# Reprogramming of TIMP-1 and TIMP-3 expression profiles in brain microvascular endothelial cells and astrocytes in response to proinflammatory cytokines

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**Abstract Cytokine-dependent regulation of tissue inhibitors of metalloproteinases (TIMPs) expression provides an important mechanism for controlling the activity of matrix metalloproteinases. We present data indicating that during inflammatory processes TIMP-1 and TIMP-3 may be involved in the proteolytic remodeling of subendothelial basement membrane of the brain microvascular system, a key step during leukocyte migration into the brain perivascular tissue. In brain endothelial cells the expression of TIMP-1 is dramatically up-regulated by major proinflammatory cytokines, with the combination of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) exhibiting the strongest synergistic stimulation. Simultaneously, IL-1β/TNFα almost completely blocks TIMP-3 expression. Both synergistic effects are dose-dependent within the concentration range 0.05–5 ng/ml of both cytokines and correlate with the expression of inducible nitric oxide synthase, an endothelial cell activation marker. Down-regulation of TIMP-3 expression is also detected in astrocytes treated with TNFα or IFN-γ, whereas oncostatin M as well as TNFα up-regulate TIMP-1 mRNA level. We propose that the cytokine-modified balance between TIMP-1 and TIMP-3 expression provides a potential mechanism involved in the regulation of microvascular basement membrane proteolysis.**



*Key words:* Endothelial cell; Astrocyte; Inflammation; Tissue inhibitor of metalloproteinases

## 1. Introduction

Endothelial cells actively participate in inflammatory reactions. In response to inflammatory cytokines they undergo profound alteration of functions referred to as endothelial cell activation [1]. A major alteration of endothelial cell characteristics upon activation is their acquisition of adhesiveness for leukocytes mediated by increased expression of some constitutive cell adhesion molecules as well as synthesis of new surface proteins such as E-selectin or VCAM-1. Integrin-dependent adhesion and transmigration of leukocytes through the endothelial cell layer have been extensively studied, whereas only limited information is available on the mechanisms which control proteolytic remodeling of subendothelial basement membrane required for further steps of leukocyte migration [2].

Unlike interstitial extracellular matrix (ECM) composed predominantly of collagen types I, II, and III, basement membranes contain collagen types IV and V structurally organized in a non-fibrillar, multilayer network [3]. The major group of enzymes controlling basement membrane ECM turnover are gelatinases (MMP-2 and MMP-9), a subclass of the matrix metalloproteinase (MMP) family including also collagenases, stromelysins, and membrane-type MMPs (MT-MMPs). MMPs are usually secreted as inactive proenzymes and can be activated extracellularly by other proteinases including active MMPs themselves [4]. Several physiological mechanisms prevent uncontrolled MMP activation. One of these is binding of MMPs by tissue inhibitors of metalloproteinases (TIMPs) [5]. By inhibition of MMP activity, TIMPs contribute to the regulation of ECM remodeling associated with various physiological and pathological conditions [6].

Apart from the role in the regulation of ECM proteolysis, at sites of inflammation MMPs and TIMPs may contribute to important immunoregulatory processes such as cytokine and cytokine receptor turnover [7]. For example, MMP-1, -2, -3, and -9 have been shown to degrade interleukin-1β (IL-1β); and this can be effectively blocked by TIMP-1 [8]. Furthermore, it has been recently described that TIMP-3 is capable of inhibiting tumor necrosis factor-α (TNFα) converting enzyme, a metalloproteinase which belongs to the ADAM (A disintegrin and metalloproteinase) family of proteins [9]. Apart from the inhibition of TNFα precursor processing, TIMP-3 can also down-regulate shedding of TNF receptor p55, IL-6 receptor gp80 and L-selectin [10–12], however proteinases involved in the later processes remain unknown.

Major proinflammatory cytokines (IL-1β, TNFα and interferon-γ (IFN-γ)) as well as IL-6 family cytokines (IL-6 and oncostatin M (OSM)) are, in turn, important regulators of MMP and TIMP expression [13]. In this paper we describe a dramatic reprogramming of TIMP-1 and TIMP-3 expression profiles in cytokine-stimulated brain endothelial cells and astrocytes, two major cell types which contribute to the synthesis and turnover of the brain microvascular basement membrane. These findings suggest that TIMP-1 and TIMP-3 may play distinct roles in the regulation of local inflammatory processes in the central nervous system.

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*Abbreviations:* ECM, extracellular matrix; EtBr, ethidium bromide; FCS, fetal calf serum; iNOS, inducible nitric oxide synthase; OSM, oncostatin M; MBE, murine brain microvascular endothelial cells; MMP, matrix metalloproteinases; MS, multiple sclerosis; MT-MMP, membrane-type MMP; Stat, signal transducer and activator of transcription; TIMP, tissue inhibitor of metalloproteinases


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## 2. Materials and methods

### 2.1. Cell culture

Primary astrocyte cultures were prepared by multiple passaging of the mixed brain cell cultures established from cortical tissue of 12–14 week old Wistar rats [14]. The cells were cultured in MEM (Life Technologies Ltd., Paisley, UK) containing 1% glucose, 1 mM sodium pyruvate, 2 mM glutamine and 10% fetal bovine serum. Prior to confluence the cell culture flasks were shaken overnight and non-adherent cells were discarded. The remaining cells were repassaged by trypsinization (dilution 1:4). This process was repeated until the cultures were >98% pure astrocytes as judged by immunocytochemistry analysis for glial fibrillary acidic protein (3–4 passages). Passages 5–10 were used for the experiments. Cultures of murine brain microvascular endothelial cells (MBE), a gift from Dr. R. Auerbach (Madison, WI, USA) were >98% pure as judged by the assay for VCAM-1-positive cells and were additionally characterized in several previously published studies [15]. The cells were cultured in medium consisting of DMEM, 20% fetal calf serum (FCS) (both from Life Technologies Ltd., Paisley, UK), endothelial cell growth supplement (30 mg/ml), 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 U/ml) (all from Sigma Chemical Co., St. Louis, MO, USA). Passages 8–12 were used in the experiments. Human hepatoma HepG2 cells, a gift of Dr. H. Baumann (Buffalo, NY, USA), were cultured in DMEM supplemented with 10% FCS and antibiotics.

### 2.2. Cytokines and cell stimulation

Cells were stimulated with 0.05, 0.5 or 5 ng/ml rhIL-1β ($1.2\times10^8$ U/mg), 25 ng/ml rhOSM ($4.7\times10^7$ U/mg) (both a gift from Immunex Corp., Seattle, WA, USA), 0.05, 0.5 or 5 ng/ml rhTNFα (purchased from R&D Systems, Minneapolis, MN, USA) and 200 U/ml rmIFN-γ (Life Technologies Ltd., Paisley, UK).

### 2.3. RNA preparation and Northern blot analysis

MBE cells and astrocytes were grown to 80–90% confluence as described above. Media were then changed into RPMI 1640 (Life Technologies Ltd., Paisley, UK) containing 2% of heat-inactivated FCS (MBE cells) or DMEM containing 2% heat-inactivated FCS (astrocytes), 2 mM glutamine and antibiotics. The tested cytokines were then added and cells were incubated for an additional 18 h. Total RNA was prepared using the phenol extraction method [15]. 5 μg samples of RNA were subjected to formaldehyde gel electrophoresis using standard procedures [16] and transferred to Hybond-N membranes (Amersham Life Science, Little Chalfont, UK), according to the manufacturer's instructions. Ethidium bromide-stained filters were photographed prior to hybridization to show the equal loading of RNA. 28S and 18S rRNA visualized in this procedure were used as indicators of gel loading since the expression of some reference genes used to standardize Northern blotting data, especially glyceraldehyde 3-phosphate dehydrogenase [17], may be considerably altered by cytokines in MBE cells. Filters were prehybridized at 68°C for 3 h in 10% dextran sulfate, 1 M sodium chloride and 1% SDS, and hybridized in the same solution with cDNA probes specific for the murine TIMP-1 and TIMP-3, murine macrophage iNOS (a gift from Drs. Q.-W. Xie and C. Nathan, New York, NY, USA), and murine VCAM-1 (a gift from Dr. L. Osborn, Cambridge, MA, USA) labeled by random priming using Megaprime DNA labeling system (Amersham). After hybridization, non-specifically bound radioactivity was removed by washing in 2×SSC at room temperature, followed by two washes in 2×SSC/1% SDS at 68°C for 20 min. The blots were then subjected to autoradiography at −70°C.

### 2.4. Nuclear extract preparation and gel retardation assays

Cells were grown as described previously to 70–80% confluence and fresh media containing 2% FCS were then added. After 24 h cells were stimulated for 15 min with indicated factors. Nuclear extracts were prepared as described [18,19]. A double stranded DNA fragment containing the high affinity sis-inducible element of human c-fos promoter (SIE) [20] was labeled by filling in 5′ protruding ends with Klenow enzyme using [α-$^{32}$P]dCTP (3000 Ci/mmol). Gel retardation assays were carried out according to published procedures [21,22]. 2–5 μg of nuclear extracts and approximately 10 fmol (10 000 cpm) of probe were used. Some samples were incubated in the presence of a 50-fold molar excess of specific unlabeled oligonucleotide probe as a competitor. Protein-DNA complexes were separated in a 4.5% polyacrylamide gel in 0.5×TBE. The gels were dried and exposed to X-ray film at −70°C.

### 2.5. Nitrite assay

Assays for nitrate production were performed as described previously [23]. Cells cultured in RPMI 1640 supplemented with 2% FCS and antibiotics were stimulated with indicated factors. After 18 h incubation, nitrite concentrations in the media were determined by a microplate assay. 80 μl aliquots of the culture media were incubated with equal volumes of Griess reagent at room temperature for 10 min. The absorbance at 540 nm was measured with an ELISA plate reader. The nitrite concentrations were determined using dilutions of sodium nitrite in water as a standard.

### 2.6. Data representation

Figures containing data from Northern blot and electrophoretic mobility shift assay (EMSA) show typical results obtained from 3–5 independent experiments. Nitrite assays are presented for the same experiments which were used to illustrate Northern blot data. Variations in cytokine-induced nitrite accumulation observed in repeated experiments were below 20% for every cytokine or cytokine combination tested.

## 3. Results

Previous reports have demonstrated that TIMPs are expressed in endothelial cells [24–26]. We used MBE cells as a model system to study the regulation of TIMP-1 and TIMP-3 expression in response to the proinflammatory cytokines IL-1β, TNFα, IFN-γ, and OSM. TIMP-2 was not included to this study since it is expressed constitutively in endothelial cells and, as in other cell types, its expression is not significantly influenced by cytokines and growth factors [24]. Both in control and in cytokine-treated MBE cells we did not detect mRNA for TIMP-4, the last of the known members of the TIMP family.

In initial experiments the TIMP-1 expression pattern was compared to cytokine-induced changes in mRNA levels of VCAM-1 and iNOS, markers of endothelial cell activation. Changes of TIMP-1 mRNA level observed in cytokine-stimulated MBE cells resembled those observed for iNOS mRNA (Fig. 1A) which, in turn, directly corresponded to the iNOS activity measured as nitrite accumulation in the culture media (Fig. 1B). The level of TIMP-1 transcript in control cells was very low. Among single cytokines tested only IL-1β significantly up-regulated TIMP-1 mRNA level, whereas TNFα or IFN-γ exhibited weaker, yet detectable stimulatory effects (compare Figs. 1A and 2, representing a prolonged TIMP-1 blot exposure time). The most dramatic stimulation of TIMP-1 mRNA was observed when the combinations of IL-1β/TNFα or TNFα/IFN-γ were used.

MBE cells constitutively expressed a high level of TIMP-3 mRNA. In contrast to the regulation of TIMP-1 expression, the level of TIMP-3 mRNA significantly decreased in MBE cells treated with IL-1β/TNFα and to some extent also with IFN-γ/IL-1β (Fig. 2A). Because synergistic stimulation of MBE cells with two major proinflammatory cytokines (IL-1β and TNFα) had the most profound effects on TIMP-1 and TIMP-3 expression we studied this regulation in more detail. Fig. 3 shows the dose-dependent effect of IL-1β and TNFα on the expression of TIMP-1 and TIMP-3. The cytokines exhibited full synergistic effects within the concentration range 0.05–5 ng/ml: the highest stimulation of TIMP-1 mRNA as well as the strongest down-regulation of TIMP-3 mRNA level was observed in MBE cells treated with 5 ng/ml of both IL-1β and TNFα. A decrease in concentration of

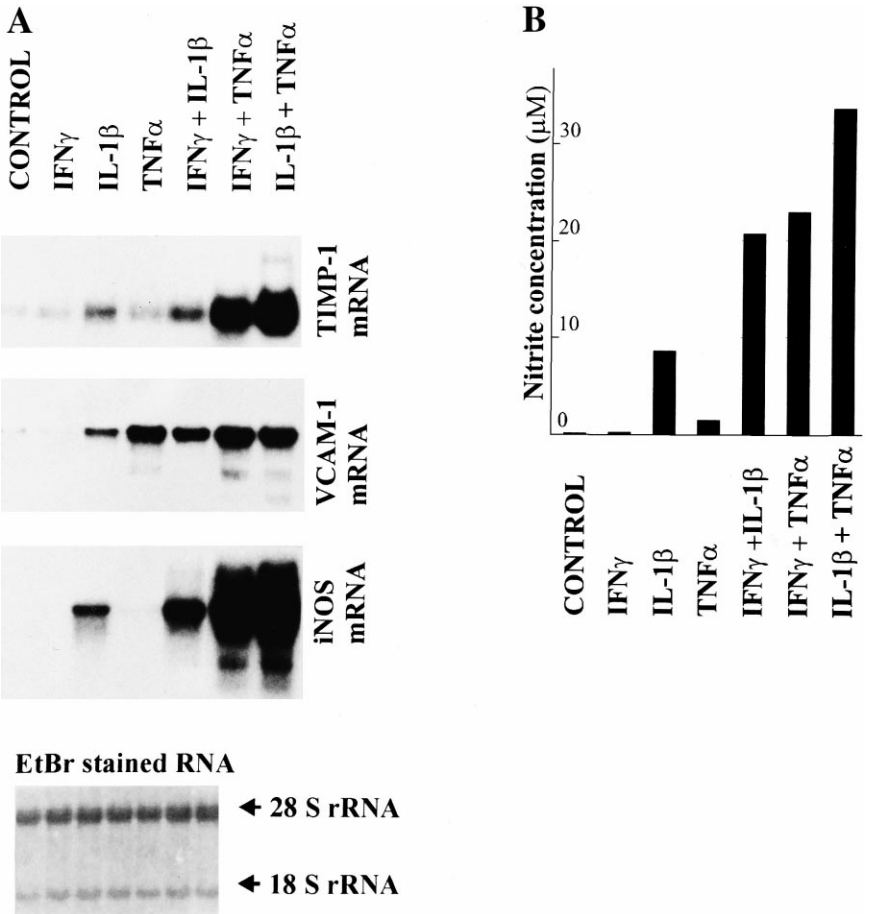


Fig. 1. Expression of TIMP-1 and endothelial cell activation markers VCAM-1 and iNOS in cytokine-treated MBE cells. A: MBE cells were supplemented with fresh medium (RPMI with 2% FCS) and treated with IL-1β (5 ng/ml), TNFα (5 ng/ml), IFN-γ (200 u/ml) or the combinations of cytokines as indicated. RNA was isolated after 18 h and subjected to Northern blot analysis with mTIMP-1, mVCAM-1, and miNOS cDNA probes. The lower panel shows an ethidium bromide (EtBr)-stained blot photographed after RNA transfer. B: The culture media collected before RNA isolation were used for nitrite measurement. Nitrite concentration was determined using Griess reagent after 18 h incubation of approximately $6\times10^6$ MBE cells in 8 ml of medium.

either cytokine resulted in a marked decrease of their synergistic action. Again, dose-dependent up-regulation of TIMP-1 and down-regulation of TIMP-3 expression correlated very well with iNOS activity (Fig. 3B) providing further evidence that reprogramming of TIMP-1 and TIMP-3 expression patterns represents an additional characteristic of endothelial cell activation in this experimental system.

Unexpectedly, OSM, which was shown to regulate TIMP-1 and/or TIMP-3 expression in fibroblasts [27], hepatocytes [28], chondrocytes [29] and synovial lining cells [30], did not significantly influence the levels of the corresponding mRNAs in MBE cells (Fig. 2A). The absence of OSM effects on TIMP-1 and TIMP-3 expression in MBE cells observed both at the mRNA level and in transient transfection experiments (data not shown) could be explained by the impaired mechanisms of signal transduction for this cytokine. To test for this possibility we performed EMSA (Fig. 4) which clearly showed that in MBE cells Stat3 is activated by OSM, whereas IFN-γ activated both Stat1 and Stat3, indicating that the functional OSM receptor complexes are present in MBE cells. Therefore, the tissue-specific pattern of the cytokine-dependent regulation of TIMP-1 and TIMP-3 expression appears to be unique for endothelial cells in comparison with previously studied cell systems [27–30].

In additional experiments we tested the profile of TIMP-1 and TIMP-3 expression in rat astrocytes, representing a major cell type in the brain perivascular tissue (Fig. 2B). In contrast to brain endothelial cells, astrocytes expressed a significant basal level of TIMP-1 mRNA and a markedly lower level of TIMP-3 mRNA. Up-regulation of TIMP-1 mRNA level was observed only in OSM- and TNFα-stimulated astrocytes but no synergistic cooperation between the tested cytokines was detected. The effects of specific cytokines on TIMP-3 expression in astrocytes were also different from those described for MBE cells: in astrocytes, TNFα alone significantly decreased TIMP-3 mRNA levels and TNFα in combination with IFN-γ almost completely suppressed TIMP-3 expression. However, in both MBE cells and astrocytes the tested proinflammatory cytokines generally exerted negative regulatory effects on TIMP-3 expression whereas TIMP-1 expression was up-regulated.

## 4. Discussion

Proteolysis of subendothelial basement membrane during leukocyte extravasation is an example of a highly coordinated local ECM remodeling process. It involves spatial and temporal regulation of MMP synthesis and activity. Expression of

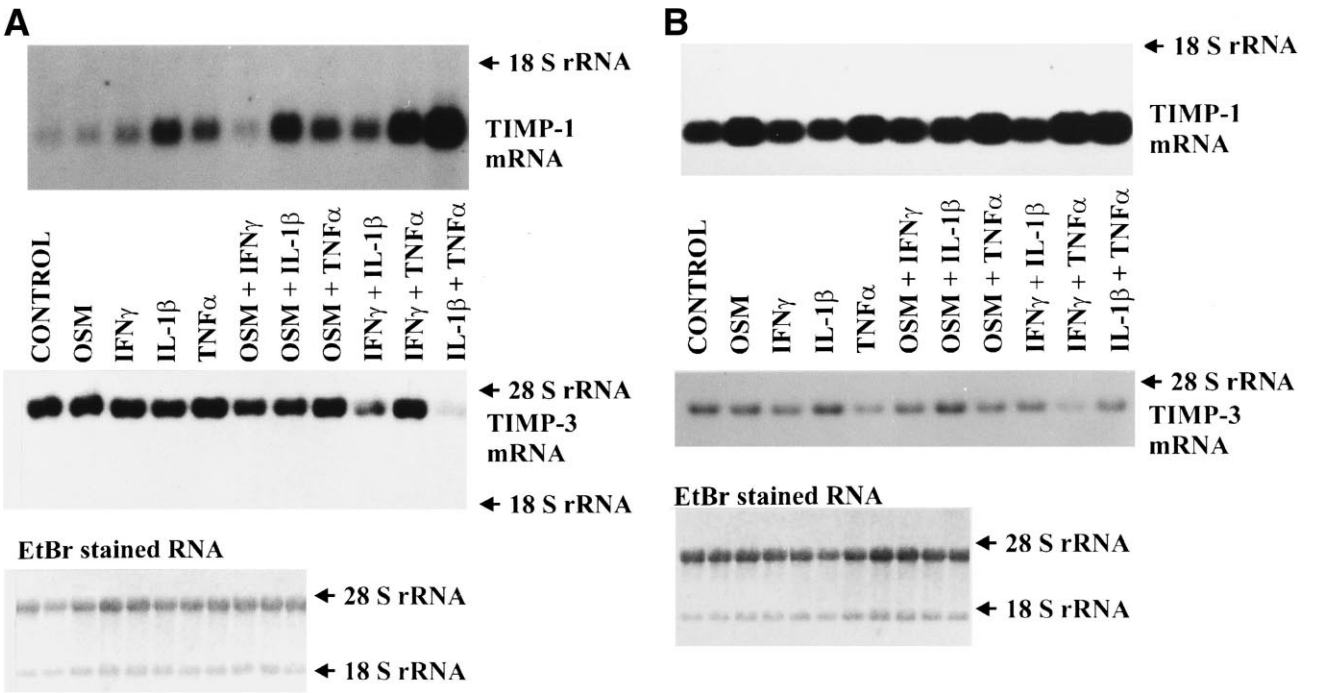


Fig. 2. Comparison of TIMP-1 and TIMP-3 expression profiles in cytokine-treated MBE cells (A) and astrocytes (B). MBE cells and astrocytes were supplemented with fresh medium and treated with IL-1β (5 ng/ml), TNFα (5 ng/ml), IFN-γ (200 u/ml), OSM (25 ng/ml) or the combinations of cytokines as indicated. RNA was isolated after 18 h and subjected to Northern blot analysis with mTIMP-1 and mTIMP-3 cDNA probes. The lower panel shows an ethidium bromide (EtBr)-stained blot photographed after RNA transfer.

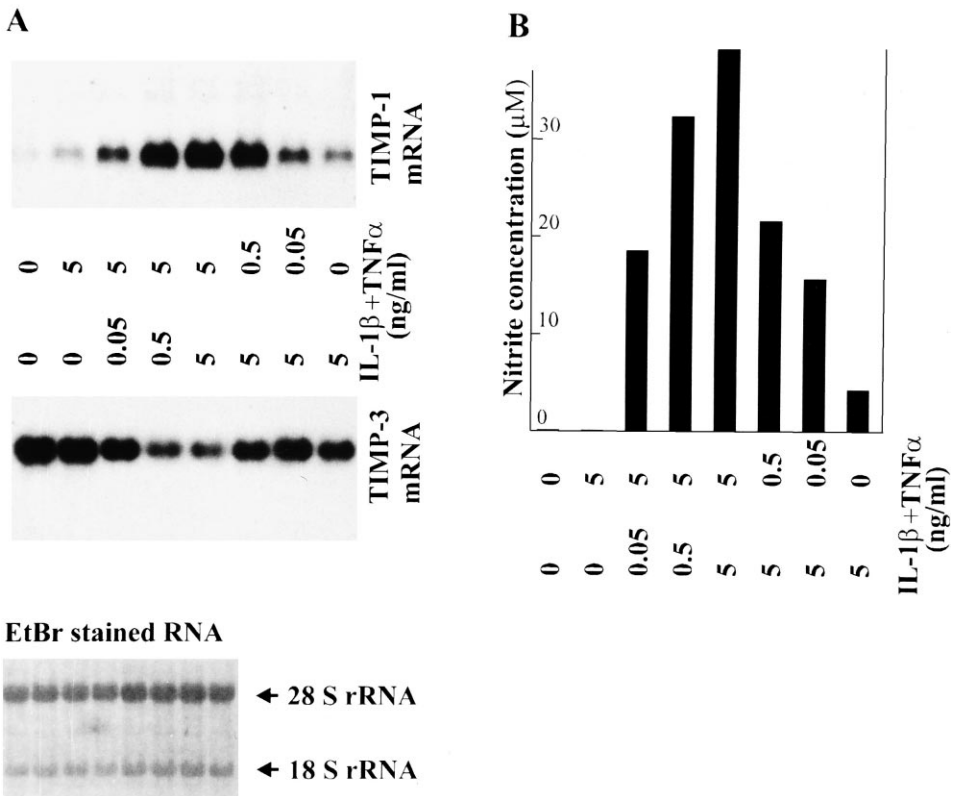


Fig. 3. Synergistic dose-dependent effects of IL-1β and TNFα on the level of TIMP-1 and TIMP-3 expression in MBE cells. A: MBE cells were supplemented with fresh medium (RPMI with 2% FCS) and treated with various concentration of IL-1β and TNFα as indicated. RNA was isolated after 18 h and subjected to Northern blot analysis with mTIMP-1 and mTIMP-3 cDNA probes. The lower panel shows an ethidium bromide (EtBr)-stained blot photographed after RNA transfer. B: The culture media collected before RNA isolation were used for nitrite measurement. Nitrite concentration was determined using Griess reagent after 18 h incubation of approximately $6\times10^6$ MBE cells in 8 ml of medium.

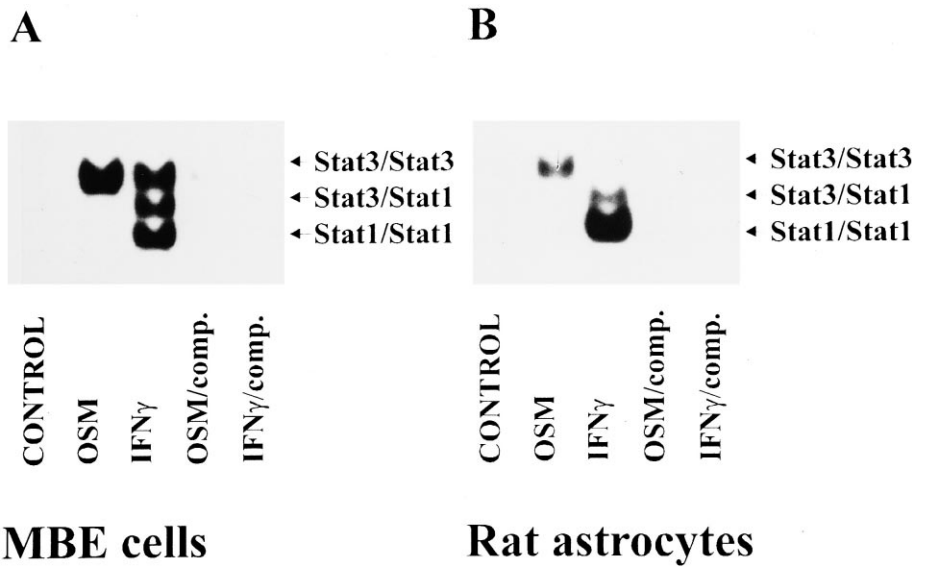


Fig. 4. IFN-γ and OSM activate Stat factors in MBE cells and astrocytes. MBE cells and primary rat astrocytes were stimulated with IFN-γ (200 U/ml) and OSM (25 ng/ml) for 15 min. Cells were collected and nuclear extracts were prepared. 5 μg of protein was incubated with the $^{32}$P-labelled SIE probe. The DNA-protein complexes were separated on a native 4.5% polyacrylamide gel. After drying the gel was exposed to an X-ray film.

several MMPs is up-regulated by IL-1β and TNFα in endothelial cells [24], macrophages and T cells [7,31]. Human T cell activation results in the stimulation of expression of both gelatinases (MMP-9 and MMP-2) [32]. Furthermore, in endothelial cells MMP-2 expression is induced by T cell adhesion and requires binding of the T cell very late activation antigen 4 (VLA-4) to VCAM-1 [33]. These data indicate that leukocyte activation and endothelial cell activation trigger increased local MMP synthesis, including synthesis of pro-gelatinases which, in their active forms, are able to degrade major subendothelial basement membrane components including collagen types IV and V.

In this paper we show that major proinflammatory cytokines cause a dramatic reprogramming of the TIMP-1 and TIMP-3 expression profiles in endothelial cells. The most spectacular effects are mediated by two key proinflammatory cytokines: IL-1β and TNFα. In MBE cells they synergistically repress TIMP-3 expression, whereas the opposite regulation is detected for TIMP-1. Fully synergistic action of these cytokines could be observed within a concentration range of 0.05–5 ng/ml, which strongly suggests that in vivo precise, cytokine- and concentration-dependent mechanisms regulate levels of these endogenous MMP inhibitors and, indirectly, microvascular basement membrane proteolysis.

The opposite regulation of TIMP-1 and TIMP-3 expression indicates that these proteins may play distinct roles in the regulation of microvascular ECM proteolysis. In contrast to other characterized TIMP family proteins which are soluble, TIMP-3 is associated with ECM [34]. TIMP-3 deposited into the microvascular basement membrane may, therefore, protect it against proteolytic degradation mediated by MMPs. Under normal conditions, characterized by high TIMP-3 and low TIMP-1 expression, the dynamic balance between synthesis and degradation of the basement membrane is maintained. In activated endothelial cells TIMP-3 expression is down-regulated and proteolysis of microvascular ECM may be subsequently enhanced. Simultaneous stimulation of TIMP-1 expression observed in response to inflammatory cytokines may, in turn, provide a compensatory mechanism to prevent uncontrolled activation of MMPs at sites of inflammation and tissue damage.

Changes in the profile of TIMP-1 and TIMP-3 expression in cytokine-stimulated endothelial cells may also enhance cell surface-associated activation of ECM proteolysis mediated by MT-MMP-1. MT-MMP-1 is expressed in vascular endothelial cells [35]. On the cell surface MT-MMP-1 acts as a broad-spectrum ECM proteinase. Recently, it has been shown that MT-MMP-1 may also initiate a cascade activation of other MMPs, including gelatinases [36]. Because MT-MMP-1 is efficiently inhibited by TIMP-3, but not TIMP-1 [37], reprogramming of TIMP-1 and TIMP-3 expression during endothelial cell activation could result in the local increase in MT-MMP-1 activity.

The newly described ability of TIMP-3 to inhibit cell surface-associated TNFα converting enzyme (ADAM-17) [9] suggests that the down-regulation of TIMP-3 expression level may also result in the acceleration of pro-TNFα processing and further amplification of local inflammation. Interestingly, we show that TNFα (alone or in combination with IFN-γ) is also capable of inhibiting TIMP-3 expression in primary astrocytes, with TIMP-1 expression being simultaneously stimulated. The coordinated regulation of TIMP-1 and TIMP-3 expression described in this paper for both endothelial cells and astrocytes could, therefore, represent an important mechanism involved in the brain ECM proteolysis under pathological conditions associated with inflammation, such as multiple sclerosis (MS); especially the TNFα-mediated stimulation of MMPs expression and synthesis is considered to be an important link between the proinflammatory cytokine network and the local increase of the proteolytic activity observed in MS and several other neurodegenerative diseases [38].

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