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Matrix-Degrading Metalloproteinases and Cytokines in Multiple Sclerosis and Ischemic Stroke

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Mit dem Wissen wächst der Zweifel

Johan Wolfgang Goethe (1749-1832)

ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system characterized by blood-brain barrier breakdown, perivascular inflammation and demyelination. In ischemic stroke, an inflammatory reaction develops shortly after arterial occlusion. Inflammatory processes, therefore, may play an important role in the clinical outcome of both MS and stroke. Professional antigen presenting cells (APC) like dendritic cells (DC) and monocytes are critical in initiation and perpetuation of inflammatory responses. Efficient functioning of APC requires the shuttling of antigen from tissues to lymphoid organs. Local secretion of matrix-degrading metalloproteinases (MMP), a group of enzymes capable of degrading virtually all components of the extracellular matrix (ECM), may provide APC with a powerful tool to cross the various barriers between tissues and lymph nodes. Cytokines encompass a group of immunomodulatory proteins secreted by a variety of cells that regulate extent, nature and duration of immune responses. MMP and cytokines are two interdependent immune variables regulating production and activation of each other. In this study, the involvement of APC in MS and stroke was examined by evaluating their MMP and cytokine production capacities.

We observed that MS is associated with high levels of monocytes secreting IL-6 and IL-12 and expressing mRNA of MMP-1, -2, -7 and -9 and of TIMP-1 compared to healthy subjects (HC). MS is also associated with high levels of immature DC expressing MMP-1, -2 and -9 and mature DC expressing MMP-2 and -3 and of TIMP-1. In parallel, we observed high enzymatic activity in culture supernatants and a higher migratory capacity over ECM-coated filters of DC generated from MS patients' blood compared to DC from HC. Treatment with IFN-β is beneficial for MS. When patients with MS were examined before vs. during IFN-β treatment, lower levels of IL-6 and TNF-α secreting and of MMP-3 and –9 mRNA expressing mononuclear cells (MNC) compared to pre-treatment levels were observed. On the contrary, numbers of IL-10 secreting MNC and TIMP-1 mRNA expressing MNC were augmented during IFN-β treatment. We conclude that MS is associated with altered levels of MMP, TIMP and cytokines. When studied separately, APC such as monocytes and DC partly mirror the systemic imbalances in MMP and cytokine profiles observed in MS suggesting their involvement in MS pathogenesis. MMP, TIMP and cytokine alterations in MS were normalized by IFN-β treatment.

Examining patients with ischemic stroke within 4 days and 1 month after onset of symptoms, we observed higher levels of blood monocytes and MNC secreting TNFα, IL-12 and IL-6 in the acute phase of stroke compared to convalescence and to HC. Levels of monocytes expressing MMP-1 and -7 and TIMP-1 mRNA were also elevated in the acute phase of stroke, as were levels of MMP-1, -2, -7 and -9 and of TIMP-1 mRNA expressing MNC. MMP activity as measured by zymography was also higher in MNC supernatants in the acute phase of stroke compared to convalescence. The acute phase of ischemic stroke is associated with elevated levels of MMP and cytokine expressing monocytes and MNC that are normalized in convalescence. Our data suggest that monocytes are involved in the transient systemic immune alterations observed in stroke.

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II Kouwenhoven M, Teleshova N, Özenci V, Press R, Link H. Monocytes: phenotype and cytokine profile. *J Neuroimmunol.* 2001;**112**:197-205.

III Kouwenhoven M, Özenci V, Gomes A, Yarilin D, Press R, Link H. Elevated expression of matrix metalloproteinases in multiple sclerosis monocytes. *J Autoimmun*. 2001;**16**:463-470.

IV Özenci V, Kouwenhoven M, Teleshova N, Pashenkov M, Fredrikson S, Link H. Multiple sclerosis: Pro- and anti-inflammatory cytokines and metalloproteinases are affected differentially by treatment with IFN-β. *J Neuroimmunol*. 2000;**108**:236-243.

V Kouwenhoven M, Özenci V, Tjernlund A, Pashenkov M, Homman M, Press R, Link H. Dendritic cells express and secrete matrix-degrading metalloproteinases and their inhibitors and are imbalanced in multiple sclerosis. *Manuscript.*

VI Kouwenhoven M, Carlström C, Özenci V, Link H. Matrix metalloproteinase and cytokine profiles in monocytes over the course of stroke. *J Clin Immunol*. In press.

CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
APC	Antigen presenting cells
BBB	Blood-brain barrier
BM	Basal membrane
CNS	Central nervous system
CSF	Cerebrospinal fluid
DC	Dendritic cells
ECM	Extracellular matrix
EDSS	Expanded disability status scale
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
IFN	Interferon
IL	Interleukin
ISH	In situ hybridization
LPS	Lipopolysaccharide
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MMP	Matrix-degrading metalloproteinases
MNC	Mononuclear cells
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
OND	Other non-inflammatory neurological diseases
PBS	Phosphate buffered saline
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tc	Cytotoxic T cell
TCR	T cell receptor
Th	Helper T cell
TIMP	Tissue inhibitors of MMP
TNF	Tumor necrosis factor

INTRODUCTION

Inflammatory processes are crucial to maintain homeostasis of the organism. Skin and mucous membranes together with their glands and secretions generally provide sufficient protection to prevent foreign pathogens to penetrate the body. If barriers are broken, the immune system is alerted and inflammatory responses are initiated as perceivable by the four cardinal symptoms of inflammation, e.g. '*rubor, dolor, calor* and *tumor*'.

The immune system is a highly specialized system composed of cells and soluble mediators that respond in an extremely efficient and flexible fashion to protect and restore the integrity of the organism. The components of the immune system (cells and soluble factors) are usually classified as belonging to the innate or adaptive immune system. The functioning of the innate immune system does not require generation of antigen (Ag) specific effector cells and molecules. Therefore, it provides a fast and potent defense mechanism that combats pathogens and initiates tissue repair without delay. Granulocytes, monocytes/macrophages, dendritic cells (DC), natural killer (NK) cells, natural killer T (NKT) cells and γδ-T cells, and a number of pre-stored or rapidly inducible soluble factors including complement, cytokines and chemokines form the critical components of this versatile system (Medzhitov and Janeway, 1997). The host elicits the adaptive immune response when innate processes fail to resolve the inflammatory stimulus. Through generation of Ag specific effector B and/or T cells, the adaptive response provides plasticity and immunological memory to the defense system. Since many immune responses originate from an intimate and essential collaboration between innate and adaptive immune system (Shi et al., 2001), the segregation between adaptive and innate immunity seems merely arbitrary. The flexibility and diversity of the immune system results in a powerful system that requires stringent regulatory autocrine, paracrine, and hormonal control mechanisms. Dysregulation of the immune response may result in either an unsuccessful elimination of the pathogen and chronic inflammation, or misdirected and undesirable tissue destruction as proposed in autoimmune disorders (Langman and Cohn, 2000).

PROFESSIONAL ANTIGEN PRESENTING CELLS PLAY A KEY ROLE IN THE INITIATION OF ADAPTIVE IMMUNE RESPONSES

The backbone of the adaptive immune response is the ability of cytotoxic T (Tc)/CD8+ and helper T (Th)/CD4+ cells to recognize Ag in its native form. Initiation and perpetuation of immune responses critically depend on presentation of antigen in the context of major histocompatibility complex (MHC) molecules by specialized cells. Depending on the localization, i.e. intracellular vs. extracellular, antigenic peptides are differentially presented by class I or II MHC molecules, respectively. Most cells have the machinery to process protein Ag and express class I MHC molecules on the cell surface and thus are competent antigen presenting cells (APC) for Tc cells. In contrast, class II MHC molecules necessary for presentation of peptides to Th cells are expressed by a limited set of cells designated as professional APC. In blood, DC, monocytes/macrophages and B cells form the major groups of professional APC, while specialized tissue APC expressing MHC class II may regulate immune responses locally (Kalinski et al., 1999)(Fig.1).

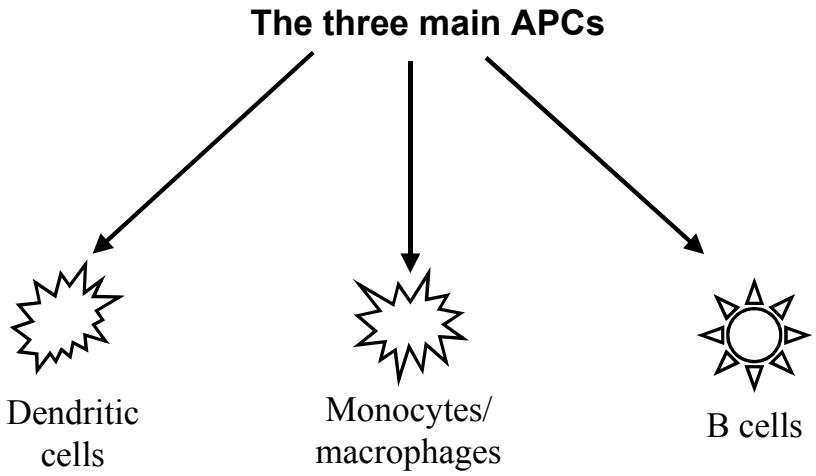


Fig.1. Professional antigen presenting cells (APC) are the specialized leukocytes responsible for antigen presentation. These include three sets of cells, the dendritic cells including Langerhans cells of the skin, the mononuclear phagocytes, and the B cells. Other cells can be made to have APC function under special conditions. APC need to have several properties in order for them to be involved in antigen presentation to CD4 and CD8 T cells: 1) the capacity to take up the antigen; 2) the capacity to internalize the antigen and process it; 3) the synthesis and expression of MHC class I and II molecules; 4) the expression of adhesion molecules, to promote and insure the APC–T cell interaction; and 5) the expression of costimulatory molecules (i.e. those molecules that promote the growth and differentiation of T cells during antigen presentation) and the release of cytokines.

Among the professional APC, DC probably are the most potent to stimulate naive Th cells due to (1) their strategic position in non-lymphoid tissues as immature cells and their ability to circulate via blood and lymph to lymphoid organs, where Ag presentation to naive T cells occurs, allowing an efficient sampling of virtually all body compartments for possible Ags (Steinman et al., 1997; Madri and Graesser, 2000; Sozzani et al., 2000); (2) an effective pinocytosis and phagocytic activity necessary for Ag sampling and (3) a rapidly inducible expression of high levels of MHC class II expression and of costimulatory and adhesion molecules upon activation (Steinman and Young, 1991).

Monocytes/macrophages, another set of professional APC, have a relatively low migratory capacity and are poor stimulators of naive T cells compared to DC (Levine and Chain 1992; Mamula and Janeway, 1993). However, expression of MHC II and costimulatory molecules by monocytes/macrophages in concert with their localization in tissue may play an important role in amplifying the local memory effector responses (O'Garra et al., 1996). Monocytes are highly responsive to inflammatory and immunological signals and thus serve as an important component of the first-line immunological defense in host cell-mediated immunity to infection, chronic and acute inflammatory processes but also tissue homeostasis through secretion of a variety of immune-regulatory cytokines (Williams et al., 1999). Monocytes are crucial in scavenging dead cells, are capable of initiating antibody-dependent cellular cytotoxicity (ADCC) and generate reactive oxygen metabolites that facilitate the elimination of pathogenic intracellular bacteria and parasites. The isolation and study of this cell population is important for the understanding of host defense and the pathogenesis of inflammation.

Finally, B cells constitute the main factors of the humoral immune response and thus may have particular importance in defense to extracellular pathogens through opsonization. B cells, in addition, form an important contributing factor to the establishment of immunological memory.

In conclusion, different subsets of APC may be dedicated to different stages of the immune response such as: (1) priming of naive CD4+ T cells; (2) amplification and diversification of the immune response; (3) skewing the immune response, i.e. cellular or humoral response; and (4) activation of memory effector type T cells.

The initiation of adaptive immune responses through APC is summarized in Fig. 2.

Initiation of adaptive immune responses requires three signals

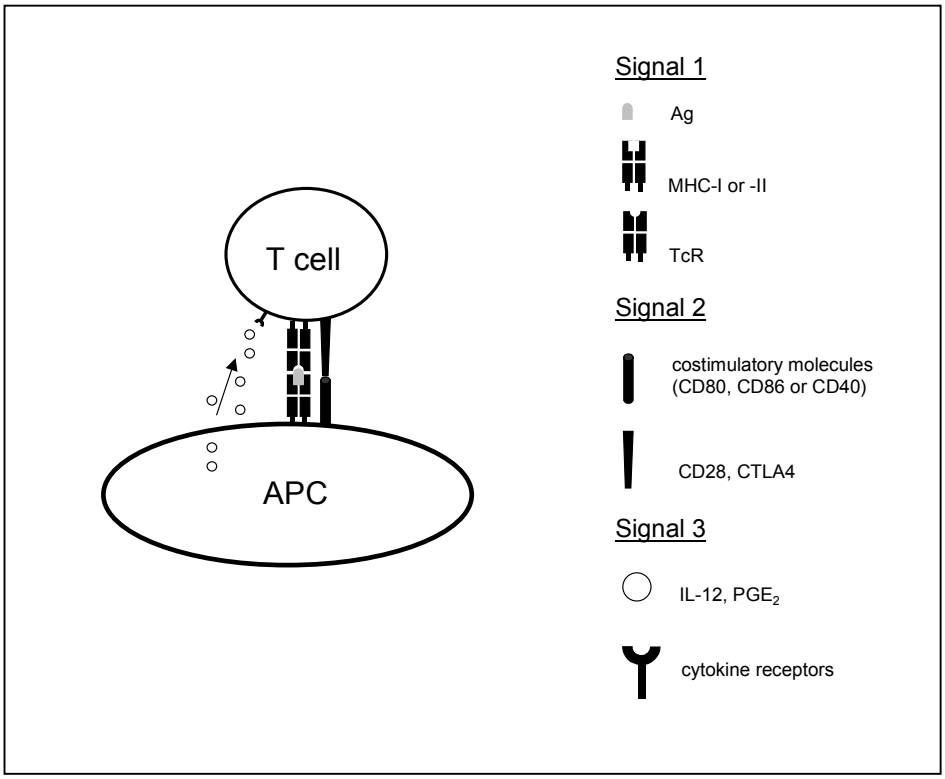


Fig. 2. Antigen presenting cells (APC) trigger the T cell receptor (TCR) through engagement of specific antigenic peptides bound to MHC class I or II molecules. (Linsey et al., 1992; McArthur and Raulet, 1993), designated as 'Signal 1'. Subsequently, APC upregulate costimulatory molecules (CD80, CD86 and CD40) on their cell surface, which interact with their ligand CD28 and CTLA-4 (for CD80, CD86) or CD40L (for CD40) expressed on T cells, thus enforcing the signal triggered by MHC-TCR engagement (Liu et al., 1991; Linsey et al., 1992; Young et al., 1992; Caux et al., 1994) (Signal 2). MHC-TCR and costimulatory signals are sufficient to activate the naive T cells. A 3rd signal from the APC may polarize the immune response by promoting cellular or humoral immunity (Kalinski et al., 1999). This 'Signal 3' probably relies on levels of the cytokine interleukin (IL)-12, locally secreted by polarized APC. Besides these classical molecules involved in T cell activation, adhesion molecules expressed by APC and T cells may be involved in the process of antigen presentation. Adhesion molecules may thus coordinate APC-T cell approximation, thereby optimising interactions between T cells and APC at the immunological synapse (Geissler et al., 1990; Ybarrondo et al., 1994).

CYTOKINES ARE IMPORTANT REGULATORS OF THE IMMUNE RESPONSE

Cytokines are soluble proteins that exert numerous effects on cells of the immune system, resulting in the orchestration of all phases of the immune response, from its initiation to its completion. Cytokines are generally produced in small quantities in response to local stimuli, such as the presence of Ags, endotoxins or the transduction of signals provided by other cytokines. Cytokines can be classified as Th1-type cytokines, e.g. IL-2, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), which promote cellular immunity by activating cytotoxic and phagocytic functions in effector cells such as Tc cells, NK cells and macrophages, and Th2-type cytokines like IL-4, -5, -6, -10 and -13 that support B cells to produce Ag-specific antibodies (Ab)(Kalinski et al., 1999). Alternatively, cytokines can be categorized according to their effects on cellular immunity. Those facilitating cellular immunity are defined as pro-inflammatory cytokines and include IFN-γ, TNF-α, TNF-α, IL-2, IL-12, IL-15, IL-17 and IL-18. Anti-inflammatory cytokines counterbalance the pro-inflammatory cytokines, and include IL-4, IL-6, IL-10 and IL-13. Balances between the different pro- and anti-inflammatory cytokines are considered of critical importance to maintain immune homeostasis of the individual.

The effects of cytokines on the immune system are determined by various factors including the expression of the corresponding cytokine receptor on target cells; the status of intracellular signalling pathways processing the 'cytokine signal'; the local microenvironment in which the cytokine acts including the presence of other related cytokines and the presence of competing or synergistic elements, i.e. soluble cytokine receptors, binding proteins and anti-cytokine antibodies (Barnes, 1998). In the present study, five cytokines were evaluated, namely IL-12, IFN-γ, TNF-α, IL-6 and IL-10. A short description of these cytokines is given below.

The functional IL-12p70 molecule is heterodimeric. It is composed of an IL-12p35 subunit that is constitutively expressed but not secreted, and an IL-12p40 subunit that is exclusively induced and secreted upon activation of APC. IL-12 is the most potent factor for Th1 polarization (Gateley et al., 1998). Besides induction of IFN-γ in T and NK cells, IL-12 also promotes the production of IL-6 and TNF-α (Caspi, 1998), facilitates cell-mediated cytotoxicity and can function as chemoattractant

for monocytes/macrophages (Ha et al., 1999).

IFN-γ is a proto-type pro-inflammatory cytokine that is produced mainly by T and NK cells. IFN-γ induces MHC class II expression on monocytes/macrophages (Mosman and Coffman, 1989) and promotes a cascade of other pro-inflammatory cytokines such as TNF-α.

TNF-α is a pro-inflammatory cytokine secreted by monocytes/macrophages, T and B cells. TNF-α facilitates the expression of cell adhesion molecules, induces the expression of several matrix-degrading metalloproteinases (MMP) (Johnatty et al., 1997; Aoudjit et al., 1998; Singer et al., 1999), up regulates MHC class II expression and is also a potent chemoattractant cytokine for monocytes and to lesser extent for neutrophils (Caspi, 1998; Ha et al., 1999). Moreover, TNF-α may favour development of Th1-type immune responses through induction of IL-12 production (Chandler et al., 1997).

IL-6 is synthesized by APC and T cells and exerts both pro- as anti-inflammatory effects (Tilg et al., 1997). IL-6 induces B cell differentiation and immunoglobulin production but may also play a role in T cell activation (Hirano, 1998). IL-6 counteracts lipopolysaccharide-(LPS) induced TNF-α production (Schindler et al., 1990), inhibits MMP but enhances the expression of tissue inhibitors of MMP (TIMP) (Lotz and Guerne, 1991).

The anti-inflammatory cytokine IL-10 is likewise produced by T cells and APC. IL-10 suppresses Ag-specific proliferation of Th1 clones and inhibits the synthesis of many Th1 related cytokines such as IFN-γ, TNF-α, TNF-β, IL-1, IL-2 and IL-6 (Singer 1999). The effects of IL-10 on professional APC include suppression of MHC class II, adhesion and co-stimulatory molecule expression (De Waal Malefyt and Moore, 1998). IL-10 also induces B cell differentiation.

MIGRATION OF ANTIGEN PRESENTING CELLS IS ESSENTIAL FOR INITIATION AND PERPETUATION OF THE IMMUNE RESPONSE

APC and T cells need to encounter each other to allow the transduction and expansion of the immune stimulus from APC to T cell(s). However, lymphocytes, the mediators of the specific, acquired immune system, are usually not exposed where foreign Ags are encountered. Thus a system must ferry these Ags from the periphery into inner tissues where lymphocytes reside

or traffic. Clearly, translocation of Ags from the periphery to the lymphoid niches is a pivotal function whereby the DC system plays an important role (Flores-Romo, 2001). Migratory ability is, therefore, an indispensable trait of APC in immune surveillance, initiation and control of inflammatory responses. During these migratory processes, cells should transverse from blood towards the site of tissue injury and possibly back into the lymphatic circulation. Several barriers should be crossed during this process including: (1) micro-vascular endothelial cells, (2) the basal membrane (BM), as well as migration through (3) the extracellular matrix (ECM). Adhesion molecules, chemokines and chemokine receptors, as well as cytokines, MMP and TIMP all play an important role and provide the basis for the tightly orchestrated process of immune cell extravasation as depicted in Fig. 3 (Muller and Randolph, 1999).

MATRIX-DEGRADING METALLOPROTEINASES DEGRADE BASEMENT MEMBRANE AND EXTRACELLULAR MATRIX PROTEINS

A supportive network of ECM surrounds all epithelia, endothelia and connective tissue cells. The BM is a specialized type of ECM, which lines all tissues and capillaries, thereby separating tissues from each other consequently providing distinct properties to each of the tissue compartments. ECM and BM thus form a major barrier to macromolecules and immune cell extravasation. To pass the BM and migrate into the ECM, cells should secrete enzymes capable of degrading components of the ECM. MMP are a group of Zn^{+}-dependent, Ca^{2+} requiring proteases that can degrade virtually all components of the ECM (Machein et al., 1997). MMP are structurally related enzymes that can be divided into six main groups according to substrate specificity as: collagenases (MMP-1, -8, -13 and -18), gelatinases (MMP-2 and –9), matrylisins (MMP-7), stromelysins (MMP-3, -10 and -11), elastase (MMP-12) and membrane-bound metalloproteinases (MMP-14 to -17) (Woessner and Nagase, 2000).

The regulation of MMP is controlled at the level of production and activation. (1) MMP transcription is mainly controlled by soluble factors such as cytokines, growth factors, extracellular matrix components (Zigrino et al., 2001) and cell-to-cell interactions (Malik et al., 1996). These regulatory mechanisms are to some extent cell type specific (Mauch, 1998). (2) Most cell types do not store MMP protein. (3) The majority of MMP are secreted as inactive zymogens, which

Leukocyte extravasation is a multistep process

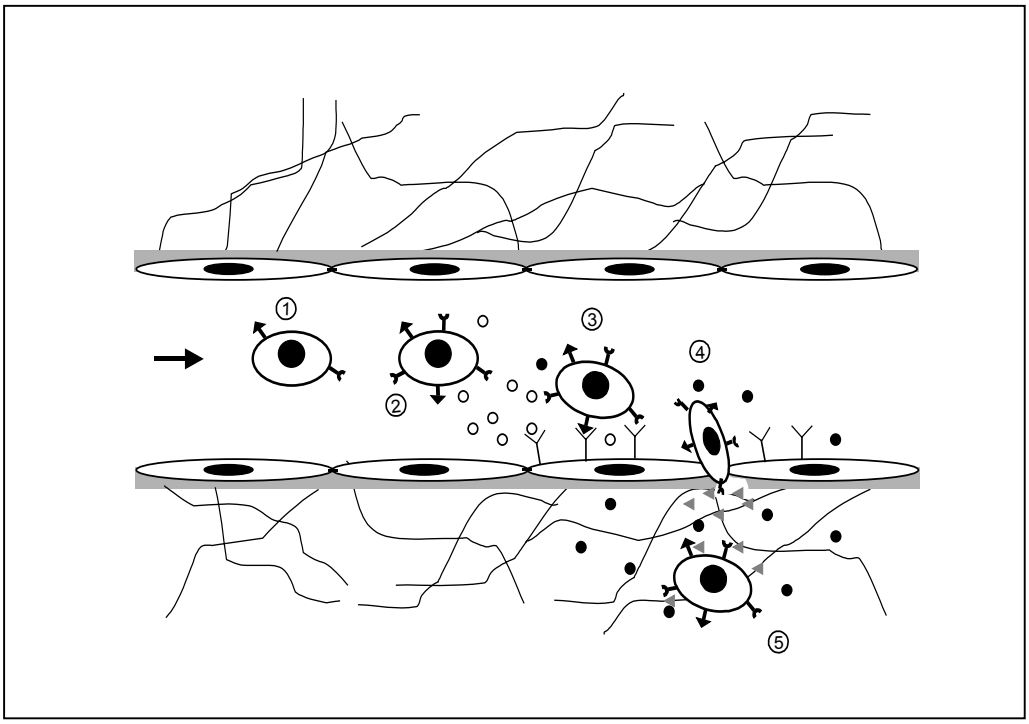


Fig. 3. The initial step in cell migration involves the upregulation of adhesion molecules on endothelial cell surface (Y) and immune cells (⬆) (1, 2) by mediators (○) released during tissue injury, e.g. cytokines like IL-1, IL-8, TNF-α, IFN-γ or chemoattractants (Campbell et al., 1997; Van Furth, 1998). (3) Cells loosely tether and, subsequently, firmly adhere to the endothelial vessel lining through coordinated expression of selectins (Ley et al., 1995), integrins and members of the immunoglobulin super family (Yednock et al., 1992). The direction of migration is determined by a concentration gradient of chemokines (●) acting upon cell type specific chemokine receptors (Y) (Murphy, 1994; Ransohoff, 1999). Most chemokines bind to ECM components or cell-surfaces and thus limit washout of the chemotactic gradient into the blood stream (Middleton et al., 1997). (4) Surpassing of the BM necessitates proteolytic cleavage by proteases (▲). (5) Upon entering the tissue, cells are directed to the site of tissue injury by combinations of adhesion molecules, chemo-attractants as well as through local ECM modification by MMP. In conclusion, cellular extravasation is a complex process, regulated by a multitude of receptor-ligand interactions and local ECM proteolysis. The combinatorial diversity of these various ligand-receptor pairs may confer important parameters to specify the nature of the inflammatory infiltrate (Springer et al., 1994).

require proteolytic cleavage by plasmin or other MMP. (4) Once activated, MMP are subjected to stochiometrical inhibition by naturally occurring TIMP. The currently known TIMP (1-4) can to a certain degree inhibit almost all MMP. More unspecific serum proteins as α_2-macroglobulins may also regulate MMP activity. (5) Through localization of MMP to receptors, adhesion sites, or invasive protrusions of cells, proteolytic events are further confined to the immediate peri-

cellular environment of cells (Woessner and Nagase, 2000). Proteolysis, therefore, can be confined to a specific area and be effective even in the presence of high concentrations of TIMP. Imbalances in MMP may in case of excessive MMP production result in disproportionate ECM breakdown or, when MMP are suppressed, in excessive deposition of ECM as observed in fibrosis (Kieseier et al., 1999).

Hitherto only limited information is available on MMP expression by APC. MMP expression by monocytes has been studied in some detail. Cell-cell contact between monocytes and activated peripheral blood T cells seems to play an important role in the induction of MMP-1 and -9 and of TIMP-1 production by monocytes (Lacraz et al., 1995). Besides cell surface molecules, soluble factors secreted by T cells like cytokines may modulate monocyte MMP and TIMP expression. IFN-γ, IL-4 and IL-10 suppress MMP production, while IL-6 and IL-10 increase TIMP production in monocytes (Zhang et al., 1998). The effects of soluble mediators on monocytes' MMP production depend on differentiation status of the monocytes. E.g., IL-4 and IL-10 (Zhang et al., 1998) decrease MMP-1 production in freshly isolated CD14+ monocytes but increase MMP-1 production in GM-CSF maturated monocytes (Chizzolini et al., 2000). Recently, also the expression of MMP-1, -3 and of TIMP-1 was described in B cells (Di Girolamo et al., 1998; Trocme et al., 1998). The expression of MMP and TIMP by DC is largely unexplored. Gelatinolytic activity was detected in supernatants of monocyte-derived DC (Sauter et al., 1996). However, the authors did not consider the purity of their DC cultures, and it is therefore not possible to conclude that these enzymes were secreted by DC and not by contaminating T or B cells or even by monocytes. Such proof requires identification of MMP or TIMP on cellular level.

The role of MMP secreting monocytes and DC in MS and stroke is thus far unexplored. Such is of great interest since monocytes form main constituents of MS as well as ischemic lesions and thus may utilize MMP to migrate into CNS. Moreover, MMP expression by DC may be crucial for DC migration and functioning and, therefore, in their efficiency of immune induction.

IMMUNE RESPONSES IN THE CENTRAL NERVOUS SYSTEM

The central nervous system (CNS) has long been considered to be an `immunologically privileged' site due to delayed rejection of xenografts and tumor allografts as well as the relative paucity of leukocytes in the CNS and of MHC class II expressing cells under physiological conditions (Carson and Sutcliffe, 1999). The presence of the blood-brain barrier (BBB), which shields the brain from fluctuations in the circulation, and absence of conventional lymphatic drainage further restrict (but does not completely prevent) the trafficking of Ag from and to the CNS (Wekerle et al., 1986; Engelhardt et al., 2001). The BBB thus hinders the quick entrance of APC to the CNS and a rapid acquisition of Ag by APC in the CNS. In addition, the egress of APC from the CNS to lymph node and subsequent activation of lymph node T cells is slowed in such way that very few cells may succeed in completing this route to initiate an immune cascade before the immune stimulus subsides (Carson and Sutcliffe, 1999). During pathological and host-defence processes as diverse as viral encephalitis, multiple sclerosis (MS), stroke and head trauma, the immune privilege of the CNS is partly compromised, allowing recruitment of blood-borne cells into the CNS (Ransohoff, 1999).

Multiple sclerosis

MS is a chronic disease of the CNS characterized by multifocal inflammation and destruction of myelin. MS is a major cause of neurological disability among young adults in the Western hemisphere. MS manifests itself usually between the 2^{nd} and 5^{th} decade of life. Females are twice as often affected as males. The primary cause of MS is unknown. Viral infection, genetic predisposition, environmental factors, and autoimmunity are all considered to contribute to the pathogenesis of the disease (Ebers et al., 1998; Trapp et al., 1998; Kurtzke, 2000). In the majority of patients, disease commences with a relapsing remitting course (RR MS) characterized by periods of exacerbations followed by complete or partial recovery. The frequency of relapses diminishes over the years, resulting in a steady, secondary chronic-progressive clinical deterioration (SCP MS). Up to 15% of the patients with MS show a primary chronic-progressive course from onset (PCP MS) of the disease. The clinical course of

MS is highly variable both within and among patients (Compston, 1998; Ebers, 1998). The random distribution and heterogeneity of lesions throughout the CNS as reported by magnetic resonance imaging (MRI), spectroscopy studies as well as pathological studies (Lee et al., 1999; Lucchinetti et al., 2000) may contribute to the wide range of signs and symptoms observed in MS patients. The diagnosis of MS is to date still critically dependent on clinical examination according standard criteria (Poser et al., 1983). The improved accessibility to MRI over recent years and classical cerebrospinal fluid (CSF) analysis may enhance the confidence of the diagnosis strongly and are now implicated in new diagnostic criteria for MS (McDonald et al., 2001).
The pathological hallmark of MS constitutes of an inflammatory demyelinating plaque composed of different blood-borne cells such as activated lymphocytes and macrophages, but also of endogenous glial cells (astrocytes and microglia). Moreover, myelin destruction, oligodendrocyte death and axonal loss are characteristic features of the lesions observed in MS and likely contribute to impaired signal transduction and clinical manifestations (Raine, 1994; Trapp et al., 1998).

Several lines of evidence indicate that immune-mediated mechanisms may play a role in MS. The intense inflammatory reaction observed in the CNS of MS patients points to the involvement of the immune system in disease pathogenesis (Navikas and Link, 1996). In addition, correlations with immune response genes in the form of certain HLA-DR haplotypes (Jersild et al., 1973), the presence of high levels of T and B cells reactive to myelin Ags in blood and in particular CSF of MS patients (Link et al., 1990; Olsson et al., 1990; Link et al., 1992) as well as the beneficial effect of the cytokine IFN-β on the course of MS (The IFNB Multiple Sclerosis Study Group, 1993) further provide evidence for immune mediated processes in the pathogenesis of MS.

The initiation of the immune response

The processes underlying initiation of the immune response in MS are not fully understood and probably depend on an unfortunate combination of environmental, genetic and immunological factors resulting in an immune attack

to supposedly CNS-derived Ags (Ludewig et al., 1999). The initiation of this response and the subsequent activation of naive T cells by APC probably occurs in the periphery, since naive T cells lack the capacity to cross the BBB unless activated via bystander mechanisms (Aloisi et al., 2000). Due to their efficient Ag processing capacity and high mobility, DC may be attractive candidates to activate these naive T cells in the secondary lymphoid organs. Whether DC acquire Ag in the periphery or in the CNS as suggested by their presence in human CSF (Pashenkov et al., 2001) and by migration studies (Randolph et al., 1998; Duddy et al., 2001) remains to be clarified (Fisher and Reichmann, 2001).

Aberrations in the expression of MHC class II and costimulatory molecules by APC could contribute to initiation of aberrant immune responses in MS. Data on the expression of these molecules by circulating monocytes in MS is subject to controversy. Lower percentages of HLA-DR expressing monocytes in MS compared to healthy subjects were reported (Baxevanis, 1989). In contrast, high density of HLA-DR on monocyte cell surface was also observed in blood of MS patients during clinical exacerbation compared to remission (Reder et al., 1998; Boylan, 1999). Concerning costimulatory molecules, both CD80 and CD86 expressing cells were observed in MS plaques (Windhagen et al., 1995). The expression of CD80 was co-localized with IL-12p40 mRNA expressing MNC in MS lesions. In MS patients' CSF, the majority of CD80 and CD86 positive cells were monocytes (Windhagen et al, 1999). In MS patients' blood, lower ratios of CD80/CD86 were observed during clinical exacerbations (Genc et al., 1997; Boylan et al., 1999), but these levels did not differ when compared to controls (Reder et al., 1998). MS is also associated with circulating DC expressing low levels of CD86 but similar levels of CD80 and HLA-DR compared to controls (Huang et al., 2001).

Cytokine imbalances have been extensively reported in blood, CSF as well as CNS lesions of patients with MS (Link et al., 1994; Raine, 1994; Martin and McFarland, 1995; Link, 1998). These imbalances may play a role in different steps of the pathophysiological process, e.g. from initiation of the immune response to upregulation of cell adhesion molecules on cerebral endothelial cells, transmigration of inflammatory cells into the CNS to demyelination and tissue damage. Cytokine imbalances may also contribute to alterations in APC phenotype, resulting in altered migration patterns, altered release of cytokines, and/or altered antigen processing. Such alterations in APC may generate DC that

directly prime autoreactive T cells, fail to prime regulatory T cell subsets, or shift a Th1/Th2 balance to an unfavourable outcome (Drakesmith et al., 2000; Huang et al., 2001). IL-12, a cytokine mainly secreted by APC, is among the most crucial cytokines to induce deviation of the immune response towards a Th1 profile (Shevach et al., 1999). IL-12 thus facilitates the secretion of Th1-like cytokine IFN-γ, which is considered detrimental in MS and causes relapses (Panitch et al, 1987). Moreover, TNF-α and IFN-γ may have myelinotoxic properties (Louis et al., 1993). Data on IL-12 in MS are partly contradictory. High levels of IL-12 mRNA expressing MNC were observed in MS lesions (Windhagen et al., 1995). High levels of IL-12p40 (Fassbender et al., 1998) and IL-12p40 mRNA expressing MNC (Matusevicius et al., 1998) were also observed in CSF of MS patients. Elevated IL-12 expression by MNC upon stimulation (Comabella et al, 1998) and increased IL-12p40 levels were also observed in blood (Van Boxel-Dezaire et al., 1999). In contrast, others reported similar levels of IL-12p40 in MS compared to controls (Fassbender et al., 1998; Heesen et al., 1999; Özenci et al., 2001). Considering different APC subsets, MS is associated with similar IL-12 production by DC derived from MS patients compared to healthy subjects (Huang et al., 2001). Thus much controversy exists on changes in APC phenotype as well as their IL-12 production in MS.

Likewise, the degree and nature of cytokine changes of other cytokines is disputed in MS as further indicated for the cytokines under study in Box 1. Much of these differences probably relate to different methodologies applied in different studies, as discussed below. Data on specific alterations in cytokine levels in individual cell types like APC are limited.

Box 1: Controversy on selected cytokines in MS

IFN-γ: INF-γ was detected in MS lesions (Cannela and Raine, 1995). High levels of IFN-γ mRNA expressing MNC in MS blood and in particular in CSF (Link et al., 1994) and increased serum levels of IFN-γ (Hohnoki et al., 1998) were observed. Others reported similar levels of IFN-γ mRNA expressing MNC (Gayo et al., 1999; Huang et al., 1999), percentages of IFN-γ expressing (Nuygen et al., 1999) or numbers of IFN-γ secreting blood MNC in MS compared to controls (Özenci et al, 1999).

TNF-α: TNF-α mRNA was identified in MS lesions (Canella and Raine, 1995). Elevated levels of TNF-α mRNA expressing blood and CSF MNC (Navikas et al., 1996; Huang et al., 1999), elevated TNF-α levels in serum (Hohnoki et al., 1998) and high numbers of TNF-α secreting blood MNC (Özenci et al., 2000) were previously reported in MS. High levels TNF-α in serum were associated with clinical exacerbations (Tsukada et al., 1991). Considering APC, increased TNF-α secretion by monocytes in culture supernatants was reported (Reder et al, 1998). In contrast, similar levels of TNF-α secreted by monocytes were also observed in MS compared to HC (Rudick and Ransohoff, 1992; Imamura et al., 1993). Finally, MS is associated with high numbers of TNF-α secreting cells in blood MNC preparations enriched for DC (Huang et al., 1999).

IL-6: IL-6 was observed in perivascular cuffs and MS lesions (Woodroofe and Cuzner, 1993). High levels of IL-6 mRNA (Navikas et al., 1996) expressing but similar levels of secreting IL-6 CSF MNC (Özenci et al., 2000) were observed in MS. Similar serum levels of IL-6 but higher levels of IL-6 in plasma (Frei et al., 1991) and high numbers of IL-6 secreting cells were also detected (Padberg et al., 1999; Özenci et al., 2000). Circulating monocytes secreted higher levels of IL-6 (Maimone et al., 1993) and higher numbers of enriched DC secreted IL-6 compared to controls (Huang et al., 1999).

IL-10: IL-10 was also detected in MS lesions (Cannela and Raine, 1995). High numbers of IL-10 mRNA expressing and IL-10 protein secreting MNC were observed in CSF compared to blood in MS (Navikas et al., 1995; and Özenci et al., 1999). In MS, low levels of IL-10 mRNA, low levels of IL-10 protein and low numbers of IL-10 secreting cells were detected in blood of patients with MS (Salmaggi et al., 1996; Huang et al., 1999; Özenci et al., 1999). In contrast, high levels of IL-10 mRNA expressing MNC in blood (Navikas et al., 1995) were also reported. Similar levels of enriched DC secreting IL-10 have been observed in MS (Huang et al., 1999).

Blood-brain barrier breakdown is a feature of MS

Local re-activation and amplification of the inflammatory immune response by activated Ag-specific T and CNS resident cells probably is an important contributing factor in the recruitment of non-specific inflammatory cells, principally macrophages, into the CNS (Ohmori et al., 1992; Becher et al., 2000; Martino et al., 2000). Cytokines such as TNF-α, IL-1β and IFN-γ, released by these activated cells can not only induce adhesion molecule expression on brain endothelial and infiltrating cells (Hofman et al., 1989; Hickey et al., 1991; Merrill et al., 1991), but also facilitate the secretion of chemokines by CNS resident cells (Ransohoff, 1999), thus further direct and specify acquisition of inflammatory cells to the MS lesion. Breakdown of the BBB, resulting in an influx of inflammatory cells, fluid and proteins, including complement and cytokines into the normally sequestered CNS is a key feature of neuroinflammatory conditions as MS (McFarland et al., 1992; Rudick et al., 1996; Waubant et al., 1999). Monocytes and T cells, the main infiltrating cell types, probably use MMP to degrade parts of the BM and penetrate into the CNS. MMP are, in general, elevated in pathological conditions characterized by an excess of activated macrophages (Yong et al, 1998). A role for MMP in MS is suggested by the presence of high levels of MMP-1, -2, -3, -7 and -9 mRNA expressing microglia and monocytes/macrophages within inflammatory MS lesions (Maeda and Sobel, 1996; Anthony et al., 1997; Cossins et al., 1997). MS is also associated with high levels of MMP-9 in CSF (Leppert et al., 1998) as well as high numbers of MMP-9 mRNA expressing CSF MNC (Özenci et al., 1999). Moreover, correlations between levels of MMP-9 in CSF and CSF cell count may yet be another indication of the involvement of MMP in leukocyte recruitment (Yushchenko et al., 2000). Systemic aberrations such as high levels of circulating MMP-3 and -9 in serum (Lichtinghagen et al., 1999) and of MMP-3 and -9 mRNA expressing blood MNC (Özenci et al., 1999) were also observed in MS. Additionally, correlations between Gd enhancement on MRI as sign of BBB leakage, clinical exacerbation and the serum content of MMP-9 were reported (Lee, 1999; Waubant et al., 1999). These data may indicate a role for MMP in MS and especially a role of MMP expressing monocytes in their recruitment into the CNS.

MMP may not only contribute to cellular migration but also play a role in tissue damage observed in MS. Recently, neurotoxic effects of MMP-1 and MMP-2 were observed in vitro (Vos et al., 2000). MMP-3 and -9 can degrade human MBP in vitro and can hence directly contribute to myelin damage (Opdenakker and Van Damme, 1994). Indirectly, MMP may contribute to expand the inflammatory response and tissue damage through generation of Ags by breakdown of myelin or conversion of inactive membrane bound TNF-α into active myelinotoxic TNF-α (English et al., 2000). Other molecules such as TGF-α, IL-6R-α, TNF receptors, L-selectin and FAS ligand may undergo similar processes by the action of MMP (Chandler et al., 1997).

TIMP are important inhibitors of MMP. Recent data indicate that, besides imbalances in MMP, MS also may be associated with changes in TIMP. High numbers of TIMP-2 mRNA expressing CSF MNC (Özenci et al., 1999) but similar levels of TIMP-1 and -2 in CSF compared to patients with other non-inflammatory neurological diseases (OND) (Leppert et al., 1998) were observed in MS. In blood, higher serum levels of TIMP-1 in patients with RR MS that decreased during clinical exacerbation were reported (Lee et al., 1999). Higher levels of TIMP-1 mRNA expressing MNC in blood were also observed in patients with MS (Özenci et al., 1999).

In conclusion, available data on expression of MMP and/or TIMP in MS are contradictory. The net effect of MMP and their TIMP imbalances probably relates to local concentrations of MMP and inhibitors as well as to the cellular content of the infiltrate. Data on the expression of MMP and TIMP by individual cell types in MS patients' blood is restricted to T cells (Leppert et al., 1996; Stuve et al., 1996). The local action of MMP warrants studies on the cellular level.

IFN-β treatment in multiple sclerosis

The treatment of MS is mainly aimed at alleviation of symptoms, preventing or modulating relapses, and slowing of disease progression. New drugs as IFN-β and glatiramer acetate (Copaxone) that modulate the course of MS beneficially are now widely available. IFN-β is an immunomodulatory cytokine that is effective to only a certain degree in MS. IFN-β has been proven to reduce

relapse rate by up to 34% (IFN-β Multiple Sclerosis Study Group, 1995; Jacobs et al., 1996) in patients with RR MS and slow disease progression (The IFNB Multiple Sclerosis Study Group, 1995). MRI data suggest that IFN-β treatment reduces the occurrence of the number of active MS lesions (Paty et al., 1993). Recent studies also show beneficial effects of IFN-β in patients with SCP MS including a delayed progression of neurological deterioration (Jacobs et al., 1996; European Study Group on Interferon β-1b in Secondary Progressive MS, 1998).

The mechanism of action of IFN-β on the immune system remains unsettled. Potential mechanisms may besides effects on molecules involved in the initiation of the immune response as MHC and co-stimulatory molecules (Yong and Antel, 1992) also include effects on cytokine production and modulation of factors involved in entry of leukocytes into the CNS such as chemokines, adhesion molecules and MMP (Yong et al., 1998).

In a limited number of studies addressing the in vivo effects of IFN-β on cytokine levels, contradictory results were obtained. Similar plasma levels of IL-6, TNF-α or IL-10 were observed when patients were treated for 3 (Duddy et al., 1999) or 9 months (Brod et al., 1997) with IFN-β compared to pretreatment levels. In contrast, increases in serum IL-10 levels upon IFN-β treatment were also reported (Rep et al., 1999; Waubant et al., 2001). In a cross sectional study, lower levels of IL-10 secreting blood MNC were observed in patients with untreated but not in IFN-β treated patients with MS compared to controls (Özenci et al., 1999). Correlation between increased CSF IL-10 levels with a favorable therapeutic response to IFN-β was observed and may indicate that upregulation of IL-10 could underlie part of the beneficial effects of IFN-β therapy (Rudick et al., 1998).

The literature on the effects of IFN-β on MMPs is sparse. In vitro addition of IFN-β decreased the migration of T cells by inhibiting MMP-9 secretion (Leppert et al., 1996; Stuve et al., 1996; Uhm et al., 1999). Serum levels of MMP-9 but not of MMP-2 were decreased during IFN-β treatment of MS (Trojano et al., 1999). In contrast, similar levels of MMP-9 in serum but upregulated TIMP-1 levels after 4 months of treatment with IFN-β were also reported (Waubant et al., 2001).

Ischemic stroke

Ischemic stroke is a major public health problem in Western society and is the second most common cause of death after ischemic heart diseases and an important cause of disability among elderly (Hankey, 1999). Ischemic stroke is defined as a clinical syndrome of permanent brain dysfunction, which can improve or worsen over time and results from an infarction. The outcome of stroke depends partly on the extent of the lesion.

The ischemic lesion is typically composed of a necrotic core, characterized by irreversible tissue damage. However, the final size of the tissue damage depends on the fate of the penumbra region surrounding the core, which can survive as long as the blood blow remains at 20% of the normal flow (Heiss and Graf, 1994). The cellular patho-mechanisms involved in the damage to this penumbra region are still incompletely understood (Stoll et al., 1998). It is thus of critical importance to delineate mechanisms, which lead to neuronal cell death following compromised blood circulation and blood supply.

Secondary to oxygen deprivation, a typical acute inflammatory reaction develops that is characterized by recruitment of polymorphonuclear cells (PMN) and monocytes/macrophages into the ischemic lesion (Kochanek and Hallenbeck, 1992). The significance of these inflammatory responses to the brain injury is not fully understood, although evidence has emerged that this reaction may be causally related both to neuronal damage and recovery (Barone and Feuerstein, 1999). Inflammation associated with stroke could aggravate brain damage by several mechanisms such as plugging surrounding capillaries, production of radical oxygen metabolites and enzymes leading to brain damage, edema and tissue destruction (Tarkowski et al., 1997). Leukocytes and their products could be involved in this process. The extent of leukocyte infiltration was found to be correlated to final lesion volume (Akopov et al., 1996). Moreover, cytokines and MMP secreted by inflammatory cells could contribute to development of an inflammatory reaction through upregulation of adhesion molecules, promotion of leukocyte-endothelium interactions, induction of chemokines (Kostulas et al., 1999), and through local proteolysis of BM resulting in BBB leakage (Hickey et al., 1991).

Stroke is furthermore associated with cytokine alterations in the CNS, CSF as well as systemically. High levels of IFN-γ and TNF-α expressing cells were previously reported in brain biopsies of patients with acute stroke (Tomimoto et al., 1996). Feurstein and colleagues (1998) demonstrated high levels of TNF-α mRNA confined to monocytes/macrophages in acute ischemic lesions, implicating these cells in the pathogenesis of stroke. In CSF, high levels of TNF-α were also observed in patients with acute stroke (Tarkowski et al., 1995; Tomimoto et al., 1996; Tarkowski et al., 1997; Zaremba et al., 2001). Systemic aberrations in cytokine levels were also observed in serum of patients with stroke in the acute phase, e.g. high levels of TNF-α and IL-6 (Fassbender et al., 1994; Beamer et al., 1995; Kim et al., 1996). Elevated IL-6 in serum correlated with infarct volume and stroke outcome (Fassbender et al., 1994). On the other hand, similar levels of IFN-γ in serum were observed in the acute phase of ischemic stroke (Kim et al, 2000). Increased levels of IL-10 in CSF (Tarkowski et al., 1997) and high numbers of IL-10 secreting MNC systemically in the acute phase of stroke (Pelidou et al., 1999) may indicate that anti-inflammatory cytokines are also involved in the early events associated with stroke.

The source of the immune aberrations observed in stroke remains to be established. Upregulated cytokine levels in the absence of overt BBB damage were reported, indicating that intrathecal production of these molecules occurs in stroke (Tarkowski et al., 1997). On the other hand, infiltration of blood-borne neutrophils and monocytes into the ischemic lesion may indicate that systemic cytokine aberrations may thus contribute to the generation of the intrathecal immune imbalance.

Data on MMP expression in stroke are limited. In stroke, MMP may be involved in damaging of the BBB (Clark et al., 1997), proteolysis of the ECM, and in facilitating cellular migration through the ECM, cleaving cytokine precursors from the cell surface (Lichtinghagen et al., 1999; Lukes et al., 1999) and exacerbating post-ischemic edema (Hamann et al., 1996). On the other hand, MMP may also have beneficial effects and contribute to angiogenesis and restoration of the blood flow. High levels of MMP-2, -7 and -9 mRNA expressing monocytes/macrophages were previously detected in ischemic brain lesions (Anthony et al., 1997; Clark et al., 1997). Recently, high levels of MMP-9 but not

MMP-2 were observed in plasma during the acute phase of stroke. MMP-9 levels were correlated to final infarct volume and neurological deficit (Montaner et al., 2001).

The involvement of monocytes in stroke has only been partially addressed in pathological studies. Whether monocyte alterations occur systemically with regard to e.g. cytokine and MMP profiles is not known.

GENERAL AIMS OF THE THESIS

Professional APC constitute a group of cells that are crucial in the initiation and perpetuation of physiological and probably also of pathological immune responses. Immune mediators produced by these cells such as cytokines and MMP may play a role in disease outcome. The general aim of this thesis was to study the involvement of professional APC in MS and stroke by studying cytokine and MMP production. In addition, the effects of IFN-β, an approved treatment for MS, on cytokines and MMP were studied.

SPECIFIC AIMS OF THE THESIS

Papers I - II: To establish sensitive enzyme-linked immunospot (ELISPOT) assays to detect blood monocytes actively secreting the cytokines IL-12, TNF-α, IL-6 and IL-10 and to investigate the phenotype and cytokine profile of monocytes in patients with untreated MS and controls.

Paper III: To study the presence and magnitude of MMP-1, -3, -7, -9 and -14 and of TIMP-1 produced by monocytes in MS.

Paper IV: To examine the effects of IFN-β treatment on the cytokines IL-6, TNF-α, IFN-γ and IL-10 and on MMP-3, -7 and –9 and on TIMP-1 longitudinally in MS patients, i.e. comparing the levels obtained from the same individual before and during IFN-β treatment.

Paper V: To study the expression of MMP-1, -2, -3 and -9 and of TIMP-1 and -2 by monocyte-derived iDC and mDC in MS.

Paper VI: To investigate changes in levels of blood monocytes and MNC expressing the cytokines TNF-α, IL-6, IL-10 and IL-12, and the proteinases MMP-1, -2, -7 and –9, and of TIMP-1 over the course of ischemic stroke.

MATERIAL AND METHODS

PATIENTS

Patients with MS

The diagnosis of MS was made if the patient fulfilled the criteria for clinically definite MS (Poser et al., 1983). An exacerbation was defined as the sudden appearance of new symptoms and signs or the abrupt reappearance or worsening of previously present signs and symptoms lasting at least 24 h and occurring within 1 month before examination (Schumacher et al., 1965). Patients with MS were subgrouped into those with an Expanded Disability Status Scale (EDSS)(Kurtzke et al., 1983) score of less than 3.0 and those with a score of 3.0 or higher. None of the patients included in the 'untreated patient with MS' group had been treated with any immunomodulatory drug during the last 6 months prior to examination. Patients in the 'IFN-β-treated patients with MS' group were at the time of examination treated either with IFN-β-1a (Avonex; Biogen, Cambridge, MA; or Rebif; Serono Nordic AB, Solna, Sweden) or IFN-β-1b (Betaferon; Schering AG, Berlin, Germany) in standard doses.

Patients with ischemic stroke

Patients had a clinical and radiological diagnosis of acute ischemic stroke. Patients were classified according to the Oxford Community Stroke Project (Bamford et al., 1991) in lacunar syndromes (LACS), partial anterior circulation syndromes (PACS), total anterior circulation syndrome (TACS) and posterior circulation syndromes (POCS). All patients were examined with computer tomography (CT)-scan within 48 h after onset of symptoms and their neurological deficit was evaluated by a neurologist adopting the National Institute of Health Stroke Scale (NIH-SS). The patients' pre-stroke and post-stroke dependency was evaluated with historical Barthel index (BI) reflecting neurological deficit and dependency of the patients. In some cases, additional diagnostic procedures (MRI, CSF-analyses) were performed in order to establish a correct diagnosis.

Patients with other neurological diseases

The diagnoses of the patients with OND enrolled into present study were as follows: amyotrophic lateral sclerosis, dementia, non-acute cerebrovascular diseases, cerebral tumor, epilepsy, tension headache, hydrocephalus, Creutzfeld

Jacobs disease, facial palsy, polyneuropathy, paraesthesia and benign intracranial hypertension.

Healthy subjects

The healthy subjects included in the study consisted of staff members from the Department of Neurology of Huddinge University Hospital, relatives of patients with ischemic stroke and blood donors.

PREPARATION OF MONONUCLEAR CELLS FROM BLOOD

Blood was obtained by venous puncture. MNC were separated by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). Cells from the interphase were collected, washed three times with Dulbecco's modification of Eagle's medium supplemented with antibiotics, 10% fetal calf serum, 1% minimal essential medium, and 1% of L-glutamine (all from Gibco, Paisley, UK).

PREPARATION OF MONOCYTES FROM BLOOD

Monocytes were obtained using discontinuous density gradient separation. Upon density gradient centrifugation of MNC as described above, $20x10^6$ MNC were collected and centrifuged at 500 x g for 10 min at 4°C. Supernatant was discarded and cells were carefully resuspended in 60% v/v Percoll (Pharmacia, Uppsala, Sweden) in complete medium. Subsequently, 47.5% and 34% Percoll in complete medium were layered upon the cell suspension (Fig. 4). Cells were centrifuged for 40 min at 1,700 x g. The low-density fraction representing monocytes was collected and washed twice with complete medium. Cells were adjusted to a concentration of $1x10^6$ cells/ml. Cell viability as measured by Trypan blue exclusion always exceeded 95%. The monocytes isolated by this procedure were >90% pure as measured by staining of the purified cells with anti-CD14 (monocytes), anti-CD3 (T cells) and anti-CD19 (B cells) mAbs. B plus T cell contamination never exceeded 10%. Purity did not differ between groups under study.

Monocyte preparation utilizing Percoll density gradients

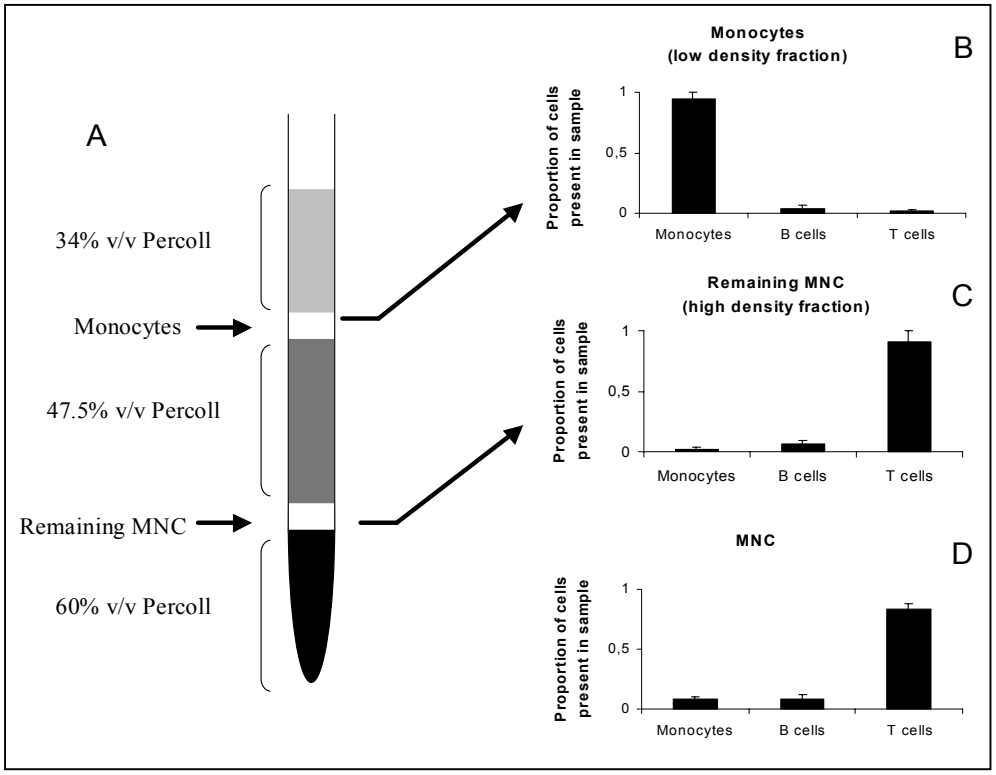


Fig.4. Mean proportions of monocytes, B and T cells among mononuclear cells (MNC) and among fractions obtained after Percoll separation (A). Monocytes were mainly confined to the low-density fraction (B). The high-density fraction (Remaining MNC) consisted mainly of B and T cells (C). The whole MNC fractions of the same samples were also examined (D). Data shown reflect mean values + one standard deviation (SD) of results from examination of 10 healthy subjects.

PREPARATION OF MONOCYTE-DERIVED DENDRITIC CELLS FROM BLOOD

Monocytes prepared by Percoll density gradient centrifugation were seeded in 24 well-culture plates (Costar, Cambridge, MA) at a density of 0.5×10^6 cells/ml. After 1.5-2 h of incubation at 37°C, the few nonadherent cells were removed by extensive washings with medium. Adherent cells were cultured in RPMI 1640 (Life Technologies Ltd, Paisley, UK) and 1% fetal calf serum (FCS; Hyclone, Logan, UT), supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF; 500 U/ml; Leukomax; Novartis, Basel, Switzerland) and IL-4 (250 U/ml; R&D Systems, Minneapolis, MN) to obtain iDC. At day 3,

the media including the supplements were refreshed. At day 6, $CD1a^+CD14^-$ $HLA\text{-}DR^+CD83^-$ iDC were obtained. In some cases, mDC were obtained by a 2-day exposure to LPS (250 ng/ml; Difco, Detroit, MI). mDC were $CD83^+CD14^-$ $HLA\text{-}DR^+CD1a^-$. iDC were kept in nonmaturing conditions during these 2 days. All subsequent tests were performed after harvesting the cells at day 8 and after removal of GM-CSF, IL-4 and LPS by extensive washings. To check the purity of DC cultures, each sample was stained by flow cytometry, using FITC conjugated mAbs to lineage markers including for CD3 (expressed on all T cells), CD14 (monocytes, macrophages, neutrophils, and eosinophils), CD16 (resting NK cells, macrophages, cultured monocytes and neutrophils), CD19 and CD20 (B cells), and CD56 (activated and resting NK cells), PerCP labelled HLA-DR, as well as PE labelled mAbs for maturation (CD83+) of DC for iDC and CD1a+ for mDC. Contamination with lineage markers or CD83+ (for iDC) or CD1a+ (for mDC) never exceeded 10%.

IN SITU HYBRIDIZATION

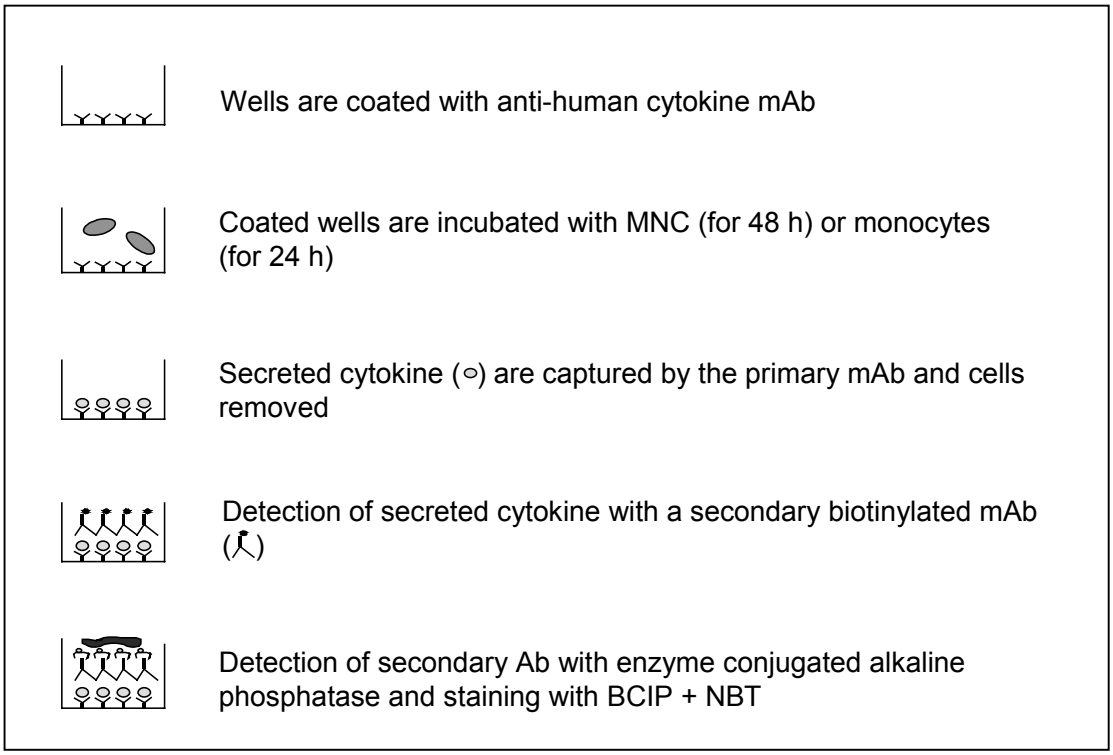
In situ hybridization (ISH) is a method for studying the expression of a gene encoding a certain molecule in individual cells. Aliquots of 10-20 μl of cell suspension containing 1×10^5 blood MNC or 1×10^4 monocytes or DC were dried onto restricted areas of electrically charged glass slides (SuperFrost/Plus, Menzel-Gläser, Braunschweig, Germany) at 55°C for 15-30 min. Slides were stored with silica in sealed boxes at -20°C until ISH. Synthetic oligonucleotide probes (KEBO, Stockholm, Sweden) were labelled with ^{35}S deoxyadenosine-5′-α-(thio)-triphosphate (Dupont Scandinavia, Stockholm, Sweden) with terminal deoxynucleotidyl transferase (Amersham, Little Chalfont, UK). For each MMP or TIMP, a mixture of equal amounts of two to three different synthetic oligonucleotide probes, each approximately 30 bases long, was used in order to increase the sensitivity of the method. The oligonucleotide sequences were obtained from GenBank and probes were designed using MacVector Software (IBI, New Haven, CT). A constant ratio of guanine/cytosine content of 60% was used. The oligonucleotide probes were checked for the absence of palindromes or long sequences of homology within the species against available GenBank data. Cells were hybridized for 16-18 h at 42°C in a humidified chamber with 10^6

c.p.m. of a labelled probe per 100 µl of hybridization mixture. Following ISH, slides were rinsed 5 × 15 min at 55°C, allowed to cool to room temperature (RT), dipped in distilled water, dehydrated through gradient ethanol (60% and 95%), air-dried in front of a fan, dipped in Kodak NTB2 emulsion, and exposed at 4°C for 14 days. Slides were developed in D19 (Kodak) and fixed in Unifix (Kodak) each for 4 min, stained with cresyl violet, and mounted with Entellan (Merck, Darmstadt, Germany). Coded slides were examined by dark-field microscopy at 100× magnification for positive cells containing >15 grains per cell in a star-like distribution. The intracellular distribution of the grains was always checked by light microscopy at 400× magnification. Labelled cells were expressed as numbers per 10^5 MNC, monocytes or DC. In many positive cells, the grains were so heavily accumulated within and around the cell that it was not possible to count every single grain. In cells judged to be negative, the numbers of grains were almost always 0-2 per cell, and the grains were scattered randomly over the cell and not distributed in a star-like fashion. There were no difficulties in differentiating between positive and negative cells. Variation between duplicates was <10%. The number of cells applied for ISH was not equal to the number of cells ultimately detected on the slides. To compensate for cell losses, the total number of cells on the slides was counted. Cell losses varied between 10% and 50% from cell application to cell counting. The preferential loss of certain cell types cannot be ruled out. With the help of a microscope grid used as a measuring unit, the radius (r) of the circular area (A) covered by cells was determined. The area A was calculated by the formula $A= r^2 \times \pi$. Cells were usually counted in two grids at the periphery and one grid at the centre of the surface covered by cells. In the case of uneven cell distribution, cells in additional grids were counted. The total number of cells was calculated by multiplying the area A with the mean value of the number of cells per grid. As control probes, the sense sequence of MMP-3 (21-50) (Saus et al., 1988) was used in parallel with the MMP probe on the cells from each subject. In no instance were any positive cells revealed.

DETECTION OF CYTOKINE SECRETING MONOCYTES AND MONONUCLEAR CELLS BY ELISPOT ASSAYS

To detect and enumerate TNF-α, IL-6, IL-10 and total IL-12 secreting monocytes and MNC as well as IFN-γ secreting MNC, ELISPOT assays as originally described (Czerkinsky et al., 1988) and modified in our laboratory to monocytes were adopted (Fig. 5). Microtitre plates with nitrocellulose bottoms (Multiscreen-HA plates; Millipore, Mulsheim, France) were coated for 2 h at 37°C with 100 µl/well of monoclonal antibody (mAb) to human IFN-γ (1-DIK), IL-6 (13A5), IL-10 (9-D7), IL-12p40 (13A5) (all from Mabtech, Stockholm, Sweden) or TNF-α (28401.11; R&D, Oxon, UK) diluted in phosphate-buffered saline (PBS, pH 7.4) to a concentration of 10 µg/ml. The IL-12p40 antibody applied in this study is capable of detecting both the IL-12p40 subunit and the bioactive IL-12p70. After removal of coating solutions by washings with PBS, 200 µl aliquots containing $1x10^3$ (TNF-α), $2x10^3$ (IL-6), $5x10^4$ (IL-10) or $1x10^5$ (IL-12) monocytes or $1x10^3$ (TNF-α), $2x10^3$ (IL-6), $2x10^5$ (IL-10, -12 and IFN-γ) MNC were applied to individual wells in duplicates. As a negative control, complete medium was also

Fig. 5. Enzyme-linked immunospot assays for cytokine detection



added to wells in the absence of cells. Plates were incubated at 37°C for 24 h (for all cytokines under study for monocyte ELISPOT assays and for MNC ELISPOT assays for the cytokines TNF-α and IL-6,) or 48 h (to detect numbers of IFN-γ, IL-10 and IL-12 secreting MNC; see also Ozenci et al., 1999) in humidified air containing 5% CO_2, then emptied and washed. 100 μl of a biotinylated mAb to human IFN-γ (7-B6-1), IL-6 (39C3), IL-10 (12G8), IL-12p40 (39C3) (all from Mabtech) or a biotinylated polyclonal Ab to TNF-α (R&D) diluted in PBS to a concentration of 1 μg/ml were added overnight at 4°C. Wells were washed, and 100 μl of streptavidin-alkaline phosphatase (ALP)(Mabtech) diluted 1:1,000 was added for 1.5 h at RT. After incubation, plates were emptied, washed and stained with BCIP-NBT (Gibco) diluted in TRIS-buffer. Immunospots, each considered to represent a cell secreting IL-6, IL-10, IL-12, IFN-γ or TNF-α, were counted with a dissection microscope under 25 x magnification. The mean values of duplicates were calculated. Results were presented as numbers of IL-6, IL-10, IL-12, IFN-γ or TNF-α secreting cells per 10^5 monocytes or MNC. Variation of duplicates was <10% for MNC. Interassay variability for monocyte ELISPOT assays was <10% for IL-6 and total IL-12, and <15% for numbers of TNF-α and IL-10 secreting monocytes.

ELISA

To detect cytokines secreted by isolated monocytes, cells were diluted to a final concentration of 10^6 cells/ml in complete medium and cultured for 24 h at 37 °C in humidified air containing 5% CO_2. The following day, cell-free supernatants were collected, centrifuged and stored at -70 °C. Each sample was tested in duplicate for IL-6, TNF-α, IL-10 and IL-12 production by ELISA according manufacturers instructions. The Abs applied for ELISA were identical to those used for ELISPOT assays. Optical densities were measured using an ELISA reader. Cytokine concentrations were calculated using standard curves that were performed for each ELISA plate. The lowest detection limits were 8 pg/ml for TNF-α, IL-12 and IL-10 and 12 pg/ml for IL-6. These measurements were repeated at three different occasions.

IMMUNOFLUORESCENCE

10^6 DC were resuspended in 1 ml of PBS, and 200 µl of the suspension was applied per cell spot by cytocentrifugation (Cytospin 3; Shandon Inc., Pittsburgh, Pa) at 350 x g for 10 min onto restricted areas of electrically charged glass slides (SuperFrost Plus). Slides were air dried and then fixed with 4% paraformaldehyde (PFA) in PBS and incubated in this fixative for 20 min at RT. Samples were washed once with PBS for 5 min. Cells were permeabilized with 1% Triton X-100 in PBS for 10 min and then washed with PBS. To block non-specific binding sites, Dako block (Life Technologies) was applied for 10 min at RT. Samples were incubated for 1 h with primary antibodies to MMP-1, -2, -3 or -9 or to TIMP-1 or -2 (Oncogen, Boston, MA) at a dilution of ~1:50 µg/ml in Dako sample dilution buffer (Life Technologies). After several rinses with PBS, the samples were incubated with secondary antibody for 30 min (Goat-anti-mouse $F(Ab)_2$ RPE; Dako). Samples were extensively washed with PBS and blocked for 10 min. Directly labelled mAb (CD1a FITC or CD83 FITC; BD, Mountain View, CA) diluted (1:25) in Dako sample buffer were used for surface staining for 1 h. After washings and drying of slides, they were mounted with ProLong Antifade Kit (Molecular Probes, Eugene, OR). A number of controls were performed to verify the specificity of the antibodies that were used in this study. No signal was detected when the primary or the secondary antibody were used alone. Isotype controls were always stained in parallel. Samples were viewed with a 510 Zeiss Laser Scanning Confocal microscope with 63× and 100× objectives (NA = 1.4).

IMMUNOSTAINING AND FLOW CYTOMETRY

For immunostaining, 80 µl of the cell suspension (containing at least $5x10^4$ cells) were incubated with a mixture of mAbs conjugated with fluorescein isothiocyanate (FITC, green fluorescence), phycoerythrin (PE, orange fluorescence) or peridinin chlorophyll protein (PerCP, red fluorescence). All mAbs were titrated in preliminary experiments. Samples were incubated with mAb for 30 min at 4°C in the dark. The cells were washed twice with PBS containing 1%

bovine serum albumin (BSA; Sigma, St Louis, MO) and centrifuged at 2,200 x g for 10 min. Cells were resuspended in 200 μl 1% BSA/PBS.

For intracellular staining of MMP or TIMP, DC were fixed with 4% ice-cold PFA for 20 min, washed and permeabilized with 0.1% saponin. Anti-MMP and TIMP mAbs were applied for 20 min at 4°C. MMP and TIMP immunoreactivity was further developed by goat anti-mouse $F(Ab)_2$ RPE-labelled Ab (Dako) for 20 min at 4°C in the dark. Subsequently, cells were washed with 0.1% saponin and PBS containing 1% BSA. DC were then incubated with FITC-labelled mAb to CD1a (iDC) or CD83 (mDC). Cells were washed and resuspended in 200 μl 1% BSA/PBS.

All samples were analysed in a Becton Dickinson FACScan flow cytometer. This instrument was calibrated twice per month for fluorescence intensity (Calibrite; Becton Dickinson Mountain View, CA). Expression of surface molecules on monocytes or DC was presented as percentage (%) and mean fluorescence intensity (MFI), which shows the proportion of stained cells and the staining signal intensity of molecules on the cells, respectively.

WESTERN BLOT

To detect secreted MMP and TIMP in supernatants as well as gelatinolytic activity of secreted MMP (see below), DC were diluted to a final concentration of 10^5 cells/ml in serum-free medium and cultured for 40 h at 37°C. Cell-free supernatants were collected, centrifuged and stored at -70°C. Equal volumes of iDC or mDC culture supernatants of the same protein content and of 2x Laemmli buffer (composed of 2% w/v SDS, 10% v/v glycerol, 5 mM EDTA, 5% v/v 2-mercaptoethanol, 10 μg/ml bromophenol blue and 62.5 mM Tris–HCl (pH 8.8)) were mixed, boiled for 3 min and centrifuged for 15 s at 20,000 x g. Culture supernatants containing 10 μg of total protein as measured with a spectrophotometer were then loaded on 10% w/v polyacrylamide gel. As a positive control, recombinant MMP or TIMP was used in each gel. After SDS-PAGE, proteins were transferred onto a nitrocellulose membrane using electroblotting. Nonspecific binding sites were blocked overnight at 4°C in PBS–

Tween 20 (0.1%) containing 5% non-fatty dry milk (Biorad, Hercules, CA). Subsequently, membranes were incubated for 1 h with mAb to MMP or TIMP according to the manufacturers instructions (Oncogene). After washings with PBS-Tween 20 (0.1%), blots were incubated with peroxidase-conjugated rabbit anti-mouse IgG (dilution 1:5,000; Dako) for 90 min at RT and developed with the enhanced chemiluminescence (ECL) system (Pharmacia).

Images of gels were captured by scanning on a flatbed scanner. A standard curve was obtained for densitometric quatitation of MMP and TIMP using the respective proteins. In each sample, MMP and TIMP concentrations were calculated using the National Institutes of Health Image V1.56 software program.

ZYMOGRAPHY

To detect gelatinolytic activity in cell culture supernatants obtained from MNC and monocytes, cells were diluted to a final concentration of 10^6 cells/ml in complete medium. The following day, cell free supernatants were collected, centrifuged and stored at -70 °C. For DC, aliquots of cell culture supernatants containing 10 μg of total protein were used. Enzymatic activity was assayed by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS) and gelatin. Briefly, 10 μl aliquots of cell culture supernatants were resuspended in 10 μl sample buffer (50 mmol/L Tris HCL, 2% SDS, 0.1% bromophenol blue, 10% glycerol, PH 6.8) and applied to 10% (wt/vol) acrylamide gels containing 1 mg/ml of gelatin (Sigma) followed by electrophoresis at 180 V, 20 mA/gel. Gels were then incubated in 2.5% (vol/vol) Triton X-100 for 30 minutes to remove SDS followed by overnight incubation at 37 °C in developing buffer (50 mmol/L Tris-HCl, 0.2 mol/L NaCl, 5 mmol/L CaCl2, adjusted to pH 7.5). Gels were fixed and stained for 45 min in 30% methanol, 10% glacial acetic acid, 0.5% Coomassie Blue G-250, destained for 45 min in 30% methanol, 10% glacial acetic acid). Zones of proteolytic activity were evident as clear bands against a dark blue background. In each gel, purified MMP-2 and –9 (a kind gift from Dr. U. Bergmann, Dept. of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden) were used as standards.

Images of gels were captured by scanning on a flatbed scanner. A standard curve was obtained for densitometric quatitation of MMP activity using purified

MMP-2 and -9. In each sample, MMP-2 and –9 activities were calculated using electrophoretic gel lane calculation option on the National Institutes of Health Image V1.56 software program.

MIGRATION ASSAYS

Cell migration was quantified with Transwell inserts (6.5 mm; Costar, Cambridge, MA) fitted with polycarbonate filters (5-μm pore size; Nuclepore, Pleasanton, CA) (Fig. 6). Filters were coated with 17.5 μl of Matrigel according to the manufacturers instructions (Collaborative Research, Bedford, MA), and then allowed to gel for 1 hour at 37°C. DC were washed,

Scheme of migration assay

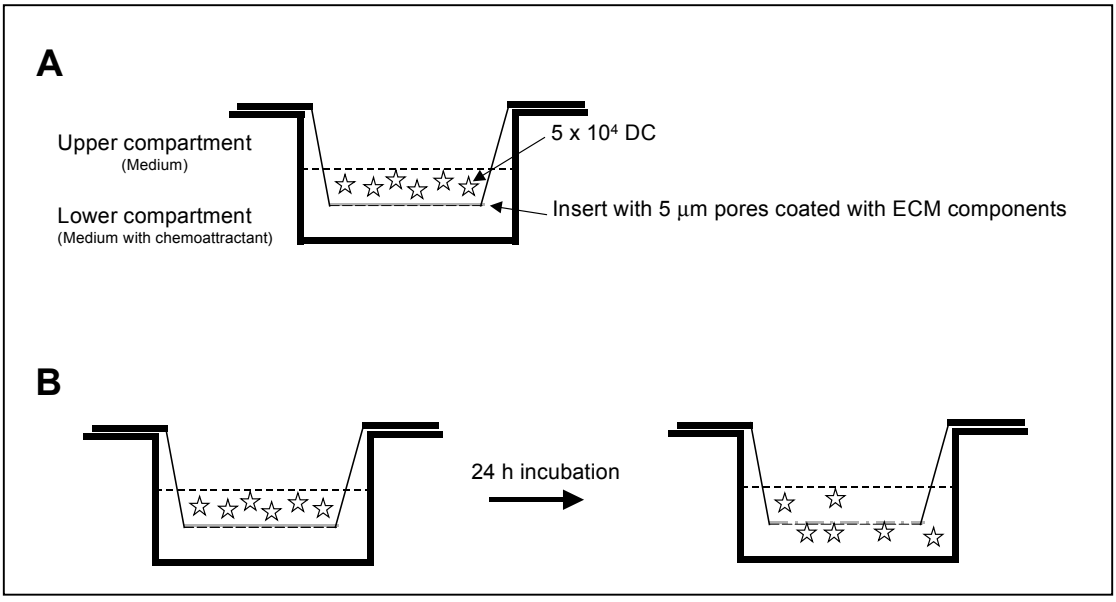


Fig.6.

Panel A: The Transwell system consists of an upper and lower compartment separated by a filter with 5 μm pores. Prior to usage filters are coated with 17.5 μl of Matrigel. Matrigel contains a variety of ECM components. Upon coating, Matrigel is allowed to gel for 1 h at 37 °C.

Panel B: 5 x 10^4 iDC or mDC in 100 ml medium are placed in the upper compartment of a Transwell system. In the lower compartment, 600 μl medium containing recombinant chemokines is added. These chemokines form a driving force, which direct cells towards the lower compartment. After 24 h of incubation in an incubator at 37 °C, the upper compartment is removed and the lower surface of the filters is stained with Giemsa. Migrated cells are counted under a light microscope (400x).

resuspended at $5x10^5$/ml in medium (RPMI with 1% FCS) and placed into upper chambers of transwells (100 μl/well), while lower chambers contained 600 μl of either medium or as a positive control recombinant human (rh) RANTES (50 ng/ml) for iDC or rhMIP-3β (500 ng/ml) for mDC. After 24 h of incubation at 37 °C, inserts were lifted, washed on their upper surface with PBS, and Giemsa-stained on their lower surface, to visualize migrated DC. Migrated cells in 10 consecutive high-power (x250) fields along the diameter of each insert were counted. Assays were done in duplicates. To check the specificity of the assay, cells were also incubated with either 10 μM EDTA or rh-TIMP-1 (10 μg/ml). Addition of these agents virtually abolished DC migration over ECM coated filters. The migratory capacity of DC was expressed as percentage of cells that migrated through the filter, compared to the initial amount of cells added to the upper chamber. Cells that migrated through the filters into the medium of the lower chamber were regularly checked but did not exceed 1% of the initial cells added to the upper chamber and were thus omitted from further analysis.

STATISTICAL ANALYSIS

Mann-Whitney's U-test was used for two-group-comparisons. The non-parametric Kruskal-Wallis ANOVA test was used to compare more than two groups simultaneously. Upon obtaining a significant p-value ($p<0.05$) for the Kruskal-Wallis ANOVA test, reflecting differences between one vs. another group(s) under study, a multiple comparisons test (Siegel and Castellan, 1988) was used to determine which groups under study were differing. A two-tailed Mann-Whitney's U-test was used for two-group-comparisons. Wilcoxon signed rank test was used when comparing samples from the same individual. Spearman's rank test was used for correlation. Reported p-values are two-tailed and considered statistically significant at $p<0.05$.

RESULTS

DETECTION OF CIRCULATING MONOCYTES ACTIVELY SECRETING CYTOKINES (PAPER I)

Blood monocytes as well as tissue-differentiated macrophages play a pivotal role in controlling immune reactions. Monocytes regulate extent, nature and duration of immune responses by secretion of cytokines. IL-6, TNF-α, IL-10 and IL-12 are of particular interest, since IL-12 shifts the immune response towards a Th1 type, facilitating the production of e.g. IFN-γ and TNF-α but also IL-6, while IL-10 counteracts Th1 responses and promotes the production of Th2 related cytokines such as IL-4. A tight regulation of these four cytokines keeps the balance and decides whether Th1 or Th2 will predominate in immune reactions.

ELISPOT assays are among the most sensitive and specific methods available for cytokine research. They permit ex vivo identification of individual cells actively secreting cytokines. In the present study we prepared monocytes from healthy subjects' blood and adapted ELISPOT assays to define optimal conditions to detect and enumerate monocytes secreting IL-6, TNF-α, IL-10 and IL-12. Optimal time for monocyte incubation was 24 h, and optimal monocyte numbers were 2,000 for IL-6, 1,000 for TNF-α, 50,000 for IL-10 and 100,000 cells/well for enumeration of IL-12 secreting monocytes.

We observed high levels of IL-6, IL-10, TNF-α ($p<0.0001$ for all comparisons) and IL-12 ($p<0.001$) secreting monocytes upon LPS stimulation compared to monocytes without such stimulation indicating that monocytes obtained by Percoll density centrifugation are capable to respond efficiently to exogenous stimuli like LPS and can be evaluated by ELISPOT assays without difficulty. Variation between duplicates was <10% in ELISPOT assays. Variation between duplicates for ELISA was 17% for TNF-α, 28% for IL-6, and 17% for IL-10, being much higher ($p<0.05$, $p<0.001$ and $p<0.0001$, respectively) compared to ELISPOT assays.

Variation between duplicates was similar for levels of IL-12 (10%) as measured by ELISA compared to numbers of IL-12 secreting monocytes by ELISPOT.

Among healthy subjects, 10% ± 5%, of the blood monocytes secreted IL-6, 12% ± 12% TNF-α, 0.1% ± 0.1% IL-10 and 0.2% ± 0.3% IL-12.

In conclusion, ELISPOT assays represent a useful tool to enumerate monocytes secreting IL-6, TNF-α, IL-10 and IL-12, and probably to enumerate monocytes secreting other cytokines and proteins as well.

PROPERTIES OF BLOOD MONOCYTES IN MS: PHENOTYPE, CYTOKINES, MATRIX-DEGRADING METALLOPROTEINASES AND THEIR INHIBITORS (PAPER II AND III)

MS is characterised by repetitive disruptions of the BBB with subsequent infiltration of MNC into the CNS. Blood-borne T cells and monocytes/macrophages constitute the major cell types in the perivascular infiltrates characteristic for MS. Circulating T cells and monocytes may be important regulators of the bursts of intrathecal inflammation observed in MS. Furthermore, these cells may facilitate BBB breakdown and their entrance into the CNS through the secretion of high levels of ECM degrading enzymes like MMP. MMP are also implicated in proteolysis of myelin components, generation of remnant antigens, and proteolytic cleavage of zymogens.

Utilising ELISPOT assays, we observed elevated levels of IL-6 and IL-12 secreting monocytes in patients with MS as well as in patients with OND compared to healthy subjects without prior stimulation of the cells in vitro. Levels of TNF-α and IL-10 secreting monocytes did not differ in patients with MS compared to the two control groups.

We observed a higher density of CD86 as reflected by MFI, on CD86 expressing blood monocytes in patients with MS compared to healthy subjects. Expression of CD80 and HLA-ABC, and density of HLA-ABC reflected by MFI were augmented on monocytes in patients with long-standing (>10 years) MS.

Similarly, percentages of CD80 expressing monocytes were higher in patients with moderate to severe disability (EDSS >3).

In conclusion, different monocyte properties in the form of elevated levels of IL-6 and IL-12 secreting monocytes and increased density of CD86 characterize circulating monocytes in MS vs. healthy subjects. An altered monocyte phenotype reflected by increased percentages of HLA-ABC and CD80 expressing monocytes seems to be associated with long duration of MS.

Applying ISH to detect monocytes expressing mRNA for MMP-1, -3, -7, -9 and –14 and of TIMP-1, we also observed that MS is also associated with high levels of MMP-1, -3, -7 and –9, and TIMP-1 mRNA expressing monocytes in blood. Whether the observed imbalances in MMP and TIMP-1 are found in MS only or in other inflammatory diseases of the CNS as well, remains to be determined.

In conclusion, the observed alterations in monocyte phenotype, cytokine and MMP profiles argue for a possible role for monocytes in MS and suggest that monocytes may contribute to the activation and perpetuation of inflammation observed in patients with MS.

EFFECTS OF IFN-β TREATMENT ON LEVELS OF MMP MRNA EXPRESSING AND CYTOKINE SECRETING MONONUCLEAR CELLS IN MULTIPLE SCLEROSIS (PAPER IV)

IFN-β is an approved treatment for MS. Its mechanisms of action are unclear. In vivo effects of IFN-β treatment on cytokines and MMP were investigated in blood MNC derived from patients with MS examined before vs. during treatment with IFN-β.

IFN-β treatment resulted in decreased numbers of IL-6 and TNF-α secreting MNC and of MMP-3 and -9 mRNA expressing MNC compared to pre-treatment levels. On the contrary, numbers of IL-10 secreting MNC and TIMP-1 mRNA expressing MNC were augmented during IFN-β therapy.

Whether these systemic immunoregulatory effects are a direct result of IFN-β or secondary to clinical stabilization in MS remains to be examined.

DENDRITIC CELLS EXPRESS AND SECRETE MATRIX-DEGRADING METALLOPROTEINASES AND THEIR INHIBITORS AND ARE IMBALANCED IN MULTIPLE SCLEROSIS (PAPER V)

DC are APC with the unique ability to initiate and control immune responses. Depending on their maturation status, DC are found at different locations and exhibit unique properties of phenotypic and functional heterogeneity and plasticity Migratory capacity of DC is crucial in their performance of their professional APC functions. The mechanisms by which cells transverse capillaries, penetrate BM, and migrate into stroma necessitate the proteolytic cleavage of ECM components, including collagens and glycoproteins. Proteolytic events in vivo are confined to the immediate pericellular environment of cells through local secretion of MMP. It is, therefore, important to study the expression of these proteins at the cellular level. Whether immature DC (iDC) and mature DC (mDC) are capable of expressing these enzymes and/or their inhibitors is not known. MMP secreting DC may be involved in MS pathogenesis since these molecules are critical for migration through physiological barriers. To understand the properties of DC, it is important to examine MMP and TIMP expressing DC in MS.

In the present study, examining several consecutive stages in protein production and enzyme activity, we demonstrated that both iDC and mDC are capable to produce, express and secrete functionally active MMP-1, -2, -3 and –9 and their inhibitors TIMP-1 and -2.

Moreover, we also evaluated the functional importance of MMP and TIMP expression by iDC and mDC. We found that spontaneous migratory capacity of both iDC and mDC are closely correlated to their MMP-1, -2 and -9 production. No such correlation was observed for TIMP, suggesting the involvement of MMP in migratory processes of DC through ECM coated filters. We found that MS is associated with high MMP-2 and TIMP-2 mRNA expressing iDC, and with high MMP-1, -2 and -9 protein production together with high MMP-1 secretion and

high MMP-2 and -9 activity by iDC. MS is also associated with high MMP-2 and –3, and high TIMP-2 protein production by mDC together with high MMP-3 secretion and high MMP-9 activity by mDC. We consider the high MMP-1, -2 and –9, and TIMP-2 produced by iDC, and the high MMP-2, -3 and -9, and TIMP-2 produced by mDC detected at different stages of protein production in MS are associated with the disease. The underlying reason of discrepant findings with different methods may be due to the sensitivity of the method and detection of different parameters by different methods, and the complex regulatory processes in MMP production and activity. Upon activation of MMP, their action is controlled by TIMP, which are often co-produced with MMP. Here we found elevated levels of TIMP-1 and TIMP-2 production by DC in MS. Whether elevation of TIMP-1 and TIMP-2 production by DC in patients with MS is a primary phenomenon or a consequence of MMP elevation remains to be determined.

We demonstrated that iDC and mDC obtained from patients with MS have higher chemokinetic capacity compared to healthy subjects. Alterations in protein expression have significant effects on in vitro migration capacity of DC. Modulation of immune responses and immune cells are considered to be a relevant way of treating several diseases including cancer and autoimmune diseases. Expression of MMP by DC is crucial in their function and could thus be targeted with treatment strategies in the future.

MATRIX METALLOPROTEINASE AND CYTOKINE PROFILES IN MONOCYTES OVER THE COURSE OF STROKE (PAPER VI)

Stroke is a common cause of death and disability in our society. Stroke is associated with changes in immune responses within the CNS as well as systemically. The cells contributing to such changes as well as the factors contributing to formation of the inflammatory infiltrate observed in stroke remain to be clarified.

In this study, blood monocytes and corresponding MNC were separated and examined in parallel within 4 days and 1-3 months after onset of ischemic stroke.

Numbers of TNF-α, IL-12, IL-6 and IL-10 secreting cells and of cells expressing mRNA for MMP-1, -2, -7 and –9, and of TIMP-1 were studied.

TNF-α, IL-12 and IL-6 secreting monocytes and MNC were elevated during the acute phase of ischemic stroke compared to healthy subjects. Such differences were not observed when stroke patients were examined during convalescence. IL-10 secreting monocytes did not change over the course of stroke. Levels of monocytes expressing MMP-1and -7 and TIMP-1 mRNA were elevated in the acute phase of stroke patients compared to convalescence and healthy subjects, as were levels of MMP-1, -2, -7 and –9, and TIMP-1 mRNA expressing blood MNC. MMP-2 and –9 activity as measured by zymography was also higher in MNC supernatants in the acute phase of stroke compared to convalescence.

The high levels of pro-inflammatory cytokines and MMP in blood monocytes and MNC further demonstrate the presence of systemic immune aberrations in the acute phase of stroke. Such changes may contribute to the influx of blood-borne immune cells into the ischemic lesions during the acute phase of stroke.

DISCUSSION

METHODOLOGICAL ASPECTS OF PURIFICATION OF MONOCYTES AND MONOCYTE-DERIVED DENDRITIC CELLS FROM BLOOD

Knowledge about the involvement of monocytes and DC in MS and stroke is incomplete and the literature is replete with contradictory data. Monocytes account for 5-20% of the MNC population in blood (Jones et al., 1989). Monocytes in the circulation are heterogeneous with regard to size, morphology, and surface antigens. Additionally, a number of monocyte subpopulations have been proposed on the basis of different levels of expression of several phenotypic markers, including CD14, CD16, CD33 and CD69 (Bennett and Breit, 1994; Ziegler-Heitbrock, 2000). Moreover, technical difficulties associated with isolating, purifying and culturing of these cells may further complicate analysis of the individual cells (Graziani-Bowering et al., 1997). In the present study, we evaluated cytokines and MMP that exert their function upon secretion and are important in evaluation of the role of monocytes in the immune response. These properties necessitate the analysis of isolated cell populations.

Several factors should be taken into account when isolating monocytes from blood, including: 1) purity of the isolated monocyte population; 2) possible subpopulation selection during the isolation procedure; 3) the activation status of the cell; 4) length and time of culture; and 5) possible influence of serum components. In addition, blood samples available from patients are necessarily restricted so that a rapid and efficient method is required to isolate these cells (Wiegand et al., 1999). To overcome these difficulties numerous isolation procedures have been developed (Denholm and Wolber, 1991) as depicted in Table 1.

Some of these methods such as counter-flow elutriation require large amounts of blood and expensive specialized equipment. Other methods, as in the case of negative selection by magnetic beads, require numerous mAbs that make routine usage expensive. Purification by adherence or positive selection by magnetic beads inevitably leads to activation of monocytes and results in upregulation of

the expression of immune regulatory proteins such as cytokines (Haskill et al., 1988; Rodenburg et al., 1998). Monocytes can be studied without prior purification in both whole blood and MNC preparations by flow cytometry. Such an approach can provide valuable information on monocyte-phenotype. However, depending on the epitope to which mAbs applied in flow cytometry are raised only 70-85% of monocytes are recognized (Todd et al., 1981; Griffin et al., 1989; Steinbach and Thiele, 1994). Presently there is no exclusive marker for monocytes available.

Table 1. Methods to study monocytes

Method	Advantages	Disadvantages
Counter-flow elutriation	High purity (up to 95%) Viable cells	Low recovery Large volumes of blood necessary Specialized equipment Time consuming
Adherence	Simple method Inexpensive	Monocyte activation Low yields Compromised cell viability
Flow cytometry	No isolation of cells necessary (whole blood, MNC preparations are sufficient) Fast	No exclusive marker for monocytes currently available
Magnetic bead separation		
- Positive selection	Pure populations of monocytes	Activates monocytes Selection of monocyte-subpopulation
- Negative selection	Fast	Requires numerous expensive mAbs
Percoll density gradient separation	Relatively pure populations of monocytes Inexpensive Fast Functional and viable cells Good recovery	Prolonged exposure to Percoll may lead to phagocytosis of the separation medium

In the present study, we chose Percoll density-gradient separation to prepare blood monocytes. The rationale behind Percoll density gradient separation is that monocytes, being less dense and larger than lymphocytes, have a higher flotation rate than lymphocytes. Monocytes isolated according to this procedure yield viable (Denholm and Wolber, 1991) and functionally competent cells, which adhere to glass and plastic, have cytolytic capabilities, phagocytose latex beads and erythrocytes as well as react on chemotactic stimuli (Gmelig-Myeling and Waldmann, 1980; Pertoft et al., 1980; Weiner and Mason, 1984). This method may circumvent many of the difficulties associated with other separation protocols, and is fast (Denholm and Wolber, 1991). Theoretically, the particulate nature of Percoll could lead to ingestion of Percoll by monocytes (Heppeston and Styles, 1967). To date evidence for such a phenomenon is however lacking. Percoll-density gradient separation of monocytes as used in the present study yielded monocytes with a purity of >90%. Most of the monocytes were recovered by Percoll separation, leading us to conclude that no major selection of a subpopulation of monocytes occurred during separation. Monocytes were functionally competent, since stimulation with LPS, a major activation stimulus for monocytes, resulted in increased secretion of all immune modulatory cytokines under study. Yields obtained by this method were typically 1,5-2,0 x 10^6 monocytes per 20 ml of blood and were sufficient to phenotype and evaluate immune properties of these cells from individual patients.

DC compromise less than 1% of the MNC population. On the other hand, DC differentiation can be driven through culturing of adherent monocytes in the presence of GM-CSF plus IL-4 or IL-13 or IFN-α (Duddy et al., 2001). The acquisition of DC characteristics by monocytes upon migration across endothelial barriers in vivo also has been described (Randolph et al., 1999; Duddy et al., 2001). Taking advantage of purification of monocytes by Percoll density-gradient separation, adherent monocytes were further cultured to obtain pure populations of monocyte-derived DC (Kalinski et al., 1999). Such an approach may be useful since the majority of contaminating MNC is eliminated prior to culturing (Thurner et al., 1999).

The reduced numbers of cells at the start of culture lead to decreased consumption of expensive recombinant cytokines, and more homogeneous phenotype of DC due to decreased content of contaminating cells possibly affecting the development of DC.

Generating DC from monocytes yielded pure populations of CD1a+HLA-DR+ cells, that were negative for lineage markers for B, T, NK cells and monocytes as well as CD83, a marker for mature DC. The purity of DC obtained by this method was >90%. This high purity allowed us to analyse DC properties including MMP production.

EVALUATION OF MATRIX-DEGRADING METALLOPROTEINASES AND THEIR INHIBITORS

During recent years, knowledge on matrix-degrading enzymes has rapidly evolved. Their involvement in migration and immunoregulatory processes has been established. These as well as many other functional activities of these enzymes have been the focus of a number of studies (Yong et al., 2001). MMP exert their action under narrowly controlled conditions employing a strict regulation of MMP production, activity and localization of MMP action to the direct pericellular environment (Fosang and Hardingham, 1989). A consequence of these properties is that measurement of MMP and TIMP in body fluids (Fig. 7) may yield only limited information on the involvement of MMP in health and disease states (Lein et al., 1997). Cellular localisation and phenotypic identification of cells producing as well as secreting MMP are, therefore, critical to understand the contribution of these enzymes in disease states (Fig. 7).

In the present study, we applied ISH to detect numbers of MMP or TIMP mRNA expressing cells. ISH is a highly sensitive and specific assay, which allows the analysis of gene expression at the cellular level in single cells (Fig. 7). The assay does not require the time-consuming development of antibodies and can be applied to new molecules as soon as the genetic code of the molecule has been described. On the other hand, MMP and TIMP mRNA expression may not necessarily reflect protein production. In addition, ISH does not allow

Methods to detect matrix-degrading metalloproteinases

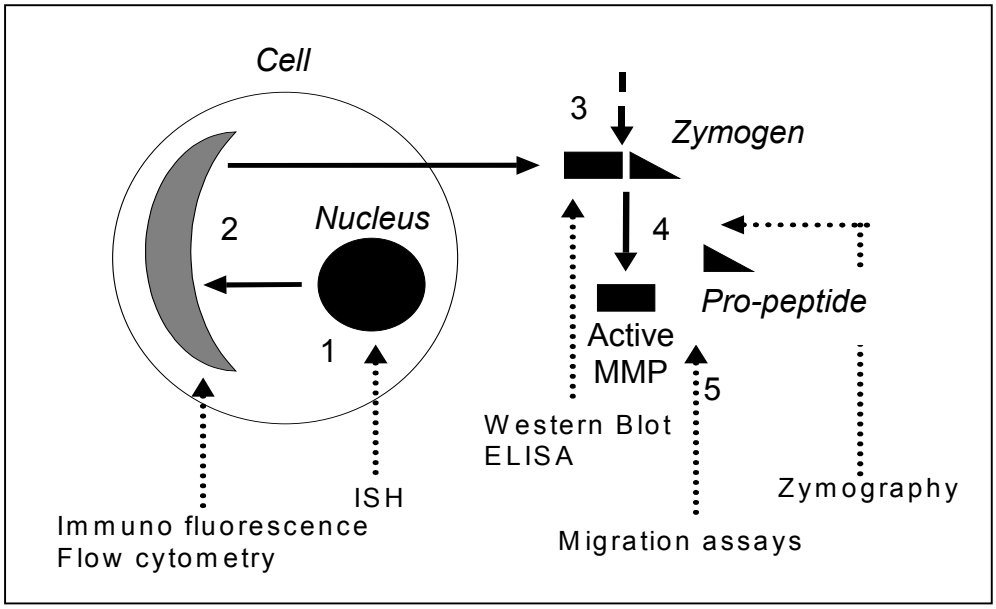


Fig. 7. Matrix-degrading metalloproteinases (MMP) and their inhibitors (TIMP) can be detected at several stages of protein production and activation. (1) Transcription of MMP can be detected at the single cell level by in situ hybridization (ISH). (2) Upon translation of MMP and/or TIMP, phenotype and MMP and/or TIMP can be detected in parallel by immunofluorescent staining or flow cytometry. (3) Either ELISA or Western blot may detect inactive MMP and TIMP secreted in the extracellular fluid. (4) MMP need to be activated by proteolytic cleavage. Zymography allows detection of both inactive as well as activated MMP. (5) Migration assays consisting of extracellular matrix (ECM) coated filters necessitate the presence of active MMP to allow migration.

simultaneous analysis of the phenotype of MMP or TIMP expressing cells thus requiring high purity grade of individual cell types. To study phenotype and MMP and TIMP protein expressing cells in parallel, flow cytometry was adapted. Detection of MMP or TIMP expressing cells by flow cytometry did not require any prior in vitro stimulation and may thus provide an attractive tool to evaluate phenotypic characteristics and proteolytic enzymes in complex mixtures of cells. To exert their function, MMP and TIMP need to be secreted and activated through proteolytic cleavage (Fig. 7). In the present study, Western blot was applied to analyse free MMP and TIMP. This method does not require the availability of antibody pairs and is sensitive for the detection of MMP (Di Girolamo et al., 1998). The functional enzyme can be analyzed by zymography (Fig. 7). Zymography is a highly sensitive method that allows the identification of the MMP of interest in a complex mixture of proteinases (Quesada et al., 1997). On the other hand, zymography may not be suitable for automatic measurements and is frequently difficult to quantify. In the present study,

therefore, the proteolytic activity of cell supernatants was compared to standard amounts of recombinant MMP, hence we presented our data as arbitrary units. Such an approach may circumvent many of the difficulties associated with quantification of MMP activity.

To evaluate whether aberrations in MMP and TIMP expression result in an altered migratory capacity of DC, a modified transwell system utilising filters coated with ECM proteins was applied (Leppert et al., 1996). A chemotactic gradient provided a driving force for cells to migrate to the lower compartment. Such passage necessitates cleavage of the ECM coating on the filters and may thus provide the means of assessment of functional activity by MMP secreting cells.

In conclusion, measurement of MMP is a complex procedure due to difficulties in methodologies and the complex biology of these enzymes. In the present study, we used several methods in parallel to analyse the production of MMP and TIMP by DC. None of these methods by itself is capable of explaining the involvement of MMP in disease processes. However, each of these methods has its own advantages and disadvantages. Moreover, they detect different parameters and thus answer different questions.

DILEMMAS IN THE EVALUATION OF CYTOKINES

The detection of cytokines is hampered by their biological properties, e.g. local secretion, rapid uptake and utilisation, and short half-life. These properties have led to the development of numerous tools to detect transcribed, translated or secreted cytokines. Different methods focus on the different stages of cytokine production and secretion. The usefulness of methods detecting either transcribed or translated proteins as Northern blot, semiquantitative reverse transcription PCR (RT-PCR) or in situ hybridization depends on correlations between mRNA levels and rates of protein secretion. Most cytokines, though, are modified both at post-transcriptional as well as post-translational level. Moreover, cytokines carry out their functions mainly after secretion of the protein, therefore, detection of cytokines before their secretion might not be a reliable reflection of the released effector molecule.

Unlike hormones, cytokines mostly exert their effects at very short distance from their source of production. Moreover, cytokines are seldom found in unbound

states but are bound to soluble cytokine receptors, anti-cytokine antibodies or other proteins. These properties may make detection of secreted cytokines in body fluids by ELISA less attractive. Moreover, the biologic relevance of determining absolute quantities of cytokines in body fluids has to date not been addressed.

Detection of cytokines at the individual cell level, i.e. the cytokine producing/secreting cell, may be more relevant in order to understand the involvement of individual cell types in the immune process. To study the involvement of particular cell types in health and disease, phenotyping and cytokine detection should, ideally, be performed in parallel. Flow cytometry allows such determinations but due to low sensitivity, this method requires usage of in vitro stimulation or Golgi blockers (Comabella et al., 1998).

Assessment of cytokine secreting-cells by ELISPOT assays circumvents most of these problems and offers the advantage of close protein-capture antibody proximity that circumvents paracrine and autocrine cytokine uptake of the cytokine. ELISPOT assays are sensitive enough to detect cytokine-secreting cells without stimulation. ELISPOT assays thus permits the detection of well-defined spots that clearly represent numbers of cytokine-secreting cells present in the circulation.

To be able to utilize ELISPOT assays in a broader perspective for monitoring of immune responses, feasibility, sensitivity and reliability of the assays should be established. Although the utilisation of ELISPOT assays were previously described for MNC (Czerkinsky et al., 1983; Özenci et al., 1999) it is important to determine optimal assay conditions for different cell types like monocytes, since these conditions may change from one effector cell to another. In addition, cells should be plated in a single cell layer in this assay. Thus, background effects and levels of the cytokine produced by the individual cells are highly dependent on interactions between the individual cells, frequency of cytokine secreting cells, incubation periods etc. Changes in assay conditions, therefore, are likely to influence the assay results considerably. For these various reasons, ELISPOT assays have to be performed under carefully defined and controlled conditions. A drawback of ELISPOT assays is the difficulty to establish the assays for new cytokines due to lack of suitable antibody pairs.

In the present study, we adapted ELISPOT assays to detect monocytes secreting TNF-α, IL-6, IL-10 and IL-12. A tight regulation of these four cytokines

keeps the balance and decides whether Th1 or Th2 cells will predominate in immune reactions (Roubenoff et al., 1998; Snijders et al., 1998), and may be altered in a number of disease states. We observed that an incubation time of 24 h yielded optimal numbers of spots. In contrast to the results obtained with ELISA, monocyte ELISPOT assays were highly reproducible with an inter-assay variability of less than 15%. In conclusion, ELISPOT assays to detect cytokine secreting cells, therefore, may be an attractive and fast tool in monocyte research.

THE INVOLVEMENT OF DENDRITIC CELLS IN MULTIPLE SCLEROSIS

The primary event initiating the immune pathogenesis of MS remains elusive. Due to the relative immune privilege of the CNS and the inability of naive T cells to cross BBB, induction of the immune response probably takes place in the periphery (Fisher and Reichmann, 2001). Since DC are considered most potent stimulators of naive T cells, it seems reasonable to assume that immune aberrations observed in MS may at least partly underlie aberrant presentation of Ag by DC, alterations in DC phenotype or DC-derived immune modulators (Link et al., 1999, 2001). Recent evidence indicates that, indeed, MS is characterized by circulating DC with altered phenotype and cytokine profiles (Bartholome et al., 1999; Huang et al., 2001; McRae et al., 2000).

Critical to the initiation of the immune response is an efficient trafficking of APC back and forth from tissue to secondary lymphoid organs. A recent study indicates that leukocytes require MMP activity to penetrate into lymph nodes (Faveeuw et al., 2001). In the present study, we provide evidence that DC express and secrete functionally active MMP and TIMP. We observed that MS is associated with high MMP-1, -2 and -9 and TIMP-2 produced by iDC, and high MMP-2, -3 and -9, and high TIMP-2 produced by mDC, detectable at different stages of protein. Increased migration capacity of DC in vitro that was virtually abolished by addition of MMP inhibitors as EDTA or rhTIMP-1.

The present data, therefore, further contribute to the notion that alterations in DC phenotype and function occur in MS and may have functional consequences in

the form of altered migration properties. Interestingly, we observed that mDC produce MMP and TIMP as well. mDC are already located in the lymphoid organs where they present Ags to T cells. However, the fate of these cells following Ag presentation is elusive (Parajuli et al., 1999). On the other hand, it is possible that mDC utilize MMP in their migration process following Ag presentation. It is also possible that iDC do not downregulate their MMP and TIMP producing capacity in the process of maturation and that mDC keep the capability of producing these molecules. In the present study, we utilized an artificial system to generate DC. Whether the observed changes in MMP also occur in vivo should be confirmed.

POSSIBLE ROLE OF MONOCYTES IN MULTIPLE SCLEROSIS

The presence of monocytes in the MS lesion is well established. Accumulated monocytes may augment the inflammatory responses observed in MS through secretion of pro-inflammatory cytokines including IL-12, TNF-α and IL-6. These cytokines may exert significant immunoregulatory effects on circulating cells, including T cells, but they may play a critical role in the regulation of the intrathecal inflammation observed in MS (Hickey and Kimura, 1988; Ransohoff et al., 1992). A further indication of the involvement of monocytes in the pathogenesis of neuroinflammation comes from animal models supposed to reflect MS. Upon induction of experimental autoimmune encephalomyelitis (EAE), monocyte depleted mice show accumulation of leukocytes in subarachnoid and perivascular spaces. These cells, though, fail to cross the BM thus abrogating disease symptoms and pathology (Tran et al., 1998). The inability of cells to cross the BBB may be due to the failure to induce the necessary chemotactic gradient or proteolytic enzymes to destruct the BM. Recent evidence indicates that intimate cell-cell contact in addition to secretion of soluble mediators not only is an important mechanism of MMP induction in T cells and monocytes but also is a major inducer of cytokine production in monocytes (Burger, 1998).

Additionally, monocytes give rise to perivascular and tissue macrophages that appear to be implicated in the final common pathway of MS-related myelin

destruction, both through phagocytosis and by secreting cytokines that may locally promote inflammation or myelin destruction (Louis, 1993; Rudick et al., 1992).

In the present study, we observed aberrations of circulating monocytes both at the phenotypic and cytokine level. We found that MS is associated with aberrations in monocytes expressing HLA as well as co-stimulatory molecules. A higher density of CD86 on monocytes, as reflected by MFI, on cell surface of blood monocytes was observed in MS compared to controls. The expression of CD80 and HLA-ABC, and density of HLA-ABC reflected by MFI, were also augmented on monocytes in patients with long-standing MS (>10 years). Similarly, percentages of CD80 expressing monocytes were higher in patients with moderate to severe disability score (EDSS >3). Whether changes in monocyte phenotype over the course of MS are a result of the disease process remains to be established. Previous studies showed that monocytes are activated prior and during clinical attacks as reflected by increased HLA-DR expression. CD80 expression was normal in MS but tends to drop compared to HC in stable disease (Reder et al., 1998). We observed that MS is also associated with high levels of IL-12 secreting monocytes, which may facilitate cellular immune responses and may shift the immune response towards Th1 type. In addition, we showed that MS is associated high levels of IL-6 secreting monocytes, which may facilitate B cell differentiation and Ig secretion. In concert with previous studies (Rudick and Ransohoff, 1992; Imamura et al., 1993; Maimone et al., 1993), we observed similar levels of IL-10 and TNF-α secreting monocytes in MS compared to controls.

Given these results, we hypothesise that monocytes contribute to immune aberrations observed in MS systemically and may play a role in replenishing the perivascular macrophage pool observed in MS.

SIGNIFICANCE OF IMBALANCES IN MMP AND TIMP IN MULTIPLE SCLEROSIS

Through the availability of MRI, the occurrence of repetitive disruptions of the integrity of the BBB appeared to be up to ten times more frequent than incidence of clinical relapses. Infiltrating activated T lymphocytes and monocytes/macrophages are likely candidates as possible mediators in BBB breakdown (McFarland et al., 1992; Kieseier et al., 1999). To gain access to the CNS, infiltrating cells need to secrete MMP. Such a notion is strengthened by studies demonstrating the involvement of MMP in migration of T cells over ECM barriers (Leppert et al. 1996; Stuve et al., 1996) and the presence of altered levels of MMP in blood, CSF and CNS (Hartung and Kieseier, 2000). In the present study, we show that MS is associated with imbalances in MMP. We observed high levels of MMP-1, -3, -7, and -9 mRNA expressing monocytes in blood of MS patients. From our studies we can conclude that MS is associated with the upregulation of a variety of MMP. Activated human peripheral blood T cells may induce MMP and TIMP production in monocytes via direct cell-cell contact in monocytes (Lacraz et al., 1994). Similar observations have been reported upon interaction of monocytes with activated endothelial cells (Amorino and Hoover, 1998; Aoudjit et al., 1998; Hojo et al., 2000). Additionally, cytokines may modulate MMP transcription. Thus, aberrations in MMP expressing monocytes may be primarily monocyte dependent but are more likely to evolve from soluble mediators or cell-cell interaction with T or other cells (Contasta et al., 1999; Yong et al., 2001). Furthermore, MMP can enforce their action through proteolytic cleavage of other zymogens (Opdenakker and Van Damme, 1994).

Besides their contribution to cellular migration, MMP may also be involved in other stages of MS pathogenesis. Proteolytic enzymes released by macrophages/ microglia and activated T cells during the demyelinating process can generate cryptic epitopes that are normally not exposed (Lehmann et al., 1992) and may thus contribute to the epitope spreading observed in MS (Tuohy et al., 1998; Hartung and Rieckmann, 1997). This is supported by the observation that human MBP exposed to proteolytic enzymes results in the appearance of new antigenic determinants generated from the degradation

process (Whitaker, 1982; Day and Potter, 1986). The inflammatory responses in MS may be further enhanced by certain MMP (MMP-7 and –17) that are efficient in mediating conversion of inactive membrane bound TNF-α into active TNF-α (Chandler et al., 1997). MMP can also contribute to direct tissue damage either by cleaving MBP or through damaging effects on neuronal cells (Vos et al., 2000).

MMP were thus far mainly implicated as detrimental factors in MS pathogenesis, MMP may also contribute to resolution of the inflammatory response and the initiation of repair processes. MMP are indispensable during physiological ECM modulation. Moreover, MMP promote neurogenesis and myelinogenesis during development and may, with the appropriate stimuli, exert such actions in adulthood (Del Bigio and Jacque, 1995; Yong et al., 2001). MMP could enable migration of progenitor cells to injured sites and replenish lost cells (Yong et al., 2001). MMP may aid localization of neurotrophins (Houweling et al., 1998; Gruenbaum and Carew, 1999) and facilitate axonal growth and angiogenesis. Finally, MMP may contribute to the termination of inflammation through breakdown of pro-inflammatory cytokines as IL-1β as well as cleavage of certain chemokines, e.g. MCP-3, thus abolishing the chemotactic gradient for leukocyte entry. Besides imbalances in MMP expressing monocytes, we also observed that MS is associated with alterations in levels of TIMP expressing monocytes. Whether elevation of TIMP production by monocytes in patients with MS is a primary phenomenon or a consequence of MMP elevation or alterations in certain cytokines as IL-6, remains to be determined. In conclusion, MS is associated with changes in MMP and TIMP expressing APC. Whether these alterations exert mainly beneficial or detrimental actions in MS warrants further studies.

IFN-β TREATMENT IN MULTIPLE SCLEROSIS

The mechanisms underlying the beneficial effects of IFN-β are largely unknown. An understanding of the mechanisms by which IFN-β acts, may improve the use of this drug, and may aid in the design of more potent drugs to treat the disease. In this study, we examined the effects of IFN-β treatment on the cytokines TNF-α, IL-6, IFN-γ and IL-10, on the metalloproteinases MMP-3, -7 and -9, and on TIMP-

1 in a longitudinal study. We found that the effects of IFN-β on individual cytokines and MMPs were selective and time-dependent. IFN-β down-modulated numbers of TNF-α secreting and MMP-3 and -9 mRNA expressing MNC within 1-3 months after treatment onset, while the augmenting effect on numbers of IL-10 secreting and TIMP-1 mRNA expressing MNC were first observed after 6-12 months of treatment. No effects of IFN-β treatment were observed on levels of IFN-γ secreting or MMP-7 mRNA expressing MNC over the course of IFN-β treatment.

The present study did not address the effects of IFN-β treatment on cytokines and MMP production in APC specifically. It is reasonable to assume that IFN-β partially exerts it function through modulation of APC function. IFN-β is postulated to interfere with Ag presentation through down regulation of MHC class II and co-stimulatory molecules (Yong et al., 1998). IFN-β enhances the secretion (Porrini et al., 1995) and expression of IL-10 by monocytes (Liu et al., 2001). Additionally, increases in expression of CD40 and CD86 on monocyte cell surface upon IFN-β treatment were reported (Liu et al., 2001). Lower HLA-DR expression on monocytes was also described after 1-year treatment (Gelati et al., 1999).
The effects of IFN-β on DC are thus far mainly limited to in vitro studies. IFN-β upregulates HLA-DR, CD80 and CD86 expression on DC (Bartholome et al., 1999; Huang et al., 2001). Upon IFN-β treatment, DC also secreted high levels of IL-10 and were relatively deficient of IL-12 (McRae et al., 1998, 2000).
Thus, the net outcome of IFN-β treatment indicates a shift towards an anti-inflammatory immune-response pattern in which APC may be involved. From our studies, it is not possible to conclude that IFN-β suppresses all Th1-type responses and induces all Th2-type responses since IFN-β did not affect the pro-inflammatory cytokine IFN-γ and decreased IL-6, which has anti-inflammatory properties. Moreover, whether the down regulatory effects on pro-inflammatory molecules and the up regulatory effects on anti-inflammatory molecules represent direct effects of IFN-β on the immune system, or whether they are secondary to the clinical stabilization of MS pathology induced by IFN-β, remains to be evaluated.

ACUTE STROKE IS ASSOCIATED WITH IMBALANCES IN MATRIX-DEGRADING METALLOPROTEINASES AND CYTOKINES SYSTEMICALLY

In stroke, an inflammatory response develops shortly after the ischemic period. The initiation of this inflammatory stimulus remains unclear but may involve 'danger' signals released by necrotic tissue present in the ischemic core of the lesion (Matzinger, 1998). Neutrophils and monocytes, components of the fast innate immune system are recruited to the CNS. Neutrophils can be detected within CSF as early as 48-72 h after onset of stroke (Sornas et al., 1972), while monocytes can be detected in ischemic lesions 2-14 days after onset of the ischemic stroke (Pozzilli et al., 1985). The mechanisms underlying the recruitment of these cells are incompletely understood but cytokines and MMP are likely to contribute (Feuerstein et al., 1998; Del Zoppo and Hallenbeck, 2000). Furthermore, inflammatory responses observed in acute stroke may be important contributing factors in determining the fate of the ischemic penumbra and, therefore, of the final brain damage and outcome caused by stroke (Tarkowski et al., 1995, 1997; Feuerstein et al., 1998; Del Zoppo and Hallenbeck, 2000). Changes in cytokine levels systemically were previously reported in stroke (Fassbender et al., 1994; Beamer et al, 1995;Tarkowski et al., 1995; Kim et al., 1996; Pantoni et al., 1998) Interestingly, some of these changes could be correlated with infarct volume and outcome of stroke (Fassbender et al., 1994; Tarkowski et al., 1997). These studies, however, did not focus on a specific cell type as PMN or monocytes. Since PMN and monocytes constitute the major inflammatory cells in ischemic lesions of the brain (Kochanek and Hallenbeck, 1992) it may be more attractive to study properties of individual cell types such as monocytes involved in lesion formation. Such an approach may provide deeper insight in the pathogenesis of ischemic stroke and provide tools for new therapeutic strategies.

In the present study, we provided further evidence for the occurrence of immune aberrations systemically during the acute phase of stroke. The acute phase of stroke is characterized by systemic aberrations in cytokines, i.e. elevated numbers of TNF-α, IL-12 and IL-6 secreting monocytes and MNC and MMP, i.e. elevated levels of MMP-1, MMP-7 and TIMP-1 mRNA expressing monocytes and high levels of MMP-1, -2, -7, -9 and TIMP-1 mRNA expressing MNC during the acute phase of stroke compared to convalescence and

healthy subjects. Such data indicates that monocytes may at least be partly responsible for the immune alterations observed in blood of patients with stroke. Moreover, our data suggests that IL-12, a potent inducer of TNF-α as well as IL-6 production, could play a crucial role in the elaboration of systemic immune imbalances in stroke. Upregulation of levels of IL-12 secreting cells in the very acute phase of stroke (<2 days after onset of stroke) was followed by upregulation of levels of TNF-α and IL-6 secreting cells (up to 4 days after onset of stroke). It is, therefore, tempting to assume that increases in levels of TNF-α and IL-6 secreting cells are a consequence of this early raise in levels of IL-12 secreting cells.

IL-12 and TNF-α are potent chemoattractant cytokines for monocytes and to lesser extent for neutrophils (Ha et al., 1999; Poggi et al., 1999). Additionally, TNF-α may increase the expression of adhesion molecules on endothelial cells of the microvasculature in the CNS (Jean et al., 1998) allowing docking of inflammatory cells to the endothelial cell surface. Moreover, TNF-α facilitates the expression of several MMP, like MMP-1 (Singer et al., 1999) and MMP-9 (Johnatty et al., 1997; Aoudjit et al., 1998). IL-6 on the other hand, may partially counterbalance the pro-inflammatory actions of TNF-α and IL-12 by enhancement of TIMP expression (Lotz and Guerne, 1991). Whether the augmented levels of TNF-α and IL-6 secreting cells are directly responsible for the upregulation of MMP mRNA expressing cells and MMP activity in monocytes and MNC systemically in patients with stroke remains to be determined.

We observed only mild increase in IL-10 secreting MNC in the acute phase of stroke, while levels of IL-10 secreting cells did not change over the course of stroke, thus suggesting that deviations of numbers of IL-10 secreting cells occurring in the acute phase of stroke are of minor dimensions. Since IL-10 is an important inhibitor of TNF-α, IL-12, and IL-6, it is, therefore, also known as monocyte de-activating factor (De Waal Malefyt and Moore, 1998). These results may yet another indication of monocyte activation during the acute phase of ischemic stroke.

High levels of MMP-2, -7 and -9 mRNA expressing monocytes/macrophages were previously detected in ischemic brain lesions (Clark et al., 1997; Anthony et al., 1997). The elevation of numbers of MMP expressing monocytes in blood

may have several implications. MMP are considered physiological mediators of cell migration and may thus be involved in the recruitment of inflammatory cells into the ischemic lesion site. Additionally, MMP may contribute to aggravation of tissue damage. Detrimental effects of MMP on stroke outcome are supported by a recent study correlating high levels of MMP-9 but not MMP-2 in plasma samples obtained 12-24 h after onset of stroke with neurological deficit and total lesion volume (Montaner et al., 2001). In the present study, we show that within 4 days after onset of stroke symptoms, a variety of MMP including MMP-2 are elevated in blood of patients with stroke. Discrepancies between these two studies probably relay on methodological differences and the slightly different time points of sampling as well as the utilised materials (plasma vs. cells). Besides detrimental actions of MMP, recent data indicate that certain MMP as MMP-2 and -9 (Pepper, 2001) and MMP-7 promote angiogenesis (Di Girolamo et al., 2001), which is considered beneficial in stroke.

In conclusion, upregulation of levels of cytokine secreting and of MMP and TIMP mRNA expressing monocytes in the acute phase, and their normalization over time add evidence to the occurrence of systemic immune aberrations and monocyte activation in patients with acute stroke. Blood monocytes may be partly responsible for the changes observed over the course of stroke and contribute to activation of cells in the periphery.

CONCLUSIONS AND FUTURE PERSPECTIVES

In science relevant answers can only be obtained by relevant questions. On the other hand, it requires strong basis, i.e. good answers to ask relevant questions. Like in many other cases in research, the present results bring more questions. **'Mit dem Wissen wächst der Zweifel'**.

The present work focussed on the possible involvement of APC in two detrimental diseases namely MS and stroke. To answer this question, we studied cytokine and MMP production by monocytes and DC.

Our findings indicate that both MS and stroke alterations in MMP and cytokines. MS is associated with high levels of monocytes secreting IL-6 and IL-12 and expressing mRNA of MMP-1, -2, -7 and -9 and of TIMP-1 compared to HC. In stroke, we observed higher levels of blood monocytes secreting TNFα, IL-12 and IL-6 and expressing mRNA for MMP-1, and -7 and TIMP-1 in the acute phase of stroke compared to convalescence and to HC.
In line with these findings in monocytes, we showed that DC are able to express and secrete functional MMP-1, -2, -3, and –9 and of TIMP-1, -2 and are elevated in MS.

Together with existing data, the present results give further evidence for the occurrence of aberrations in APC in MS and ischemic stroke.

As mentioned above interesting questions on the current findings are brought up. Including (i) the functional implications of elevated MMP and cytokines secreted by monocytes and DC; (ii) development of more advanced methods to study MMP and cytokines, and (iii) the possible usage of this information in developing treatments to MS and stroke.

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