

Imaging of Inflammatory Regions in the Brain of Rats with Experimental Autoimmune Encephalomyelitis using High Resolution MRI

K. BAETEN^{1,2}, P. ADRIAENSSENS², F. VANDENABEELE¹, E. THEUNISSEN¹, J. GELAN² AND P. STINISSEN¹

¹ *Biomedical Research Institute (BIOMED) and* ² *Institute of Material Research (IMO), Limburgs Universitair Centrum, Diepenbeek, Belgium*

Background and Purpose: Magnetic Resonance Imaging (MRI) is widely used in multiple sclerosis (MS) to detect blood-brain barrier leakage and demyelinated regions. Recently this technique has also been used to study the pathophysiology of autoimmune demyelination in several animal models for MS. A widely utilized animal model for MS is experimental autoimmune encephalomyelitis (EAE) in rodents. Earlier studies indicated that conventional T1, T2 and spin density images (SD) were not sensitive enough to detect inflammatory regions. Ultrasmall particles of iron oxide (USPIO) are therefore used to provide the needed contrast. USPIOs accumulate in cells from the mononuclear phagocytic system. The goal of this study was to identify the optimal parameters for the detection of inflammatory sites in both the brain and the spinal cord of EAE rats using USPIOs visualised by high resolution MRI (9 tesla).

Methods: Acute EAE was induced in eight week old female Lewis rats by immunisation with guinea pig myelin basic protein together with adjuvants. Injection of the contrast agent USPIO and imaging was performed at several time points during the disease. Imaging was performed at 9.4 Tesla with T1, T2 and SD weighted sequences. Afterwards, brain and spinal cord were drained into Unifix for histological examination.

Results: The sites of inflammation, as detected by histology, were clearly visible with USPIO-enhanced T2 weighted images. Optimal visualization of inflammatory infiltrates was obtained when USPIOs were injected when the first disease symptoms appear. A relative long TE (XXX ms) and short TR (XXX ms) were optimal for detection of inflammatory regions in the brain of EAE rats. A detailed characterization of the inflammation in the spinal cord however required a shorter TE (XXX ms), necessary for a clear distinction between white and grey matter in the spinal cord. Histological examination revealed the presence of perivascular cuffs and macrophages at the sites of USPIO accumulation in the white matter of the brain.

Conclusion: We determined the optimal imaging parameters for the identification of USPIO accumulation in brain and spinal cord of EAE rats. We revealed that the timing of USPIO injection is crucial for obtaining representative information about the inflammatory process. In conclusion, high resolution MRI (9 Tesla) can be used to detect USPIO particles in the brain and spinal cord of EAE rats, indicative for sites of inflammation.

Reduced esterase activity is an early marker of apoptosis in oligodendroglial cell lines: flow cytometry and CLSM measurements using calcein-AM and propidium-iodide

SZ. BARON^{1,2}, E. GIELEN¹, I. SMETS¹, M. VANDEVEN¹, P. STEELS¹ AND M. AMELOOT¹

¹Laboratory of Physiology, Limburgs Universitair Centrum/transnationale Universiteit Limburg, Biomedisch Onderzoeksinstituut, Diepenbeek, Belgium and

²Department of Botany, Szeged University, Szeged, Hungary

In multiple sclerosis, a chronic inflammatory disease of the central nervous system, the immune system attacks the myelin around the nerves. It is thought that lymphocytes and their products (e.g. cytokines) may be involved in this attack. This autoimmune disease leads to oligodendrocytes (OLG) injury and loss. OLGs are often lost via the apoptotic cell death pathway (BURGMAIER *et al.*, 2000). The aim of this study was to develop a reliable protocol for flow cytometry to detect early apoptosis in OLG.

Apoptosis was induced in HOG and OLN-93 oligodendroglial cell lines by the application of the cytokines TNF- α and INF- γ or staurosporine (RAINE, 1995, BERTRAND *et al.*, 1994). Well-accepted fluorescence-based apoptosis detection methods, such as annexin-V-FITC/propidium-iodide (VERMES *et al.*, 1995), JC-1 (COSSARIZZA *et al.*, 1993) and the TUNEL method (NEGOESCU *et al.*, 1998) were compared with a new assay: the combination of calcein-AM and propidium-iodide. Confocal and standard fluorescence microscopy at 37 °C were used to verify the results obtained by flow cytometry.

Reduction of the cytosolic esterase activity, as measured by the loss of calcein fluorescence, and a decrease of mitochondrial membrane potential, as detected by JC-1 occurred simultaneously in the early phase of apoptosis in OLGs. Both events preceded DNA strand breaks. Changes in plasma membrane asymmetry, as reflected by annexin-V-FITC, occurred rather late in the apoptotic process. Furthermore, the combination of calcein-AM and propidium iodide assay is suitable for obvious differentiation of apoptotic cells from living and necrotic cells, unlike JC-1 or the TUNEL method.

Consequently, calcein-AM can be used as a reliable marker of rather early processes in the apoptotic pathway in OLGs. As the translocation of phosphatidylserine occurs rather late in the apoptotic process the annexin-V-FITC assay might underestimate the amount of apoptosis.

This work was financially supported by a bilateral research collaboration program between Flanders and Hungary.

References

- BURGMAIER, G., SCHONROCK, L. M., KUHLMANN, T., RICHTER LANDSBERG, C. AND BRUCK, W. (2000) *J Neurochem* **75**: 2270-2276.
- RAINE, CS., (1995) *Nat Med* **1**: 211-214.
- BERTRAND, R., SOLARY, E., O'CONNER, P., KOHN, K. W., AND POMMIER, Y. (1994) *Exp Cell Res* **211**: 314-321.
- VERMES, I., HAANEN, C., STEFFENS-NAKKEN, H., AND REUTELINGSPERGER, C. (1995) *J Immunol Methods* **184**: 39-51.
- COSSARIZZA, A., BACCARANI-CONTRI, M., KALASHNIKOVA, G., AND FRANCESCHI, C. (1993) *Biochem Biophys Res Commun* **197**: 40-45.
- NEGOESCU, A., GUILLERMET, C., LORIMIER, P., BRAMBILLA, E., AND LABAT-MOLEUR, F. (1998) *Biomed Pharmacother* **52**: 252-258.

Interferon-alpha response in the central nervous system during viral infection.

S. DELHAYE AND T. MICHIELS.

Christian de Duve Institute of Cellular Pathology, Université Catholique de Louvain, MIPA-VIRO 74-49, 74, avenue Hippocrate, B-1200, Brussels, Belgium.

Interferon(IFN)-alpha belongs to the type-I IFNs cytokines family which were identified by their ability to protect cells against viral infection. In the periphery, the major IFN-alpha/beta producers were identified as plasmacytoid dendritic cells¹. Dendritic cells are considered to be absent from the central nervous system (CNS) parenchyma. Little is known about induction of IFNs type-I response in this organ.

In this work, we tried to identify, in the context of the CNS, the cells that produce IFN-alpha and the cells that can respond to type-I IFNs. As model of brain infection, we used the neurovirulent strain (GDVII) of Theiler's virus, which induces an acute lethal necrotizing encephalitis in the mouse.

We first analyzed the response to IFNs, by using Mx protein as a marker. Mx protein production is specifically and strongly induced by type-I IFNs. Balb/Mx mice which express the Mx1 gene, were infected with the neurovirulent strain of Theiler's virus. We performed double immunohistochemistry using an antibody directed against Mx protein and antibodies which recognize the different CNS cell types (oligodendrocytes, astrocytes, neurons, macrophages and endothelial cells). Our data show that all cell types analyzed can respond to type-I IFNs by producing the Mx protein.

We are currently analyzing the cells producing IFN-alpha. Preliminary data show that IFN-alpha production occurs in infected area. IFN appears to be produced by infected resident cells rather than by invading non-infected inflammatory cells. The identification of producing cells is in progress.

References

1. MOTOYA et al. (2002). Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* 99 (9): 3263-3271.

PLAC24/eIF3k interacts with the human 5-HT_{7(a)} receptor

K. DE MARTELAERE, B. LINTERMANS, G. HAEGEMAN AND P. VANHOENACKER
Laboratory of Eukaryotic Gene Expression and Signal Transduction, Ghent University - UGent, K. L. Ledeganckstraat 35, 9000 Gent, Belgium.
Kim.DeMartelaere@UGent.be

One of the most recently identified serotonin receptors is the 5-HT₇ receptor. Current studies indicate that this receptor has a role in sleep and thermoregulation as well as a possible involvement in depression, anxiety, pain, schizophrenia, mood disorders and memory. Alternative splicing of the mRNA causes the formation of three isoforms in rat (5-HT_{7(a)}, (b), (c)) and in human (5-HT_{7(a)}, (b), (d)), which differ in their C-terminal sequence. The C-terminus of G-protein coupled receptors has an important role in the signaling, localisation and regulation of the receptor (VANHOENACKER *et al.*, 2000). It was therefore of interest to identify possible interacting partners for the 5-HT₇ receptor.

In order to do so, we performed a yeast two hybrid screening in which we used the C-terminus of the 5-HT_{7(a)} variant against a human fetal cDNA bank. Amongst several candidates, PLAC24 emerged as a possible interacting protein. PLAC24 is a 'Dynein-binding protein', which localizes at the membrane upon cell-to-cell contact (KARKI *et al.*, 2002). In addition, PLAC24 is also known as eIF3k which represents subunit 11 of the eukaryotic initiation of translation factor eIF3 (MAYEUR *et al.*, 2003).

Interaction of PLAC24 with the different receptor isoforms was reconfirmed in mammalian cell lines by coimmunoprecipitation. By labeling of membrane proteins before lysis and immunoprecipitation we could demonstrate that the interaction between both proteins occurs at the level of the membrane. Additionally, preliminary studies in CHO K1 cells revealed a possible relocalisation of PLAC24 from the perinuclear sites of the cell towards the membrane upon coexpression of the 5-HT₇ receptor. In addition, coexpression of PLAC24 in HEK293 cells, stably expressing the 5-HT_{7a} receptor, caused a threefold augmentation in expression levels of the receptor. In conclusion, we have provided definite evidence of an existing interaction between PLAC24 and 5-HT₇. Further studies are needed to gain more insight into the possible role of PLAC24 in regulation or signaling of the 5-HT₇ receptor.

This work was financially supported by grants from the IWT (Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen) (project 990173) and Janssen Pharmaceutica N.V., Beerse, Belgium.

References

- VANHOENACKER, P., HAEGEMAN, G., and LEYSEN, J. E. (2000) *Trends Pharmacol. Sci.* **21**: 70-77.
- MAYEUR, G. L., FRASER, C. S., PEIRETTI, F., BLOCK, K. L., and HERSHEY, W. B. (2003) *Eur. J. Biochem.* **270**: 4133-4139.
- KARKI, S., LIGON, L. A., DESANTIS, J., TOKITO, M., and HOLZBAUR, E. L. F. (2002) *Mol. Biol. Cell* **13**: 1722-1734.

PMF1N: a possible interaction partner of the human 5-HT_{7(a)} receptor?

K. DE MARTELAERE, B. LINTERMANS, G. HAEGEMAN AND P. VANHOENACKER
Laboratory of Eukaryotic Gene Expression and Signal Transduction, Ghent University - UGent, K. L. Ledeganckstraat 35, 9000 Gent, Belgium.
Kim.DeMartelaere@UGent.be

The 5-HT₇ receptor is a recently identified serotonin receptor of which current studies indicate a role in sleep and thermoregulation. A possible involvement in depression, anxiety, pain, schizophrenia, mood disorders and memory has also been postulated for this receptor subtype. As a result of alternative splicing of the mRNA, three isoforms, which differ in their C-terminal sequences, are formed in rat (5-HT_{7(a)}, (b), (c)) and human (5-HT_{7(a)}, (b), (d)). As the C-terminus of G-protein-coupled receptors plays an important role in the signaling, localisation and regulation of the receptor (VANHOENACKER *et al.*, 2000), the aim of our study was to identify potential differences in interacting proteins between the different 5-HT₇ receptor isoforms.

Using a yeast two hybrid approach, in which we used the C-terminus of the 5-HT_{7(a)} variant against a human fetal cDNA bank, we isolated a new isoform of PMF1 (which we refer to as PMF1N) as a possible partner of the 5-HT_{7(a)} receptor. PMF1 is involved in the transcriptional regulation of the spermidine/spermine N¹-acetyltransferase (SSAT) gene (WANG *et al.*, 2001). SSAT overexpression induces hypoactivity and impairment of spatial learning in mice (KAASINEN *et al.*, 2004). PMF1N differs in its C-terminal sequence from PMF1 and PMF1S, another isoform of PMF1.

Colocalisation studies of PMF1N with the different receptor isoforms demonstrated that PMF1N might be interacting differently with the three isoforms. More precisely, PMF1N colocalizes with the 5-HT_{7(a)} receptor at the cell membrane, while it mostly forms clusters with the 5-HT_{7(b)} receptor. On the other hand, it does not seem to colocalize with the 5-HT_{7(d)} receptor. Interaction between PMF1N with the 5-HT_{7(a)} receptor was reconfirmed in mammalian cell lines by coimmunoprecipitation. Taken together, these results indicate that PMF1N interacts with the 5-HT_{7(a)} receptor. Further studies are needed to dissect whether PMF1N might be interacting differently with the three receptor isoforms.

This work was financially supported by grants from the IWT (Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen) (project 990173) and Janssen Pharmaceutica N. V., Beerse, Belgium.

References

- VANHOENACKER, P., HAEGEMAN, G., and LEYSEN, J. E. (2000) *Trends Pharmacol. Sci.* **21**: 70-77.
- KAASINEN, S. K., OKSMAN, M., ALHONEN, L., TANILA, H., and JÄNNE, J. (2004) *Pharmacol. Biochem. Behav.* **78**: 35-45.
- WANG, Y., DEVEREUX, W., STEWART, T. M., and CASERO, R. A. JR (2001) *Biochem. J.* **355**: 45-49.

CREB is involved in the mitochondrial dysfunction-induced gene expression modifications and triglyceride accumulation.

A. DE PAUW*, S. VANKONINGSLOO*, A. HOUBION*, E. DELAIVE*, S. TEJERINA*, F. DE LONGUEVILLE§, P. RENARD*, M. RAES* AND T. ARNOULD*.

* : *Laboratoire de Biochimie et Biologie Cellulaire, University of Namur (F.U.N.D.P), 61 rue de Bruxelles, 5000 Namur, Belgium* § : *Eppendorf Array Technologies, 20 rue du séminaire, 5000 Namur, Belgium. e-mail : thierry.arnould@fundp.ac.be*

Numerous mitochondrial diseases result from mutations or deletions in the mitochondrial genome (1). A major consequence of a mitochondrial disorder is an inhibition of oxidative phosphorylation (OXPHOS). Among the various symptoms observed in the pathologies associated with mitochondrial dysfunction, alterations in lipid metabolism is frequently observed (2). The molecular retrograde communication is a relatively new concept dealing with organelle dysfunction that activates signaling pathways leading to gene expression modifications. We have previously shown that a signaling pathway initiated by impaired mitochondria activates CREB (cyclicAMP-responsive element binding protein), a transcription factor with pleiotropic actions in cell proliferation, cell growth and differentiation that could be involved in the modifications of gene expression in response to mitochondrial dysfunction (3). In order to study the effect of a mitochondrial activity inhibition on lipid metabolism, we set up a cellular model in which murine 3T3-L1 preadipocytes are incubated for several days with antimycin A, a complex III inhibitor of the mitochondrial respiratory chain. After 8 days of incubation with 10 nM antimycin A, 3T3-L1 phenotype is modified as cells accumulate numerous small vesicles of triglycerides. To characterize the 3T3-L1 cell response to antimycin A, we analyzed gene expression modifications with a low density DNA microarray (ADIPO-ATHEROCHIP) that allows gene expression profiling for 89 genes specifically chosen for their role in adipogenesis. Results were compared with gene expression modifications that occur during *in vitro* adipogenesis induced by a standard pro-adipogenic cocktail (insulin, dexamethasone and dibutyryl-cAMP) Moreover, CREB, a master transcription factor involved in adipogenesis, is activated in 3T3-L1 cells in response to an antimycin A treatment. Using siRNA to inhibit CREB expression, we thus looked for the potential role of this factor in the accumulation of triglycerides and identified several CREB-target genes that are differentially expressed in 3T3-L1 incubated with antimycin A. We found that CREB is necessary for the accumulation of neutral lipid vesicles in 3T3-L1-treated with antimycin A and we identified mitochondrial GPD, CHOP-10, PAI-1 and mtCLIC as genes potentially regulated by CREB. Taken together, these results show that while triglyceride accumulation is observed in differentiating cells and in cells with impaired mitochondrial activity, genes that are differentially expressed in both conditions are different. Furthermore, our data illustrate that the combination of siRNA and cDNA microarray technologies is a very powerful approach to establish gene expression profiles in differentiating cells and to determine the role of transcription factors in this process.

T. Arnould is a Research Associate of FNRS (Fonds National de la Recherche, Brussels, Belgium). S. Vankoningsloo was a recipient of a FRIA fellowship and S. Tejerina is funded by a scholarship of the CUD (Coopération Universitaire au Développement-Bolivia)

References :

- (1) Hancock MR. *Ann Clin Biochem.* 39 (2002):456-63.
- (2) Mootha-VK et al. *Nat Genet.* 34(2003):267-73.
- (3) Arnould T. et al. *EMBO J.* 21 (2002)1-10

Characterization of the multiple sclerosis CSF proteome and identification of potential disease markers

D. DUMONT, J.-P. NOBEN, P. STINISSEN, J. RAUS AND J. ROBBEN

Biomedisch Onderzoeksinstituut (BIOMED), Limburgs Universitair Centrum and School of Life Sciences, Transnational University Limburg, Diepenbeek, Belgium

The cerebrospinal fluid (CSF) fills the ventricles and external surfaces of the central nervous system (CNS). The circulating fluid is considered as a “third circulation” conveying substances secreted into the CSF to many brain regions and draining waste products of cerebral metabolism. CSF homeostasis depends directly on the epithelial blood-CSF barrier located at the choroid plexuses and the outer arachnoid membrane, and indirectly on the endothelial blood-brain barrier via the interstitial compartment of the brain. Neurological diseases such as multiple sclerosis (MS) often provoke changes in the functioning of these barriers and give rise to disease-associated alterations of the CSF proteome, as has been demonstrated in numerous neuroproteomic studies. Hope exists that neuroproteomics will indicate paraclinical disease markers and will give insight in brain disease initiation, propagation and recovery. In the present study, the goal is the construction of a database of CSF proteins from MS patients. First, individual unfractionated CSF samples of MS patients were analysed with two-dimensional gel electrophoresis coupled to on-line reversed-phase LC-ESI-MS/MS. Second, pooled CSF samples from relapsing-remitting MS patients and from a selection of control patients were prefractionated by centrifugal ultrafiltration. The resulting partial (5-50 kDa) proteome was fractionated by off-line strong cation exchange chromatography coupled to on-line reversed-phase LC-ESI-MS/MS. Third, prefractionated samples were analysed directly by LC-ESI-MS/MS and gas-phase fractionation. The application of these proteomic techniques on the CSF proteome resulted in the identification of 200 proteins. Seventy of the encountered proteins were previously unnoticed in other gel-free or gel-based CSF proteomic studies. A preliminary analysis of the CSF protein profile of MS patients compared to control patients revealed a panel of candidate protein markers. Further investigation will provide additional information on whether these proteins are implicated in the MS pathogenesis. Potential markers will be validated for their use as paraclinical disease markers of diagnostic, prognostic or therapeutic value.

D. Dumont holds a fellowship from the Belgian ‘Wetenschappelijk Onderzoek Multiple Sclerosis’ (WOMS) Foundation.

References

DUMONT, D., NOBEN, J.P., RAUS, J., STINISSEN, P., AND ROBBEN, J. (2004) *Proteomics* 4:1117-1124.

***In vivo* long-term cell marking of neural stem cells in adult rodent brain by lentiviral vectors.**

¹M. GERAERTS, C. VALLEJO, ¹S. WILLEMS, ²V. BAEKELANDT, ¹Z. DEBYSER

¹Laboratory for Molecular Virology and Gene Therapy, KULeuven and KULAK, Flanders, Belgium. ²Laboratory for Neurobiology and Gene Therapy, KULeuven, Flanders, Belgium

In adult mammalian brain, continuous neurogenesis takes place in the subventricular zone (SVZ) of the lateral ventricles (LV) and in the dentate gyrus of the hippocampus. Neural progenitor cells (NPCs) from the SVZ migrate tangentially through the rostral migratory stream (RMS) towards the olfactory bulb (OB) and differentiate into local interneurons. Current labeling and gene marking methods of NPCs face technological hurdles. Bromodeoxyuridine labeling is hampered by toxicity and lacks specificity while transduction by retroviral vectors is restricted to dividing NPCs. Since lentiviral vectors do integrate into both dividing and non-dividing cells, we evaluated their potential for cell marking of both quiescent and slowly dividing stem cells as well as more rapidly dividing progenitor cells. In this study lentiviral vectors encoding for enhanced Green Fluorescent Protein (eGFP) were injected stereotactically in the SVZ or in the LV of adult C57bl/6 mice. Until six months after injection, eGFP-positive cells in the SVZ and in the RMS continued to express markers characteristic for both immature and migrating NPCs. Moreover, the number of eGFP-expressing granular and periglomerular interneurons in the ipsilateral OB increased over time. Interestingly, lentiviral vector injection into the LV not only resulted in stable gene expression in ependymal cells but also in type B astroglial-like stem cells, since eGFP-positive interneurons were detected in the ipsilateral and contralateral OB. In conclusion, lentiviral vector-mediated gene delivery is an efficient method for long-term *in vivo* cell marking of endogenous neuronal stem cells that may be used for developmental studies and therapeutic applications.

Study of the membrane organization of OLN-93 oligodendroglial cells by means of fluorescence correlation spectroscopy: raft versus non-raft

E. GIELEN¹, J. SYKORA², J. HUMPOLICKOVA², J. VERCAMMEN³, M. VANDEVEN¹, A. BENDA², N. HELLINGS¹, M. HOF², Y. ENGELBORGH³, P. STEELS¹ AND M. AMELOOT¹.
¹*Biomedisch Onderzoeksinstituut, Limburgs Universitair Centrum (LUC) and transnationale Universiteit Limburg (tUL), Universitaire Campus Bldg. D and A, B-3590 Diepenbeek, Belgium;* ²*J. Heyrovsk_ Institute of Physical Chemistry, Acad. of Sciences of the Czech Republic, Dolej_kova 3, Cz-18223, Prague 8, Czech Republic;* ³*Biochemistry Department, K.U.Leuven, Celestijnenlaan 200D, B-3001 Heverlee, Belgium.*

Differentiated oligodendrocytes (OLGs) enwrap the axons of the central nervous system with myelin, a fatty material that allows the nerve cell to transmit its impulses rapidly. Based on detergent extractions and co-localization studies, several myelin proteins have been identified in cholesterol/sphingolipid-rich membrane microdomains, so-called rafts. Our current research efforts focus on the lateral diffusion of raft and non-raft components in living OLGs. Therefore OLN-93 oligodendroglial cells were labeled with the raft marker Bodipy FL-C5 sphingomyelin (SM) or with the non-raft marker 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD). Cells were grown in 8-well glass-bottom LabTek chambers. Confocal laser scanning microscope imaging (CLSM) and fluorescence correlation spectroscopy (FCS) were carried out on these cells at 24°C with a Zeiss LSM 510 META CLSM and a Zeiss Confocor 2 setup. Data were collected at the membrane close to the glass cover slip. Other measurement conditions were: 488 nm Ar ion laser at 0.1% maximum power (~5 μW at the sample position) and 633 nm red HeNe laser at 0.1% maximum power for SM and DiD respectively, and a 40x, NA 1.2 water immersion objective. Data were analyzed by free and anomalous diffusion models using Zeiss Confocor and Globals (LFD, UIUC) software packages and a home-made macro (A. Benda) in Origin (OriginLab corporation). The motion of SM can be described by two diffusion coefficients (order of magnitude 2 μm²/s and 0.3 μm²/s) with about 80% of the slowly diffusing component. Depletion of cholesterol with methyl-β-cyclodextrin results in a shift towards the faster diffusing component. Colchicine (disruption of microtubules) has no effect on the mobility of SM. In addition to the measurements on lipids, OLN-93 cells were transiently transfected with a pMOG-EGFP construct by means of lipofection. MOG (myelin oligodendrocyte glycoprotein) is thought to be located outside rafts. Results indicate that the motion of MOG-EGFP can be described by two diffusion coefficients (order of magnitude 10 μm²/s and 0.2 μm²/s) with about equal weight. The data can also be described in terms of anomalous diffusion where the mean-square displacement $\langle r^2 \rangle$ is proportional to t^α . In the case of MOG-EGFP, α is about 0.5, whereas it is about 0.9 in the case of the fluorescent lipids.

This work has been supported by the Research Council of the LUC and tUL, the K.U.Leuven (GOA) and a bilateral program between Flanders and the Czech Republic.

Identification of new molecular markers for multiple sclerosis using phage cDNA display

C. GOVARTS, K. SOMERS, P. STINISSEN AND V. SOMERS

Biomedisch Onderzoeksinstituut, Limburgs Universitair Centrum and School of Life Sciences, Transnational University Limburg, Diepenbeek, Belgium

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system that involves immune reactivity against several myelin components. In the past few years, an important role of autoreactive antibodies and B cells in the pathogenesis of MS has been demonstrated. To fully explore the complex information present within the antibody repertoire of MS patients, we have applied a method, called serological antigen selection (SAS), to molecularly define MS antigens recognized by the humoral immune response. SAS is a phage display-based method that involves the display of a cDNA library as a fusion protein with a filamentous phage minor coat protein, pVI. The cDNA library used is a normalized cDNA library (10^6 primary recombinants) prepared from active chronic MS plaques, with varying degrees of demyelination and inflammatory activity. We cloned this library in the MSpSP6 vector. This resulted in a cDNA display library of 1.1×10^7 colony forming units. This cDNA display library was enriched on 2 separate pools of cerebrospinal fluid from 10 patients with relapsing remitting MS. Next, PCR and fingerprint analysis were used to determine the selective enrichment of the clones. The first CSF pool revealed 8 different antigens out of 52 clones while the second pool revealed 7 different antigens out of 86 clones.

The frequency of the antibody response of the antigens were further evaluated for their MS specificity by means of a phage ELISA procedure in which individual CSF from 65 MS patients and 74 controls was used. Forty percent of the MS CSF samples from the first pool showed reactivity to at least 1 of the 8 selected antigens while no or significantly lower reactivity was found for the control group. The antigens that were selected from the second CSF pool are currently being analysed.

In conclusion, our results indicate that this novel molecular approach can be used to study the humoral immune response in MS and may lead to the identification of MS-related antigens that can be used as diagnostic or prognostic markers.

No Association of Leukemia Inhibitory Factor (LIF) DNA Polymorphisms with Multiple Sclerosis

N. HELLINGS, J. VANDERLOCHT, J. BLEUS, J. RAUS AND P. STINISSEN

Biomedisch Onderzoeksinstituut, Limburgs Universitair Centrum, Diepenbeek, Belgium

Pro-inflammatory cytokines play a crucial role in inducing immune mediated oligodendrocyte cell death in multiple sclerosis (MS) lesions. Recently, neuropoietins such as leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) have been shown to ameliorate experimental autoimmune encephalomyelitis (EAE) and promote oligodendrocyte survival *in vivo*. Our *in vitro* studies confirmed that both LIF and CNTF have potential protective effects on tumor necrosis factor alpha (TNF α) induced oligodendrocyte damage. In this study we tested whether two known DNA polymorphisms in the LIF gene are associated with MS and rheumatoid arthritis (RA). We designed a restriction fragment length polymorphism (RFLP) based assay to genotype these SNPs in a group of MS patients (n=110), RA patients (n=120) and healthy controls (HC, n=109). SNP G2680A (Val64Met) is located in exon 3 in a region thought to be highly important for interaction with the LIF receptor. SNP C3951T is located in the 3' untranslated region and could play a role in mRNA stability. For C3951T, allele C was overrepresented as compared to allele T in either MS (C: 0.68; T: 0.32), RA (C:0.70; T:0.30) and HC (C: 0.66; T: 0.34). No significant differences were found in allele and genotype frequencies between the different study populations. For SNP G2680A, almost all patients and controls were homozygous for the G allele. Only 2 HC, 3 MS patients and 3 RA patients were heterozygous for the A allele. Similar allele and genotype frequencies were found for all study groups.

In conclusion, no association was found between the studied LIF DNA polymorphisms and the prevalence of the autoimmune diseases MS and RA indicating that these polymorphisms are not involved in determining susceptibility to these diseases

This work was supported by grants from the Belgian 'Nationaal Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO)' and the Limburgs Universitair Centrum (LUC). J.V. holds a fellowship from the 'Bijzonder Onderzoeksfonds'-LUC.

Flavonoids Influence Monocytic GTPase Activity and Are Protective in Experimental Allergic Encephalitis

J. J.A. HENDRIKS^{1#}, J. ALBLAS¹, S. M.A. VAN DER POL¹, E. A.F. VAN TOL², C. D. DIJKSTRA¹, AND H. E. DE VRIES¹

¹ *Department of Molecular Cell Biology and Immunology, Vrije Universiteit Medical Center (VUMC), 1007 MB Amsterdam, Netherlands. Phone: +31-20-444-8077; Fax: +31-20-444-8081; email: he.devries@vumc.nl # New address: Biomedical Research Institute (BIOMED), Limburgs Universitair Centrum, University Campus, B-3590 Diepenbeek, Belgium. Phone: +32-1126-9293, email: Jerome.Hendriks@luc.ac.be ² Biomedical Research Department, Numico Research B.V., 6704 PH Wageningen, Netherlands*

In the chronic disabling disease multiple sclerosis (MS), migration of monocytes across the blood-brain barrier is a crucial step in the formation of new lesions in the central nervous system (CNS). Infiltrating monocyte-derived macrophages secrete inflammatory mediators such as oxygen radicals, which contribute to axonal demyelination and damage, resulting in neurological deficits. Flavonoids are compounds occurring naturally in food, which scavenge oxygen radicals and have antiinflammatory properties. To investigate whether they might suppress clinical symptoms in MS, we treated rats sensitized for acute and chronic experimental allergic encephalomyelitis, an experimental model of MS, with flavonoids. We demonstrated that the flavonoid luteolin substantially suppressed clinical symptoms and prevented relapse when administered either before or after disease onset. Luteolin treatment resulted in reduced inflammation and axonal damage in the CNS by preventing monocyte migration across the brain endothelium. Luteolin influenced migration by modulating the activity of Rho GTPases, signal transducers involved in transendothelial migration. Oral administration of luteolin also significantly reduced clinical symptoms.

Conditional knockout of peroxisomes in the brain

L. HULSHAGEN¹, O. KRYSKO¹, S. HUYGHE¹, R. D'HOOGHE², P. P. DE DEYN² AND M. BAES¹

¹Laboratory of Clinical Chemistry, K.U.Leuven, Leuven, Belgium and ²Laboratory of Neurochemistry and Behavior, University of Antwerp, Antwerp, Belgium

Defects in cortical neuronal migration are one of the hallmarks of peroxisome biogenesis disorders in men and in mice (BAES *et al.*, 1997). Whether peroxisomes are also important in the adult brain is unclear since men and mice with peroxisome deficiency die in the postnatal period.

In order to investigate the importance of peroxisomes for the maintenance of the adult brain, mice with selective elimination of functional peroxisomes from brain were generated. Therefore, mice with a floxed *Pex5* gene (BAES *et al.*, 2002), encoding an essential component of the peroxisomal matrix import pathway, were crossed with *Nestin-Cre* mice. In the latter mice the Cre recombinase is selectively expressed in all central nervous system progenitor cells (TRONCHE *et al.*, 1999).

Inactivation of *Pex5* in the brain of *Nestin-Cre/ Pex5- LoxP* mice was confirmed at the level of DNA, protein and peroxisomal function. Southern blot showed a complete and selective recombination of *Pex5* in the brain. Western blot analysis revealed that peroxisomal matrix proteins were not processed, indicative of an import defect. This resulted in severely reduced activity of DHAPAT, a peroxisomal enzyme which is very sensitive to an ectopic localisation.

Nestin Cre/ Pex5- LoxP mice were macroscopically observed and appeared to have an extended pathology. They exhibited a postnatal growth retardation, cataract, dyskinesia of the limbs, infertility and all died within 6 months of age. Behavioral studies such as the open field, dark light transition box, rotarod, wire suspension, 23h activity and passive avoidance tests were performed at 7, 13 and 19 weeks of age. Mice with deficiency of peroxisomes in brain displayed impaired motoric performance, exploratory behavior and cognition.

At the microscopic level, diverse brain lesions were observed. From the age of 6 weeks, astrogliosis and microgliosis were noted which became more severe with age. In most brain areas lipid accumulations were observed which were most prominent in the molecular layer of the cerebellum and the ependymal lining of the cerebral ventricles.

These findings illustrate the importance of peroxisomes in adult mouse brain. Interestingly, the phenotype of *Nestin-Cre/ Pex5- LoxP* mice shows strong similarity with that of mice with multifunctional protein-2 (MFP-2) deficiency, a pivotal enzyme in peroxisomal α -oxidation (HUYGHE *et al.*, 2005). This indicates that a dysfunction of MFP-2 within the central nervous system is causing the brain abnormalities.

This work was supported by grants from the EU (BMH4- 98-3569 and QLG1-CT2001-01277), FWO (G.0235.01) and GOA (99/09 and 2004/08).

References

- BAES, M ET AL.. (1997) Nat. Genet. **17**: 49-57.
- BAES, M., ET AL. (2002) Genesis. **32**: 177-178.
- TRONCHE, F., ET AL.. (1999) Nat. Genet. **23**: 99-103.
- HUYGHE, S., ET AL. (2005) Am. J. Pathol. submitted

Liver peroxisomes are necessary for cortical neuronal migration and normal development of the cerebellum.

O. KRYSKO¹; P. GRESSENS³; M. ESPEEL² AND M. BAES¹

¹Laboratory of Clinical Chemistry, K.U. Leuven, ²Department of Human Anatomy, Embryology, Histology and Medical Physics, Ghent University, Belgium and

³Laboratory of Developmental Neurology, Hospital Robert-Debré, Paris, France

Zellweger syndrome is an inherent peroxisomal disorder characterized by neuronal migration abnormalities in the cerebral cortex, cerebellum and abnormal formation of the inferior olive. Patients have severe psychomotor retardation, hypotonia, neonatal seizures with life expectancy less than 1 year (GOULD *et al*, 2000). Recently, mouse models were generated, which mimic the human phenotype, i.e. they are hypotonic at birth, display a neuronal migration defect in the cortical plate and die within 24-48 hours (BAES *et al*, 1997; FAUST *et al* 1997). A major question is whether the brain malformations of peroxisome deficient mice are caused by the local absence of peroxisomes in the brain or by a more generalised deletion of peroxisomal function, in which the liver might play an important role. To approach this, mice with liver selective elimination of peroxisomes were generated by inactivation of the PEX5 gene (receptor for peroxisomal matrix proteins) in hepatocytes. *Alfp Pex5*^{-/-} mice were obtained by crossing *Pex5*^{FL/FL} mice with mice expressing Cre recombinase under the control of albumin promoter and alpha-fetoprotein enhancers during embryonic development starting at E9.5-E10.5 (KELLENDONK *et al*, 2000). Elimination of functional peroxisomes from liver of *Alfp Pex5*^{-/-} mice was demonstrated by the impairment of peroxisomal enzymes (urate oxidase, DHAPAT) whose activity is dependent on the intraperoxisomal localisation. The disappearance of normal peroxisomes was also proven at the ultrastructural level by cytochemical methods (catalase activity). The process of cortical neuronal migration was analyzed by cresyl violet staining of coronal sections of E18 knockout embryos, revealing altered cell densities in the inner zone of neocortical plate and in the underlying intermediate zone (prospective white matter), consistent with a delay of neuronal migration. BrdU pulse chase experiments at E18.5 (injecting at E13.5), confirmed that significantly higher numbers of labeled cells were present in the intermediate zone of the cerebral cortex at E18.5. Analysis of P5 pups by BrdU and NeuN immunocytochemistry revealed arrested neurons in the intermediate zone of the cortex and disordered lamination of the layers IV and V. Increased apoptotic cell death in the layers II-III of the cortical plate by cleaved caspase 3 immunocytochemistry was found. Cerebellar abnormalities of *Alfp Pex5*^{-/-} mice including reduced size, altered foliation with absence of the intercrural fissure, declival sulcus, ulular sulcus and sulcus that separate folia 4 and 5; reduced dendritic arborisation of Purkinje cells and increased cell death of granule neurons were observed. In conclusion, these data provide evidence that the normal function of peroxisomes in liver is crucial for the normal neuronal migration process in cerebral cortex and for the normal development of the cerebellum.

This work was supported by grants from the EU (BMH4- 98-3569 and QLGI-CT2001-01277), FWO (G.0235.01) and GOA (99/09 and 2004/08).

References

GOULD SJ, RAYMOND GVAND VALLE D (2000) The metabolic and molecular bases of inherited disease 3181-3217.

BAES M, GRESSENS P ET AL. *Nat Genet* (1997) 17:49-57.

FAUST PL. *J Comp Neurol* (2003) 461:394-413.

KELLENDONK C, OPPERK C et al. *Genesis* (2000) 26:151-3.

Cloning and characterization of three mouse 5-HT₇ receptor splice variants

K. LAENEN, K. DE MARTELAERE, A. MATTHYS, B. LINTERMANS, G. HAEGEMAN AND P. VANHOENACKER

Laboratory of Eukaryotic Gene Expression and Signal Transduction, Ghent University - UGent, K. L. Ledeganckstraat 35, 9000 Gent, Belgium.

Peter.Vanhoenacker@ugent.be

Serotonin or 5-hydroxytryptamine (5-HT) acts on a diverse group of receptors, fourteen in total, which underscores the importance of 5-HT and the need for fine-tuning of its actions (HOYER *et al.*, 2002). This diversity is even more amplified by the existence of several variants (isoforms) for some members of this family.

The most recently identified serotonin receptor is the 5-HT₇ receptor (VANHOENACKER *et al.*, 2000). Recent studies suggest that this receptor is involved in thermoregulation, circadian rhythm, sleep, hippocampal signaling, learning and memory and endocrine regulation. Furthermore, a potential involvement in mood regulation suggests that this receptor might represent another potential target for the treatment of depression (HEDLUND *et al.*, 2004). This receptor has been cloned from several species, but so far the existence of receptor variants has only been reported for rat and man. These receptor isoforms are the consequence of alternative splicing of the mRNA, which gives rise to three receptors in rat [r5-HT_{7(a)}, r5-HT_{7(b)}, r5-HT_{7(c)} and] and man [h5-HT_{7(a)}, h5-HT_{7(b)} and h5-HT_{7(d)}] (HEIDMANN *et al.*, 1998). A fourth isoform has been reported for rat [r5-HT_{7(e)}] (LIU *et al.*, 2001), but until today no pharmacological characterization of this isoform has been reported. These receptors differ in the amino acid sequence of the intracellular C-terminal tail, a region of the receptor that plays an important role in the signalization and regulation of the receptor.

We report on the cloning of two C-terminal splice variants from mouse total brain. The isoforms, which we refer to as m5-HT_{7(b)} and m5-HT_{7(c)}, have C-terminal amino acid sequences which are almost identical to their rat counterparts. The receptors have a high affinity for 5-HT and are positively coupled to adenylate cyclase. As has been reported for the human variants, these mouse isoforms are also able to activate the Erk1/2 pathway. Finally, immunofluorescence experiments revealed a clear membrane localization of the three variants.

To our knowledge, these results are the first to report on the functional characterization of two additional mouse 5-HT₇ receptor splice variants.

This work was financially supported by grants from the IWT (Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen) (project 990173) and Janssen Pharmaceutica N.V., Beerse, Belgium.

References

- HEDLUND, P. B. and SUTCLIFFE, J. G. (2004) *Trends Pharmacol. Sci.* **25**: 481-486.
HEIDMANN, D. E. A., *et al.*, (1998) *Neuropharmacol.* **37**: 1621-1632.
HOYER, D., HANNON, J. P., and MARTIN, G. R. (2002) *Pharmacol. Biochem. Behav.* **71**: 533-554.
LIU, H., IRVING, H. R. and COUPAR, I. M. (2001) *Life Sci.* **69**: 2467-75.
VANHOENACKER, P., HAEGEMAN, G., and LEYSEN, J. E. (2000) *Trends Pharmacol. Sci.* **21**: 70-77.

Non-invasive imaging of neuropathology in a rat model for Parkinson's disease based on overexpression of alpha-synuclein

E LAUWERS¹, D. BEQUÉ², K. VAN LAERE², J. NUYTS², G. BORMANS³, L. MORTELMANS², L. VERCAMMEN¹, O. BOCKSTAEL⁶, B. NUTTIN⁴, Z. DEBYSER⁵ AND V. BAEKELANDT¹

¹Laboratory for Neurobiology and Gene Therapy, ² Division of Nuclear Medicine, University Hospital Leuven, ³ Laboratory for Radiopharmaceutical Chemistry, ⁴ Laboratory for Experimental Neurosurgery and Neuroanatomy, ⁵ Laboratory for Molecular Virology and Gene Therapy; K.U.Leuven, Leuven, Belgium

⁶Laboratory of Experimental Neurosurgery, Institute of Interdisciplinary Research, Faculty of Medicine, Université Libre de Bruxelles

Parkinson's disease is a neurodegenerative disorder affecting the dopaminergic neurons in the substantia nigra. Aggregation of alpha-synuclein appears to play a central role in the pathogenesis. Novel animal models for neurodegeneration have been generated by lentiviral vector-mediated locoregional overexpression of disease-associated genes in the adult brain.

We have used lentiviral vectors to mediate overexpression of α -synuclein in the substantia nigra of the rat. Stereotactic injection of lentiviral vectors encoding a clinical mutant of alpha-synuclein, A30P, in the rat substantia nigra induced time-dependent cytoplasmic and neuritic accumulation of alpha-synuclein and neurodegeneration. Amphetamine-induced rotational tests were performed at several time points after gene delivery. A subgroup of the rats developed asymmetric rotational behavior after administration of amphetamine. At 46 weeks after injection, 5 of these rats and 5 age-matched control rats were injected with ¹²³I-FP-CIT to evaluate the dopamine transporter binding in the striatum by microSPECT. All animals of the alpha-synuclein overexpression group displayed 15-42 % reduction in dopamine transporter binding (mean 27% +/- 12 % SD; p= 0.009) visualized by ¹²³I-FP-CIT microSPECT imaging. The behavioral and microSPECT data were validated by histological analysis. The number of nigral tyrosine hydroxylase-positive neurons was quantified by stereological counting. In the alpha-synuclein-transduced rats dopaminergic cell loss in the substantia nigra varied between 22 and 46% (mean 37 % +/- 13 % SD; p = 0.02) as compared to the contralateral side. There was a strong correlation between the reduction of dopaminergic neurons in the substantia nigra and the reduction of dopamine transporter binding in the striatum.

In conclusion, lentiviral vector-mediated overexpression of alpha-synuclein in the substantia nigra of the rat results in degeneration of dopaminergic neurons and a corresponding reduction of dopamine transporter activity in the striatum. MicroSPECT imaging enables non-invasive imaging of the neurodegeneration allowing longitudinal follow-up in this new animal model for Parkinson's disease and the evaluation of neuroprotective drugs.

Synovial Fluid NKT Cells Display Different Properties Compared to Peripheral Blood NKT Cells in Rheumatoid Arthritis

L. LINSEN, M. THEWISSEN, V. SOMERS, P. GEUSENS, J. RAUS AND P. STINISSEN
Biomedisch Onderzoeksinstituut, Limburgs Universitair Centrum, Diepenbeek, Belgium

Natural killer T cells (NKT) are a population of regulatory T cells that co-express an invariant T cell receptor as well as NK cell markers. Several studies have shown that NKT cells are decreased or dysfunctional in autoimmune conditions such as insulin-dependent diabetes mellitus, systemic sclerosis, systemic lupus erythematosus and multiple sclerosis. Significant therapeutic effects of α -GalactosylCeramide (α -GalCer), a synthetic antigen of NKT cells, have been demonstrated in animal models of autoimmunity. NKT cells have therefore been implicated to participate in the regulatory immune mechanisms controlling autoimmunity. However, their role in the pathogenesis of rheumatoid arthritis (RA) remains unclear. To this end, we studied the frequency, cytokine profile and heterogeneity of NKT cells in peripheral blood mononuclear cells (PBMC) of 23 RA patients and 22 healthy controls, which included paired PBMC-synovial fluid (SF) samples of 7 and paired PBMC-synovial tissue (ST) samples of 4 RA patients, respectively. Using flow cytometry, a decreased NKT cell frequency was observed in blood of RA patients compared to healthy controls. In addition, direct ex vivo ELISPOT analysis revealed a reduced IL-4/IFN- γ ratio in NKT cells of RA patients. The invariant T cell receptor sequence was detected in paired SF and ST samples. NKT cells of all healthy controls, but only of 53.8% of the RA patients (responders) expanded upon in vitro stimulation. However, reactivity towards α -GalCer was observed in NKT cells isolated from SF of both responder and non-responder RA patients. Intracellular FACS analysis of the cytokine profile of CD4⁺ and CD4⁻ PBMC derived NKT cell lines of RA patients revealed that both produced significantly less IL-4 compared to those of healthy controls. In contrast, SF derived NKT cell lines displayed a Th0 phenotype comparable to that of healthy controls. These findings suggest that SF NKT cells are functional, even in patients with non-responding NKT cells in the blood.

In conclusion, our data demonstrate that NKT cells are decreased and biased towards a Th1 phenotype in blood, but are not impaired in SF of RA patients. This indicates NKT cells that might be functionally related to resistance or progression of rheumatoid arthritis.

This work was financially supported by a grant of the 'Bijzonder Onderzoeksfonds, LUC'.

Measuring protein-protein interactions inside living cells using single color fluorescence correlation spectroscopy. Application to human immunodeficiency virus type 1 integrase and LEDGF/p75.

G. MAERTENS^{1,2}, J. VERCAMMEN^{1,2}, Z. DEBYSER² AND Y. ENGELBORGH¹

¹Laboratory of Biomolecular Dynamics, Katholieke Universiteit Leuven, and

²Molecular Virology and Gene Therapy, Katholieke Universiteit Leuven

Recently we described the interaction of human immunodeficiency virus type 1 (HIV-1) integrase (IN) with a cellular protein, lens epithelium-derived growth factor/transcription co-activator p75 (LEDGF/p75) which seems to play an important role in the nuclear localization of IN (MAERTENS *et al.*, 2003; CHEREPANOV *et al.*, 2003). Here we present the study of the diffusion behavior of three independent domains of IN (i.e. the N-terminal, the C-terminal part and the core) and LEDGF/p75, using fluorescence correlation microscopy (FCS). In FCS the diffusion of fluorescent molecules is studied in a confocal microscope (RIGLER *et al.*, 1993; SCHWILLE, 2001). Here we use the Confocor II from Zeiss. Each time a fluorescent molecule passes through the confocal volume a light pulse is produced. The time width of this pulse represents the residence time of the molecule in the confocal volume. From the average residence time of many molecules, the average diffusion coefficient, and therefore the size of the molecule can be deduced. We constructed different fusion proteins of Enhanced Green Fluorescent Protein (EGFP) with IN and its domains, and expressed them in HeLa cells. We show that diffusion of these proteins in the cell is rather variable from spot to spot and in each spot has to be described by two components (fast and slow) with different fractions, reflecting the complexity of the cellular medium. We observe that good quality average parameters can be obtained and that these average parameters are comparable in the nucleus and in the cytoplasm. In addition we demonstrate that specific interaction between EGFP-fused HIV-1 IN and LEDGF/p75, results in a downward shift in the average diffusion coefficient ($\langle D \rangle$). The opposite shift was observed in an IN-deletion mutant that does not exhibit LEDGF/p75 binding or in a LEDGF/p75 knock-down experiment using siRNA. We thus demonstrate that protein-protein interactions can be studied in living cells, using single-color FCS.

This work was financially supported by a grant from the K.U.Leuven (GOA2001/2) and the I.W.T. (SBO 030239). G.M. is aspirant of the F.W.O. Flanders. This work is in press in FASEB J.

References

- CHEREPANOV, P., MAERTENS, G., PROOST, P., DEVREESE, B., VAN BEEUMEN, J., ENGELBORGH, Y., DE CLERCQ, E., AND DEBYSER, Z. (2003) *J Biol Chem* **278**, 372-381
- MAERTENS, G., CHEREPANOV, P., PLUYMERS, W., BUSSCHOTS, K., DE CLERCQ, E., DEBYSER, Z., AND ENGELBORGH, Y. (2003) *J. Biol. Chem.* **278**, 33528-33539.
- RIGLER R., M. U., WIDENGREN J., AND KASK P. (1993) *European Biophysics Journal* **22**, 169-175
- SCHWILLE, P. (2001) *Cell Biochem Biophys* **34**, 383-408.

TGF- β 1 mediates the organization and expression of cytoskeletal proteins in astrocytes

M. MOREELS, F. VANDENABEELE, D. DUMONT, J. ROBBEN AND I. LAMBRICHTS
Biomedisch Onderzoeksinstituut, Limburgs Universitair Centrum and School of Life Sciences, Transnational University Limburg, University Campus, Diepenbeek, Belgium.

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the human CNS. The pathological changes include inflammation, demyelination, oligodendrocyte cell death, reactive gliosis and astroglial scar formation, and axonal degeneration. The glial scar is largely formed by activated astrocytes which acquire a distinctive "reactive" shape. This phenomenon is not only restricted to MS lesions. Whenever the CNS is damaged (wound, MS, ischemia ...) it undergoes an injury response, usually called reactive gliosis or glial scarring. The response is broadly the same whatever the source of injury. Activated astrocytes become hypertrophied and produce high levels of the intermediate filament (IF) GFAP. It has been shown that transforming growth factor-beta 1 (TGF- β 1) is implicated in astrocyte activation and glial scar formation (LOGAN *et al.*, 1992). Recent studies revealed that reactive astrocytes also increase other IF such as nestin and vimentin (HOLLEY *et al.*, 2003, YAMADA *et al.*, 1991). Furthermore, upregulation of microfilaments (actin) in brain injury is also recently described (BOUKHELIFA *et al.*, 2003).

This study investigates the role of TGF- β 1 in the organization and expression of GFAP, nestin (embryonic IF) and alpha-smooth muscle actin (α -SMA, actin microfilament) in astrocytes *in vitro*. Primary cultures of astrocytes obtained from rat neonatal forebrain were used as a model system. The organization of the cytoskeletal proteins was investigated by immunocytochemistry whereas the protein expression was followed by western blot analysis.

TGF- β 1 induces morphological changes in astrocytes accompanied by cytoskeletal rearrangements (GFAP, nestin, α -SMA). The cells gradually enlarged, exhibited nuclear hypertrophy and were spread onto the substratum. Both nestin and GFAP became organized in well-developed bundles. α -SMA polymerised in parallel stress-fibers. Furthermore, western blot analysis revealed that TGF- β 1 up-regulates nestin expression and induces α -SMA expression in astrocytes in a dose-dependent manner.

We can conclude that TGF- β 1 promotes major morphological changes in the astrocyte cytoskeleton (IF bundles, α -SMA stress fibers) *in vitro*, associated with an increased synthesis of α -SMA and nestin. Our *in vitro* results support the idea that in astrocytes both α -SMA and nestin are targets of TGF- β 1. The presence of the contractile iso-actin α -SMA in reactive astrocytes might be involved in cell migration and motility during reactive gliosis. Furthermore, the expression of the embryonic IF nestin in astrocytes could implicate an embryonic reversion of the cells *in vitro*.

References

- LOGAN, A., FRAUTSCHY, S. H., GONZALEZ, A. M., SPORN, M. B. AND BAIRD, A. (1992) *Brain Res.* **587**:216-25.
HOLLEY, J. E., GVERIC, D., NEWCOMBE, J., CUZNER, M. L. AND GUTOWSKI, N. J. (2003) *Neuropathol. Appl. Neurobiol.* **29**:434-44.
YAMADA, T., KAWAMATA, T., WALKER, D. G. AND MCGEER, P. L. (1992) *Acta Neuropathol.* **84**:157-62.
ABD-EL-BASSET, E. M. AND FEDOROFF, S. (1997) *J Neurosci Res.* **49**:608-16

Reversal of mitochondrial Na/Ca exchangers in metabolically inhibited MDCK cells: confocal microscopic study of mitochondrial calcium and sodium

I. SMETS¹, SZ. BARON^{1,2}, A. CAPLANUSI³, ZS. MOLNAR², M. RADU⁴, M. VANDEVEN¹, M. AMELOOT¹ AND P. STEELS¹

¹Limburgs Universitair Centrum/transnationale Universiteit Limburg –Biomedical Research Institute, Diepenbeek, Belgium; ²University of Szeged, Szeged, Hungary;

³Carol Davila University of Medicine and Pharmacy, Bucharest, Romania and

⁴Horia Hulubei National Institute for Physics and Nuclear Engineering, Bucharest, Romania

In ischemic or hypoxic tissues, elevated calcium levels have emerged as one of the main mechanisms of cellular injury. Since mitochondria play a key role in the maintenance of cellular Ca²⁺ homeostasis, alterations in the mitochondrial Ca²⁺ content ([Ca²⁺]_m) were monitored in addition to changes in the cytosolic Ca²⁺ concentration ([Ca²⁺]_i) in renal epithelial Madin-Darby canine kidney (MDCK) cells. Since the mode of action of mitochondrial Na⁺/Ca²⁺ exchangers (NCE) is determined by both Ca²⁺ and Na⁺ gradients, intracellular ([Na⁺]_i) and mitochondrial ([Na⁺]_m) concentrations were also determined.

To mimic renal ischemia, metabolic inhibition (MI) with NaCN and 2-deoxyglucose was used as an *in vitro* model. [Ca²⁺]_i and [Ca²⁺]_m were monitored on confocal and standard fluorescence microscopes via, respectively, Fura-2 and Rhod-2 measurements (SMETS *et al.*, 2004). Changes in intracellular ([Na⁺]_i) and mitochondrial ([Na⁺]_m) were detected using, respectively, SBFI and CoroNa Red.

MI induced an increase in [Ca²⁺]_i reaching 631±78 nM in ~20 min followed by a decrease to 118±9 nM in the next ~25 min. A rapid increase of [Na⁺]_i in the first 20 min of MI excluded Ca²⁺ efflux in the second phase via plasma membrane NCE. Mitochondrial Rhod-2 intensities increased to 434±46% of the control value during MI, indicating that mitochondria sequester Ca²⁺ during MI. Under Na⁺-free conditions, or when CGP37157, a specific inhibitor of the mitochondrial NCE, was used, no drop in [Ca²⁺]_i was seen during MI, while the MI-induced increase in mitochondrial Rhod-2 fluorescence was strongly reduced. In the first 15 min of MI, a twofold increase of [Na⁺]_m was observed reaching 105±7 mM. In the next 45 min of MI, [Na⁺]_m dropped to 84±7 mM. The striking rise in [Na⁺]_m is likely sufficient to sustain the driving force for mitochondrial Ca²⁺ uptake via the NCE. When CGP37157 was applied during MI, the second-phase drop of [Na⁺]_m was completely abolished. This confocal microscopic study of cytosolic and mitochondrial Ca²⁺ and Na⁺ indicates that mitochondria in MDCK cells take up Ca²⁺ via the NCE in the reverse mode.

References

SMETS, I., CAPLANUSI, A., DESPA, S., MOLNAR ZS., RADU, M., VANDEVEN, M., AMELOOT, M. AND STEELS, P. (2004) *Am J Physiol Renal Physiol* **286**: F784-F794.

The hypothalamic AMPK-mediated stimulation of food intake is lacking in Lou/C rats, a strain resistant to obesity

N. TALEUX^{1,2}, B. GUIGAS¹, G. LACRAZ², R. FAVIER², X. LEVERVE² AND L. HUE¹
¹*Hormone and Metabolic Research Unit, Institut of Cellular Pathology, University of Louvain Medical School, Brussels, Belgium ;* ²*Laboratory of Fundamental and Applied Bioenergetic, University of Joseph Fourier, Grenoble, France.*

The hypothalamus plays a central role in the control of food intake and, recently, the AMP-activated-protein-kinase (AMPK) was found to be involved in this regulation. The activity of hypothalamic AMPK is controlled by circulating levels of leptin, glucose, insulin and nutritional state. It regulates the levels of neuropeptides and consequently food intake. Lou/C rats are naturally resistant to obesity (Couturier et al., 2002). Their caloric intake is 40 % lower than that of age related Wistar and remains unexplained. The purpose of this present work was to investigate if AMPK was implicated in the spontaneous lower food intake of the Lou/C rats.

We extracted hypothalami of fed and 24-hours-starved Wistar (control) and Lou/C rats and immediately froze them. The hypothalami were further lysated and AMPK activity was measured by incorporation of ³²P into the peptide SAMS after immunoprecipitation of $\alpha 1$ and $\alpha 2$ AMPK.

In agreement with previous reports (Minokoshi et al., 2004 ; Andersson et al., 2004), our results show that starvation increase AMPK activity in both isoforms (35 %, 47 % $p < 0.05$ for $\alpha 1$ and $\alpha 2$ respectively) in the hypothalamus of control rats. This increase was not observed in the Lou/C group. Intraperitoneal injection of mercaptoacetate (68 mg/kg, 4 h before sacrifice), an inhibitor of hepatic beta-oxidation known to activate food intake, increased AMPK activity in control rats as did starvation. Again, this activation was not observed in Lou/C rats. In keeping with this, the phosphorylation state of eukaryotic elongation factor 2 and acetyl-CoA carboxylase, two downstream targets of AMPK, followed the changes in AMPK activity and confirmed these differences.

We conclude that food intake is related to the activation of AMPK in the hypothalamus, a regulation lacking in the Lou/C rats.

This work was financially supported by a French grant of the Région Rhône-Alpes (EURODOC fellowship, N Taleux) and by INSERM. B. Guigas is recipient of the ICP Michel de Visscher Fellowship.

References

- COUTURIER K, SERVAIS S, KOUBI H, SEMPORÉ B, SORNAY-MAYET MH, COTTET-EMARD JM, LAVOIE JM AND FAVIER R (2002) *Obes Res.* 10(3):188-95.
MINOKOSHI Y, ALQUIER T, FURUKAWA N, KIM YB, LEE A, XUE B, MU J, FOUFELLE F, FERRE P, BIRNBAUM MJ, STUCK BJ AND KAHN BB (2004) *Nature* 428(6982):569-74.
ANDERSSON U, FILIPSSON K, ABBOTT CR, WOODS A, SMITH K, BLOOM SR, CARLING D AND SMALL CJ (2004) *J. Biol Chem.* 279(13):12005-8.

Study of the hTERT Expression in PBMC of Rheumatoid Arthritis Patients

M. THEWISSEN, L. LINSEN, P. GEUSENS, J. RAUS AND P. STINISSEN

Biomedisch Onderzoeksinstituut, Limburgs Universitair Centrum (LUC) and transnational University Limburg (iUL), Universitaire Campus building A, Diepenbeek, Belgium

The progressive shortening of telomeres, during cell division, has been proposed to act as a mitotic clock that monitors cell division and provides a measure of residual replicative capacity of cells. Telomerase is a large ribonucleoprotein complex which synthesizes telomere repeats to maintain telomere length. Germ line cells and the majority of malignant tumor cells express telomerase. In other somatic cell populations, such as lymphocytes, there is a highly regulated, transient expression of telomerase. Some autoimmune diseases, like rheumatoid arthritis (RA) and multiple sclerosis (MS), are considered to be T-cell mediated. A disturbance in the naive T cell receptor (TCR) repertoire has been observed with a loss of TCR diversity and multiclonal growth of a high proportion of T-cells. It remains unclear whether telomerase or telomerase (dys)regulation is part of the pathogenic immune response in RA. In this study, we evaluated the expression kinetics of the catalytic subunit of telomerase, hTERT, after mitogenic stimulation in peripheral blood mononuclear cells (PBMC) of RA patients and controls.

The hTERT expression kinetics in PBMC of 8 early, untreated RA (eRA) patients and 8 chronic RA (cRA) patients were compared with those of 8 MS patients and 8 healthy controls (HC). PBMC were stimulated with anti-CD3 (2 µg/ml) for 7 days. At day 0 (after isolation), 4 and 7, flow-cytometric (FACS) analysis was performed. hTERT expression was determined using the PCR LightCycler Telo *TAGGG* hTERT Quantification kit (Roche Diagnostics, Belgium) and telomerase activity using the telomerase PCR ELISA^{plus} (Roche Diagnostics, Belgium).

The direct *ex vivo*, hTERT expression was low or even undetectable in 3/8 HC, 6/8 cRA, 8/8 eRA, and 2/8 MS patients. After anti-CD3 stimulation, hTERT expression gradually increased, peaked briefly and slowly decreased again. We found a different hTERT expression pattern in eRA and MS patients compared to HC ($p < 0.01$). The hTERT expression peaked at day 3 in the group of the HC, but one day later (day 4) in the cRA patients. The level of maximal hTERT expression was comparable in the HC and the cRA patients, but was significantly lower in the eRA and MS patients compared to the HC ($p < 0.01$). The maximal activation state of PBMC of eRA and MS patients was, however, not different from that of PBMC of HC.

hTERT expression kinetics, following antigenic challenge, are disturbed in eRA patients, but also in MS patients. This indicates that telomerase dysregulation is not specific for RA. Moreover, these kinetics seem to normalize later in the disease process of RA. It remains, however, unclear whether this deviated hTERT expression, early in the disease process of RA, is a cause or a result of the disease. Further studies are necessary to reveal the contribution of telomerase and its regulation in the pathogenesis of RA.

Folding efficiency is rate-limiting in dopamine D4 receptor biogenesis

K. VAN CRAENENBROECK^{1,2}, S CLARK², G. HAEGEMAN¹, P. VANHOENACKER¹ AND H.H.M. VAN TOL²

¹*Laboratory of Molecular Biology, Ghent University, Ghent, Belgium*

²*Laboratory of Molecular Neurobiology, Centre for Addiction and Mental Health, Toronto, Canada*

Synthesis and maturation of G protein-coupled receptors (GPCRs) are complex events that require an intricate combination of processes including protein folding, post-translational modifications and transport through distinct cellular compartments. Here we provide data for the maturation of the dopamine D4 receptor (D4DR), a polymorphic 7-transmembrane (TM) receptor that shows high affinity for antipsychotics. We show that the D4DR expression is subject to strong post-transcriptional regulation. Pre-exposure with chemical (glycerol, DMSO) and pharmacological (D4DR ligands) chaperones upregulates the expression of the receptor in stably transfected CHO cells. All tested membrane-permeable agonists and antagonists facilitate maturation of the receptor. The upregulation is particularly well seen with D4DR mutants (M345A, S or T). M345 is located close to TM6 in the third intracellular loop of the D4.4DR and it is proposed that this region may form an extra turn of the TM6 α -helix. As ligand binding is almost completely disrupted and functional activity is abolished in these mutants it is likely that the D4.4DR(M345) mutation affects the structural integrity of the receptor. Pre-exposure of cells, expressing these mutants, with chaperones results in a significant membrane expression of functional receptor. We further provide evidence that the increase in membrane expression is not due to increased mRNA synthesis but rather to a stabilization effect of the ligand on the newly synthesized receptor in the endoplasmic reticulum (ER). The ER controls the fidelity of gene expression at the post-transcriptional level and routes a significant amount of receptor to the degradation pathway, mediated by the 26S proteasome. Ligands can rescue the receptor from this degradation pathway. Understanding the D4DR maturation and steady state expression levels are important to obtain better insight into the clinical effects of exogenously administered drugs.

Expression of a specific calcium-binding protein of the S100 protein family, S100A6 (calcylin) in multiple sclerosis plaques.

F. VANDENABEELE, M. MOREELS, I. LAMBRICHTS, M. AMELOOT , P. STINISSEN

Biomedisch Onderzoeksinstituut, Limburgs Universitair Centrum and School of Life Sciences, Transnational University Limburg, University Campus, Diepenbeek, Belgium.

S100A6 (calcylin) is a specific calcium-binding protein of the S100 family, considered to activate several processes along the calcium signal transduction pathway including the regulation of cell growth, proliferation, secretion, and exocytosis. S100A6 has been shown to be overexpressed in amyotrophic lateral sclerosis (ALS), a neurodegenerative disease characterized by selective degeneration of motoneurons (HOYAUX *et al.*, 2000; HOYAUX *et al.*, 2002). Vulnerability of motoneurons in ALS has been attributed to their low calcium buffering capacity as a result of low calcium-binding protein expression, making them particularly sensitive to disturbed calcium homeostasis (LIPS *et al.*, 1998; PALECEK *et al.*, 1999). The molecular mechanisms that underlie axonal loss in MS are not clearly understood. Electrophysiological data demonstrated that influx of damaging levels of intra-axonal calcium is implicated in white matter axonal injury (WAXMAN, 1992; CRANER *et al.*, 2004). Differential calcium buffering capacities in neurons and glial cells have been implicated in the vulnerability of the central nervous system (CNS) and the pathogenesis of MS (BENJAMINS and NEDELKOSKA, 1996; KNAPP *et al.*, 1999, MA *et al.*, 2002). Up to date, no data are available on the expression of the calcium-binding protein S100A6 within the CNS during MS lesion. Therefore, we have examined the expression of S100A6 in post-mortem human brain tissue obtained from MS patients. Specific patterns of S100A6 expression could be observed within active and chronic active MS lesions. Strongest S100A6 immunoreactivity was observed in reactive hypertrophic astrocytes within the hypercellular rim of chronic active MS lesions. Moreover, perivascular macrophages and blood monocytes were strongly immunoreactive for S100A6 in active MS lesions. Although our results do not give any clue about the beneficial or detrimental role played by S100A6 within the CNS during MS lesion, the high S100A6 expression in reactive astrocytes and activated macrophages/microglia reflects a high calcium buffering capacity and could play a protective role in the cascade of events leading to axonal loss and oligodendroglial cell death.

References

- WAXMAN S.G. (1992) *J Neurotrauma* **9**: 105-117.
BENJAMINS J.A. AND NEDELKOSKA L. (1996) *Neurochem Res.* **21**: 471-479.
LIPS M.B. AND KELLER B.U. (1998) *J Physiol.* **511**: 105-117.
KNAPP P.E ET AL. (1999) *Brain Res.* **847**: 332-337.
PALECEK J., LIPS M.B. AND KELLER B.U. (1999) *J Physiol. (Lond.)* **520**: 485-502.
HOYAUX D., ET AL.. (2000) *Biochim. Biophys. Acta* **1498**: 264-272.
HOYAUX D. ET AL. (2002) *J Neuropathol. Exp. Neurol.* **61**: 736-744.
MA H., ET AL. (2002) *Chin J Traumatol.* **5**: 32-35.
CRANER M.J., ET AL. (2004) *Brain* **127**: 294-3003.

Leukemia inhibitory factor is produced by myelin reactive T-cells and protects against cytokine induced oligodendrocyte cell death

J. VANDERLOCHT¹, N. HELLINGS¹, F. VANDENABEELE¹, M. MOREELS¹, M. BUNTINX¹, JP. ANTEL², J. RAUS¹ AND P. STINISSEN¹

*Biomedisch Onderzoeksinstituut, Limburgs Universitair Centrum, Diepenbeek, Belgium, and *Montreal Neurological Institute, Mc Gill University, Montreal, Canada*

Leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) are members of the neuropoietic family of neurotrophins and ameliorate experimental autoimmune encephalomyelitis (EAE). We examined whether human T-cells specific for myelin basic protein (MBP) and myelin oligodendrocyte protein (MOG) are able to secrete LIF. Six out of 19 myelin reactive T-cell lines/clones (TCL) from 8 multiple sclerosis (MS) patients and 5/11 TCL from 5 healthy controls (HC) produced LIF upon stimulation. The mean net LIF secretion of MS TCL did not significantly differ from HC TCL. LIF expression was confirmed by RT-PCR. In addition, TCL specific for tetanus toxoid, monocytes, CD4⁺ and CD8⁺ T-cells, but not B-cells secreted LIF. LIF producing T-lymphocytes and macrophages could also be identified immunohistochemically in active and chronic active MS lesions. We further demonstrated dose-dependent protective effects of both CNTF and LIF on TNF-alpha induced apoptosis in HOG cells and rat oligodendrocyte cultures. At concentrations of 10-20 ng/ml both neurokines completely protected oligodendrocytes against cytokine induced apoptosis. Post-treatment with neurokines was as effective as pre-treatment. In conclusion, our in vitro studies indicate that LIF and CNTF are possible candidates for therapeutic interventions in MS. Factors that enhance the production of neurotrophins by T cells may provide new tools for MS therapy.

This work was supported by grants from the Belgian 'Nationaal Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO)', the Belgian WOMS-foundation and the Limburgs Universitair Centrum (LUC). J.V. holds a fellowship from the 'Bijzonder Onderzoeksfonds'-LUC.

On the presence of N-acetyl-sphinganine (C₂-ceramide) in mammalian brain, possible relationship to etherphospholipids and phosphorylation by ceramide kinase.

H. VAN OVERLOOP, M. BAES* AND P.P. VAN VELDHOVEN

*Afdeling Farmacologie, Faculteit Geneeskunde and *Labo Klinische Chemie, Faculteit Farmaceutische Wetenschappen, Katholieke Universiteit Leuven, Belgium.*

Ceramides (N-acyl-sphinganine) belong to the group of sphingolipids, ubiquitous constituents of eukaryotic cells with essential roles in cell growth, survival and death¹. Ceramide, the initial product of the sphingomyelin cycle, functions as a second messenger in a variety of biological processes like cell differentiation and apoptosis. To facilitate the study of these processes, C₂-ceramide (N-acetyl-sphinganine) has been introduced as a water-soluble ceramide analogue. This lipid however, was previously reported to be formed by an acetyl-transfer from Platelet Activating Factor (PAF, 1-O-hexadecyl-2-acetyl-glycero-3-phosphocholine) to sphinganine, a reaction catalyzed by a membrane-bound PAF:sphingosine acetyltransferase that transfers the acetylgroup from PAF to sphinganine in a CoA-independent way². The synthesis of etherlipids (like PAF) is dependent on the presence of functional peroxisomes and in patients with Zellweger syndrome, a fatal disease due to the absence of peroxisomes, vinyl etherphospholipids (plasmalogens) are severely reduced and the synthesis of PAF is disturbed³. With regard to the catabolism of C₂-ceramide, not much is known. As for ceramide, it can (hypothetically) be hydrolyzed by ceramidase(s) to produce sphinganine or be phosphorylated by ceramide kinase (CERK) to produce a truncated ceramide-1-phosphate (Cer1P). Here we addressed following questions : i) is C₂-ceramide present in mammalian brain; ii) is its formation linked to etherphospholipids; iii) is it a substrate for CERK ? To quantify C₂-ceramide in tissue samples (~100 mg), a method was developed, based on extraction, separation by NH₂-SPE columns, and GC-MS. By this method the presence of C₂-ceramide could be established in brain (±10 pmol /g) and liver (± 25 pmol /g) from control mice. To reveal a possible link between etherlipids and C₂-ceramide, the levels were analyzed in *Pex5*^{-/-} mice which lack peroxisomes⁴. Instead of the expected reduction or absence of C₂-ceramide in *Pex5*^{-/-} mice, there was no significant difference between control and *Pex5*^{-/-}. With regard to the phosphorylation of C₂-ceramide, the influence of the N-acyl chain length of ceramide as substrates for human CERK was tested. C₂-ceramide was a good substrate when albumin is used as a carrier. Also in CHO cells, overexpressing CERK and uploaded with ³²P, phosphorylation of exogenously added C₂-ceramide could be demonstrated. Hence, our data indicate that C₂-ceramide is present in brain (and other tissues) and as we demonstrated a high CERK activity in cerebrum, C₂-ceramide-1-phosphate is likely to be present as well. The function of CERK and role of (truncated) Cer1P in brain is not clear. For Cer1P, a regulatory function in the secretion of neurotransmitters, by promoting membrane fusion, has been suggested.

This work was supported by the Flemish government (Geconcerteerde Onderzoeksacties GOA/2004/08), the Belgian Ministry of 'Federaal Wetenschapsbeleid' (Interuniversitaire Attractiepolen IAP-P5/05) and the Flemish 'Fonds voor Wetenschappelijk Onderzoek' (G.0405.02). H.V.O. is an aspirant from the Flemish 'Fonds voor Wetenschappelijk Onderzoek'.

References

1. RUVOLO, P.P. (2001) *Leukemia*. 15, 1153-1160
2. LEE T.C., *et al.* (1996) *J. Biol. Chem.* 271, 209-217
3. STURK A. *et al.* (1987) *Blood* 70, 460-463
4. BAES, M., *et al.* (1997). *Nature Genet.* 17, 49-56

CD4⁺CD25⁺ regulatory T cells isolated from patients with relapsing-remitting multiple sclerosis show an impaired suppressive capacity and reduced FOXP3 expression

K. VENKEN¹, N. HELLINGS¹, K. HENSEN², J.-L. RUMMENS², R. MEDAER¹, J. RAUS¹
AND P. STINISSEN¹

¹*Biomedisch Onderzoeksinstituut, Limburgs Universitair Centrum and School of Life Sciences, Transnational University Limburg, Diepenbeek, Belgium*

²*Clinical Laboratory of Experimental Hematology, Virga Jesse Hospital, Hasselt, Belgium*

Accumulating evidence indicates an immunosuppressive role for CD4⁺CD25⁺ regulatory T cells (Tregs) in autoimmune diseases. A member of the forkhead/winged-helix family of transcription factors, Foxp3 has recently been identified as a key regulator of Treg development and function. In mice, the expression is almost completely restricted to CD4⁺CD25⁺ Tregs and induction of transgenic Foxp3 expression confers suppressive activity to otherwise non-regulatory CD4⁺CD25⁻ T cells. Mutation in the Foxp3 gene leads to scurfy in mice, a wasting disease characterized by hyperactive T cells and multi-organ autoimmune manifestations as a result of the absence of Treg activity.

In the present study, the phenotypic and functional characteristics of Tregs from the peripheral blood of patients with relapsing-remitting (RR), chronic progressive (CP) multiple sclerosis (MS) and age matched healthy controls (HC) were investigated. CD25⁺ (Tregs) and CD25⁻ (responders) CD4⁺ T cells were isolated by magnetic beads and studied for their phenotypic expression by flow cytometry and for FOXP3 expression by real-time PCR. Responder cells were cultured with irradiated PBMC and stimulated with anti-CD3 antibody in the presence of varying amounts of Tregs. The suppressive capacity of Tregs was measured as inhibition of proliferation of the responder cells in coculture relative to the maximal proliferation of the responders alone.

No significant frequency or phenotypic abnormalities in Tregs from MS patients were detected as compared to HC. However a reduced suppressor function of Tregs was found in the majority of patients with RR-MS (n=16) in contrast to CP-MS patients (n=7) that showed Treg function comparable to HC (n=15). Interestingly, the relative FOXP3 mRNA expression of Tregs was significantly lower in RR-MS as compared to CP-MS and HC. There was a clear correlation between Treg suppressive function and FOXP3 levels further indicating that a reduced FOXP3 expression in Tregs of MS patients may influence immune function of these cells. To study whether the reduced FOXP3 expression of bead-sorted CD4⁺CD25⁺ T cells of RR-MS patients was due to the presence of non regulatory CD4⁺CD25^{int} T cells, we also analyzed the FOXP3 mRNA expression in FACS-sorted CD4⁺CD25^{hi} T cells and CD4⁺CD25^{int} T cells. FOXP3 expression was observed in CD4⁺CD25^{int} T cells, although at much lower levels than in CD4⁺CD25^{hi} T cells. CD4⁺CD25^{hi} T cells of RR-MS patients showed a reduced FOXP3 expression, confirming our initial observations. No difference in FOXP3 mRNA expression was found between CD4⁺CD25^{int} T cells of both study groups

Taken together, our data indicate that Tregs of RR-MS patients have a decreased FOXP3 expression correlated with their suboptimal *in vitro* suppressive capacity, suggesting an impairment of Treg function in RR-MS patients.

Modulation of Trp53 dependent and independent transcripts in the X-ray irradiated developing mouse brain.

J. VERHEYDE^{1,2}, L. DE SAINT-GEORGES¹, L. LEYNS², M. MERGEAY¹ AND M.A. BENOTMANE¹

¹Laboratory of Radiobiology, Belgian Nuclear Research Centre (SCK•CEN), Boeretang 200, Mol B-2400, Belgium and ²Laboratory for Cellular Genetics, Free University of Brussels (VUB), Pleinlaan 2, Brussel B-1000, Belgium

Ionizing radiation induces some types of cellular damage that can result in cell death either by apoptosis or reproductive death and involve the alteration of specific genes, resulting in chromosome rearrangements, point mutations or changes in expression levels.

The present work evaluates the effect of 0,5 Gy dose of X-ray ionizing radiation on the differential gene expression in three stages of the developing mouse brain (E13, E15, and E18). More specific, we try to decipher the Trp53 dependent and independent genes involved in the radiation response in the central nervous system.

High throughput gene expression analysis, using cDNA microarrays (22.000 clones) combined with real-time PCR has been used to evaluate the molecular basis of the radiation response in the developing brain. Statistical analysis of the microarray data, based on developing day E13, resulted in 2758 differentially expressed transcripts in the Trp53 wild-type model and 2115 in the Trp53 null mutant model. A more focused analysis based on gene function reduced the number of biologically interesting differentially expressed transcripts to 758 in the Trp53 wild-type and 962 in the Trp53 null mutant model. Additionally, 139 transcripts were found to be differentially expressed in both models, including those with complementary expression patterns.

In the Trp53 wild-type genotype, a set of Trp53 dependent genes appear to be upregulated upon ionizing radiation exposure and include *Ccng1*, *Mdm2*, *Cdkna1* and *Trp53inp1*. Other modulated mechanisms are related to the interferon- γ (IFN- γ) and insulin growth factor-1 (*Igf1*) inducing signaling pathways.

In irradiated Trp53 null mutants, Trp53 dependent transcripts showed no modulation, while downregulation of various cyclins and cyclin dependent kinases could still be observed and may suggest activation of cell cycle arrest mechanisms or even apoptosis. The strongest effect of ionizing radiation is observed at E13 and might be related with the early onset of neuron proliferation and migration. Several development related transcripts are also modulated in both wild-type and Trp53 null mutant genotypes suggesting a direct effect of ionizing radiation during development which could provide a link between the underlying molecular mechanisms and the induction of late physiologic and behavioral malformations.

The presence of four iron-containing superoxide dismutase isozymes in Trypanosomatidae : characterization and subcellular localization in *Trypanosoma brucei*.

C. YERNAUX¹, D. GERBOD², F. R. OPPERDOES¹ AND E. VISCOGLIOSI².

¹*Christian de Duve Institute of Cellular Pathology, B-1200 Brussels, Belgium and*

²*Institut Pasteur, Inserm U547, F-59019 Lille cedex, France*

Superoxide dismutases (SODs) form part of a defense mechanism that helps protect obligate and facultative aerobic organisms from oxygen toxicity and damage. We report the presence in the trypanosomatid genomes of four SOD genes: *Fesoda*, *Fesodb1* and *Fesodb2* and a newly identified *Fesodc*. All four genes of *Trypanosoma brucei* have been cloned, sequenced and overexpressed in *Escherichia coli* and shown to encode active dimeric FeSOD isozymes. Homology modelling of the structures of all four enzymes using available X-ray crystal structures of homologs showed that the four TbSOD structures were nearly identical. Subcellular localization using GFP-fusion proteins in procyclic insect trypomastigotes shows that FeSODB1 is mainly cytosolic, with a minor glycosomal component, FeSODB2 is mainly glycosomal with some activity in the cytosol and FeSODA and FeSODC are both mitochondrial isozymes. Phylogenetic studies of all available trypanosomatid SODs and 106 dimeric FeSODs and closely related cambialistic dimeric SOD sequences suggest that the trypanosomatid SODs have all been acquired by more than one event of horizontal gene transfer, followed by events of gene duplication.