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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TABLE OF CONTENTS

CHRISTIAN DE DUVE INSTITUTE OF CELLULAR PATHOLOGY (ICP) 1
ICP: an International Biomedical Research Institute 3
ADMINISTRATION AND GENERAL SERVICES 5
DEVELOPMENT AND EXPANSION COUNCIL (DEC) 6
ACKNOWLEDGMENTS 7

CARBOHYDRATE METABOLISM 8
Emile VAN SCHAFTINGEN, Member
Maria VEIGA-da-CUNHA, Associate Member
Protein deglycation 8
Fructosamine metabolism in bacteria 10
Regulation of Vitamin C synthesis 11
Metabolism of L- and D-2-hydroxyglutarate 12
Inborn errors of metabolism 12
Selected publications 13

PURINE METABOLISM 14
Françoise BONTEMPS, Associate Member
Marie-Françoise VINCENT, Associate Member
Adenylosuccinate lyase deficiency 14
A new defect in the purine biosynthesis 15
Antileukaemic properties of 2-chloro-2'-deoxyadenosine 16
Selected publications 17

TISSUE-SPECIFIC TRANSCRIPTION FACTORS IN DEVELOPMENT 19
Guy G. ROUSSEAU, Member
Frédéric P. LEMAIGRE, Member
Control and molecular mode of action of HNF-6 20
Control of pancreas development by HNF-6 20
Control of liver development by HNF-6 21
Role of OC-2 and OC-3 in the development of endoderm derivatives 22
Selected publications 22

SIGNAL TRANSDUCTION AND PROTEIN PHOSPHORYLATION 24
Louis HUE, Member
Mark H. RIDER, Member
Insulin signalling 25
AMP-activated protein kinase 25
Control of smooth muscle contraction 27
Selected publications 27

ENDOCYTOSIS 29
Pierre J. COURTOY, Member
Marie-France VANDENBROUCKE, Member
Oncogene-induced macropinocytosis in fibroblasts 29
Regulation of endocytosis by v-Src in polarized cells 29
Relation between endocytosis and cell motility 30
Regulation of transcytosis 31
Apical endocytosis regulates thyroid hormone production in human disease; molecular dissection *in vitro* 31
Alterations in the endocytic apparatus of mice lacking renal chloride channel, ClC-5, account for proteinuria in Dent's disease 31
Selected publications 32

EXTRACELLULAR MATRIX BREAKDOWN 33
Pierre J. COURTOY, Member
Yves EECKHOUT, Member
Etienne MARBAIX, Member
Regulation of the expression of endometrial MMPs and cytokines 33
Selective binding of active MMP-7 to human epithelial cell membrane 34
Receptor-mediated endocytic clearance of proMMP-2:TIMP-2 complexes 34
Role of matrix metalloproteinases in abnormal endometrial bleeding 35
Selected publications 35

CONNECTIVE TISSUE AND ARTHRITIS 37
Daniel MANICOURT, Member
Effects of nonsteroidal anti-inflammatory drugs on the overall metabolism of articular cartilage ... 37
Markers of connective tissue metabolism in health and disease 38
Role of the subchondral bone in the initiation and progression of the osteoarthritic disease process 38
Towards a better understanding of the metabolism of hyaluronan in connective tissues 38
Selected publications 39

METABOLIC COMPARTMENTATION IN TRYPANOSOMES 40
Fred R. OPPERDOES, Member
Paul A.M. MICHELS, Member
Enzymes of carbohydrate metabolism 41
Enzymes of the hexose monophosphate pathway (HPM) 43
The presence of plant traits in the *Trypanosomatidae* 43
Analysis of the control of the glycolytic flux 44
Biogenesis of glycosomes 44
Analysis of glycosomal membrane solute 45
Selected publications 45

GENETICS OF HUMAN CARDIOVASCULAR ANOMALIES, SKELETAL DISORDERS AND CEREBRAL TUMORS 46
Miikka VIKKULA, Member
Venous malformations, glomuvenous malformations (“glomangiomas”) and Maffucci syndrome. 46
Lymphedema 47
Vascular anomalies affecting capillaries 47
Cardiopathies 48
Cleft lip and palate 48
Other cutaneous disorders 49
Cerebral tumors 49
Conclusions 49
Selected publications 50

THEILER'S ENCEPHALOMYELITIS VIRUS 51
Thomas MICHIELS, Associate Member
Analysis of viral proteins involved in Theiler's virus escape of the host immune response 52
Inhibition of type-I interferon production by the leader protein 52
Influence of the L* protein on macrophage infection and viral persistence 52
Characterization of the type-I IFN response 53
Selected publications 53

ANTIVIRAL IMMUNITY AND PATHOGENESIS 54
Jean-Paul COUTELIER, Associate Member
Viral infections result in a dramatic increase in the proportion of IgG2a 54
Activation of natural killer cells 54
Activation of macrophages 55
Autoimmune thrombocytopenia 55
Selected publications 55

SIGNAL TRANSDUCTION AND GENE REGULATION BY PLATELET-DERIVED GROWTH FACTORS 57
Jean-Baptiste DEMOULIN, Assistant Member
Gene regulation by PDGF 58
Regulation of the PDGF receptor kinase activity by its C-terminal tail and NHERF 58
Role of Gab1 in PDGF signal transduction 58
Selected publications: 59

HUMAN TUMOR IMMUNOLOGY* 60
Pierre G. COULIE, Member
Correlation between tumor regression and T cell responses in melanoma patients vaccinated with a MAGE antigen 60
Tumor regressions observed after vaccination: a possible role for tumor-specific cytolytic T lymphocytes that do not recognize the vaccine antigens 62
Selected publications 63

LUDWIG INSTITUTE FOR CANCER RESEARCH (LICR) 65

ADMINISTRATION AND GENERAL SERVICES 68
Administration and secretariat 68
Pre-clinical Research Resources Group: 68
Technical support 68

TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING 69
Benoît VAN DEN EYNDE, Member
Differential processing of tumor antigens by the standard proteasome and the immunoproteasome 70
An antigenic peptide produced by peptide splicing in the proteasome 70
Identification of new antigens recognized by autologous CTL on human melanoma 71
A novel tumor immune escape mechanism based on tryptophan degradation by indoleamine 2,3 dioxygenase 71
Development of an inducible mouse melanoma model for immunotherapy 72
Antigen presentation by dendritic cells in Systemic Lupus Erythematosus 72
Selected publications 73

GENES EXPRESSED IN CANCER AND GERMLINE CELLS 74
Etienne De PLAEN, Assistant member
Isolation and characterization of new cancer-germline genes 74
MAGE-A1 acts as a transcriptional repressor 74
Genome hypomethylation in the activation of cancer-germline genes in tumors 75
Selected publications 75

IDENTIFICATION OF HUMAN TUMOR ANTIGENS 77
Pierre van der BRUGGEN, Member
New MAGE antigens recognized by $CD8^+$ and $CD4^+$ T cells 77
A novel approach to identify antigens recognized by CD4 T cells using complement-opsonized bacteria expressing a cDNA library 80
The normal anti-MAGE-3.A1 repertoire 80
A reversible functional defect of $CD8^+$ T lymphocytes involving loss of tetramer labeling 80
Detection of anti-vaccine CD4 T cell response in vaccinated patients 81
Selected publications 81

THERAPEUTIC VACCINATION OF CANCER PATIENTS WITH TUMOR-SPECIFIC ANTIGENS 83
Marie MARCHAND, Senior Clinical Investigator
Therapeutic vaccination with MAGE tumor antigens 83
Selected publications 85

ANALYSIS OF T CELL RESPONSES OF VACCINATED CANCER PATIENTS * 86
Aline VAN PEL, Associate Member
Methods for evaluation of T-cell responses in vaccinated cancer patients 86
A melanoma patient vaccinated with a MAGE-3 recombinant canarypox virus 87
A melanoma patient vaccinated with dendritic cells pulsed with a MAGE-3 peptide presented by HLA-A1 88
Selected publications 89

CYTOKINES IN IMMUNITY AND INFLAMMATION 90
Jean-Christophe RENAULD, Member
Interleukin 9 90
IL-9 receptor and signal transduction 91
Anti-apoptotic activity of I-309receptor and signal transduction 92
IL-9-induced genes 92
LICR2: a new cytokine receptor mediating antiviral activities 93
Selected publications 93

SIGNAL TRANSDUCTION GROUP: STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS 95
Stefan CONSTANTINESCU, Associate Member
Determination of the interface and orientation of the activated erythropoietin receptor dimer 95
Structural studies on the transmembrane and juxtamembrane cytosolic sequences of the EpoR 97
Signaling by the thrombopoietin receptor 97
Signaling by the IL9R via the common γ chain (γ_c) and traffic of cytokine receptors to the cell-surface 97
Sequence–specific interactions between transmembrane domains 98
Constitutive activation of JAK-STAT signaling pathways and genes targeted by STAT5 in transformed hematopoietic and patient-derived leukemia cells 98
Selected publications 99

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. But 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.

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In 1978 the Ludwig Institute for Cancer Research decided to base its Belgian branch within the walls of ICP. A happy 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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ACKNOWLEDGMENTS

In 2004, the ICP has attracted major gifts from several foundations, companies and individuals who have been very generous. These sponsors are providing the resources which enable our scientists to better understand and treat diseases that afflict people around the world. Gifts are the lifeblood of new research initiatives and private resources are crucial in underwriting the costs of new laboratories. On an annual basis, fund-raising from private sources has nearly tripled during the past decade over levels achieved previously and now support 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 Katharina KUBATZKY

the "Pierre Lacroix" fellowship to Anders KALLIN

the "Umicore" fellowship to Delphine GERBOD

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, Member
Younes ACHOURI, 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

Fructosamine 3-kinase

Chronic elevation of the blood glucose concentration in diabetes appears to be responsible for the long-term complications of this disease. The link between the elevated concentration of glucose and the development of these complications is not yet clear. One of the theories on this link emphasizes the role of fructosamines. These are formed through a spontaneous reaction (known as 'glycation') of glucose with primary amines, followed by an Amadori rearrangement. Fructosamine 3-kinase (FN3K) is a recently identified enzyme 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.

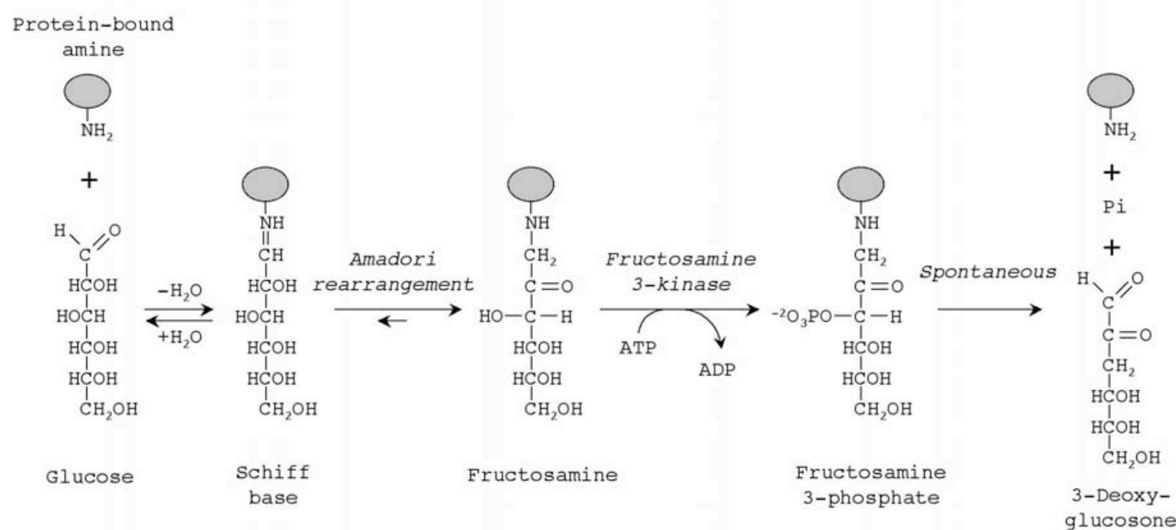

Figure 1. Role of fructosamine 3-kinase in the deglycation of proteins

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 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 [4]. 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.

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, and they both have a similar 6-exon structure. Northern blots indicate high level of expression of both genes in bone marrow, brain, kidney and spleen. Human FN3K-RP was transfected in HEK cells and the expressed protein was partially purified by chromatography on Blue-Sepharose. Unlike FN3K, FN3K-RP did not phosphorylate fructosamines (fructoselysine, deoxymorpholino-fructose, lysozyme glycated with glucose). Both FN3K-RP and FN3K were found, however, to phosphorylate ketoamines with a D configuration in C3 (psicoselysine, deoxymorpholinopsicose, deoxymorpholino-ribulose, lysozyme glycated with allose or with

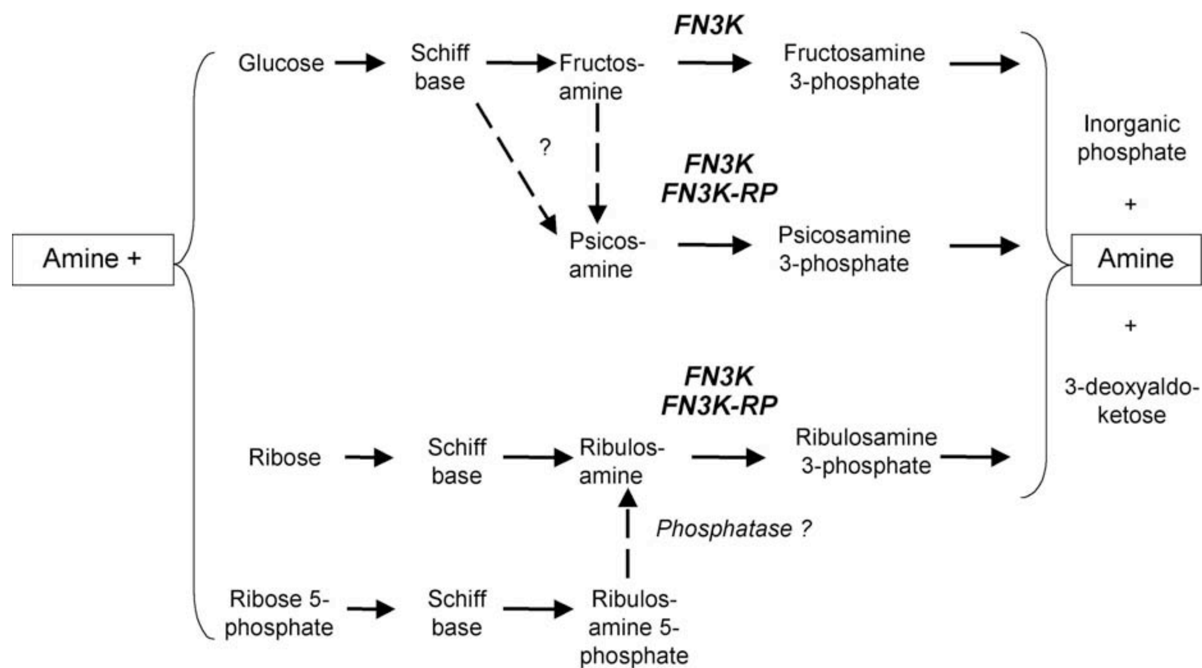

Figure 2. Role of FN3K and FN3K-RP in protein deglycation. FN3K-RP phosphorylates psicosamines and ribulosamines whereas FN3K also phosphorylates fructosamines. Psicosamines may in principle arise from the condensation of amines with allose (not shown), but, since this sugar is absent from mammalian cells, a more likely source is the epimerisation of fructosamines. Ribulosamines may arise from a condensation of amines with ribose or derivatives thereof such as ribose 5-phosphate.

ribose). Tandem mass spectrometry and NMR analysis of the product of phosphorylation of.deoxymorpholinopsicose by FN3K-RP indicated that FN3K-RP also phosphorylates the third carbon of the sugar moiety. These results indicate that FN3K-RP is a ketoamine-3-kinase, which presumably plays a role in the removal of ribulosamines and psicosamines from proteins. This role is shared by FN3K, which has, in addition, the unique capacity to phosphorylate fructosamines.

To investigate the possibility that FN3K-RP also participates in protein deglycation [4], we used human erythrocytes. First, we verified that these cells also contain FN3K-RP, and found indeed that this enzyme is about three times as active as FN3K in these cells when the phosphorylation of psicosamines is assayed. We verified that deoxymorpholinopsicose (a substrate and competitive inhibitor of FN3K-RP) penetrated intact erythrocytes. We also determined that protein-bound psicosamine 3-phosphates and ribulosamine 3-phosphates were unstable, decomposing at pH 7.1 and 37°C with half-lives of 8.8 h and 25 min, respectively, as compared to 7.0 h for fructosamine 3-phosphates. The proof that FN3K-RP could act as a deglycating enzyme could therefore be established by incubating erythrocytes with 50 mM allose or 10 mM ribose for 24 h in the absence or in the presence of deoxymorpholinopsicose. The presence of the latter caused an about 1.9-2.6-fold higher accumulation of glycated hemoglobin, an effect that was not mimicked by deoxymorpholino-fructose, a specific inhibitor of FN3K. 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. Our present hypothesis is that the physiological substrates of FN3K-RP are ribulosamines and that these arise from the reaction of proteins with the potent glycating agent ribose 5-phosphate.

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* [5]. 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 (see below); 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.

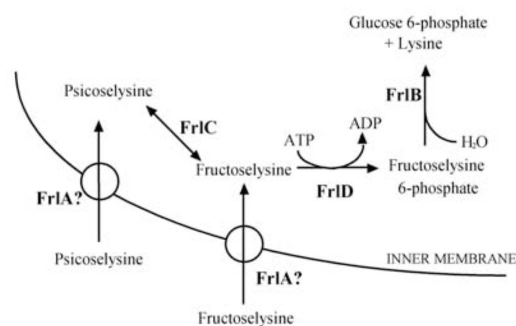

Figure 3. Metabolism of fructoselysine and psicoselysine in *Escherichia coli*.

BLAST searches indicated that FrlC (see above) shares ≈ 20 % identity with tagatose 3-epimerase, an enzyme that isomerizes D-tagatose to D-sorbose, as well as D-fructose to D-psicose. This finding suggested that FrlC could catalyse the interconversion of fructoselysine with its C3 epimer, psicoselysine. FrlC was overexpressed and found indeed to catalyse this reaction. The enzyme can be easily assayed through the formation of tritiated water from [3-^{3}H]fructoselysine, which indicates that the reaction proceeds through an enediol intermediate. Psicoselysine was also found to support the growth of *E. coli*, causing the induction of the three enzymes of the *Frl* operon [6]. The existence of a specific enzyme for the metabolism of psicoselysine suggests the occurrence of this unusual Amadori compound in nature. It most likely originates from the condensation of the rare sugar allose with lysine or from the epimerisation of fructoselysine residues during food processing.

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.

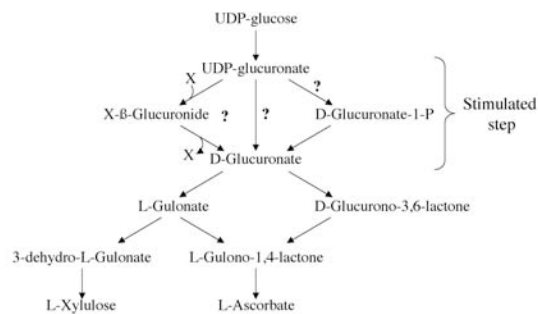

Figure 4. Formation of L-ascorbate and L-xylulose from UDP-glucuronate in liver. The formation of glucuronate from UDP-glucuronate is stimulated by aminopyrine and other agents. The precise mechanism of this formation is still unknown.

Using a simple enzymatic assay of glucuronate that we have developed [7], we recently investigated the mechanism by which aminopyrine and other agents stimulate the formation of vitamin C in isolated rat hepatocytes [8]. 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.

Metabolism of L- and D-2-hydroxyglutarate

Y. Achouri, R. Rzem, G.Noël, M. Veiga-da-Cunha, E. Van Schaftingen in collaboration with D. Vertommen and M. Rider, Horm unit

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. The biochemical lesions responsible for these disorders are not identified, largely due to the fact that the enzymes responsible for the utilization of these two α-hydroxyacids are not well characterized and that their molecular identity is unknown.

In order to characterize enzymes involved in the metabolism of these compounds, we have prepared DL-2-hydroxy[2-^{3}H]glutarate, which was likely to be a convenient substrate for the development of sensitive enzymatic assays. Extracts of frozen rat liver catalysed the formation of tritiated water from DL-2-hydroxy[2-^{3}H]glutarate. Chromatography of a liver extract on DEAE-Sepharose separated two major peaks of enzyme activities. The first corresponded to an enzyme acting on L-2-hydroxyglutarate and the second to an enzyme acting on D-2-hydroxyglutarate, as indicated by competitive inhibition of the detritiation of the racemic radioactive compound by the unlabelled L- and D-isomers, respectively. The enzyme acting on the D-form was further characterized. It was independent of NAD or NADP and converted D-2-hydroxyglutarate to α-ketoglutarate, transferring electrons to artificial electron acceptors. It also oxidized D-lactate, D-malate, and *meso*tartrate, and was stimulated by Zn^{2+}, Co^{2+} and Mn^{2+}, but not by Mg^{2+} or Ca^{2+}. Subcellular fractionation indicated that it was present in the mitochondrial fraction. The enzyme was further purified by chromatography on Blue Trisacryl and Phenyl-Sepharose, up to a stage where only a few bands were still visible by SDS-PAGE. Four candidate polypeptides were analysed by trypsin digestion and mass spectrometry. One of them corresponded to a predicted mitochondrial protein homologous to FAD-dependent D-lactate dehydrogenase. The corresponding human protein was expressed in HEK 293 cells and shown to catalyse the detritiation of DL-2-hydroxy[2-^{3}H]glutarate with similar properties as the purified rat enzyme. In conclusion, we have identified the enzyme that is responsible for the metabolism of D-2-hydroxyglutarate in mammalian tissues. This enzyme is most likely deficient in D-2-hydroxyglutaric aciduria [9].

Inborn errors of metabolism

T. de Barsy, J.F. Collet, G. Noël, M Veiga da Cunha, E. Van Schaftingen

In 2003, samples from about 100 patients were analysed, allowing the diagnosis of carnitine palmitoyl transferase deficiency (3 cases), of aldolase B deficiency (3 cases), of various forms of glycogen storage disease (9 cases) and of phosphomannomutase deficiency (7 cases). We have also identified the mutations in the phosphoserine phosphatase gene resulting in deficiency of this enzyme in the unique case of phosphoserine phosphatase deficiency identified until now [10].

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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, S. Marie, V. Race, T. Timmerman,

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.

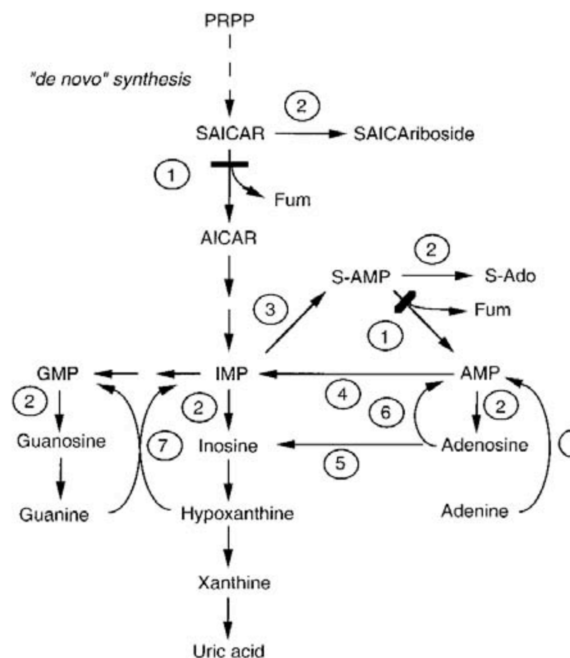

Figure 1. Pathways of purine metabolism. The *ten-step synthetic route*, often termed 'de novo' pathway, leads from phosphoribosyl pyrophosphate (PRPP) to IMP. From IMP, the nucleoside monophosphates, AMP and GMP, and the corresponding di- and triphosphates (not shown) are formed. The *catabolic pathway* 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 MF. 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, 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'-deoxy-adenosine (CdA), was started with the Department of Haematology of the University Hospital Saint-Luc. This adenosine deaminase-resistant deoxyadenosine analogue displays remarkable therapeutic properties in indolent lymphoid malignancies including hairy cell leukaemia and B-cell chronic lymphocytic leukaemia (B-CLL). Nevertheless, resistance is also observed, and CdA does not confer a survival advantage when compared to more conventional therapies such as alkylating agents. The aims of the project are to understand the mechanisms that lead to resistance to CdA, and to improve its therapeutic efficacy by searching for synergisms with other compounds.

To exert is antileukaemic effect, CdA has to be phosphorylated by deoxycytidine kinase (dCK) into CdAMP, followed by conversion into CdADP and CdATP. The latter, the active metabolite of CdA, has been shown to inhibit a variety of enzymes involved in DNA synthesis, including ribonucleotide reductase and DNA polymerase α. Moreover, CdATP can be incorporated into newly synthesised DNA, causing chain termination. Together, these actions result in arrest of DNA synthesis and in the progressive accumulation of DNA strand breaks, leading to apoptosis by mechanisms which are not yet entirely clear. 2-Chloroadenine, the major catabolite of CdA, was found to be actively phosphorylated, but poorly cytotoxic (6).

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 continous 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 IC_{50} (5 – 10 µM) (7). 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. This led us to study the effect of CdA on proteins regulating the G1/S checkpoint of the cell cycle, and firstly on the phosphorylation of the retinoblastoma (Rb) protein, which is increased during the G1/S transition. We have observed that CdA enhances the phosphorylation of Rb in EHEB cells. Additional preliminary experiments have shown that CdA also increases the activity of the cyclin-dependent kinase 2 (cdk2), a kinase that phosphorylates Rb and plays a crucial role in the progression of cells from G1 to S phase. The expression of p21, a key inhibitor of cdk2, tends to decrease after CdA, despite an increase of the level of p53. The p53 status of this cell line was determined and found unmutated. In conclusion, we show a new mode of cellular response to CdA, implying modification of components involved in cell cycle regulation. We are currently studying if the activation of cell cycle by CdA in EHEB cells is correlated with the onset of apoptosis.

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 (8). 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 (9).

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 appear 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. We plan now to verify if dCK can be effectively labelled with [^{32}P]orthophosphate and if this labelling can be modulated by effectors 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 (10) or UV-light (11), 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 (12).

We are currently analysing if efficacy of CdA could be strengthened by combination with MAP kinase inhibitors. MAP kinases indeed regulate important cellular processes, including cell proliferation and survival, and programmed cell death, which explains that inhibitors of these enzymes are being explored as anticancer agents.

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.

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TISSUE-SPECIFIC TRANSCRIPTION FACTORS IN DEVELOPMENT

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We are studying the role of the Onecut (OC) transcription factors in tissue-specific gene expression and in development. These factors, discovered in the laboratory, define a new class (1) of conserved homeoproteins, with three members in mammals : HNF-6 or OC-1, OC-2 and OC-3. They display a restricted tissue distribution and the classes of genes that they regulate overlap, but do not superimpose. HNF-6 (Hepatocyte Nuclear Factor-6), the OC prototype, was identified (2) as a factor that controls the liver-specific transcription of PFK2/FBPase2. HNF-6 stimulates transcription by at least two different mechanisms depending on the nucleotide sequence of the DNA binding site (3). By interacting with DNA-bound glucocorticoid receptor, HNF-6 can also inhibit glucocorticoid-induced gene transcription in a target-specific way. In adult liver, HNF-6 stimulates the transcription of genes coding for enzymes of glucose metabolism, for P450 cytochromes and for secreted proteins. In the embryo, HNF-6 is expressed in the liver and pancreas where it regulates genes coding for other transcription factors. The role of HNF-6 in development has been addressed by studying Hnf6 knockout mice generated in our laboratory. The aim of our current work is to determine the mechanisms by which the OC transcription factors control development of the pancreas, liver and gastrointestinal tract, and to investigate the role of OC factors in diseases of these organs.

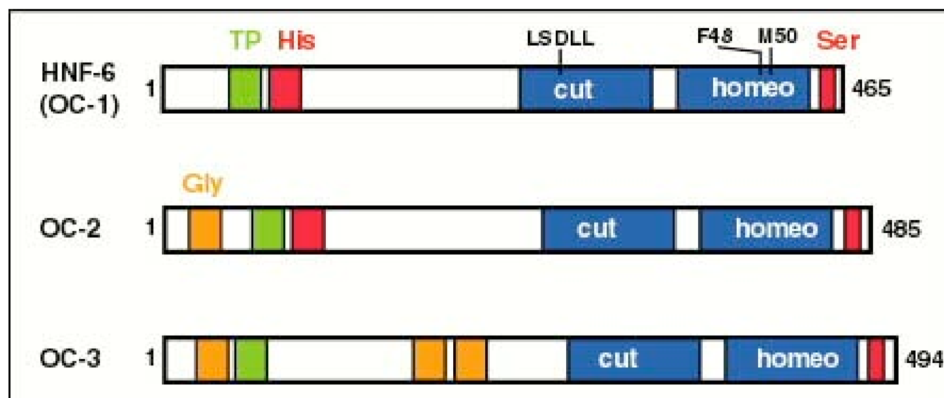

Figure 1. Stucture of human Onecut proteins. The DNA-binding domain consists of a cut domain and a homeodomain. The cut domain contains a LXXLL motif (LSDLL) that contacts transcriptional co-activators. The homeodomain diverges from other homeodomains by the presence of a phenylalanine at position 48 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.

Control and molecular mode of action of HNF-6

N. Plumb, A. Poll, and J.-B. Beaudry

OC proteins contain a divergent homeodomain in which residue 48 is a Phe instead of the canonical Trp and residue 50 is a Met, an amino acid never found at this conserved position in classical homeodomains. They also contain a single cut domain, as opposed to the Drosophila cut protein and its mammalian homologues, which have a classical homeodomain and three cut domains. In addition to the homeo and cut domains, which bind DNA on the sequence DRTCMATND, OC proteins contain other OC-specific conserved regions that modulate their activity (Fig. 1).The Hnf6 gene is made of three exons separated by more than 10 kb. The cut domain is coded by exon 1, and the homeodomain by exon 3. Exon 2 (78 bp) codes for a spacer between these two DNA-binding domains. Differential splicing yields two isoforms with distinct DNA-binding properties, HNF-6α (no spacer) and HNF-6β (with spacer) (1). The study of Hnf6 gene regulatory sequences suggest that the gene contains more than one promoter. We showed that the liver promoter is a target of STAT5 and in this way HNF-6 plays a role in the effects of growth hormone in the liver. Our current objective is to identify the promoter and regulatory sequences that are active in the pancreas and liver, and the transcription factors that bind to them. These regulatory sequences have been cloned upstream of the β-gal reporter gene and their tissue-specific activity is being tested in transgenic mice and in the endoderm of developing embryos.

By stimulating transcription of the Hnf1β, Hnf4 and Hnf3β genes and by being itself controlled by C/EBPα, HNF-6 participates to the network of liver transcription factors. We are further studying this network, as we have evidence that HNF-6 inhibits the stimulation of the Hnf3α gene by TGFβ.

Stimulation of gene transcription by HNF-6 involves co-activators and depends on the integrity of the N-terminal region of HNF-6 (3). We are characterising the transcriptional partners of HNF-6 that are recruited by this region in view of investigating their role in the mechanism of action of HNF-6.

Control of pancreas development by HNF-6

P. Jacquemin, C. Pierreux, J. van Eyll

Hnf6 knockout mice have an hypoplastic pancreas devoid of islets of Langerhans (Fig. 2). We have shown that HNF-6 is required for the differentiation of pluripotent prepancreatic cells into endocrine precursors. This involves the stimulation by HNF-6 of the proendocrine transcription factor NGN-3 (4). At birth, the knockout mice have too few pancreatic endocrine cells and they become diabetic. We have now found that this glucose intolerance also results from a defective liver expression of the glucokinase gene, which is a target of HNF-6. Type II diabetic patients were screened for mutations in the Hnf6 gene, but none has been identified so far.

Embryonic stem cells, when grown as embryoid bodies, spontaneously generate insulin-producing cells that could be used in therapy of diabetes mellitus. Does differentiation of cells in embryoid bodies mimic that of pancreatic β cells in embryos!? To address this question we verified if the differentiation of the insulin-producing cells in embryoid bodies requires HNF-6. No difference was observed in the expression of insulin between wild-type and Hnf6-/- embryoid bodies. In both cases insulin was expressed in the outer layer of cells, which is similar to the visceral endoderm. In wild-type embryoid bodies HNF-6 was transiently expressed in the outer layer of cells, but was not co-expressed with insulin. The expression of genes that are targets of HNF-6 in developing pancreas was unaffected in Hnf6-/- embryoid bodies. Thus, the differentiation mechanism of insulin-producing cells in embryoid bodies differs from that of the β cells and it is likely to resemble that of insulin-producing cells in the visceral endoderm.

As to the pancreas hypoplasia of Hnf6 knockout mice, our data point to a decrease in the number of endodermal precursors specified to a pancreatic fate by the transcription factor Pdx-1. Indeed, in the foetal foregut endoderm, where it is expressed, HNF-6 initiates the expression of Pdx-1, thereby controlling the timing of pancreas specification (5) (Fig. 3).

As Pdx-1-expressing cells eventually appear in the Hnf6-/- embryos, we are investigating the role of other genes in pancreas development. In

the course of these studies we have discovered, using cultured pancreatic explants, that embryonic pancreas can be differentiated, via a Shh-dependent mechanism, into intestinal tissue by activin A (6).

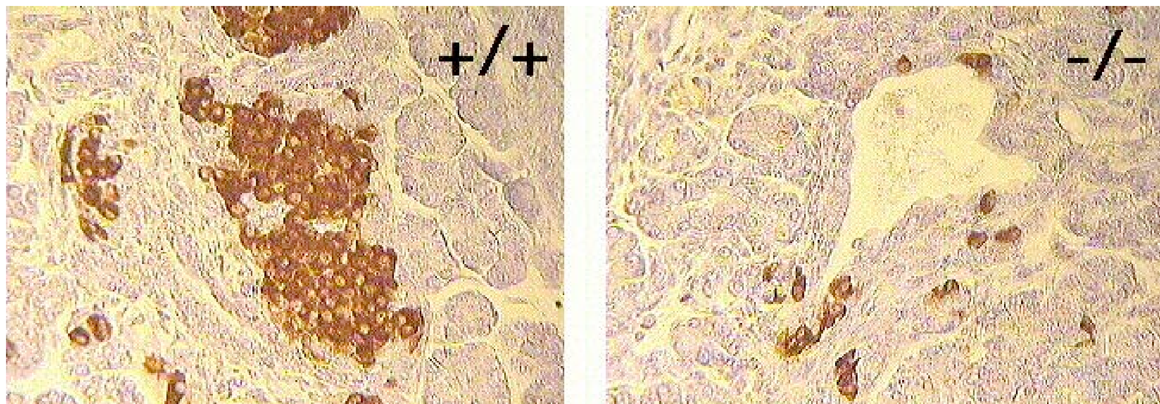

Figure 2. Abnormal development of the endocrine pancreas in HNF-6 knockout mice. Immunohistochemistry of tissue sections four days after birth shows that only a few insulin-producing cells (brown) are found near pancreatic ducts in Hnf6-/- (right panel), instead of being organized in islets as in Hnf6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

Control of liver development by HNF-6

F. Clotman, S. Margagliotti, N. Plumb

During liver development, hepatoblasts differentiate into hepatocytes or into biliary epithelial cells (BEC) 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 BEC. In Hnf6-/- mice, the gallbladder does not develop and the extrahepatic bile ducts are abnormal. The mice suffer from cholestasis and display a phenotype that resembles human biliary diseases called "ductal plate malformations", which are related to biliary atresia. Indeed, the differentiation and morphogenesis of the intrahepatic bile ducts are perturbed (Fig. 4). These disorders involve the transcription factor HNF-1β, which we showed to be a target of HNF-6 and whose expression is downregulated in Hnf6-/- fetal liver (7).

Moreover, this primary defect in liver development was associated with anomalies of the hepatic artery (8), providing a model for studying such anomalies in humans, where they are often associated with ductal plate malformations.

We are further studying the mechanisms of this biliary phenotype by microarray analysis and by exploiting an ex vivo model, the immortalised bipotential mouse embryonic liver (BMEL) cells, which can differentiate into either BEC or hepatocytes.

The developing liver harbours haematopoietic precursors which differentiate into the different blood cell lineages in response to signals sent by hepatoblasts. We have discovered a severe B lymphopenia in young Hnf6-/- mice and have shown that this results from a liver defect, thereby identifying HNF-6 as the first non cell-intrinsic transcription factor known to control B lymphopoiesis specifically in fetal liver.

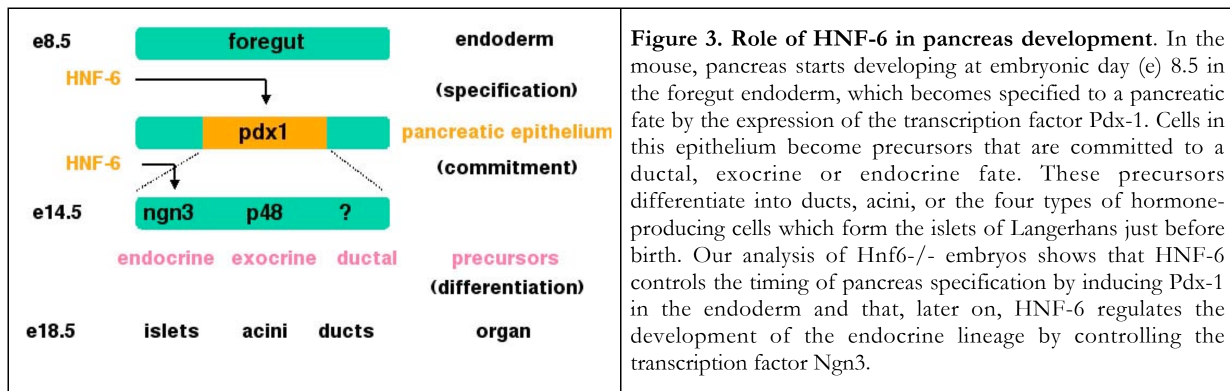

Figure 3. Role of HNF-6 in pancreas development. In the mouse, pancreas starts developing at embryonic day (e) 8.5 in the foregut endoderm, which becomes specified to a pancreatic fate by the expression of the transcription factor Pdx-1. Cells in this epithelium become precursors that are committed to a ductal, exocrine or endocrine fate. These precursors differentiate into ducts, acini, or the four types of hormone-producing cells which form the islets of Langerhans just before birth. Our analysis of Hnf6-/- embryos shows that HNF-6 controls the timing of pancreas specification by inducing Pdx-1 in the endoderm and that, later on, HNF-6 regulates the development of the endocrine lineage by controlling the transcription factor Ngn3.

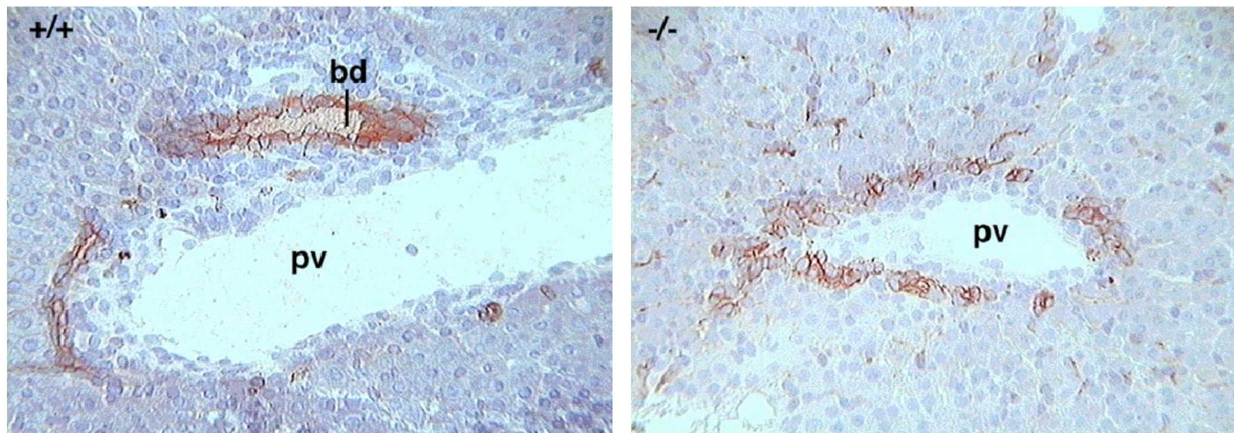

Figure 4. Abnormal development of the intrahepatic bile ducts in HNF-6 knockout mice. Immunohistochemistry of liver sections 10 days after birth shows in normal mice (left panel) a typical bile duct (bd) delineated by biliary cells (stained red) embedded in mesenchyme. In contrast, biliary cells do not form bile ducts in Hnf6-/- mice (right panel) and remain dispersed as a layer around a branch of the portal vein (pv).

Role of OC-2 and OC-3 in the development of endoderm derivatives

P. Jacquemin, V. Vanhorenbeeck, C. Pierreux

We have cloned OC-2 (9) and OC-3 (10) and have shown they have properties similar to those of HNF-6 in terms of DNA-binding and transcriptional activation. In the adult mouse, the patterns of expression of these paralogues are tissue-restricted and partially overlapping with those of HNF-6 (10). While they are all expressed in brain and foregut endoderm, HNF-6 is also expressed in the testis, liver and pancreas, OC-2 in the liver, gut and stomach, and OC-3 in the gut and stomach. In addition, OC-2 is expressed in skin melanocytes where it controls differentiation by regulating the microphthalmia-associated transcription factor (MITF) gene. We have now generated OC-2 and OC-3 knockout mice and are studying their phenotype.

As the three mammalian Onecut genes are expressed in the foregut endoderm, we are setting up cultures of this tissue from normal or knockout mouse embryos in view of exploring *ex vivo* the respective roles of the Onecut factors in pancreas, liver, stomach and gut development.

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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 6-phosphofructo-2-kinase (PFK-2). Its hydrolysis to fructose 6-phosphate and Pi is catalysed by FBPase-2. These two activities 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 (1). 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. The concentration of fructose 2,6-bisphosphate changes in response to metabolites, hormones, growth factors, and oncogene activation (1).

Over recent years, we made a detailed study of the molecular mechanisms responsible for the activation of heart PFK-2 by insulin and showed that protein kinase B was probably not necessary. More recently, we studied the AMP-activated protein kinase (AMPK) which phosphorylates and activates heart PFK-2 in ischaemia, providing a new explanation for the Pasteur effect. We characterised new actions of AMPK on cellular processes. Its activation not only leads to the stimulation of glycolysis in heart and monocytes, but also induces apoptosis in certain cells, inhibits mTOR signalling by amino acids and inhibits protein synthesis at elongation. The latter can be explained by the phosphorylation-induced inactivation of eukaryotic elongation factor-2 (eEF2). The target of AMPK is not eEF2 itself but its upstream eEF2 kinase which is phosphorylated and activated by AMPK. We are currently investigating the upstream regulation of AMPK and novel downstream targets.

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. In mice lacking 3-phosphoinositide-dependent protein kinase-1 (PDK1) in cardiac muscle, insulin did not activate PKB and PFK-2, nor did it increase fructose 2,6-bisphosphate (3), consistent with PDK1 mediating these processes. We also tested the role of PKB in the activation of PFK-2 by insulin using a 'dominant-negative' construct and found that the activation of PFK-2 by insulin did not require PKB, but was mediated by another protein kinase located downstream of PDK1 (4). 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 participate in the insulin-induced activation of heart PFK-2.

Disruption of the gene for the 5'-phosphatase acting on phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) known as SHIP2, increases insulin sensitivity (6). When co-transfected with heart PFK-2 in CHO-IR cells, SHIP2 blunted insulin-induced PFK-2 activation, emphasizing the requirement of PtdIns(3,4,5)P_3 for WISK activation.

AMP-activated protein kinase

S. Horman, D. Vertommen, B. Guigas, D. Leroy, N. El-Najjar, M.H. Rider, L. Hue, 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, and T. Walliman and U. Schlattner, Zurich

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 when the oxygen supply is restricted 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. AMPK switches off energy-consuming biosynthetic pathways, thereby conserving ATP.

Stimulation of heart glycolysis by ischemia.

Ischemia or anoxia stimulates glycolysis (Pasteur Effect) which involves increased glucose transport and PFK-2 activation in heart. We investigated whether AMPK could mediate this phenomenon. AMPK phosphorylated heart PFK-2 on Ser 466 which led to its activation (7). In perfused hearts, ischemia induced an activation of AMPK, which correlated with PFK-2 activation and with an increase in Fru-2,6-P2 concentration. In cultured HEK-293 cells transfected with heart PFK-2, a dominant negative construct of AMPK abolished both the phosphorylation and activation of transfected PFK-2 induced by oligomycin, an inhibitor of oxidative phosphorylation (7). Therefore, heart PFK-2 is a new substrate of AMPK and its activation is involved in the Pasteur Effect.

AMPK activation inhibits protein synthesis

Protein synthesis, in particular peptide chain elongation, consumes a large proportion of intracellular ATP. Therefore, we investigated whether AMPK activation could inhibit protein synthesis via the phosphorylation of regulatory components of the translation machinery. In anoxic rat hepatocytes or in hepatocytes treated with AICA-riboside, AMPK was activated and protein synthesis was inhibited. The inhibition of protein synthesis was associated with the phosphorylation of eEF2. In HEK-293 cells, transfection of a dominant negative AMPK construct abolished the oligomycin-induced inhibition of protein synthesis and eEF2 phosphorylation. Lastly, eEF2 kinase (eEF2K), the kinase that phosphorylates eEF2, was activated in anoxic or AICA riboside-treated hepatocytes and in ischaemic hearts. Moreover, phosphorylation of recombinant eEF2K by

AMPK correlated with eEF2K activation, providing a novel mechanism for the inhibition of protein synthesis (8,9).

Incubation of hepatocytes with amino acids, such as glutamine and leucine, leads to an activation of biosynthetic pathways, such as glycogen synthesis, lipogenesis and protein synthesis. Under these conditions, p70 ribosomal S6 kinase (p70S6K), a protein kinase that participates in the control of protein synthesis and is activated in response to hormones, mitogens and nutrients, becomes activated via activation of the mammalian target of rapamycin (mTOR) by an unknown mechanism.

Pretreatment of hepatocytes with phosphorylating target(s) in the mTOR AICA riboside prevented the activation and phosphorylation of p70S6K (10). Therefore, it is likely that AMPK inhibits p70S6K activation by signalling pathway.

Sustained activation of AMPK triggers apoptosis in liver cells

We studied the effect of long-term AMPK activation on liver cell survival. AMPK activation was maintained in FTO2B cells treated with AICA riboside or by adenoviral transfection of hepatocytes with constitutively active AMPK. Sustained AMPK activation triggered apoptosis through an activation pathway involving c-Jun kinase and caspase-3 (11).

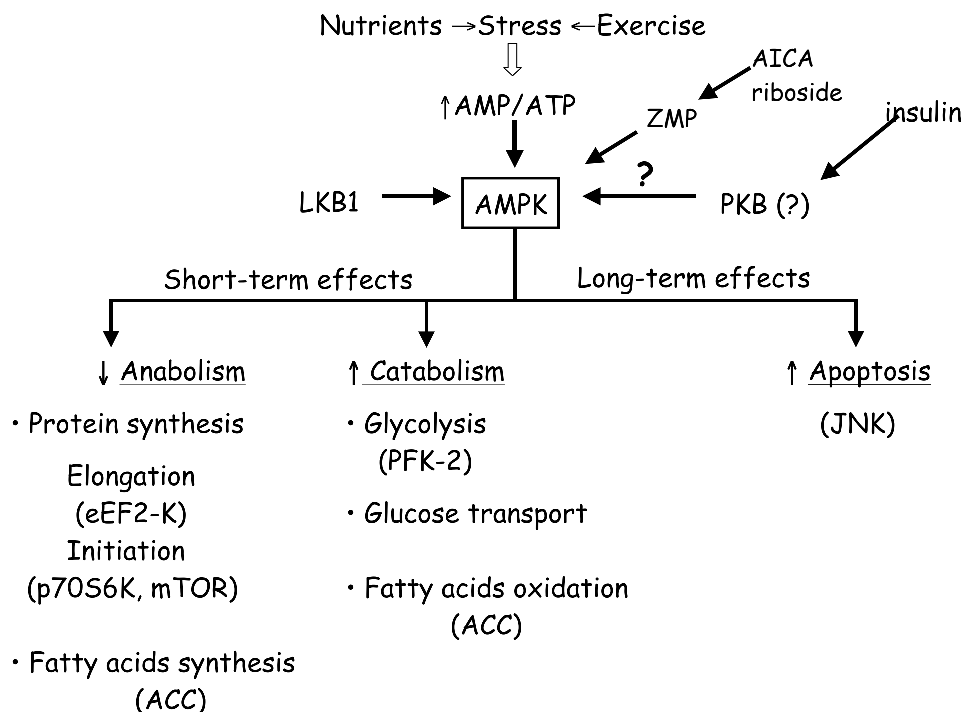

Figure 1. Mechanism of activation and targets of AMP-activated protein kinase (AMPK). AMPK is phosphorylated and activated by AMPKK when the AMP/ATP ratio increases as a result of metabolic stresses. It can also be activated by an AMP analogue, ZMP, which is formed from AICA-riboside. Insulin antagonises AMPK activation by a mechanism which might involve phosphorylation of regulatory sites in the catalytic α-subunits by PKB. The targets of AMPK, that we discovered and which are responsible for some of its short-and long-term effects, are indicated. AMP, adenosine monophosphate; ATP, adenosine triphosphate; AICA riboside, 5-aminoimidazole-4-carboxamide riboside; ZMP, AICA ribotide; AMPK, AMP-activated protein kinase, AMPKK, AMPK-kinase; eEF2K, eukaryotic elongation factor-2-kinase; p70S6K, p70 ribosomal protein S6 kinase; mTOR, mammalian target of rapamycin; ACC, acetyl-CoA carboxylase; PFK-2, 6-phosphofructo-2-kinase; JNK, c-jun kinase.

AMPK activation by upstream kinases

AMPK activation requires phosphorylation in the activation loop at Thr172 of its catalytic α-subunit by an upstream kinase, AMPK-kinase (AMPKK). An upstream kinase phosphorylating Thr172 for AMPK activation was identified independently by the groups of Carling and Hardie as the Peutz-Jegher's protein, LKB1. Using partially purified AMPKK to phosphorylate bacterially expressed AMPK heterotrimers, we have identified upstream kinase phosphorylation sites in AMPK by mass spectrometry. In addition to confirming the phosphorylation of Thr 172, new sites (Thr258 and Ser485/Ser491) have been identified in the α1/2 catalytic subunits (12). Mutagenesis studies showed that phosphorylation at the novel sites is not essential for AMPK activation but might affect subcellular localisation or play a modulatory role. We are currently trying to identify the AMPKK(s) responsible for phosphorylation at the Thr258 and Ser485/Ser491 sites.

We studied the effect of insulin on AMPK activation. We observed that AMPK activation in ischaemic hearts was antagonised by a pre-treatment of the hearts with insulin. The effect of insulin was blocked by wortmannin, an inhibitor of PI 3-kinase and resulted in a decreased phosphorylation state of Thr172 in AMPK (13). In addition, the insulin effect was unrelated to changes in the AMP/ATP ratio, thus demonstrating that AMPK activity could be modified by a mechanism independent of the AMP/ATP ratio in cardiomyocytes. Our recent studies suggest that PKB might mediate the antagonism of AMPK by phosphorylation of the novel regulatory sites (Ser485/Ser491) in the catalytic α-1/2 subunits.

Control of smooth muscle contraction

D. Vertommen and M.H. Rider in collaboration with E. Waelkens, KUL, P. Gailly, UCL, Brussels and M. Walsh, Calgary, Canada

In earlier work, we used nanoelectrospray- and on-line capillary- electrospray ionisation mass spectrometry (ESI-MS) to identify autophosphorylation and regulatory phosphorylation sites in the protein kinase D (PKD)(14). However, there are very few physiological substrates recognised for this protein kinase. We have shown that in various smooth muscles stimulated with vasoconstrictors, such as vasopressin, PKD becomes activated. Moreover, we have found that a thin filament regulatory protein is a new *in vitro* substrate for PKD. Its phosphorylation could be implicated in the prolonged phase of muscle contraction when Ca^{2+}-dependent P-light chain phosphorylation of myosin is not involved in force generation. Using mass spectrometry, we are looking whether the phosphorylation sites phosphorylated by PKD *in vitro* are phosphorylated in response to agonists *in vivo*. This work has potential for the understanding of hypertension and could lead to new treatments for this condition.

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ENDOCYTOSIS

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Olga MEERT, Technician (half-time)
Francisca N'KULI, Technician
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 fibroblasts

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, 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

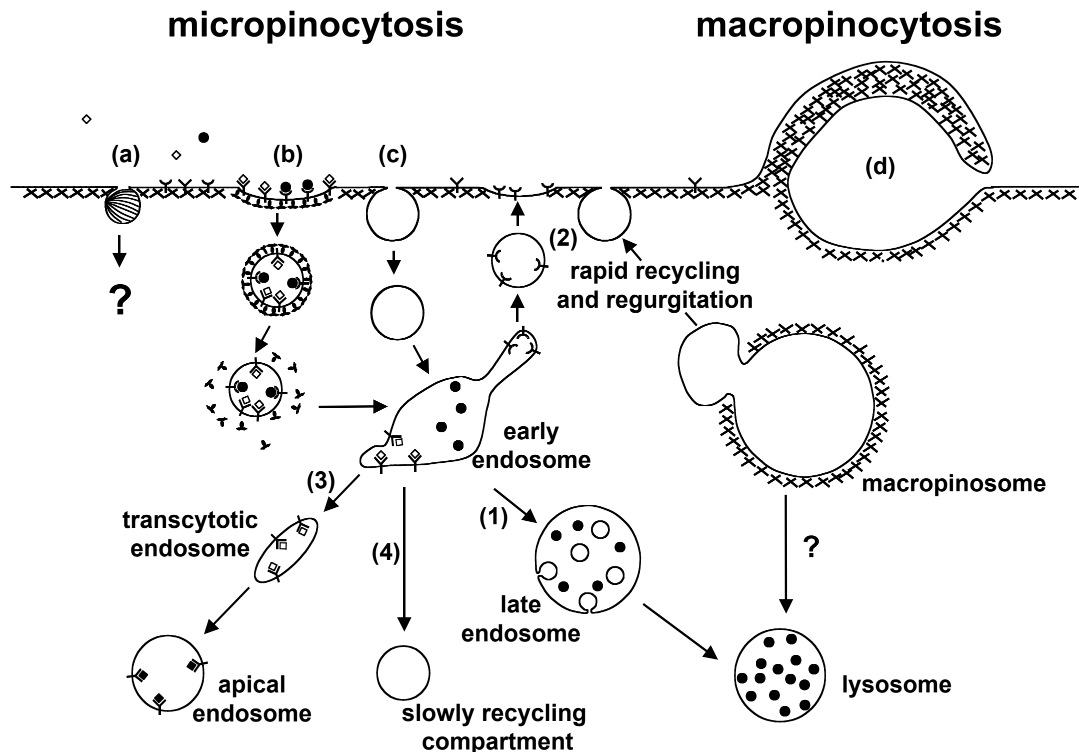

Figure 1. Pathways of endocytosis. This scheme represents four possible modes of vesicular entry of solutes into cells : (a) caveolae; (b) clathrin- and dynamin-associated pits, or "coated pits"; (c) clathrin- and dynamin-independent micropinocytic pits; and (d) macropinocytosis. Crosses represent cortical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

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. Surprisingly, 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. We currently test whether this loss of a polarized actin response results from diffuse activation of PI3K, PLC and/or PLD ("excess noise") (10).

Regulation of transcytosis

P.J. Courtoy, in collaboration with J.P. Vaerman, MEXP

We have continued our long-lasting collaboration with Dr. J.P. Vaerman to address the regulation of transcytosis in transfected MDCK cells bearing the rabbit polymeric Ig-receptor. Receptor occupancy by polymeric IgA, which accelerates transcytosis, was found to activate PLC, as shown by an increased level of inositol 3,4,5-trisphosphate. In the absence of added polymeric IgA, transcytosis of polymeric Ig-receptor was also accelerated by the co-operative effect of the two downstream arms of the PLC response, i.e. by increasing cytosolic calcium concentration with ionomycin, and activation of protein kinase C via phorbol esters. Thus, PLC is involved in signalling, not only of v-Src-induced apical endocytosis (see above), but also in a presumably late step of basolateral-to-apical transcytosis of the polymeric Ig-receptor. It is likely that both effects are mediated by the actin cytoskeleton.

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 currently investigate the possible effect of the cAMP cascade on the expression of appropriate Rab GEFs and GAPs 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

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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). 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. 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

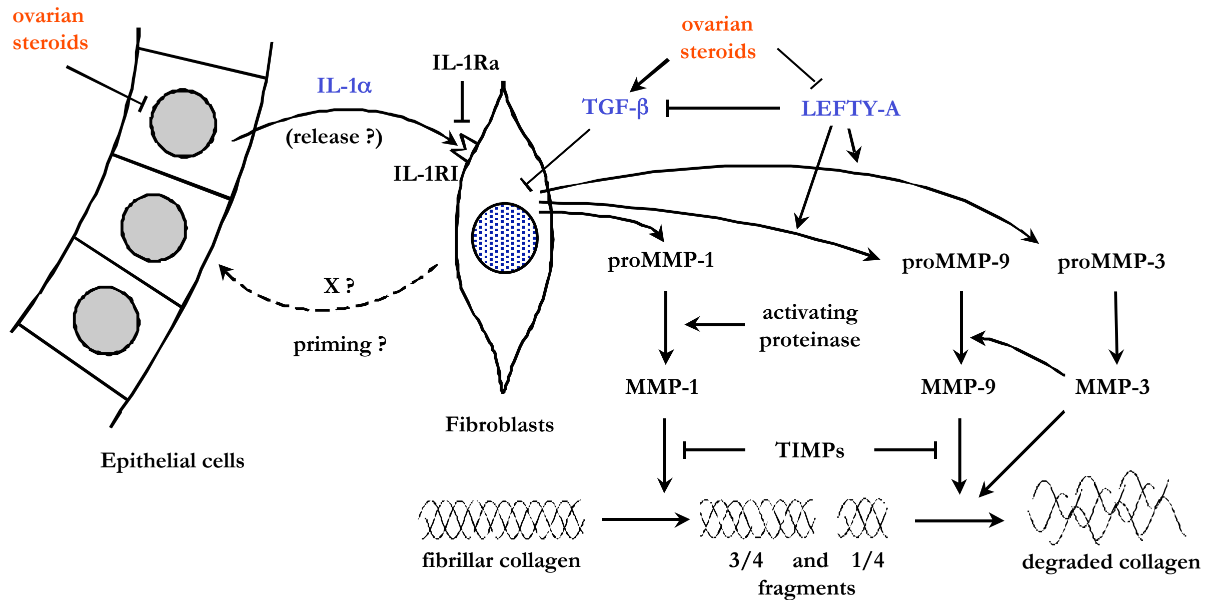

Figure 1. Regulation of MMPs in the human endometrium : current model. Upon ovarian steroids withdrawal, LEFTY-A and epithelial IL-1α are released and trigger the production by adjacent fibroblasts of various proMMPs, including interstitial procollagenase-1/proMMP-1, progelatinase B/proMMP-9 and prostromelysin-1/proMMP-3. Fibroblast activation involves interleukin-1 receptor (IL-1RI) and is opposed by various soluble factors, such as interleukin-1 receptor antagonist (IL-1Ra). TGF-b 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.

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 diffuse cytoplasmic staining with 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-associated 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 (9). Besides tissue inhibitors of metalloproteinases (TIMPs), MMPs may be inhibited by various natural compounds, including unsaturated long-chain fatty acids (10). 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. 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 (11). 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

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Hafida EL AJJAJI, Postgraduate
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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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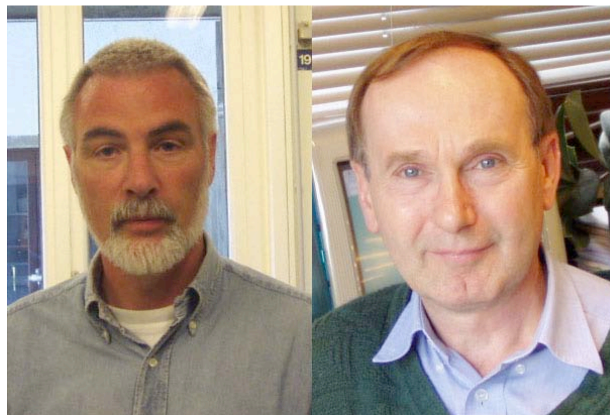

Trypanosomes are responsible for human sleeping sickness in tropical Africa and for a similar disease called 'nagana' in cattle. These are very serious diseases, with fatal outcome if left untreated. The presently available drugs are not very efficient and cause serious side effects. Moreover, development of drug resistant parasites is becoming a major problem. Therefore new drugs are badly needed.

Trypanosoma brucei, when it resides in the mammalian bloodstream, relies entirely on glycolysis for its ATP supply. Moreover, the parasite is characterized by a unique form of metabolic compartmentation; the majority of the enzymes of the glycolytic pathway is sequestered in peroxisome-like organelles called glycosomes (1). For the above reasons the glycolytic pathway is considered a validated and promising target for new drugs to be designed. Since many years we study the kinetic and structural properties of the glycolytic enzymes of T. brucei and closely related parasites such as Trypanosoma cruzi and Leishmania mexicana, and use the collected information for the design of effective and selective inhibitors by structure-based and catalytic mechanism-based approaches (2).

In addition our research aims at understanding what controls the glycolytic flux in vivo. The flux control is being studied in a quantitative manner by using a mathematical model prepared on the basis of the experimentally determined kinetic properties of all enzymes constituting the pathway, and by in vivo experiments in which the activity of different enzymes of the pathway is varied by either biochemical or genetic means. Such experiments could provide both insight into the consequences of the compartmentation of the pathway and information as to which enzymes of the pathway are the best targets for drugs.

Several enzymes of another pathway of carbohydrate metabolism: the hexose monophosphate pathway, involved in the generation of intermediates essential for cell growth, cell division and protection against oxidative stress, are associated with the glycosomes as well. This triggered our interest in their function as glycosomal proteins. Not only the role of the glycosome in trypanosomatid metabolism is the topic of our research, but also the assembly of the organelle. We are studying the proteins, called peroxins, involved in glycosome biogenesis and particularly the mechanism by which they accomplish the import of matrix proteins.

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

In previous years, genes of all enzymes of the pathway have been cloned and characterized in our laboratory (3). In addition, we have cloned and sequenced the genes for two 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 possibly the major regulator of glycolysis in trypanosomatids. Also genes for a number of glycolytic enzymes of *Leishmania* species and *T. cruzi* have been cloned and sequenced; in 2001 the genes for *L. mexicana* phosphoglycerate mutase (PGAM) and *L. donovani* phosphofructokinase (PFK) were characterized (4). The predicted primary structure of almost all trypanosomatid glycolytic enzymes appeared quite different from their mammalian counterparts and some enzymes are even not homologous, rendering several of these enzymes highly promising targets for anti-parasite drugs. Most of the *T. brucei* glycolytic enzymes have been expressed in *Escherichia coli* and purified and their kinetic properties have been determined.

In collaboration with C. López and J.L. Ramirez (Universad Central de Venezuela, Caracas) the gene encoding *L donovani* PFK was cloned and sequenced and its bacterially expressed gene product studied (4). The deduced polypeptide contains a C-terminal type 1 peroxisome-targeting signal (PTS1), -SKV. Like that of the previously characterized *T. brucei* PFK (70 % identity), the sequence showed the highest similarity to inorganic pyrophosphate (PP_i)-dependent PFKs, despite being ATP-dependent. Its kinetic properties were similar to those of the *T. brucei* enzyme. Modeling studies and site-directed mutagenesis were employed to shed light on the structural basis for the unique AMP effector specificity and on ATP/PP_i specificity among PFKs.

The predicted amino-acid sequence of *L. mexicana* PGAM is 74 % identical to that of the *T. brucei* mutase. Both trypanosomatid PGAMs belong to the class of cofactor 2,3-bisphosphoglycerate independent mutases, contrary to the non-homologous cofactor-dependent enzyme of humans. The parasite enzymes have been expressed in *E. coli,* and, upon purification, their kinetic properties have been determined.

During previous years, Prof. W. Hol (University of Washington, Seattle, USA) and Dr. L. Gilmore (University of Edinburgh, UK) and their coworkers have, in collaboration with us, established the crystal structure of several trypanosomatid glycolytic enzymes: aldolase (ALD), triosephosphate isomerase (TIM), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate kinase (PGK), glycerol-3-phosphate dehydrogenase (GPDH) and pyruvate kinase (PYK). Based on these crystal structures, and on insight in the kinetic properties and catalytic mechanisms, inhibitors of each of these enzymes have been designed and synthesised by our colleagues Prof. J. Périé (Université Paul Sabatier, Toulouse, France) and Prof. M. Gelb (University of Washington, Seattle, USA). Promising inhibitors, selective for parasite GAPDH and ALD, with K_i's in the nanomolar range and inhibiting growth of parasites in culture without affecting growth of cultured human cells have already been obtained.

In collaboration with Dr. M. Willson and Prof. J. Périé (Université Paul Sabatier) we also synthesized a number of glucosamine analogues which inhibit both the yeast and the *T. brucei* hexokinase (HXK) (5). The most potent inhibitor of the trypanosome HXK, *m*-bromophenyl glucosamide, did not affect the activity of its yeast counterpart. This compound also effectively inhibited growth of *in vitro* cultured trypanosomes. The structure of the *T. brucei* enzyme, in complex with its *m*-bromophenyl glucosamide inhibitor, was modelled using the crystal structure of the *Schistosoma mansoni* HXK - glucose complex. This model allowed us to explain the mode of action of this inhibitor on the trypanosome HXK.

Also in collaboration with our colleagues in Toulouse, the kinetic mechanism of *T. brucei* PFK has been studied in detail, and its active site has been explored by using a variety of inhibitors derived from the fructose 6-phosphate analogue

2,5-anhydromannitol (6). The best inhibitor was a compound with an electrophilic isothiocyanate group at position 1; it displayed an irreversible inactivation pattern with a K_i value of 133 μM. The residue involved in the specific inactivation of the parasite enzyme was identified by site-directed mutagenesis, Lys227. Based on this promising result, other compounds are now being developed.

The cloning of the *T. brucei* enolase (ENO) gene was reported last year. The encoded polypeptide has 59 – 62 % identity with the different human enolases.

The kinetic properties of bacterially expressed *T. brucei* ENO are very similar to those of the mammalian enzymes. Furthermore, structure modelling (in collaboration with Dr. D. Rigden, (CENARGEN/EMPRAPA, Brasilia, 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 crystal structures are available. However, there are some atypical residues (one Lys and two Cys residues) close to the *T. brucei* active site. These residues could possibly be exploited for the irreversible binding of selective inhibitors. The accessibility of these residues for inhibitors is currently being studied in collaboration with Dr. D. Vertommen (HORM Unit).

The activity of trypanosomatid PYK is allosterically regulated by fructose 2,6-bisphosphate (F-2,6-P_2), contrary to the PYKs from other eukaryotes that are usually stimulated by fructose 1,6-bisphosphate (F-1,6-P_2). The molecular basis of the specificity for the allosteric effector was studied in more detail in collaboration with Drs. D. Rigden (Brasilia) and L. Gilmore (Edinburgh)(7). Based on the comparison of the three-dimensional structure of *Saccharomyces cerevisiae* PYK crystallized with F-1,6-P_2 present at its effector site (R-state) and the *L. mexicana* enzyme crystallized in the T-state, two residues (Lys453 and His480) were proposed to bind the 2-phospho group of the effector. This hypothesis was tested by site-directed mutagenesis. The allosteric activation by F-2,6-P_2 appeared to be entirely abrogated in the mutated enzymes confirming our predictions. In addition, we have prepared two mutants for use as tools to screen the large number of compounds we anticipate from ligand docking, database mining and combinatorial chemistry. Wild-type trypanosomatid PYK has no tryptophan residues, and we have introduced this residue into two different positions near the effector site (F442W and E451W). Both mutants show fluorescence quenching in response to substrates and effectors, and will thus play an important role in screening combinatorial libraries.

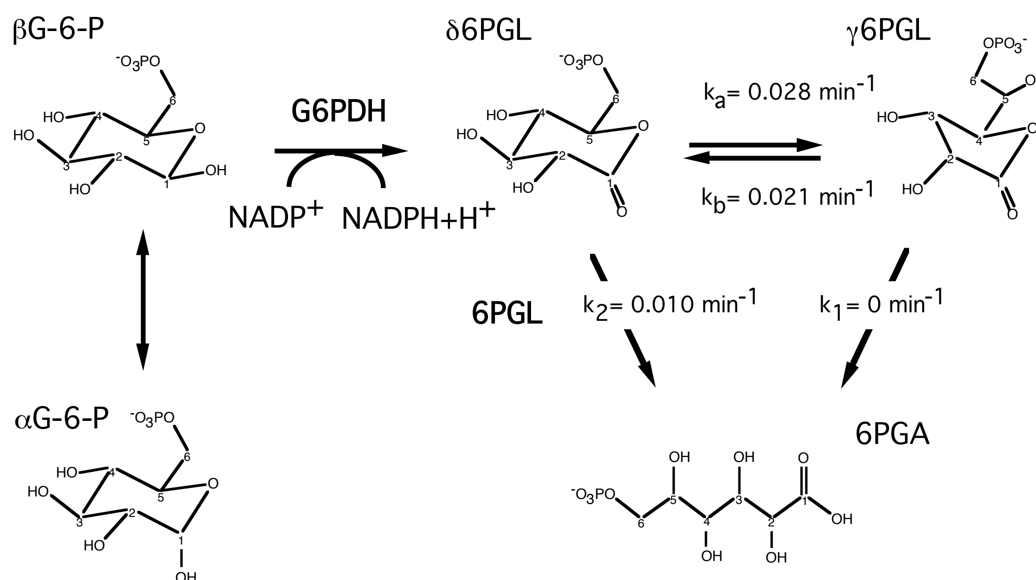

Figure 1. Proposed scheme for the first two steps of the HMP. Glucose 6-phosphate (G-6-P) oxidation by G6PDH leads to the formation of d-6-phosphogluconolactone (d-6-P-G-L). The two 6-P-G-Ls are in exchange, characterized by k_a, the rate constant of the conversion of d-6-P-G-L into g-6-P-G-L, and k_b, for the reverse reaction. No spontaneous hydrolysis of g-6-P-G-L was measured.

Enzymes of the hexose monophosphate pathway (HPM)

Glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase

E. Saavedra, P. Michels and F. Opperdoes, in collaboration with F. Duffieux, Université de Paris Sud, France

Previously, we reported the cloning and characterization of *T. brucei* glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconolactonase (6PGL). We have now also cloned, characterized and expressed the corresponding genes of *L. mexicana*.

In collaboration with Dr. Duffieux (Paris) the three-dimensional structure of the *T. brucei* 6PGL is being solved using the technique of nuclear magnetic resonance. So far, the role of this enzyme in metabolism was still questionable, because 6-phosphogluconolactones were believed to undergo rapid spontaneous hydrolysis. By using both ^{13}C and ^{31}P-nuclear magnetic resonance spectroscopy we have characterized the chemical scheme and kinetic features of the oxidative branch of this pathway (Fig. 1) (8). The δ form of the lactone is the only product of glucose 6-phosphate oxidation. It leads to the spontaneous formation of the γ form by intramolecular rearrangement. However, only the δ form undergoes spontaneous hydrolysis, the γ form being a "dead end" of this branch. The δ form is the only substrate for 6PGL. Therefore, the activity of this enzyme accelerates hydrolysis of the δ form, thus preventing its conversion into the γ form. Furthermore, 6PGL guards against the accumulation of δ-6-phosphogluconolactone, which may be toxic through its reaction with endogenous cellular nucleophiles.

The presence of plant traits in the *Trypanosomatidae*

V. Hannaert, J.-P. Szikora, P. Michels and F.!Opperdoes, in collaboration with D. Rigden, CENARGEN/EMPRAPA, Brasilia, Brazil

While searching for fructose-1,6-bisphosphatase in trypanosomatid genome databases, we have recently identified in *T. brucei* a complete open-reading frame encoding a homologue of a related enzyme, sedoheptulose-1,7-bisphosphatase (SBPase). The gene has been cloned and sequenced and contains a PTS1, making it a glycosomal protein. SBPase is an enzyme typical of the Calvin cycle of photosynthetic organisms and hence (so far) only encountered in the chloroplasts of green algae and plants. Phylogenetic analysis shows that the closest affiliation of the trypanosome enzyme is with that of the chlorophyte *Chlamydomonas reinhardtii.*

Our recent observation that the glycosomal fructose-bisphosphate aldolase is also closely related to its homologues from plants, which all have a broad substrate specificity and are able to synthesize (and cleave) sedoheptulose 1,7-bisphosphate, suggests to us that these two enzymes must function in tandem in the trypanosomatid HMP. A closer inspection of other genes available in the trypanosome genome database revealed many more sequences with either plant or chloroplast/cyanobacterial affiliation. Most of these enzymes fulfil usually functions in either the Calvin cycle, in glycolysis or in the HMP. We hypothesize that these genes of carbohydrate metabolism probably entered the trypanosomatid ancestor from an algal endosymbiont and that their gene products have later been relocated from the endosymbiont to the host, often to its peroxisomes, after which the remainder of the endosymbiont was lost (9). This may explain the enigmatic presence of glycosomes (i.e. peroxisomes specialized in carbohydrate metabolism) in these organisms.

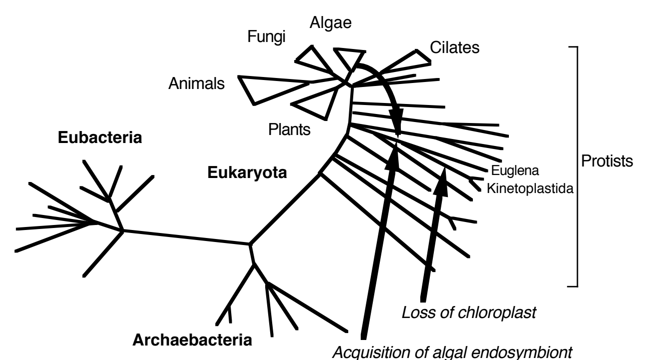

Figure 2. The "tree of life" based on 16S ribosomal RNA sequences, as modified from Sogin.
Indicated are the supposed acquisition of an algal endosymbiont by an organism ancestral to both euglenoids and kinetoplastids. The subsequent loss of this endosymbiont (and its chloroplast) from the kinetoplastid (*Trypanosoma* and *Leishmania*) lineages took place after their separation from the euglenoids, which still have chloroplasts.

Analysis of the control of the glycolytic flux

M.-A. Albert and P. Michels in collaboration with B. Bakker and H. Westerhoff, Vrije Universiteit Amsterdam, The Netherlands, and S. Helfert and C.!Clayton, Universität Heidelberg, Germany

Previously, a mathematical model of trypanosome glycolysis was developed based on the kinetic data available for the enzymes involved. This model was able to predict successfully the experimentally determined fluxes and metabolite concentrations in trypanosomes. Our present experiments focus on the experimental determination of the flux control coefficients of the various steps of glycolysis either by their titration with inhibitors, or by the regulation of the expression of the genes coding for the respective glycolytic enzymes. To this end *T. brucei* cell lines have been created in which the expression of HXK, PFK, TIM and glycerol-3-phosphate oxidase (GPO), the mitochondrial enzyme responsible for oxidising the glycolytically produced NADH, can be decreased by RNA interference (RNAi). For that purpose, double-stranded RNA corresponding to the mRNA is synthesised from a transgene under the control of a promoter that is regulated by exogenously added tetracycline. Using these cell lines, it was shown that the growth rate is halved when the TIM level is decreased to 15%; lower TIM levels are lethal. Indeed, simulation by the model predicted that the flux decreases when the enzyme activity drops to about 30%. Upon lowering the level of GPO mRNA, the oxygen consumption was reduced 4-fold and the rate of trypanosome growth was halved. Similarly, reducing HXK expression by RNAi leads to reduction of the glycolytic flux and the growth rate of the cells. Currently, experiments are in progress to establish the quantitative relationship between HXK and PFK expression on the one hand, and the flux and growth rate on the other hand. Similar experiments are being performed with conditional knockout cells which have been created by disruption of the endogenous HXK and PFK genes after the introduction of an newly introduced additional gene copy under the control of an inducible promoter. These experiments will also reveal if the *in vitro* determined kinetic properties of these enzymes apply also to *in vivo* conditions, and/or if additional regulatory mechanisms occurring in the intact cell have to be invoked.

Biogenesis of glycosomes

J. Moyersoen, H. Krazy, V. Hannaert and P. Michels

Glycosomal matrix proteins are synthesized in the cytosol and imported post-translationally. The translocation of these matrix proteins across the peroxisomal membrane involves a variety of peroxins. Inhibitors interfering with peroxin interactions in trypanosomatids are expected to prevent the synthesis of functional glycosomes and thus kill the parasites. The design of selective inhibitors seems feasible because of the very low level of conservation of peroxins. Previously, we reported the cloning and characterization of two cytosolic peroxins, Pex5 and Pex7. These peroxins act as receptors for proteins to be imported into the glycosomal matrix. Pex5 recognizes the PTS1, a signal specified by the three C-terminal amino acids (10), and Pex7 interacts with the PTS2, a nonapeptide motif close to the N-terminus. Both *T. brucei* peroxins, have been expressed as recombinant proteins in *E. coli*. The functional identity of the 70 kDa *T. brucei* Pex5 has been established *in vitro*; the purified protein recognized glycosomal PGK with high affinity.

We have now also cloned and sequenced the *T. brucei* homologues of Pex6, 10, 12 and 14. Their sequences have 32, 21, 25 and 26% identity with the corresponding human peroxins. Pex14 is part of the receptor-docking complex at the glycosomal membrane. Indeed, we could show, by *in-vitro* experiments, its specific interaction with Pex5. The vital importance of Pex14 for bloodstream-form *T. brucei* was demonstrated by RNAi; induced expression of double-stranded Pex14 mRNA resulted in growth arrest of the cells. Pex10 and Pex12 are possibly involved in the translocation process of the receptors Pex5 and Pex7, charged with their ligand, across the organellar membrane, and/or the dissociation of the receptor-cargo complexes at the matrix site of the membrane. These peroxins contain a C-terminal zinc-binding RING domain known to be involved in protein-protein interactions. Pex6 belongs to the family of AAA-proteins (ATPases Associated with a variety of cellular Activities). It is a large (>100 kDa) protein thought to be either involved in membrane fusion processes which lead to the formation of mature organelles or in the receptor recycling from the organellar matrix to the cytosol. So far, we have expressed in *E. coli* the separate ATPase domain of *T. brucei* Pex6.

Crystallization trials are currently being performed with this trypanosome Pex6 domain and with various forms of Pex14 and Pex5, and with combinatons of these latter two peroxins. (J. Choe and Prof. W. Hol).

Analysis of glycosomal membrane solute

Transporters

C. Yernaux and P. Michels

We have started an investigation of glycosomal membrane proteins that might be involved in the transport of glycolytic intermediates or other solutes across the membrane. We have cloned and sequenced two genes coding for putative *T. brucei* glycosomal membrane transporters (TbGAT1 and 2). The amino-acid sequences encoded by these genes are only 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 peroxisomal ABC transporters. Segments of both polypeptides have been expressed in *E. coli* and are being purified; they will be used for antisera production. Investigation of the subcellular localisation and topology of the transporters is in progress. These studies involve transfection of trypanosomes with constructs encoding fusions of segments of the transporters and fluorescent reporter proteins.

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GENETICS OF HUMAN CARDIOVASCULAR ANOMALIES, SKELETAL DISORDERS AND CEREBRAL TUMORS

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The basic aim of our research is to get insights into the molecular mechanisms behind certain human diseases, 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, and 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 (15).

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 (2). 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 (16). This allows an easly genetic diagnosis for individuals thought to have GVM. The rest, 30%, have a unique mutation.

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 (4), but almost exclusively in vascular smooth muscle cells (14).

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 (4). 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 (4). 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. As the construct contains loxP sites within the glomulin sequence, we can decide the time and tissue for glomulin deficiency. Murine embryos containing such deficiency would be used to study the role of glomulin in development and angiogenesis.

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 (Meige's disease) or at puberty (Milroy 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 (5). In vitro expression studies demonstrated that the mutated receptor has lost its autophosphorylation capacity (5). 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. A dominant SOX18 mutation was identified in two other families, with individuals having varying degree of lymphedema. 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.

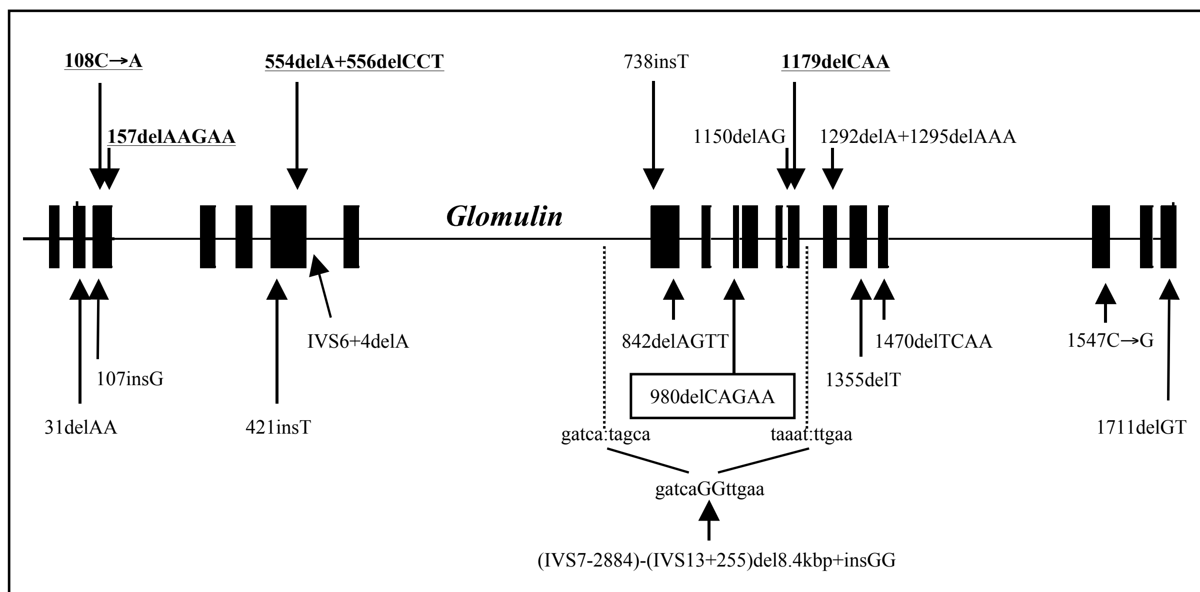

Figure 1. Schematic representation of the *glomulin* gene summarizing all known mutations (15). Mutations found in more than one family are underlined. Somatic second hit is boxed.

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 (6). 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 (7). Screening of positional functional candidate genes led to the identification of mutations in the RASA1 gene, a modifier of Ras signaling pathway (9). This implies that RAS signaling pathway modulators may serve as a novel therapy for these patients in the future.

Cardiopathies

I. Gutierrez-Roelens, A. Irrthum 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 conduction defect, we identified two novel mutations in the *CSX/Nkx2.5* gene (8), 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., unpublished).

Cleft lip and palate

M. Ghassibé, N. Revencu, M. Vikkula, in collaboration with D. Manicourt, 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) (13). Based on this, we have initiated a collaboration with Prof. J. Murray, University of Yowa, to study IRF6 association in cleft lip and palate.

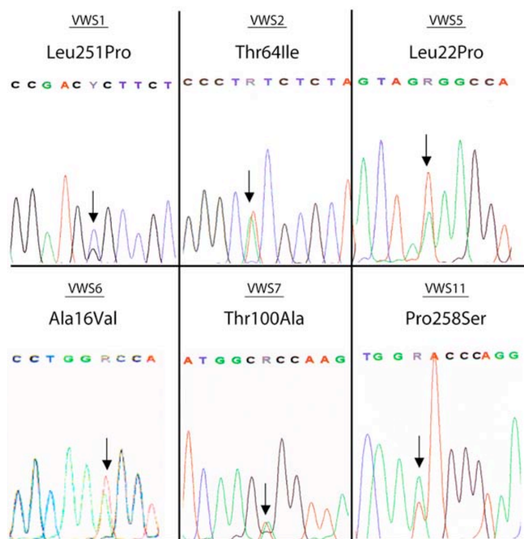

Figure 2. Electropherograms of detected IRF6 heterozygous mutations.
Arrow, site of mutation (12).

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 (12). 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 (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.

Another clinically important cutaneous problem is the formation of keloids in some individuals after wounding. The tendancy to make grossly hypertrophic scars is sometimes hereditary, and in collaboration with Prof. B. Olsen we try to identify the mutated genes to understand their pathophysiology.

Cerebral tumors

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

Morphological characterization and classification of tumors is not always clear. Thus, better (molecular) criteria are needed. In addition, the causative genes are often unknown. We are especially interested in two types of cerebral tumors: oligodendrogliomas and ependymal tumours. Using DNA, extracted from formalin-fixed and paraffin-embedded tissues, we have performed loss-of-heterozygosity testing. A restricted screening was performed in a number of oligodendroglial tumours as well as in a large series of ependymal tumours. For oligodendrogliomas, this allowed us to identify and define specific histological characteristics for tumors that have lost chromosome 1p and 19q and that are known to have a preferable response to chemotherapy (10). This data had direct clinical relevance. In addition, we identified methylation differences in ependymomas depending on their age and location (11).

Conclusions

With the genetic approach described, our group 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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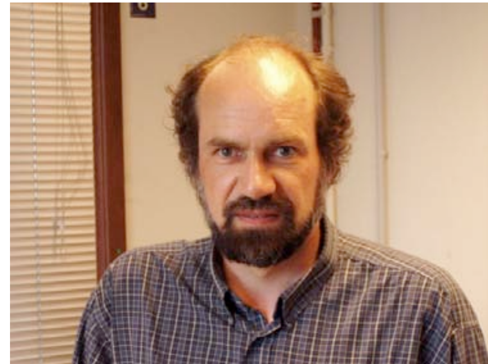

Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) is a murine picornavirus responsible for infections of the central nervous system. Strains of Theiler's virus have a striking ability to persist in the central nervous system in spite of a specific cellular and humoral immune response. 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.

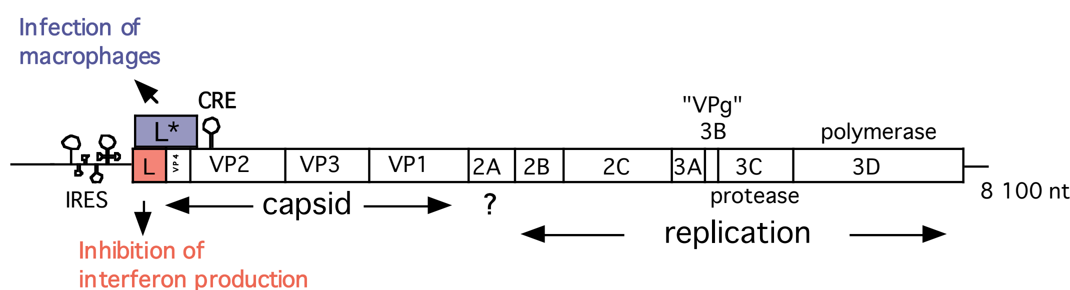

Figure 1. Genome of Theiler's virus. A large open reading frame encodes a 2000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, into 12 mature proteins. An additional protein (L*) is encoded by an alternative open reading frame. Translation of both ORFs is driven by an Internal 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

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 dramatically impair viral persistence in the 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 transcritpional activation of IFN genes. IRF-3 is present in the cytoplasm of non-infected cells. Upon viral infection, IRF-3 is activated and translocated to the nucleus where it activates the transcription of the IFN genes.

We recently showed that the leader protein interfered with nucleo-cytoplasmic trafficking of host cell proteins, and notably of IRF-3 (9). Perturbaation 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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ANTIVIRAL IMMUNITY AND PATHOGENESIS

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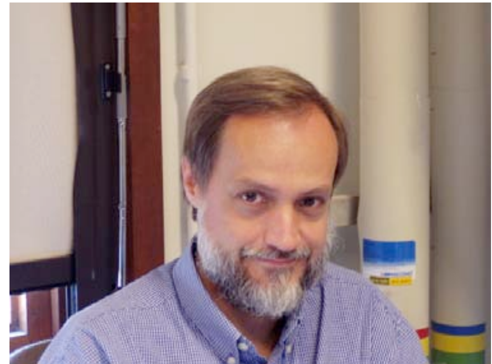

The possibility for evoluted organisms to survive viral infections depends on the ability of their immune system to eliminate the infectious agent. Therefore, numerous mechanisms, involving different types of immune cells such as cytolytic lymphocytes, T helper and B lymphocytes and macrophages, the 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 frequent mutations or latency, or even to impair this system, which often leads to diseases such as autoimmunity or immune deficiencies. Our project is to investigate, 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 activate the complement system, in contrast to IgG2a, another major isotype 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 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, 6). Indeed, different subpopulations of those cells, called Th1 and Th2, respectively, are distinguished in particular by their capability of producing selectively interferon-γ (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 (7), 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-γsecretion affects the early and rapid viral replication that follows LDV inoculation (8).

Activation of macrophages

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 (9). We have thus analysed whether it was possible to modulate such an activation by treating infected mice with clodronate-containing liposomes. Administration of anti-erythrocyte monoclonal autoantibody to mice resulted in the development of a transient hemolytic anemia. Infection with LDV simultaneously with autoantibody injection was followed by a dramatic enhancement of the anemia, 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. Treatment with total immunoglobulin G (IVIg) attenuated the autoantibody-induced disease in uninfected mice, but not in LDV-infected animals (10). In contrast, administration of clodronate-containing liposomes resulted in a delay and a decrease of anemia in LDV-infected mice. This treatment decreased also the *in vitro* phagocytosis of autoantibody-coated red cells by macrophages from LDV-infected animals. Thus, regulation of macrophage activation results in modulation of autoantibody-mediated anaemia and may be considered as a possible treatment for autoimmune diseases that involve phagocytosis as a pathogenic mechanism.

Autoimmune thrombocytopenia

To further analyse this effect of viral infections that could be relevant to human pathology, a new model of autoimmune disease, against platelets, was developped (11). 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 kDa mouse platelet antigen recognized in CBA/Ht, but not in BALB/C animals. This immunization protocol may serve as a model of anti-platelet autoimmune response, and especially of post-transfusion purpura.

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SIGNAL TRANSDUCTION AND GENE REGULATION BY PLATELET-DERIVED GROWTH FACTORS

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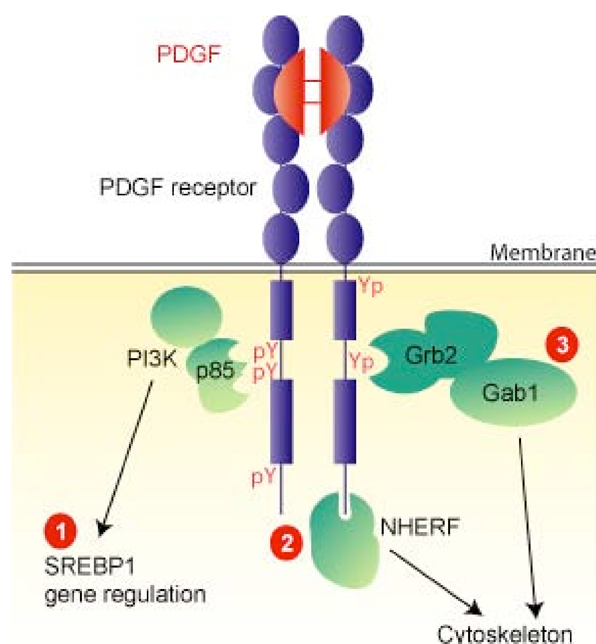
Platelet-derived growth factors (PDGF) are hormone-like proteins that stimulate cell proliferation and migration during wound healing and the development of the embryo and. PDGF acts through two specific receptor tyrosine kinases, the PDGF a- and b-receptors, which are targeted by several anticancer drugs. Uncontrolled PDGF production or receptor activation is indeed associated with the development of cancers such as dermatofibrosarcoma, glioblastoma and certain types of leukemia.

The growth factor group, which started in collaboration with Prof. Carl-Henrik Heldin at the Ludwig Institute for Cancer Research (Uppsala, Sweden), focuses on the mechanism of action of PDGF. The cytoplasmic domain of the PDGF receptors contains a tyrosine kinase domain that is activated upon PDGF binding and phosphorylates signal transduction molecules. Our present lines of research are : (1) the role of SREBP transcription factors in gene regulation by PDGF in fibroblasts and tumor cells, (2) the regulation of the PDGF b-receptor activity by its C-terminal tail and the NHERF protein, and (3) the role of Gab1 in PDGF signal transduction and the reorganization of the cell cytoskeleton, as illustrated below.



Gene regulation by PDGF

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 been previously 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, and their expression correlated with increased membrane lipid production. 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. 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 tyrosines that bind p85. Fibroblast growth factors (FGF-2 and FGF-4) mimicked the effects of PDGF on NIH3T3 and human fibroblasts. In conclusion, our results suggest that growth factors induce membrane lipid synthesis via the activation SREBP and PI3K (1). The role of SREBP in growth factor signaling and tumour development will be further analyzed in the near future.

Regulation of the PDGF receptor kinase activity by its C-terminal tail and NHERF

In an effort to better understand the activation of the PDGF receptor tyrosine kinase, we investigated the role of the C-terminal tail of the PDGF β-receptor in the control of the receptor kinase activity. First, we showed that NHERF is recruited to the C-terminal end of the receptor (2). By contrast to published data, NHERF binding did not modify the receptor activity. 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 proline- and glutamic acid-rich motif, increased the activity of the receptor in the absence of ligand, compared to wild-type receptors (3). 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 presented 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.

Role of Gab1 in PDGF signal transduction

Gab1 is a scaffolding/docking protein that has been suggested to play a role in signal transduction downstream of platelet-derived growth factor (PDGF) receptors. We found that PDGF induced a rapid Gab1 phosphorylation, which depended on the recruitment of Grb2, indicating that Grb2 acts as a bridge between Gab1 and the PDGF β-receptor. PDGF also enhanced the binding of Gab1 to the phosphatase SHP-2, but not to p85. To further study the role of Gab1 in PDGF signaling, we transfected porcine aortic endothelial cells with a doxycyclin-inducible Gab1 construct. Increased Gab1 expression enhanced the recruitment and activation of SHP-2, as well as the phosphorylation of the mitogen-activated protein kinases Erk and p38 by PDGF. Gab1 expression also enhanced the formation of lamellipodia and cellular protrusions. In Gab1-deficient mouse embryonic fibroblasts, the same phenotype was induced by restoring the expression of wild-type Gab1, but not a mutant Gab1 that was unable to associate with SHP-2. Finally, Gab1-deficient fibroblasts showed a decreased chemotactic response towards gradients of PDGF as compared to wild-type cells. In conclusion, Gab1 plays a selective role in regulation of the mitogen-activated protein kinases Erk and p38 downstream of the PDGF β-receptor, and contributes to cytoskeletal reorganization and chemotaxis in response to PDGF (4).

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HUMAN TUMOR IMMUNOLOGY*

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

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 (1-5). 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 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 (3, 6-8).

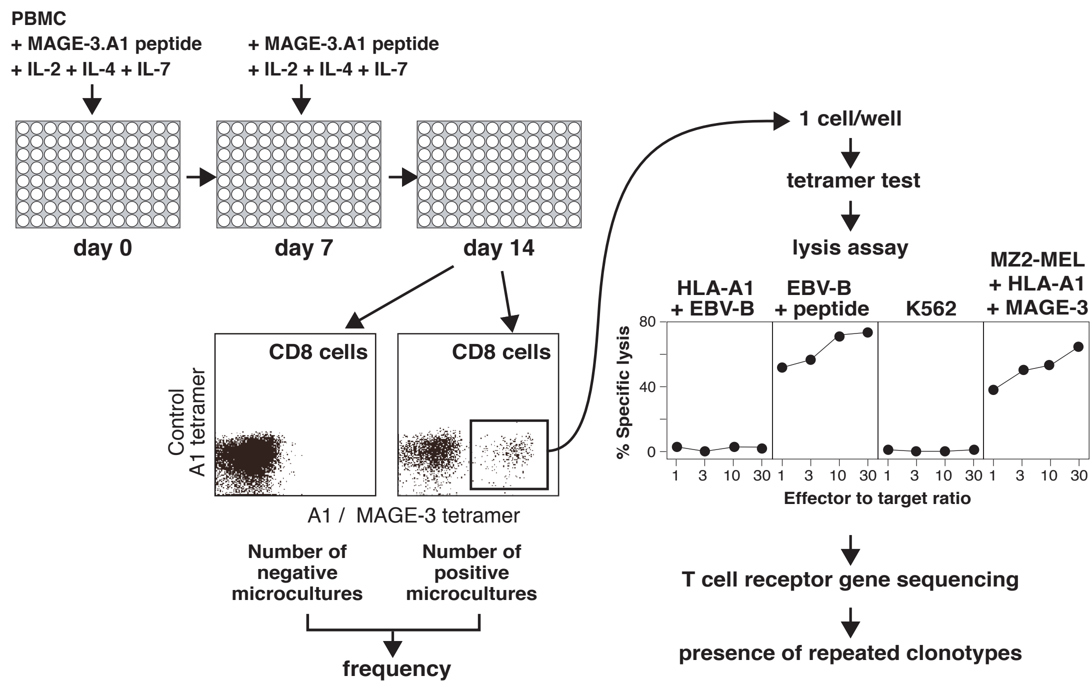

Figure 1. Overview of the procedure to evaluate CTL responses of vaccinated patients. Typically, 250,000 peripheral blood mononuclear cells (PBMC) were distributed in each microwell.

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 non-cancerous individual. Such a study was undertaken by the group of P. van der Bruggen and the results indicate that the frequency of the naïve anti-MAGE-3.A1 CTL precursors is about $4x10^{-7}$ of the blood CD8 T lymphocytes (9).

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.

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

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.

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 (10). 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 2.5×10^{-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 9×10^{-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.

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.

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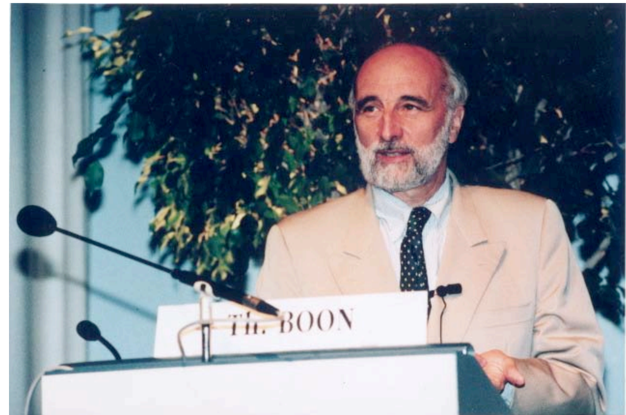
Ludwig Institute for Cancer Research (LICR),

Brussels branch

The Ludwig Institute for Cancer Research

Cancer is a major concern in human health. The prospects for bringing cancer under control require linked innovative basic and clinical research. In this view, Daniel K. Ludwig created in 1971 the Ludwig Institute for Cancer Research, an international organization bringing together scientists and clinicians from around the world. Ludwig investigators are recognized leaders in many areas of science, involving genetics, bioinformatics, immunology, virology, cell biology and signal transduction.



Thierry Boon

Faithful to the organizing principles laid down by Mr Ludwig, the Institute conducts its research through ten Branches, located in seven countries. The Branch structure allows the Institute to interact with a number of different research and clinical environments. Each Branch is focused on a research program defined by the Branch Director in relation with the overall objectives of the Institute. The Branches are established in association with University Hospitals, to stimulate close collaborations between research laboratories and the clinic. By organizing and controlling its own clinical trials programs, the Institute has indeed created a continuum that integrates laboratory and clinical research.

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The central research theme of our group is the study of tumor antigens recognized by T lymphocytes. Besides our continued effort to identify additional antigens of interest, we mainly want to address a number of fundamental or mechanistic issues that have a direct impact on the utilization of such antigens as cancer vaccines in human patients. These antigens 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". We are currently studying the processing of several human tumor antigens by the proteasome, and we are particularly interested by the processing differences we have observed between the standard proteasome, which is present in most cells, and the immunoproteasome which is found in some dendritic cells and in cells exposed to interferon-gamma.

We have also recently described a new aspect of the proteasome function in antigen processing, which is the production of antigenic peptides made of two non-contiguous segments. The production of such peptides involves the excision of a peptidic segment and the splicing of the two fragments. We showed that this excision and splicing process is done by the proteasome, presumably by a mechanism of transpeptidation. The study of additional peptides produced by splicing is ongoing.

We are also studying a mouse preclinical model of cancer immunotherapy, where we try to define the optimal conditions to induce effective anti-tumor responses by various vaccination approaches against defined antigens. This led 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 frequently expressed in tumors. The resulting local tryptophan shortage appears to prevent the proliferation of lymphocytes at the tumor site. Treatment of mice with inhibitors of indoleamine-2,3 dioxygenase can counteract this tumor resistance mechanism. These results suggest that a treatment based on the inhibition of this enzyme could be combined with approaches of cancer immunotherapy in order to increase the tumor response rate.

To obtain the most relevant information from such preclinical models, we are building a new mouse melanoma model where tumors expressing a given antigen can be induced, using a transgenic system based on Cre-lox recombination. This should recapitulate the long-term host/tumor relationship that occurs in humans when a tumor slowly develops within a normal tissue.

Differential processing of tumor antigens by the standard proteasome and the immunoproteasome

J. Chapiro, V. Stroobant, B. Guillaume, W. Ma, F. 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 combination of antigens in order to minimize the risk of tumor escape by proteasome switching.

An antigenic peptide produced by peptide splicing in the proteasome

N. Vigneron, V. Stroobant, J. Chapiro (in collaboration with A. Ooms and G. Degiovanni, Université de Liège, Belgium)

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. This is done in collaboration with two research groups from USA.

Identification of new antigens recognized by autologous CTL on human melanoma

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

Melanoma patient EB81 was vaccinated with a MAGE-type antigen and showed regression of all cutaneous metastases. Blood lymphocytes collected after the regression were stimulated with autologous tumor cells, and CTL clones were obtained. Surprisingly, none of these clones was directed against the antigen used for vaccination. Using a cDNA expression cloning approach, we identified the antigens recognized by three of them. These antigens correspond to three distinct peptides all derived from *MAGE-C2*, a gene with a cancer-germ-line expression pattern, which is expressed in about 40% of melanomas and 30% of bladder carcinomas. Two peptides are presented by HLA-A2, and one by HLA-B57 (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 (6). 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.

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

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

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

We have observed that many human tumors express IDO in a constitutive manner (7). To determine whether IDO expression provides tumor cells with a survival advantage by allowing

their escape from immune rejection in vivo, we used the well-characterized model system of mouse tumor P815, where the antigen encoded by gene P1A is the major target of the rejection response. We observed that expression of IDO by P815 tumor cells prevents their rejection by pre-immunized mice. This effect can be partly reverted by systemic treatment of mice with an inhibitor of IDO, in the absence of noticeable toxicity. 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

I. Huijbers (in collaboration with P. Krimperfort (NKI, Amsterdam) and A.-M. 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* locus, which encodes two distinct tumor suppressor proteins $p16^{INK4A}$ and $p14^{ARF}$. Genetic disruption of this locus 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 generated transgenic mice in which the deletion of the *Ink4a/Arf* genes 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 locus 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-2d 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

B. Lauwerys (in collaboration with F. 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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GENES EXPRESSED IN CANCER AND GERMLINE CELLS

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Human tumors express specific antigens arising from the activation of genes, such as MAGE genes, 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. We are trying to identify new genes that present the same pattern of expression as MAGE genes. Additionally, efforts are devoted to determining both the function of "cancer-germline" genes, and the mechanisms leading to their activation in tumors.

Isolation and characterization of new cancer-germline genes

Screening procedures based on differential expression profiling allowed the isolation of several genes with cancer and germline specific expression (1-4). Most of these genes are located on the X chromosome, and have their normal site of expression in spermatogonia, the pre-meiotic stage of sperm development. This restricted expression is likely related to the chromosomal location of these genes, as the X chromosome is inactivated in post-meiotic sperm cells.

MAGE-A1 acts as a transcriptional repressor

MAGE-A1, the first characterized cancer-germline gene, belongs to a family of twelve genes located on the X chromosome in region q28 (5,6). To analyze the functions of MAGE-A1, we searched to identify protein partners of this protein. Using yeast two-hybrid screening, we found interaction between MAGE-A1 and transcriptional regulator SKIP. SKIP is an adaptor protein that connects DNA-binding proteins such as Smad3, the Vitamin D receptor, CBF1 or MyoD, to proteins that activate or repress transcription. A repression complex including histone deacetylases (HDAC) is known to bind to SKIP and CBF1. In the presence of the intracellular part of Notch1 (Notch1-IC), the repression complex is detached from SKIP by Notch1-IC and recruitment of an activation complex including histone acetyltransferases (HAT) is facilitated by SKIP. To examine whether MAGE-A1 could interfere with a signalling pathway involving SKIP, we expressed MAGE-A1 in mammalian cells in which Notch1-IC binds to SKIP fused to the Gal4 DNA-binding domain and activates a *CAT* reporter gene containing Gal4-binding sites near its promoter (Fig. 1).

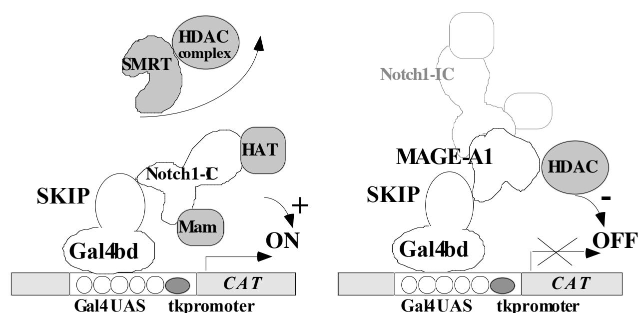

Figure 1. MAGE-A1 counteracts Notch1-IC transactivation and recruits histone deacetylases

We found that MAGE-A1 inhibited the Notch-1/SKIP transcriptional activation. Deletion analysis indicate that binding to SKIP is required to observe MAGE-A1-mediated repression of Notch1-IC transactivation. Moreover, MAGE-A1 was found to actively repress transcription by binding and recruiting histone deacetylase 1. Our results suggest that by binding to SKIP and by recruiting histone deacetylases, MAGE-A1 protein present in the nucleus could repress genes implicated in development and spermatogenesis. We are now trying to identify the genes that are regulated by MAGE-1 by using an inducible transfected *MAGE-A1* gene and the microarray technology.

Genome hypomethylation in the activation of cancer-germline genes in tumors

Studies on the transcriptional regulation of cancer-germline genes, such as *MAGE-A1,* showed that CpG methylation is an essential component of their repression in normal somatic tissues (7,8). Activation of these genes in tumors is associated with the genome-wide DNA demethylation process which often accompanies tumorigenesis (9). Recently, we found that hypomethylated CpGs in tumors are not randomly distributed but are clustered into specific regions such as the promoter region of *MAGE-A1* (10). This site-specific hypomethylation is not due to a persistent targeted demethylating activity, but appears to result from a transient overall demethylation process followed by a permanent local inhibition of remethylation due to the binding of transcriptional activators (Fig. 2).

Using the antisense oligonucleotide technology, we are currently trying to identify the enzymes that are normally involved in maintaining CpG methylation within cancer-germline genes, with a special emphasis on the DNA methyltransferases DNMT1, DNMT3a and DNMT3b.

These studies should bring insight into the processes that lead to DNA methylation changes in cancer. Moreover, understanding the mechanisms that activate cancer-germline genes may help designing procedures to induce the expression of specific antigens on tumors, thereby facilitating their elimination by the immune system.

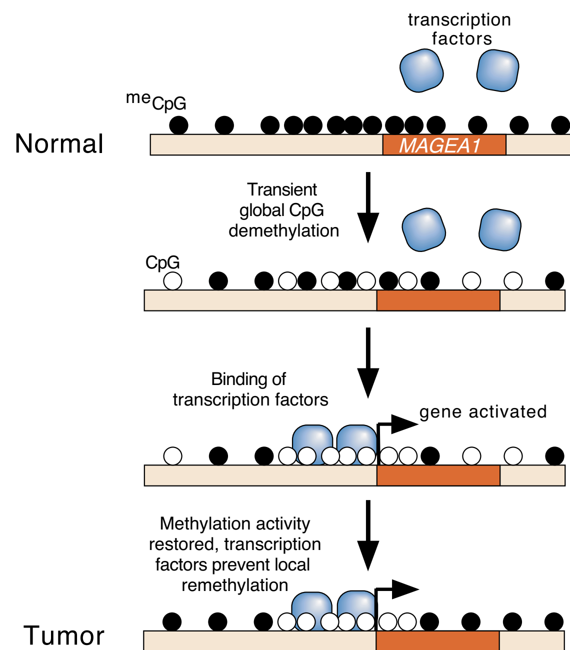

Figure 2. Model for the stable activation of *MAGE-A1* in tumors.

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IDENTIFICATION OF HUMAN TUMOR ANTIGENS

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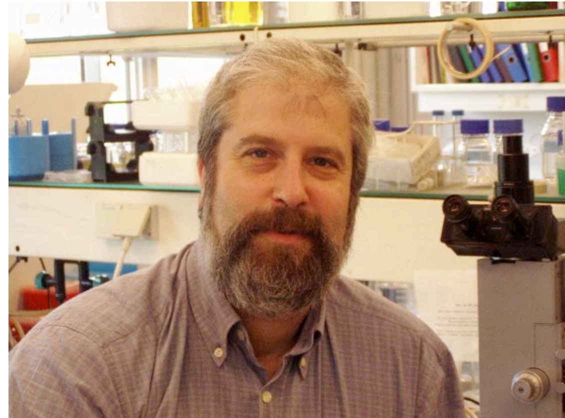
Bénédicte TOLLET, Technician



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. The monitoring of the anti-vaccine T cell frequencies in patients vaccinated with protein is even more complex. The responses could be directed against a large number of HLA-peptide combinations, including many that are presently unknown. The group has undertaken a systematic effort to develop a reproducible monitoring approach with high specificity and sensitivity. The group is also involved in the study of functional defects of T cells.

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

Y. Zhang, V. Stroobant, Z. Sun, S. 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). 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 (2). As a result, a vast number of sequences are known that can code for tumor-specific shared antigens, but most of the encoded antigenic peptides have not been identified yet. The identification of a large number of antigenic peptides presented by HLA class I and class II molecules is likely to be important for the future of clinical trials with defined antigenic peptides. A large set of peptide/HLA combinations will alleviate HLA restriction and widen the set of eligible patients. It will also facilitate the design of concurrent immunizations against several antigens. Such immunizations could increase the primary anti-tumor efficacy of the vaccine and also decrease the risk of tumor escape by loss of antigen expression.

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

We stimulated CD8⁺ T lymphocytes with dendritic cells transduced with viral vectors containing complete MAGE-coding sequences. As this requires the processing of the antigen by the dendritic cells, we surmised that the peptides that would be identified would also be processed in the tumors expressing the MAGE genes. A difficulty of the use of recombinant viruses is the activation of CTL precursors directed against viral antigens. We circumvented this problem by using different vectors for the stimulation of the

microcultures, for the lytic assay with the responder T cells, and for the cloning step. This procedure is summarized in 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, 5). After three weekly stimulations, the responder cells were tested for lysis on autologous EBV-B cells infected with vaccinia-MAGE. Positive microcultures were cloned. To identify the antigenic peptide, the resulting CTL clone was tested for lysis of autologous EBV-B cells pulsed with a complete set of peptides of 16 amino acids that overlap by 12. When a peptide scored positive, shorter peptides were synthesized to identify the shortest optimal peptide. To identify the HLA presenting molecule, the CTL clones were tested for stimulation by cells transfected with the MAGE cDNA together with cDNAs coding for the possible HLA presenting molecules. Finally, relevant tumor targets were used in a lysis assay to ascertain that the antigen was also processed by tumor cells.

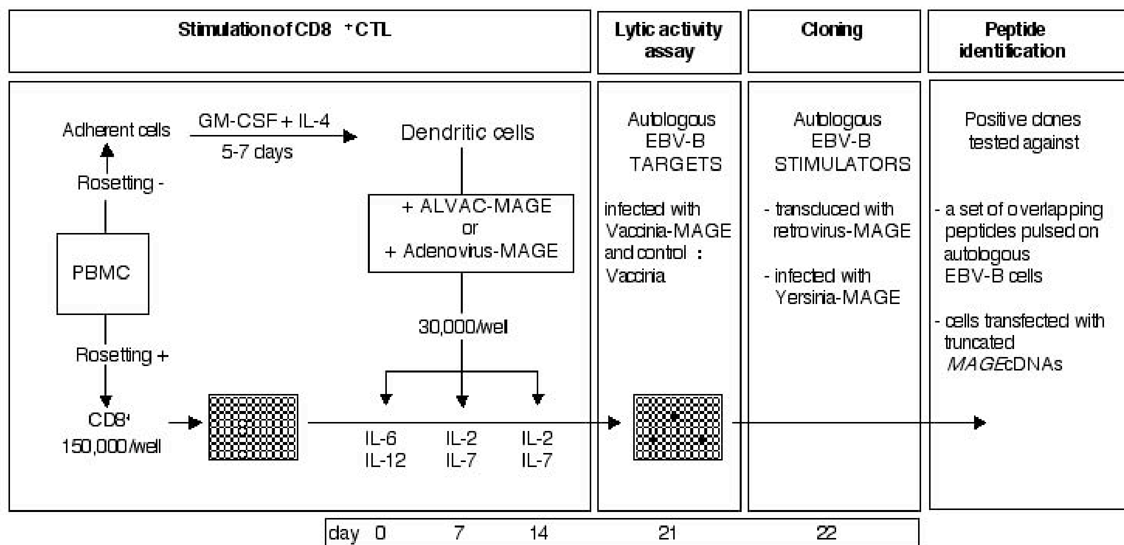


Figure 1. Overview of the procedure to obtain anti-MAGE CD8+ CTL clones by stimulation with dendritic cells infected with viral vectors carrying a MAGE coding sequence.

We have listed in a database class I-restricted antigenic peptides that are encoded by cancer-germline genes (http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm). MAGE-1 and MAGE-3 antigenic peptides are available for more than 90% of Caucasians.

We have identified a MAGE-3.B40 antigen which is the first example of a tumor-specific antigen exclusively presented by tumor cells expressing the immunoproteasome (6). This work was done in collaboration with the group of Benoît Van den Eynde.

We have found that MAGE-1 peptide SAYGEPRKL is recognized by CTL clones that are restricted either by HLA-Cw3, Cw6 or Cw16 (3). The presentation of the same peptide by different HLA molecules may be frequent for HLA-C molecules, because they are more closely related to each other in the peptide-binding region than HLA-A and B molecules. But for HLA-A and B also this may occur more frequently than usually thought. MAGE-3 peptide MEVDPIGLY is presented to different CTL by HLA-B*4402, B*4403, and B*1801 (3). MAGE-1 peptide EADPTGHSY was found to be recognized by different CTL on HLA-A1 and B35 molecules, and to bind to HLA-A29 (3). The same was found for the MAGE-3 homologous peptide EVDPIGHLY.

These results have consequences for the monitoring of the immune response of patients vaccinated with such tumor-specific shared peptides. A number of HLA-A1 patients were

injected with MAGE-3.A1 peptide EVDPIGHLY, at a time when we did not know that it could be presented by B35 and A29 (3). The immune response was evaluated with HLA-A1 tetramers folded with the MAGE-3 peptide. Thus, A29 or B35-restricted responses against the peptide may have been missed.

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 (7). 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. The procedure is summarized in Figure 2.

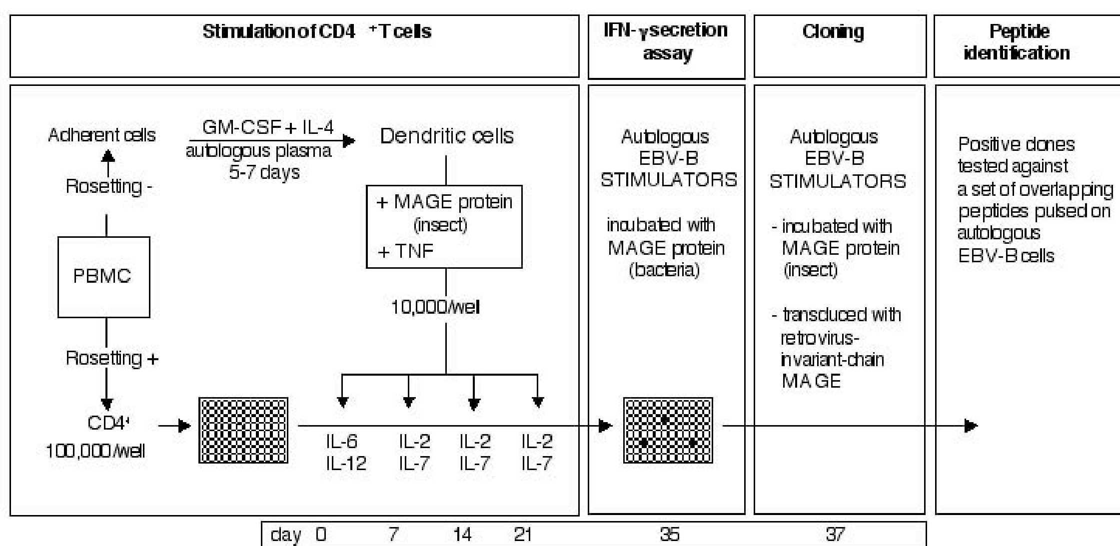


Figure 2. Overview of the procedure to obtain anti-MAGE CD4+ T cell clones by stimulation with dendritic cells loaded with a whole protein.

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. Because a large number of the CD4+ T cells that were obtained in our first experiments appeared to be directed against bacterial contaminants, we chose to alternate the sources of protein used at the various stages of the procedure. For example, to stimulate the lymphocytes, we used a MAGE protein produced in insect cells, and to test the specificity of the responder lymphocytes, we used a protein produced in bacteria. Microcultures that specifically produced IFN-γ after stimulation with the MAGE protein were cloned by limiting dilution using autologous EBV-B stimulator cells either loaded with the MAGE protein used during the stimulation step, or transduced with a retroviral construct encoding a truncated human invariant chain (Ii) fused with the MAGE protein.

MAGE-1 and MAGE-3 antigenic peptides identified by this procedure are listed in a database (http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm) (8). They include a MAGE-3 peptide presented by HLA-DP4, which is expressed by more than 70% of Caucasians. This peptide could not have been found by a peptide stimulation approach because

no consensus anchor residue was known for HLA-DP4.

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

P. van der Bruggen (in collaboration with L. van de Corput from Leiden University, and P. 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 (6).

The normal anti-MAGE-3.A1 repertoire

S. Ottaviani, C. Wildmann (a project started by C. Lonchay)

Cancer-germline gene *MAGE-3* codes for tumor-specific antigens recognized on many tumors by T lymphocytes. A MAGE-3 antigen presented by HLA-A1 has been used in several vaccination trials on metastatic melanoma patients. Only a small minority of patients have shown evidence of tumor regression. Attempts to correlate the tumor rejections with the CTL response against the vaccine have been hampered by the low level of these responses. To estimate not only the frequency but also the T cell receptor diversity of the naive precursors directed against MAGE-3.A1, we developed an approach aimed at obtaining a large number of CTL clones from very low frequency precursors. More than three billion peripheral blood mononuclear cells (PBMC) were obtained from several blood samples of a non cancerous individual. An HLA-A1/MAGE-3 tetramer was used to label large numbers of T cells and the positive cells were enriched by magnetic sorting. The selected fraction was then passed through a flow cytometer to further enrich the A1/MAGE-3 tetramer-positive cells among the CD8 cells. A number of positive cells were cloned. A fraction of the proliferating clones were tetramer-positive and showed lytic activity on cells pulsed with the peptide. These CTL clones invariably lysed the melanoma cells also, indicating that their avidity was high enough to recognize the level of antigen expressed by tumor cells. This led to an estimate of $4x10^{-7}$ of the CD8 T cells for the anti-MAGE-3.A1 CTLp in this individual (9). To evaluate the diversity of the naive anti-MAGE-3.A1 repertoire, we examined the TCR sequences of the 24 lytic 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. The results indicate that it is very likely that the repertoire comprises more than 100 clonotypes. On this basis it is possible to use not only the frequency of CTL precursors in the blood but also the presence of dominant clonotypes to ascertain in patients the existence of anti-MAGE-3.A1 responses as low as 10^{-6} of CD8.

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

N. Demotte, S. Ottaviani, C. Wildmann

We have observed that human CTL clones lose their specific cytolytic activity and cytokine production under certain stimulation conditions, while retaining an antigen-dependent growth pattern. 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 (10). The tetramer-negative cells recover tetramer staining and cytolytic activity after stimulation with tumor cells in the presence of a supernatant of activated lymphocytes. Our results suggest the existence of a new type of functional defect of CTL. They also indicate that tetramers may fail to reveal some CTL bearing the relevant TCR, even when such functionally arrested CTL retain the potential to participate in immune responses because their defect is reversible. We will analyze the ability of CTL to be labelled by tetramer during the days following

the antigenic stimulation. We also plan to analyze the composition of lipid rafts of tetramer-positive and tetramer-negative cells. In addition, we will analyze tetramer-positive and tetramer-negative cells for differential expression of genes by microarray analyzes.

Detection of anti-vaccine CD4 T cell response in vaccinated patients

Y. Zhang, N. Renkvist, Z. Sun, H. Nicolay, S. Ottaviani, in collaboration with D. 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. HLA class II tetramers have been more difficult to obtain than HLA class I tetramers. Didier Colau has recently succeeded in obtaining a DP4.MAGE-3 tetramer, which was produced in insect cells. It stained specifically relevant CD4 clones. We have evaluated the anti-vaccine T cell frequencies in melanoma patients, who have been injected with dendritic cells pulsed with the MAGE-3 peptide presented by HLA-DP4. Blood cells from vaccinated patients were labeled *ex vivo* with the multimers and the multimer-positive CD4 T cells were sorted by flow cytometry, multiplied in clonal conditions and tested for their specific recognition of the MAGE-3 antigen. In one patient, the frequency of anti-MAGE-3.DP4 T cells peaked at 1/1,400 of the CD4 T cells, representing at least an increase of 3,000-fold of the frequency found before immunization. TCR analysis of the anti-MAGE-3.DP4 clones from this patient established that the response was highly polyclonal.

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. The monitoring of the anti-vaccine T cell frequencies in patients vaccinated with protein is complex. The responses could be directed against a large number of HLA-peptide combinations, including many that are presently unknown. Therefore, the tetramer approach used for the detection of CD8 or CD4 is not appropriate. We have undertaken a systematic effort to develop a reproducible monitoring approach with high specificity and sensitivity. It combines sorting of living blood T cells producing IFN-γ after a short antigenic stimulation and detailed functional analyses of the cells amplified in clonal conditions. Using this approach, we have estimated the anti-vaccine CD4 T cell frequencies in the five patients who showed tumor regressions after injection of a MAGE-3 protein without adjuvant. Blood cells were stimulated overnight with autologous dendritic cells loaded with protein MAGE-3. The IFN-γ-secreting CD4 T cells were sorted by flow cytometry, multiplied in clonal conditions and tested for their specific recognition of MAGE-3. Anti-MAGE-3 CD4 T cells were detected in one patient after vaccination. Surprisingly the 13 anti-MAGE-3 clones, which corresponded to five different TCR clonotypes, recognized the same peptide presented by HLA-DR1. 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 were confirmed in experiments where, instead of the protein, the peptide was used to stimulate the blood cells. The frequency of the dominant CD4 clonotype was also confirmed by a direct PCR evaluation on cDNA extracted from blood cells. The blood cells of the four other clinical responders were screened using our specific and sensitive monitoring approach, but no anti-MAGE-3 T cell was detected. We are now defied to explain why several patients with evidence of tumor regression following vaccination have no detectable anti-vaccine T cells in their blood.

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THERAPEUTIC VACCINATION OF CANCER PATIENTS WITH TUMOR-SPECIFIC ANTIGENS

Marie MARCHAND, Senior Clinical Investigator

Nicolas van BAREN, Clinical Investigator
Jérôme DEGUELDRE, Clinical Research Associate
Christian VERFAILLE, Project Manager
Sylvie HOPPE, Secretary

Tumor cells carry antigens such as MAGE antigens that are absent from normal tissues, and that can be targeted by cytolytic T lymphocytes (CTL) (1). 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 timepoints 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 330 patients have been included in these multicentric 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 on a 4 week basis to 5 patients, without any evidence of melanoma 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 a future clinical trial, the MAGE-3.A1 peptide will be mixed with an immunostimulatory CpG-containing oligonucleotide to try to increase its immunogenicity. This new and promising adjuvant activates antigen presenting cells after binding to Toll-like receptor 9, and is thought to enhance CTL responses.

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 complete response lasting for more than 1 year. 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. 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 20 %) show regression of metastatic lesions following immunization, whatever the MAGE vaccine used. About 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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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 tetrameric 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 3×10^7 PBMC of normal donor and they were highly specific for the given TCR insofar as no product was amplified with cDNA prepared from 3×10^7 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, that remained stable during the peptide vaccinations, rose to 1.1×10^{-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. 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 $\sim 3.5 \times 10^{-5}$ 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 and ~ 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.

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 3.4×10^{-7} before vaccination to 9.3×10^{-7} after 3 vaccinations, to 2.6×10^{-6} after 6 vaccinations and 5.4×10^{-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 2.2×10^{-4} before and 2×10^{-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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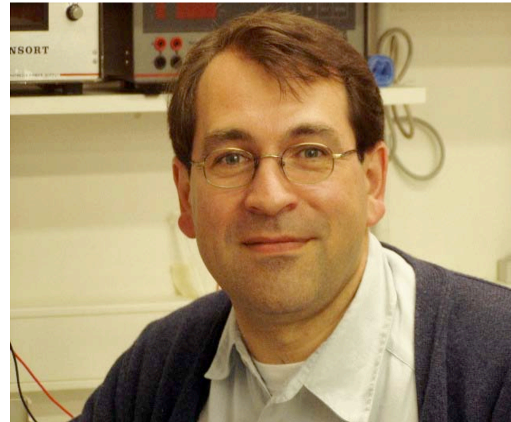

The cytokine group studies the biology of Interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is a TH2 cytokine that plays a role in immune responses against intestinal parasites and asthma. IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, upregulates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

J.-C. Renauld, J. Louahed, L. Knoops, V. Steenwinckel, B. de Lestré, M. Stevens, E. 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 and in the lungs of IL-9 transgenic mice. This observation is reminiscent of mice that are prone to the development of diseases that are characterized by the production of autoantibodies, such as Systemic Lupus Erythematosus, and suggests that IL-9 might play a role in some autoimmune processes (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. Current studies indicate that IL-9 promotes sthma through both IL-13-dependent and IL-13-independent pathways. 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. Phase I clinical trials using anti-IL-9 antibodies produced in our laboratory have been initiated in collaboration with Medimmune.

IL-9 receptor and signal transduction

J.-C. Renauld, L. Knoops, M. Stevens, E. 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.

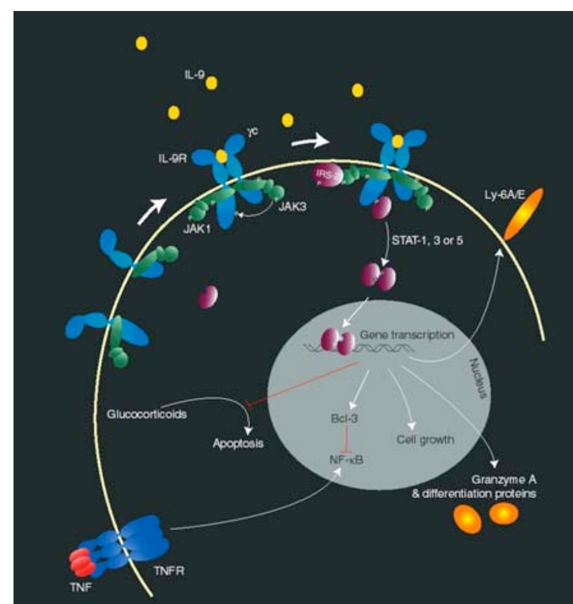

Figure 1.

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

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

Incidentally, our studies of this particular model of the regulation of cell death by cytokines, lead them to purify another protein called I-309, originally described as a human chemotactic factor, and that turned out to exert a significant anti-apoptotic activity for thymic lymphomas (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

J.-C. Renauld, J. Van Snick, L. Dumoutier, J. Louahed, L. Knoops, A. Tounsi, M. Stevens, E. 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) and protection against experimental heatitis (L. Dumoutier, unpublished results). Recombinant human IL-22 was produced (with D. Colau, LICR) and its crystallograhic structure solved. Despite its structural homology with IL-10, IL-22 fails to recapitulate any of IL-10 biological activities.

Analysis of the genome databases leads 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.

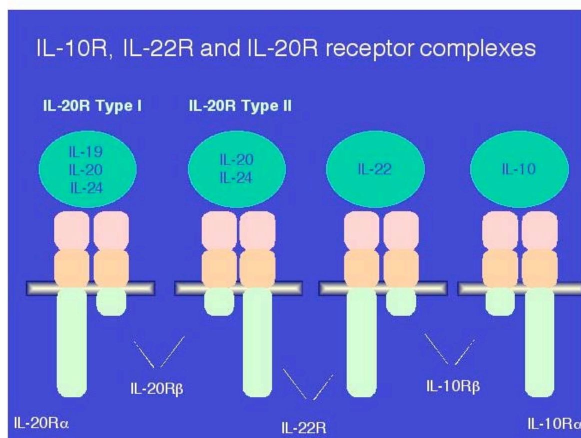

Figure 2.

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-20R ß, the second short chain member of the IL-10R-related receptor family. This complex mediates STAT-1 and –3 activation by IL-20 and IL-24, but not by IL-22 (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 (see also ref. 12 for a review of this new cytokine family).

LICR2: a new cytokine receptor mediating antiviral activities

J.-C. Renauld, L. Dumoutier

Type II cytokine receptors include receptors for type I and Ii interferons (IFNs) and for IL-10-related cytokines. These transmembrane proteins are almost exclusively related by their extracellular part, which consists of tandem fibronectin type II domains, whereas the cytoplasmic domain is associated with a tyrosine kinase of the Janus Kinase (JAK family).By screening genomic databases for similarity with the extracellular domain of these receptors, we identified a new receptor that we called LICR2 (Likely Interleukin or Cytokine receptor 2). This receptor binds new cytokines designated IFN-λ1-3, and mediates the same activities as those mediated by the receptors for IFN-α and ß, including antiviral and antiproliferative activities (13), raising the possibility of therapeutic applications in viral infections and cancer.

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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. We also study the mechanisms by which STAT proteins become constitutively activated and how they function in transformed hematopoietic or patient-derived leukemia cells.

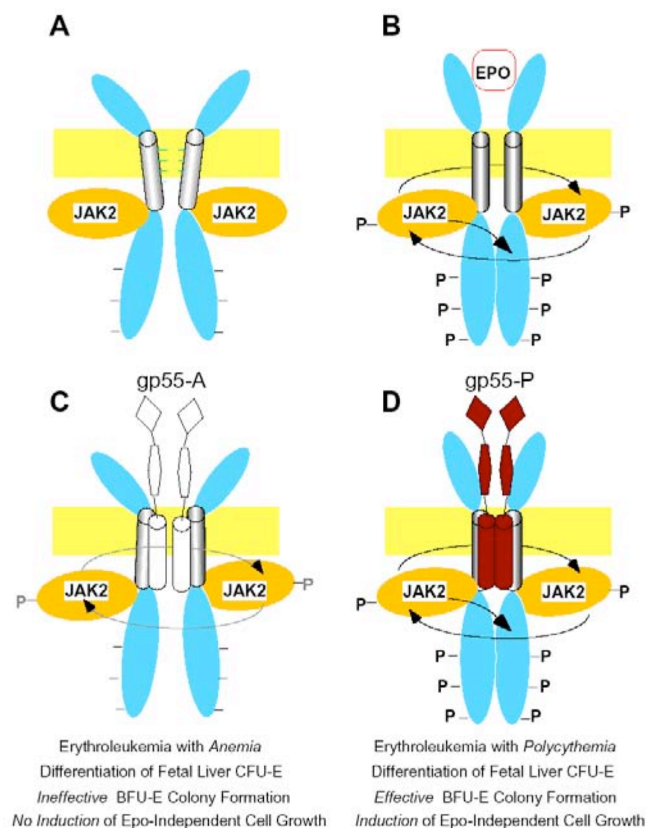
Determination of the interface and orientation of the activated erythropoietin receptor dimer

N. Seubert, Y. Royer, J. Staerk, K. Kubatzky, V. Moucadel

The determination of the interface of the active erythropoietin receptor (EpoR) dimer has been a priority during the last year (1). 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 (2). 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 (3). In collaboration with Lily Huang and Harvey Lodish, Whitehead Institute, Cambridge, MA, USA, we have identified a number of key residues in the EpoR cytosolic JM domain, which are required for switching on the activity of JAK2 and initiate signaling (4).

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 (1).
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 (1). 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.



(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)*.

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

K. Kubatzky, J. Staerk, M. Li, A. Dusa

To define the interfaces of the active and inactive EpoR dimers we performed cysteine scanning mutagenesis of the extracellular juxtamembrane and TM domains (5). 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 (5). The same residues were found to be in the interface of the active coiled-coil-EpoR fusion proteins (1). 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. Due to our previous work showing that the junction between the EpoR TM and intracellular domain is rigid (3) 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

J. 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

Y. 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 on the cell surface is totally different than the one of the IL9R or the EpoR. The γ_c forms big patches whereas the IL9R and the EpoR exhibit a normal diffuse distribution, as we have shown in collaboration with Pierre Courtoy. Paraformaldehyde fixation was required for the appearance of the big patches in the case of the γ_c, suggesting that the receptor is

localized in membrane regions where formaldehylde can not block diffusion and aggregation of membrane proteins. This observation could reveal different compartmentalization of receptors on the cell-surface.

Traffic and cell-surface expression of the EpoR critically depend on the ability of EpoR to bind JAK2 intracellularly (4). In the absence of JAK2, the receptor does not get transported from the endoplasmic reticulum (ER) to the Golgi apparatus and does not acquire EndoH resistance. We have observed that, in hematopoietic cells, overexpressing 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

A. Dusa

Two transmembrane viral envelope proteins (gp55-P and gp55-A) belonging to the polycythemic (P) and anemic (A) Spleen Focus Forming Virus (SFFV) strains, can activate the EpoR when co-expressed in the same cell (6). 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

V. Moucadel, J. 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) [reviewed in 9]. 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 absence of any cytokine. 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 (12). 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 (12).

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 that allow wide expression of cDNAs at physiologic levels.

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