Research at

the Christian de Duve

Institute of Cellular Pathology (ICP)

and at

the Brussels Branch of

the Ludwig Institute for Cancer Research (LICR)

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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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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

CARBOHYDRATE METABOLISM

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

Younes ACHOURI, Postdoctoral fellow
Jean-François COLLET, Postdoctoral Fellow
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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, E. Wiame, G. Connerotte, K. Peel, M. Veiga-da-Cunha, E. Van Schaftingen in collaboration with M.H. Rider and D. Vertommen, Horm Unit and Vincent Stroobant, LICR

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

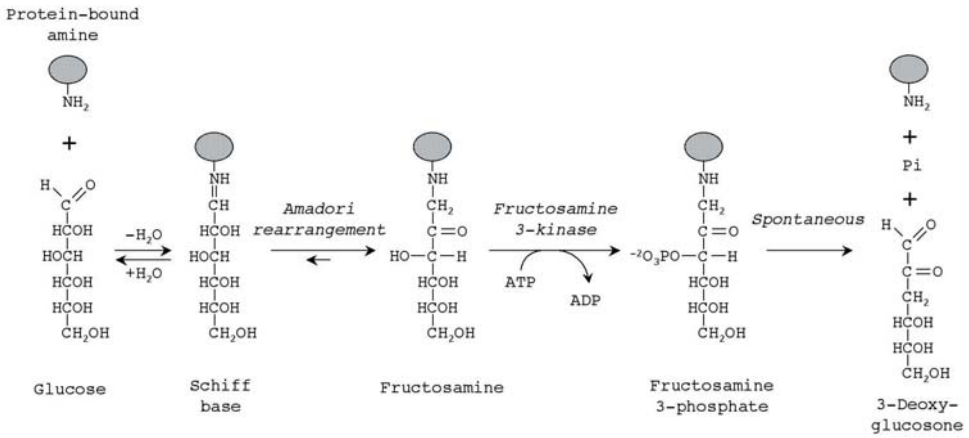

Figure 1. Role of fructosamine 3-kinase in the deglycation of proteins

of fructosamines. These are formed through a spontaneous reaction (known as 'glycation') of glucose with primary amines, followed by an Amadori rearrangement. Fructosamine 3-kinase (FN3K) is a recently identified enzyme that phosphorylates both low-molecular-weight and protein-bound fructosamines [1]. Fructosamine 3-phosphates are unstable, breaking down spontaneously to 3-deoxyglucosone, inorganic phosphate and the amino compound that originally reacted with glucose (Fig. 1). FN3K is therefore a 'deglycating' enzyme. Evidence has been provided that this enzyme indeed removes some of the fructosamine residues present on hemoglobin in erythrocytes, a cell type in which FN3K is particularly active and from which it was first isolated. Taken together all these findings indicate that FN3K initiates a new protein repair mechanism.

It is often stated that proteins, being easily renewed, do not need to be repaired, unlike DNA where mutations can threaten the life of the cell. However, two types of protein repair mechanisms have been quite well established. These are the reduction of methionine-S-oxide residues to methionyl residues and the conversion of L-isoaspartate residues to L-aspartate residues by a methyl transferase. Inactivation

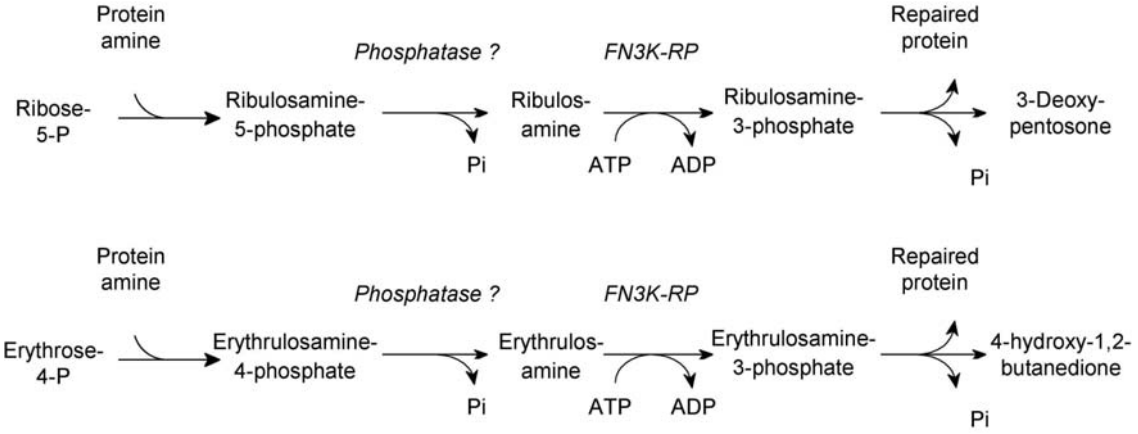

Figure 2. Hypothetical role of FN3K-RP in protein deglycation. Ribulosamines and erythrulosamines, the best substrates of FN3K-RP, are possibly formed from ribose 5-phosphate and erythrose 4-phosphate, two extremely potent glycating agents. Removal of the phosphate group in position 4 or 5 by a hypothetical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

of these repair mechanisms in mice results in a diseased state and in a decreased lifespan, underlining their physiological importance. Phosphorylation of fructosamine residues by FN3K represents a third protein repair mechanism, the physiological significance of which is presently evaluated. The results of our previous studies indicated that only part of the fructosamines that form on hemoglobin can be removed as a result of the action of FN3K in intact erythrocytes. We have recently determined the identity of these fructosamine residues [2]. This was done by comparing the glycation sites of hemoglobin derived from erythrocytes incubated with an elevated glucose concentration in the absence or in the presence of deoxymorpholinofructose, a (substrate and) competitive inhibitor of FN3K. The identification of the residues was facilitated by their prior tagging by *in vitro* incubation of hemoglobin with FN3K and [γ-^{32}P]ATP. After reduction of fructosamine 3-phosphates with borohydride, the labeled hemoglobin was digested with trypsin. Peptides were separated by reverse-phase HPLC and the radioactive peaks were analyzed by mass spectrometry. This type of analysis indicated that fructosamines bound to Lys-α-16, Lys-α-139 and Lys-β-17 were readily deglycated as a result of the action of FN3K in intact cells. By contrast, the amount of fructosamines bound to Lys-α-61, Val-β-1, Lys-β-59 and Lys-β-66 was unaffected by the presence of the FN3K inhibitor, indicating a poor clearing of these residues in intact erythrocytes. Accessibility to FN3K seems to be the major factor driving the potential deglycation of lysine residues in hemoglobin.

FN3K is particularly active in brain, heart, kidney and skeletal muscle of various species. Its activity is also relatively elevated in erythrocytes from man, rat and mouse, but barely detectable in erythrocytes from chicken and pig, correlating therefore with the known intracellular concentration of glucose (the precursor of fructosamines) in erythrocytes from these species. This is in keeping with the specific role of FN3K to repair protein damage caused by glucose [5].

Fructosamine-3-kinase related protein

We have also cloned human and mouse cDNAs encoding proteins sharing 65 % sequence identity with FN3K [3]. The gene encoding fructosamine-3-kinase related protein (FN3K-RP) is present next to the FN3K gene on human chromosome 17q25. Unlike FN3K, FN3K-RP does not act on fructosamines, but it does phosphorylate ketoamines with a D configuration in C3 (ribulosamines, erythrulosamines and, with a lower affinity, psicosamines). This is also true for FN3K, which has therefore a broader substrate specificity than FN3K-RP. Tandem mass spectrometry and NMR analysis indicated that FN3K-RP also phosphorylates the third carbon of the sugar portion of its substrates, being therefore also a 'ketoamine 3-kinase'. The ketoamine 3-phosphates that are formed by this enzyme are also unstable, decomposing with half lives of 8 hours (psicosamine 3-phosphates), 25 min (ribulosamine 3-phosphates) and < 5 min (erythrulosamine 3-phosphates). 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 [4], which are quite rich in FN3K-RP. Inhibition of FN3K-RP with deoxymorpholinopsicose (a substrate and competitive inhibitor of FN3K-RP) increases the amount of glycated hemoglobin that forms when red blood cells are incubated with 50 mM D-allose or with 10 mM D-ribose. Furthermore, incubation with 50 mM allose also caused the accumulation of ketoamine 3-phosphates, which was inhibited by deoxymorpholinopsicose but not by deoxymorpholinofructose. These data indicated that FN3K-RP can phosphorylate intracellular, protein-bound psicosamines and ribulosamines, thus leading to deglycation.

It is quite unlikely that the physiological substrates of FN3K-RP are formed through a reaction of amines with free ribose or erythrose, because these sugars are presumably present at very low concentration (< 10 μM) in tissues. Furthermore, D-allose is not a physiological sugar 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 ≥ 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 presumably formed from phosphorylated intermediates are possibly converted to their dephosphorylated form by an unknown phosphatase before being phosphorylated on their third carbon by FN3K-RP, and thereby destabilized and removed from proteins.

The idea 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. 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 [11].

Database searches indicate the presence of both FN3K and FN3K-RP in various mammals and in chicken, but of only one homologous gene, closer to FN3K-RP than to FN3K, in fishes and the urochordate Ciona intestinalis, suggesting that a gene duplication event occurred after the fish radiation and that the FN3K gene evolved more rapidly than the FN3K-RP gene.

Fructosamine metabolism in bacteria

E. Wiame, E. Van Schaftingen

Until recently, the only enzymes known to be involved in the metabolism of low-molecular-weight fructosamines were oxidases that sever one of the bonds between the aglycone and sugar portions of these compounds. We reported recently an entirely different metabolism in E. coli. This bacterium, which was found to grow on fructose-ε-lysine, phosphorylates this fructosamine on the sixth carbon of its fructose moiety thanks to a 'fructoselysine 6-kinase'. Fructoselysine 6-phosphate is then hydrolysed to lysine and the glycolytic intermediate glucose 6-phosphate by a 'deglycase' (Fig. 3). Both enzymes are encoded by the same operon, now termed Frl operon, which comprises four open-reading-frames: FrlA, encoding a transporter related to cationic amino acid transporters; FrlB, encoding fructoselysine 6-phosphate deglycase; FrlC, an epimerase that specifically converts psicoselysine to its C3 epimer, fructoselysine; and FrlD fructoselysine 6-kinase. Free fructoselysine is most probably produced through proteolysis of glycated proteins. It is apparently not absorbed by the gut, but degraded in the intestine by the microbial flora. Our data indicate that E. coli is one of the bacteria that participates in this metabolism. In addition to this, fructoselysine 6-kinase and fructoselysine 6-phosphate deglycase are useful tools to assay fructoselysine.

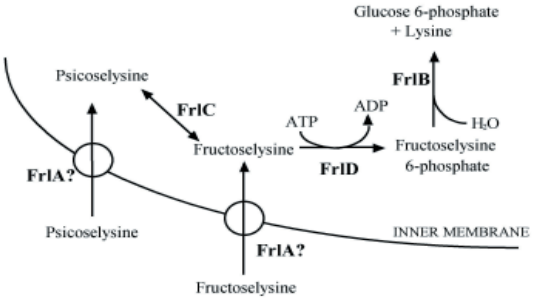

Figure 3. Metabolism of fructoselysine and psicoselysine in Escherichia coli.

BLAST searches indicate that several other bacterial genomes comprise homologs of fructoselysine 6-kinase (FrlD) and fructoselysine 6-phosphate deglycase (FrlB). We expressed the B. subtilis homologues in E. coli and purified them. YurL, the homologue of fructoselysine 6-kinase, phosphorylated the α-glycated amino acids fructosevaline and fructoseglycine with

catalytic efficiencies that were at least 30-fold higher than that observed with fructoselysine. This was in marked contrast with the E. coli fructoselysine 6-kinase, which showed a 3000-fold higher catalytic efficiency for fructoselysine than for its second best substrate, the α-glycated dipeptide, fructoseglycylglycine. Similarly, the deglycase of Bacillus substilis acted with a much higher catalytic efficiency on fructosevaline or fructoseglycine 6-phosphate than on fructoselysine 6-phosphate, whereas the opposite was true for the E. coli enzyme. This indicates that the B. subtilis fructosamine operon encodes a set of proteins involved in the metabolism of fructosamines that are bound to the alpha amino group of amino acids rather than to the epsilon amino group of lysine [6].

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 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. The mechanism and the physiological significance of this effect are unknown.

Using a simple enzymatic assay of glucuronate that we have developed, we recently investigated the mechanism by which aminopyrine and other agents stimulate the formation of vitamin C in isolated rat hepatocytes [7]. We found that aminopyrine and several other agents (antipyrine, chloretone, clotrimazole, metyrapone, proadifen, barbital) induced in a few minutes an up to 8-fold increase in the formation of glucuronate, which was best observed in the presence of sorbinil, an inhibitor of glucuronate reductase. They also caused an about 2-fold decrease in the concentration of UDP-glucuronate, but little if any change in the concentration of UDP-glucose. Depletion of UDP-glucuronate with galactosamine or resorcinol markedly decreased the formation of glucuronate both in the presence and in the absence of aminopyrine, confirming the precursor-product relationship between UDP-glucuronate and free glucuronate. Most of the investigated agents did not induce the formation of detectable amounts of glucuronides, indicating that the formation of glucuronate is not due to a glucuronidation-deglucuronidation cycle. To the exception of barbital (which inhibits glucuronate reductase), all of the above-mentioned agents caused also an increase in the concentration of ascorbic acid in the absence of sorbinil. They had little effect on glutathione concentration and their effect on glucuronate and vitamin C formation was not mimicked by glutathione-depleting agents such as diamide and buthionine sulfoximide. It is concluded that the stimulation of vitamin C synthesis exerted by some xenobiotics is mediated through a rapid increase in the conversion of UDP-glucuronate to glucuronate, which does not apparently involve a glucuronidation-deglucuronidation cycle. Our present aim is to identify the enzyme responsible for the formation of D-glucuronate from UDP-glucuronate.

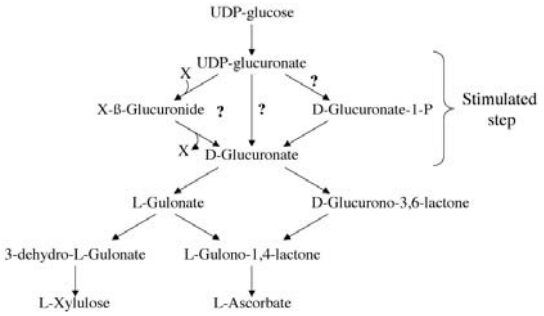

Figure 4. Formation of L-ascorbate and L-xylulose from UDP-glucuronate in liver. The formation of glucuronate from UDP-glucuronate is stimulated by aminopyrine and other agents. The precise mechanism of this formation is still unknown.

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

Y. Achouri, 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. To identify the biochemical defects underlying these diseases, we have undertaken the identification of enzymes involved in the metabolism of these compounds.

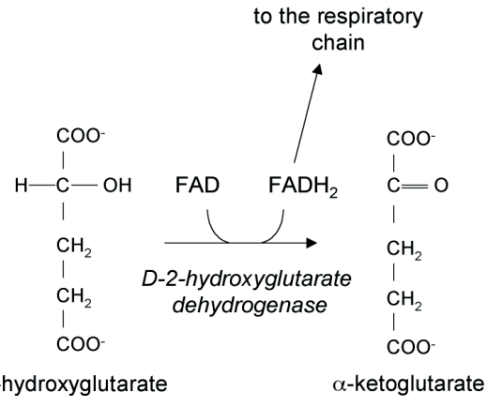

Figure 5. Conversion of D-2-hydroxyglutarate by D-2-hydroxyglutarate dehydrogenase. L-2-hydroxyglutarate is similarly converted to α-ketoglutarate by another FAD-linked dehydrogenase.

We reported last year [8] the identification of an FAD-linked D-2-hydroxyglutarate dehydrogenase, which is present in mitochondria and is presumably linked to the respiratory chain via 'electron transfer flavoprotein'. This enzyme shows a very low Km for its substrate (≈ 3 μM) and catalyses 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 [10]. 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. Since this enzyme produces α-ketoglutarate, a common metabolic intermediate, the neurological phenotype and malformations observed in D-2-hydroxyglutaric aciduria are most likely the consequence of the accumulation of D-2-hydroxyglutarate.

We have also identified the gene mutated in L-2-hydroxyglutaric aciduria [9]. Biochemical investigations on rat liver demonstrated the presence of a membrane-bound, mitochondrial FAD-linked, L-2-hydroxyglutarate dehydrogenase. This enzyme is mainly expressed in liver and kidneys, but also at lower levels in heart, brain and other tissues. Based on this information, a database search led to the identification of a gene encoding a human hypothetical protein homologous to bacterial FAD-dependent malate dehydrogenases and targeted to mitochondria. The gene encoding this protein, present on chromosome 14q22.1, was found to be mutated in patients with L-2-hydroxyglutaric aciduria from three consanguineous families. Two of the mutations found replaces a highly conserved residue (Lys71Glu; Glu176Asp) whereas the third one removes a full exon (exon 9). From these observations, we could conclude that L-2-hydroxyglutarate is normally metabolised to α-ketoglutarate in mammalian tissues by an FAD-linked L-2-hydroxyglutarate dehydrogenase and that L-2-hydroxyglutaric aciduria is caused by mutations in the gene that most likely encodes this enzyme [9].

In 2004, samples from about 100 patients were analysed, allowing the diagnosis of or carnitine palmitoyl transferase deficiency (5 cases), of various forms of glycogen storage disease (5 cases) and of phosphomannomutase deficiency (7 cases).

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

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PURINE METABOLISM

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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, and the mechanisms of action of select synthetic purine nucleoside analogues which possess therapeutic, mainly anticancer and antiviral properties.

Adenylosuccinate lyase deficiency

M.-F. Vincent, T. Timmerman, S. Marie.

Collaboration with the Department of Paediatrics of the University Hospital Gasthuisberg in Leuven has led us to the discovery, in 1984, of adenylosuccinate lyase (adenylosuccinase, ADSL) deficiency, the first enzyme deficiency described on the 'de novo' pathway of purine synthesis in man. This disorder causes accumulation in cerebrospinal fluid and urine of two normally undetectable compounds, succinylaminoimidazolecarboxamide riboside (SAICA-riboside) and succinyladenosine (S-Ado). These are the dephosphorylated derivatives of the two substrates of ADSL, SAICA-ribotide (SAICAR) and adenylosuccinate (S-AMP), respectively (see Figure 1). Affected children display variable, but mostly profound psychomotor delay, often epilepsy and/or autistic features, occasionally growth retardation and muscular wasting (1). We study the mutations that lead to ADSL deficiency (2-4), and the pathophysiologic mechanisms of the disorder.

Mutation analysis

ADSL deficiency has been diagnosed in more than 60 patients worldwide. In accordance with the variability of the clinical picture, 40 different mutations in the ADSL gene have been identified to date in 38 unrelated families (see Adenylosuccinate Lyase Mutations Database at http://www.icp.ucl.ac.be/adsldb/). The majority are missense mutations. In about half of the families, the patients are compound heterozygotes. Most frequently encountered, accounting for about one third of the alleles investigated, is a R426H mutation, which has been found in 13 families.

Pathogenetic studies

The symptoms of ADSL deficiency could a priori be due to a distal deficiency of purine, particularly adenine nucleotides, and/or to a toxic effect of proximally accumulating SAICAR and S-AMP, and/or of their dephosphorylated derivatives, SAICA-riboside and S-Ado, respectively.

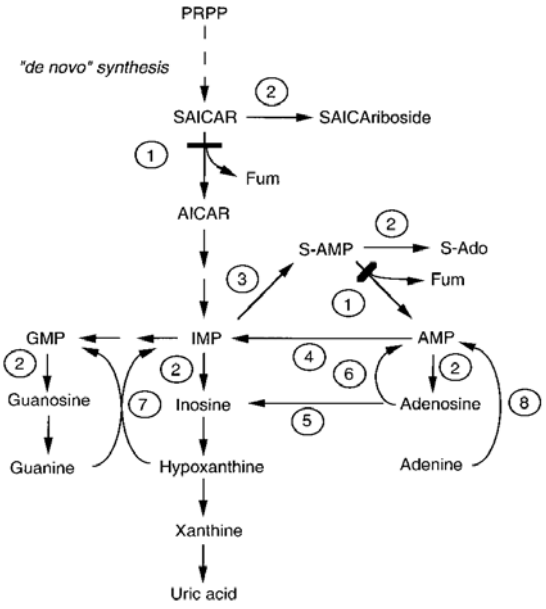

Figure 1. Pathways of purine metabolism. The ten-step synthetic route, often termed 'de novo' pathway, leads from phosphoribosyl pyrophosphate (PRPP) to IMP. From IMP, the nucleoside monophosphates, AMP and GMP, and the corresponding di- and triphosphates (not shown) are formed. The catabolic pathway starts from the nucleoside monophosphates and, in man, produces uric acid, a poorly soluble compound. In lower mammals, uricase (not shown) converts uric acid into allantoin, which is much more soluble. The salvage pathway, composed of two enzymes, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase, converts the purine bases, guanine, hypoxanthine and adenine, into the corresponding nucleoside monophosphates. Adenosine kinase can also be considered a salvage enzyme. AICAR, aminoimidazolecarboxamide ribotide; Fum, fumarate, S-Ado, succinyladenosine; SAICAR, succinylaminoimidazolecarboxamide ribotide; S-AMP, adenylosuccinate. (1) adenylosuccinate lyase; (2) cytosolic 5'-nucleotidase; (3) adenylosuccinate synthetase; (4) AMP deaminase; (5) adenosine deaminase; (6) adenosine kinase; (7) hypoxanthine-guanine phosphoribosyltransferase; (8) adenine phosphoribosyltransferase. Bars indicate the defect in adenylosuccinate lyase deficiency.

The observation that the levels of SAICA-riboside are comparable in severely and mildly retarded patients, whereas those of S-Ado are markedly higher in the latter, has led to the hypothesis that SAICA-riboside is the neurotoxic compound, and that S-Ado could counteract its noxious effects. However, we could not demonstrate a cytoxic effect of the succinylpurines on cultured rat neurones.

These findings have led us to consider the possibility that the intracellular accumulation of the substrates of the enzyme might be toxic. To test this hypothesis, construction of ADSL-deficient models was initiated. Several approaches are under way to obtain ADSL-deficient cells: creation of dominant negative mutants by overexpression of mutated ADSL in mammalian cells, expression of antisense RNAs to repress endogenous ADSL, and inhibition of ADSL by compounds such as adenylophosphonopropionate. We are also in the process of creating an ADSL-deficient neuronal cell line by knock-in through insertion of the most frequent mutation, R426H, in the murine gene.

Our studies were also devoted to the mechanisms involved in the dephosphorylation of the two substrates of ADSL, giving new insight in the pathophysiology of the neurologic symptoms of the deficiency

A new defect in the purine biosynthesis

S. Marie, T. Timmerman and M.F. Vincent

In a female infant with dysmorphic features, severe neurological defects, and congenital blindness, a positive urinary Bratton-Marshall test led to identification of a massive excretion of 5-amino-4-imidazolecarboxamide (AICA)-riboside, the dephosphorylated counterpart of AICAR (also termed «ZMP»), an intermediate of de novo purine biosynthesis. ZMP and its di- and triphosphate accumulated in the patient's erythrocytes. Incubation of her fibroblasts with AICA-riboside led to

accumulation of AICAR, not observed in control cells, suggesting impairment of the final steps of purine biosynthesis, catalyzed by the bifunctional enzyme AICAR transformylase/ IMP cyclohydrolase (ATIC). AICAR transformylase was profoundly deficient, whereas the IMP cyclohydrolase level was 40% of normal. Sequencing of ATIC showed a K426R change in the transformylase region in one allele and a frameshift in the other. Recombinant protein carrying mutation K426R completely lacks AICAR transformylase activity (5).

Antileukaemic properties of 2 chloro-2'-deoxyadenosine

F. Bontemps, S. Cardoen, A. Delacauw, S. Lisart, C. Smal, E. Van Den Neste, in collaboration with A. Ferrant, Department of Haematology, University Hospital Saint-Luc

In 1997, a collaborative study of the antileukaemic nucleoside, 2-chloro-2'-deoxyadenosine (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.

Effects of CdA in EHEB cells

To improve our understanding of the mechanisms by which CdA induces apoptosis in B-CLL cells, we investigate 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 1000-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 (5 – 10 μM) (6). 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 contribu-

te to CdA-induced apoptosis, as suggested by the antagonism between CdA and roscovitine.

Regulation of dCK activity

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 quantity of enzyme. 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 (7).

To further unravel its regulation, dCK was overexpressed in HEK-293 cells as a His-tag fusion protein. Western blot analysis showed that purified overexpressed dCK appears as doublet protein bands. The slower band disappeared after treatment with protein phosphatase lambda in parallel with a decrease of dCK activity, providing additional arguments in favor of both phosphorylated and unphosphorylated forms of dCK. In addition, we have found conditions in which dCK could be labelled with [^{32}P]orthophosphate. Work is in progress to identify the phosphorylation sites of dCK.

Search for potentiation of antileukaemic effect of CdA

In recent years, we have shown that combination of CdA with DNA-damaging agents, such as cyclophosphamide (CP) derivatives (8) or UV-light (9), resulted in synergistic cytotoxicity in B-CLL lymphocytes. The *in vitro* synergy between CdA and CP derivatives have provided the rationale for a clinical trial of this combination, which gives encouraging results (10).

We are currently analysing if efficacy of CdA could be strengthened by combination with inhibitors of the MAPK/ERK pathway, which plays a major role in cell proliferation and survival, and has been shown to exert an anti-apoptotic effect.

Drug sensitivity profiles

With standard treatments of B-CLL, clinical response is variable, depending on drug and patient. To tailor therapy on a more individual basis, we develop an *in vitro* assay which evaluates the sensitivity of the patients' lymphocytes to various drugs (nucleoside analogues, alkylating agents, steroids, anthracyclin, etc.). The lymphocytes will also be characterised by conventional cytogenetics, hybridisation in situ (FISH), and molecular biology techniques. By revealing correlations between chemoresistance profiles and genetic anomalies, these studies might also allow identification of mechanisms of resistance.

Other inborn errors of metabolism

M.-F. Vincent, S. Marie, T. Timmerman

In 2004, 995 analyses were performed for the diagnosis and follow-up of inborn errors of metabolism by measurements of accumulating metabolites (purines and pyrimidines, mucopolysaccharides, sialic acid, intra-leukocytic cystine) and/or various enzyme activities. Thiopurine methyltransferase deficiency was diagnosed in 2 patients and 13 patients were heterozygotes. Two other patients were found to have mucopolysaccharidosis type VI. Each of the following diseases was diagnosed in one patient: mucopolysaccharidosis type I, mucopolysaccharidosis type III, classical galactosaemia, partial galactosaemia, Niemann-Pick disease, cystinosis, and ADSL deficiency.

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TISSUE-SPECIFIC TRANSCRIPTION FACTORS IN DEVELOPMENT

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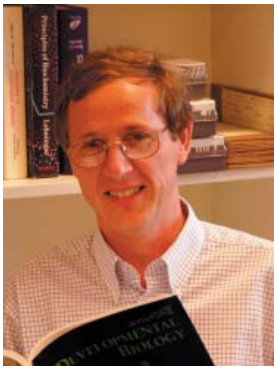
Sabine CORDI, Technician
Jean-François CORNUT, Technician
Marie-Agnès GUEUNING, Technician (half-time)
Matthieu GERADIN, Animal Caretaker (half-time)
Vivien O'CONNOR, Secretary (half-time)



A number of human diseases are characterized by malformations or functional deficiencies of cells in the liver or pancreas. The understanding of such diseases and their treatment by pharmacological or cell therapy-based approaches requires extensive knowledge of the mechanisms that govern cell differentiation. The aim of the research in the group is to determine the mechanisms by which Onecut transcription factors control development of the liver and pancreas, and to investigate their role in diseases of these organs. The Onecut factors, discovered in our laboratory, define a new class of conserved homeoproteins, with three members in mammals : HNF-6 or OC-1, OC-2 and OC-3 (1-4). The role of the Onecut factors in development has been addressed by studying knockout mice generated in our laboratory.

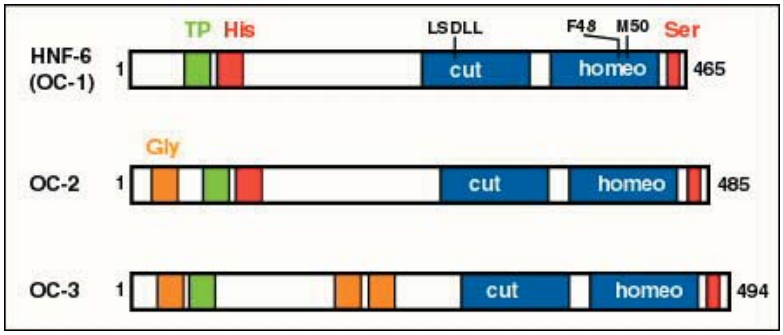


Figure 1. Structure of the human Onecut proteins. The DNA-binding domain consists of a cut domain and a homeodomain. The cut domain contains a LXXLL motif (LSDLL) that contacts transcriptional co-activators. The homeodomain diverges from other homeodomains by the presence of a phenylalanine at position 48 and a methionine at position 50. These two amino acids are important for recruitment of co-activators. The TP, His, Gly and Ser domains are rich in Threonine/Proline, Histidine, Glycine, and Serine, respectively. The TP domain is involved in transcriptional activation, the His and Ser domains in transcriptional repression.

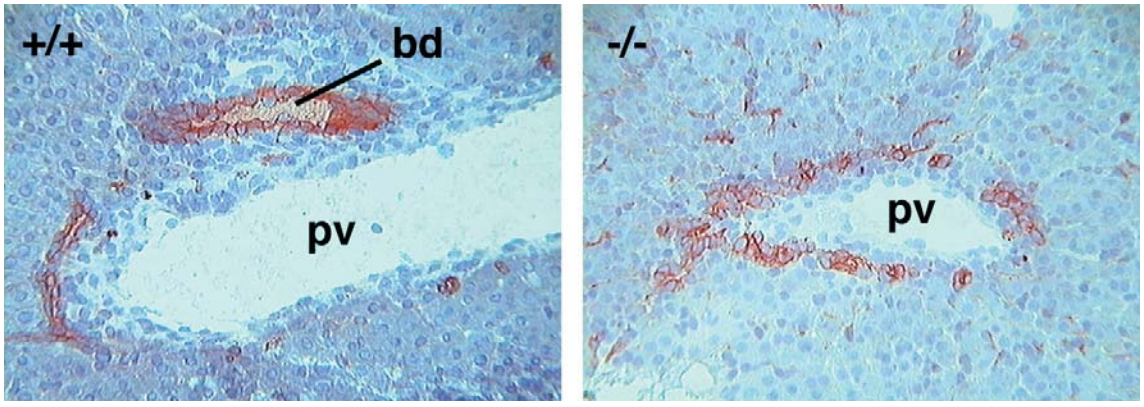


Figure 2. Abnormal development of the intrahepatic bile ducts in HNF-6 knockout mice. Immunohistochemistry of liver sections 10 days after birth shows in normal mice (left panel) a typical bile duct (bd) delineated by biliary cells (stained red) embedded in mesenchyme. In contrast, biliary cells do not form bile ducts in Hnf6-/- mice (right panel) and remain dispersed as a layer around a branch of the portal vein (pv).

Molecular mode of action of HNF-6

Jean-Bernard Beaudry, Christophe Pierreux

Onecut proteins contain a bipartite DNA-binding domain consisting of a homeodomain and a cut domain. The homeodomain is non canonical, since its residue 48 is a Phe instead of the conserved Trp and its residue 50 is a Met, an amino acid never found at this position in classical homeodomains. In addition to the homeo and cut domains, Onecut proteins share other regions that modulate their activity or interact with transcription factors and transcriptional co-activators (Fig. 1; refs 5, 6). We are currently characterizing proteins that interact with HNF-6 to stimulate transcription, in view of understanding the molecular mode of action of HNF-6 in development of the liver and in the regulation of hepatic glucose metabolism (7).

Control of liver development by Onecut factors

Frédéric Clotman, Patrick Jacquemin, Sabrina Margagliotti, Christophe Pierreux, Nicolas Plumb-Rudewiez

During liver development, hepatoblasts, (the liver precursor cells), differentiate into hepatocytes, or into biliary cells which delineate the intrahepatic and extrahepatic bile ducts and the gallbladder. We have shown that HNF-6 is expressed not only in hepatoblasts, but also in the hepatocytes and biliary cells.

In *Hnf6* knockout mice, the gallbladder does not develop and the bile ducts are abnormal (Fig. 2). The mice suffer from cholestasis and display a phenotype that resembles human biliary diseases called "ductal plate malformations", which are related to biliary atresia (8). The *Hnf6* knockout mice also lack hepatic artery branches, an anomaly which can be found associated with human biliary diseases (9). Therefore, our findings indicate that HNF-6 is essential for biliary development.

We also found that HNF-6 controls a network of transcription factors in developing liver. This network comprises HNF-1β, which is also required for biliary development, as well as HNF-4α and HNF-3α, which are known to control expression of genes in hepatocytes (8, 10, 11). The control exerted by HNF-6 on the transcription factor network is complex, and we have shown that it relies both on direct regulation of transcription factor genes, and on indirect regulations implicating a control of the TGFβ signalling pathway by HNF-6 (10).

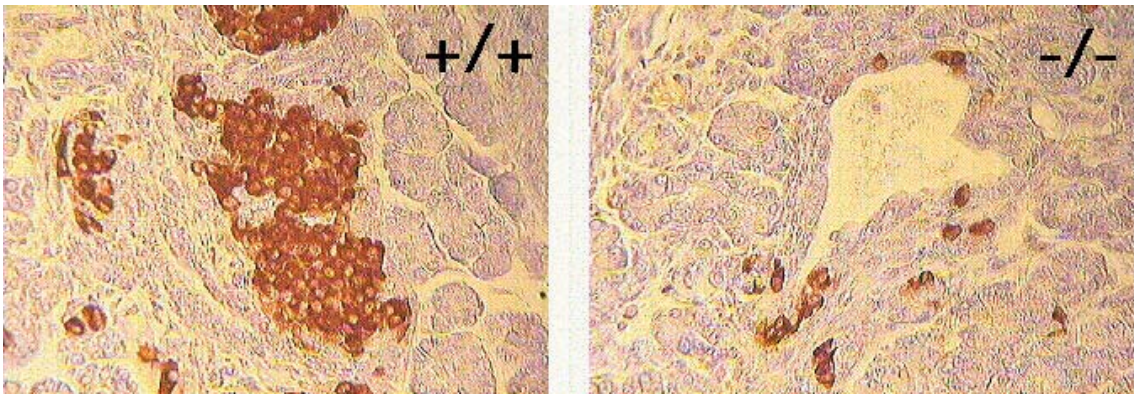


Figure 3. Abnormal development of the endocrine pancreas in HNF-6 knockout mice. Immunohistochemistry of tissue sections four days after birth shows that only a few insulin-producing cells (brown) are found near pancreatic ducts in Hnf6-/- (right panel), instead of being organized in islets as in Hnf6 +/+ littermates (left panel).

The Onecut factors OC-2 and OC-3 are expressed in the developing liver. We are currently investigating if HNF-6, OC-2, OC-3, and genes regulated by HNF-6 play a role in human congenital diseases of the liver and biliary tract.

Control of pancreas development by Onecut factors and secreted proteins

Jonathan van Eyll, Patrick Jacquemin, Christophe Pierreux, Alexandru Simion, Vinciane Van Horenbeeck

During pancreas development, cells lining the primitive gut (endoderm) start to express pancreas-specific genes. These cells, which are pluripotent pancreatic precursors, 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 controls the development of the pluripotent pancreatic precursor cells. In *Hnf6* knockout mice the number of these precursors is reduced and this leads to pancreas hypoplasia (12). HNF-6 is also requi-

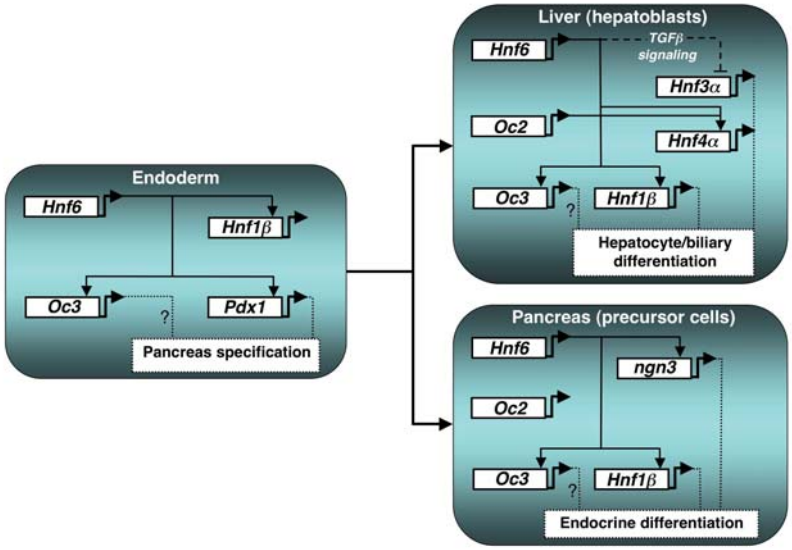


Figure 4. HNF-6 controls differentiation and morphogenesis of the endoderm, liver and pancreas by regulating a network of transcription factors.

red for differentiation of endocrine precursors, since the pancreas of *Hnf6* knockout mice is devoid of islets of Langerhans (Fig. 3). HNF6 controls pancreas precursors by stimulating the transcription factor Pdx-1 in the endoderm (12, 13), and endocrine development by stimulating the transcription factor Ngn-3 in the endocrine precursors (14). The latter finding prompted us to investigate if Onecut factors also control the differentiation of enteroendocrine cells. We are addressing this question by analyzing the phenotype of *Oc2* and *Oc3* knockout mice.

When studying pancreas development we have discovered, using cultured pancreatic explants, that embryonic pancreas can be differentiated into intestinal tissue by the secreted protein activin A, revealing an unexpected differentiation plasticity of the pancreatic cells (15). This explant culture system is now being used to investigate the role of other signalling molecules in pancreas development.

Control of endoderm development by Onecut factors

Patrick Jacquemin, Christophe Pierreux, Aurélie Poll, Alexandru Simion

The endoderm is a cell layer that lines the primitive gut and gives rise to the liver and pancreas. To understand the initial steps of this developmental process, one needs to characterize the transcription factor network involved. To this end we are currently studying the *Hnf6* gene regulatory sequences that direct expression of HNF-6 in the endoderm, as well as the genes that are regulated by HNF-6 in this tissue. We have cloned *Hnf6* regulatory sequences upstream of the β-gal reporter gene and are testing their endoderm-specific activity in transgenic embryos.

We have investigated the genes regulated by HNF-6 in the endoderm by studying the gene expression profile in *Hnf6* knockout embryos. This project 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 (16). This approach allowed us to demonstrate that HNF-6 controls expression of OC-3 in the endoderm (13). The electroporation technique is now implemented for the study of *Hnf6* gene regulatory regions.

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 are organized in a network that controls cell differentiation and organ formation (Fig. 4). Our findings open perspectives for understanding the pathophysiology of liver and pancreatic congenital diseases. The application of our findings to the programmed differentiation of cultured cells should help developing cell therapy of hepatic deficiencies and of pancreatic diseases such as diabetes.

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SIGNAL TRANSDUCTION AND PROTEIN PHOSPHORYLATION

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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 hydrolysed 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) in heart (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 in heart and monocytes, but also inhibits protein synthesis at elongation. We also observed that insulin inhibits AMPK activation in heart and that this occurred via hierarchical phosphorylation .

Insulin signalling

V. Mouton, D. Vertommen, L. Hue, M.H. Rider, in collaboration with L. Bertrand, UCL, Brussels, C. Erneux and D. Bléro, ULB, 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. The recombinant heart PFK-2 isozyme is a substrate of several protein kinases, especially protein kinases of the insulin signalling pathways (2), 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 (3,4). However, this last result was not confirmed in CHO-IR cells transfected with PKB RNAi. Also in these cells, overexpression of SHIP2, the phosphatase that hydrolyses PIP3 which is the intracellular messenger of insulin action, led to a parallel reduction in PKB and PFK-2 activity. These results confirm the importance of PIP3 for 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 (5). Our recent work indicated that WISK contains protein kinase C zeta (PKCζ), which could also participate in the insulin-induced activation of heart PFK-2. Another candidate protein kinase downstream of PDK1 and with properties consistent with WISK is the serum- and glucocorticoid-induced protein kinase-3 (SGK3). We have developed antibody tools to study SGK3 activation by insulin in heart to study whether this kinase could also participate in insulin-induced PFK-2 activation. Figure 1 summarizes the protein kinases from different signalling pathways that phosphorylate heart PFK-2.

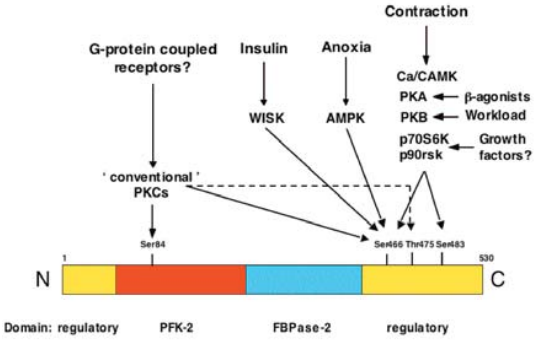

Figure 1. Protein kinases of signalling pathways that converge on heart PFK-2. The numbering of residues refers to the bovine H1 isoform.

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, K. Storey, Ottawa, X. Levèvre, 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 (6). 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

(7,8 and see below). We are currently engaged in identifying other 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, providing a novel mechanism for the inhibition of protein synthesis (7,8). We are presently studying whether adiponectin treatment and electrical stimulation of rat skeletal muscles could inhibit protein synthesis via eEF2 phosphorylation or inhibition of the mammalian target of rapamycin (mTOR)/p70 ribosomal S6 kinase (p70S6K) pathway (see Fig. 2).

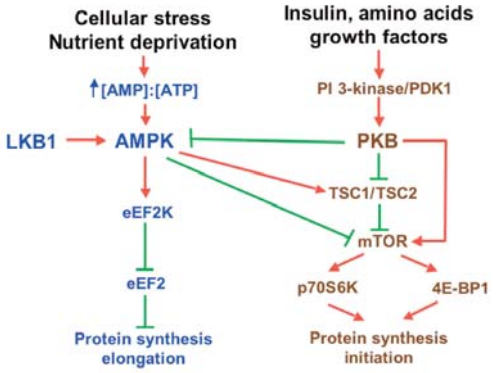

Figure 2. Cross talk between the insulin/PKB/mTOR and AMPK signalling pathways with respect to the control of protein synthesis

Evaluation of the role of AMPK and its downstream targets in mammalian hibernation

Mammalian hibernation requires an extensive reorganization of metabolism that typically includes a greater than 95% reduction in metabolic rate, selective inhibition of many ATP-consuming metabolic activities and a change in fuel use to a primary dependence on the oxidation of lipid reserves. We investigated whether AMPK could play a regulatory role in this reorganization (9). AMPK activity and the phosphorylation state of multiple downstream targets were assessed in five organs of thirteen-lined ground squirrels (Spermophilus tridecemlineatus) comparing euthermic animals with squirrels in deep torpor. AMPK was activated in white adipose tissue but not in liver, skeletal muscle, brown adipose tissue or brain of hibernators. The phosphorylation state of eEF2 increased in white adipose tissue, liver and brain of hibernators, but not in other tissues. Acetyl-CoA carboxylase phosphorylation was markedly increased in brown adipose tissue from hibernators, whereas no change was seen in white adipose tissue or liver. No change was seen in the level of phosphorylation of hormone-sensitive lipase in adipose tissues. Therefore, AMPK does not appear to participate in the metabolic re-organization and/or the metabolic rate depression that occurs during ground squirrel hibernation.

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 (10). We investigated whether the direct phosphorylation of AMPK by PKB might participate in this effect (11). 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. 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 by a hitherto unknown mechanism.

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, P. Michels, UCL, E. Waelkens, KUL, J. Van Lint, KUL

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 approach for general proteomics. We have continued to collaborate with other laboratories both in the Institute and abroad. Along with the group of E. Van Schaftingen, we characterized glycated residues in haemoglobin by mass spectrometry (12,13) and identified a dehydrogenase acting on D-2-hydroxyglutarate (14). In collaboration with the team of P. Michels, we identified essential histidine residues involved in catalysis in the co-factor-independent phosphoglycerate mutase from Leishmania mexicana (15). With J. Van Lint, we identified caspase cleavage sites responsible for protein kinase D activation (16).

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ENDOCYTOSIS

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Yves MARCHAND, Secretary

Endocytosis is a central activity of all eukaryotic cells, that 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 is also increasingly recognized. This research group has made significant contributions in the dissection of endocytic pathways (1) and in unravelling its contribution to physiopathology (6,9), parasitology (2,4) as well as pharmacology (7,8). We are currently addressing the molecular machineries controlling the endocytic activity at the apical surface of epithelia 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 (3,5,10), specifically at the apical domain of polarized MDCK cells; the role of apical endocytosis in the regulated production of thyroid hormones (6); and the elucidation of a deficit of apical endocytosis in a genetic form of kidney stones (9).

Oncogene-induced macropinocytosis in fibroblast

M. Amyere, M. Mettlen, Ph. de Diesbach and P.J. Courtoy

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 (3). These alterations were found to depend on the constitutive sequential activation of phosphoinositide 3-kinase (PI3K) and phospholipase C (PLC). Noticeably, there was no effect of v-Src, K-Ras and overall activation of PI3K and PLC on the receptor-mediated endocytosis of transferrin by fibroblasts, underscoring the difference in the molecular machinery supporting micropinocytosis via clathrin-coated pits (i.e. receptor-mediated endocytosis of transferrin) and macropinocytosis upon closure of membrane ruffles (5). Recent investigations further involve phospholipase D (PLD) in macropinocytosis, downstream of PI3K. Therefore, the role of the small GTPase, Arf-6, is also analyzed.

Regulation of endocytosis by v-Src in polarized cells

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

Since most cancers are of epithelial origin, and since apical endocytosis depends on actin,

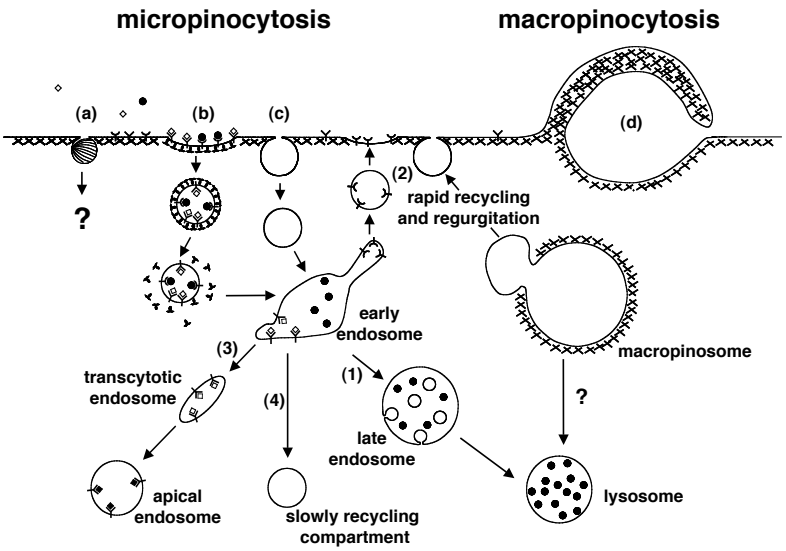

Figure 1. Pathways of endocytosis. This scheme represents four possible modes of vesicular entry of solutes into cells : (a) caveolae; (b) clathrin- and dynamin-associated pits, or «coated pits»; (c) clathrin- and dynamin-independent micropinocytic pits; and (d) macropinocytosis. Crosses represent cortical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

we examined whether v-Src would similarly trigger fluid-phase endocytosis in MDCK cells and whether apical endocytosis would be selectively affected. However, because transformation causes a rapid loss of epithelial polarity, we resorted to MDCK cells bearing a thermosensitive v-Src kinase (MDCK/tsLA31 cell line). When MDCK/tsLA31 cells were plated at high density on a permeable support and cultured at 40°C, a polarized epithelial monolayer could be established, with closely apposed tight junctions, and ~ 90 % of transferrin-receptors being exposed at the basolateral surface. This epithelium showed a 2-fold faster rate of fluid-phase endocytosis at the basolateral than at the apical surface, and a similar rate of receptor-mediated endocytosis of transferrin at both membrane domains. Shifting from the non-permissive temperature (40°C) to the permissive temperature for v-Src kinase (34°C) dramatically accelerated fluid-phase endocytosis at the apical surface (up to 6-fold), but basolateral endocytosis was not affected. This was accompanied by the induction of a single, huge (> 5 μm) apical endocytic vacuole, presumably due to coalescence or swelling of the subapical compartment. The selective acceleration of apical endocytosis and the appearance of the apical vacuole depended on PI3K, PLC and PLD, as shown by inhibition by wortmannin, NCDC and 1-butanol, respectively. v-Src activation also induced a PI3K-, PLC-, and PLD-dependent apical ruffling. 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.

Relation between endocytosis and cell motility

A. Platek, M. Mettlen and P.J. Courtoy

It has been proposed that endocytosis contributes to cell motility by allowing for 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 examine whether these two processes are linked and depend on the same regulatory machinery. To this aim,

we made use of fibroblasts and MDCK cells harbouring thermosensitive v-Src kinase (Rat-1/tsLA29 and MDCK/tsLA31 cells). In both cell lines, v-Src activation led to a 2-fold acceleration of cell motility, 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 suggest a link between accelerated motility and endocytosis. In addition, v-Src activation abrogated directionality of cell motility in response to chemotactic growth factor (GF) gradients. v-Src down-regulated GF-receptors by about two-fold, but half receptor occupancy in non-transformed cells was sufficient to induce directional motility (10). Several recent lines of evidence suggest that this loss of a polarized actin response results from diffuse activation of downstream Src effectors all around the cell surface ("excess noise").

Apical endocytosis regulates thyroid hormone production in human disease; molecular dissection in vitro

K. Croizet, P.J. Courtoy and M.F. van den Hove

The production of thyroid hormones by thyrocytes results from apical endocytosis of thyroglobulin stored in the colloid, followed by intracellular proteolysis. 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. To test this hypothesis, we have followed two approaches. First, we found that the increased level of expression of the rate-limiting endocytic catalysts, Rab5a and Rab7, in autonomous hyperactive adenomas, closely correlates with (i) a decrease in residual thyroglobulin content; and (ii) an increased recovery of particulate iodine towards more distal compartments (i.e. the most active proteolytic organelles of the degradative pathway). Second, having established polarized human thyrocytes that are competent for selective basolateral delivery of thyroid hormone, we found that TSH stimulation, or direct activation of the cAMP cascade, are sufficient to increase Rab5a and Rab7 expression (6). Rabs membrane recruitment was also enhanced, indicating their further activation. Accordingly, we recently found that the cAMP cascade induces the expression of Rab GEFs in this physiopathological system.

Alterations in the endocytic apparatus of mice lacking renal chloride channel, ClC-5, account for proteinuria in Dent's disease

C. Auzanneau and P.J. Courtoy, in collaboration with O. Devuyst, NEFR

To elucidate the molecular basis of Dent's disease, an X-linked familial form of nephrolithiasis linked to low-molecular weight proteinuria, we have analysed the endocytic apparatus in knock-out (KO) mice for the kidney-specific chloride channel, ClC-5. These investigations are carried out in collaboration with Dr. W. Guggino (John Hopkins, Baltimore, MD, USA) and Dr. E.I. Christensen (Aarhus, DK). The comparison of the steady-state level of several major constituents and regulators of the apical endocytic apparatus, their immunolocalisation at the ultrastructural level, and the follow-up by analytical subcellular fractionation of an apical endocytic tracer, at various intervals of uptake, showed that defective ClC-5 in Dent's disease patients and KO mice leads to a major trafficking defect of the low-molecular weight protein receptors, megalin and cubilin (9). To address this defect, we currently examine the role of ClC-5 in the acidification of apical endosomes. In collaboration with Dr. H. de Jonge (Amsterdam, NL) and Dr. J.J. Cassiman (KUL, Belgium), we also compare, for their role and partners, ClC-5 with the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) in KO and ΔF508 transgenic mice.

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EXTRACELLULAR MATRIX BREAKDOWN

Pierre J. COURTOY, Member (right)
Etienne MARBAIX, Member (middle)
Yves EECKHOUT, Emeritus Member

Patrick HENRIET, Member
Hervé EMONARD, Guest Investigator
Christine GALANT, Assistant Member
Alix BERTON, E.U. Marie Curie Fellow
Patricia CORNET, Graduate Student
Vassil VASSILEV, Graduate Student
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Pascale LEMOINE, Technician
Olga MEERT, Technician
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 (1, 2). We have demonstrated that menstrual bleeding in women is due to the expression and activation of some MMPs (3). 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 (4) with local environmental changes (5, 6); 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 (7). 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.

Regulation of the expression of endometrial MMPs and cytokines

P. Henriet, P. Cornet, V. Vassilev, P.J. Courtoy, Y. Eeckhout and E. Marbaix

Both endocrine and paracrine factors participate in controlling the expression and activity of MMPs involved in menstrual breakdown of the human endometrium (4, 6). Several genes encoding MMPs and cytokines present maximal endometrial mRNA concentrations around menstruation (reviewed in 1). They substantially diverge, however, in their expression profile during the other phases of the cycle, suggesting differential regulation by estradiol and progesterone.

To directly measure the effect of the ovarian steroids, mRNA amounts of selected genes were quantified in a large collection of endometrial samples collected throughout the menstrual cycle and stored before or after their culture as explants. We first focused on gelatinase B/MMP-9 and on the TGFβ-related cytokine, LEFTY-A/EBAF (8, 9). A major upregulation in MMP-9 mRNA expression occurred *in vivo* in endometria showing signs of menstrual breakdown; this followed a larger increase in LEFTY-A mRNA, pointing to a regulatory role of this cytokine. Differential response to the ovarian steroids was observed in explant culture. LEFTY-A mRNA proved more sensitive than MMP-9 mRNA to the presence of endogenous as well as exogenous progesterone.

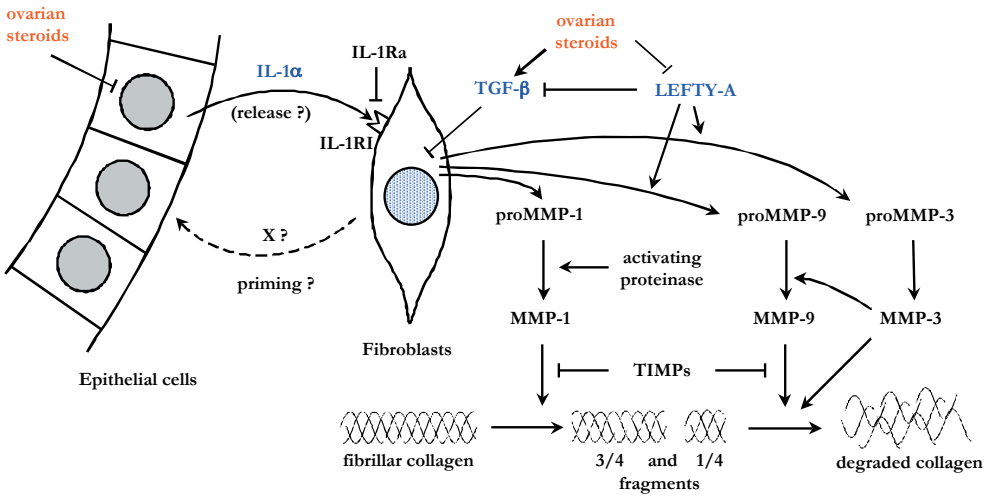

Figure 1. Regulation of MMPs in the human endometrium : current model. Upon ovarian steroids withdrawal, LEFTY-A and epithelial IL-1α are released and trigger the production by adjacent fibroblasts of various proMMPs, including interstitial procollagenase-1/proMMP-1, progelatinase B/proMMP-9 and prostromelysin-1/proMMP-3. Fibroblast activation involves interleukin-1 receptor (IL-1RI) and is opposed by various soluble factors, such as interleukin-1 receptor antagonist (IL-1Ra). TGF-β inhibits MMPs production, but this brake is relieved by LEFTY-A. Expression of proMMPs by stimulated fibroblasts is further blocked downstream by ovarian steroids (dual brake). Secreted proMMPs need activation by proteinases, including MMP-3. Active MMP-1, if not neutralised by tissue inhibitors (TIMPs), cleaves fibrillar collagens at position 3/4 of the distance from the amino-terminus. The fragments and other matrix proteins are then further degraded by other proteinases, including MMP-9 and MMP-3.

Moreover, addition of recombinant LEFTY-A to explant culture further increased the amounts of selected menstrual MMPs, including MMP-9, a response that was blocked by the ovarian steroids. Altogether, these observations suggest that, *in vivo*, different pathways finely tune in space, time and amplitude, the global control of estradiol and progesterone on the expression of genes required for menstrual ECM breakdown. The approach will be extended to other relevant menstrual MMPs and cytokines.

Selective binding of active MMP-7 to human epithelial cell membrane

A. Berton, H. Emonard, P.J. Courtoy and E. Marbaix

Most normal and cancerous epithelial cells express matrilysin-1/MMP-7. In addition to degrading extracellular matrix substrates, activating other MMPs and inactivating enzyme inhibitors, MMP-7 also cleaves cell surface proteins such as E-cadherin, β4 integrin, proTNF-α and Fas ligand. Whereas proMMP-7 tightly binds to heparan sulfate proteoglycans at the surface of rat endometrial epithelial cells, only active MMP-7 binds to colon cancer cells. In our model of endometrial cultured explants, we observed a preferential accumulation of MMP-7 in the tissue whereas proMMP-7 was the major form detected in the conditioned medium. In cryosections of human endometrium, proMMP-7 was immunolocalized in the apical cytoplasm of all epithelial cells, whereas MMP-7 was detected in focally distributed epithelial cells, showing a strongly enhanced peripheral signal, which suggested association with the plasma membrane. Recombinant MMP-7, but not proMMP-7, showed significant binding to endometrial epithelial cells, through a single class of receptors. Cell surface-asso-

ciated MMP-7 was found to be functionally active. Targeting of active MMP-7 to the plasma membrane could represent a relevant mechanism to regulate various cellular functions by activating, inactivating or releasing structural or signaling membrane proteins.

Receptor-mediated endocytic clearance of proMMP-2:TIMP-2 complexes

H. Emonard, A. Berton, and P.J. Courtoy, in collaboration with CNRS, Reims, France

Activity of MMPs is controlled at different levels. Once synthesized as proenzyme species, proMMPs must be activated to exert full proteolytic activity. MMP activation may depend on other MMPs, as illustrated for proMMP-9 activation in the endometrium tissue (10). Besides tissue inhibitors of metalloproteinases (TIMPs), MMPs may be inhibited by various natural compounds, including unsaturated long-chain fatty acids (11). Plasma membrane binding does not only contribute to MMPs concentration on discrete spots at the cell surface, as illustrated above for MMP-7, but can as well silence MMPs activities by promoting their intracellular degradation. ProMMP-2/gelatinase A is predominantly secreted in association with TIMP-2. The low-density lipoprotein receptor-related protein (LRP), a scavenger receptor, mediates the endocytic clearance of various proteins, including proteinases and proteinase-inhibitor complexes. Upon treatment with RAP, a natural LRP ligand antagonist, we showed that HT1080 human fibrosarcoma cells accumulated both proMMP-2 and TIMP-2. We further demonstrated that RAP inhibited endocytosis and lysosomal degradation of ^{125}I-proMMP-2:TIMP-2 complexes, but had no effect on binding of ^{125}I-proMMP-2:TIMP-2 complexes to the cell surface (12). These data indicate that clearance of proMMP-2:TIMP-2 complexes is a two-step process, involving initial binding to the cell membrane in a RAP-independent manner, and subsequent LRP-mediated internalisation and degradation.

Role of matrix metalloproteinases in abnormal endometrial bleeding

C. Galant, Y. Eeckhout, 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 pathological conditions characterized by excessive, prolonged or irregular bleeding without organic lesion. Up to one fourth of hysterectomies are due to such functional menstrual disorders. Endometrial biopsies from patients irregularly bleeding at the time of sampling were compared to biopsies sampled during non-bleeding intervals and to control biopsies from normally menstruating women. Irregular bleeding was clearly associated with menstrual-like stromal breakdown in foci containing low levels of ovarian steroids receptors, and with increased expression and activation of several MMPs, together with decreased production of TIMP-1 (13). These results confirmed ***in vivo*** and in various hormonal conditions our previous findings obtained with cultured explants from patients on long-term progestinic contraception (7). A recent collaboration with Dr. J.L. Brun addresses whether excessive menstrual bleeding and recurrence of bleeding after surgical treatment could be linked to the same molecular mechanisms. To clarify the reason for these local disorders, we currently look for altered expression or regulation of ovarian steroid receptors and appropriate cytokines.

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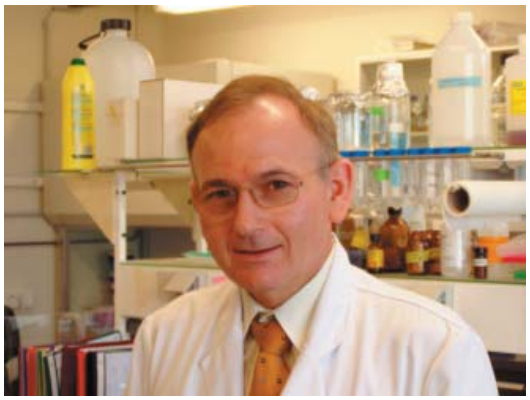
CONNECTIVE TISSUE AND ARTHRITIS

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

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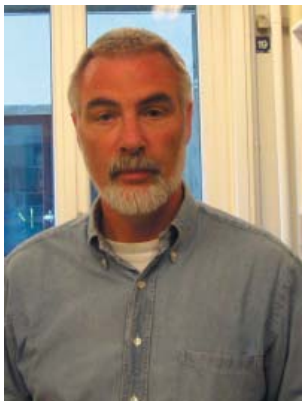

Sleeping sickness or African trypanosomiasis is a parasitic disease in humans. Caused by protozoa of the genus Trypanosoma and transmitted by the tsetse fly, the disease is endemic in certain regions of Sub-Saharan Africa; 60 million people in 36 countries are at risk. It is estimated that half a million people are infected, and about 40,000 die every year. Symptoms begin with fever, headaches, and joint pains. If untreated, symptoms spread to the central nervous system (CNS). Without treatment, the disease is fatal, with progressive mental deterioration leading to coma and death. All available drugs have many undesirable side-effects, and the treatment regimen is often difficult to enforce. It is for these reasons that new and better drugs are urgently needed. Trypanosomes, when in the blood and CNS, rely entirely on glycolysis for their ATP supply. Moreover, the parasites are characterized by a unique form of metabolic compartmentation; the majority of the enzymes of the glycolytic pathway is sequestered inside peroxisome-like organelles called glycosomes. The glycolytic pathway of these organisms has recently been validated as a drug target.

Since many years we study the physicochemical properties of the glycolytic enzymes of Trypanosoma brucei and their closely related relatives such as 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. Furthermore we aim at the complete understanding of what controls in the trypanosome the glycolytic flux. For this we use a mathematical model with the kinetic properties of all the enzymes of the glycolytic pathway, combined with biochemical and genetic approaches which allow us to manipulate the glycolytic flux. Also another pathway of carbohydrate metabolism, the hexose monophosphate pathway, has attracted our attention since it turns out that several of its enzymes are associated with the glycosomes as well. We also study glycosome assembly and degradation. Turnover of the organelles appears of utmost importance during cell differentiation. Especially peroxins, proteins involved in glycosome biogenesis, are the subject of our studies.

The recent completion of the sequencing of three trypanosomatid (TriTryp) genomes has allowed us the make a comprehensive inventory of TriTryp metabolic capacities and to identify not only metabolic differences between the various trypanosomatids, but also to establish essential differences between the parasites and their host.

Enzymes of carbohydrate metabolism

Cloning and characterization of genes for glycolytic enzymes, expression and characterization of recombinant enzymes and inhibitor development.

V. Hannaert, D. Guerra, M.-A. Albert, F. Opperdoes and P. Michels

Essentially all genes of the enzymes of the glycolytic pathway have been cloned and characterized in our laboratory. In addition, we have characterized the genes for four isoenzymes of the 6-phosphofructo-2 kinase/fructose-2,6 bisphosphatase (PFK2/FBPase2), enzymes responsible for the synthesis and hydrolysis of fructose 2,6 bisphosphate, a potent allosteric effector of pyruvate kinase (PYK) and the major regulator of glycolysis in trypanosomatids. In a number of cases the predicted primary structure of trypanosomatid enzymes appeared quite different from their mammalian counterparts and some enzymes are even not homologous, rendering them promising drug targets. In collaboration with Dr. Linda Gilmore, Prof. Malcolm Walkinshaw and Prof. Nick Turner (University of Edinburgh, UK) with whom we succeeded in the elucidation of the crystal structures of phosphofructokinase (PFK), PYK and phosphoglycerate mutase (PGAM), novel drugs exploiting these differences are being developed.

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 (in collaboration with Dr. Casimir Blonski (University of Toulouse, France) and Prof. Jurgen Sygush (University of Montreal, Canada) a potent, quasi-irreversible, inhibitor with high selectivity for the parasite enzyme. Pro-drugs have been developed to facilitate uptake in cells and these were able to kill cultured parasites with an ED50 value of approximately 2.5 microM. We intend to optimize these inhibitors by successive rounds of structure-ligand interaction analyses and chemical synthesis.

The kinetic properties of bacterially expressed T. brucei enolase (ENO) are very similar to those of the mammalian enzymes. Furthermore, crystal structure determination (in collaboration with Dr. Daniel Rigden, (University of Liverpool, UK) and Dr. Richard Garrett (University of São Paulo, Brazil) indicated that the overall conformation of the active site of the trypanosomal enzyme is very similar to those of the enzyme from yeast and lobster for which also crystal structures are available. However, there are two atypical Cys residues located in a water-filled cavity near the T. brucei active site. The accessibility of these residues for small compounds has been demonstrated in collaboration with Dr. Didier Vertommen (HORM Unit). Therefore, these residues could possibly be exploited for the irreversible binding of selective inhibitors. Such inhibitors are currently being developed in the laboratory of Dr. Blonski (Toulouse).

The activity of trypanosomatid PYK is allosterically regulated by fructose 2,6-bisphosphate (F 2,6 P2), contrary to the PYKs from other eukaryotes that are usually stimulated by fructose 1,6-bisphosphate (F 1,6 P2). The binding site for the allosteric effector F 2,6 P2 was selected as a potential drug target. In collaboration with Drs Gilmore and Walkinshaw (Edinburgh), a structure model was made based on the previously determined low substrate affinity T-

state structure of L. mexicana PYK, without any bound ligands, and the R-state structure of Saccharomyces cerevisiae PYK complexed with its effector F 1,6 P2. Specific residues were identified that may confer the observed high affinity and specificity of the trypanosomatid enzyme for F 2,6 P2 over F 1,6 P2. The crucial importance of these residues for distinguishing between the two fructose-bisphosphates was confirmed by site-directed mutagenesis. The structure of L. mexicana PYK in the R-state has now also been solved. Four different R-state structures, with different ligands in active or effector sites were determined. The superposition of R- and T-state structures provides insight in the conformational changes occurring upon activation. The LmPYK crystal structures confirm the striking differences at the effector-binding site compared to PYKs from other organisms, thus the possibility to use this site for drug targeting. The precise mode of binding and mechanism of inhibition have been determined for several of fructose-bisphosphate analogues using both the wild-type LmPYK, and mutants in which the critical residues for F 2,6 P2 binding had been substituted. This analysis has provided important information that is being taken into account in the design of new and better molecules.

Analysis of the control of the glycolytic flux

M.-A. Albert, D. Guerra and P. Michels in collaboration with B. Bakker and H. Westerhoff, Vrije Universiteit Amsterdam, The Netherland

Previously (in 1997), we reported the development of a mathematical model of trypanosome glycolysis using the kinetic data, as then available, for individual enzymes of the pathway. This model reliably predicted the experimentally determined fluxes and metabolite concentrations in trypanosomes. We have now optimized and extended the model on the basis of recently determined kinetic data and information about enzyme activities in lysates of *in vitro* cultured, growing trypanosomes. Using this model, which now includes all enzymes of the pathway, the existence of a large overexpression was predicted for hexokinase (HXK), PFK and PYK. Our present experiments focus on the experimental determination of the flux control of individual steps of glycolysis by knockdown of gene expression by the RNA interference (RNAi) approach. So far, the intracellular levels of five enzymes were varied by RNAi: HXK, PFK, PGAM, ENO and PYK. The data obtained support the conclusion from the in silico analysis that HXK, PFK and PYK are in excess, albeit less than predicted. Furthermore, several observations made when the expression of PFK and ENO was varied suggest the existence of novel regulatory mechanisms operating in glycolysis of bloodstream-form trypanosomes.

We have now also collected many data on subcellular distribution, activity and kinetic properties of enzymes involved in carbohydrate and energy metabolism in insect-stage (procyclic) T. brucei and (promastigote) L. mexicana. These data have been used to create preliminary computer models of the metabolism of these cells. These models will be used to perform flux-control analyses similar to our studies on glycolysis of bloodstream-form T. brucei.

Biogenesis of glycosomes

N. Galland, H. Krazy, V. Hannaert, E. Verplaetse, C. Yernaux and P. Michels

Peroxins (abbreviated 'PEX') are involved in the synthesis of glycosomes, the peroxisome-like organelles that harbour the major part of the glycolytic pathway of trypanosomatids. Eight peroxins (PEX1, 5, 6, 7, 10, 12, 14 and 19) have so far been identified by us and most of them have now been expressed as recombinant proteins (in collaboration with Prof. Wim Hol, University of Washington, Seattle) and the importance of most of them has been assessed by RNAi experiments. PEX5, 6, 10, 12 and 14 are all essential. Glycosomal proteins with different types of targeting signals (PTS-1, PTS-2 and I-PTS) all require these peroxins

for their import into glycosomes. The low sequence identities between peroxins of T. brucei and their homologues in the human host, and the vital importance of proper glycosome biogenesis for the trypanosome, strongly support the notion that it will be feasible to design peptido-mimetic compounds that will prevent interactions between peroxins without interfering with human peroxisome formation. Such compounds are currently being prepared by Drs Hol and Fan (Seattle). We are in the process of identifying other peroxins which may play a crucial role as well, such as homologues of the yeast PEX3, PEX8 and PEX13.

Analysis of glycosomal membrane solute transporters

C. Yernaux and P. Michels

We have identified and characterized three T. brucei genes coding for solute transporters of the glycosomal membrane. The amino-acid sequences of these genes (denoted TbGAT1, 2 and 3) are a mere 30% identical to each other. They are so-called half ABC transporters, containing only a single ATP-binding cassette in their C-terminal half. They are homologous to the ABC transporters found in peroxisomal membranes in yeast and mammalian cells. Expression of TbGAT1 and 2 in S. cerevisiae cells of which the two homologous transporters, involved in fatty-acid uptake into peroxisomes were disrupted, resulted in complementation of the yeast's affected ability to grow on oleate (in collaboration with Dr. Ben Distel, University of Amsterdam). Simultaneous expression of both TbGAT1 and 2 was required, strongly suggesting that they function as a hetero-oligomer. The glycosomal localization of the three TbGAT isoforms was confirmed by immunofluorrescence using confocal microscopy, after transfection of trypanosomes with constructs coding for TbGATs fused to Green Fluorescent Protein (GFP). The targeting determinants in TbGAT1 and 2 for the glycosomal membrane were similarly determined using fusion constructs of segments of the transporters with GFP. From peroxisomal studies it has been concluded by others that PEX19 acts as a chaperone and import receptor for newly synthesized peroxisomal membrane proteins. Indeed, we could demonstrate a specific interaction between the glycosomal targeting domain of TbGAT1 and 2 and a T. brucei PEX19 homologue, using a bacterial two-hybrid system (in collaboration with Dr. Marc Fransen, KUL). The interaction was also observed when human PEX19 was used instead of the trypanosomal protein. The region of human PEX19 responsible for interaction with TbGAT could be mapped to its C-terminus; this domain has been shown to be also responsible for interaction with human peroxisomal membrane proteins. Our results suggest, therefore, a conservation of PEX19 function throughout evolution.

Degradation of glycosomes

M. Herman and P. Michels

We have continued our investigations into the adaptation of metabolism during differentiation by degradation of old glycosomes (pexophagy). Using markers of specifc classes of glycosomes and of lysosomes, differentiating trypanosomes were analyzed with biochemical and immunofluorescence techniques and the abundance and co-localization of both types of organelles followed during differentiation (in collaboration with Prof. Etienne Pays, ULB). Transformation from long-slender to short-stumpy bloodstream forms leads to a co-localization of glycosomal and lysosomal markers suggesting a degradation of glycosomes inside lysosomal structures. Transformation of the short-stumpy forms into procyclics leads to a rapid degradation of preexisting glycosomes and the appearance of a new class of organelles rich in the procyclic marker pyruvate phosphate dikinase (PPDK).

Metabolic capacities of trypanosomatids

F. Opperdoes and J.P. Szikora

The recent completion of the sequencing of the TriTryp genomes has allowed us to analyse the metabolic capacities of the three organisms and to compare them in detail with each other. In summary, 265 genes coding for the enzymes of the major metabolic pathways in L. major have been identified. Thirty of them, or 11%, were unique to L. major in that orthologues could not be detected in the other two trypanosomatids. Of these 30 proteins at least seven showed a clear bacterial affiliation when subjected to phylogenetic analysis. 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, 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 TriTryp organisms, followed by T. cruzi. T. brucei is the most crippled of the three. Differences are mainly found at the level of fatty acid oxidation and amino-acid metabolism. Details of these analyses have been submitted to the TriTryp genome sequencing consortium for future publication. To obtain an idea of the glycosomal proteome of trypanosomatids all sequences were searched for the presence of glycosomal import signals of type 1 and type 2 (PTS1 and PTS2). The presence of such signals in the three orthologues of the TriTryp and in the majority of the enzymes belonging to an entire metabolic pathway was considered as compelling evidence for their glycosomal localisation. Using these data a chart of glycosomal metabolism has been put together. New are the observations that L. major glycosomes, as an adaptation to the sandfly vector, which feeds not only on blood but also on plant nectar, are capable of degrading many sugars other than glucose, fructose and mannose. Moreover, glycosomes fulfill a role in the hexose monophosphate pathway and are involved in fatty-acid oxidation, ether-lipid and, pyrimidine synthesis, purine salvage and protection against oxidant stress.

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

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

Venous malformations, glomuvenous malformations ("glomangiomas") and Maffucci syndrome

P. Brouillard, M. Amyere, B. McIntyre, V. Aerts, V. Wouters, 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 and O. Enjolras, Hôpital Lariboisière, Paris, France

Venous malformations (VM) are bluish-purple cutaneous and mucosal 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 (14).

We have previously identified that hereditary venous malformations can be caused by

an activating mutation in the receptor tyrosine kinase TIE2/TEK (1). In contrast to inherited VMs, inherited glomuvenous malformations do not link to the TIE2/TEK gene. Instead, they link to VMGLOM on chromosome 1p21. Characterization of the positional candidate genes led to the identification of the mutated gene that we named "glomulin" (3). 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 (15). This allows an easy genetic diagnosis for individuals thought to have GVM. The rest, 30%, have a unique mutation. During the year 2004 we purchased a DHPLC system for mutation detection. With this, screenings of these private mutations is easier, faster and more sensitive.

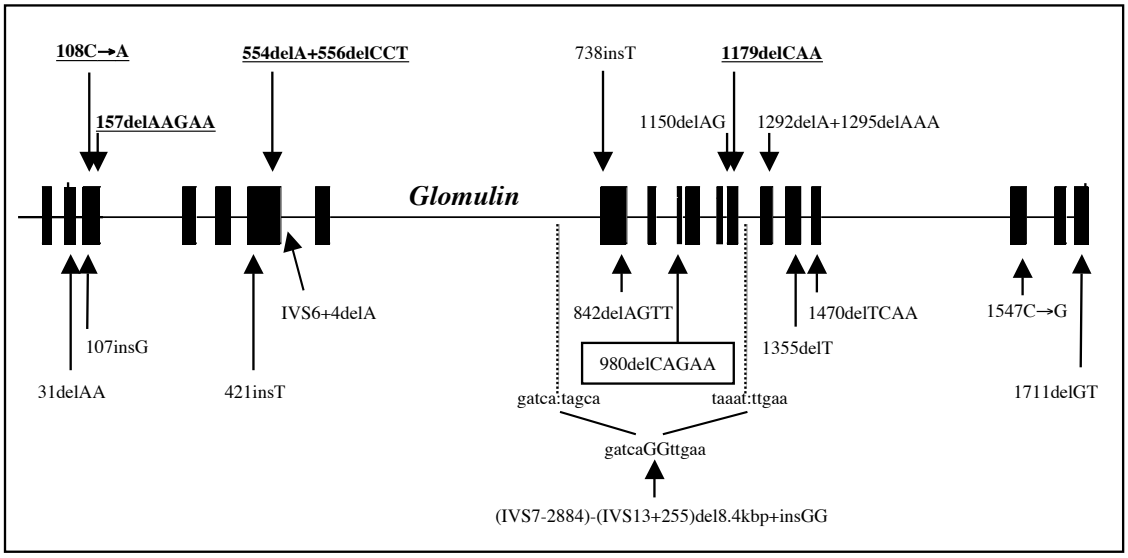

Figure 1. Schematic representation of the glomulin gene summarizing all known mutations (adapted from [13]). Mutations found in more than one family are underlined. Somatic second hit is boxed.

Glomulin does not have sequence identities to known proteins, nor does it contain known functional domains. Thus, its molecular function is unknown. To unravel at least partially the function of glomulin, we have studied its expression. Glomulin was found in almost all tissues (2), but almost exclusively in vascular smooth muscle cells (11).

As most of the identified mutations cause premature STOP codons in the coding sequence of glomulin (Fig. 1), loss-of-function is the most likely mechanism causing GVMs (2). 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 (2). To further study glomulin function, we have cloned 20kb of the murine glomulin gene. This has been 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. To understand the pathophysiology, we have initiated genetic studies. We identified a family in which primary lymphedema was present at birth in several family members. Genetic studies confirmed linkage to 5q33-34 and led to the identification of a mutation in the VEGFR3 gene (3). *In vitro* expression studies demonstrated that the mutated receptor has lost its autophosphorylation capacity (3). The continued studies have led to the identification of a transcription factor gene, SOX18, to be mutated in a family with recessively inherited congenital lymphedema (4). A dominant SOX18 mutation was identified in two other families, with individuals having varying degree of lymphedema (4). All individuals with a SOX18 mutation had also a hypothrichosis. This study described a new target for lymphedema therapy, the third human gene known to cause lymphedema, a disorder currently without cure.

Vascular anomalies affecting capillaries

N. Revencu, I. Eerola, L.M. Boon and M. Vikkula in collaboration with J.B. Mulliken, Children's Hospital, Boston, USA, S. Watanabe, Showa University School of Medicine, Tokyo, Japan, A. Dompmartin, CHU de Caen, France 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, like 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 suggests that KRIT1, a possible intracellular signaling molecule, is important not only for cerebral but also for cutaneous vasculature. 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.

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 are collecting samples from families with possibly hereditary cardiopathies. In two families, in which atrial septal defect is associated with progressive atrioventricular conduc-

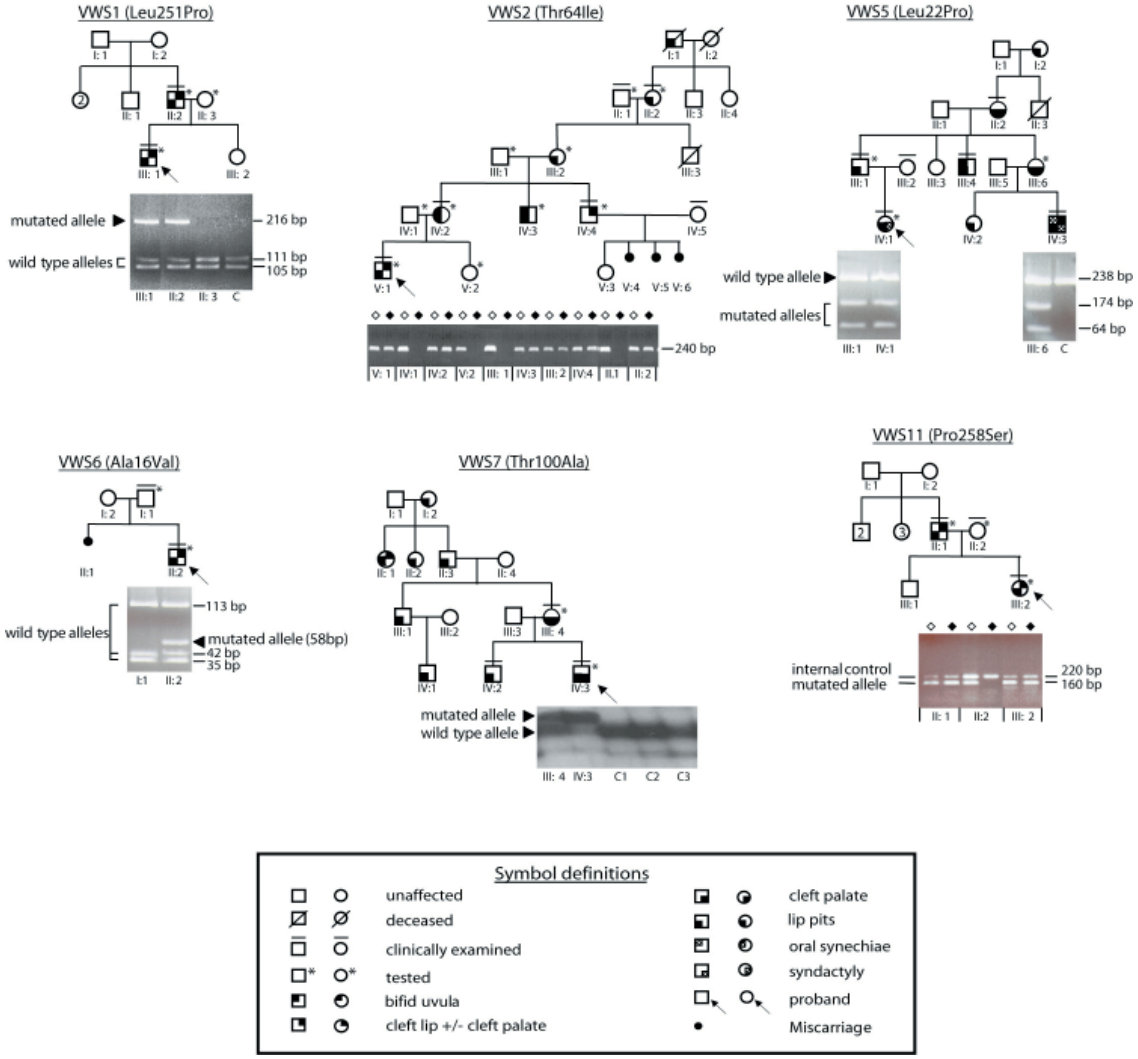

Figure 2. Pedigrees of 6 families with VWS/PPS syndrome. A photograph of an agarose gel underneath each pedigree shows the segregation of the allele harbouring the substitution. VWS1, VWS5, VWS6 : restriction enzyme digestion; VWS2, VWS11 : allele specific PCR. VWS7 : SSCP analysis. ◇, Wt allele; **u**, mutant allele. (adapted from [10])

tion defect, we identified two novel mutations in the CSX/Nkx2.5 gene (5), 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 used the DNA-chip based approach to perform whole-genome linkage analysis, and identified a possible locus for a gene causing heterotaxia, situs inversus (Gutierrez et al., manuscript in preparation).

Cleft lip and palate

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

Our main project in collaboration with Centre labio-palatin, St Luc, is to unravel the molecular background of syndromic and non-syndromic cleft lip and/or palate. Numerous blood samples of affected individuals, and their parents and siblings have been collected.

They are used for association studies. In addition, collaboration with the cleft lip and palate center of the CHRU Lille (Prof. Ph. Pellerin) has been initiated. These studies have recently led to the identification of IRF6 mutations causing Van der Woude syndrome (Fig. 2) (10). Based on this, we have initiated a collaboration with Prof. J. Murray, University of Yowa, to study IRF6 association in cleft lip and palate.

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 recently 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 (9). 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. manuscript 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 (7). This data had direct clinical relevance. In addition, we identified methylation differences in ependymomas depending on their age and location (8).

Conclusions

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

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THEILER'S ENCEPHALOMYELITIS VIRUS

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Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) is a murine picornavirus responsible for infections of the central nervous system. Strains of Theiler's virus have a striking ability to persist in the central nervous system 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. The genome of Theiler's virus is an 8 kb-long positive strand RNA molecule (Fig. 1).

Theiler's virus is an outstanding model to analyze the basic mechanisms of viral persistence and demyelination. i) It replicates and persists in the central nervous system in the face of a specific humoral and cellular immune response. ii) It induces chronic demyelination in mice. iii) It is a natural pathogen of the mouse and allows the experimental analysis of a natural host-pathogen interaction. iv) Its genome is cloned and can be manipulated by the tools of molecular biology.

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 and cellular determinants of tropism and persistence, notably the interferon α/β response and the inhibition of this response by Theiler's virus, in the context of the central nervous system.

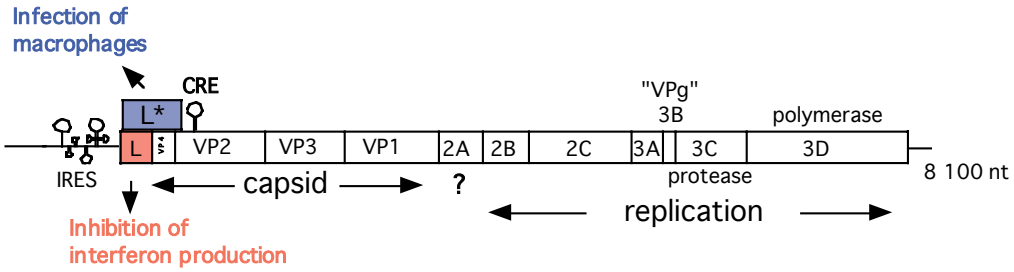

Figure 1. Genome of Theiler's virus. A large open reading frame encodes a 2000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, into 12 mature proteins. An additional protein (L*) is encoded by an alternative open reading frame. Translation of both ORFs is driven by an Internal Ribosome Entry Site (IRES) present in the 5' non-coding region of the genome (1). Protein L* was shown to facilitate the infection of macrophages and viral persistence (2). Protein L inhibits type-I interferon production (3). The role of protein 2A is unknown (4). 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» (5).

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

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

The viral capsid was found to play an important role in determining the pathology induced by the virus. For instance, exchanging the capsid of neurovirulent and persistent Theiler's virus strains resulted in phenotype swapping, though not complete.

The isolation of cellular mutants that became resistant to the neurovirulent GDVII virus, but that retained susceptibility to the persistent DA virus, showed that the capsids of neurovirulent and persistent strains interacted with different receptors or co-receptors on the target cell (6).

Despite many efforts, the cellular receptor for Theiler's virus has not yet been identified. Persistent strains but not neurovirulent strains of Theiler's virus were found to bind sialic acid. Interaction with sialic acid involves protein loops exposed at the surface of the viral capsid, which were found to modulate viral tropism as well as antigenicity and pathogenesis (7).

Proteins L and L*

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 interferon production by the leader protein

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

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 (3). Mutation of the zinc-finger was sufficient to abolish the anti-IFN activity of the L protein *in vitro* and to central nervous system of SJL/J mice. However, IFN production inhibition was not complete *in vivo*. Modulation rather than blockade of the IFN response might be viewed as a better viral strategy toward long-term persistence in the host.

Inhibition of IFN production was found to occur at the transcriptional level (8). Thus, a potential target of the leader protein is IRF-3, a factor known to be required for transcriptional 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 interfered with nucleo-cytoplasmic trafficking of host cell proteins, and notably of IRF-3 (9). Perturbation of nucleo-cytoplasmic trafficking by viruses could be viewed as a fast way to inhibit early cell defense mechanisms, including the production of cytokines.

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

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

We 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 (2).

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.

Characterization of the type-I IFN response

C. Sommereyns, S. Delhaye, 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 (10).

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. However, the same set of IFN genes was activated in L929 cells in response to different stimuli such as poly(I-C) transfection and viral infection (10).

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VIRAL IMMUNITY AND PATHOGENESIS

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

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

Of particular interest is the fact that all antibody responses are not equal. Indeed, depending on their isotype, immunoglobulins display various properties. For example, IgG1, one of the major IgG subclass in mice, cannot fix the complement, contrary to IgG2a, another major component of murine immunoglobulins. Such a difference may lead to dramatic variations in the functional effect of antibodies, as their ability to lyse cells they have bound. During the last few years, we found that the isotype of antibody responses was influenced by concomitant viral infections. The effect of the virus resulted in a dramatic increase in the proportion of IgG2a, not only in antiviral antibodies, but also in immunoglobulins with an antigenic target unrelated to viral proteins. A dual regulation of antibody responses by gamma-interferon (IFN-γ) and interleukin-6 explains this isotypic bias (1, 2). In the case of antiviral antibodies, a possible explanation for this phenomenon could be the selection by the infected host of the most appropriate response against the virus. Using a model of infection with lactate dehydrogenase-elevating virus (LDV), 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 (5). 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 (6), 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 (7).

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 (8). Immunization of CBA/Ht mice with rat platelets was followed by a transient thrombocytopenia. Platelets were opsonized by autoantibodies that recognized both rat and mouse normal platelets. Absorption experiments indicated that these autoantibodies reacted with epitope(s) shared by rat and mouse platelets. In contrast, BALB/C mice similarly immunized with rat platelets did not develop thrombocytopenia. The ability of BALB/C mice to produce anti-rat platelet antibodies and to eliminate antibody-coated platelets was comparable with that of CBA/Ht animals. However, the specificity of the antibody response elicited in these two mouse strains differed markedly, with a 145-155 kDa mouse platelet antigen, corresponding to 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 was followed by severe thrombocytopenia and by the appearance of petechiae similar to those observed in patients with immune thrombocytopenic purpura (9). A similar exacerbation of anti-platelet-mediated thrombocytopenia was induced by mouse hepatitis virus. This enhancement of anti-platelet antibody pathogenicity by LDV was not observed with F(ab')2 fragments, suggesting that phagocytosis was involved in platelet destruction. Similarly, administration of anti-erythrocyte monoclonal autoantibody to mice resulted in the development of a transient hemolytic anemia that was dramatically enhanced by a simultaneous infection with LDV, leading to the death of most animals. This viral infection induced an increase in the ability of macrophages to phagocytose *in vitro* autoantibody-coated red cells, and an enhancement of erythrophagocytosis in the liver (10).

Treatment of thrombopenic or anemic mice with clodronate-containing liposomes and with total IgG indicated that opsonized platelets and erythrocytes were cleared by macrophages. Administration of clodronate-containing liposomes decreased also the *in vitro* phagocytosis of autoantibody-coated red cells by macrophages from LDV-infected animals. The increase of thrombocytopenia triggered by LDV after administration of anti-platelet antibodies was largely suppressed in animals deficient for IFN-γ receptor. Together, these results suggest that viruses may exacerbate autoantibody-media-

ted 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.

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SIGNAL TRANSDUCTION BY GROWTH FACTOR RECEPTORS

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Growth factors are soluble proteins that stimulate cell proliferation and migration through receptor tyrosine kinases. Our group is mainly interested in Platelet-derived growth factors (PDGF), which play an important role during wound healing and embryonic development. Four PDGF isoforms have been described (A, B, C and D), which bind with different affinities to two specific receptor tyrosine kinases, the PDGFα- and β-receptors. The 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.

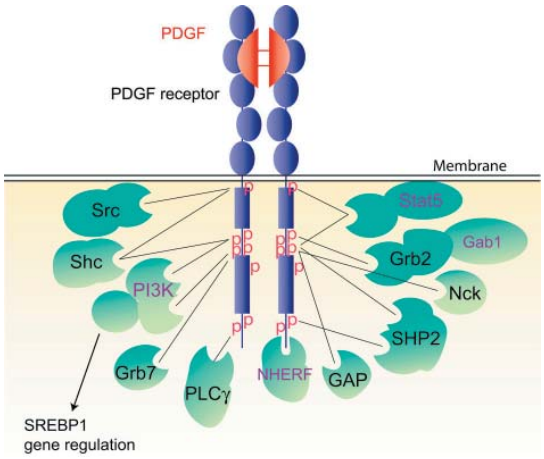

Figure 1. Signal transduction by the PDGF receptors. Signaling molecules of special interest for our group are indicated in purple. The receptor (blue) contains five extracellular immunoglobulin-like domains and an intracellular tyrosine kinase domain (boxes). p, phosphorylated tyrosine residue.

PDGF receptor activation

A. Kallin, F. Chiara, and J.B. Demoulin, in collaboration with C.H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden; Y. Malka and Y. Henis, University of Tel-Aviv, Israel; and S. Constantinescu, ICP.

PDGF receptors have an unusually 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).

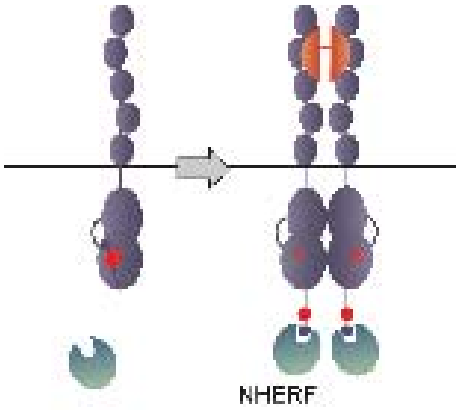

Figure 2. Role of the PDGF receptor C-terminal domain. In the inactive conformation, a Pro/Glu-rich acidic motif (red disk) blocks the kinase domain. Our data suggest that the C-terminal tail is released upon receptor activation and the NHERF binding site (blue disk) becomes accessible.

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.

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 hydroxy-methylglutaryl-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, 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, de-

ficient 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.

Selected publications

1. Demoulin JB, Seo J, Ekman S, Grapengiesser E, Hellman U, Ronnstrand L, Heldin CH. *Ligand-induced recruitment of Na+/H+-exchanger regulatory factor to the PDGF (platelet-derived growth factor) receptor regulates actin cytoskeleton reorganization by PDGF.* **Biochem J** 2003;376:505-10.

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HUMAN TUMOR IMMUNOLOGY*

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Tomoko SO, Post-doctoral fellow
Rim BENMAAMAR, Graduate student
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Véronique CORBIERE, Graduate student
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*In association with the Ludwig Institute: see **Analysis of T cell responses of vaccinated cancer patients** (research at LICR, Brussels)

Human tumor cells bear antigens that are not present on normal cells and that can be specifically recognized by autologous CD4 or CD8 T lymphocytes. We contributed to the identification of several tumor-specific antigens, present on melanoma or lung carcinoma cells (1-3). 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 if a better understanding of the immune responses of the patients is acquired. Our group focuses on such analyses.

Correlation between tumor regression and T cell responses in melanoma patients vaccinated with a MAGE antigen.

V. Corbière, T. Connerotte, Y. Dodoo in collaboration with P. van der Bruggen, D. Colau, D. Godelaine, A. Van Pel, and N. van Baren, Brussels branch of the Ludwig Institute for Cancer Research, K. Thielemans, Laboratory of Physiology, Vrije Universiteit Brussel, and G. Schuler, Department of Dermatology, University Hospital of Erlangen, Germany.

Shared tumor-specific antigens encoded by cancer-germline genes such as those of the MAGE family have been used for therapeutic vaccination of cancer patients. A number of small clinical trials on metastatic melanoma patients have been performed with the MAGE-3 antigenic peptide EVDPIGHLY, which is presented by HLA-A1. Evidence of tumor regression was observed in about 20% of the patients, but clinical benefit was limited to about 10% of the patients. As these vaccination trials have not included randomization with an untreated arm, one cannot rigorously exclude the possibility that the regressions observed are not due to the vaccine. Occasional spontaneous regressions of melanoma have been observed, but the frequency reported is at least 20 times lower than the frequency observed in the vaccination trials.

Our initial work suggested that in most vaccinated patients, even in those who displayed tumor regression, it was difficult to ascertain the existence of an anti-vaccine T cell response. We nevertheless felt that it was crucial to

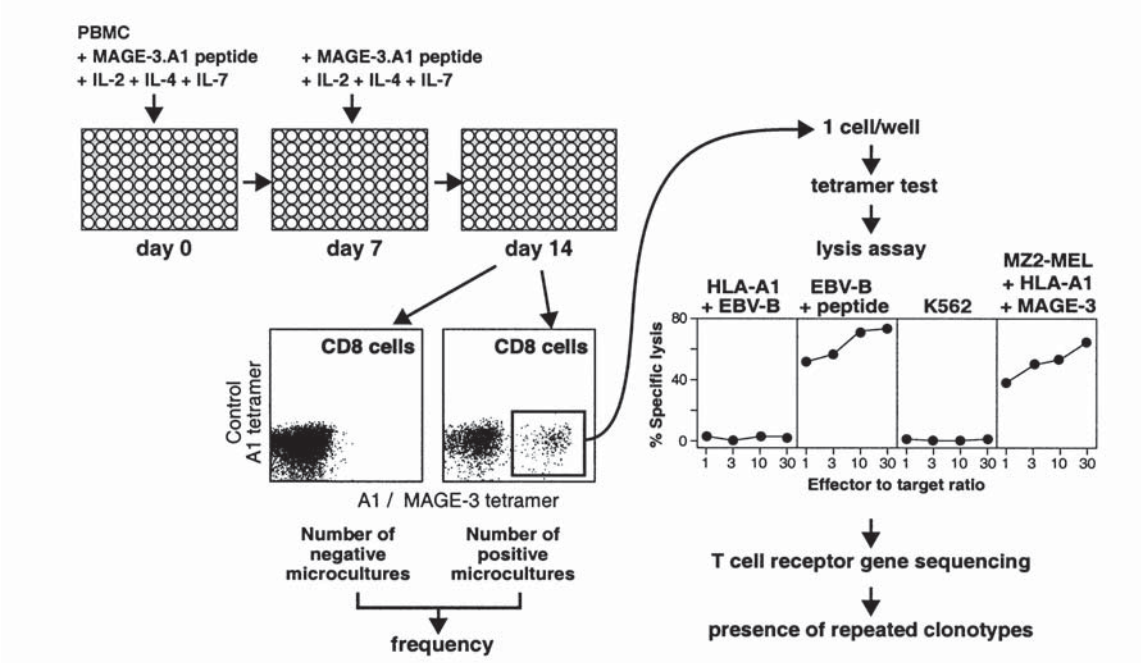

Figure 1. Overview of the procedure to evaluate CTL responses of vaccinated patients. Typically, 250,000 peripheral blood mononuclear cells (PBMC) were distributed in each microwell.

know whether or not low-level responses had occurred and whether such cytolytic T lymphocytes (CTL) responses showed a correlation with tumor regression, in order to understand why most patients failed to show any evidence of regression. We therefore developed a sensitive approach based on *in vitro* restimulation of blood lymphocytes with the antigenic peptide over two weeks, followed by labeling with tetramers. To evaluate precursor frequencies, these mixed lymphocyte-peptide cultures were conducted under limiting dilution conditions. Cells that were labeled with the tetramer were cloned, the lytic specificity of the clones was verified, and their diversity was analyzed by T cell receptor (TCR) sequencing (2, 4-6).

As the interpretation of these analyses was based both on the frequency and on the diversity of the anti-MAGE-3.A1 CTL clones, it was necessary to evaluate these parameters in a noncancerous individual. Such a study was undertaken by the group of P. van der Bruggen and the

Table 1. Summary of anti-MAGE3.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

results indicate that the frequency of the naïve anti-MAGE-3.A1 CTL precursors is about $4x10^{-7}$ of the blood CD8 T lymphocytes (7).

To evaluate the diversity of this naïve anti-MAGE-3.A1 repertoire, we examined the TCR sequences of the 23 anti-MAGE-3.A1 CTL clones obtained in this individual. Among the total of 23 clones, only two had the same TCRα and β sequences, indicating that the size of the anti-MAGE-3.A1 repertoire is very likely to be above 50, with a maximum likehood at 250. On the basis of this evaluation of the diversity of the anti-MAGE-3.A1 naïve TCR repertoire, it is possible to ascertain low level CTL responses, provided TCR sequence analysis indicates that the same clonotypes are obtained in several independent microcultures.

Table 1 presents a summary of the anti-MAGE-3.A1 CTL responses that we observed in patients who showed evidence of tumor regression and in patients who did not. Among patients vaccinated with ALVAC-MAGE, a recombinant poxvirus containing a minigene encoding the MAGE-3.A1 peptide, a CTL response was found in 3/4 patients who showed regression and 1/11 patients who did not. Among patients vaccinated with dendritic cells loaded with peptide MAGE-3.A1 (G. Schuler, Erlangen), a CTL response was found in 3/3 patients who showed regression and 0/3 patients who did not. These are statistically significant correlations. Only one out of seven patients vaccinated with the MAGE-3.A1 peptide, showed a CTL response. The correlation between CTL responses and tumor regression observed in the ALVAC and dendritic cell trials supports the notion that the rejection is caused by the vaccine.

Tumor regressions observed after vaccination: a possible role for tumor-specific cytolytic T lymphocytes that do not recognize the vaccine antigens

C. Germeau, in collaboration with W. Ma, C. Lurquin, N. Vigneron, F. Brasseur, B. Lethé, E De Plaen, Brussels branch of the Ludwig Institute for Cancer Research, and T. Velu, Medical Oncology, Erasme Hospital, Brussels.

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.

We selected five metastatic melanoma patients who had been vaccinated with MAGE antigens. They were selected because it had been possible to derive a permanent cell line from their tumor cells. We set up mixed lymphocyte-tumor cell cultures (MLTC), to estimate the blood frequencies of their CTL directed specifically against the tumor. We will refer to the lytic effectors detected in these MLTC as 'anti-tumor' CTL to distinguish them from the 'anti-vaccine' CTL, which recognize the vaccine antigen. For all five patients, anti-tumor CTL were found at high frequencies, i.e. from 10^{-4} to 3×10^{-3} of the CD8 T cells, in the blood after vaccination. Unexpectedly, they were already present at similar high frequencies before vaccination. The frequency of anti-tumor CTL observed after vaccination was considerably higher than that of the anti-vaccine CTL, ranging from 12 fold to 20,000 fold higher (8).

Because T cells directed at other tumor antigens than the vaccine antigen could make an important contribution to the tumor regressions that are observed occasionally following vaccination, we felt that it was necessary to define the precise nature of their target antigens, particularly with regard to their tumor specificity. We focused our effort on patient EB81, who had shown complete regression of a large number of cutaneous metastases following vaccination with ALVAC-MAGE. The antigens recognized by the anti-tumor CTL of patient

EB81 turned out to belong to the main classes of tumor antigens that have been defined previously. A majority of anti-tumor CTL clones recognized antigens encoded by MAGE-C2, a cancer-germline gene (9). Others recognized an antigen encoded by gp100, a melanocytic differentiation gene. Another anti-tumor CTL clone of patient EB81 recognized an antigen encoded by an ubiquitously expressed gene which had undergone a point mutation in the tumor. 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 occasional tumor rejections that occur 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 an invaded lymph node. An anti-tumor CTL recognizing an antigen encoded by MAGE-C2 showed a considerably higher enrichment: whereas in the blood the frequency of this CTL was 9 x 10^{-5}, it was about 1,000 times higher in the invaded lymph node. Several other anti-tumor T cell clonotypes also had frequencies above 1% and appeared to constitute the majority of the T cells present in this site. Similar findings were made on a regressing cutaneous metastasis (10).

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. New naive T cells may be stimulated in the course of this process, as we observed that new anti-tumor CTL clonotypes emerged following vaccination and were present in the metastases at a very high frequency. An implication of this wide T cell response triggered by the vaccine is that loss of the vaccine antigen by a number of tumor cells would not ensure tumor escape.

Functions of tumor-specific T cells

T. Connerotte, W. Fink.

Our results suggest that, in some vaccinated patients, low numbers of anti-vaccine CTL may reverse a local immunosuppression within the tumor, enabling other anti-tumor CTL to be activated. As a first step in the analysis of the functional properties of the 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. This result indicates that these 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.

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Boon T, Coulie PG. *A human endogenous retroviral sequence encoding an antigen recognized on melanoma by cytolytic T lymphocytes*. **Cancer Res.** 2002;62:5510-6

4. Coulie PG, Karanikas V, Lurquin C, Colau D, Connerotte T, Hanagiri T, Van Pel A, Lucas S, Godelaine D, Lonchay C, Marchand M, Van Baren N, Boon T. *Cytolytic T-cell responses of cancer patients vaccinated with a MAGE antigen.* **Immunol Rev** 2002;188:33-42

5. Coulie PG, Bruggen P. *T-cell responses of vaccinated cancer patients*. **Curr Opin Immunol** 2003;15:131-76.

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8. Germeau C, Ma W, Schiavetti F, Lurquin C, Henry E, Vigneron N, Brasseur F, Lethé B, De Plaen E, Velu T, Coulie PG. *High frequency of anti-tumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens.* **J Exp Med** 2005;201:241-248.

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DNA DAMAGE REPAIR AND TELOMERE MAINTENANCE

Anabelle DECOTTIGNIES, Associate Member

Armelle DUQUENNE, Student (till september 2004)
Gaëlle TILMAN, 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 small mtDNA sequences in chromosomes, suggesting that mtDNA integration occurred during evolution and is probably an ongoing process.

In order to investigate DNA repair mechanisms in fission yeast, I set up a new simple extrachromosomal DSB repair assay allowing to monitor HR, NHEJ and MMEJ-dependent end-joining [1]. The assay revealed that DSB repair was associated with the capture of endogenously produced mtDNA fragments in nearly 30 % of repair events [1], supporting the hypothesis that DSB repair is a universal muta-

genic mechanism responsible for the insertion of linear mtDNA molecules into chromosome breaks, providing a novel mechanism of human inherited disease. 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 [1], a condition resembling most of the cells of multicellular organisms since: 1) most energy comes from mitochondrial respiration and 2) cells have exited from the cell cycle, i.e., have entered the G0 phase. I am currently investigating the mechanisms involved in production and/or transfer of mtDNA fragments to the nucleus. I also screened the S. pombe nuclear genome available in the databases for the presence of mtDNA and found 38 sequences (22-358 bp-long) [1], a value close to that reported in budding yeast. For comparison, the human nuclear genome comprises more than 600 mtDNA sequences. Hence, it appears that capture of mtDNA fragments at naturally occurring DSBs took place during evolution in eukaryotic cells, remodeling the nuclear genome. Next, using the same assay, I found that some genomic DNA sequences from either human or salmon are preferentially captured at fission yeast DSBs. These included microsatellite repeat sequences at high frequency [1]. Capture of microsatellite DNA at mammalian DSBs had been previously reported experimentally and was also detected at the breakpoints of lymhoid tumour-specific translocations. Insertion of microsatellite DNA at DSBs provides a source of genomic instability as DNA repeats are prone to expansions/contractions, a feature at the basis of some diseases including Huntington disease or Friedreich's ataxia. Hence, yeast provides a useful tool to understand the molecular basis of DSB preference for microsatellite DNA.

Finally, the fission yeast DSB repair assay is actually used to characterize the MMEJ DNA repair pathway for which genetic requirements are largely unknown.

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). 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 hTERT telomerase-encoding gene 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 resistant ALT cells in the culture. In that respect, a recent study reported the emergence of ALT cells after inhibition of telomerase with a dominant negative hTERT. We have chosen to inhibit hTERT expression by stable RNA interference in TEL^+ cancer cell lines. The system has been set up and gives a reduction of hTERT mRNA of about 70% in 293 HEK cells. We screened a series of cancer cell lines available in the institute to

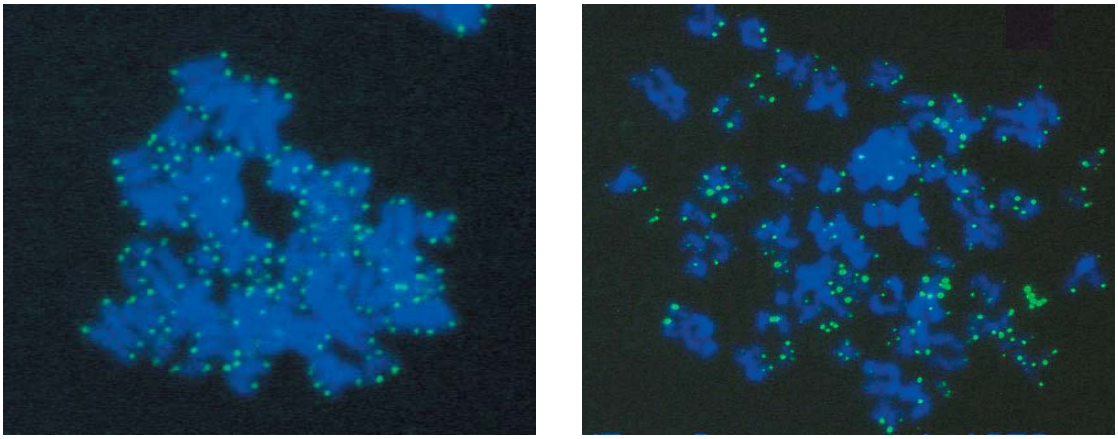


Figure 1. Telomere-specific fluorescence in situ hybridization (FISH) on metaphase chromosomes of telomerase-positive and ALT cancer cells. Telomeres are hybridized with a fluorescent telomeric probe (green) and DNA is stained with DAPI (blue). In ALT cells, telomeres are very heterogeneous and not always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

find the best candidate(s) for telomerase inhibition i.e. a cell line with low level of hTERT mRNA and not refractory to transfection. Clones expressing sihTERT have been obtained and are currently under investigation. We follow telomere length evolution by Southern blot and Q-FISH. If we obtain ALT survivors after inhibition of hTERT, we plan to compare the global expression profile of ALT clones with the original cell line by screening on cDNA microarrays. We also plan to inhibit hTERT by overexpression of a dominant negative allele of the gene. In a second approach, we wish to obtain ALT clones after *in vitro* immortalization of telomerase-negative human fibroblasts with SV40 DNA. Such a procedure has indeed been reported to lead to the formation of an average of 40 % ALT and 60 % TEL^+ survivors after the crisis (Fig. 2). Crisis occurs after about 80 population doublings when telomeres have reached a critical length (Fig. 2). Screening of the respective expression profiles of both types of surviving clones would then be performed on cDNA microarrays. Human BJ fibroblasts have already been transfected with SV40 DNA and telomere length evolution is followed regularly.

Publication

1. Decottignies, A. *Capture of extranuclear DNA at fission yeast double-strand breaks.* **Submitted.**

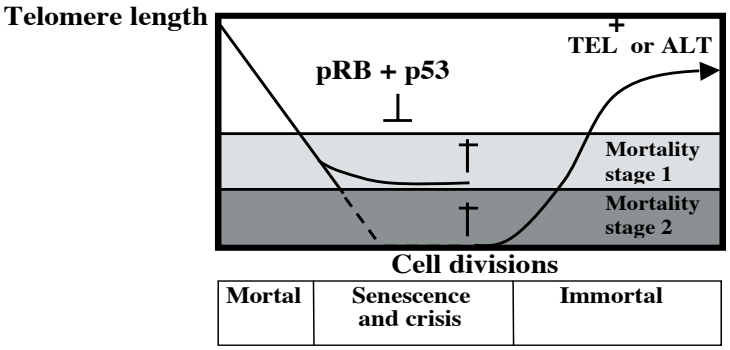
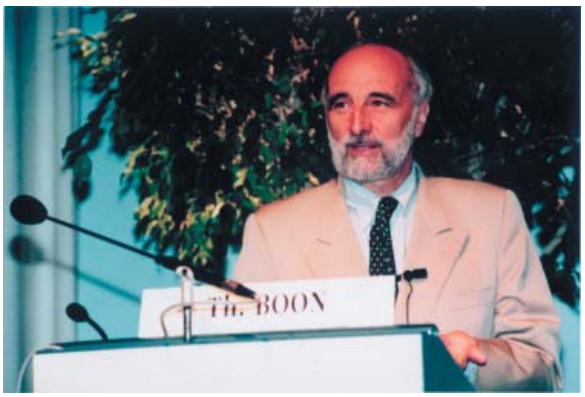


Figure 2. Telomere length in the course of cellular transformation. In telomerase-negative cells, telomere length decreases with continuous replication. At a critical telomere length, cells enter M1 through the action of p53/pRb and eventually die. Upon inactivation of p53/pRb, cells continue to divide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

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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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, Fanny Piette

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, as a result of the 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. In other cases, the difference lies in the efficiency of cleavage at the C-terminal end of the antigenic peptide.

Our observations indicate that the pool of antigenic peptides presented at the cell surface may differ substantially according to the proteasome type that is predominant in the cell. This may have major implications for immunotherapy, particularly for cancer immunotherapy, as it means that the peptide repertoire presented by tumor cells may differ from the repertoire presented by antigen-presenting cells (3). The peptide repertoire of tumor cells themselves may vary according to the localization of the tumor (e.g. primary tumor versus lymph node metastasis) and its level of exposure to IFNγ. Therefore, it appears essential for the success of cancer immunotherapy to study those processing differences in detail, so as to define the most effective vaccination strategy for each epitope and to use the appropriate combina-

tion of antigens in order to minimize the risk of tumor escape by proteasome switching.

Antigenic peptides produced by peptide splicing in the proteasome

Nathalie Vigneron, Vincent Stroobant, Jacques Chapiro (in collaboration with Edus Warren, Fred Hutchinson Cancer Research Center, Seattle, USA)

By stimulating blood lymphocytes of a melanoma patient with autologous tumor cells, we isolated a clone of CD8 cytolytic T lymphocytes that recognizes an antigenic peptide presented by HLA-A32 and derived from glycoprotein gp100. This peptide, whose sequence is RTKQLYPEW, is present on several melanoma lines and was found to be composed of two non-contiguous segments of the gp100 protein. Its production requires the excision of four amino acids and splicing of the residual fragments (4). The antigenic peptide could be produced ***in vivo*** by electroporating the 13-amino acid precursor RTKAWNRQLYPEW into EBV-B cells. Proteasome inhibitors lactacystin and epoxomicin prevented the recognition of target cells electroporated with the 13-amino acid peptide, indicating that proteasome activity was required for the splicing of this precursor. Moreover, the digests produced after incubation of the 13-amino acid precursor with highly purified proteasomes strongly stimulated IFNγ production by the CTL. Analysis of these digests by tandem mass spectrometry clearly demonstrated the presence of the spliced peptide in the reaction mixture. Thus, the proteasome appears to produce the antigenic peptide RTKQLYPEW by excision and splicing. By incubating proteasomes with sets of two distinct fragments each containing a different portion of the precursor peptide, we could show that the energy required for the creation of the new peptide bond was recycled from one of the bonds that are cleaved during the excision process. These data suggest that the splicing occurs via a transpeptidation mechanism involving an acyl-enzyme intermediate. Our results reveal an unanticipated aspect of the proteasome function, which increases the diversity of antigenic peptides presented to T cells.

We are currently studying in more details this novel activity of the proteasome. We are also extending our observation to additional peptides that are also produced by splicing. In collaboration with Edus Waren (Seattle, USA) we recently found another peptide produced by splicing. This peptide, which is a human minor histocompatibility antigen corresponding to a polymorphism in gene SP110, is made of two peptide fragments that are non-contiguous in the parental protein. The two fragments are rearranged before splicing, so that they end up inverted in the spliced antigenic peptide. The proteasome appears responsible for this rearrangement/splicing, presumably by the same transpeptidation mechanism as above.

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-germline expression pattern, which is expressed in about 40% of melanomas and 30% of bladder carcinomas. Two peptides are presented by HLA-A2, and one by HLA-B57 (5). 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.

Melanoma line LG2-MEL also expresses several antigens recognized by autologous CTLs. We previously reported the molecular definition of three distinct antigens recognized by some of these CTL clones. One of them consists of a peptide derived from tyrosinase and presented by HLA-B35. Another is a peptide corresponding to a mutation in a gene expressed ubiquitously, named OS9. A third is a peptide produced by splicing of two non-contiguous fragments of melanocytic protein gp100 (4) (see above). We have now identified a fourth antigenic peptide expressed by this melanoma line and recognized by a CTL clone restricted by HLA-B35. The antigenic peptide, which is 9-amino acid long, has the sequence LPHSSSHWL and is also derived from melanocyte differentiation protein gp100 (6).

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. These results suggest that the efficacy of therapeutic vaccination of cancer patients could be improved by concomitant administration of an IDO inhibitor.

Development of an inducible mouse melanoma model for immunotherapy

Ivo Huijbers (in collaboration with Paul Krimperfort (NKI, Amsterdam) and Anne-Marie Schmitt-Verhulst (CIML, Marseille)

Immunotherapy represents an attractive approach for the treatment of cancer, in particular melanoma. Preclinical studies of various strategies of immunotherapy rely on model systems where tumor cells grown *in vitro* are inoculated into syngeneic mice. However, this does not recapitulate the long-term host-tumor relationship that occurs in patients during tumor development. In order to have a model system more relevant to the human situation, we are developing a mouse strain in which we can induce melanomas expressing a tumor antigen of interest.

One of the most common site of genetic lesions in human melanoma is the INK4A/ARF gene, which encodes two distinct tumor suppressor proteins $p16^{INK4A}$ and $p14^{ARF}$. Genetic disruption of this gene predisposes mice to the formation of various tumor types, but is not sufficient to induce melanoma unless the Ras pathway is specifically activated in melanocytes. In order to have a fully controlled model system for melanoma, we have genera-

ted transgenic mice in which the deletion of the Ink4a/Arf gene and the melanocyte-specific expression of both activated Harvey-Ras^{G12V} and a well characterized antigen is spatially and temporally regulated by a fusion protein between the Cre-recombinase and the tamoxifen responsive hormone-binding domain of the estrogen receptor (CreER$^{\Delta D}$). The antigen is encoded by P1A, a gene expressed in several tumors but silent in normal tissues except testis and placenta. The tumor induction in these mice will be performed by topical administration of tamoxifen, which should be sufficient to induce the essential genetic rearrangements in melanocytes necessary to establish neoplastic transformation.Eighteen transgenic lines were generated by three different strategies based on the use of a construct containing the tyrosinase promoter, a CreER$^{\Delta D}$ fusion gene flanked by loxP sites, a V12 mutated H-Ras gene, an internal ribosomal entry site and the P815A-antigen encoding gene, P1A. After crossing with a mouse strain containing a conditional INK4A/ARF gene flanked by loxP sites, one transgenic line was found to develop cutaneous melanomas after subcutaneous injection of tamoxifen, in 8 out of 33 treated animals. The induced tumors are deleted for INK4A/ARF, express the activated Ras and express P1A.

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.

Antigen presentation by dendritic cells in Systemic Lupus Erythematosus

Bernard Lauwerys, Anne-Lise Maudoux (in collaboration with Frédéric Houssiau, Unité de Rhumatologie)

Systemic lupus erythematosus (SLE) is an autoimmune disorder that is characterized by overt polyclonal B cell activation and T cell-driven autoantibody production against nuclear antigens. We are investigating the involvement of dendritic cells (DC) in the impaired peripheral tolerance leading to the activation of autoreactive CD4 T cells in SLE. Studying strains of mice that are congenic for different SLE susceptibility loci and were developed by E.K. Wakeland (University of Texas, Southwestern Medical Center at Dallas), we observed that DC from one of these strains (Sle3) significantly impact CD4 T cell activation and apoptosis as compared to control DC. Further work will focus on the identification of the genetic mechanisms underlying these observations, taking advantage of the availability of newly produced subcongenic strains, carrying smaller intervals of the Sle3 region.

In parallel, the availability of large amounts of PBMC collected from untreated patients with active disease gives us the opportunity to carry on experimental procedures aiming at the identification of new physiopathological targets in SLE, using high-density cDNA microarrays (Affymetrix). In order to increase the sensibility of that procedure, PBMC from SLE patients are sorted by flow cytometry in lymphocyte subsets, prior to RNA extraction and hybridization of the slides. Interpretation of the results will be facilitated by the knowledge of numerous SLE susceptibility regions in the human genome that contain genes of particular interest.

Finally, we recently identified a new gene encoding a potential decoy receptor for MIF (Macrophage migration Inhibitory Factor), a cytokine that plays important roles in inflammatory and tumoral responses. We currently investigate the functions of that novel molecule, targeting its potential inhibitory activity of MIF actions in inflammation and tumor growth.

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TUMOR GENETICS GROUP

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Human tumors express specific antigens arising from the activation of genes, such as MAGE, BAGE, GAGE and LAGE/NY-ESO1, that 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 is trying to identify new genes that are specifically expressed in tumors and germ cells. Screening procedures based on differential expression profiling allowed the isolation of several genes with cancer and germline specific expression (1). 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. Additionally, efforts are also devoted to determining the function of «cancer-germline» genes.

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 (2). 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. The group is now trying to identify the genes that are regulated by MAGE-A1 by using an inducible transfected MAGE-A1 gene and the microarray technology.

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. The group is now focusing on the mechanisms of demethylation of these genes in tumors. 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. Transient down-regulation of DNMT1 induced stable activation of cancer-germline genes, supporting the view that hypomethylation of these genes in tumors results from a historical event of demethylation.

Finally, the group investigates the gene expression profile of tumor samples and tumor cell lines obtained from melanoma patients who received experimental cancer vaccines. Using microarray and quantitative RT-PCR, the group is trying to identify genes involved in the resistance of tumors to their rejection by T lymphocytes.

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IDENTIFICATION OF HUMAN TUMOR ANTIGENS

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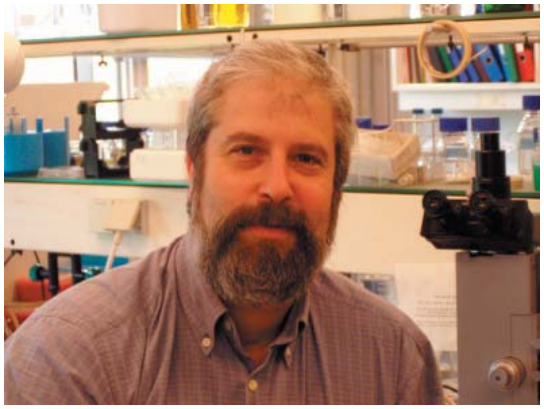

The group led by Pierre van der Bruggen is defining antigenic peptides encoded by genes such as those of the MAGE family. Therapeutic vaccination of cancer patients with MAGE peptides is now in progress, and the identification of additional antigenic peptides is important to increase the range of patients eligible for therapy with peptides and to provide tools for a reliable monitoring of the immune response. Efforts are currently devoted to set up assays that accurately monitor CD4$^+$ T cell responses to cancer vaccines. For patients vaccinated with a peptide, we recently validated the use of the first HLA-DP4 tetramer, which was folded with a MAGE-3 peptide. 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. The group is also 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

Yi Zhang, Vincent Stroobant, Zhaojun Sun, Sabrina Ottaviani

"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 (1-2). 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 eli-

gible 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» (3). 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

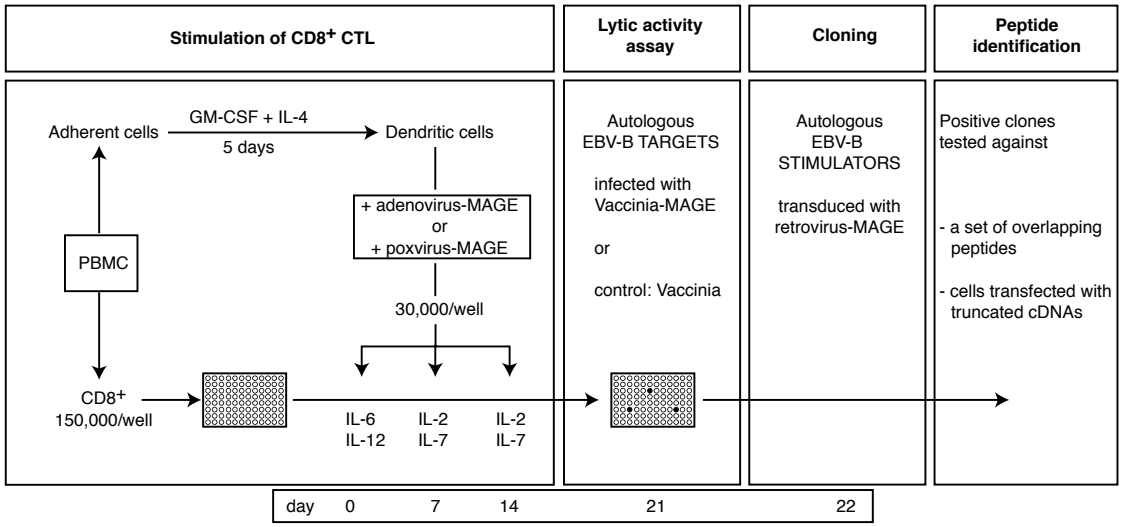

Figure 1. Overview of the procedure to obtain anti-MAGE $CD8^+$ CTL clones by stimulation with dendritic cells infected with viral vectors carrying a MAGE coding sequence

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 Figure 1. Dendritic cells were infected with either an adenovirus, a canarypoxvirus or a lentivirus, and they were used to stimulate microcultures of autologous $CD8^+$ T lymphocytes (4). After three weekly stimulations, the responder cells were tested for lysis on autologous EBV-B cells infected with vaccinia-MAGE. Positive microcultures were cloned. To identify the antigenic peptide, the resulting CTL clone was tested for lysis of autologous EBV-B cells pulsed with a complete set of peptides of 16 amino acids that overlap by 12. When a peptide scored positive, shorter peptides were synthesized to identify the shortest optimal peptide. To identify the HLA presenting molecule, the CTL clones were tested for stimulation by cells transfected with the MAGE cDNA together with cDNAs coding for the possible HLA presenting molecules. Finally, relevant tumor targets were used in a lysis assay to ascertain that the antigen was also processed by tumor cells.

In a second approach, a large number of

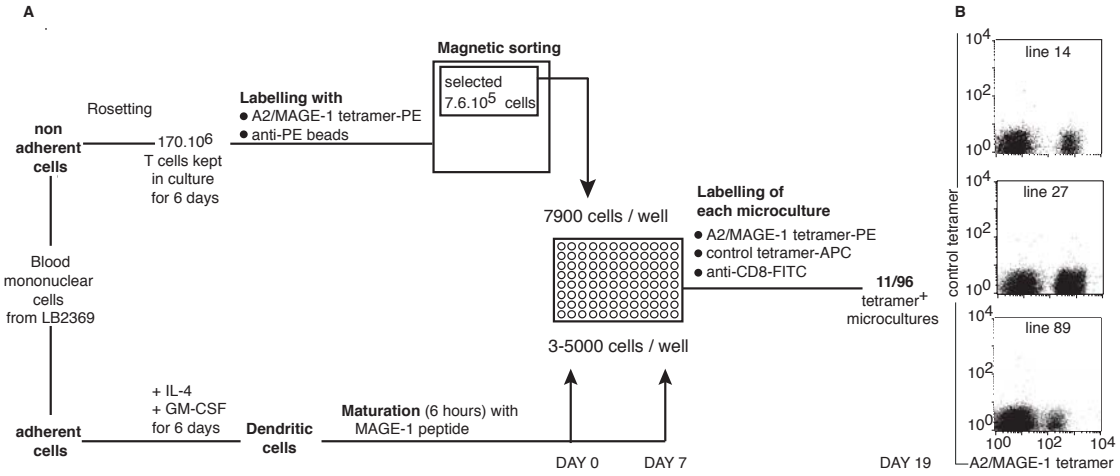

Figure 2. Overview of the procedure using HLA-peptide fluorescent multimers to isolate rare anti-MAGE-1 CD8⁺ CTL clones.

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) (5).

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 (6). 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 libray 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 (7).

Detection of CD4 T cell response in vaccinated cancer patients

Yi Zhang, Nicolina Renkvist, Zhaojun Sun, Hugues Nicolay, Sabrina Ottaviani, in collaboration with Didier Colau

Patients injected with class II-restricted peptides

For therapeutic vaccination trials, the vaccine can consist of defined antigenic peptides. This approach greatly facilitates the monitoring of the T cell response, because the presumed target of the T cells is completely defined. It allows the use of HLA-peptide tetramers to detect T cell responses in patients. Although the production of soluble MHC class I multimers has rapidly expanded since their first use in 1996, the development of MHC class II complexes has proved to be more difficult probably due to the intrinsic structural instability of soluble class II molecules. We have developed a very sensitive approach using fluorescent HLA class II/peptide multimers to detect antigen specific CD4 T cells in vaccinated cancer patients. We have produced HLA-DP4 multimers loaded with the MAGE-$3_{243\text{-}258}$ peptide and used them 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-$3_{243\text{-}258}$ peptide (8). The multimer positive CD4 T cells were sorted and amplified in clonal conditions. Specificity of the clones was assessed by their ability to secrete IFN-γ 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. A detailed analysis of one patient showed an anti-MAGE 3.DP4 CD4 T cell amplification of at least 3,000-fold upon immunization. T cell receptor analysis of the clones from this patient demonstrated a polyclonal response against the MAGE-3 peptide. One advantage of the multimer staining approach is that antigen-specific CD4 T cells are detected independently of their effector functions. We have started to analyze several patients injected with different vaccines containing the MAGE-3.DP4 peptide: peptide with or without adjuvant, dendritic cells pulsed with peptides. We intend to correlate the type of vaccine and the functional phenotype of the anti-vaccine T cells isolated from the vaccinated cancer patients.

Patients injected with a protein

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

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

ning of these cells, and evaluation of the number of T cell clones that secrete IFN-γ upon stimulation with the antigen (9). 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 regression. 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 (5). 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, such as IL-10. 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

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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 (10).

These first observations are now confirmed with thirteen CD8 T cell clones and twenty 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 therefore compared for their TCR distribution in the lipid raft domains of the membrane and for the TCR spreading by confocal 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.

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THERAPEUTIC VACCINATION OF CANCER PATIENTS WITH TUMOR-SPECIFIC ANTIGENS

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Tumor cells carry antigens such as MAGE antigens that are absent from normal tissues, and that can be targeted by cytolytic T lymphocytes (CTL) (1). While 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, T lymphocytes and tumor samples collected at different time points during vaccination can be analyzed in detail, which improves our understanding on what happens in patients who experience regression of metastatic lesions, and which may explain why this does not happen in the majority of patients with overall disease progression. This knowledge can then be used to design new vaccination modalities.*

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, 1 partial response and 3 mixed responses (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 an immunostimulatory CpG-containing oligonucleotide to try to increase its immunogenicity (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 an other ongoing study (LUD 03-007). Results of both studies will be compared in terms of therapeutic efficacy and immune response.

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 will now mix this recombinant protein with adjuvant AS15 containing an immunostimulatory CpG nucleotide, and combine 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).

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. We plan to investigate in a new trial whether increasing the dose of ALVAC would result in improved immunological and clinical 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 to 10% 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).

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ANALYSIS OF T CELL RESPONSES OF VACCINATED CANCER PATIENTS *

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*In association with the group of Pierre Coulie, ICP: see **Human tumor immunology** (research at ICP).

The identification of antigens recognized on human tumors by autologous T lymphocytes has opened the way for therapeutic vaccination strategies involving defined tumor antigens such as the MAGE antigens (1, 2). Tumor regressions have been observed in a minority of treated melanoma patients. Such clinical responder patients have been found following immunization with peptides and recombinant ALVAC viruses. In some patients, a monoclonal T cell response was observed and the level of the response appeared to be stable during the course of the vaccination protocol (3, 4). Since detectable T cell responses occured more frequently in patients who show signs of tumor regression than in those who did not, we consider the possibility that the limiting factor for the anti-tumor effect of the vaccine is the intensity of the CTL response to this vaccine (5, 6). Improving the efficacy of such vaccines would thus critically depend on their capacity to trigger a robust immune response. A novel approach to vaccination is to exploit the potentiality of dendritic cells that are widely accepted to be particularly effective in presenting antigens to T cells and immunize cancer patients with a sample of their autologous dendritic cells charged with tumor antigens. We initiated a collaboration with G. Schuler and B. Schuler-Thurner at the University of Erlangen (Germany), who vaccinated advanced stage IV melanoma patients with mature, monocyte-derived dendritic cells pulsed with MAGE peptides and observed regression of some metastases(7). As shown in our previous report, an immune response was found in a few regressing patients(8). These results prompted us to initiate a new collaboration with K. Thielemans (VUB, Belgium), who vaccinated less advanced patients with the same protocol.

Methods for evaluation of T-cell responses in vaccinated cancer patients

To establish whether there is a correlation between tumoral regressions and T-cell responses against the vaccine antigen, we evaluated the responses of patients vaccinated with a MAGE-3 antigenic peptide, with a recombinant virus coding for this peptide or with MAGE peptide-pulsed dendritic cells.

The recent development of tetrame-

ric peptide- MHC class I and class II complexes (tetramers) allows the direct identification of antigen-specific T-cells. The technology has been introduced and is still being developed by Didier Colau.

To detect low-level responses, blood lymphocyte microcultures were stimulated with the antigenic peptide in limiting dilution conditions (Mixed Lymphocyte Peptide Culture, MLPC), followed by tetramer analysis, cloning of the tetramer-positive cells, and T-cell receptor (TCR) sequence analysis of the cytolytic T lymphocyte (CTL) clones that showed strict specificity for the vaccine antigen (for method, see P. Coulie, ICP report).

Christophe Lurquin and Bernard Lethé focused their efforts on detailed analysis of frequencies of characterized T-cell clones in blood, metastases and non tumoral tissue samples, using 'clonotypic' polymerase chain reaction (PCR) amplifications specific for the Vα and Vβ rearrangements of relevant TCR. These PCR amplifications on cDNA were sensitive enough to detect one CTL expressing a given TCR mixed with $3x10^7$ PBMC of normal donor and they were highly specific for the given TCR insofar as no product was amplified with cDNA prepared from $3x10^7$ PBMC of 5 unrelated donors.

Previous results showed that a monoclonal CTL response against a MAGE-3 antigen presented by HLA-A1 was observed by the *in vitro* tetramer analysis in a melanoma patient who showed partial rejection of a large metastasis after treatment with a vaccine containing only the tumor-specific antigenic peptide. Our results proved that the vaccination induced at least a 100-fold amplification of an anti-MAGE-3.A1 CTL and that vaccines containing only a tumor-specific antigenic peptide can elicit a CTL response (3)

A melanoma patient vaccinated with a MAGE-3 recombinant canarypox virus

Patient EB81 had about 70 cutaneous metastases when she was vaccinated with a recombinant canarypox virus of the ALVAC type containing a minigene encoding the MAGE-3.A1 peptide. Repeated injections of ALVAC were followed by vaccinations with the MAGE-3 peptide. Ten months after the first vaccine, all the metastases had become undetectable except an enlarged lymph node which was resected. A similar vaccination protocol alternating virus and peptide injections was carried on until now.

Blood lymphocytes collected before vaccination were analyzed with the MLPC-tetramer method. No anti-MAGE-3 CTL could be detected among 10^7 $CD8^+$ cells, suggesting a frequency similar to that found in normal donors (4). Two different anti-MAGE-3.A1 CTL clones were identified in postimmune blood . One, CTL 35, was found in more than 95% of the analyzed independent microcultures, suggesting that this MAGE-3.A1 response was essentially monoclonal.

PCR amplifications specific for the TCR 35 Vα and Vβ rearrangements were applied directly to cDNA obtained from groups of PBMC. In the first post-ALVAC sample, the frequency of CTL C35 rose to 3.6×10^{-6} of the $CD8^+$, suggesting that the ALVAC vaccination induced at least a 30 times amplification of anti-MAGE-3.A1 T cells. This frequency, which remained stable during the peptide vaccinations, rose to $1.1x10^{-5}$ after a boost of ALVAC vaccinations given one year later and remained stable thereafter.

By stimulating blood lymphocytes from melanoma patient EB81 with autologous tumor cells (MLTC), a series of CTL clones that specifically lysed autologous melanoma cells were isolated (9). Some of these CTL clones recognized peptides presented by HLA-A2 and encoded by gene MAGE-C2, another cancer-germline gene which is expressed at high level by the melanoma cells of the patient. We analyzed the TCR Vβ gene expression of this set of anti-MAGE-C2 CTL clones. One of them, named CTL 16, seemed to be amplified after

vaccination since it was retrieved many times as independent clones from the postimmune blood but was not found in preimmune blood samples. Clonotypic RT-PCR amplifications of TCR 16 Vα and Vβ rearrangements indicated an average frequency of the corresponding CTL in blood throughout the whole time of vaccination of ~3.5 x 10^{-5} among the $CD8^+$.

To investigate the involvement of the MAGE-C2-specific CTL in the tumor regression process observed in this patient after vaccination against a MAGE-3 antigen, we have analyzed the frequencies of the anti-MAGE-3.A1 CTL 35 and the anti-MAGE-C2.A2 CTL 16 in the resected metastatic lymph node, which presented histological signs of regression. Sections of 7 μm thick, ~80 mm^2 fragments of such sections, and groups of cells excised from sections with laser microdissection were tested by RT-PCR for the presence of TCR 35 and TCR 16. We obtained a frequency of ~1/32,500 CD8 for anti-MAGE-3 CTL, which represents a 20-fold enrichment relative to blood frequency at the time of the metastasis resection. Moreover, the anti-MAGE-C2 CTL was at least 275-times enriched in the resected sample with a frequency higher than 1/100 CD8 in the lymph node tissue and a frequency >1/5 CD8 in tissue regions strongly invaded by tumoral cells.

During this year, we confirmed and extended these results by analyzing TCRß cDNA libraries performed on the RNA of various metastases of patient EB81. These assays allowed the estimation of the frequencies of various anti-tumor CTL within metastases. They also enabled us to considerably enlarge the diversity of activated T lymphocyte clones repeatedly found in either the same metastasis, or in other metastases resected before and after vaccination. T lymphocyte clones above a frequency of 1% of all the T cells were not rare. Some of these clonotypes were not found before vaccination neither in blood nor in tumor. Among the frequent clones, one appeared to be directed against a mutated antigen, but most of them were directed against various MAGE-C2 epitopes, accounting for up to 20% of the T cells present in the metastasis. In this patient who is still disease-free four years after the onset of vaccinations, some of the intra-tumor clonotypes were enriched up to 1000-fold relative to their frequencies in the blood (10).

A melanoma patient vaccinated with dendritic cells pulsed with a MAGE-3 peptide presented by HLA-A1

Melanoma patient MMB02 had about 20 cutaneous metastases on her right leg when she was vaccinated by K. Thielemans and his coworkers at the VUB with mature, monocyte-derived dendritic cells pulsed with MAGE-3.A1 peptide according to a protocol set up by Gerold Schuler at Erlangen (Germany). At the end of the first cycle of 6 vaccinations, some metastases started to regress while new ones appeared. Vaccinations were continued and the patient kept showing evidence of a mixed response. To improve our understanding of the process leading to occasional tumor regressions that occur following vaccination, we investigated the presence of anti-vaccine and anti-tumor CTL in the blood and inside metastases collected at various time points.

In the blood, the frequency of anti-MAGE-3.A1 T cells among CD8 cells raised from less than 3.4x10^{-7} before vaccination to 9.3x10^{-7} after 3 vaccinations, to 2.6x10^{-6} after 6 vaccinations and 5.4x10-6 after another cycle of 6 vaccinations. At this time point, only one anti-MAGE-3.A1 clonotype was detected, a monoclonality that contrasts with the polyclonality observed in Schuler's patients (8). On the other hand, the frequency of anti-tumor CTL, i.e. lytic effectors that recognize the autologous melanoma cells but not autologous B cells nor NK target K562, was 2.2x10^{-4} before and 2x10^{-4} after 6 vaccinations. Thus, in the same patient, the frequency of blood anti-tumor CTL was more than two orders of magnitude above the frequency of anti-vaccine CTL and was already present before vaccination.

Cutaneous metastases removed after the second cycle of vaccinations allowed the isolation of a few TIL clones able to specifically recognize the autologous tumor cells. None of these clones was directed against the vaccine antigen. One of them was shown to be directed against a new tumor-specific antigen encoded by gene MAGE-C2 and presented to CTL by HLA-B44: peptide SESIKKKVL. This anti-MAGE-C2.B44 CTL was also found in the blood taken after 6 vaccinations. RT-PCR specific for TCR sequences of that anti-MAGE-C2-B44 CTL clone is being set up and will allow estimation of the frequency of that clone in the blood samples taken before and after immunization, as well as in cutaneous metastases resected before and after vaccination. This should help to evaluate the potential contribution of that clone to the tumor regressions that are observed occasionally after vaccination.

If the frequency and the diversity of the anti-vaccinal and anti-tumoral CTL are important features to understand the mechanisms underlying tumor regressions, the functional activity of these CTL is another important aspect to be analyzed. In this prospect, we have undertaken the analysis of anti-MAGE-3.A1 CTL from vaccinated patients for expression of some phenotypic markers (CD25, CD69, CD28, CD27, CCR7, CD45...) that have been associated to the CTL activation status or particular levels of maturation, in order to search for a correlation with CTL expansion capacities, lysis and secretion profile of cytokines.

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CYTOKINES IN IMMUNITY AND INFLAMMATION

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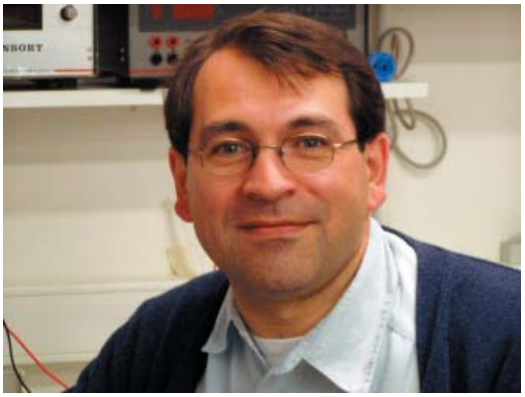

The cytokine group studies the biology of Interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is a TH2 cytokine that plays a role in immune responses against intestinal parasites and asthma. IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, upregulates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

Interleukin 9

Jean-Christophe Renauld, Jamila Louahed, Laurent Knoops, Valérie Steenwinckel, Brigitte de Lestré, Monique Stevens, Emiel Van Roost

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 (1). 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 (2).

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 (3).

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.

IL-9 receptor and signal transduction

Jean-Christophe Renauld, Laurent Knoops, Monique Stevens, Emiel Van Roost

Analysis of the mode of action of IL-9 at the molecular level was initiated in 1992 by the cloning of the murine and human IL-9 receptor (IL-9R) cDNAs (4). By further dissecting the signal transduction cascade triggered by IL-9, we showed that, upon IL-9 binding, the IL-9R associates with a co-receptor protein called 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*** (5).

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

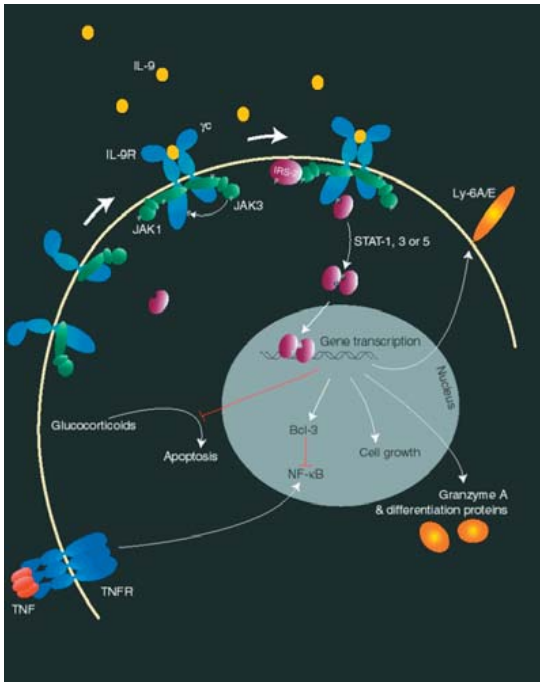

Figure 1.

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

Jean-Christophe Renauld, Jacques Van Snick, Jamila Louahed

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 (6). 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, Jamila Louahed, Laurent Knoops, Monique Stevens, Emiel Van Roost

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 (7). 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 (8).

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 (9). 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 (10). 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 (9). 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.

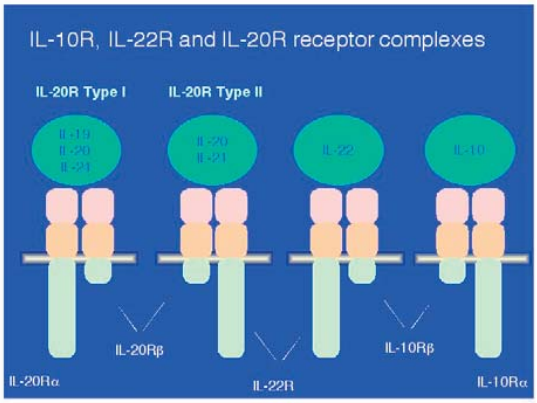

Figure 2.

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 (11). 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.

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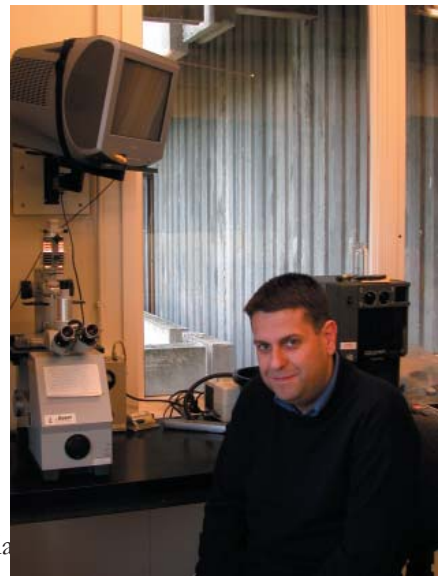
SIGNAL TRANSDUCTION GROUP: STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS



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Cytokines and their receptors are critical for the formation of mature blood cells and for the function of the immune system. Signaling by cytokine receptors is triggered by ligand-induced changes in receptor dimerization/oligomerization, which induces the activation of cytosolic Janus tyrosine kinases (JAK). These kinases phosphorylate downstream proteins, like receptors themselves, signal transducers and activators of transcription (STAT) proteins and a variety of other signaling proteins. 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 (IL9) and their involvement in diseases such as Polycythemia Vera or leukemias. 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.

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

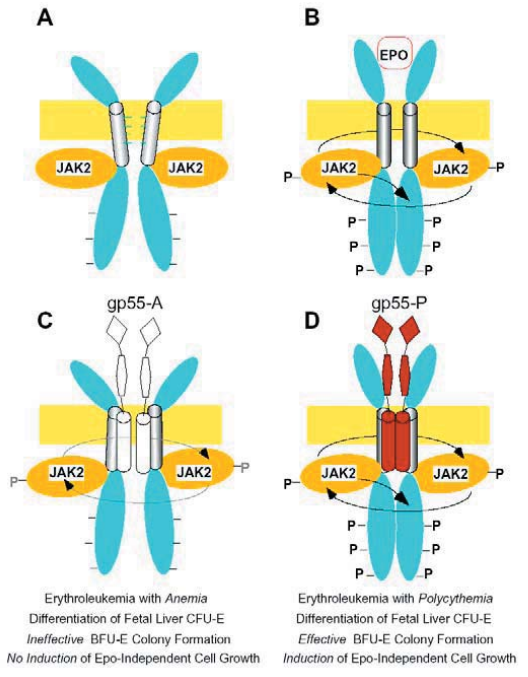
Judith Staerk

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 recep-

tors and are switched-on upon ligand binding to the receptors' extracellular domains (1). In addition to their role in signaling, JAKs appear to play chaperone roles for promoting traffic of cytokine receptors to the cell-surface (2).

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

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 a novel point mutant of JAK2, where a unique V617F mutation in the pseudokinase domain renders the enzyme constitutively active (3). The mutation alters a physiologic inhibition exerted by the pseudokinase domain on the kinase domain (3). This mutant is found in >80% of PV patients and in 50% of Essential Thrombocythemia and Idiopathic Myelofibrosis (IMF), two other diseases that belong to the myeloproliferative syndromes. Current projects include the determination of downstream signaling proteins activated by the mutant JAK2, and the characterization of cytokine receptor signaling in the



(A) The erythropoietin receptor (EpoR) is an inactive dimer on the cell-surface in the absence of ligand due to interactions between the transmembrane (TM) domains (interrupted line). Cytosolic Janus kinase 2 (JAK2) is bound to the receptor juxtamembrane domain and stimulates receptor folding and traffic to the cell-surface.

(B) Erythropoietin (Epo) binding induces a conformational change in the extracellular domain which is transmitted via the α-helical TM domain to receptor residues in the juxtamembrane domain that contact JAK2 and switch its activity on. Activated JAK2 phosphorylates (-P) itself, the receptor cytosolic domain and many other signaling proteins leading to survival, proliferation and differentiation of erythroid progenitors into mature red blood cells.

(C) Co-expression of the EpoR with the gp55-A envelope protein of the Spleen Focus Forming Virus results in cell-surface complex formation due to specific interactions between the TM domains. The EpoR transmembrane dimer, which maintains the EpoR inactive, is disrupted by the interaction with gp55-A. The receptor acquires a conformation, which allows weak constitutive activation resulting in erythroleukemia with low numbers of red blood cells (anemia)

(D) Co-expression of the EpoR with the viral gp55-P envelope protein results in cell-surface complex formation due to specific interactions between the TM domains. In this complex the EpoR acquires a dimeric conformation very similar to that induced by Epo, which results in strong constitutive activation of EpoR signaling leading to erythroleukemia and massive production of mature red blood cells (polycythemia).

presence of the mutant and wild type JAK2. These results suggest that point mutations in JAK proteins may be involved in different forms of cancers and autoimmune diseases.

Determination of the interface and orientation of the activated erythropoietin receptor dimer

Katharina Kubatzky, Judith Staerk, Yohan Royer, Virginie Moucadel

The determination of the interface of the active erythropoietin receptor (EpoR) dimer has been a priority during the last year (4). Epo binding to the erythropoietin receptor (EpoR) results in survival, proliferation and differentiation of erythroid progenitors into mature red blood cells. We have shown that, in the absence of Epo, the cell-surface EpoR is dimerized in an inactive conformation, which is stabilized by interactions between the TM sequences (5). Epo binding to the extracellular EpoR domain induces a conformational change of the receptor, which results in the activation of cytosolic JAK2 proteins (Figure 1A and B). The α-helical orientation of the(TM) and cytosolic JM domains is crucial for receptor activation (1).

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 (4). 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.

This allows the prediction of the positions (on an α-helix) of the residues that will be in the interface of the activated EpoR dimer. We have generated seven different constructs where all seven possible orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR. All seven fusion proteins are expressed when transduced in cytokine-dependent cell lines and reach the cell-surface. However, only two of the seven fusion proteins showed activity represented by stimulation of proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells (4). The predicted dimeric interfaces of the two active fusion proteins are very close, emphasizing the notion that a unique dimeric EpoR conformation is 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. This approach of exploring orientation-dependent signaling is now applied in our group for the determination of the active interfaces of the thrombopoietin and G-CSF receptors.

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

Katharina Kubatzky, Judith Staerk, Mingli Li, 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 (6). 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 (6). The same residues were found to be in the interface of the active coiled-coil-EpoR fusion proteins (4). These data show that in the native structure the TM domains of the EpoR are closely interacting with each other.

The structure of the cytosolic domains of cytokine receptors remains a mystery. We hypothesized that the important structured segments of the cytosolic domain must contain the cytosolic JM segment. We have cloned the cDNAs

coding for the seven coiled-coil-EpoR fusion proteins in the pET31b vector in order to produce recombinant fusion proteins in quantities amenable for biophysical and structural studies. In collaboration with the group of Steven Smith, SUNY, Stony Brook, NY, USA, we will determine the NMR structure of the EpoR TM and cytosolic JM domains in the active and inactive coiled-coil-EpoR fusion proteins.

Signaling by the thrombopoietin receptor

Judith Staerk

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. In contrast, only EpoR can support efficient formation of mature red cells. Since both EpoR and TpoR are members of the homodimeric class of cytokine receptors we have started to compare signaling and gene expression induced by these two receptors as well as the orientation of their intracellular domains in the activated state. We have already identified a major difference between TpoR and the EpoR, namely that several orientations of the TpoR are compatible with inducing cell proliferation but only a restricted dimeric conformation is compatible with the induction of intercellular adhesion. By constructing chimeric receptors we attempt to identify the relevant sequences which confer to the TpoR and EpoR their specific biologic activities in immature hematopoietic progenitors and committed erythroid progenitors, respectively.

Signaling by the IL9R via the common γ chain (γ_c) and traffic of cytokine receptors to the cell-surface

Yohan Royer

γ_c is a cytokine receptor that is shared by the receptor complexes of several cytokines, such as IL2, IL4, IL7, IL9, IL15 and IL21. γ_c binds and activate JAK3. Humans that lack the γ_c or have mutations in JAK3 develop severe combined immunodeficiency (SCID). We study the regions important in the IL9R and the γ_c for JAK1 and JAK3 interactions. For both of them, the region comprising Box1 and Box2 is necessary for the JAKs binding. In contrast, the region between the juxtamembrane domain and Box1 has quite different sequence requirements. While IL9R is rather similar to the EpoR (3), γ_c does not require hydrophobic residues before Box1. Finally, by confocal microscopy, the distribution of the γ_c in the cell is totally different from the one of the IL9R or the EpoR. The γ_c is localized in big intracellular patches (colocalizing with LAMP1), whereas the IL9R and the EpoR exhibit a normal diffuse distribution, as we have shown in collaboration with Pierre Courtoy.

We have observed that, in hematopoietic cells, over-expressing cognate JAK proteins, leads to enhanced cell-surface localization of several cytokine receptors (i.e. TpoR, IL9R, IL2R, γ_c). 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. Our working hypothesis is that the N-terminus FERM domain of JAK proteins exerts a generic pro-folding effect on cytosolic domains of cytokine receptors. We are testing this hypothesis on several different cytokine receptors and are investigating the link between proper folding in the ER and transport to the cell-surface.

Sequence–specific interactions between transmembrane domains

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 (7). 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 (Figure 1C and D)(7). 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 (8). 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 represent a powerful selection.

Constitutive activation of JAK-STAT signaling pathways and genes targeted by STAT5 in transformed hematopoietic and patient-derived leukemia cells

Virginie Moucadel, Judith Staerk

Cytokine stimulation of cytokine receptors, induces transient activation of the JAK-STAT pathway. In contrast several mutant forms of cytokine receptors have been isolated that signal constitutively (i.e. EpoR R129C or TpoR S498N). Such receptors are permanently dimerized in an activated state and induce the biologic effects of the wild type receptors as well as leukemic cell transformation. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously. In the 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 remain 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 not only to N3 sites, which is characteristic of ligand-activated STAT5 (9). Furthermore, while cytokines such as Epo activate both STAT5A and STAT5B, we have recently observed that in transformed hematopoietic cells it is mainly STAT5B that is constitutively active (9).

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 gene 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 expressed in bicistronic retroviral vectors (10) that allow wide expression of cDNAs at physiologic levels.

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