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Cytotoxicity by Matrix Metalloprotease-1 in Organotypic Spinal Cord and Dissociated Neuronal Cultures

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Extracellular matrix (ECM) proteins, including collagens and laminins, are critical to the structure of the neuronal synapse and may also be involved in cell survival. In the present study, we therefore examined the possibility that select ECM degrading proteins might be toxic to organotypic spinal cord and dissociated neuronal cultures. Of those proteins tested, including MMP-1, -7, and -9, we observed that MMP-1 was toxic to spinal cord cultures as determined by release of lactic acid dehydrogenase as well as uptake of propidium iodide. Pretreatment of cell cultures with 50 μM α-tocopherol partially reversed these effects. We also observed that MMP-1 was toxic to human neurons grown in dissociated cultures and that increased amounts of MMP-1 were released by astrocytes following their stimulation with IL-1β. These results suggest that further studies may be warranted to determine whether MMP-1 contributes to neurodegenerative conditions in which activated astrocytes may play a role.

INTRODUCTION

MMPs belong to a family of structurally similar, zinc containing endopeptidases and typically act to degrade specific components of the extracellular matrix (ECM), including collagen, fibronectin, elastin, and laminin (29). Furthermore, through their convertase actions, they may also function to activate membrane bound cytokines and receptors (7, 26, 29). While there is some overlap is substrate specificity, MMPs differ not only with respect to cellular source and inducibility, but with respect to the efficiency with which they can act on specific substrates (29). Typically, the MMPs are grouped according to those substrates upon which they exert their main activity. These groups include the interstitial collagenases (MMP-1 and -8), which cleave collagen types I–III, the stromelysins (MMP-3, -7, -10, and -11) with some specificity for laminin, the membrane type (MT)-MMPs, which cleave pro-MMP-2 as well as some collagens, and the gelatinases (MMP-2 and -9), which most efficiently cleave type IV collagen. MMPs also differ with respect to their posttranslational regulation. For example, the activity of MMPs is affected both by proenzyme activation and by interactions with specific tissue inhibitors of metalloproteinases (TIMPs) (29).

Matrix metalloproteinases (MMPs) have a number of physiologically important roles in processes including wound healing and angiogenesis. When their production is dysregulated, however, they may also contribute to disease. For example, excessive activity of MMPs has been implicated in tumor metastasis (25, 29), atherosclerosis (24), rheumatoid arthritis (27), and emphysema (13). Interestingly, in emphysema, it has been shown that a macrophage-derived proteinase is primarily responsible for tissue destruction (13).

Most MMPs are not constitutively active but are inducible by stimuli that increase the production of reactive oxygen intermediates (ROIs) and/or the DNA binding activity of select transcription factors including NF-κB and AP-1 (2, 29). Consistent with this, in a number of cell types it has been shown that both phorbol esters and proinflammatory cytokines can influence the expression of MMPs produced by monocytes and/or astrocytes (1, 10–12, 25). It has also been shown that prostaglandins, which can affect the activity of cAMP and may therefore affect the expression of AP-1 family members through the activation of the cAMP response element binding protein, can increase both MMP-1 (31) and -9 (17, 27, 30) production. In addition, it has been shown that stimulation of brain-derived cells with β-amyloid protein, which can increase NF-κB binding in astrocytes, will increase the release of select MMPs (6).

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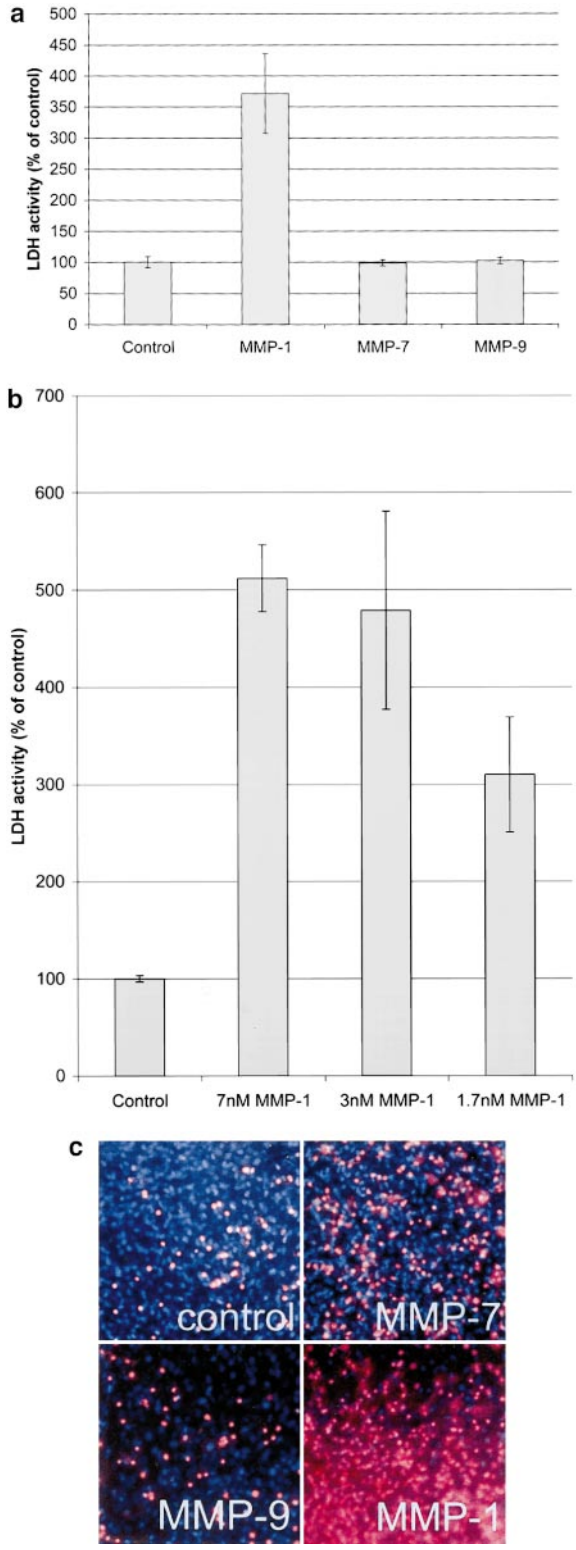

FIG. 1. (a) Relative activity of LDH in the supernatants of spinal cord organotypic cultures treated with MMP buffer in a volume (25 μl/ml) similar to that used for MMP treated cultures (control), 7 nM MMP-1 (MMP-1), 100 nM MMP-7 (MMP-7), or 10 nM MMP-9 (MMP-9) for 48 h as indicated. Data represent the mean ± SEM of two experiments which were done in triplicate. (b) Relative activity of LDH in the supernatants of spinal cord organotypic cultures treated with 7, 3 or 1.7 nM MMP-1 as indicated. Data represent the mean ± SEM of an experiment that was done in triplicate. (c) Propidium iodide (red) and Hoescht 33342 (blue) uptake in spinal cord organotypic cultures treated with MMP buffer (control), 7 nM MMP-1, 100 nM MMP-7, or 10 nM MMP-9 for 48 h as indicated. Figures shown are representative sections of slides which were scanned and collated using Adobe Photoshop. For quantitative data, please see the Results section.

Inflammatory diseases of the central nervous system (CNS), including HIV dementia, are associated with increased levels of stimuli which would be expected to increase MMP production. Consistent with this possibility, elevated levels and activities of several MMPs have been detected in association with such conditions (4, 5, 14, 15, 21).

A number of studies suggest that those substrates that are targeted by inflammatory disease-associated MMPs may play an important role in CNS function. For example, laminin has been shown to contribute to synaptic structure (16, 20). In addition, dendritic arborization, which is reduced in HIV dementia (18), may be dependent on the presence of select ECM proteins. Also, fibrillar collagens and chondroitin sulfate proteoglycans, which are both targets of MMP-1, may be important to neuronal survival (3, 19). In the present study, we have therefore examined the possibility that select MMPs may be toxic to cultured neural cells. We focused on those MMPs that are known to be produced by activated astrocytes and/or monocyte-derived cells, since activated astrocytes and monocyte-derived cells are increased in association with CNS diseases, including HIV and Alzheimer's dementia. We used not only dissociated, but organotypic cultures, because the latter would be expected to retain much of the ECM architecture that exists *in vivo*.

MATERIALS AND METHODS

Cell Culture

Spinal cord organotypic cultures. Organotypic spinal cord cultures were prepared from lumbar spinal cords of 8-day-old rat pups as described previously (23). Lumbar spinal cords were collected under sterile conditions and sectioned transversely into 350-μm slices with a McIvain tissue chopper. Slices were cultured in Millicell CM semipermeable culture inserts at a density of five slices/well in an incubator at 37°C (5% CO_2, 95% humidity). Under these conditions, >95% of cultures retained cellular organization, and a stable population of neurons persisted in excess of 3 months. Culture medium (Neurobasal medium with 1 × B27 and N2 supplements (GIBCO)) was changed twice weekly. Of note, culture inserts were not coated with extracellular matrix protein.

Dissociated neuronal cultures. Dissociated neuronal cultures were prepared from human fetal brain specimens of 12 to 15 weeks' gestational age obtained with approval of the Human Ethics Committee at the University of Kentucky. The meninges and blood vessels were removed and the specimens were washed in OptiMEM (GIBCO). The tissue was mechanically dissociated by repeated trituration with a 20-gauge needle and syringe and pelleted at 270*g* for 10 min. The cells were resuspended in OptiMEM with 1% heat-inactivated fetal bovine serum, 1% N2 supplement (GIBCO), and 0.1% antibiotic solution (10^3 units of penicillin G/ml, 10 μg of streptomycin/ml, and 25 μg of amphotericin B/ml in 0.9% NaCl). Cells were plated in 96-well microtiter plates, and maintained in culture for at least 4 weeks before use in neurotoxicity assays. Cultures used in experiments were positive by immunostaining for the neuronal marker MAP-2 ($\geq$70% of cells), and the astrocyte marker glial fibrillary acidic protein ($\leq$30%).

Astrocyte cultures. Cultured human astrocytes were prepared from temporal lobe sections of adults who underwent surgery for the treatment of intractable epilepsy. Myelin was dissected from sections, which were then washed in phosphate-buffered saline and triturated through a 19-gauge needle. Tissue was then placed in 0.05% trypsin at 37°C and stirred for 45 min. Following trituration and trypsinization, cells were resuspended in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum. Cultured cells were characterized by immunocytochemistry using specific antibodies to glial fibrillary acidic protein (GFAP) (Dako Z0334, Carpinteria, CA) and CD68 (Dako MO876). Only those cultures which were >98% GFAP-positive were used for experiments. Astrocytes used for experiments were grown at 10^5 cells per milliliter media and were plated so that they were near confluence (>90%).

Cytokines and matrix metalloproteinases. Recombinant human IL-1β was obtained from R & D Systems (Minneapolis, MN). Recombinant active MMP-1 was obtained from Chemicon (Temecula, CA) and Amersham (Arlington Heights, IL). Recombinant active MMP-7 and -9 were obtained from Calbiochem (La Jolla, CA). All proteins were stored in aliquots at −70°C. Of note, MMPs were dissolved in buffer as follows. MMP-1 buffer: 50 mM Tris, 5 mM $CaCl_2$, and 200 mM NaCl, MMP-7 and -9 buffer: 10 mM Hepes, 5 mM $CaCl_2$, 150 mM NaCl. MMP-1 buffer was used as a control in cytotoxicity experiments.

Cytotoxicity Assays

Assessment of lactic acid dehydrogenase release. Lactic acid dehydrogenase (LDH) levels were determined using a standard assay (Tox-7, Sigma Chemical, St. Louis, MO), which was performed according to the manufacturer's instructions.

Propidium iodide uptake. Cultures were washed in phosphate-buffered saline (PBS) and then treated with a solution of Hoechst (bisBenzimide/Hoechst 33342) and propidium iodide (10 μg/ml Hoechst, 9 μg/ml propidium iodide, and 25 mM glucose in PBS) for 10 min at 37°C. Cultures were then viewed and photographed at 40× using fluorescence microscopy with a broad spectrum filter so as to visualize both dyes. Hoechst and propidium iodide were from Sigma Chemical.

Trypan blue uptake. Cultures were assayed for neuronal cell counts, which were determined from six fields at predetermined coordinate locations. Each field was photographed, coded and counted without knowledge of its experimental identity. At least 200 cells were counted in each field. Two independent experiments were performed. The means ± standard error of means were calculated from these data and analyzed by Student's *t* test.

Immunoabsorption of MMP-1. Mouse anti-MMP-1 (R & D Systems) was bound to protein G–Sepharose (Amersham-Pharmacia) and incubated with MMP-1 for 45 min at room temperature followed by a 20-min spin in a desktop eppendorf centrifuge at 14,000 rpm. The supernatant was then tested for cytotoxicity using the LDH assay described above.

ELISA. ELISA was performed using a commercially available kit (Amersham), according to the manufacturer's instructions. This ELISA recognizes both pro- and active MMP-1 as well as MMP-1 which is associated with TIMPs.

RESULTS

MMP-1 Is Toxic to Cells Grown in Organotypic Spinal Cord Cultures

To determine whether select MMPs may be cytotoxic, we treated spinal cord organotypic cultures with 7 nM recombinant active MMP-1, 100 nM recombinant active MMP-7, or 10 nM recombinant active MMP-9 for 48 h. Cell culture supernatants were then sampled and tested for LDH activity. The results, expressed as the mean ± standard error of two experiments which were performed in triplicate, are shown in Fig. 1a. From this figure it can be appreciated that as compared to MMP-7 and -9, MMP-1 treatment of organotypic cultures was associated with a significant increase in LDH release. With a two-tailed *t* test, the *P* values for the difference in LDH release between control and MMP-1-treated cultures was 0.0004. The difference in LDH release between control and MMP-7- or -9-treated cultures was not statistically significant ($P = 0.78$ and 0.7, respectively). Figure 1b shows LDH results from a similar study except that varied concentrations of MMP-1 were assessed. With a two-tailed *t* test the *P*

values for the difference in LDH release between control and 1.7, 3, and 7 nM MMP-1 were 0.09, 0.09, and 0.025, respectively.

Figure 1c shows increased propidium iodide uptake by cells that were treated with MMP-1. Photographic slides were prepared from two separate experiments that were performed in triplicate and included control, MMP-1-, -7-, and -9-treated cultures. From the subsequently projected images, the number of PI-positive cells, which were in the focal plane of a same-sized and equally magnified region, were counted. Control cultures showed an average of 55 positive cells per region with an SEM of 29. MMP-1-treated cultures showed an average of 173 PI positive cells per region with an SEM of 1.9. With a two-tailed *t* test, these differences were statistically significant ($P < 0.05$). MMP-7- and -9-treated cultures did not differ significantly from control when analyzed by the same techniques.

For the results shown in Fig. 1, MMP-1 was obtained from Chemicon. In subsequent experiments, however, we observed that MMP-1 from an alternative source (Amersham) had a similar effect and that immunoabsorption of MMP-1 blocked the effect (not shown).

While we were not able to test the activity of MMP-1 or -7, the activity of MMP-9 was confirmed through the use of gelatin substrate zymography (not shown). It should also be noted that while MMPs differ between species, the target specificity of select MMPs does not.

α-Tocopherol Inhibits MMP-1-Associated Cytotoxicity

To determine whether oxidant stress, which has been implicated in numerous paradigms of cell death, might play a role in MMP-1 associated toxicity, select cultures were pretreated with 50 μM α-tocopherol. Lactic acid dehydrogenase release from such cultures is shown in Fig. 2. In this figure, MMP-1 was associated with a lesser fold increase than that observed in the experiment shown in Fig. 1, possibly because the cultures used for this experiment had been prepared from a different animal and at a different time. Data represent the mean ± standard error of one experiment that was performed in triplicate. With a two-tailed *t* test, the differences between control and MMP-1 were significant ($P = 0.013$) as were the differences between MMP-1 and MMP-1 + vitamin E ($P = 0.014$). The difference between control and MMP-1 + vitamin E was not statistically significant.

MMP-1 Is Toxic to Neurons Grown in Dissociated Cultures

Because experimental results may be dependent on the nature of the system that has been studied, it was decided to test the effects on MMP-1 in a system that would extend our observations to human cells grown in dissociated cultures. While such cultures may not have the ECM architecture that would be found in the intact CNS, cultured neurons might produce select ECM proteins which may in turn affect their survival.

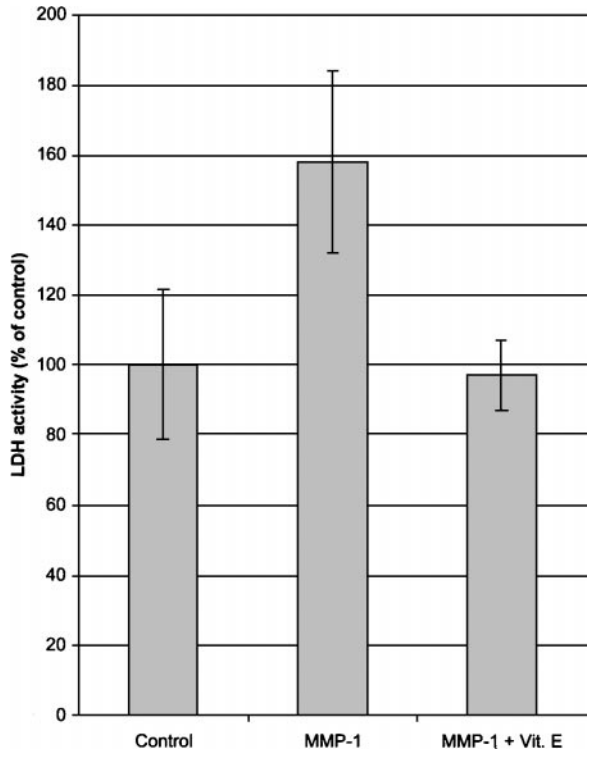

FIG. 2. Relative activity of LDH in the supernatants of spinal cord organotypic cultures treated for 48 h with MMP buffer (control), 7 nM MMP-1, or 7 nM MMP-1 20 min following the administration of 50 μM α-tocopherol (MMP-1 + Vit E). Data represent the mean ± SEM of an experiment which was performed in triplicate.

Due to the relatively small number of cells used in these experiments, toxicity was determined by the uptake of trypan blue rather than by LDH release and propidium iodide uptake. Results, expressed as the mean and SEM, are shown in Fig. 3a. From this figure it can be appreciated that the administration of 7 nM MMP-1 to dissociated cultures of human neurons was associated with a significant increase in cell death as determined by trypan blue uptake ($P = 0.003$ with a two-tailed *t* test). A representative photograph of the type of culture which was used in these experiments is shown in Fig. 3b.

Of note, a similar experiment was performed in triplicate on cultured human fetal astrocytes (>95% GFAP positive) but did not show a significant difference (control: 2.3%, MMP-1: 1.4%, $P = 0.37$ with a two-tailed *t* test) in cell death between control and MMP-1-treated cultures (C. Anderson and A. Nath, unpublished observations).

The Proinflammatory Cytokine IL-1β Increases MMP-1 Release from Cultured Astrocytes

Figure 4 demonstrates the results of ELISA analysis of human brain-derived astrocyte supernatants. Data

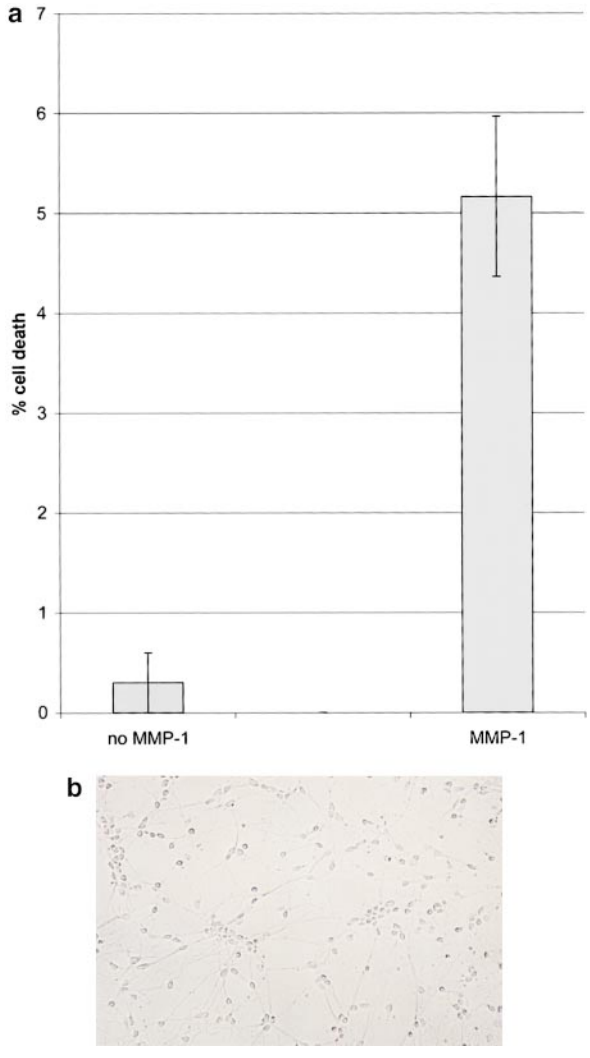

FIG. 3. (a) Percentage cell death in dissociated neuronal cultures treated for 48 h with control buffer (no MMP-1) or 7 nM MMP-1 (MMP-1). Data represent the mean ± SEM of two experiments that were performed in triplicate. (b) Representative photograph of the type of culture which was used for those experiments with results represented in a.

represent the mean ± standard error of an experiment that was performed in triplicate. From this figure it can be appreciated that cultured astrocytes produce MMP-1 and that such production is increased following their stimulation with IL-1β. With a two-tailed t test, this difference was significant at $P < 0.005$. Of note, relatively high basal expression may reflect activation of astrocytes when grown in tissue culture.

DISCUSSION

Matrix metalloproteinases have been implicated in numerous processes, including blood–brain barrier degradation (22) and activation of cytokines, including IL-1β (26, 29). Such activities could contribute to neuronal dysfunction in the setting of CNS inflammation. For example, increased blood–brain barrier permeability is thought to allow for an increase in the CNS ingress of inflammatory cells, and may also allow for the ingress of potentially neurotoxic serum proteins, including thrombin (28). In the present study, however, we examined the possibility that select MMPs may be more directly neurotoxic.

The results described in the present study suggest that MMP-1 may be neurotoxic. The mechanism(s) by which MMP-1 is neurotoxic is/are, however, unknown. It could be that MMP-1 targets one or more extracellular matrix proteins that are critical to neuronal survival. One possibility would be that MMP-1 exerts its effects through the destruction of chrondroitin sulfate proteoglycans. These proteins are targeted by MMP-1 and have been shown to protect cultured neurons from excitotoxic cell death (19). Yet another possibility would be that MMP-1 is toxic through its ability to degrade collagen type I. This ECM protein has been shown to promote the survival of neuroblastoma cells through integrin receptor mediated effects (3).

Other mechanisms by which MMPs may be neurotoxic include their potential to affect membrane bound receptors/cytokines as well as soluble cytokines. For example, it has been shown that MMP inhibitors prevent truncation of the nerve growth factor receptor (7),

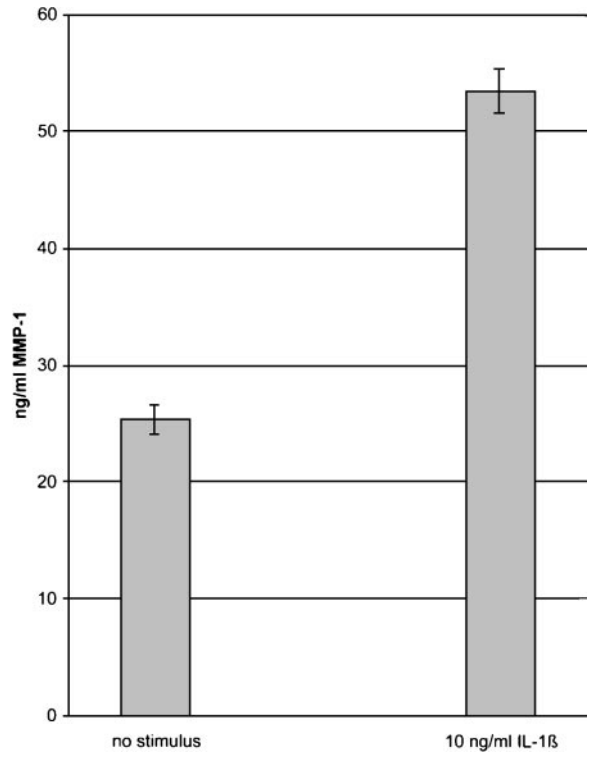

FIG. 4. MMP-1 release, as determined by ELISA, into the supernatants of cultured human astrocytes, stimulated for 24 h as indicated. 25 and 53 ng/ml correspond to 0.49 and 1.04 μM, respectively.

that MMPs may affect chloride flux (8), and that MMPs may be involved in the activation of the potential neurotoxin IL-1β (26).

While further studies will be necessary to elucidate those mechanisms responsible for MMP-1-associated neurotoxicity, the finding that this particular MMP can be toxic could have relevance to a number of neuropathological conditions. As shown herein, MMP-1 may be produced by astrocytes that are activated either by the conditions of cell culture or by stimulation with IL-1β. We have also observed that in the setting of both HIV dementia and Rasmussen's encephalitis, reactive astrocytes show MMP-1 immunoreactivity by immunohistochemistry (C. Pardo, unpublished observations). Of note, astrocytes are the most numerous cells in the CNS and activation of this cell type is observed in numerous conditions including Alzheimer's disease and HIV-1-associated dementia. Interestingly, MMP-1 may also be produced by monocyte-derived cells and its production by such cells inhibited by nonsteroidal anti-inflammatory drugs (31).

An interesting consideration has to do with the possibility that the activity of MMPs, including MMP-1, may also increase with normal aging. While this possibility has not been examined with respect to the CNS, it has been shown that MMP-1 production by human diploid fibroblasts increases with age (2) and that the quantity of this MMP in aorta (24), as detected by immunohistochemistry, also increases with age.

In summary, MMPs have complex effects that may be both helpful and harmful to an organism. The overall balance may depend on where and when such proteins are expressed. The results presented herein, however, suggest that determining whether these proteins, and MMP-1 in particular, may be neurotoxic in the setting of adult CNS inflammation warrants further investigation.

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