature TpoR, which co-localizes with recycling transferrin (1). JAK2 and Tyk2 exert similar effects on TpoR traffic, while JAK2 is 10 fold more potent in transmitting a signal from the TpoR (1). We are examining the possibility that at different stages of megakaryocyte differentiation, the ratio of JAK2 to Tyk2 may vary in order to modulate TpoR signaling. Furthermore, we are examining the mechanisms by which patients with myeloproliferative diseases have defective TpoR expression on their megakaryocytes and platelets.

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 attemtping to clone novel proteins that can regulate traffic and stability of TpoR.

Random mutagenesis approaches to study interactions between receptor transmembrane domains and the structure and function of JAK2 V617F

A. Dusa

Two transmembrane viral envelope proteins (gp55-P and gp55-A) belonging to the polycythemic (P) and anemic (A) Spleen Focus Forming Virus (SFFV) strains, can activate the EpoR when co-expressed in the same cell. In collaboration with Yoav Henis, Tel-Aviv University, Israel, we have shown that both the gp55-A and gp55-P TM domains specifically interact with the TM domain of the EpoR. gp55-A weakly activates the receptor leading to erythroleukemia with low number of red blood cells (anemia). gp55-P fully activates the EpoR inducing erythroleukemia with elevated levels of red cells (polycythemia). The basis for this difference between gp55-P and gp55-A is represented by differences in specific binding of the TM domains to the TM domain of the EpoR. Taking advantage of this specific interaction we are constructing a genetic system where the TM sequence of gp55-P is randomized and tested for the ability to bind and activate the EpoR. In this system activation of EpoR signaling will result in cell survival and proliferation, which represents a powerful selection.

Second, we employ a random mutegensis approach coupled to retroviral gene transduction in order to examine the sequence requirements at position V617 of JAK2 for constitutive activation. Mutants of JAK2 V617 to each of the other 19 amino acid residues will be tested for constitutive kinase activity, for induction of cell proliferation and activation of STAT5. Since the V617F mutation also activated JAK1 and Tyk2 (4), we will test whether any of the potentially activating mutations may also activate JAK1 and Tyk2. We expect these results to shed light on the uniqueness of the JAK2 V617F in patients and on the structural requirements at position V617 of the JH2 region for activation.

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. In these transformed cells many of the transient signaling events induced by cytokines are detectable permanently, i.e. ligand-independent phosphorylation of JAK and STAT proteins or high levels of nuclear activated STATs especially STAT5 and STAT3. A similar picture has been noted in patient-derived leukemia cells. The critical questions we would like to answer concern the mechanisms by which the JAK-STAT pathway remains permanently activated in transformed cells and which genes are regulated by constitutively active STAT proteins in leukemic cells. Using chromatin immuno-precipitation and sequencing of native promoters bound by STAT5 we noted that in transformed cells STAT5 can also bind to low affinity N4 sites (TTC-NNNN-GAA), and not only to N3 sites, which are characteristic of ligand-activated STAT5 (10). We are attempting to identify the promoters actually bound by STAT pro-

teins in living cells in physiologic and pathologic situations. We are using a modified version of the chromatin immunoprecipitation assay pioneered by Alex Varshavsky in conjunction with DNA microarray genomic profiling. The isolated genomic fragments are screened for the presence of STAT-binding sites and tested for the ability to regulate transcription of reporter genes. Newly identified genes regulated by such genomic sequences will be tested for function by cloning their cDNA expressed in bicistronic retroviral vectors that allow wide expression of candidate proteins at physiologic levels.

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