Research at

the Christian de Duve

Institute of Cellular Pathology (ICP)

and at

the Brussels Branch of

the Ludwig Institute for Cancer Research (LICR)

August 2006

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Christian de Duve Institute of Cellular Pathology (ICP)

ICP: an International Biomedical Research Institute

When Christian de Duve founded the Institute of Cellular Pathology (ICP) in 1974, 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 ICP.

Emile Van Schaftingen

Benoît Van den Eynde

The main commitment of the members of ICP 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 ICP. 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 ICP and the Ludwig Institute is extremely close and the sharing of resources is considerable.

ICP 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 ICP and in the Ludwig Institute, assisted by a technical and administrative staff of about 80 members. Despite this relatively small size, ICP 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 ICP to stand at the forefront of European Research.

Miikka Vikkula

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Contact:

ICP 75.50, Avenue Hippocrate 75, 1200 Bruxelles, Belgium

Tel: +32 (2) 764 7550
Fax: +32 (2) 764 7573

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ACKNOWLEDGMENTS

In 2004, the ICP has attracted major gifts from several foundations, companies and individuals who have been very generous. These sponsors are providing the resources which 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 ICP's budget.

The appeal for sponsoring postdoctoral fellowships was also widely followed. In 2004 the ICP has been able to allocate the following fellowships, entirely supported by our donors :

the «Haas-Teichen» fellowship to Tomoko SO

the «Michel de Visscher» fellowship to Bruno GUIGAS

the «Philippe Delori» fellowship to Nicolina RENQVIST

the «Pierre Lacroix» fellowship to Anders KALLIN

the «Umicore» fellowship to Artur CORDEIRO

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

Jean PETERBROECK,
President of the Development and Expansion Council

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For many years, the main interest of our group has been the regulation of carbohydrate metabolism in mammals. Two important contributions have been the discovery of fructose 2,6-bisphosphate (in 1980, in collaboration with L Hue, HORM unit, and HG Hers, former head of this group) and that of the regulatory protein of glucokinase. Our laboratory has also identified several "new" enzymatic deficiencies in patients with inborn errors of metabolism (serine biosynthesis and degradation defects; phosphomannomutase deficiency, in collaboration with prof. J. Jaeken, KULeuven) and the identification of the gene mutated in glycogen storage disease type Ib. As a result of this, part of our work has been devoted to the biochemical characterization of some of the enzymes involved in these deficiencies. More recently, the study of the mechanism of formation of the intriguing phosphate ester, fructose 3-phosphate, has led us to identify fructosamine 3-kinase. This has brought us into a very different field, that of protein repair.

Protein deglycation

Gh. Delpierre, F. Collard, J. Fortpied, R. Gemayel, A. Preumont, G. Connerotte, 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 fructosami-

nes. These are formed through a spontaneous reaction (known as 'glycation') of glucose with primary amines, followed by an Amadori rearrangement. Fructosamine 3-kinase (FN3K) is a recently identified enzyme that phosphorylates both low-molecular-weight and protein-bound fructosamines. Fructosamine 3-phosphates are unstable, breaking down spontaneously to 3-deoxyglucosone, inorganic phosphate and the amino compound that originally reacted with glucose (Fig. 1).

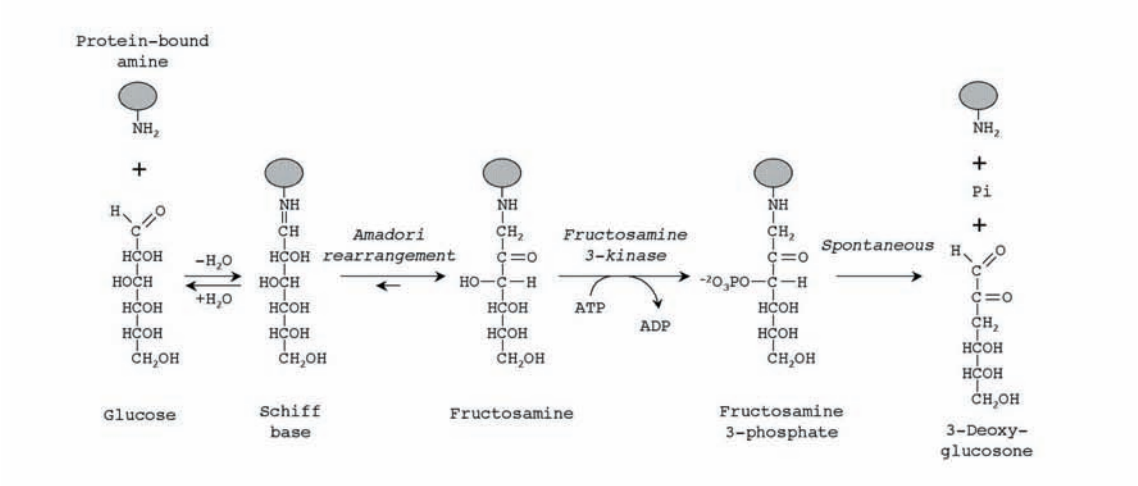

Figure 1. Role of fructosamine 3-kinase in the deglycation of proteins

That FN3K indeed acts as a 'deglycating' enzyme was first provided by experiments in which erythrocytes were incubated *ex vivo* with an elevated concentration of glucose, with or without a competitive inhibitor of FN3K. These studies showed also that only part of the fructosamines—those that are accessible to FN3K— are cleared in this way. The role of FN3K in deglycation was confirmed and extended by analysis of a mouse FN3K knockout model that we created (Veiga-da-Cunha et al. submitted).

We also determined the FN3K activity in erythrocytes from normoglycaemic subjects (n = 26) and Type 1 diabetic patients (n = 31). No significant difference in the mean activity was observed between the two groups. However, we noted that, in both groups, the FN3K activity is variable from subject to subject in an about fourfold range (from about 1 to 4 mU/g haemoglobin). This variability is stable with time and it is associated ($p < 0.0001$) with two single nucleotide polymorphisms in the promoter region and in exon 6 of the *FN3K* gene, indicating that it is largely due to sequence differences in this gene. Low FN3K activity was found to be associated with a higher glycation level at a specific site in hemoglobin. The fructosamine that is present at this site is apparently only slowly phosphorylated by FN3K. Glycation at other sites, which are either well accessible to FN3K or not accessible at all to this enzyme, were not detectably affected by the variability in the FN3K activity, which has therefore no detectable impact on the levels of HbA1c or total glycated hemoglobin [1].

Fructosamine-3-kinase related protein

Fructosamine-3-kinase related protein (FN3K-RP) shares about 65 % sequence identity with FN3K and is encoded by a gene that is present next to the *FN3K* gene on human chromosome 17q25. A similar gene arrangement is found in other mammals and in chicken, although not in fishes, indicating that a gene duplication event occurred during or after the fish radiation. FN3K-RP is also a ketoamine

3-kinase, acting on ribulosamines, erythrulosamines and with a lower affinity, psicosamines, but not on fructosamines. By contrast FN3K acts on the four types of substrates including fructosamines. All ketoamine 3-phosphates are unstable and their spontaneous decomposition leads to the regeneration of a free amino group, indicating that FN3K-RP is also a protein repair enzyme. This role has been confirmed in human erythrocytes incubated *ex vivo* with D-allose or D-ribose and an inhibitor of FN3K and FN3K-RP.

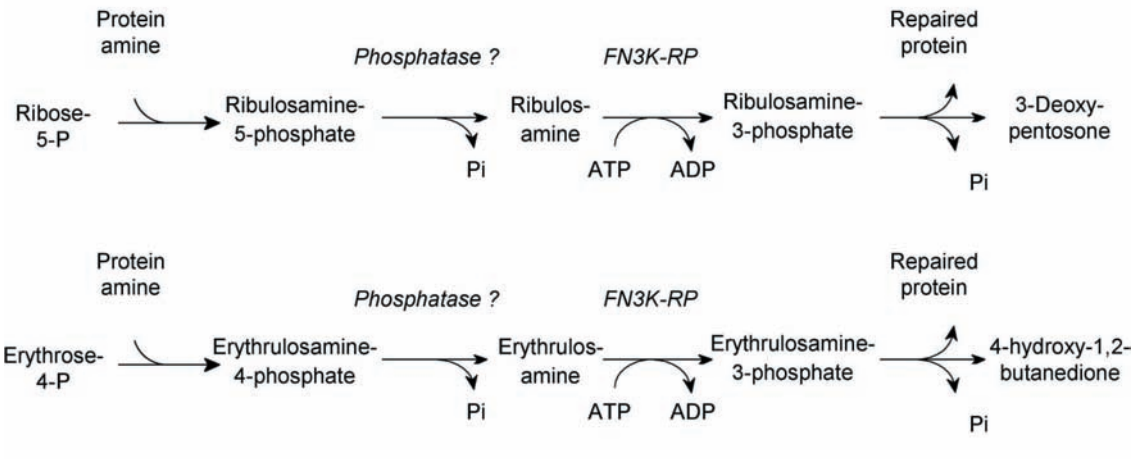

Figure 2. Hypothetical role of FN3K-RP in protein deglycation. Ribulosamines and erythrulosamines, the best substrates of FN3K-RP, are possibly formed from ribose 5-phosphate and erythrose 4-phosphate, two extremely potent glycating agents. Removal of the phosphate group in position 4 or 5 by a hypothetical phosphatase is required before phosphorylation of the third carbon by FN3K-RP.

It is unlikely that the physiological substrates of FN3K-RP are formed through a reaction of amines with free ribose or erythrose, because these sugars are present at very low concentration (< 10 μM) in tissues. Furthermore, D-allose is not a physiological compound in vertebrates. 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 ≈ 250 and 1000-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. The identity of the phosphatase catalyzing this reaction is unknown.

The hypothesis that FN3K-RP is involved in the repair of protein damage caused by ribose 5-phosphate and erythrose 4-phosphate is also consistent with the observation that spinach leaves contain a highly active ribulosamine/erythrulosamine 3-kinase, with a specific activity more than 2 orders of magnitude higher than that of FN3K-RP in human erythrocytes [2]. Ribose 5-phosphate and erythrose 4-phosphate, intermediates of the Calvin cycle, are expected to be abundant in chloroplasts. The plant enzyme is an interesting tool to detect protein-bound ribulosamines.

Fructosamine metabolism in bacteria

E. Wiame, E. Van Schaftingen

Fructosamines are metabolized by some fungi and some bacteria through two different types of pathways. A first one involves H2O2-producing oxidases that (generally) catalyze the cleavage of the bond between the amino group and the first carbon of the sugar portion, releasing glucosone. A second type of pathway, described in *Escherichia coli* and *Bacillus subtilis*, is initiated by the phosphorylation of the 6th carbon of the deoxyfructose moiety by an ATP-dependent kinase belonging to the PfkB family. The resulting fructosamine 6-phosphate derivative is next cleaved to a free amino acid and glucose 6-phosphate by a «deglycase» (FrlB) distantly related to the isomerase domain of glucosamine-6-phosphate synthase. Psicoselysine, a compound resulting from a glycation reaction with allose is metabolized after enzymatic epimerization to fructoselysine (Fig. 3). Until now, no enzyme is known to metabolize compounds that result from a condensation of amines with fructose. Due to the rearrangement that takes place after Schiff base formation, these compounds have predominantly a 'glucosamine-like' structure.

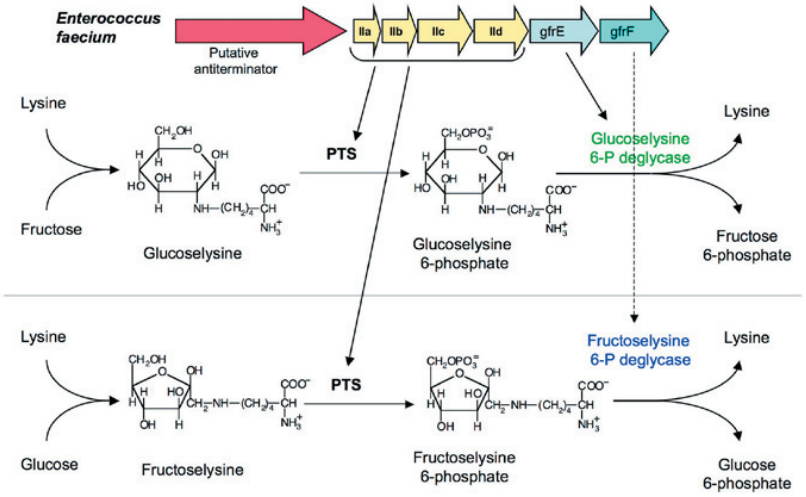

Figure 3. Metabolism of fructoselysine and glucoselysine in *Enterococcus faecium*.

While searching bacterial genomes for putative fructosamine-6-phosphate deglycases, we noted the existence of operons comprising (1) a homologue of fructoselysine-6-phosphate deglycase; (2) a second homologue of the isomerase domain of glucosamine-6-phosphate synthase, more closely related to it; (3) components of a novel phosphotransferase system, but no FrlD homologue. The FrlB homologue (which we designated GfrF) and the closer glucosamine-6-phosphate synthase homologue (GfrE) encoded by an *Enterococcus faecium* operon were expressed in *E. coli* and purified. Like FrlB, GfrF catalyzed the reversible conversion of fructoselysine 6-phosphate to glucose 6-phosphate and lysine. When incubated with fructose 6-phosphate and elevated concentrations of lysine, GfrE catalyzed the formation of a compound identified as 2-ε-lysino-2-deoxy-6-phospho-glucose (glucoselysine 6-phosphate) by NMR. GfrE catalyzed also the reciprocal conversion, i.e. the formation of fructose 6-phosphate (but not glucose-6-phosphate) from glucoselysine 6-phosphate. The equilibrium constant of this reaction (0.8 M) suggests that the enzyme serves to degrade glucoselysine 6-phosphate. In conclusion,

GfrF and GfrE serve to metabolise glycation products formed from lysine and glucose (fructoselysine) or fructose (glucoselysine), via their 6-phospho-derivatives. This is the first description of an enzyme catalyzing the metabolism of a fructation product [10].

N-Acetylneuraminate synthesis

P. Maliekal, E. Van Schaftingen

Sialic acids are nine-carbon sugars with a carboxylate group that are found as components of many glycoproteins, glycolipids and polysaccharides in animals, viruses and bacteria. The main form of sialic acid, N-acetylneuraminate (Neu5Ac), is often present as the terminal sugar of N-glycans on glycoproteins and glycolipids and plays an important role in protein-protein and cell-cell recognition. This compound is synthesized from N-acetylmannosamine in bacteria and from N-acetylmannosamine 6-phosphate in higher eukaryotes in an enzymatic reaction in which the pyruvate moiety of phosphoenolpyruvate is added to the first carbon of the aldohexosamine or of its 6-phospho-derivative. Neu5Ac-9-P, which is formed in mammals, needs to be dephosphorylated before it can be used by CMP-Neu5Ac synthase. Evidence has been provided for the fact that removal of the phosphate is catalyzed by a specific phosphatase, which is present in the cytosol and shows a wide tissue distribution, but the sequence of this enzyme has never been identified.

To identify the gene encoding Neu5Ac-9-Pase [6], we have purified this enzyme more than 1000-fold from rat liver. Its dependency on Mg^{2+} and the fact that it was inhibited by vanadate and Ca^{2+} suggested that it belonged to the haloacid dehalogenase family of phosphatases. Trypsin digestion and mass spectrometry analysis of a polypeptide of ≈ 30 kDa that co-eluted with the activity in the last purification step indicated the presence of a protein designated 'Haloacid-dehalogenase-like-hydrolase domain containing 4'. The human orthologue of this protein is encoded by a 2-exon gene present on chromosome 20p11. The human protein was overexpressed in *E. coli* as a fusion protein with a polyHis tag and purified to homogeneity. It displayed a > 230-fold higher catalytic efficiency on Neu5Ac 9-phosphate than on its next best substrate (fructose 1,6-bisphosphate). Its properties were similar to those of the enzyme purified from rat liver. The identification of the gene encoding Neu5Ac-9-Pase will facilitate studies aimed at testing its potential implication in unexplained forms of glycosylation deficiency.

Regulation of Vitamin C synthesis

C. Linster, E. Van Schaftingen

Vitamin C synthesis in animals proceeds from glucuronate, which is first reduced to L-gulonate. The latter is lactonized and then oxidized by L-gulonolactone oxidase, an enzyme that is deficient in man. Vitamin C formation in animals (and, in man, the formation of the pentose L-xylulose, also derived from glucuronate) is known to be enhanced by a series of xenobiotics, including aminopyrine and chloretone. In this context, we have previously reported that metyrapone, aminopyrine, and other xenobiotics cause a rapid increase in the conversion of UDP-glucuronate to glucuronate in isolated rat hepatocytes. However, the precise mechanism by which free glucuronate is formed remains unclear. It is usually stated that glucuronate formation from UDP-glucuronate is the result of two successive reactions consisting in hydrolysis of UDP-glucuronate to glucuronate 1-phosphate and UMP by a pyrophosphatase, followed by dephosphorylation of glucuronate 1-phosphate. However, neither the pyrophosphatase nor the phosphatase implicated in these reactions have been identified.

Experiments in cell free systems were performed to solve these questions [5]. Metyrapone and other xenobiotics stimulated by about 3-fold the formation of glucuronate from

UDP-glucuronate in liver extracts enriched with ATP-Mg, but did not affect the formation of glucuronate 1-phosphate from UDP-glucuronate, or the conversion of glucuronate 1-phosphate to glucuronate. This and other data indicated that glucuronate 1-phosphate is not an intermediate in glucuronate formation from UDP-glucuronate, suggesting that this reaction is catalyzed by a 'UDP-glucuronidase'. UDP-glucuronidase was mainly present in the microsomal fraction, where its activity was stimulated by UDP-N-acetylglucosamine, known to stimulate UDP-glucuronosyltransferases by enhancing the transport of UDP-glucuronate across the endoplasmic reticulum membrane. UDP-glucuronidase and UDP-glucuronosyltransferases displayed a similar sensitivity to a series of detergents, which stimulated at low concentrations and generally inhibited at higher concentrations. Substrates of glucuronidation inhibited UDP-glucuronidase activity, suggesting that the latter is contributed by UDP-glucuronosyltransferase(s). Inhibitors of ß-glucuronidase and esterases did not affect the formation of glucuronate, excluding the involvement of a glucuronidation-deglucuronidation cycle. The sensitivity of UDP-glucuronidase to metyrapone and other stimulatory xenobiotics was lost in washed microsomes, even in the presence of ATP-Mg, but it could be restored by adding a heated liver high-speed supernatant or CoASH. In conclusion, glucuronate formation in liver is catalysed by a UDP-glucuronidase which is closely related to UDP-glucuronosyltransferases. Metyrapone and other xenobiotics stimulate UDP-glucuronidase by antagonizing the inhibition exerted —presumably indirectly— by a combination of ATP-Mg and CoASH (Fig. 4).

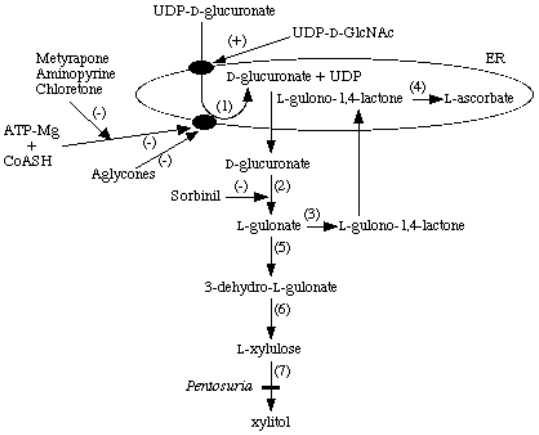

Figure 4. Pathways of vitamin C, L-xylulose and glucuronate 1-phosphate formation. (1), UDP-glucuronidase; (2), glucuronate reductase; (3), aldonolactonase; (4), L-gulono-1,4-lactone oxidase; (5), L-gulonate 3-dehydrogenase; (6), 3-dehydro-L-gulonate decarboxylase; (7), L-xylulose reductase (from [5] with modifications).

Metabolism of L- and D-2-hydroxyglutarate and other inborn errors of metabolism

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. We reported in 2004 the identification of a mitochondrial, FAD-linked D-2-hydroxyglutarate dehydrogenase. This enzyme shows a very low Km for its substrate (≈ 3 μM) and catalyzes an irreversible reaction under physiological conditions, being therefore well suited to eliminate D-2-hydroxyglutarate. In collaboration with the group of C. Jakobs (Amsterdam, The Netherlands), we have now found that this enzyme is mutated in several cases of D-2-hydroxyglutaric aciduria [9]. By overexpressing the mutated proteins in embryonic kidney cells, we showed that all mutations found in patients markedly decreased the enzymatic activity. These results indicate therefore that D-2-hydroxyglutarate dehydrogenase deficiency is a cause of D-2-hydroxyglutaric aciduria.

We also studied an enzyme (hydroxyacid-oxoacid transhydrogenase, HOT) that produces D-2-hydroxyglutarate. This enzyme is very peculiar in catalyzing the transfer of reducing equivalents from a metabolite (4-hydroxybuty-

rate) onto another metabolite (alpha-ketoglutarate), leading to the formation of succinate semialdehyde and D-2-hydroxyglutarate. This reaction is easily reversible *in vitro*, but it is driven towards the consumption of 4-hydroxybutyrate by the elevated alpha-ketoglutarate/D-2-hydroxyglutarate ratio that is maintained by D-2-hydroxyglutarate dehydrogenase. 4-Hydroxybutyrate (also named γ-hydroxybutyrate, GHB), a compound known for its anesthetic properties and an abuse drug, is used as a sleep regulator in the treatment of catalepsy. It is endogenously produced in mammals through reduction of succinate semialdehyde, a product of γ-aminobutyrate deamination. It is also formed by enzymatic oxidation of 1,4-butanediol, an industrial solvent, or through hydrolysis of γ-butyrolactone. HOT is likely to be the main enzyme responsible for 4-hydroxybutyrate metabolism. Surprisingly, its sequence has never been determined.

To identify this sequence, we have purified HOT from rat liver and obtained partial sequences of proteins coeluting with the enzymatic activity in the last purification step. One of the identified proteins corresponded to 'iron-dependent alcohol dehydrogenase', an enzyme encoded by a gene present on human chromosome 8q13.1 and distantly related to bacterial 4-hydroxybutyrate dehydrogenases. The identification of this protein as HOT was established by showing that overexpression of the mouse homologue in human embryonic kidney cells resulted in the appearance of an enzyme catalyzing the α-ketoglutarate-dependent oxidation of 4-hydroxybutyrate to succinate semialdehyde [4]. HOT belongs to the same protein family as a number of NAD-linked dehydrogenases and the NAD-binding residues are conserved in it. HOT most likely has a tightly bound NAD that serves as an intermediate electron acceptor in a Ping-Pong reaction. The availability of the HOT sequence will facilitate studies aimed at determining the physiological role of this enzyme.

We reported also last year the identification of the gene mutated in L-2-hydroxyglutaric aciduria [7]. Biochemical investigations on rat liver had demonstrated the presence of a membrane-bound, mitochondrial, FAD-linked L-2-hydroxyglutarate dehydrogenase, that oxidizes its substrate to α-ketoglutarate. Based on this information, a database search led us to identify a gene encoding a human protein of

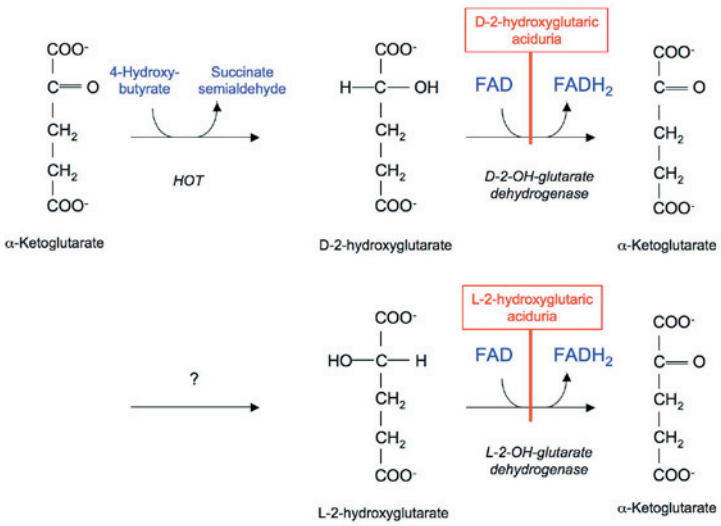

Figure 5. Enzyme defects in D- and L-2-hydroglutaric acidurias. The figure also shows the reaction catalysed by hydroxyacid-oxoacid transhydrogenase. See Text.

unknown function, homologous to bacterial FAD-dependent L-malate dehydrogenases and targeted to mitochondria. This gene, present on chromosome 14q22.1, was found to be mutated in patients with L-2-hydroxyglutaric aciduria. We have now overexpressed the protein encoded by this gene in HEK cells and found that it indeed corresponds to L-2-hydroxyglutarate dehydrogenase. The mutations identified in patients abolished its activity. These studies indicate that L-2-hydroxyglutaric aciduria is due to a deficiency in L-2-hydroxyglutarate dehydrogenase and point to L-2-hydroxyglutarate as a toxic metabolite [8].

In 2005, samples from about 80 patients were analyzed, allowing the diagnosis of or carnitine or carnitine palmitoyl transferase deficiency (2 cases), of various forms of glycogen storage disease (9 cases) and of phosphomannomutase (6 cases) and phosphomannose isomerase (1 case) deficiency.

Oxidative protein folding

J.F. Collet, H. El Hajjaji

This project has been initiated by Jean-François Collet during his post-doctoral stay in the laboratory of J. Bardwell (University of Michigan, Ann Arbor). Oxidation of two cysteine residues leads to the formation of a disulfide bond and the concomitant release of two electrons. The formation of disulfide bonds is a required step in the folding pathway of many secreted proteins. It takes place in the eukaryotic endoplasmic reticulum or the bacterial periplasm. In contrast, 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 of the thioredoxin and glutaredoxin systems.

We use the bacterium *Escherichia coli* to study the mechanisms involved in disulfide bond formation, isomerization or reduction. First, we are working on a membrane protein called DsbD. The role of DsbD is to provide electrons to 2 protein disulfide isomerases that are active in the periplasm, DsbC and DsbG. These 2 proteins are required to allow the correct folding of proteins with multiple cysteines. The electrons given by DsbD to DsbC and DsbG originate from the cytoplasmic pool of NADPH. The function of DsbD is therefore to transport electrons across the membranes. This is a very unique activity as electrons are usually transported laterally within the membranes. The mechanism of action of DsbD is essentially unknown.

Second, we are working on Trx2, a thioredoxin present in the cytoplasm. Thioredoxins are small redox proteins present in many eukaryotic and prokaryotic genomes. They all share a similar 3-dimensional structure and possess a conserved WCGPC catalytic motif. The main function of thioredoxins is to reduce disulfide bonds formed between cysteine residues as a result of either a catalytic activity or oxidative stress.

Two thioredoxins have been described in *E. coli*. The first one, TrxA, has been identified 40 years ago and is well characterized. The second thioredoxin, Trx2, coded by the gene trxC, has been discovered only recently. Trx2 has two striking characteristics that differentiate it from TrxA and suggest that it has a specific role to play in response to oxidative stress. Our goal is to discover this role.

Selected publications

1. Delpierre G*, Veiga-da-Cunha M*, Vertommen D, Buysschaert M, Van Schaftingen E. *Variability in erythrocyte fructosamine 3-kinase correlates with polymorphisms in the FN3K gene and impacts on haemoglobin glycation at specific sites.* **Diabetes Metab** 2006;32:31-40.

2. Fortpied J, Gemayel R, Stroobant V, Van Schaftingen E. *Plant ribulosamine/erythru-*

losamine 3-kinase, a putative protein-repair enzyme. **Biochem J** 2005;388:795-802.

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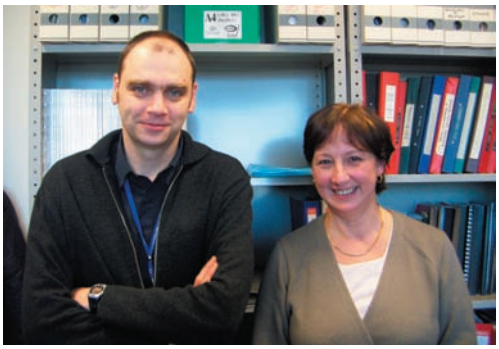
PURINE METABOLISM

Françoise BONTEMPS, Associate Member
Eric VAN DEN NESTE, Clinical Investigator
Georges VAN DEN BERGHE, Emeritus Member

Sabine CARDOEN, Graduate Student (till April 2005)
Caroline SMAL, Graduate Student
Stéphanie LISART, Graduate Student (till June 2005)
Laurent BASTIN-COYETTE, Graduate student
Angélique AERTS, Technician
Ilse SCHMIDT, Secretary
Karim ACHERKI, Technical Staff



Purine metabolism is essential to the body: it provides components of the nucleic acids, DNA and RNA, and the energy currency of the cell, ATP. Purine catabolism leads to the formation of a poorly soluble compound, uric acid, which can precipitate when elevated, and thereby causes gout. Our major present interests are the genetic defects of purine metabolism (1), and the mechanisms of action of purine nucleoside analogues, and particularly of 2-chloro-2'-deoxyadenosine that possess potent antileukaemic properties.

In 1997, a collaborative study of the antileukaemic 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 is 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, S. Cardoen, S. Lisart, C. Smal, L. Bastin-Coyette, 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 IC50 (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 phosphorylation of Rb and the activity of cyclin-dependent kinase 2 (cdk2), a kinase that phosphorylates Rb and 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.

Although the mechanism of action of CdA has been extensively investigated in leukaemic cells, the possibility that this nucleoside analogue interacts with the mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) pathway had never been explored. We have shown that CdA, at concentrations close to the IC50, activated the ERK pathway in EHEB cells. Because activation of this pathway is assumed to exert anti-apoptotic effect, we combined CdA with inhibitors of the ERK pathway. The latter were found to enhance CdA-induced apoptosis. These results suggest that the efficacy of CdA could be strengthened by combination with inhibitors of the ERK pathway (4)

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

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

In recent years, we have shown that combination of CdA with DNA-damaging agents, such as cyclophosphamide (CP) derivatives (5) or UV-light (6), 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 (7).

We are currently analysing if efficacy of CdA in B-CLL lymphocytes could be strengthened by combination with inhibitors of the MAPK/ERK pathway, as it has been observed in EHEB cells.

Regulation of dCK activity

C. Smal, E. Vanden 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 is 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 by CdA, we first investigated the effect of a variety of activators and inhibitors of protein kinases on the basal activity of dCK and on its activation by CdA. We discovered that dCK can be activated by several inhibitors of protein kinases, including genistein, an unspecific inhibitor of protein tyrosine kinases, AG-490, an inhibitor of the protein tyrosine kinase JAK-2 and JAK-3, and PD-98059 and U0126,

two specific inhibitors of the MAPK/ERK pathway. We also observed that these inhibitors potentiated the activating effect of CdA. On the other hand, we have shown that dCK activity can be markedly increased in intact EHEB cells by incubation with okadaic acid, an inhibitor of protein phosphatase PP2A. Taken together, these results do not allow to identify the pathway by which dCK is activated, but clearly indicate that its activity can be regulated by protein kinase(s) and phosphatase(s). This was confirmed by our observation that dCK, activated or not by CdA, can be inactivated in a crude cell extract by purified PP2A. This last result also demonstrates that activation of dCK results from its phosphorylation (8).

To further unravel its regulation, dCK was overexpressed in HEK-293 cells as a His-tag fusion protein. We showed that dCK was labelled after incubating the cells with [32P]orthophosphate. Tandem mass spectrometry allowed the identification of four in vivo phosphorylation sites, Thr3, Ser11, Ser15 and Ser74 (9). 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. Phosphorylation of Ser74 was also detected on endogenous dCK in leukemic cells, in which the Ser74 phosphorylation state was increased by agents that enhanced dCK activity. These results provided direct evidence that dCK activity can be controlled by phosphorylation in intact leukemic cells (10).

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TISSUE-SPECIFIC TRANSCRIPTION FACTORS IN DEVELOPMENT

Frédéric P. LEMAIGRE, Member
Patrick JACQUEMIN, Associate Member

Guy G. ROUSSEAU, Emeritus Member
Frédéric CLOTMAN, Assistant Member
Christophe PIERREUX, Assistant Member
Peggy RAYNAUD, Assistant Member
Aline ANTONIOU, Graduate Student
Jean-Bernard BEAUDRY, Graduate Student
Eric HEINEN, Graduate Student
Sabrina MARGAGLIOTTI, Graduate Student
Nicolas PLUMB-RUDEWIEZ, Graduate Student
Aurélie POLL, Graduate Student
Alexandru SIMION, Graduate Student
Jonathan van EYLL, Graduate Student
Vinciane VANHORENBEECK, Graduate Student
Freddy ABRASSART, Technician (half-time)
Sabine CORDI, Technician
Jean-François CORNUT, Technician
Marie-Agnès GUEUNING, Technician (half-time)
Matthieu GERADIN, Animal Caretaker (half-time)
Vivien O'CONNOR, Secretary (half-time)

A number of human diseases are characterized by malformations or functional deficiencies of cells 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, Jean-Bernard Beaudry, Frédéric Clotman, Sabrina Margagliotti, Christophe Pierreux, Nicolas Plumb-Rudewiez

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, 3), and this led to the conclusion that hepatic cell differentiation is controlled by Onecut factors via a modulation of TGFβ signaling.

In mice in which only the *Hnf6* gene is inactivated, hepatic cell differentiation is affected, but less severely than in double HNF-6/OC-2 knockout mice. 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 (4). The *Hnf6* knockout mice also lack hepatic artery branches, an anomaly which can aldo be found associated with human biliary diseases (5). Therefore, our findings indicate that HNF-6 is essential for bile duct development.

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 differentiation.

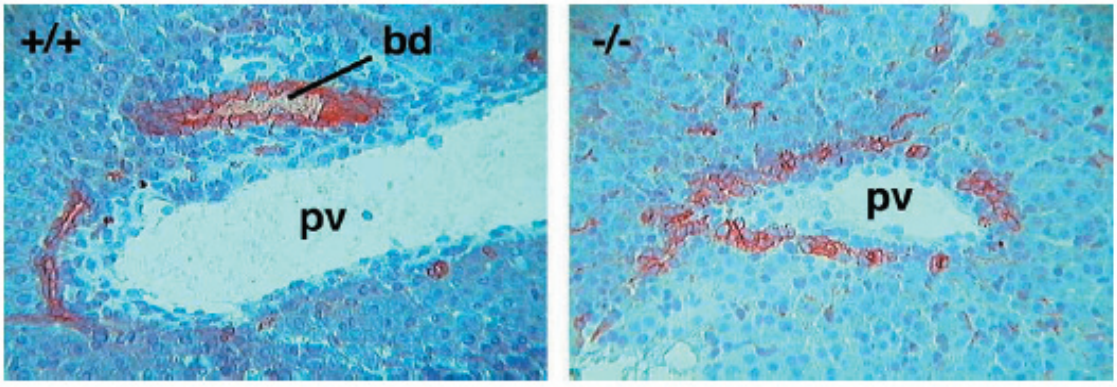

Figure 1. Abnormal development of the intrahepatic bile ducts in HNF-6 knockout mice. Immunohistochemistry of liver sections 10 days after birth shows in normal mice (left panel) a typical bile duct (bd) delineated by biliary cells (stained red) embedded in mesenchyme. In contrast, biliary cells do not 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

Eric Heinen, Alexandru Simion, Vinciane Vanhorenbeeck

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). The latter finding prompted us to investigate if Onecut factors also control the differentiation of endocrine cells in the gut (enteroendocrine cells), which also depend on Ngn-3. We are addressing this question by analyzing the phenotype of Oc2 and Oc3 knockout mice.

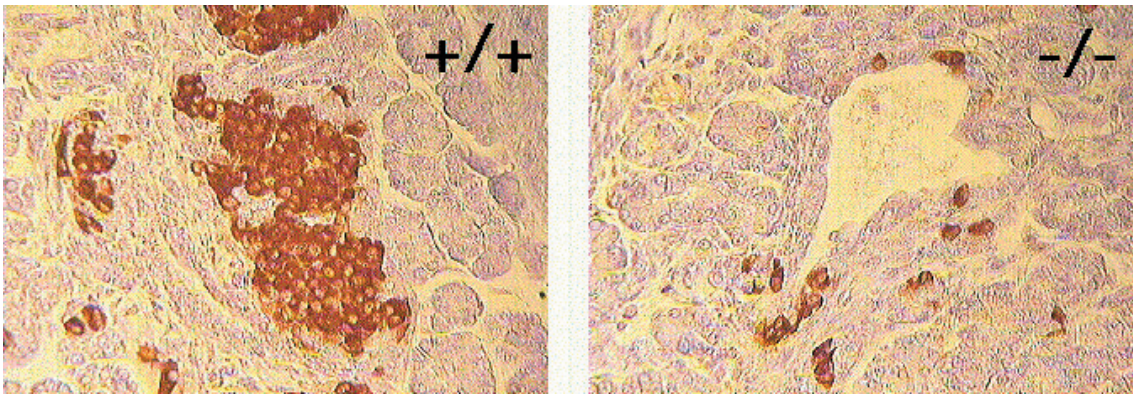

Figure 2. Abnormal development of the endocrine pancreas in HNF-6 knockout mice. Immunohistochemistry of tissue sections four days after birth shows that only a few insulin-producing cells (brown) are found near pancreatic ducts in *Hnf6* knockout mice (-/- ; right panel), instead of organized endocrine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

Control of pancreatic duct development by Onecut factors

Jonathan van Eyll, Christophe Pierreux, Aurélie Poll

We found that HNF-6 is required for development of the pancreatic ducts (7). 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.

When studying pancreas development we have also described the expression profile of ephrins and of their receptors. In addition, using cultured pancreatic explants, we showed that embryonic pancreas can be differentiated in vitro. This explant culture system is now being used to investigate the role of signalling molecules in pancreas development, in particular in the formation of the pancreatic ducts.

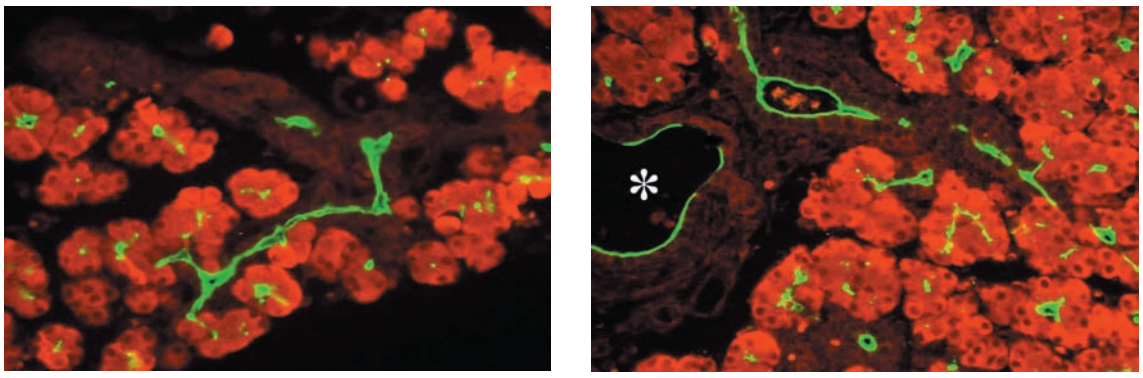

Figure 3. Formation of cysts in the pancreatic ducts in *Hnf6* knockout mice. Immunofluorescence analysis of tissue sections two days before birth shows that specific segments of the pancreatic ducts develop cysts (asterisks). The ducts are stained with an anti-Mucin-1 antibody (green) and the exocrine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

Control of endoderm development by Onecut factors

Christophe Pierreux, Aurélie Poll, Alexandru 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

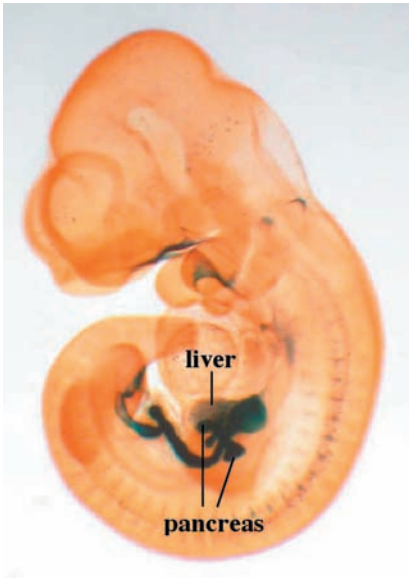

Figure 4. *Hnf6* gene regions drive expression of beta-galactosidase in liver and pancreas of transgenic embryos at embryonic day 10.5.

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

Louis HUE, Member
Mark H. RIDER, Member

Isabelle DE POTTER, Assistant Member
Bruno GUIGAS, Assistant Member
Sandrine HORMAN, Assistant Member
Didier VERTOMMEN, Assistant Member
Lisa MIRANDA, Graduate Student
Véronique MOUTON, Graduate Student
Yurda OZKAN, Graduate student
Nellie TALEUX, Graduate student
Martine DE CLOEDT, Technician (half-time)
Nusrat HUSSAIN, Technician
Liliane MAISIN, Technician (part-time)
Vivien O'CONNOR, Secretary (half-time)
Freddy ABRASSART, Technical Staff (part-time)

Our research concerns the role of protein phosphorylation in the control of metabolism by nutrients, hormones and various stresses. As a model system, we started out by studying 6-phosphofructo-2-kinase (PFK-2) / fructose-2,6-bisphosphatase (FBPase-2). This bifunctional enzyme catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, a potent stimulator of glycolysis. Fructose 2,6-bisphosphate was discovered in this Institute by Van Schaftingen, Hue and Hers in 1980 and is the most potent stimulator of 6-phosphofructo-1-kinase (PFK-1), a key enzyme of glycolysis. Fructose 2,6-bisphosphate is synthesised from fructose 6-phosphate and ATP by PFK-2 and hydrolysis to fructose 6-phosphate and Pi by FBPase-2. The PFK-2 and FBPase-2 reactions are catalysed at separate sites of a bifunctional enzyme (PFK-2/FBPase-2) composed of two identical subunits.

We characterised several PFK-2/FBPase-2 isoforms in mammalian tissues and cloned the corresponding mRNAs, showing that they originate from at least two genes. The isoforms differ in PFK-2/FBPase-2 activity ratio, kinetic properties and response to phosphorylation by protein kinases. The C-terminus of the heart (H) isozyme, contains phosphorylation sites for several protein kinases. These sites are not present in the other isozymes, such as the liver (L) isozyme, which, by contrast, contains a single phosphorylation site for the cyclic AMP-dependent protein kinase (PKA) at the N-terminus.

Our interest in the regulation of heart PFK-2 by phosphorylation led to the study of insulin-stimulated protein kinases and AMP-activated protein kinase (AMPK) (1). These results are described below. Briefly, we found that the molecular mechanisms responsible for the activation of heart PFK-2 by insulin involve protein kinase B (PKB) along with other insulin-stimulated protein kinases. In addition, we demonstrated that AMPK phosphorylates and activates heart PFK-2 in ischaemia, providing a new explanation for the Pasteur effect. AMPK activation not only leads to the stimulation of glycolysis, but also inhibits protein synthesis at elongation, and, as our recent work indicated, it is inhibited by insulin through a refined mechanism of hierarchical phosphorylation. AMPK activation also participates in the control of food intake and energy expenditure, and our recent work showed that lack of AMPK activation in the hypothalamus explains food restriction in a strain of rats resistant to obesity.

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 CHO-IR cells co-transfected with heart PFK-2 and PKB RNAi. Experiments with insulin-perfused hearts from PKB isoform knockout mice are envisaged to definitively address the role of PKB in 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. Figure 1 summarizes the protein kinases from different signalling pathways that phosphorylate heart PFK-2.

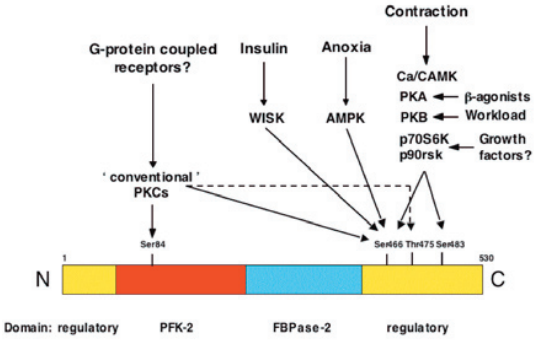

Figure 1. Protein kinases of signalling pathways that converge on heart PFK-2. The numbering of residues refers to the bovine H1 isoform.

AMP-activated protein kinase

S. Horman, D. Vertommen, B. Guigas, L. Miranda, N. Taleux, Y. Ozkan, L. Hue, M.H. Rider, in collaboration with J.-L. Vanoverschelde and L. Bertrand, UCL, Brussels, 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. In certain cells, AMPK can also be activated by 5-aminoimidazole-4-carboxamide (AICA)-riboside, which enters cells to be phosphorylated into ZMP, an analogue of AMP. The role of AMPK in the cell is to maintain ATP by stimulating ATP-producing pathways and inhibiting energy-consuming biosynthetic pathways. We contributed to the development of this concept by the discovery of new substrates of AMPK. We demonstrated that the activation of PFK-2 by AMPK participates in the stimulation of heart glycolysis by ischaemia. 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 (2 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 (2). 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 (3). 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 80% during contraction, there was no increase in Thr56 eEF2 phosphorylation in spite of the fact that AMPK was activated 5-fold. Moreover, the inhibition of protein synthesis during contraction could not be explained by inhibition of the mammalian target of rapamycin (mTOR)/p70 ribosomal S6 kinase (p70S6K)/4E-BP1 pathway (see Fig. 2). Therefore, it is likely that AMPK has targets other than eEF2/eEF2K and mTOR signalling in the translation machinery.

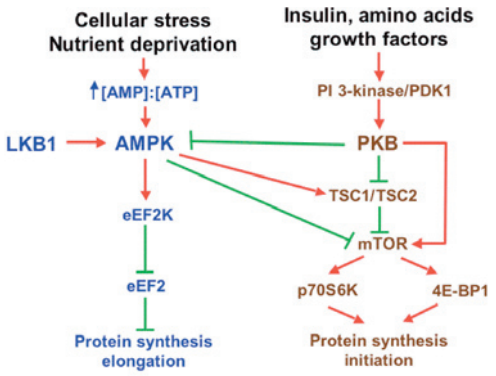

Figure 2. Cross talk between the insulin/PKB/mTOR and AMPK signalling pathways with respect to the control of protein synthesis.

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

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 phospho-specific 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.

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 (4). 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 (5). 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.

Lack of AMPK activation by starvation in the hypothalamus of the Lou/C rats, a strain resistant to obesity

AMPK is involved in the control of food intake by the hypothalamus. We investigated if hypothalamic AMPK was implicated in the spontaneous food restriction of the Lou/C rats, a strain resistant to obesity. Starvation activated both isoforms of AMPK in the hypothalamus of control rats, but not of the Lou/C rats. The starvation-induced decrease in hypothalamic mRNA levels of the anorexigenic neuropeptides (POMC and CART) was also totally prevented in the Lou/C rats. Analysis of the concentrations of circulating substrates and hormones known to regulate hypothalamic AMPK indicated that the starvation-induced changes in ghrelin, adiponectin and, above all, leptin were not observed in Lou/C rats and remained similar to those observed in the fed state. We conclude that food intake is related to the activation of AMPK in the hypothalamus, a regulation lacking in the Lou/C rats.

Control of glycolysis during differentiation in human keratinocytes

De Potter and L. Hue in collaboration with L'Oréal, Paris

To study the relationship between glycolysis and proliferation/differentiation, we developed a model of human keratinocytes in autocrine cultures that switch from proliferation to differentiation in 10 days. Glycolysis was maximal during proliferation and was 4-fold lower in differentiated cells. The mechanism responsible for this decrease was the down-regulation of the glucose transporter GLUT1. Retinoic acid, known to inhibit differentiation, stimulated glycolysis, whereas stimulation of glycolysis in differentiated cells led to an inhibition of differentiation. These findings underline the relationship between glycolysis and the control of differentiation.

Mass spectrometry

D. Vertommen and M.H. Rider in collaboration with E. Van Schaftingen, UCL, F. Bontemps, UCL

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 identifying new AMPK substrates and establishing a 2-D LC-MS gel-free proteomics approach for identifying proteins in complex mixtures and for studying changes in protein expression. We have continued to collaborate with other laboratories in the Institute. Along with the group of E. Van Schaftingen, we identified N-acetylneuraminate 9-phosphate phosphatase, an enzyme potentially involved in protein glycosylation deficiency (6) and hydroxyacid-oxoacid transhydrogenase, an enzyme that metabolizes 4-hydroxybutyrate (7). Also, we showed that in fructosamine 3-kinase knockout mice, the glycation of haemoglobin was affected in vivo but only at specific sites (8) that we had previously identified *in vitro* (9). Lastly, in collaboration 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. The major phosphorylation site identified, Ser74, was shown to be important for the control of enzyme activity (10).

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ENDOCYTOSIS

Pierre J. COURTOY, Member
Marie-France VAN DEN HOVE, Member

Patrick VAN DER SMISSEN, Assistant Member
Céline AUZANNEAU, Postdoctoral Fellow
Philippe de DIESBACH, Postdoctoral Fellow
Donatienne TYTECA, Postdoctoral Fellow
Sarah CARPENTIER, Graduate Student
Thierry MEDTS, Graduate Student
Marcel METTLEN, Graduate Student
Anna PLATEK, Graduate Student
Wânia REZENDE-LIMA, Graduate Student
Geneviève DOM, Research Associate
Thanh LAC, Technician (half-time),
Michèle LERUTH, Technician
Benoît MARIEN, Technician
Olga MEERT, Technician (half-time)
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) and in unravelling the contribution of endocytosis to physiopathology (4,7,10), parasitology (3) and pharmacology (5,6). 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: the elucidation of the signalling cascade whereby the paradigmatic oncogenes, v -Src and K-Ras, control the actin cytocortex (2,8), specifically at the apical domain of polarized MDCK cells (13); the fine tuning of apical endocytosis leading to regulated production of thyroid hormones (4); and the elucidation of a deficit of apical endocytosis in a genetic form of kidney stones (7).

Regulation of endocytosis by v-Src in polarized cells

M. Mettlen, A. Platek, P. Van Der Smissen, S. Carpentier, Ph. de Diesbach, D. Tyteca and 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

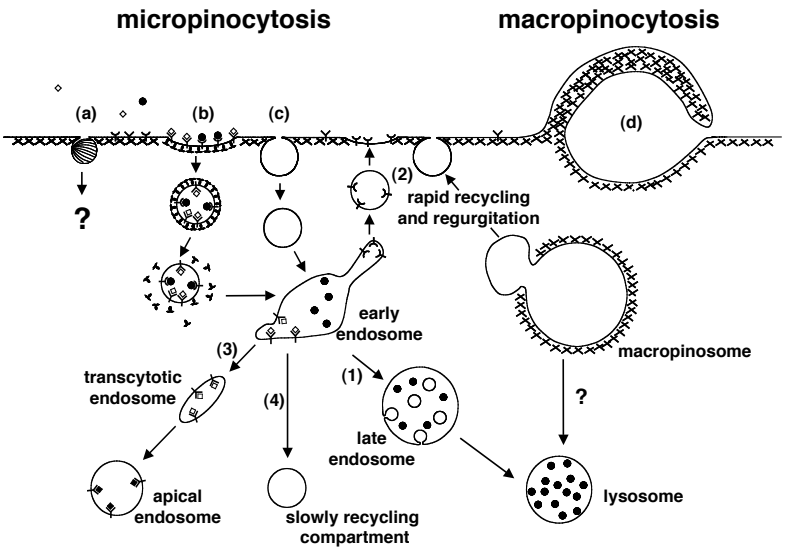

Figure 1. Pathways of endocytosis. This scheme represents four possible modes of vesicular entry of solutes into cells : (a) caveolae; (b) clathrin- and dynamin-associated pits, or «coated pits»; (c) clathrin- and dynamin-independent micropinocytic pits; and (d) macropinocytosis. Crosses represent cortical 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.

be established, with a 2-fold faster rate 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). 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 (9).

Relation between endocytosis and cell motility

A. Platek, M. Mettlen, D. Tyteca, Ph. de Diesbach and 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 acce-

lerated 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 (8).

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 (9). 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".

The rate of apical endocytosis regulates thyroid hormone production

P.J. Courtoy, K. Croizet and 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. 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.

A role for ClC-5 in the thyroid gland

M.F. van den Hove, K. Croizet and P.J. Courtoy, in collaboration with O. Devuyst, NEFR

Genetic inactivation of ClC-5, a voltage-gated chloride/proton antiporter prominently expressed in the kidney, leads to proteinuria because of defective apical endocytosis in proximal tubular cells (see below, 7). Since thyroid hormone secretion depends on apical endocytosis of Tg, we investigated whether ClC-5 is expressed in the thyroid gland and affects its endocytic activity, using *Clcn5*-deficient (KO) mice. These mice also allowed to test whether ClC-5 could provide a significant iodide conductance. We found that ClC-5 is well expressed in wild-type (WT) mouse thyroid (~ 40% of mRNA kidney level). The protein was immunolocalized at the apical pole of thyrocytes. In Percoll gradients, ClC-5 overlapped with plasma membrane and early endosome markers, but best co-distributed with the late endosomal marker, Rab7. ClC-5 KO mice were euthyroid but developed a goiter. Their ^{125}I-thyroid uptake after 1 h was doubled, but incorporation into Tg was decreased by ~ 2-fold. Enhanced ^{125}I-efflux upon perchlorate and restriction of ^{125}I-Tg at follicle periphery demonstrated delayed iodide organification. Endocytic trafficking of ^{125}I-Tg towards lysosomes was not inhibited. Expression of pendrin, an I^-/Cl^- exchanger involved in apical iodide efflux, was selectively decreased by 60% in KO mice at mRNA and protein levels. Thus, ClC-5 is well expressed in the thyroid but is not critical for apical endocytosis, contrary to the kidney. Instead, the goiter associated with ClC-5 KO results from impaired rate of apical iodide efflux, associated with down-regulation of pendrin expression (10).

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

C. Auzanneau, W. Rezende-Lima and P.J. Courtoy

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). In contrast, fluid-phase endocytosis was largely preserved, indicating that the basal endocytic machinery was not affected by ClC-5 absence. We therefore focused on 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 (7).

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 elec-

trogenic 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. Indeed, ClC-3 was found to be selectively upregulated by 2-3-fold in ClC-5 KO mice. 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.

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 2005, significant outcomes are 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 in kidney (Jouret et al., 2005, J. Am. Soc. Nephrol. 16:3235-46); of the subcellular trafficking of thrombopoietin receptor (see report by S. Constantinescu: Royer et al., 2005, J. Biol. Chem. 280:27251-61) and of the amyloid precursor protein, APP (Feyt et al., 2005, J. Biol. Chem. 280:33220-7); and the ultrastructural analysis of differentiating hepatoblasts (see report by F. Lemaigre: Clotman et al., 2005, Genes Dev. 19:1849-54).

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EXTRACELLULAR MATRIX BREAKDOWN

Etienne MARBAIX, Member
Patrick HENRIET, Member

Pierre J. COURTOY, Member
Hervé EMONARD, Guest Investigator
Christine GALANT, Assistant Member
Alix BERTON, E.U. Marie Curie Fellow (until Feb.)
Héloïse GAIDE CHEVRONNAY, Graduate Stud.
Chrystel PRETTO, Graduate Student
Charlotte SELVAIS, Graduate Student
Vassil VASSILEV, Graduate Student (until Dec.)
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). Our group has a long-standing expertise in the biochemistry and molecular biology of collagenase and related MMPs. We have demonstrated that menstrual bleeding in women is due to the expression and activation of some MMPs. 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 (1). Recently, our group has entered a new field of research, investigating how local MMP activity may be controlled by cells through plasma membrane binding or endocytosis.

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

V. Vassilev, C. Pretto, H. Gaide Chevronnay, P.J. Courtoy, E. Marbaix and 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 present 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 (MMP-1, -3, -7, -8, -9, -10, TIMPs-1, -2 -3, EBAF, IL-1 and TGF-β) were quantified in a large collection of endometrial samples collected throughout the menstrual cycle and stored before or after their culture as explants (4, 7, 9). Different expression patterns were identified *in vivo* according to the time frame, sharpness and magnitude of the changes. Differential response to the ovarian steroids was quantitatively mimicked in explant culture. Altogether, these observations suggest that, *in vivo*, different pathways finely tune in space, time and amplitude the global control by estradiol and progesterone of the expression of genes required for menstrual ECM breakdown.

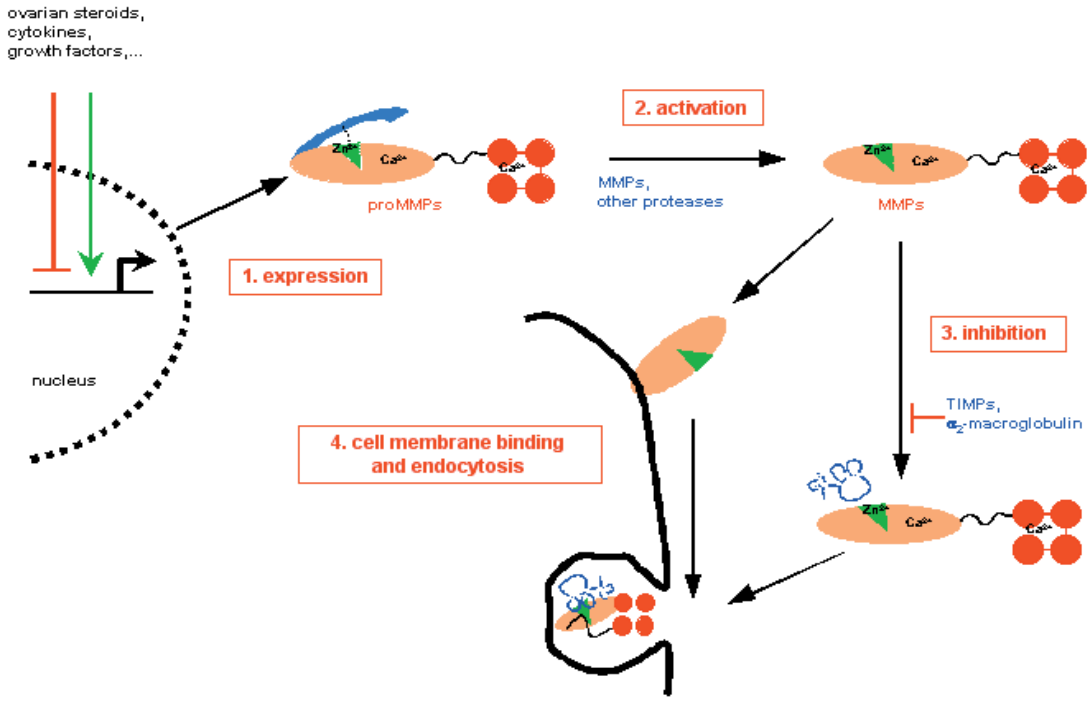

Figure 1. Regulation of MMPs activity in the human endometrium : current model. Proteolytic activity must be tightly controlled, both globally and locally, in order to allow blastocyst implantation and menstrual breakdown while preventing undesired collateral damage. The MMPs activity is overall controlled at 4 different levels : (i) expression, (ii) secretion and activation of zymogens, (iii) inhibition of active forms by physiological inhibitors (TIMPs) and (iv) membrane binding and endocytosis. (i) **Expression** : Ovarian steroids, their intracellular receptors and cytokines interact to form an integrated system that differentially controls the expression of endometrial MMPs and TIMPs. (ii) **Activation** : Most MMPs are secreted as inactive proforms that require proteolytic cleavage to acquire full activity. (iii) **Inhibition** : MMPs activity can be blocked upon occupancy of their catalytic site by TIMP molecules. (iv) **Cell membrane binding and endocytosis** : MMP binding to membrane receptors allows formation of pericellular proteolytic clusters and, at the same time, prepares for MMP endocytosis and lysosomal degradation. Our present research is focused on levels (i) and (iv).

Molecular mechanisms modulating MMP and TIMP expression upon ovarian steroids withdrawal

V. Vassilev, P.J. Courtoy, E. Marbaix and P. Henriet

Endometrial fibroblasts were used to further investigate the molecular mechanisms responsible for the differential response of MMPs and TIMPs to menstrual-like withdrawal of ovarian steroids. Changes in MMP-1 and TIMP-3 expression were measured in cells primed with estradiol and progesterone and suddenly deprived of hormones.

Upon hormone withdrawal, the mRNA amounts of MMP-1, -3, -8 and -10 quickly increased whereas those of TIMP-3 mRNA progressively decreased in proportions similar to MMP-1 mRNA increase. Dual luciferase assays demonstrated that sequences within the proximal promoters of MMP-1 and TIMP-3 were sufficient to retain the inverse response to hormones, suggesting indirect interactions between specific transcription factors. Addition of IL-1 at the time of hormone withdrawal further increased MMP mRNA upregulation through a MAP kinase-mediated pathway, suggesting crosstalks between MAP kinases and the ovarian steroids receptors. Finally, confo-

cal microscopy was used to follow the changes in progesterone receptor nucleocytoplasmic shuttling upon hormone withdrawal and/or IL-1 addition and their dependance on MAP kinase pathways. Altogether, these observations show that the differential regulation of MMP and TIMP genes depends on crosstalks between nuclear receptors and MAP kinase pathways.

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

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

The low-density lipoprotein receptor-related protein (LRP)-1 mediates the endocytic clearance of numerous ligands. We recently demonstrated that proMMP-2:TIMP-2 complexes and free TIMP-2 were endocytosed by LRP-1 in a HT1080 human fibrosarcoma cell line and that such a process involved primary TIMP-2 binding to an unidentified receptor (5). The identification of the primary receptor and its cell-membrane localization are in progress. Beside endocytic activities, LRP-1 is involved in transducing various extracellular signals (reviewed in 8). TIMP-2 abrogates the EGF-stimulating effect on cell proliferation of various origins, but the nature of its cell-surface receptor remains speculative. Upon treatment with RAP, a natural LRP-1 ligand antagonist, we showed that inhibition of EGF-enhanced HT1080 cell proliferation by TIMP-2 was removed, suggesting that LRP-1 could mediate TIMP-2 inhibiting signals, by a pathway we are currently investigating.

Addition of RAP to endometrial explant cultures considerably increased the amount of MMP-2 and MMP-9. This observation suggests that LRP-mediated endocytosis of MMPs could represent an additive way of post-traductional regulation of MMPs activity in endometrium during menstrual cycle. This approach will be extended to other menstrual MMPs and TIMPs. Also, regulation of LRP-1 and RAP expression in endometrium during menstrual cycle will be analyzed.

Role of matrix metalloproteinases in abnormal endometrial bleeding

C. Galant, P. Henriet, P.J. Courtoy and E. Marbaix, in collaboration with J.L. Brun, Bordeaux, France

Since matrix metalloproteinases (MMPs) play a key role in initiating normal menstrual breakdown, we looked for their contribution in two pathological conditions characterized by abnormal bleeding without organic lesion. Up to one fourth of hysterectomies are due to such functional menstrual disorders. We demonstrated that irregular dysfunctional bleeding or metrorrhagia was associated with menstrual-like stromal breakdown in foci containing low levels of ovarian steroids receptors, ER alpha and PR, and with increased expression and activation of several MMPs, mainly MMP-1, -2, -3 and -9 together with decreased production of TIMP-1 (6). Inflammatory cells, in particular neutrophils, are recruited in the same sites and release their abundant content of proMMP-8 and -9, which, when activated, increase the proteolysis of the matrix. These results confirmed *in vivo* our previous findings obtained with cultured explants from patients on long-term progestinic contraception (1). Menorrhagia, *i.e.* excessive menstrual blood loss, is the other gynaecological disorder we investigated. It affects up to 20 % of women in their reproductive age. Menstrual expression and activity of several MMPs are increased in menorrhagic women. Thermocoagulation of the endometrium with an intra-uterine balloon containing hot water eliminates bleeding or efficiently decreases its amount. Actually, endometrium regenerates, presumably from the uterine cornuae and isthmus or from adenomyotic lesions, and menstrual bleeding reappears in 10-20 % of the treated patients. The reason for the lower amount of bleeding remains unclear, although we observed an increased

production of TIMPs in such condition. To clarify the mechanisms responsible for these local disorders, we currently look for altered expression or regulation of ovarian steroid receptors and appropriate cytokines. In addition, we are 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 apprehend their role in physiological and abnormal endometrial bleeding, as well as in endometrial angiogenesis and vessel maturation through pericyte recruitement (10).

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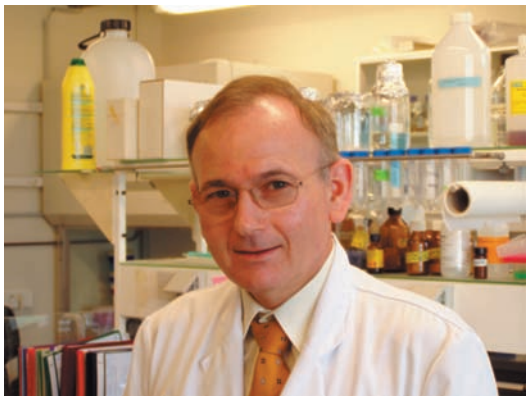
CONNECTIVE TISSUE AND ARTHRITIS

Daniel MANICOURT, Member

Hafida EL AJJAJI, Postgraduate
Annette MARCELIS, Technician
Anne van EGEREN, Technician



The major research efforts of the group are focused on the pathophysiology of osteoarthritis (OA), the most prevalent of joint disorders. In this disease process, the anatomy and composition of both articular cartilage and subchondral bone are altered by a complex combination of degradative and reparative processes which depend upon an interplay between, on the one hand, local biomechanical factors acting on the joint and, on the other hand, generalized factors making a predisposition to the disease. However, relatively little is known about which of the disease processes and etiological factors control progression of the disease process to OA. Further, although it is clear that the initiation of cartilage damage and the progression to full cartilage loss may involve separate pathophysiological mechanisms, it is unclear whether OA changes occur first in cartilage, in bone or are concurrent.

Effects of nonsteroidal anti-inflammatory drugs on the overall metabolism of articular cartilage

Because they inhibit cyclo-oxygenase (COX), and hence the production of prostaglandins (PGs), nonsteroidal anti-inflammatory drugs (NSAIDs) are widely prescribed in patients suffering from arthritides. Two isoforms of COX have been identified thus far: COX-1, which is constitutively expressed in most tissues and COX-2, which is highly inducible in response to proinflammatory cytokines and mitogens. It is generally believed that the beneficial effects of NSAIDs are related to their ability to inhibit COX-2 whereas the gastrointestinal and renal toxicity of these drugs results from their inhibition of COX-1, a contention that has provided the basis for the development of highly selective COX-2 inhibitors. It should be however stressed that COX-1-derived PGs can contribute to the inflammatory response and that COX-2-derived PGs perform physiologically important roles such as the maintenance of normal renal function. Furthermore, COX-2-derived PGs, in addition to their anti-inflammatory properties, have been implicated in the protection of the gastrointestinal tract from injury.

Although NSAIDs undeniably produce relief of pain and improvement of joint mobility in patients suffering from arthritides, *ex vivo* and ***in vivo*** studies have shown that some NSAIDs inhibit the synthesis of cartilage proteoglycans whereas others do not. This differential effect of NSAIDs on cartilage metabolism is most relevant to clinical practice since any drug, that suppresses proteoglycan synthesis and impairs the chondrocyte to repair its already damaged extracellular matrix, could potentially accelerate the breakdown of the cartilage tissue. On the other hand, although hyaluronan (HA) plays a central structural role in the supramolecular organization of proteoglycans and, hence on the biomechanical properties of articular cartilage, the possible effects of NSAIDs on the metabolism of this glycosaminoglycan has so far focused little investigative attention.

We therefore investigated the action of celecoxib (a strong selective COX-2 inhibitor), on the metabolism of newly synthesized HA and proteoglycan molecules in explants from human OA cartilage (1). In contrast to classical NSAIDs, this COX-2 selective inhibitor had a positive effect on the overall metabolism of both proteoglycans and hyaluronan, two major components of the extracellular matrix of cartilage. This effect, which is independent of the inhibition of prostaglandin production, is under investigation as it might be of great biological and therapeutic significance in arthritides.

Markers of connective tissue metabolism in health and disease.

(In collaboration with E. Thonar, Rush-Presbyterian-St Luke's Medical Center, Chicago, USA).

Nowadays, several biochemical molecules derived from the joint components can be quantified in body fluids (joint fluid, blood and urine). Theses molecules termed "metabolic markers" or simply "markers" appear as important tools to disclose *in vivo* important changes occurring during both the preclinical and clinical stages of various joint diseases, including osteoarthritis. There is also evidence that these markers may prove helpful in determining whether a therapeutic regimen is effective or not, and this in a relatively short period of time. Indeed, in the absence of markers, the efficacy of treatment in joint disorders relies mainly on radiographic changes, an approach that takes years before one can reach meaningful results.

The markers that are most currently used are hyaluronan, a marker of synovial proliferation and inflammation, antigenic keratan sulfate, a marker of proteoglycan metabolism, cartilage oligo-matrix protein, a marker of cartilage matrix remodeling, and the telopeptides of type II collagen, a marker of the breakdown of cartilage collagen. There is indeed good agreement that this panel of markers helps diagnose, monitor or prognosticate osteoarthritic changes.

Role of the subchondral bone in the initiation and progression of the osteoarthritic disease process

So far, the possible role of subchondral bone in the initiation and/or progression of osteoarthritis (OA) has focused little investigative attention. We have therefore explored this topic in an animal model of osteoarthritis. In this model, severing of the anterior cruciate ligament of the knee increases dramatically the biomechanical forces applied to the internal compartment of the knee joint and results in the progressive apparition of OA lesions in the operated joint which closely resemble those seen in human OA.

During the first weeks following joint destabilization, we have observed a dramatic decrease in the density and volume of the trabecular subchondral bone. These changes increased with time post-surgery and were restricted to the internal compartment of the operated joint whereas no significant changes in bone density and volume could be disclosed in the external compartment of the unstable joint.

Obviously, theses changes reflect an adaptation of the bone to absorb the enhanced biomechanical forces imposed upon it. On the other hand, these changes concomitantly induce a dramatic increase in the tensile and shearing forces upon the overlying articular cartilage and, in so doing, contribute to the degradation of the cartilage tissue. Our working hypothesis is supported by the finding that animals receiving drugs known to inhibit bone resorption do not show up any change in the volume and density of the trabecular subchondral bone of the operated knee and, more importantly, exhibit a dramatic decrease in the severity of cartilage OA lesions (2).

These findings open a new approach in the

therapeutic regimen of OA and studies are currently conducted in human OA.

Towards a better understanding of the metabolism of hyaluronan in connective tissues

Research efforts are also devoted to the regulation of hyaluronan metabolism both in health and disease. In skin, which contains 50 % of total body hyaluronan, the half-life of hyaluronan is about one day, and even in as seemingly inert tissue as cartilage, hyaluronan turns over with a half-life of one to three weeks. In the blood stream, the half-life of hyaluronan is two to five minutes. All such catabolism is presumably a result of hyaluronidases. What is the nature of the control mechanisms that orchestrate such vastly different rates of turnover? The hyaluronan of vertebrate organisms can exist in many states, in a variety of sizes, in extracellular forms, free in the circulation, loosely associated with cells and tissues, tightly intercalated within proteoglycan-rich matrices such as that of cartilage, bound by receptors to cell surfaces, or even in several intracellular locations. Superimposed on these many states are the panoply of binding proteins, or hyaladherins, that decorate the hyaluronan molecule. How do mechanisms of catalysis differ among this wide range of physical and chemical states of the hyaluronan substrate? It is unlikely that hyaluronidase activity is retained *in vivo* in an active form within the extracellular matrix where it could cause great havoc. If it is found within the extracellular matrix, it may be in an inactive or suppressed form, perhaps bound to an inhibitor. Such a situation would parallel the relationship between the metalloproteinases and the tissue inhibitors of metalloproteinases or TIMPs that exert exquisite control over metalloproteinase activity.

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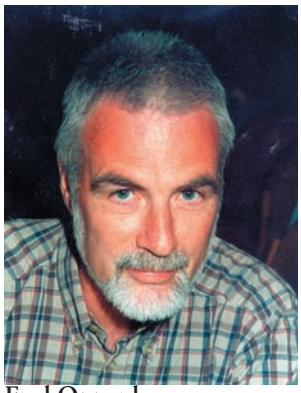
METABOLIC COMPARTMENTATION IN TRYPANOSOMES

Fred R. OPPERDOES, Member
Paul A.M. MICHELS, Member

Véronique HANNAERT, Assistant Member
Jean-Pierre SZIKORA, Assistant Member

Artur CORDEIRO (from April 2005), ICP fellow
Mariana IGOILLO-ESTEVE (from July 2005), ICP fellow
Marie-Astrid ALBERT (until April), Graduate Student
Ana CACERES (from June), Graduate Student
Daniel GUERRA (until November), Graduate Student
Nathalie GALLAND, Graduate Student,
Murielle HERMAN, Graduate Student,
Hanane KRAZY, Graduate Student,
Emilie VERPLAETSE, Graduate Student,
Cédric YERNAUX (until September), Graduate Student
Freddy ABRASSART, Technician
Nathalie CHEVALIER, Technician
Christian VAN LANGENHOVE, Technician
Joris VAN ROY, 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 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 ICP is trying to better understand the biochemistry of these organisms in order to develop new and better drugs. Trypanosomes rely on glycolysis for their ATP supply and are characterized by a unique form of glycolytic compartmentation where the majority of the enzymes 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. Because of its unique compartmentation, glycolysis in trypanosomes requires a type of regulation and flux control that is entirely different from that in other organisms. To better understand the control of this pathway in these organisms we use a detailed mathematical model of the glycolytic pathway in combination with enzyme inhibition studies and gene expression knock-down experiments. 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. 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.

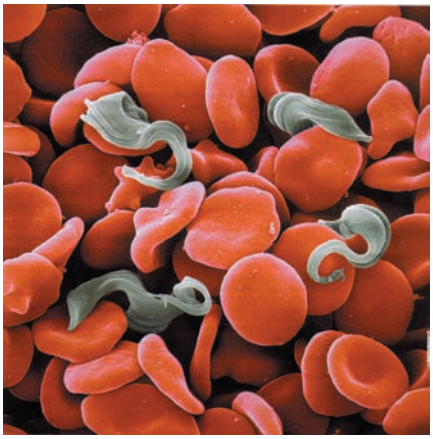


Figure 1. Trypanosomes surrounded by red blood cells.

Pathways and enzymes of carbohydrate metabolism

Energy metabolism

F. Opperdoes, V. Hannaert and P. Michels, in collaboration with A. Tielens, University of Utrecht, The Netherlands and F. Bringaud, Université Victor Segalen, Bordeaux, France

The energy metabolism of *T. brucei* differs significantly from that of its hosts and changes drastically during the life cycle. Despite the presence of all citric acid cycle enzymes in the procyclic insect-stage, citric acid cycle activity is not used for energy generation. Recent investigations on the influence of substrate availability on the type of energy metabolism showed that absence of glycolytic substrates did not induce a shift from a fermentative metabolism to complete oxidation of substrates. Apparently, insect-stage *T. brucei* uses parts of the citric acid cycle for other purposes than for complete degradation of mitochondrial substrates. Parts of the cycle are used for (i) transport of acetyl-CoA units from the mitochondrion to the cytosol for the biosynthesis of fatty acids, (ii) degradation of proline and glutamate to succinate, (iii) generation of malate, which can then be used for gluconeogenesis. Apparently the citric acid cycle in trypanosomes does not function as a cycle.

Control of glycolysis

M.-A. Albert, V. Hannaert, D. Guerra, A. Caceres and P. Michels, in collaboration with B. Bakker, University of Amsterdam, The Netherlands

Previously a mathematical model of glycolysis in bloodstream-form trypanosomes has been constructed on the basis of experimentally determined enzyme kinetics data. This model predicted correctly the fluxes and cellular metabolite concentrations in non-growing trypanosomes and the rate-limiting role of the plasma-membrane glucose transporter. It also predicted a large overcapacity for hexokinase (HXK), phosphofructokinase (PFK), and pyruvate kinase (PYK). The precise contribution of these enzymes to the control of glycolytic flux was now experimentally determined. After having optimized our model with more precise kinetic data obtained for enzymes in lysates of growing trypanosomes, the concentrations of seven glycolytic enzymes (HXK, PFK, aldolase, glyceraldehyde-phosphate dehydrogenase, phosphoglycerate mutase, enolase, and PYK) in trypanosomes were changed by the method of RNA interference (RNAi). Data thus obtained still support our original conclusion that HXK, PFK, and PYK are present in excess, albeit less than originally predicted. Interestingly, depletion of PFK and enolase had an effect on the activity of some other glycolytic enzymes located both in glycosomes and in the cytosol. A specific and significant decrease in PYK concentration was observed upon depletion of almost any of the six other enzymes. These data suggest the existence of novel regulatory mechanisms operating in trypanosome glycolysis.

Preliminary mathematical models have now also been developed for the more elaborate glycosomal system by which glucose is catabolized in insect-stage *T. brucei* and *Leishmania* spp. These models too will be used in guiding our experimental work aimed towards a better understanding of the energy metabolism of the parasites.

Fructose-bisphosphate aldolase

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

Previously we have identified important differences in active-site structure and catalytic mechanism of aldolases from mammalian cells and two trypanosomatids, *T. brucei* and *L. mexicana*. Together, these data indicate that aldolase has high promise as drug target. These differences allowed us to design and synthesize a potent, quasi-irreversible inhibitor (hydroxynaphthaldehyde phosphate) with high selectivity for the parasite enzyme. This inhibition involves Schiff base formation by the inhibitor with a catalytic lysine followed by reaction of the resultant Schiff base with a second serine residue. Pro-drugs have been developed to facilitate uptake in cells and these were able to kill cultured parasites with an ED_{50} value of approximately 2.5 μM. We intend to optimize these inhibitors by successive rounds of structure-ligand interaction analyses and chemical synthesis.

Phosphofructokinase and pyruvate kinase

V. Hannaert and P. Michels, in collaboration with L. Fothergill-Gilmore, M. Walkinshaw and N. Turner, University of Edinburgh, Scotland

High-resolution crystal structures have been determined for two allosteric enzymes, *T. brucei* PFK and *Leishmania mexicana* PYK, each in its R-state with high-affinity for the substrate fructose 6-phosphate and phospho*enol*pyruvate, respectively, and the low-affinity T-state. These crystal structures revealed several unique features that might be used for developing selective inhibitors of the trypanosomatid enzymes. Because of the fortuitous fact that fructose-bisphosphate is both a product of PFK and the effector of PYK, compounds were synthesized using a designed fructose analogue as chemical template for the preparation of combinatorial libraries that would exploit the unique structural features of the targeted ligand-binding sites of respective enzymes. Accordingly, several trypanosomatid enzyme-specific inhibitors have been obtained displaying IC_{50} values in the low micromolar range and killing cultured bloodstream-form trypanosomes with similar ED_{50} values. Further improvement of these inhibitors by detailed analysis of their structure-activity relationships is ongoing, with the aim to develop lead trypanocidal drugs.

Malate dehydrogenase

F. Opperdoes, in collaboration J.J. Cazzulo University of San Martin and C. Nowicki, University of Buenos Aires, Buenos Aires, Argentina and A.D. Uttaro, University of Rosario, Argentina

In *T. brucei* three different malate dehydrogenase isozymes were characterized. The enzymes were identified as the mitochondrial, glycosomal and cytosolic isozymes, respectively. The latter is only expressed in the bloodstream forms. The respective genes were cloned and the recombinant enzymes were functionally expressed in *Escherichia coli* cultures, purified and characterized.

Glucose-6-phosphate dehydrogenase

A. Cordeiro, M. Igoillo-Esteve and P. Michels in collaboration with J.J. Cazzulo, University of San Martin, Argentina

Previously, we reported the cloning and characterization of *T. brucei* and *L. mexicana* glucose-6-phosphate dehydrogenase (G6PDH), the first enzyme of the hexose monophosphate pathway. The enzymes of both trypanosomatids have been expressed as recombinant proteins and are now being used in crystallization trials, with the aims of structure determination and inhibitor design. Also different isoforms of the *T. cruzi* G6PDH, previously characterized by M. Igoillo-Esteve and J. Cazzulo, are used for crystallization. Furthermore, it has been shown that the enzyme is essential in bloodstream-form trypanosomes; the trypanosomes die upon depletion of G6PDH by RNAi.

Superoxide dismutase

D. Gerbod, C. Yernaux and F. Opperdoes, in collaboration with E. Viscogliosi, Institut Pasteur, Lille, France

We have identified in *T. brucei* the presence of four superoxide dismutase (SOD) genes: SODA, SODB1, SODB2 and SODC. The genes were cloned, sequenced, and overexpressed in *E. coli* and shown to encode active dimeric Fe-dependent SOD isozymes. All four *T. brucei* SODs are very similar in structure. SODB1 is mainly cytosolic, with a minor glycosomal component, SODB2 is mainly glycosomal with some activity in the cytosol, and SODA and SODC are both mitochondrial isozymes. Phylogenetic studies suggested that the trypanosomatid SODs have all been acquired by events of horizontal gene transfer, followed by events of gene duplication.

Glycosome biogenesis

H. Krazy, N. Galland, E. Verplaetse and 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 2005 several additional *T. brucei* orthologues of yeast and mammalian peroxins involved in import of peroxisomal matrix proteins: PEX6, PEX7, PEX10 and PEX12. 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 different types of glycosome-targeting signals. These experiments confirmed the identity of all these trypanosomal proteins as peroxins. Furthermore, strong support was obtained for the notion that PEX7, the cytosolic import receptor for proteins with a glycosome/peroxisome-targeting signal type 2 (PTS2) interacts with PEX5, the cytosolic receptor for proteins containing a type-1 signal (PTS1). PEX7 was co-purified with a C-terminally Tap-tagged form of PEX5, expressed from an ectopic gene copy in insect-stage trypanosomes, and a sequence characteristic for PEX7-binding proteins was identified in the N-terminal half of *T. brucei* PEX5.

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 and P. Michels, in collaboration with M. Fransen, Katholieke Universiteit Leuven

We have started an investigation of glycosomal membrane proteins that might be involved in the transport of glycolytic intermediates or other solutes across the membrane. We have cloned and sequenced three *T. brucei* genes coding for ABC transporters which we located to the glycosomal membrane. The amino-acid sequences encoded by these genes are only 30% identical to each other. These proteins, designated GAT1-3 (for Glycosomal ABC Transporter), are so-called half ABC transporters, containing only a single ATP-binding cassette in their C-terminal half. The glycosomal localization was shown by immunofluorescence microscopy of trypanosomes expressing fusion constructs of the transporters with Green Fluorescent Protein. By expression of fluorescent deletion constructs, the glycosome-targeting determinant of two transporters was mapped to different fragments of their respective primary structures. Interestingly, these fragments share a short sequence motif and contain adjacent to it one - but not the same - of

the predicted six transmembrane segments of the transmembrane domain. We also identified the *T. brucei* homologue of peroxin PEX19, which is considered to act as a chaperonin and/or receptor for cytosolically synthesized proteins destined for insertion into the peroxisomal membrane. By using a bacterial two-hybrid system, it was shown that glycosomal ABC transporter fragments containing an organelle-targeting determinant can interact with both the trypanosomatid and human PEX19, despite their low overall sequence identity.

In order to analyze the functions of the different GAT proteins, experiments were performed to determine whether they could functionally complement yeast mutants deficient for the homologous proteins. Preliminary results suggest that GAT2 might be involved in the metabolism of fatty acids. Other preliminary experiments involving hybridization studies using RNA blots and immunoanalysis of protein blots using antisera raised against purified fragments specific for each transporter expressed in *E. coli*, showed that the GAT transporters are differentially regulated in different stages of the *T. brucei* life cycle.

Turnover of glycosomes

M. Herman and 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.

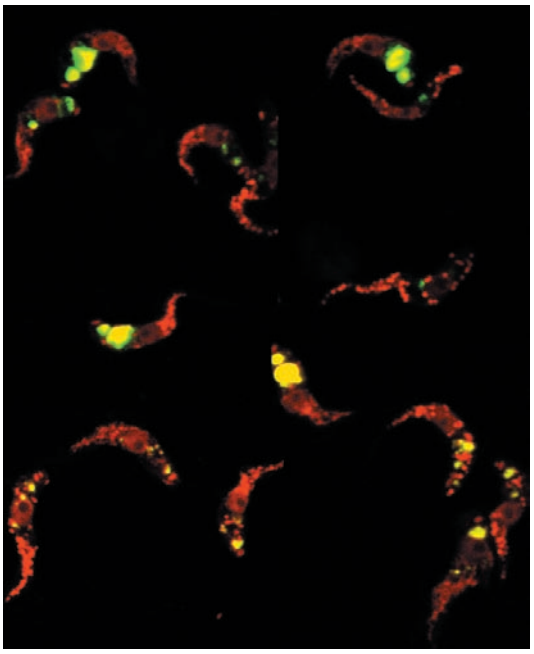


Figure 2. Autophagy of glycosomes inside lysosomes. Immunofluorescence image of trypanosomes in the process of digesting glycosomes during the differentiation from bloodstream to procyclic form *in vitro*. The lysosome (stained in green) is enlarged and colocalizes with some glycosomes (stained with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

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

The recent completion 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 trypanosomes, 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* glycosomes seem 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.

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6-Phosphofructo-2-kinase and fructose-2,6-bisphosphatase in Trypanosomatidae. Molecular characterization, database searches, modelling studies and evolutionary analysis. **FEBS J** 2005;272:3542-60.

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

Miikka VIKKULA, Member
Laurence BOON, Plastic Surgeon,
Part time Research Associate

Mustapha AMYERE, Postdoctoral Fellow
Pascal BROUILLARD, Postdoctoral Fellow
Ilse GUTIERREZ-ROELENS, Postdoctoral Fellow
Nisha LIMAYE, Postdoctoral Fellow
Nicole REVENCU, Pediatrician, Genetics Resident
Virginie AERTS, Graduate Student
Laurence DESMYTER, Graduate Student
Arash GHALAMKARPOUR, M.D., Graduate Student
Michella GHASSIBE, Graduate Student
Brendan MCINTYRE (until October), Graduate Student
Thomas PALM, Graduate Student
Vinciane WOUTERS, Graduate Student
Anne Van EGEREN, Technician
Dominique COTTEM, Technician
Antonella MENDOLA, Technician
Matthieu GERADIN, Part-time Technical Assistant
Claude MAUYEN, Part-time Technical Assistant
Liliane NICULESCU, Secretary

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, B. McIntyre, V. Aerts, V. Wouters, N. Limaye, L.M. Boon and M. Vikkula, in collaboration with B.R. Olsen, Harvard Medical School, Boston, USA; J.B. Mulliken and S. Fishman, Children's Hospital, Boston, USA; O. Enjolras, Hôpital Lariboisière, Paris, France 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 and 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. Amyere, N. Limaye and M. Vikkula in collaboration with J.B. Mulliken, Children's Hospital, Boston, USA, S. Watanabe, Showa University School of Medicine, Tokyo, Japan, A. Dompmartin, CHU de Caen, France 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 and symptoms (Revencu et al, in preparation).

Cardiopathies

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

The cardiovascular system 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 (Gutierrez et al, submitted), 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).

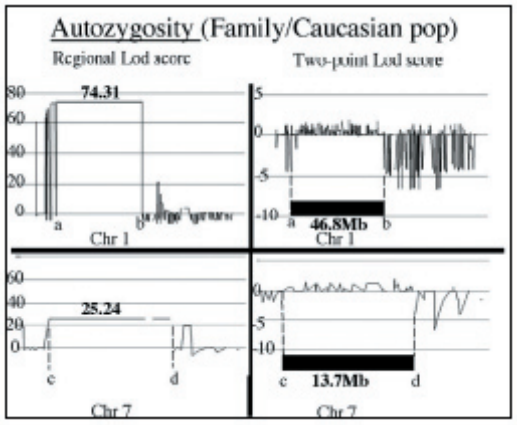

Figure 1. Regional and two-point Lod scores calculated by Genespring GT based on a Caucasian population. The highest regional Lod scores of 74.31 and 25.24, when comparing the family data with a Caucasian control population were obtained, in chromosome 1 and 7 respectively. (adapted from [8]).

Cleft lip and palate

M. Ghassibé, L. Desmyter, N. Revencu, M. Vikkula, in collaboration with Y. Gillerot, B. Bayet, R. Vanwijck, 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).

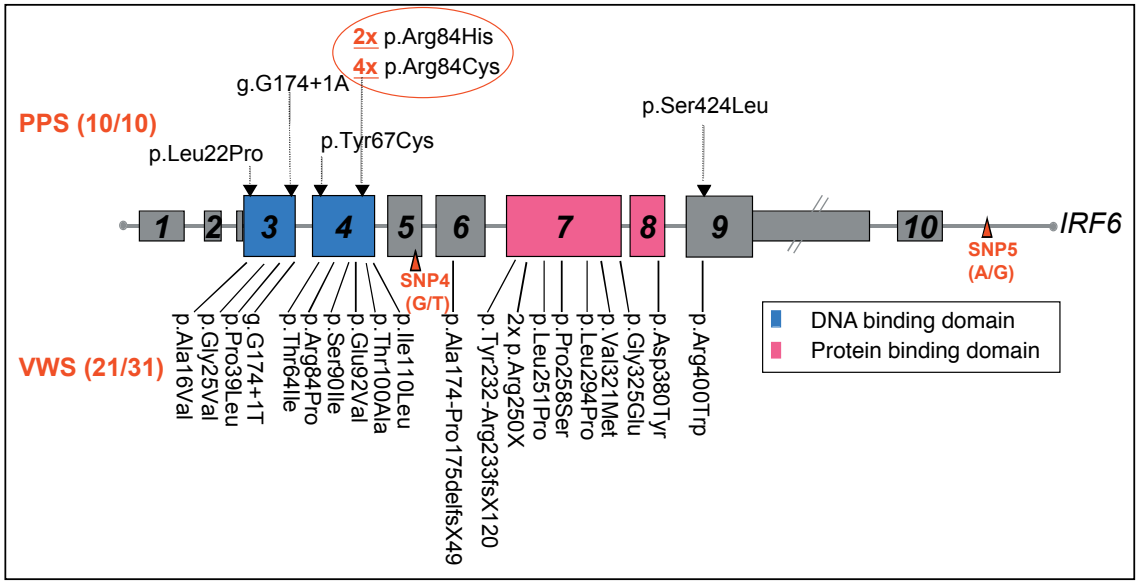

Figure 2. Mutation frequency in the IRF6 gene in Europe.

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

Morphological characterization and classification of tumors is not always clear. Thus, better (molecular) criteria are needed. 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 testing. 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. In the more distant future, we hope that this knowledge will help develop novel targeted therapies.

Selected publications

1. Boon LM, Mulliken JB, Enjolras O, Vikkula M. *Glomuvenous malformation ("glomangioma") and venous malformation are distinct clinicopathologic and genetic entities.* **Arch Dermatol** 2004;140:971-76.

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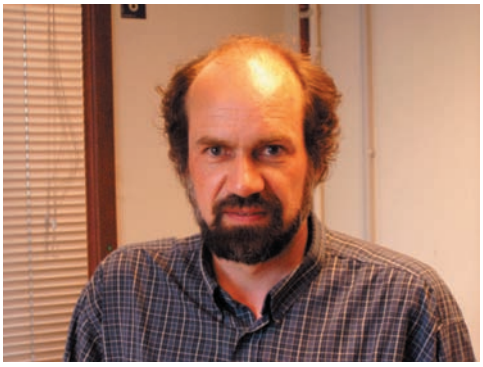
VIRAL PERSISTENCE AND INTERFERON RESPONSE

Thomas MICHIELS, Associate member

Sophie PAUL, Graduate student
Céline RICOUR, Graduate student
Caroline SOMMEREYNS, Graduate student
Frédéric SORGELOOS, Graduate student
Muriel MINET, Technician



Our work focuses on the interplay between viral infecctions 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 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 of an immunocompetent host, thus evading the immune response. We analyze viral determinants contributing to escape the immune response and leading to the demyelinating disease

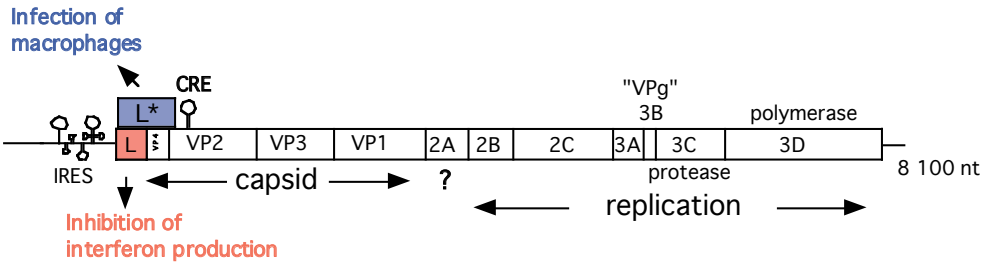


Figure 1. Genome of Theiler's virus. A large open reading frame encodes a 2000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, into 12 mature proteins. An additional protein (L*) is encoded by an alternative open reading frame. Translation of both ORFs is driven by an Internal Ribosome Entry Site (IRES) present in the 5' non-coding region of the genome. Protein L* was shown to facilitate the infection of macrophages and viral persistence (1). Protein L inhibits type-I interferon production (2). The role of protein 2A is unknown. 60 copies of proteins VP1 to VP4 assemble to form the viral capsid. 3B (also termed 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» (3).

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

C. Ricour, F. Sorgeloos, 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 were 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.

Inhibition of IFN production was found to occur at the transcriptional level (5). Thus, a potential target of the leader protein is IRF-3, a factor known to be required for transcritpional activation of IFN genes. IRF-3 is present in the cytoplasm of non-infected cells. Upon viral infection, IRF-3 is activated and translocated to the nucleus where it activates the transcription of the IFN genes.

We recently showed that the leader protein interferes with nucleo-cytoplasmic trafficking of host cell proteins, and notably of IRF-3 (6). Perturbation of nucleo-cytoplasmic trafficking can also constitute a way to inhibit early cell defense mechanisms. Indeed, the induction of many genes involved in early defenses, such as genes for cytokines and chemokines, depends on the nuclear translocation of transcription factors.

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 (8).

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. Our data show that, 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 nucleocytoplasmic trafficking of host-cell proteins (9).

Interestingly, analysis of viral RNA replication of the recombinant viruses raised the hypothesis that L proteins of TMEV and EMCV diverged during evolution to adapt to the different replication fitness of these viruses (9).

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

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

We analyzed the phenotype of L* mutant viruses carrying either an AUG to ACG mutation of the initiation codon or a stop codon mutation introduced in the L* ORF. Our data suggest that L* can be expressed from an ACG initiation codon. Thus, neurovirulent strains which have ACG instead of AUG, can also produce the L* protein. This would be the first

example of picornavirus IRES-driven non-AUG translation initiation (1).

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.

Type-I Interferons

Characterization of the murine IFN-α family

C. Sommereyns, S. Paul, and T. Michiels

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.

As some confusion existed in the identity and characteristics of mouse IFN-α subtypes, availability of data from the mouse genome sequence prompted us to characterize the murine IFN-α family. A total of 14 IFN-α genes were detected in the mouse genome, in addition to three IFN-α pseudogenes (7).

All IFN-α subtypes were found to be stable at pH2 and to exhibit antiviral activity. Interestingly, some IFN subtypes showed higher biological activity than others. Most murine IFN-α turned out to be N-glycosylated. However, no correlation was found between N-glycosylation and activity.

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.

Glycosylation of IFNs

C. Sommereyns and 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.

We are now analyzing the influence of IFN N-glycosylation on the biological activity of these cytokines. We also characterize the type-I IFN response to viral infections in the particular context of the central nervous system.

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VIRAL IMMUNITY AND PATHOGENESIS

Jean-Paul COUTELIER, Associate Member

Thao LE THI PHUONG, Graduate Student
Gaëtan THIRION, Graduate Student
Laurent **DETALLE**, Graduate Student
Nadia OULED HADDOU, Technician
Thérèse BRIET (part time), Technician
Claude MAUYEN (part time), Technician
Anick DELCHAMBRE, Secretary

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), we could demonstrate that IgG2a antiviral antibodies are indeed more efficient than other isotypes to protect mice against a fatal polioencephalomyelitis induced by the virus (3, 4). 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 (5).

Activation of macrophages and autoimmune diseases

Activation of cells of the innate immune system includes also macrophages and leads to an enhanced phagocytic activity, with potential detrimental consequences for ongoing autoimmune diseases. Our analysis has been focused on autoantibody-mediated blood autoimmune diseases. A new experimental model of anti-platelet response was developed in the mouse (6). Immunization of CBA/Ht mice with rat platelets was followed by a transient thrombocytopenia and production of autoantibodies that react with epitope(s) shared by rat and mouse platelets. In contrast, BALB/C mice similarly immunized with rat platelets did not develop thrombocytopenia. 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 (7). Similarly, administration of anti-erythrocyte monoclonal autoantibody to mice resulted in the development of a transient hemolytic anemia that was dramatically enhanced by a simultaneous infection with LDV, leading to the death of most animals. This viral infection induced an increase in the ability of macrophages to phagocytose *in vitro* autoantibody-coated red cells, and an enhancement of erythrophagocytosis in the liver (8,9).

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).

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SIGNAL TRANSDUCTION BY GROWTH FACTOR RECEPTORS

Jean-Baptiste DEMOULIN, Associate Member

Anders KALLIN, ICP post-doctoral fellow (Lacroix fellowship)
Ahmed ESSAGHIR, graduate student
Federica TOFFALINI, graduate student
Axelle NZOKIRANTEVYE student
Catherine MARBEHANT, technician
Anick DELCHAMBRE, secretary (part time)

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.

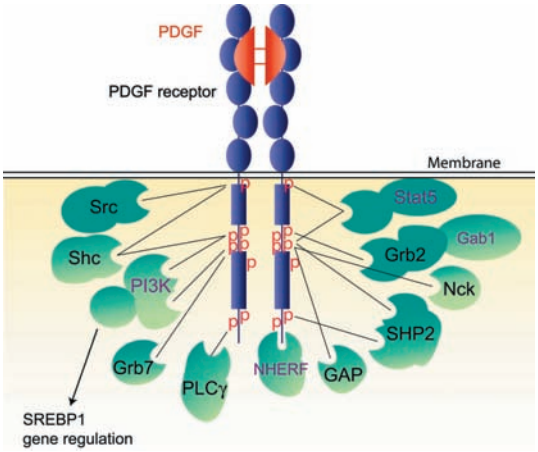

Figure 1. Signal transduction by the PDGF receptors. Signaling molecules of special interest for our group are indicated in purple. The receptor (blue) contains five extracellular immunoglobulin-like domains and an intracellular tyrosine kinase domain (boxes). p, phosphorylated tyrosine residue.

PDGF receptor activation

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

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 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-receptor 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).

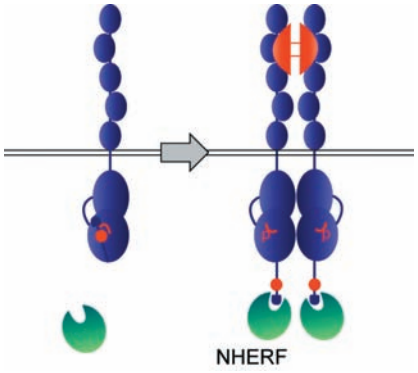

Figure 2. Role of the PDGF receptor C-terminal domain. In the inactive conformation, a Pro/Glu-rich acidic motif (red disk) blocks the kinase domain. Our data suggest that the C-terminal tail is released upon receptor activation and the NHERF binding site (blue disk) becomes accessible.

The activated PDGF receptor complex comprises two PDGF receptor molecules (α and/or β) bound to one molecule of dimeric PDGF ligand (Fig. 2). Whether the PDGF receptors form monomers or dimers in the absence of ligand is still a matter a debate. This question is also relevant for oncogenic mutated PDGF receptors which are active in the absence of ligand. In collaboration with Yoav Henis, Yaniv Malka and Stefan Constantinescu, this issue will be addressed using the co-patching method with HA- and FLAG-tagged receptors.

Gene regulation by PDGF and other growth factors

J.B. Demoulin, A. Kallin, C. Nyiraneza, 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.

Role of stearoyl-CoA desaturase, a gene regulated by PDGF

J.B. Demoulin, C. Marbehant, in collaboration with Catherine Godfraind, Cliniques Universitaires Saint-

Luc, UCL, Marie-Paule Mingeot, Ecole de pharmacie, UCL, and Charlotte Rorsman, Ludwig Institute for Cancer Research, Uppsala, Sweden.

As mentioned above, we found in a microarray screen that PDGF strongly regulates the expression of stearoyl-coenzyme-A desaturase (SCD), which catalyses the synthesis of monounsaturated fatty acids. A role for this enzyme has been suggested in tumor growth based on the following observations: (i) analysis of a limited number of tumor RNA samples indicated that it may be over-expressed in cancer cells; (ii) some tumor types show an increased unsaturated fatty acid content; and (iii) treatment of mice with lipid mixtures that block SCD activity decreased tumor growth. These effects have been ascribed to increased membrane fluidity, a likely consequence of increased unsaturated fatty acid production.

We are now measuring the expression of SCD by quantitative PCR and immunohistochemistry in tumors types associated with PDGF receptor activation.

In parallel, we transfected porcine aortic endothelial cells with an artificial SCD gene controlled by a tetracycline-inducible promoter. SCD expression in these cells strongly enhanced unsaturated fatty acid synthesis. We observed a weak increase in membrane fluidity when SCD expression was induced, but no effect on PDGF receptor signaling, proliferation and chemotaxis induced by PDGF.

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 Carl-Henrik 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, A. Kallin, J.B. Demoulin, in collaboration with Hélène 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*

Pierre G. COULIE, Member
Sophie LUCAS, Senior Investigator

Wolfram FINK, Post-doctoral fellow (till December 2005)
Tomoko SO, Post-doctoral fellow (till December 2005)
Rim BENMAAMAR, Graduate student (till October 2005)
Thierry CONNEROTTE, Graduate student
Véronique CORBIERE, Graduate student
Ygierne DODOO, Graduate student (till October 2005)
Thérèse AERTS, Technician
Gérald HAMES, Technician
Catherine MULLER, Technician
Suzanne DEPELCHIN, Secretary

*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 (7, 8). 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

G. Hames, T. Connerotte, V. Corbière and P.G. Coulie, in collaboration with A.-M. Feyens, Oncology unit, UCL, J.-F. Baurain, Department of Medical Oncology, Cliniques Universitaires St Luc, M. Marchand and N. van Baren, Brussels branch of the Ludwig Institute for Cancer Research.

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 and 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 x 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 (7).

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, 7). 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 x 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 x 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 (8). 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, and 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 (8). 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 and 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*.

Unexpected result from the monitoring of anti-vaccine T cell responses

T. So and P.G. Coulie, in collaboration with D. Colau and J. Chapiro, Brussels branch of the Ludwig Institute for Cancer Research.

Antigenic peptides encoded by gene *MAGE-A3* and presented by HLA class I molecules have been identified using CD8 lymphocytes stimulated with cells that either expressed gene *MAGE-A3* or were pulsed with candidate peptides. One antigen identified with the latter method is peptide MAGE-$A3_{195-203}$ IMPKAGLLI, presented by HLA-A24 molecules. It has been used to vaccinate advanced cancer patients, mostly in Japan where HLA-A2 is the most frequently expressed HLA class I allele, being present in about 60% of the population. We have used HLA/peptide tetramers to detect T cells recognizing this peptide. Their frequency was estimated to be 2 x 10^{-8} of the blood CD8 cells in non cancerous HLA-$A24^+$ individuals, which is 10-fold lower than the reported frequencies of T cells against other MAGE peptides.

In the blood of a patient vaccinated with MAGE-A3 the estimated frequency was 5 x 10^{-7} of the CD8 cells. Anti-MAGE-3.A24 cytolytic T cell clones were derived, that lysed peptide-pulsed cells with half-maximal effect at the low concentration of 500 pM. However, these CTL did not recognize a panel of HLA-$A24^+$ tumor cells that expressed *MAGE-A3* at levels similar to those found in HLA-$A1^+$ tumor cells recognized by anti-MAGE-3.A1 CTLs. Furthermore, 293-EBNA cells transfected with *MAGE-A3* and *HLA-A24* constructs were hardly recognized by the anti-MAGE-3.A24 CTL clones, even though this approach ensures high levels of expression of the transfected genes. These results suggest that peptide MAGE-$A3_{195-203}$ is poorly processed and is not an appropriate target for cancer immunotherapy (10).

Clonal analysis of regulatory T cells from cancer patients

S. Lucas, W. Fink, T. Aerts, T. Connerotte, and 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 mouse models. 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. Recently, T_{reg} clones directed against tumor-specific peptides were isolated from lymphocytes infiltrating human melanoma tumors (6). This formally demonstrated the existence of tumor-specific suppressive immune responses in cancer patients, and encouraged us to further investigate the implication of T_{regs} in the context of anti-tumor immunotherapy. We derived numerous 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 specific antigens.

We are using these T_{reg} clones as tools to identify mechanisms underlying the suppressive function of human T_{regs}. We have initiated a micro-array based approach to identify genes expressed differentially in activated T_{reg} clones in comparison to conventional $CD4^+$ clones. We are focusing on a set of 24 genes that are specifically up-regulated in activated T_{reg} clones. Genes whose over-expression in T_{regs} is confirmed by an independent method are further characterized to determine whether the proteins they encode participate to the suppressive phenotype. Two confirmed candidates are transcription factors that have no described function in Treg cell physiology. We have cloned the genes encoding these two transcription factors in a retroviral vector routinely used in Dr Stefan Constantinescu's laboratory. We will derive $CD4^+$ effector T cell clones transduced with these recombinant retroviruses and examine whether ectopic expression of these T_{reg}-specific factors induces a suppressive phenotype.

A second micro-array based approach is simultaneously pursued to identify genes that are differentially regulated in suppressed T cell clones versus controls. Results generated by this approach already allowed us to develop a robust RT-PCR assay to measure suppression of target T cells.

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DNA DAMAGE REPAIR AND TELOMERE MAINTENANCE

Anabelle DECOTTIGNIES, Associate Member

Gaëlle TILMAN, Graduate Student

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 three major mechanisms of DNA repair: homologous recombination (HR), non-homologous end-joining (NHEJ) and microhomology-mediated end-joining (MMEJ). 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, I screened the *S. pombe* nuclear genome for the presence of mtDNA and found 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.

Having set up a new simple extrachromosomal DSB repair assay in fission yeast, I found that repair was 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. Competition assays revealed that microsatellite repeat sequences from either human or salmon genomic DNA decrease the capture of yeast endogenous mtDNA, suggesting that such sequences are good substrates for DSBs [1]. This provides another source of genomic instability as microsatellite DNA is very unstable. Capture of microsatellite DNA at mammalian DSBs had been previously reported experimentally and was also detected at the breakpoints of lymhoid tumour-specific translocations.

Next, using a series of yeast mutants, I showed that mtDNA insertion at DSBs was dependent on the NHEJ machinery. The evolutionary conserved Mre11 complex was also required for efficient capture of mtDNA fragments [1]. Strikingly, capture of mtDNA fragments was highly increased in yeast cells grown to stationary phase (post-diauxic shift) [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 G_0 phase. I found that carboxypeptidase Y was required for the increased capture of mtDNA at DSBs in stationary phase yeast cells, suggesting that vacuolar turnover of mitochondria may be involved. However, this occurred independently of cellular macroautophagy and was not observed in nitrogen-starved cells either. Live observation of mitochondria by fluorescence microscopy revealed that, although mitochondria co-localize with microtubules in exponentially-growing cells, tubular organization is lost in post-diauxic shift cells. In these cells, mitochondria fluorescence localized to cytoplasmic spots, a fraction of which being enriched around the nucleus, raising the hypothesis that this may help the transfer of nucleic acids from mitochondria to the nucleus. This is currently under investigation.

Using the same extrachromosomal DSB repair assay in fission yeast, I am currently investigating genetic requirements for microhomology-mediated end- joining, a DNA repair process poorly characterized so far. Construction of a series of yeast mutants revealed that mismatch repair genes are involved in this process.

Alternative mechanism(s) of telomere maintenance

G. Tilman, A. Decottignies

Activation of a telomere maintenance mechanism seems to be indispensable for the immortalization of human cells. Most cancers and cancer cell lines maintain their telomeres via the telomerase. 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.

These two pathways of telomere maintenance are very distinct phenotypically (Fig. 1). In telomerase-expressing cells (TEL^+), telomere length is very homogenous (around 5 kb) and telomeres are found at the end of every chromosome. However, in ALT cells, telomeres are very heterogeneous, ranging from 0 to 50 kb and some chromatids lack telomeres (Fig. 1).

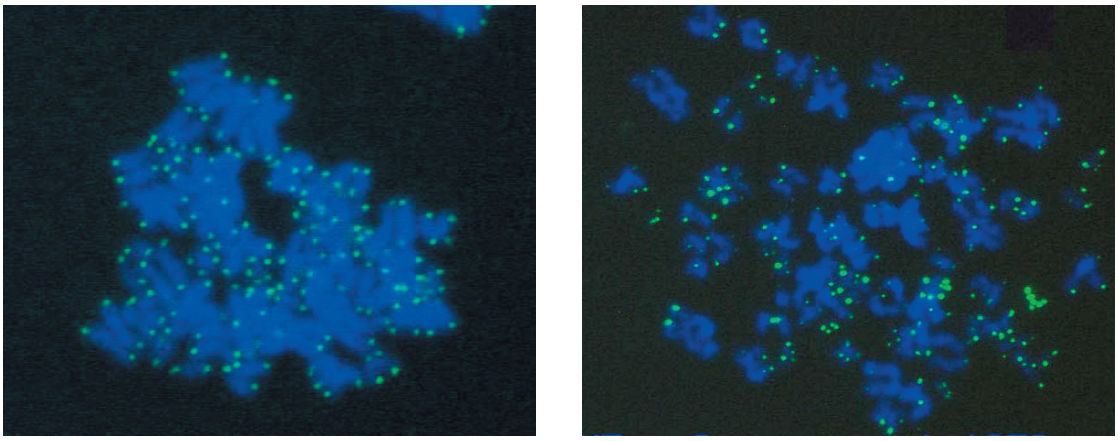

Figure 1. Telomere-specific fluorescence in situ hybridization (FISH) on metaphase chromosomes of telomerase-positive and ALT cancer cells. Telomeres are hybridized with a fluorescent telomeric probe (green) and DNA is stained with DAPI (blue). In ALT cells, telomeres are very heterogeneous and not always present at chromosome ends (arrowheads). ALT cells are further characterized by the presence of extrachromosomal telomeric DNA (arrow).

The other peculiarity of ALT cells is the presence of extrachromosomal telomeric repeats in the nucleus, which may serve as template for recombination-based synthesis of ALT telomeres (Fig. 1).

Although the h*TERT* gene encoding the catalytic subunit of human telomerase has been well characterized, genes involved in the activation and/or propagation of ALT remain largely elusive. Our study aims to identify such genes. We have developed two approaches. In the first approach, we wish to inhibit telomerase expression in TEL^+ cells with the expectation that this would lead to the emergence of ALT resistant cells in the culture. In that respect, a recent study reported the emergence of ALT cells after inhibition of telomerase with a dominant negative h*TERT*. We have chosen to inhibit h*TERT* expression by stable RNA interference in TEL^+ cancer cell lines. The system has been set up and gives a reduction of h*TERT* mRNA of about 70% in 293 HEK cells correlated with a reduction of telomere length. This reduced expression of h*TERT* gene has been maintained over a period of about 1 year 1/2 and the cells recently went through a crisis after 60 passages. About 50 survivor clones appeared after crisis and are currently being characterized to know whether they are ALT survivors or not. If we obtain ALT survivors after inhibition of h*TERT*, we plan to compare the global gene expression profile of ALT clones with the original TEL^+ cell line by RDA (Representational Difference Analysis). In addition, we screened a series of cancer cell lines available in the institute to find the best candidate(s) for telomerase inhibition, *i.e.* a cell line with low level of h*TERT* mRNA and not refractory to transfection, to repeat the RNA interference against h*TERT* in another background. Clones expressing sih*TERT* have been obtained and are currently under investigation. We have also constructed retroviral plasmids for RNA interference against h*TERT* and h*TR* (the RNA subunit of human telomerase) to circumvent poor transfection efficiency problems. Retroviral particles are being produced. In a second approach, we wish to analyze ALT clones obtained after *in vitro* immortalization of telomerase-negative human fibroblasts with SV40 DNA. 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. 2). We received SV40-immortalized fibroblasts of both types (ALT and TEL^+) and have started the global gene expression profile comparisons by RDA.

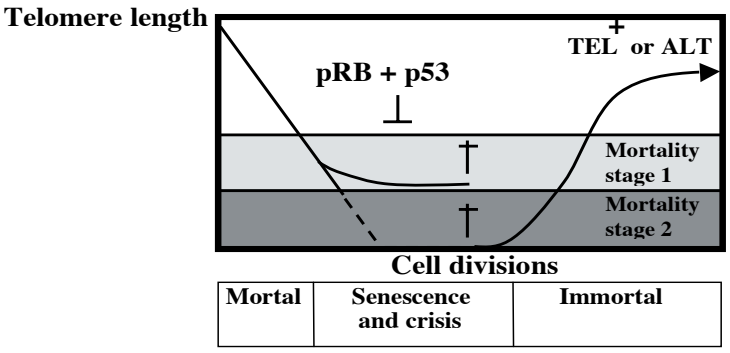

Figure 2. Telomere length in the course of cellular transformation. In telomerase-negative cells, telomere length decreases with continuous replication. At a critical telomere length, cells enter M1 through the action of p53/pRb and eventually die. Upon inactivation of p53/pRb, cells continue to divide and enter M2 characterized by huge genomic instability. A small percentage of cells survive by re-activation of a telomere maintenance mechanism.

Publication

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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. Ludwig investigators are recognized 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 84 members and is headed by Thierry Boon, Branch Director.

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Encarnacion MARIANINI, Laboratory Helper

Rafaëla REYES PRIETO, Laboratory Helper

Contact:

UCL7459, Avenue Hippocrate 74, 1200 Bruxelles, Belgium

Tel: +32 (2) 764 7459
Fax: +32 (2) 762 9405

TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

Benoît VAN DEN EYNDE, Member

Bernard LAUWERYS, Senior Investigator
Catherine UYTTENHOVE, Senior Investigator
Guy WARNIER, Veterinarian, Associate Investigator
Luc PILOTTE, Research Assistant
Jacques CHAPIRO, Postdoctoral Fellow, until February
Vincent STROOBANT, Postdoctoral Fellow
Ivan THEATE, Postdoctoral Fellow
Alexandre DALET, Graduate Student
Benoît GUILLAUME, Graduate Student
Nicolas PARMENTIER, Graduate Student
Grégory PARVIZI, Student
Dominique DONCKERS, Technician
Anne-Lise MAUDOUX, Technician
Bénédicte TOLLET, Technician

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

Tumor antigens recognized by Cytolytic T Lymphocytes (CTL) consist of peptides that are presented by MHC molecules at the cell surface and derive from intracellular proteins that are degraded by the proteasome. The intracellular pathway leading from the protein to the peptide/MHC complex is known as "antigen processing". Our group focuses on the proteasome and recently described a new mode of production of antigenic peptides by the proteasome, based on cutting and pasting peptide fragments to form a new spliced peptide. The first example was a peptide derived from human melanocyte protein gp100. This antigenic peptide is nine-amino acid long and is produced by the splicing of two fragments that were initially non-contiguous in the parental protein. The splicing is made by the proteasome, is tightly coupled to the proteolytic reaction, and appears to occur by transpeptidation involving an acyl-enzyme intermediate. We are currently working on a second example of spliced peptide, where 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 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 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 the standard proteasome and the immunoproteasome

Jacques Chapiro, Vincent Stroobant, Benoît Guillaume, Benoît Vanholle, Wenbin Ma, Grégory Parvizi

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 (3). 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 (4). 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

Nathalie Vigneron, Vincent Stroobant, Jacques Chapiro, Alexandre Dalet (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) (5). 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 now identified a second antigenic peptide produced by peptide splicing in the proteasome. 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

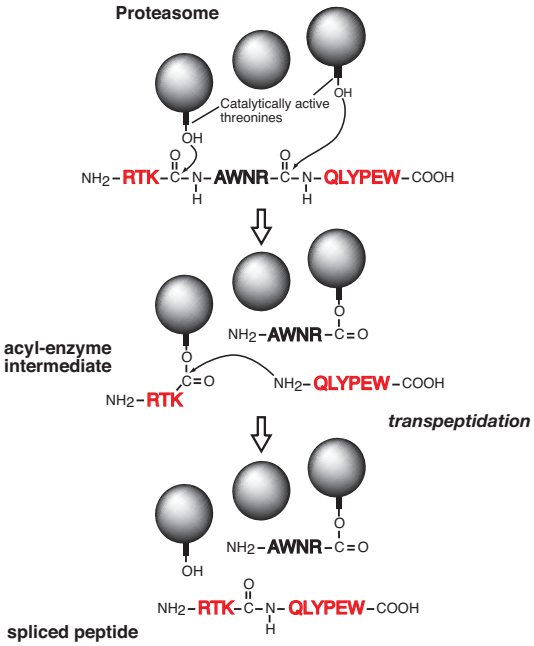

Figure 1. Model of the peptide-splicing reaction in the proteasome. The active site of the catalytic subunits of the proteasome is made up of the side-chain of a threonine residue, which initiates proteolysis by performing a nucleophilic attack on the carbonyl residue of the peptide bond. An acyl-enzyme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

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 antigenic 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

Wenbin Ma, Nathalie 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..

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

Catherine Uyttenhove, Luc Pilotte, Ivan Théate, Dominique Donckers, Nicolas Parmentier, Vincent Stroobant, Didier 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

Ivo Huijbers (in collaboration with Paul Krimperfort (NKI, Amsterdam) and Anne-Marie 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.

This line is now being backcrossed to the H-2^d background in order to be used as a model for immunotherapy. 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)

Bernard Lauwerys, Anne-Lise Maudoux (in collaboration with Frédéric 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 confirmed the presence of a strong type 1 interferon signature in SLE cell subsets, i.e. the presence of numerous interferon-induced genes, as previously observed in SLE PBMC. However, many interferon-induced genes were also induced in RA cells and only a few (including *IFI27*, *IFI44*, *IFI44L* and *EIF2AK2*) were more specifically over-expressed in SLE CD4 T and B cells. Interestingly, we also found 3 non-interferon induced genes (*SLAMF1*, *BRDG1* and *RASGRP1*) that were exclusively over-expressed in SLE B cells. These genes are known to play an important role in the regulation of B cell activation and 2 of these genes (*SLAMF1* and *BRDG1*) are located in disease susceptibility loci, thereby suggesting that they could play a causative role in the polyclonal B cell activation characteristic of SLE. 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

Etienne De PLAEN, Assistant Member
Charles DE SMET, Assistant Member

Axelle LORIOT, Postdoctoral Fellow
Olga KHOLMANSKIKH, Graduate Student
Claudine BLONDIAUX, Technician

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 determining the function of "cancer-germline" genes and deciphering the mechanism leading to their activation in tumor cells.

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 (1). 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 (2), the group is now trying to evaluate whether promoters could be repressed by MAGE-A1 in the presence of Dnmt.

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 (3). Antisense-mediated knock-down experiments indicated that DNMT1 is the primary DNA methyltransferase to maintain methylation of cancer-germline genes (4), and that transient down-regulation of this enzyme suffices to induced 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 (4). 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.

Finally, in collaboration with Nicolas Van Baren and Francis Brasseur, the group is presently analyzing the molecular mechanisms by which IFN-γ inhibits the expression of mela-

nocyte differentiation genes. Preliminary data indicate that this action is mediated by a soluble autocrine factor released by the IFN-γ-treated melanoma cells. The group is in the process of isolating and characterizing this factor.

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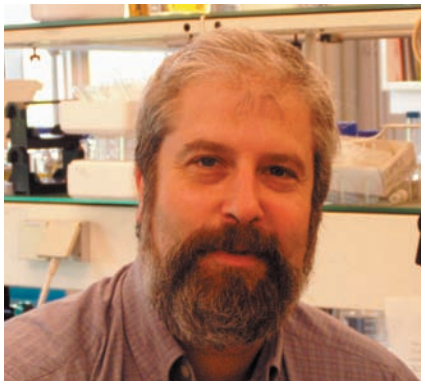
IDENTIFICATION OF HUMAN TUMOR ANTIGENS

Pierre van der BRUGGEN, Member

Didier COLAU, Senior Investigator
Nicolina RENKVIST, Postdoctoral Fellow (to December 05)
Zhaojun SUN, Postdoctoral Fellow
Yi ZHANG, Visiting Scientist (to December 2004)
Claude WILDMANN, Research Associate
Sabrina OTTAVIANI, Research Associate
Nathalie DEMOTTE, Ph.D. Student
Violaine FRANÇOIS, Ph.D. Student (from November 05)
David BASTIN, Technician



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

Sabrina Ottaviani, Zhaojun Sun and Vincent 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 anti-tumor 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.

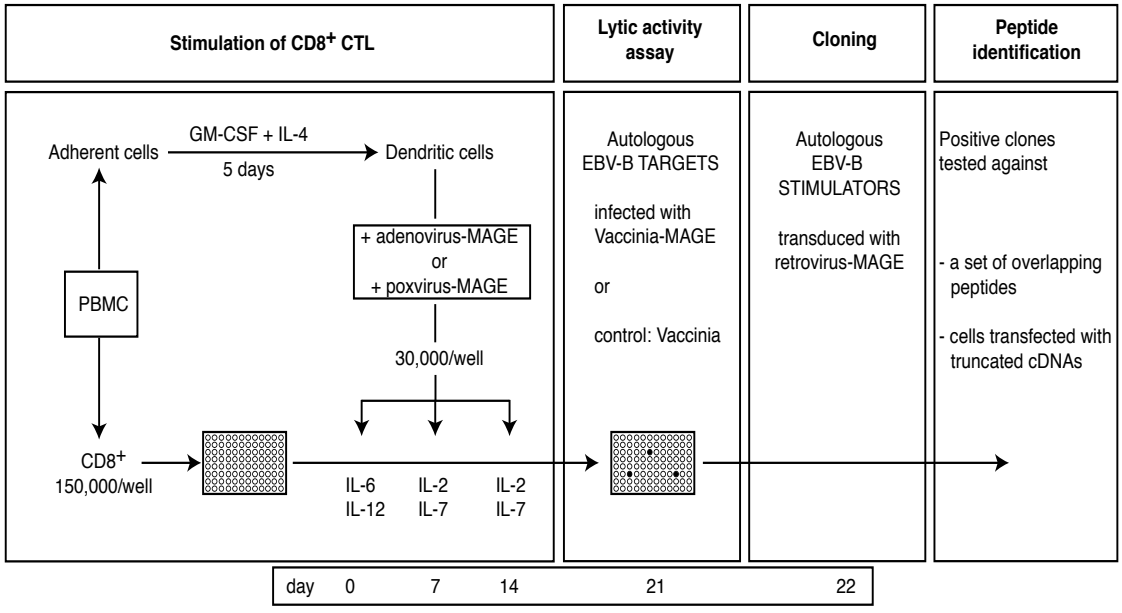


Figure 1. Overview of the procedure to obtain anti-MAGE $CD8^+$ CTL clones by stimulation with dendritic cells infected with viral vectors carrying a MAGE coding sequence.

In a second approach, a large number of 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).

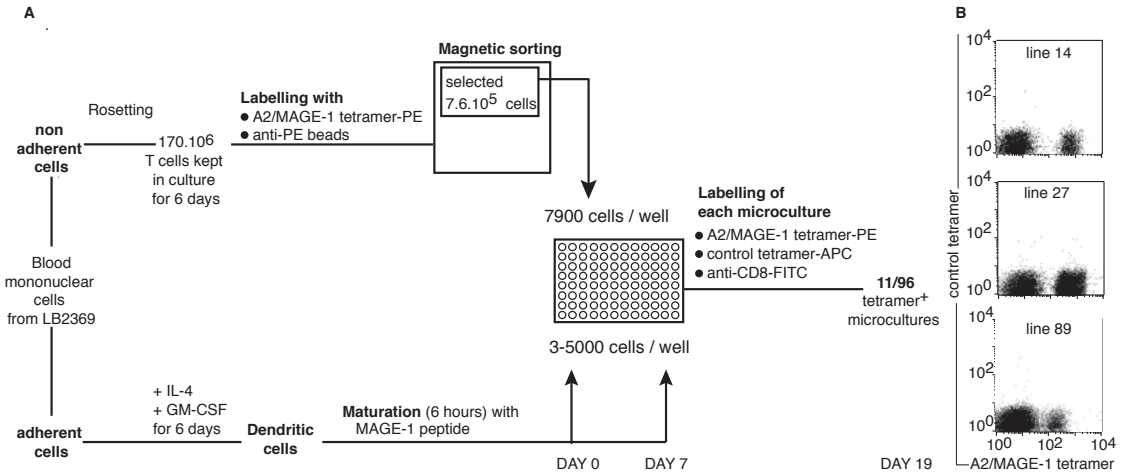


Figure 2. Overview of the procedure using HLA-peptide fluorescent multimers to isolate rare anti-MAGE-1 CD8⁺ CLTL clones.

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).

A novel approach to identify antigens recognized by CD4 T cells using complement-opsonized bacteria expressing a cDNA library

Pierre van der Bruggen (in collaboration with Lisette van de Corput from Leiden University, and Pascal Chaux)

We propose a new sensitive and rapid approach using the exogenous pathway to take up and process proteins encoded by a cDNA library expressed in bacteria. We hypothesized that, after opsonization with complement, recombinant bacteria can be endocytosed via receptor-mediated uptake by Epstein-Barr virus immortalized B cells to allow protein processing and presentation. To validate this approach, we made use of a minor histocompatibility antigen encoded by the human male-specific gene DBY. A recombinant bacteria library was constructed and screened with a DBY-specific CD4 T cell clone. We were able to identify bacteria expressing DBY diluted into a 300-fold excess of bacteria expressing a non-relevant gene. Screening of a bacterial library using a DBY-specific CD4 T cell clone resulted in the isolation of several DBY cDNAs (6).

Detection of CD4 T cell response in vaccinated cancer patients

Patients injected with class II-restricted peptides

Didier Colau, Violaine François, Sabrina Ottaviani and Nicolina Renkvist

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 produced by the addition of a biotinylated anti-Fc IgG2a antibody and PE labeled streptavidin.

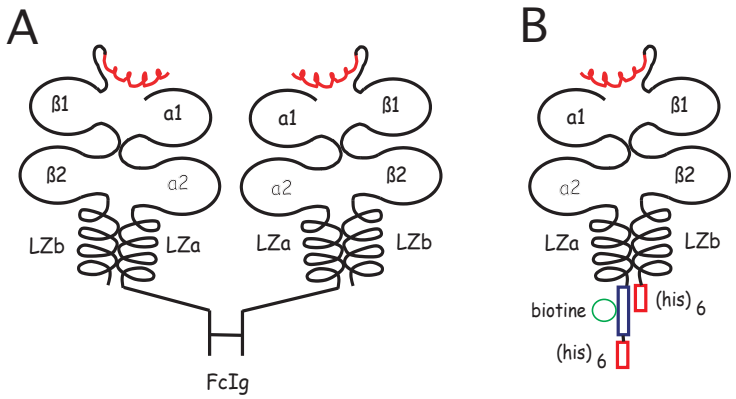


Figure 3. Schematic structures of DP4.MAGE-3 complexes.

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. The majority of anti-vaccine T cells isolated from one patient were either producing IL-10 or IFN-γ. Surprisingly, we also isolated anti-vaccine tetramer-positive CD4 T cells that did not produce any cytokine. These cells had a phenotype of regulatory T cells and suppressed the proliferation of other T cells. They are not found in blood samples collected before vaccination.

Patients injected with a protein

Yi Zhang and Zhaojun Sun

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). An important criterion is that T cell clones must recognize not only stimulator cells loaded with the protein but also stimulator cells transduced with the *MAGE-3* gene, so as to exclude the T cells that recognize contaminants generated by the protein production system. We analyzed the frequencies of anti-vaccine CD4 T cells in five metastatic melanoma patients, who have been injected with a MAGE-3 protein without adjuvant and showed evidence of tumor regres-

sion. Anti-MAGE-3 CD4 T cells were detected in one out of the five patients. The frequency of the anti-MAGE-3 CD4 T cells was estimated at 1/60,000 of the CD4 T cells in post-vaccination blood samples, representing at least an 80-fold increase of the frequency found before immunization. The frequencies of one anti-MAGE-3 CD4 T cell clonotype was confirmed by PCR analysis on blood lymphocytes. The 13 anti-MAGE-3 clones, which corresponded to five different TCR clonotypes, recognized the same peptide presented by HLA-DR1.

This monitoring procedure has the potential to detect the complete set of T lymphocytes that recognize the vaccine-derived peptides on various HLA molecules. Frequencies as low as 1 per million specific CD4 T cells can be measured with frozen samples corresponding to less than 50 ml of blood, whereas other techniques, such as *ex vivo* ELISPOT assays, reach their limit of detection at 1/25,000 of the CD4 T cells. The availability of T cell clones ensures a strict assessment of the specificity of the T cells, including their ability to recognize both cells loaded with the protein and cells transduced with the protein coding sequence. It also makes it possible to define the T cell receptor sequence of the anti-vaccine T cells and, therefore to analyze the TCR diversity and establish the presence of repeated clonotypes, an essential criterion to assess the occurrence of a response when the frequency is low. In addition, a direct quantitative evaluation of the frequency of certain clonotypes can be obtained by PCR performed on RNA extracted from blood lymphocytes and tumor samples.

A limitation of our experiments is that only those anti-MAGE-3 CD4 T cells that produce IFN-γ can be detected. However, our approach could easily be extended by using a "cytokine secretion assay" for the detection of cells producing another cytokine. It could also be extended to the detection of specific CD8 T cells, provided stimulator cells are used that are able to activate both CD8 and CD4 T cells. Various autologous cells, either electroporated with RNA constructs or infected with recombinant viral vectors, are currently tested.

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

Nathalie Demotte and Claude Wildmann

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,

The Institute for Genomic Research (TIGR), Rockville, USA,
Merial, Lyon, France,
The Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom,
The Centre for Tropical Veterinary Medicine, Edinburgh, United Kingdom,
The Ludwig Institute for Cancer Research, Brussels, Belgium

Didier Colau, Claude Wildmann and Pierre van der Bruggen

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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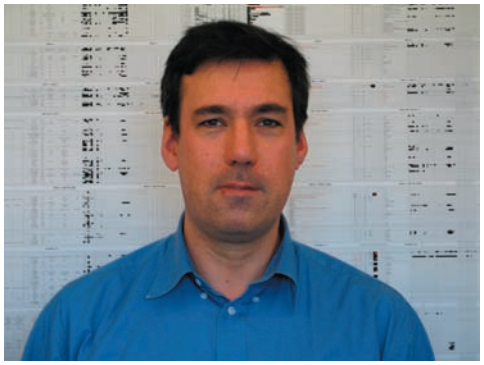
THERAPEUTIC VACCINATION AND TUMOR GENE EXPRESSION PROFILING GROUP



Nicolas van BAREN, Senior Investigator
Marie MARCHAND, Senior Clinical Investigator

Francis BRASSEUR, Associate Investigator
Jérôme DEGUELDRE, Clinical Research Associate
Christian VERFAILLE, Project Manager
Marjorie MERCIER, Technician
Madeleine SWINARSKA, Technician
Julie KLEIN, Secretary

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

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.

In an ongoing study, the MAGE-3.A1 peptide is mixed with CpG 7909, an immunostimulatory CpG-containing oligonucleotide, to try to increase the immunogenicity of this peptide (Study LUD 02-001). This new and promising adjuvant activates antigen presenting cells after binding to Toll-like receptor 9, and is thought to enhance CTL responses. Likewise, CpG 7909 is mixed with 8 HLA-A2 pepides in another ongoing study (LUD 03-007). In both studies, cohorts of 14 patients will receive 6 vaccinations by i.d. and s.c. routes, at 2-week intervals. Results of both studies will be compared in terms of therapeutic efficacy and immune response. So far, one patient has been included in the first study (LUD 02-001). His melanoma progressed under vaccination. Fourteen patients have been included in the second study (LUD 03-007). So far, 11 patients are evaluable for CTL- and tumor responses. The assessment of the CTL response is ongoing (P. Coulie). No objective tumor response (CR or PR) was observed. Ten patients displayed tumor progression, including 2 patients who had a MxR to treatment, and one patient displayed stabilization of a lung metastasis and of mediastinal metastatic lymph nodes lasting for more than 7 months since the beginning of vaccinations. We will start soon including patients in a second cohort of 14 patients. Study treatment is identical in both cohorts, except for one parameter. In this 2nd cohort, patients will receive the same 8 HLA-A2 peptides as in the 1st cohort, but CpG 7909 will be replaced by another immunological adjuvant, Montanide ISA 51. In addition, for patients of this cohort who will receive more than 6 vaccinations, low dose interferon-alpha (IFN-α) will be given s.c. for a 14-week period. During that period, 3 maintenance vaccinations will take place at 6-week intervals. IFN-α was shown to increase expression of HLA class I molecules by tumors and hence, of tumor antigens recognized by T lymphocytes. The presence of IFN-α in the melanoma metastases of vaccinated patients could sensitize the tumor to in situ CTL, trigger cytolysis and cause tumor regression.

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 study LUD 99-003. 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 (Study LUD 02-002). Of the 11 patients who were included in that study before its early closure, 10 were evaluable for T lymphocyte- and tumor responses. The assessment of the T lymphocyte response is ongoing (P. van der Bruggen). Neither objective tumor response (Partial or Complete response) nor tumor stabilization was observed in these 10 patients. They all displayed tumor progression including 3 patients who had a mixed response to treatment.

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 MAGE peptides, the MAGE-3 recombinant protein or the ALVAC recombinant viral vector, is devoid of significant toxicity. A minority of melanoma patients (about 10 to 20 %) show regression of metastatic lesions following immunization, whatever the MAGE vaccine used. About 5 % of the patients show complete or partial clinical responses. Some of these lasted for several years. 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. CTL responses can be detected in a minority of patients vaccinated either with peptide or ALVAC virus. The responses appear to be weak and are mainly monoclonal. The relative frequency of CTL responders versus non-responders is higher in patients who had tumor regressions (4).

Gene expression profiling of tumor samples from vaccinated patients

Using microarray technology, we have established the gene expression profile of a series of pre-vaccination tumor samples, mainly cutaneous metastases, obtained from melanoma patients included in our clinical trials. Comparative analysis between samples from patients who experienced either tumor regression 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.

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ANALYSIS OF T CELL RESPONSES OF VACCINATED CANCER PATIENTS

Thierry BOON, Member

Aline VAN PEL, Associate Member
Danièle GODELAINE, Senior Investigator
Vinh HA THI, Technician
Maria PANAGIOTAKOPOULOS, Technician

Christophe LURQUIN, Associate Investigator
Bernard LETHE, Associate Investigator
Marie-Claire LETELLIER, Technician

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. Similar findings were made with all the other patients who were analyzed. 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.

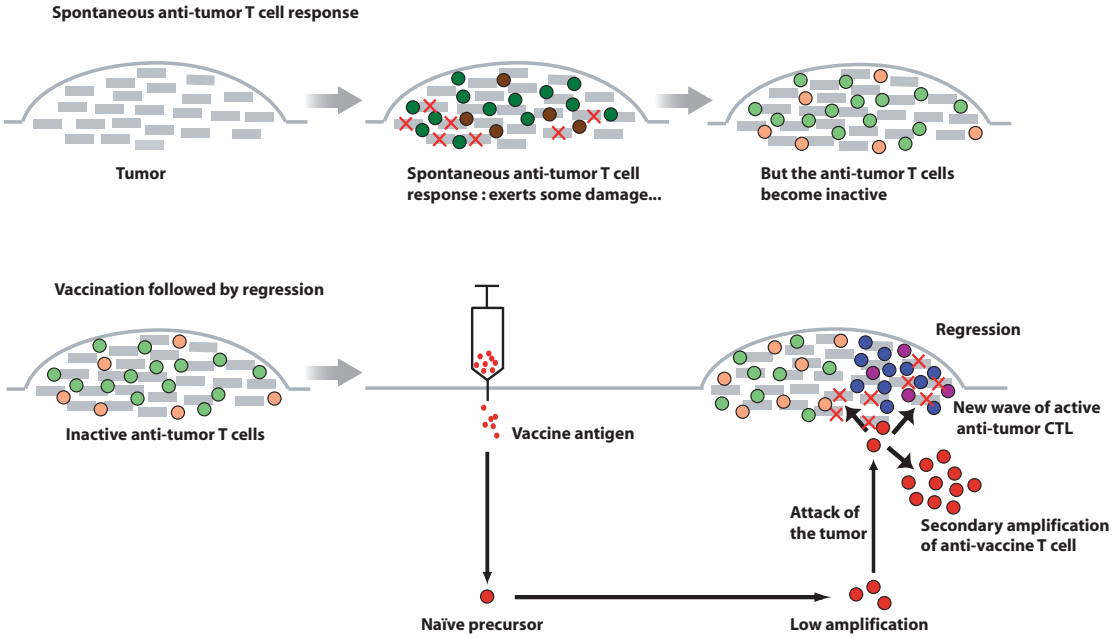

Figure 1

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.

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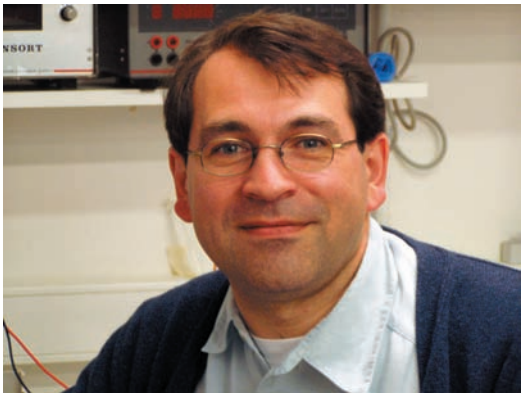
CYTOKINES IN IMMUNITY AND INFLAMMATION

Jean-Christophe RENAULD, Member

Jacques VAN SNICK, Member
Laure DUMOUTIER, Postdoctoral Fellow
Laurent KNOOPS, Postdoctoral Fellow
Tekla HORNAKOVA, Student
Valérie STEENWINCKEL, Student
Amel TOUNSI, Student
Isabelle BAR, Technician
Emilie HENDRICKX, Technician
Monique STEVENS, Technician



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

Jean-Christophe Renauld, Jacques Van Snick, Laurent Knoops, Valérie Steenwinckel, Monique 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

Jean-Christophe Renauld, Laurent Knoops, Tekla Hornakova, Monique 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.

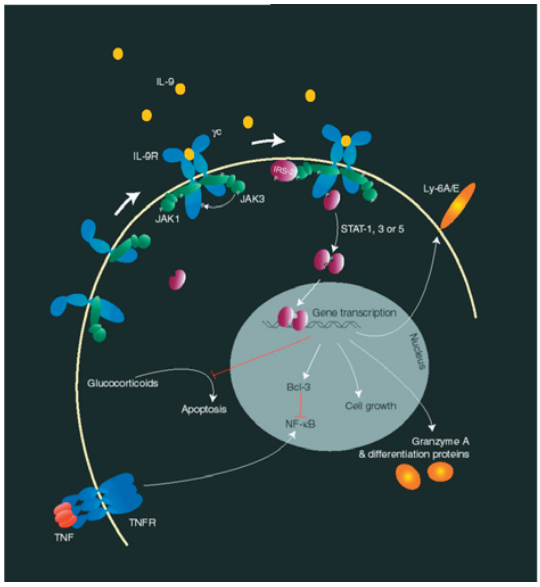


Figure 1.

Anti-apoptotic activity of I-309 receptor and signal transduction

Jean-Christophe Renauld, Amel Tounsi, Jacques 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

Jean-Christophe Renauld, Jacques Van Snick, Laure Dumoutier, Laurent Knoops, Amel Tounsi, Monique 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.

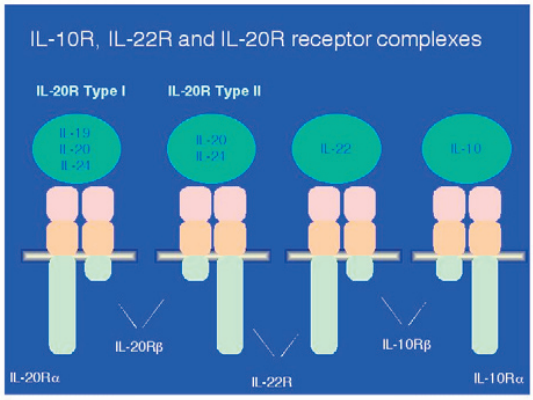


Figure 2.

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 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

Jean-Christophe Renauld, Laure Dumoutier

Type II cytokine receptors include recep-

tors 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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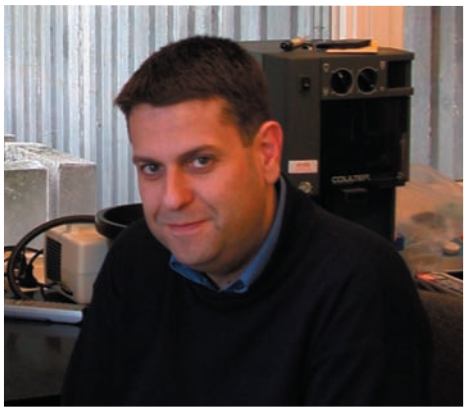
SIGNAL TRANSDUCTION GROUP: STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

Stefan N. CONSTANTINESCU, Member

Carmen DIACONU, Postdoctoral Fellow
Michael GIRARDOT, Postdoctoral Fellow
Christian PECQUET, Postdoctoral Fellow (since Feb.06)
Alexandra DUSA, PhD Student
Judith STAERK, PhD Student
Yohan ROYER, PhD Student (till Sept. 05)
Yan YIN, Research Assistant
Julie KLEIN, Administrative Assistant



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), interleukin 2 (IL2) and interleukin 9 (IL9R). 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 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

Judith Staerk, Christian 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

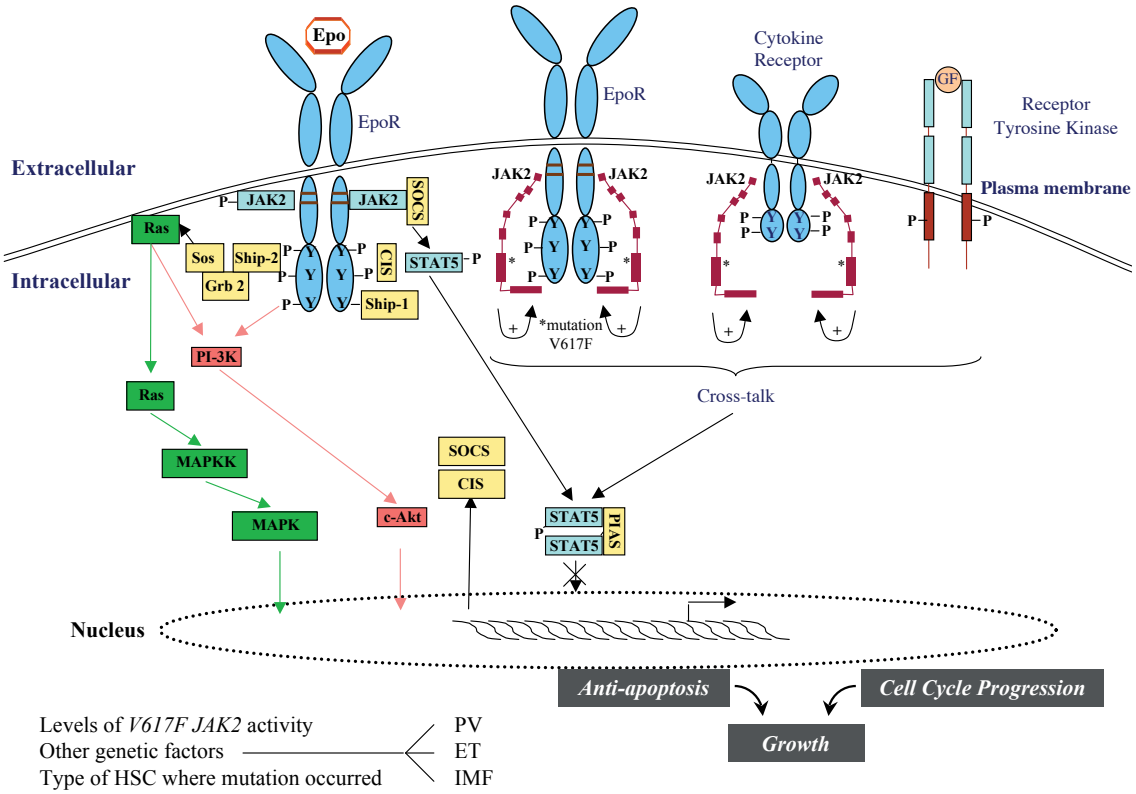


Figure 1. Cytokine receptors such as the erythropoetin receptor (EpoR) arrive at the cell surface after JAK2 had bound to the cytosolic domains early on in the secretory pathway. (**A**). When ligand (i.e. Epo for the EpoR) binds to the extracellular domain of the EpoR, the prebound JAK2 molecules trans-phosphorylate 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

to the myeloproliferative syndromes (Figure 1). Current projects include the determination of downstream signaling proteins activated by the mutant JAK2, and the characterization of cytokine receptor signaling in the presence of the mutant and wild type JAK2 (Figure 1A and D). 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

Judith Staerk, Michael Girardot

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 (5). 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 performs under the auspices of an ARC grant (Action de Recherche Concertée of the Université catholique de Louvain) with the St Luc Hospital deprtaments 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

Judith Staerk, Alexandra Dusa

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 (6-8).

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 conforma-

tion appears to be required for activation of signaling. In this active conformation TM residues L241 and L244 and JM residue W258 are predicted to be in the interface.

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

Katharina Kubatzky, Alexandra 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

Yohan Royer, Carmen Diaconu, Christian 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 hypo-

thesis 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 transmembrane domains and structure of JAK2 V617F

Alexandra 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

Michael 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) not only to N3 sites, which are characteristic of ligand-activated STAT5 (10). We are attempting to identify the promoters actually bound by STAT proteins in

living cells in physiologic and pathologic situations. We use a modified version of the chromatin immunoprecipitation assay pioneered by Alex Varshavsky in conjunction with DNA microarray genomic profiling. 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.

Selected publications

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