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Novel Role of TGF- β in Differential Astrocyte-TIMP-1 Regulation: Implications for HIV-1-Dementia and Neuroinflammation

Alok Dhar^{1,2,3}, Jessica Gardner^{1,2,3}, Kathleen Borgmann^{1,2,3}, Li Wu^{1,2,3}, and Anuja Ghorpade^{1,2,3,4,*}

¹Laboratory of Cellular Neuroimmunology, University of Nebraska Medical Center, Omaha, Nebraska

²Center for Neurovirology and Neurodegenerative Disorders, University of Nebraska Medical Center, Omaha, Nebraska

³Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska

⁴Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska

Abstract

Astrocyte production of tissue inhibitor of metalloproteinase (TIMP)-1 is important in central nervous system (CNS) homeostasis and inflammatory diseases such as HIV-1-associated dementia (HAD). TIMPs and matrix metalloproteinases (MMPs) regulate the remodeling of the extracellular matrix. An imbalance between TIMPs and MMPs is associated with many pathologic conditions. Our recently published studies uniquely demonstrate that HAD patients have reduced levels of TIMP-1 in the brain. Astrocyte-TIMP-1 expression is differentially regulated in acute and chronic inflammatory conditions. In this and the adjoining report (Gardner et al., 2006), we investigate the mechanisms that may be involved in differential TIMP-1 regulation. One mechanism for TIMP-1 downregulation is the production of anti-inflammatory molecules, which can activate signaling pathways during chronic inflammation. We investigated the contribution of transforming growth factor (TGF)-signaling in astrocyte-MMP/TIMP-1-astrocyte regulation. TGF- β 1 and 2 levels were upregulated in HAD brain tissues. Co-stimulation of astrocytes with IL-1 α and TGF- β mimicked the TIMP-1 downregulation observed with IL-1 α chronic activation. Measurement of astrocyte-MMP protein levels showed that TGF- β combined with IL-1 α increased MMP-2 and decreased proMMP-1 expression compared to IL-1 α alone. We propose that one of the mechanisms involved in TIMP-1 downregulation may be through TGF-signaling in chronic immune activation. These studies show a novel extracellular regulatory loop in astrocyte-TIMP-1 regulation.

Keywords

transforming growth factor; neurodegeneration; chronic inflammation; HIV-1-associated dementia; extracellular matrix; astrocyte-activation

Over the last few years, a keen interest has developed in the role of glia in neuronal injury and human immunodeficiency virus (HIV)-1-associated dementia (HAD) (Gardner and Ghorpade, 2003; Suryadevara et al., 2003). Mononuclear phagocyte (MP) activation and

astrocyte interaction play key roles in the pathologic events of neuro-inflammatory diseases such as HAD. Increased macrophage infiltration through the blood brain barrier is also involved in the pathogenesis of neuro-inflammatory processes (Persidsky and Ghorpade, 2001). Other factors involved in disease progression include neurotoxin secretion and pro-inflammatory mediator recruitment (Heyes et al., 1989; Tyor et al., 1992; Wesselingh et al., 1993; Gelbard et al., 1994; Griffin et al., 1994; Bukrinsky et al., 1995). A good understanding of the mechanisms of the glial anti-inflammatory response in neurodegenerative diseases remains incomplete.

Regulated by matrix metalloproteinases (MMPs) and their tissue inhibitors, extracellular matrix (ECM) degradation is involved in HAD pathogenesis (Dhawan et al., 1995; Berman et al., 1999; Conant et al., 1999; Dezzutti et al., 1999; Liuzzi et al., 2000; Sporer et al., 2000; Ghorpade et al., 2001). Astrocytes produce MMP inhibitors known as tissue inhibitors of metalloproteinases (TIMPs), with TIMP-1 linked to inflammation. TIMP-1 upregulation occurs with phorbol esters, interleukin (IL)-1, retinoids, epithelial growth factor, and IL-6 (Gomez et al., 1997). TIMP-1 is secreted extracellularly during reactive astrogliosis, which follows central nervous system (CNS) damage (Mucke and Eddleston, 1993; Ridet et al., 1997). We previously observed differential TIMP-1 regulation in IL-1-activated primary human astrocytes, and cerebrospinal fluid and brain samples from HAD patients (Suryadevara et al., 2003). TIMP-1 is upregulated acutely, promoting damaged ECM repair and protection. In contrast, chronic inflammation downregulates TIMP-1 leading to failure in TIMP-1-mediated protection. We propose that in addition to direct IL-1 intracellular signaling, astrocyte-TIMP-1 downregulation may be indirectly mediated by anti-inflammatory molecules such as transforming growth factor (TGF)- β .

TGF- β is a key modulator of neurodegeneration and is involved in cell proliferation, ECM component production, chemotaxis, immunosuppression, and cell death regulation (Corder et al., 1998; Roberts et al., 1990; Bottner et al., 2000). TGF- β controls the molecular profile of reactive astrocytes in neurologic diseases (da Cunha and Vitkovic, 1992; Lindholm et al., 1992; da Cunha et al., 1993; Eddleston and Mucke, 1993). Three closely related isoforms TGF- β 1, 2, and 3 are known. Despite similar biologic functions, in vivo expression patterns of TGF- β isoforms vary. An immunoreactive product, TGF- β 1, has been detected in autopsied brains of individuals with CNS diseases (da Cunha et al., 1993). The anti-inflammatory (Schluesener, 1990; Panek et al., 1995; Lee et al., 1997) and immunosuppressive role of TGF- β in CNS lesion response and disease is important for glial survival. TGF- β protects glial cells from injury by nitric oxide synthase (Bottner et al., 2000). Previous evidence suggests that TGF- β may be induced by IL-1 in glial cells by both autocrine and paracrine mechanisms and TGF- β 1 production in vitro varies with cell type and isoform of IL-1 (Morganti-Kossmann et al., 1992). TGF- β operates as a master switch for the modulation of several cytokines (Bottner et al., 2000) and affects gene expression. The proximal promoters of MMP-1 and TIMP-1 genes contain an activator protein-1 (AP-1) element responsive to TGF- β regulation (Hall et al., 2003).

Our previous work with primary human astrocytes during chronic IL-1 stimulation showed TIMP-1 reduction and MMP-1 elevation (Suryadevara et al., 2003). The present investigation focuses on potential mechanisms for this effect. Our results demonstrate that chronic activation results in exacerbated TGF- β secretion, which affects a regulatory feedback loop, leading to TIMP-1 downregulation. We propose that TGF- β may play a novel role during inflammation and neurodegenerative diseases by serving as the extracellular link for astrocyte-TIMP-1 downregulation in chronic inflammation.

MATERIALS AND METHODS

Primary Human Astrocytes

All procedures were carried out in full compliance with the ethical guidelines of the NIH and the University of Nebraska Medical Center. Primary human astrocytes were isolated from elective abortus specimens from the late first and early second trimesters of pregnancy. The cells were obtained after mechanical dissociation of the dissected tissue and plated at a density of 2×10^7 cells/150 cm² as described previously (Suryadevara et al., 2003). Astrocytes were removed by trypsin treatment and subsequently cultured in astrocyte media for a week (i.e., passaged) to obtain greater than 99% pure astrocyte preparations. Immunocytochemical staining for glial fibrillary acid protein was then carried out (Suryadevara et al., 2003). The astrocytes were routinely examined under the microscope during chronic experiments to check for cell adherence and to ensure that there were no floating cells in the media.

Brain Tissue Lysate Preparation

Brain specimens were obtained from the Center for Neurovirology and Neurodegenerative Disorders brain bank and Rapid Autopsy Program at the University of Nebraska Medical Center. The NIH-National Neuro-AIDS Tissue Consortium also provided samples for this work. Brain tissue extracts were prepared from frontal cortex specimens. Tissues were obtained from control donors, HIV-1-seropositive patients without cognitive impairment (HIV+), and HAD patients. Brain tissue specimens were homogenized in lysis buffer containing 1% Triton-X 100 and 1 mM phenyl methyl sulfonyl fluoride in Mg⁺²/Ca⁺²-free phosphate-buffered saline. The supernatants were collected after centrifugation at $15,000 \times g$ for 10 min at 4°C. Protein concentration of the extract was determined by standard bicinonic acid (BCA) method as suggested by the manufacturer (Pierce, Rockford, IL).

RNA Isolation

Total RNA was isolated from primary fetal astrocytes and human brain tissue samples using the standard TRIZol method (Chadderton et al., 1997). Purity of isolated RNA was determined using the ratio of absorbance at 260–280 nm in a spectrophotometer and by denaturing agarose gel electrophoresis. The samples were stored in small aliquots at –80°C and diluted with DEPC water before real-time polymerase chain reaction (PCR).

Real-time PCR

TaqMan 5' nuclease real-time PCR assays were carried out using an ABI Prism 7900 sequence-detection system (PE Applied Biosystem, Foster City, CA). Various concentrations (0.1–1 µM) of forward and reverse primers for target genes (hTIMP-1, hTGF- 1, hTGF- 2) were used to optimize the PCR conditions. The TaqMan probes for target genes and the control gene, hGAPDH, were labeled with two different fluorescent receptors (FAM and JOE). GAPDH was used as an internal control to normalize respective mRNA expression values. Standard GAPDH and other custom-designed probes and primers were purchased from Applied Biosystems and were used at manufacturer's suggested concentrations. The total volume in the reaction was 25 µl, which consisted of Universal Mastermix (PE Applied Biosystems), 200 nM target primers, 500 nM probes, and 100 ng of diluted template RNA. The efficiency of amplification was determined each time by separately amplifying five-fold serial dilutions of template RNA (200–0.32 ng). The probes were designed from NCBI nucleotide database using Primer Express software (Applied Biosystems). The primers and probes used to detect human TIMP-1, TGF- 1 and TGF- 2 are summarized in Table I. The reactions were carried out at 48°C for 30 min, 95°C for 10

min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min in a 96-well plate. Each sample was analyzed in triplicate.

Measurement of TIMP-1, TGF- β 1, TGF- β 2, MMP-2, and proMMP-1

TIMP-1 in the astrocyte supernatants was measured at different time points using a commercial ELISA kit (Amersham Biosciences, Piscataway, NJ). MMP-2, proMMP-1, TGF- β 1, and TGF- β 2 levels in astrocyte supernatants or brain extracts were measured using commercial ELISA kits (R&D Systems, Inc., Minneapolis, MN). The TGF- β molecules were stimulated to the immunoreactive form by acid activation and neutralization, and the samples were diluted as per manufacturer's recommendation. The results were normalized to cell number based on MTT activity (Manthorpe et al., 1986). For brain extracts, the ELISA results were normalized to their total protein content. The protein determination was done using a BCA method (Pierce, Rockford, IL). Statistical analyses were carried out using GraphPad Prism 4.0 software, with one-way analysis of variance (ANOVA) and Newman-Keuls post-test for multiple comparisons.

Collection of Cell Supernatants and RNA Samples

For acute and chronic immune activation studies, the cells were stimulated with IL-1 (20 ng/ml). For mRNA preparation, 8-hr post-activation represented the acute condition; whereas, for protein analyses, supernatants were collected at 24-hr post-activation. For RNA and protein analyses, 7, 14, and 21 days of IL-1 activation represented chronic exposure to IL-1.

Astrocytes at passage 3 or higher were exposed to TGF- β 1 or TGF- β 2 (20, 100, and 250 ng/ml) with and without 20 ng/ml IL-1 for 24 hr to study if TGF- β stimulation of astrocytes could mimic TIMP-1 downregulation in chronic astrocyte activation. The cells were immune-activated for 2 hr in the presence of IL-1 (20 ng/ml) in conjunction with TGF- β 1 or TGF- β 2 (20, 100, or 250 ng/ml) after which media was replaced by fresh media with or without TGF- β 1 or TGF- β 2. Parallel controls were run in the absence of IL-1. The supernatants were collected after 24 hr from each experimental set. The corresponding total RNA samples were collected for TIMP-1 quantitation by real-time PCR.

RESULTS

TIMP-1 Expression in Acute and Chronic IL-1 β Immune Stimulation

To study the mechanism of differential TIMP-1 regulation in activated astrocytes, we first measured TIMP-1 mRNA levels in astrocytes activated for various periods of time to mimic both acute immune activation and sustained exposure to pro-inflammatory cytokines. IL-1 was used to mimic the status of immune activation present in neuroinflammation. We used 20 ng/ml IL-1 for all experiments in accordance with our previous observations (Suryadevara et al., 2003). Total RNA was extracted from IL-1-activated astrocytes at 8 hr, 7, and 14 days post-activation to measure TIMP-1 mRNA expression for both acute and chronic immune activation. Normalized TIMP-1 levels were expressed as a ratio of IL-1-stimulated astrocytes to the unstimulated controls (Fig. 1). The results demonstrate an approximate seven-fold increase in TIMP-1 mRNA at 8 hr followed by about 90% downregulation of TIMP-1 at both 7 days ($P < 0.001$) and 14 days ($P < 0.001$). Three independent donors were examined and real-time PCR analysis was carried out in triplicate. This data confirms that in astrocytes stimulated with IL-1, TIMP-1 levels are significantly higher. However, a reversal of this acute protective response by astrocytes occurs with prolonged and sustained exposure to the same pro-inflammatory cytokine.

Astrocyte TGF- β 1 and TGF- β 2 Expression in Acute and Chronic IL-1 β -Activation

Our premise for the next series of experiments was that because IL-1 stimulation leads to a variety of effects in astrocytes, it is possible that the downregulation of TIMP-1 during chronic astrocyte activation may be a feedback effect of an anti-inflammatory cytokine produced in response to the IL-1 stimulation. To investigate whether an extracellular loop exists for chronic TIMP-1 downregulation, we examined TGF- β 1 and TGF- β 2 mRNA and protein levels in astrocytes with and without IL-1 activation (Fig. 2). For mRNA preparation, 8-hr post-activation represented the acute condition, whereas, for protein analyses, supernatants were collected at 24-hr post-activation. For RNA and protein analyses, IL-1 treatments of 7 days or greater represented chronic activation. No significant changes were observed in TGF- β 1 mRNA levels at 14 days (Fig. 2A). A significant increase in TGF- β 2 mRNA levels was observed at 14 days post-activation compared to controls (6 fold, $P < 0.001$; Fig. 2A). The mRNA expression patterns for TGF- β 1 and TGF- β 2 in activated astrocytes demonstrated this trend in three independent astrocyte donors.

To measure TGF- β 1 and TGF- β 2 protein levels, cell supernatants collected from acute and chronic IL-1-stimulated astrocytes were analyzed by ELISA (Fig. 2B,C). Elevated TGF- β 1 levels were observed at 7 days (although not significant) and 14 days post-activation ($P < 0.01$; Fig. 2B). Overall, we observed greater upregulation of TGF- β 2 levels (Fig. 2) compared to TGF- β 1. TGF- β 2 protein levels were significantly higher at 7 days ($P < 0.01$) and 14 days ($P < 0.001$) post-IL-1-activation (Fig. 2C). Acute activation of astrocytes did not lead to any significant changes in TGF- β 1 and TGF- β 2 protein levels. These results along with our previous TIMP-1 expression data (Fig. 1) demonstrate a unique pattern of TIMP-1 downregulation accompanied by elevated TGF- β 1 and TGF- β 2 levels after chronic IL-1 stimulation.

TGF- β 1 and TGF- β 2 Expression in the Brain

Our results indicated that TGF- β 1 and TGF- β 2 levels in immune-activated astrocytes are different during acute and chronic immune activation. To determine the biologic relevance of these findings in HAD, we measured TGF- β 1 and TGF- β 2 expression in brain tissue and compared these levels to our previous data on TIMP-1 levels in HIV+ and HAD brains (Suryadevara et al., 2003). Protein lysates were prepared from frontal cortex white matter of control donors, HIV+ and HAD patients. Total proteins levels were assayed using the standard BCA method. Real-time PCR was carried out to measure TGF- β 1 and TGF- β 2 mRNA expression normalized to GAPDH levels.

Increased TGF- β 1 mRNA levels were observed in HIV+ and HAD patients when compared to nearly negligible amounts in control brain tissue, although the increase was statistically significant only for HAD patients ($P < 0.05$, data not shown). Low levels of TGF- β 2 mRNA (Fig. 3A) were also detected in control brain tissue ($n = 8$). In contrast, TGF- β 2 mRNA was increased in HAD brains by 10-fold ($n = 7$, $P < 0.01$) compared to control brains. The expression of TGF- β 2 mRNA was also higher in HIV+ brain specimens ($n = 4$, 2.2-fold), yet did not reach statistical significance in the HIV+ group. It is noteworthy that TGF- β 2 levels in HAD brain tissue were also significantly higher compared to the control group ($P < 0.01$), suggesting a stronger correlation to chronic CNS inflammation.

TGF- β 1 and TGF- β 2 protein levels were also compared between normal and diseased brains. The TGF- β 1 protein profile showed increases for HIV+ and HAD brain tissue although this trend did not reach statistical significance (data not shown). TGF- β 2 protein levels (Fig. 3B) were highest in HAD samples ($n = 9$) with a value three-fold more than control ($n = 8$, $P < 0.001$), similar to the significant differences seen in TGF- β 2 mRNA levels. TGF- β 2 levels in HIV+ ($n = 8$) brain tissue specimens were 1.8-fold more than control ($n = 8$, $P < 0.05$).

Previous work from our group showed diminished TIMP-1 mRNA and protein levels in HAD and HIV+ brains in comparison to control samples (Suryadevara et al., 2003). Consistent with our findings in primary human brain astrocytes findings in chronically activated primary human brain astrocytes, our data from biologic specimens showed upregulated TGF- β 1 and TGF- β 2 in HAD brain tissues. To determine the role of TGF- β 1 and TGF- β 2 in astrocyte-TIMP-1 downregulation during chronic CNS inflammation, we investigated the combined effects of IL-1 β with TGF- β 1 and TGF- β 2 on TIMP-1 production by activated astrocytes.

TGF- β 1 and TGF- β 2 Mimic Chronic Astrocyte TIMP-1 Downregulation

Astrocytes were acutely activated with TGF- β 1 and TGF- β 2 either in the presence or absence of IL-1 β . We expected that if our hypothesis was correct, the addition of exogenous TGF- β would counter the effects of IL-1 β stimulation and significantly reverse the IL-1 β -mediated elevation in TIMP-1 mRNA and protein levels. Astrocytes were treated with IL-1 β in combination with three independent doses of TGF- β 1 and TGF- β 2 (20, 100, and 250 ng/ml) to evaluate the dose-response in TIMP-1 downregulation. No significant differences in TIMP-1 were observed between 100 and 250 ng/ml of TGF- β 1 and TGF- β 2 treatments as the effects of both plateaued at 100 ng/ml (data not shown). Thus, all further experiments were carried out at 100 ng/ml concentration. As expected, and consistent with our previous data, IL-1 β stimulation alone significantly upregulated TIMP-1 transcripts (4.7-fold increase, $P < 0.001$; Fig. 4A, representative data). Addition of TGF- β 1 or TGF- β 2 at the same point of IL-1 β activation, however, resulted in significant reduction of this TIMP-1 upregulation. Specifically, TIMP-1 mRNA levels were significantly reduced by 10% and 40% in the presence of TGF- β 1 ($P < 0.001$) and TGF- β 2 ($P < 0.001$), respectively (Fig. 4A). TIMP-1 protein analysis further supported these observations. The measurement of TIMP-1 after MTT normalization showed a 3.4-fold increase with IL-1 β stimulation (Fig. 4B, $P < 0.001$) compared to untreated controls. This increase in TIMP-1 was significantly reduced (57% reduction, $P < 0.001$) in the presence of TGF- β 1. Addition of TGF- β 2 with IL-1 β showed a similar reduction in astrocyte-TIMP-1 (29% reduction, $P < 0.001$). This reduction was observed in four independent donors, although the magnitude these effects were sensitive to passage number and cell density. Addition of TGF- β 1 and TGF- β 2 alone did not have a significant effect on TIMP-1 expression (Fig. 4A,B). Overall, this data suggests that elevated TGF- β 1 and TGF- β 2 in the brain may contribute to TIMP-1 downregulation in disease.

TGF- β 1 and TGF- β 2 Differentially Regulate MMP-2 and proMMP-1

The function of TIMP-1 is tightly linked to MMP biology in the expression in the tissue microenvironment. Thus, we measured MMP-2 and proMMP-1 protein levels in the astrocyte supernatants by ELISA (Fig. 4C,D). Alone, IL-1 β , TGF- β 1 and TGF- β 2 did not have a significant effect on MMP-2 expression (Fig. 4C). Combined with IL-1 β , however, TGF- β 1 significantly increased MMP-2 expression compared to IL-1 β alone (1.6-fold, $P < 0.05$). Similarly, TGF- β 2 in combination with IL-1 β significantly increased MMP-2 production compared to IL-1 β alone (1.8-fold, $P < 0.01$) and compared to TGF- β 2 alone (1.4-fold, $P < 0.05$).

When proMMP-1 levels were measured on the same astrocyte supernatants, TGF- β had the opposite effect on its expression (Fig. 4D). As we reported previously (Suryadevara et al., 2003), IL-1 β significantly upregulated proMMP-1 expression compared to the control ($P < 0.001$). Compared to IL-1 β treatments, however, addition of TGF- β 1 with IL-1 β significantly decreased proMMP-1 expression by 1.2-fold ($P < 0.05$). Likewise, TGF- β 2 combined with IL-1 β decreased proMMP-1 levels by 1.3-fold as compared to IL-1 β alone ($P < 0.05$). Thus, although TGF- β alone does not seem to affect the expression of matrix regulators, in the

presence of proinflammatory cytokine IL-1, TGF- β differentially regulates TIMP-1, MMP-2, and proMMP-1 production. Because inflammation in the brain is typically associated with increased IL-1 expression, these findings are biologically relevant, and particularly highlight the role of TGF- β in regulating matrix proteins during disease states.

DISCUSSION

An imbalance in the MMP/TIMP axis is implicated in a variety of neuro-inflammatory diseases (Gardner and Ghorpade, 2003). We reported previously that neuro-inflammatory diseases, such as HAD, have elevated levels of IL-1 in the CNS, leading to reduced TIMP-1, yet enhanced MMP levels. Such dysregulation leads to exacerbated ECM breakdown in the CNS. In the accompanying manuscript (Gardner et al., 2006), we evaluated several potential mechanisms that could contribute to lower TIMP-1 levels in astrocytes during chronic activation. Because our data suggests that the major pathway of TIMP-1 downregulation may involve transcription control and loss of mRNA stabilization, the present study focuses on the existence of an extracellular link that may facilitate this process. Our current results indicate the existence of an extrinsic loop through TGF- β activation that contributes directly to TIMP-1 down-regulation. Both transcript and protein levels of TGF- β 1 and TGF- β 2 were upregulated in brain tissue from HAD patients. In addition, we could mimic this extracellular feedback regulatory loop in vitro through acute TGF- β activation of astrocytes. Stimulating astrocytes with a combination of IL-1 and TGF- β resulted in a significant reduction in IL-1-induced TIMP-1 activation.

It is well established that during inflammation, anti-inflammatory cytokines including IL-4, IL-10, and TGF- β are expressed during a natural repair response (Koeberle et al., 2004; Wei and Jonakait, 1999). Diseases such as Alzheimer's disease (AD) (Eikelenboom and van Gool, 2004), HAD (Benveniste, 1994), abdominal aortic aneurysms (Liapis and Paraskevas, 2003), chronic obstructive pulmonary disease (Barnes et al., 2003), cardiovascular disease (Ito and Ikeda, 2003), atherosclerosis (Fan and Watanabe, 2003), and end-stage renal disease (Caglar et al., 2002) are a few examples where this phenomenon occurs. Among the anti-inflammatory cytokines, the TGF family was our first choice for a regulatory loop in activated astrocytes because TGF- β upregulation is implicated in human neurologic diseases ranging from AD (Zetterberg et al., 2004) to chronic schizophrenia (Vawter et al., 1997). In the brain, TGF- β is expressed by both astrocytes and microglia and may be regulated through an autocrine loop on injury (Stoll et al., 2004; Yun et al., 2002). In addition, TGF- β is also involved in the immunopathogenic processes responsible for CNS dysfunction in HIV+ patients (Wahl et al., 1991) and plays a major role in HIV-1 gene transcription in astrocytes (Coyle-Rink et al., 2002). Consistent with our hypothesis, we found upregulated TGF- β and downregulated TIMP-1 levels after chronic astrocyte activation in both RNA and protein analyses. Our data, obtained using biologic specimens and primary human cells, demonstrated consistent patterns for TGF- β 1 and TGF- β 2 expression and the opposite patterns for TIMP-1 expression.

Because TGF- β is expressed in several tissues, it makes sense that it can exert diverse effects on different cell types. In contrast to its known growth factor properties, reduced endogenous TGF- β increases proliferation of developing adrenal chromaffin cells. In addition, TGF- β contributes to development of hydrocephalus after subarachnoid hemorrhage (Kitazawa and Tada, 1994). In meningitis, TGF- β decreases elevated intracranial pressure (Pfister et al., 1992). Upregulation of TGF- β was the main factor leading to the microvascular changes seen in fibrosis resulting from leg ulcers (Quatresooz et al., 2003). These examples illustrate the complex effects of TGF- β regulation. In our studies, we examined the effect of TGF- β treatments on IL-1-induced acute TIMP-1 upregulation to further decipher the link in opposing expression trends of TIMP-1 and TGF-

. Our data showed that the addition of TGF- β to our acute immune activation experimental paradigm mimicked the chronic downregulation in TIMP-1 observed during chronic activation. Consequently, we propose a model for an extracellular link in TIMP-1 regulation. In this model, enhanced TIMP-1 levels are observed after acute IL-1 stimulation, which protects against extracellular degradation in the tissue microenvironment. During chronic inflammatory conditions, a profound increase in ECM degradation occurs through an upregulation of TGF- β by an autocrine mechanism.

Our data also demonstrate differential regulation of MMP-2 and proMMP-1 when TGF- β was added in combination with IL-1. On closer examination of the effects of TGF- β on MMP expression, it seems that this regulation is quite complex and depends on multiple factors including dose, time, isoform, passage number and cell-type variables. For example, TGF- β 1 was found to increase MMP-2 and MMP-9 levels in human monocytic THP-1 cells (Lee et al., 2005), murine embryonic palate mesenchymal cells (Greene et al., 2003), human prostate cancer cells (Sehgal and Thompson, 1999), and primary bovine retinal endothelial cells (Behzadian et al., 2001). By contrast, MMP-9 induction by TNF- α was suppressed by TGF- β in primary mouse microglia (Paglinawan et al., 2003) and MonoMac-6 monocytic cells (Vaday et al., 2001), and TGF- β reduced MMP-9 and MMP-3 in pancreatic stellate cells (Shek et al., 2002). Decreased MMP-1 mRNA expression in response to TGF- β was observed in periodontal derived fibroblasts (Alvares et al., 1995) and chondrocytes from osteoarthritic lesions (Shlopov et al., 2000), whereas TGF- β 1 synergistically increased MMP-1 protein when combined with IL-1 in human fibroblast-like synoviocytes from rheumatoid arthritis patients (Cheon et al., 2002). We found that TGF- β decreased IL-1 activation of proMMP-1 levels, yet increased MMP-2 levels. These differences in MMP expression may be attributable to the different forms of MMPs measured by the ELISA. The proMMP-1 ELISA only measures the inactivated form of MMP-1, whereas the MMP-2 ELISA measures both pro, active, and TIMP-bound MMP-2 levels. Additionally, because overall the levels of proMMP-1 measured (<1 ng) were so much lower than that of MMP-2 protein (10–18 ng), the observation of decreased proMMP-1 although statistically significant, may not be biologically relevant in contributing to the pool of proMMPs ready to be activated in the immediate tissue microenvironment.

There is evidence for a multidimensional role of TGF- β in ECM repair regulation. TGF- β upregulates the expression of a chondroitin sulfate-type proteoglycan in primary neural and glial-derived cultures (Schnadelbach et al., 1998). In HIV disease, alteration in lymph node architecture is regulated by changes in fibronectin expression (Pal and Schnapp, 2004), which is partly mediated through increased TGF- β 1 secretion. TGF- β can promote glioma cell migration through enhanced MMP-2 and reduced TIMP-2 levels (Wick et al., 2001). MMPs, the enzymes that primarily degrade ECM components, and TIMP-1, both harbor an AP-1 site in their promoters that is regulated by TGF- β . This AP-1 site leads to TIMP-1 regulation (Clark et al., 1997) and MMP-1 suppression (Hall et al., 2003). The reciprocal role of TGF- β on TIMP-1 and MMP-1 expression supports its role in matrix homeostasis. The molecular mechanism by which TGF- β leads to such diverse effects remains unknown. A complicated picture emerges regarding the role of TGF- β in ECM maintenance, degradation, and remodeling in the CNS.

Our data unravel yet another complexity in glial inflammatory functions. During CNS inflammation, the initial glial activation could be mediated by IL-1, which has an autocrine loop and generates more IL-1. Together, this leads to chronic astrocyte activation and decreased TIMP-1 production over time, which may contribute to ECM degradation through an MMP/TIMP imbalance. Our data demonstrate that the differences in expression profiles of TIMP-1 and MMPs in acute and chronic immune astrocyte activation may be through TGF- β . Because astrocytes express the receptors for TGF- β , they are susceptible to the

actions of this cytokine. In inflammatory conditions, most likely a cocktail of cytokines with both pro- and anti-inflammatory properties is present. Thus, the temporal order of activation events may play a critical role in the net effects of these processes. It has been shown that inflammatory mediators like TGF- β affect the innate immune response of microglia by antagonizing IL-1 when administered simultaneously with IL-1 (Basu et al., 2002). We propose that elevated TGF- β elicits an autocrine, feedback loop, leading to downregulation of TIMP-1, an important ECM regulator. A crosstalk between intracellular signaling molecules such as the Smad pathway (Bottner et al., 2000) can modify the TGF- β response, which can directly affect ECM regulation in chronic inflammation. We envision this scenario in the diseased CNS, such as in HAD and other diseases. These data have implications for a variety of neuro-inflammatory diseases pertaining to glial inflammation and CNS ECM remodeling.

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Fig. 1.

Differential TIMP-1 mRNA levels in immune activated astrocytes. Primary human fetal astrocytes were activated with IL-1 (20 ng/ml), and total RNA was isolated at 8 hr, 7 days, and 14 days post-activation. TIMP-1 mRNA levels were measured by real-time PCR using TaqMan probes and the values were normalized to GAPDH. TIMP-1 expression is shown as a fold change between IL-1-activated and control cells. Representative data obtained using three independent astrocyte donors is shown. Significant downregulation of TIMP-1 was demonstrated ($P < 0.001$) in samples after 7 and 14 days (7 d, 14 d) of IL-1 stimulation. Error bars = SEM. Statistical analysis was carried out with GraphPad Prism 4.0 using one-way ANOVA.

Fig. 2.

Astrocyte TGF- 1 and TGF- 2 expression. Astrocytes were activated with 20 ng/ml IL-1 for 8 hr, 7, and 14 days. Total RNA was isolated and TGF- 1 and TGF- 2 mRNA expression was measured by real-time PCR. The results were normalized to GAPDH values. Each panel shows the representative result of the experiments from three independent donors. **A:** Shows that no significant differences in TGF- 1 mRNA expression were observed. TGF- 2 mRNA expression at 14 days was significantly higher as compared to controls and 8 hr post-IL-1 activation ($P < 0.001$). TGF- 1 and TGF- 2 protein levels were measured by ELISA in IL-1 -activated astrocyte supernatants and were normalized to MTT activity. **B:** Depicts a significant increase in TGF- 1 levels at 14 days ($P < 0.01$) post-activation. **C:** Depicts significant increases in TGF- 2 at 7 ($P < 0.01$) and 14 ($P < 0.001$) days post-activation. Error bars = SEM. Statistical analysis was carried out with GraphPad Prism 4.0 using one-way ANOVA.

Fig. 3.

TGF- β 2 levels in human brain tissue. Protein extracts and total RNA were isolated from human autopsy brain tissues obtained from control donors, and HIV+ and HAD patients. TGF- β 2 mRNA levels were measured in triplicate by real-time PCR and normalized to GAPDH. The corresponding protein levels were determined by ELISA and normalized for protein content using the BCA method. **A:** Shows that TGF- β 2 mRNA levels are significantly higher in 4) HAD ($n = 7$, $P < 0.01$) as compared to HIV+ samples ($n = 4$) and controls ($n = 8$). **B:** Depicts the results from TGF- β 2 ELISA. TGF- β 2 was significantly higher in HIV+ patients ($n = 8$, $P < 0.05$) and HAD patients ($n = 9$, $P < 0.001$) as compared to controls. TGF- β 2 levels in HAD were also significantly different as compared to HIV+ specimens ($P < 0.01$). Error bars = SEM. Statistical analysis was carried out with GraphPad Prism 4.0 using one-way ANOVA.

Fig. 4.

TGF- differentially regulates IL-1 -mediated TIMP-1, MMP-2 and proMMP-1 expression. Total RNA was collected from astrocytes treated with IL-1 , TGF- 1, TGF- 2 and combinations thereof. TIMP-1 mRNA levels were measured by real-time PCR. Data is representative of three experiments with three independent donors analyzed in triplicate. **A:** Demonstrates that 8 hr stimulation with IL-1 induced TIMP-1 mRNA expression, although this induction was significantly reduced in the presence of TGF- 1 ($P < 0.001$) and TGF- 2 ($P < 0.001$). Astrocyte culture supernatants were collected at 24 hr for TIMP-1, MMP-2, and proMMP-1 measurement. **B:** Shows a significant downregulation of IL-1 -induced TIMP-1 production in the presence of TGF- 1 ($P < 0.001$) and TGF- 2 ($P < 0.001$). **C:** Demonstrates a significant increase in MMP-2 levels when IL-1 was combined with TGF- 1 ($P < 0.05$) and TGF- 2 ($P < 0.01$) compared to IL-1 alone. **D:** Depicts the results for measuring proMMP-1 protein levels. IL-1 alone significantly increased proMMP-1 levels ($P < 0.001$), however this increase was significantly reduced in the presence of TGF- 1 and TGF- 2 ($P < 0.05$). The results for protein measurements were normalized to MTT activity. Error bars = SEM. Statistical analysis was carried out with GraphPad Prism 4.0 and one-way ANOVA.

TABLE I**Primer and Probe Sequences**

TIMP-1

Forward: 5' CTG ACA TCC GGT TCG TCT ACA C 3'

Reverse: 5' GGT TGT GGG ACC TGT GGA AGT 3'

Probe: FAM-CGC CAT GGA GAG TGT CTG CGG A-TAMRA

TGF- 1

Forward: 5' GCC CAC TGC TCC TGT GAC A 3'

Reverse: 5' CGG TAG TGA ACC CGT TGA TGT 3'

Probe: FAM-CAGGGATAACACACTGC-TAMRA

TGF- 2

Forward: 5' GCT GAG CGC TTT TCT GAT CCT 3'

Reverse: 5' CGA GTG TGC TGC GGT AGA CA 3'

Probe: FAM-CTG GTC ACG GTC GCG CTC AGC-TAMRA