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Transcriptional Suppression of Matrix Metalloproteinase-2 Gene Expression in Human Astroglioma Cells by TNF-α and IFN-γ[1]

Hongwei Qin,* Jason D. Moellinger,† Alan Wells,† L. Jack Windsor,‡ Yi Sun,§ and Etty N. Benveniste[2]*

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that function in the turnover of extracellular matrix components during development. In addition, MMPs also contribute to pathological conditions associated with inflammation, angiogenesis, and tumor invasion. A 72-kDa type IV collagenase, also referred to as gelatinase A or MMP-2, has been proposed to potentiate the invasion and metastasis of malignant tumors. In particular, MMP-2 activity has been shown to constitute an important component of human astroglioma invasion. We investigated the influence of various cytokines, both proinflammatory and immunosuppressive, on MMP-2 gene expression in two human astroglioma cell lines (U251-MG and CRT). Our results indicate that the cell lines constitutively express high levels of MMP-2 mRNA, protein, and bioactivity as assessed by ribonuclease protection assay, immunoblotting, and zymography assays, respectively. The proinflammatory cytokines TNF-α and IFN-γ individually can inhibit constitutive MMP-2 expression, and function in an additive manner for near-complete inhibition of MMP-2 expression. Inhibition of MMP-2 mRNA levels by TNF-α and IFN-γ is not due to destabilization of the MMP-2 message; rather, inhibition is mediated at the transcriptional level. Furthermore, TNF-α/IFN-γ inhibition of MMP-2 expression results in decreased invasiveness of the human astroglioma cells through an extracellular matrix. These results raise the possibility that TNF-α and IFN-γ may have beneficial effects in attenuating astroglioma invasive properties. ***The Journal of Immunology,* 1998, 161: 6664–6673.**

The matrix metalloproteinases (MMPs)[3] are a family of structurally related zinc-dependent endopeptidases that are capable of degrading almost all extracellular matrix (ECM) components (for review see Refs. 1–4). The family of MMPs includes collagenases, gelatinases, stromelysins, membrane-type metalloproteinases (MT-MMP), matrilysin, and metalloelastase. MMPs are usually secreted as soluble, latent proenzymes and are then activated in vitro by proteolytic cleavage of an amino-terminal domain. Serine proteases have been implicated in the proteolytic activation of many secreted MMPs, although recently MT-MMPs have been identified as another mechanism of activating MMPs, specifically the 72-kDa type IV collagenase MMP-2 (5–7). A recent publication has demonstrated intracellular activation of MMP-2 in fibroblasts, which is mediated by an activator that localizes to the Golgi membrane fraction (8). In addition, the activity of MMPs can be regulated by forming complexes with a class of endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (9–11).

A hallmark of invasive tumors is their ability to degrade the surrounding ECM, resulting in a compromised matrix organization and disruption of tissue boundaries. Numerous studies have examined MMP expression and correlated levels of expression with the invasive ability or metastatic potential of the tumor. Both in vitro and in vivo studies have documented a direct correlation between high levels of expression of MMPs and an increased invasive capacity of a large number of tumor cell lines (2, 12–14). Glioblastoma multiforme is a highly malignant central nervous system (CNS) tumor that is extremely refractory to therapy, due in part to the aggressive tendency of the tumor cells to invade (for review see Refs. 15, 16). In fact, the invasiveness of human gliomas is considered to be an important cause of mortality in patients afflicted with this form of cancer. MMP expression has been documented both in vitro and in vivo in human gliomas. In vitro, human glioma cell lines express a variety of MMPs, in particular the type IV collagenases MMP-2 and MMP-9 (17–23). In vivo studies have documented the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 in human gliomas (19, 20, 24–27). MMP-2 and MMP-9 expression was highest in high-grade gliomas (glioblastoma, anaplastic astroglioma) compared with noninvasive low-grade astrogliomas and normal brain (19, 26–28). Furthermore, low levels of TIMP-1 and TIMP-2 appear to correlate with the aggressive behavior of human gliomas (25). Studies to assess the invasiveness of glioma cells in vitro have demonstrated a strong correlation between glioma invasion and high levels of MMP-2 expression (17, 20, 22, 23). In addition, MT-MMP-1 expression correlates with the expression and activation of MMP-2 during malignant progression of gliomas in vivo (20).

Departments of *Cell Biology, †Pathology, and ‡Biochemistry and Molecular Genetics, University of Alabama, Birmingham, AL 35294; and §Department of Molecular Biology, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, MI 48105

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[2] Address correspondence and reprint requests to Dr. Etty N. Benveniste, Department of Cell Biology, Room 350 MCLM, University of Alabama, Birmingham, AL 35294-0005. E-mail address: ebenven@cellbio.bhs.uab.edu

[3] Abbreviations used in this paper: MMPs, matrix metalloproteinases; CHX, cycloheximide; CNS, central nervous system; ECM, extracellular matrix; MT-MMPs, membrane type-matrix metalloproteinases; RPA, ribonuclease protection assay; TIMPs, tissue inhibitors of metalloproteinases; nt, nucleotide.

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The activity of MMP-2 is regulated by several mechanisms, including gene expression, proenzyme activation by TIMP-2 and MT-MMPs, and inhibition of enzyme activity (for review see Refs. 5, 29, 30). Previous work has demonstrated that MMP-2 expression can be transcriptionally regulated (31–34). In addition, MMP-2 expression in human tumor cell lines as well as untransformed cells is modulated in a cell-type and stimulus-specific manner by a variety of immunological agents such as TGF-β, IFN-γ, IL-1β, TNF-α, PMA, and PGE_2 (19, 23, 31, 35–43). The down-regulation of MMP-2 expression in astroglioma cells may inhibit the invasion of these tumor cells, an event that would be beneficial in treatment of malignant brain tumors. Toward this end, we have investigated the ability of a number of cytokines, both proinflammatory and immunosuppressive, to modulate MMP-2 expression in human glioma cell lines. Surprisingly, we found that two proinflammatory cytokines, TNF-α and IFN-γ, were potent inhibitors of MMP-2 gene expression in astroglioma cells, and that this inhibition of MMP-2 expression occurred at the transcriptional level. Also, the cytokine-mediated reduction in MMP-2 expression correlated with decreased invasiveness of these cells.

Materials and Methods

Cell lines

The CRT astroglioma cell line, derived from a neoplastic frontal lobe lesion, was grown in RPMI 1640 medium supplemented with 10 mm HEPES, pH 7.2, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS as previously described (44). The U251-MG, U373-MG, and CH235-MG astroglioma cell lines were grown in DMEM/Ham's F-12 medium supplemented with 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 10 μg/ml streptomycin, and 10% FBS as described previously (45, 46).

Reagents

Human rTNF-α was the generous gift of Genentech, Inc. (South San Francisco, CA), and human rIFN-γ was a gift of Biogen (Cambridge, MA). Human rIL-4, rIL-10, and rIL-13 were purchased from R&D Systems (Minneapolis, MN). LPS and gelatin were purchased from Sigma (St. Louis, MO). Recombinant human TIMP-2 was prepared by Dr. L. J. Windsor (University of Alabama at Birmingham, Birmingham, AL). Mouse anti-human MMP-2 mAb was the generous gift of Dr. J. Engler (University of Alabama at Birmingham), and polyclonal anti-MT-MMP-1 Ab was purchased from Chemicon International (Temecula, CA). The secondary peroxidase conjugated Abs and enhanced chemiluminescence reagents were from Amersham (Arlington Heights, IL).

Gelatin substrate gel zymography

Zymography was performed by a minor modification of the procedure described by Overall et al. (47). The human cell lines were resuspended in their respective media containing 10% FBS and plated at 1×10^6 cells/100-mm^2 dish. Dishes were incubated until ~80% confluent, then the media was aspirated and fresh serum-free medium was added to each dish, with and without cytokine treatment. Supernatants were collected after a 48-h incubation and concentrated by an equal volume of 100% ethyl alcohol. Concentrated supernatants (750 μl) were mixed with SDS sample buffer without reducing agent, and proteins were subjected to SDS-PAGE in 8% polyacrylamide gels that were copolymerized with 1 mg/ml of gelatin. After electrophoresis, the gels were washed several times in 2.5% Triton X-100 for 1 h at room temperature to remove the SDS, then incubated for 24–48 h at 37°C in buffer containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$. The gels were stained with Coomassie blue (0.25%) for 30 min, then destained for 1 h in a solution of acetic acid and methanol. The proteolytic activity was evidenced as clear bands (zones of gelatin degradation) against the blue background of stained gelatin. Quantitation was performed on the Bio-Rad Gel Doc 1000 using the Molecular Analyst Program (Bio-Rad, Richmond, CA).

Immunoblot analysis

The same supernatants obtained for zymography were used in immunoblot analysis for MMP-2 protein. Concentrated supernatants (750 μl) were boiled for 5 min in Laemmli's sample buffer and electrophoresed in 8% SDS-PAGE gels. Proteins were transferred to nitrocellulose and the membrane was then blocked in 1% BSA in Tris-buffered saline (TBS) with 0.01% Tween 20 for 1 h. The blots were incubated with anti-MMP-2 Ab (5 μg/ml) in Ab dilution buffer (0.5% Tween 20, 1% BSA, 10% glycerol, and 1 M glucose in TBS) at 4°C overnight. Blots were washed four times in TBS with 0.01% Tween 20, and subsequently incubated in sheep anti-mouse peroxidase-conjugated Ab (1:3000) in Ab dilution buffer. After a 45-min incubation at room temperature, the blots were washed four times, and enhanced chemiluminescence reagents were used for development as previously described (48). Quantitation was performed as described above. MT-MMP-1 protein was detected using total cell lysates as previously described (46). Then, 50 μg of total protein was electrophoresed, transferred to nitrocellulose, blocked as described above, incubated with anti-MT-MMP-1 Ab (1:2000), washed, and then incubated in donkey anti-rabbit peroxidase-conjugated Ab (1:3000) as above.

Preparation of plasma membranes

Plasma membranes were prepared as described by Strongin et al. (49). Confluent cultures of U251-MG cells in serum-free medium in the absence or presence of TNF-α and IFN-γ were incubated for 48 h, then collected and homogenized using a Dounce homogenizer (Kontes Glass, Vineland, NJ). The whole-cell homogenate was centrifuged at $1500 \times g$ for 10 min to pellet nuclei and unbroken cells, then the supernatant was collected. The supernatant was then centrifuged at $50{,}000 \times g$ for 45 min at 4°C, and the membrane pellet was collected. The pellet was resuspended in 25 mM HEPES/sucrose buffer and centrifuged again at $50{,}000 \times g$ for 45 min. The pellet was dissolved in 50 μl of 25 mM HEPES/sucrose buffer, pH 7.4, containing 0.1 mM $CaCl_2$ to achieve a final protein concentration between 1–2 mg/ml. A total of 15 μg of protein was used for immunoblotting for MMP-2 as described above.

RNA isolation and RNase protection assay (RPA)

Total RNA was isolated from confluent monolayers of astroglioma cell lines that had been incubated with or without cytokines in serum-free medium for various time periods as previously described (44). Human MMP-2 cDNA (a gift of Dr. W. G. Stetler-Stevenson, National Cancer Institute, Bethesda, MD) was digested with *Sac*I/*Pst*I, and a 324-bp fragment corresponding to 1500–1824 nucleotides (nt) was subcloned into the *Sac*I/*Pst*I polylinker site of the pGEM4Z vector (Promega, Madison, WI). The construct was linearized by *Eco*RI and used to generate a radiolabeled anti-sense RNA probe of 354 nt with T7 RNA polymerase. Human TIMP-2 cDNA (a gift of Dr. W. G. Stetler-Stevenson) was digested with *Pst*I/*Kpn*I, and a 530-bp fragment corresponding to 352–882 nt was subcloned into the *Pst*I/*Kpn*I polylinker site of the pGEM3Z vector (Promega). The construct was linearized by *Hin*dIII and used to generate a radiolabeled anti-sense RNA probe of 559 nt with T7 RNA polymerase. A pAMP-1 vector containing a fragment of the human GAPDH cDNA (corresponding to 43–531 nt) was linearized with *Nco*I, and used to generate a radiolabeled anti-sense RNA probe of 290 nt with T7 polymerase. Then, 15 μg of total RNA was hybridized with MMP-2 or TIMP-2 (50×10^3 cpm) and GAPDH (25×10^3 cpm) riboprobes at 42°C overnight. The hybridized mixture was then treated with RNase A/T1 (1:200) at room temperature for 1 h, analyzed by 5% denaturing (8 M urea) PAGE, and the gels were exposed to x-ray film. The protected fragments of the MMP-2, TIMP-2, and GAPDH riboprobes are 324, 530, and 230 bp in length, respectively. Quantitation of protected RNA fragments was performed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Values for MMP-2 and TIMP-2 mRNA expression were normalized to GAPDH mRNA levels for each experimental condition. GAPDH mRNA was used as a control gene as its levels are not affected by cytokine treatment.

Plasmids, transfection, and luciferase/β-galactosidase assays

A luciferase reporter plasmid driven by 1716 bp of the human MMP-2 promoter (34) was used in this study, and the reporter vector pTKβ (Clontech, Palo Alto, CA) expressing β-galactosidase under control of the herpes simplex virus thymidine promoter was used as an internal reference plasmid. Transient transfection of human astroglioma cells was performed by electroporation with 10 μg of the MMP-2 reporter construct and 5 μg of the pTKβ construct per 3×10^6 cells as previously described (50). After electroporation, transfected cells were pooled together, plated on 60-mm^2 dishes, and allowed to recover overnight before stimulation. Transfected cells were either unstimulated or stimulated for 24 h with TNF-α (50 ng/ml), IFN-γ (100 U/ml), or both cytokines. Cells were also transfected with a promoterless vector control (pGL2-basic) and pTKβ and stimulated as described above.

Cells were lysed with 200 μl of lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM diaminocyclohexane tetraacetic acid (DCTA), 10% glycerol, and 1% Triton X-100). Extracts were assayed in triplicate for

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luciferase and β-galactosidase enzyme activity. The luciferase activity of each sample was normalized to β-galactosidase activity before calculating the fold activation value. The luciferase activity from the vector control was arbitrarily set at 1 for the calculation of fold activation.

Invasion assay

The in vitro invasion assay was performed with minor modifications as described in Xie et al. (51). Briefly, the 6-well invasion chambers consist of two compartments separated by a filter (8 μM pore size) precoated with Matrigel (100 μg/cm^2), a reconstituted ECM preparation consisting of collagen type IV, laminin, heparan sulfate proteoglycan, entactin, vitronectin, and growth factors (Biocoat, Beckon Dickinson, Bedford, MA). Glioma cells were labeled with [^{3}H]TdR (1 μCi/ml) for 24 h; during the last 8 h of labeling, TNF-α (50 ng/ml) and IFN-γ (100 U/ml) were added to one set of cells. After 24 h, the cells were washed free of unincorporated thymidine, trypsinized, and seeded onto the Matrigel-coated filters (500,000 cells/well in 1 ml of DMEM plus 1% FBS). The lower chamber contained DMEM with 10% FBS (2 ml) as a chemoattractant. The cells in the upper chamber were allowed to adhere for 24 h in the absence or presence of TNF-α/IFN-γ, after which the medium in the upper chamber was replaced with DMEM (without FBS) in the absence or presence of TNF-α/IFN-γ for an additional 48 h. At this time, cells were harvested from the lower chamber and the underside of the filter, and quantitation of cells was performed by scintillation counting. Similar experiments were performed to test the effect of rTIMP-2 on cell invasion; cells in the upper chamber adhered for 24 h in the absence or presence of TIMP-2 (0–10 μg/ml), then the medium in the upper chamber was replaced in the absence or presence of TIMP-2 for an additional 48 h. All experiments were performed in triplicate.

Statistical analysis

Levels of significance for comparisons between samples were determined using Student's *t* test distribution.

Results

TNF-α and IFN-γ inhibit MMP-2 protein expression by human astroglioma cell lines

A characteristic feature of human astroglioma cells is constitutive expression of MMP-2; in fact, MMP-2 expression correlates with malignant progression in vivo (19, 20, 23, 24, 27). We wished to determine whether cytokines known to modulate various biological functions of astroglioma cells could regulate MMP-2 expression and activity. U251-MG astroglioma cells were incubated in the absence or presence of TNF-α, IFN-γ, IL-4, IL-10, IL-13, or LPS for 48 h, and conditioned medium was harvested, then analyzed by gelatin zymography. The cytokine concentrations used have been shown by our laboratory to induce functional changes in glioma cells, such as expression of the adhesion molecules ICAM-1 and VCAM-1, and expression of class II MHC Ags (44, 45, 52–54). LPS was included because it has been reported to slightly enhance MMP-2 expression in astrocytes (55). As shown in Figure 1, gelatinolytic activity at 72 kDa corresponding to the molecular mass of MMP-2 in its proform (ProMMP-2) is detected in conditioned media from unstimulated cells. The addition of TNF-α (50 ng/ml) reduced MMP-2 activity by ~60%, while IFN-γ (100 U/ml) inhibited activity by ~25%. All the other cytokines tested were without effect, while LPS induced a slight increase in MMP-2 activity (Fig. 1). Dose-response studies were conducted using TNF-α at 0.5–100 ng/ml and IFN-γ at 1–1000 U/ml to determine the optimal concentrations for inhibition; the results indicated that maximal inhibition was observed using 50 ng/ml of TNF-α and 100 U/ml of IFN-γ (data not shown).

To further investigate the surprising finding that two proinflammatory cytokines could inhibit MMP-2 activity, we tested the action of TNF-α and IFN-γ on MMP-2 protein and mRNA expression. U251-MG cells were incubated with medium, TNF-α, IFN-γ, or both cytokines for 48 h, then MMP-2 protein expression was assessed by immunoblotting. TNF-α and IFN-γ individually inhibited constitutive ProMMP-2 protein expression, and the inclusion of both cytokines inhibited MMP-2 expression by to a greater

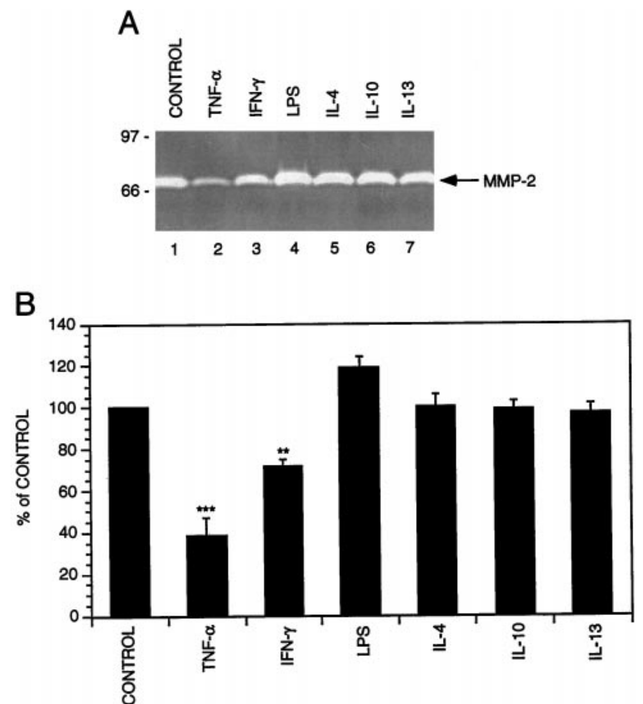

FIGURE 1. Effect of cytokines on MMP-2 enzyme activity. U251-MG cells were incubated with serum-free medium (*lane 1*), TNF-α (50 ng/ml; *lane 2*), IFN-γ (100 U/ml; *lane 3*), LPS (100 ng/ml; *lane 4*), IL-4 (2 ng/ml; *lane 5*), IL-10 (2 ng/ml; *lane 6*), or IL-13 (2 ng/ml; *lane 7*) for 48 h. Supernatants were harvested and subjected to zymography as described in *Materials and Methods* (*A*). Quantitation of the above experiment and two others is shown in *B*. Control MMP-2 enzyme activity was set at 100%, and the cytokine treatments represented as the percent of Control (mean ± SD). **, $p < 0.01$; ***, $p < 0.001$.

extent, indicating that TNF-α and IFN-γ function additively to suppress MMP-2 (Fig. 2*A*). Quantitation of the data from three experiments is shown in Fig. 2*B*; the results indicate that TNF-α, IFN-γ, and TNF-α plus IFN-γ exert statistically significant inhibitory effects on MMP-2 protein expression. The additive influence of TNF-α plus IFN-γ on inhibition of MMP-2 gelatinolytic activity as assessed by zymography was also observed (data not shown). A kinetic analysis of the inhibitory effect of TNF-α/IFN-γ was performed to determine at what time point MMP-2 protein expression was maximally inhibited. U251-MG cells were incubated in medium alone or with TNF-α plus IFN-γ, then harvested after 24, 48, and 72 h. Constitutive levels of MMP-2 protein increase over time (Fig. 2*C*, *lanes 1*, *3*, and *5*); however, the combination of TNF-α plus IFN-γ potently inhibits MMP-2 expression at all time points tested (*lanes 2*, *4*, and *6*). Inhibition of MMP-2 expression by TNF-α plus IFN-γ at 24, 48, and 72 h was ~64%, ~65%, and ~73%, respectively (Fig. 2*C*).

We next wished to determine whether the inhibitory effect of TNF-α plus IFN-γ was reversible. For these studies, U251-MG cells were incubated with medium alone or with TNF-α plus IFN-γ for 48 h. Also, the cells were incubated with TNF-α plus IFN-γ for 24 h, washed extensively to remove the cytokines, then either incubated with medium alone or the same cytokine(s) for an additional 24 h. Cells in the continuous presence of TNF-α plus IFN-γ for 48 h are strongly inhibited (~75%) with respect to ProMMP-2 expression (Fig. 3*A*, compare *lanes 1* and *2*). The removal of TNF-α plus IFN-γ after 24 h results in slightly less inhibition (~69%) compared with that seen in the continued presence of the cytokines (Fig. 3*A*, compare *lanes 2* and *4*), but the

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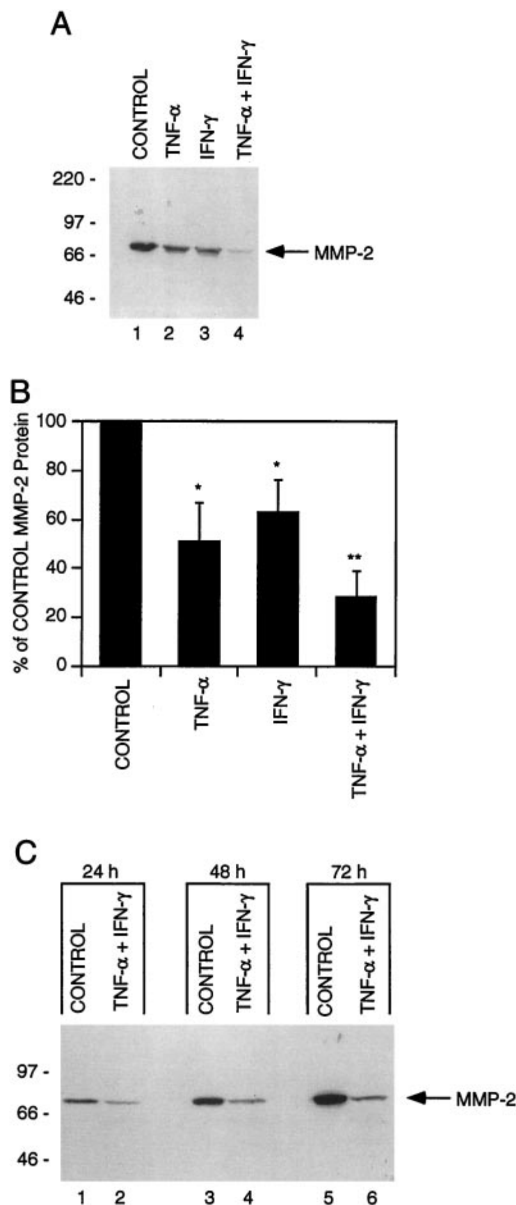

FIGURE 2. TNF-α and IFN-γ inhibition of MMP-2 protein expression in U251-MG cells. Cells were incubated with serum-free medium (*lane 1*), TNF-α (50 ng/ml; *lane 2*), IFN-γ (100 U/ml; *lane 3*), or both TNF-α plus IFN-γ (*lane 4*) for 48 h. Supernatants were harvested and immunoblotting performed using a mAb to human MMP-2. Molecular mass markers are shown on the left (*A*). Quantitation of the above experiment and two others is shown in *B*. Control MMP-2 protein expression was set at 100%, and the cytokine treatments represented as the percent of Control (mean ± SD). *, $p < 0.05$; **, $p < 0.01$. U251-MG cells were incubated with serum-free medium or TNF-α plus IFN-γ for various time periods (24, 48, and 72 h), at which point supernatants were harvested and MMP-2 protein expression assessed by immunoblotting (*C*). Representative of three experiments.

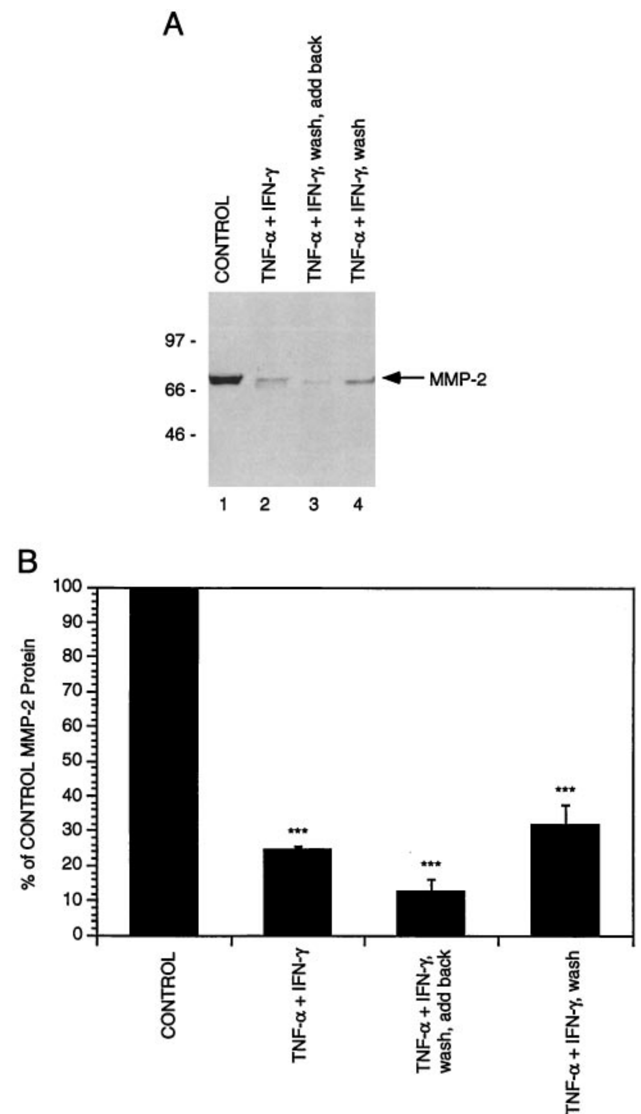

FIGURE 3. The inhibitory effect of TNF-α and IFN-γ is not reversible. U251-MG cells were incubated with serum-free medium (*lane 1*) or TNF-α (50 ng/ml) plus IFN-γ (100 U/ml) (*lane 2*) for 48 h. Samples shown in *lanes 3* and *4* were incubated with TNF-α plus IFN-γ for 24 h, washed extensively, and TNF-α plus IFN-γ added back for an additional 24 h (*lane 3*), or serum-free medium added back for 24 h (*lane 4*). Supernatants were harvested and immunoblotting performed using a mAb to human MMP-2. Molecular mass markers are shown on the left (*A*). Quantitation of the above experiment and two others is shown in *B*. Control MMP-2 protein levels were set at 100%, and the cytokine treatments represented as the percent of Control (mean ± SD). ***, $p < 0.001$.

difference is not statistically significant. These results indicate that the inhibitory effect of TNF-α plus IFN-γ is not reversible under the conditions tested in this experiment (Fig. 3*B*). Interestingly, the repeated addition of TNF-α plus IFN-γ at 24 h results in the most pronounced inhibitory effect (~88%) (Fig. 3*A*, *lane 3*).

TNF-α and IFN-γ inhibit levels of activated MMP-2 protein

The results shown in Figs. 1, 2, and 3 document TNF-α and IFN-γ inhibition of the 72-kDa ProMMP-2. Analysis of cell supernatants by either zymography or immunoblotting failed to consistently detect the 62-kDa active form of MMP-2. Because MMP-2 has been localized to the outside of the plasma membrane, particularly after activation, we attempted to determine whether the active form of MMP-2 could be detected in plasma membranes, and if TNF-α plus IFN-γ inhibited expression of activated MMP-2. Plasma membranes were extracted from U251-MG cells incubated in the absence or presence of TNF-α, IFN-γ, or TNF-α plus IFN-γ for 48 h, then analyzed by immunoblotting for MMP-2 expression. As shown in Fig. 4*A*, the activated form of MMP-2 (62 kDa) is detected in unstimulated cells (*lane 1*), and expression is inhibited in the presence of TNF-α, IFN-γ, or TNF-α plus IFN-γ (*lanes 2–4*). Quantitation of three experiments is shown in Fig. 4*B*. Thus, these

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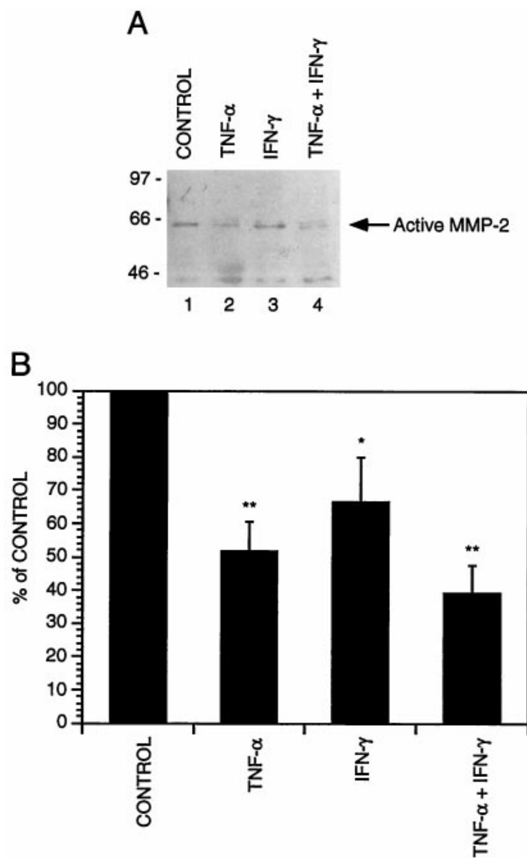

FIGURE 4. The active form of MMP-2 is inhibited by TNF-α and IFN-γ. U251-MG cells were incubated with serum-free medium (*lane 1*), TNF-α (50 ng/ml; *lane 2*), IFN-γ (100 U/ml; *lane 3*), or TNF-α plus IFN-γ (*lane 4*) for 48 h, then plasma membranes prepared as described in *Materials and Methods*. A total of 15 μg of protein was subjected to immunoblotting using a mAb to human MMP-2. Molecular mass markers are shown on the left (*A*). Quantitation of the above experiment and two others is shown in *B*. Control MMP-2 protein expression was set at 100%, and the cytokine treatments represented as the percent of Control (mean ± SD). *, $p < 0.05$; **, $p < 0.01$.

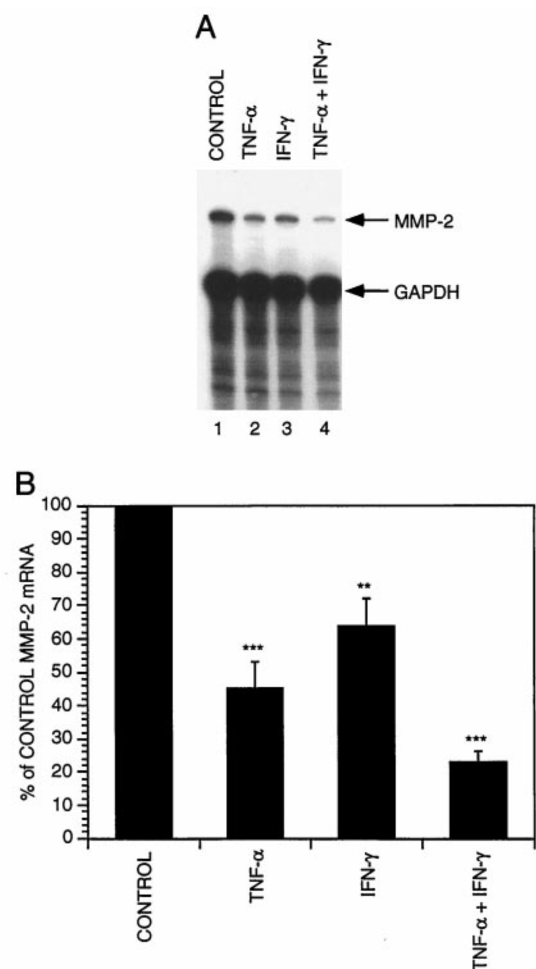

FIGURE 5. TNF-α and IFN-γ inhibition of MMP-2 mRNA expression. U251-MG cells were incubated with serum-free medium (*lane 1*), TNF-α (50 ng/ml; *lane 2*), IFN-γ (100 U/ml; *lane 3*), or both TNF-α and IFN-γ (*lane 4*) for 24 h. RNA was isolated and analyzed by RPA for MMP-2 and GAPDH mRNA expression (*A*). Quantitation of the above experiment and four others is shown in *B*. Control MMP-2 mRNA levels were set at 100%, and the cytokine treatments represented as the percent of Control (mean ± SD). **, $p < 0.01$; ***, $p < 0.001$.

results demonstrate that the active form of MMP-2 can be detected in the plasma membrane of U251-MG cells, and that cytokine treatment inhibits expression. The activated form of MMP-2 was also detected in the supernatants from the above treated cells by immunoblotting; however, a very long exposure to x-ray film was required to detect the band at 62 kDa. Also, the band depicting the 72-kDa ProMMP-2 from the supernatant was very intense and tended to obscure the band at 62 kDa (data not shown). The 62-kDa form of MMP-2 detected in the supernatant was also inhibited by TNF-α plus IFN-γ (data not shown).

Modulation of MMP-2 mRNA expression by TNF-α and IFN-γ

Our results thus far demonstrated an inhibitory effect of TNF-α and IFN-γ on MMP-2 protein expression (both the proform and active form). To determine whether MMP-2 mRNA expression was affected, RPA was performed. We had previously observed that constitutive levels of MMP-2 protein increase over time (Fig. 2*C*), thus we initially performed a kinetic analysis to determine when levels of MMP-2 mRNA were optimally expressed. Analysis of mRNA isolated at 12, 24, 48, and 72 h revealed that constitutive MMP mRNA levels were highest at 24 h (data not shown). Using the 24-h time point, the influence of TNF-α and IFN-γ on MMP-2 mRNA expression was assessed. MMP-2 mRNA expression is detected in unstimulated cells (*lane 1*), and the inclusion of TNF-α (*lane 2*), IFN-γ (*lane 3*), or TNF-α plus IFN-γ (*lane 4*) inhibits MMP-2 mRNA expression (Fig. 5*A*). Quantitation of five experiments is shown in Fig. 5*B*; TNF-α, IFN-γ, and TNF-α plus IFN-γ all significantly inhibit MMP-2 mRNA expression. Inhibition of MMP-2 gene expression by the cytokines TNF-α and IFN-γ is comparable at both the mRNA (Fig. 5) and protein level (Fig. 2).

Inhibition by TNF-α plus IFN-γ requires ongoing protein synthesis

To determine whether the inhibitory effect of TNF-α/IFN-γ on MMP-2 mRNA expression required de novo protein synthesis, experiments were conducted using the protein synthesis inhibitor cycloheximide (CHX). U251-MG cells were incubated with medium, CHX (5 μg/ml), TNF-α/IFN-γ, or CHX plus TNF-α/IFN-γ for 12 h, then RNA was extracted and analyzed by RPA. We have previously determined that CHX at 5 μg/ml inhibits protein synthesis by ~92% in astroglioma cells (53). The 12-h time point was used because longer exposure of the cells to CHX decreased cell viability. CHX alone had a minimal inhibitory effect on constitutive MMP-2 mRNA expression (Fig. 6*A*, *lane 2*; ~7% inhibition).

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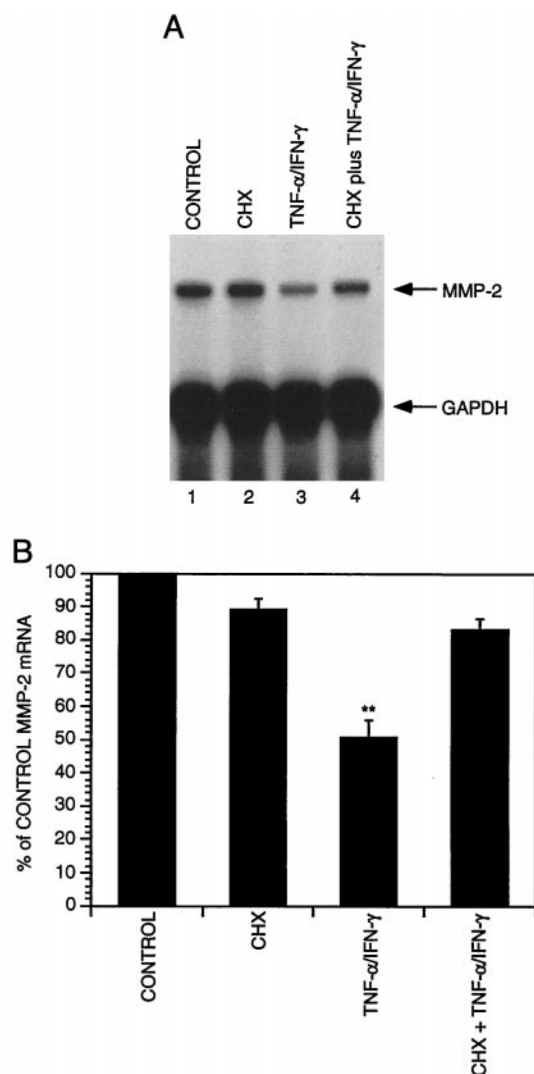

FIGURE 6. TNF-α/IFN-γ inhibition of MMP-2 mRNA requires protein synthesis. U251-MG cells were incubated with serum-free medium (*lane 1*), CHX (5 μg/ml; *lane 2*), TNF-α/IFN-γ (*lane 3*), or CHX plus TNF-α/IFN-γ (*lane 4*) for 12 h. RNA was isolated and analyzed by RPA for MMP-2 and GAPDH mRNA expression (*A*). Quantitation of the above experiment and two others is shown in *B*. Control MMP-2 mRNA levels were set at 100%, and the treatments represented as the percent of Control MMP-2 mRNA expression (mean ± SD). **, $p < 0.01$.

TNF-α/IFN-γ treatment inhibited MMP-2 mRNA expression by ~48% (*lane 3*), and the inclusion of CHX reversed the inhibitory effect of TNF-α/IFN-γ from ~48% to ~16% (*lane 4*). Thus, for optimal inhibition of MMP-2 mRNA expression by TNF-α/IFN-γ, de novo protein synthesis is required.

MMP-2 message is not destabilized by TNF-α/IFN-γ treatment

To assess if MMP-2 steady-state mRNA levels were inhibited by destabilization of the MMP-2 message, $t_{1/2}$ experiments were performed. U251-MG cells were incubated with medium alone or TNF-α/IFN-γ for 24 h, then actinomycin-D (5 μg/ml) was added for an additional 12 h. RNA was isolated at the indicated time points and analyzed for MMP-2 and GAPDH mRNA levels by RPA. As shown in Fig. 7, the constitutive MMP-2 message is very stable and did not degrade to any appreciable extent over the 12-h time course examined. The inclusion of TNF-α/IFN-γ inhibited MMP-2 mRNA expression by ~78% (compare *lanes 1* and *6*), but did not affect MMP-2 message stability (Fig. 7*B*). These results indicate that the cytokines TNF-α and IFN-γ do not influence MMP-2 gene expression at the posttranscriptional level.

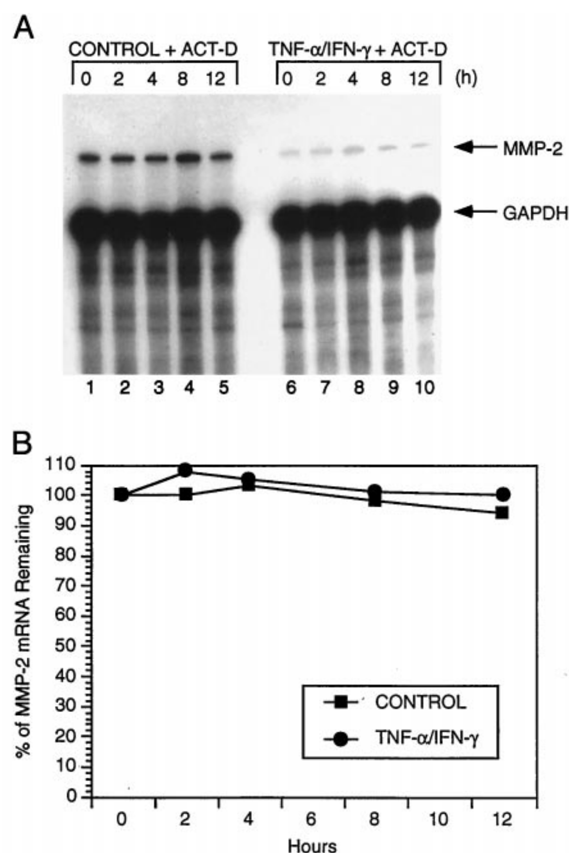

FIGURE 7. TNF-α/IFN-γ treatment does not affect the stability of the MMP-2 message. U251-MG cells were incubated with serum-free medium or TNF-α/IFN-γ for 24 h, then actinomycin D (ACT-D; 5 μg/ml) was added, cells were harvested at the indicated times (2–12 h), and RNA was subjected to RPA (*A*). MMP-2 mRNA values were normalized for GAPDH hybridization within each sample. MMP-2 mRNA at Time 0 (before addition of ACT-D) was plotted as 100% (*B*). Representative of three experiments.

TNF-α and IFN-γ inhibit transcription of the MMP-2 gene

To examine the possibility that the inhibitory effect of TNF-α/IFN-γ was mediated at the transcriptional level, a luciferase reporter driven by the MMP-2 promoter sequence was transiently transfected into U251-MG cells, and activation of the MMP-2 promoter was assessed in the absence and presence of the cytokines. As illustrated in Fig. 8, the MMP-2 promoter is constitutively active in U251-MG cells, with luciferase expression activated up to 37-fold compared with the promoterless vector control. Inclusion of TNF-α or IFN-γ inhibited MMP-2 promoter activity by ~42% and 34%, respectively, while treatment with both cytokines inhibited promoter activity by ~67% (Fig. 8). We also tested two other cytokines, IL-4 and IL-10, which had no effect on MMP-2 protein and mRNA expression, and they were without effect on MMP-2 promoter activity (data not shown). The degree of cytokine inhibition of MMP-2 promoter activity is comparable to that seen for TNF-α/IFN-γ inhibition of MMP-2 mRNA expression (Fig. 5). These results indicate that TNF-α and IFN-γ act at the transcriptional level to inhibit MMP-2 gene expression.

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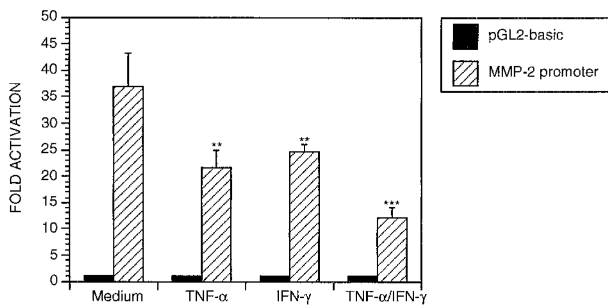

FIGURE 8. TNF-α/IFN-γ inhibit MMP-2 promoter activity. U251-MG cells were transiently transfected with either the promoterless vector control pGL2-Basic or the MMP-2 promoter construct, and luciferase activity was determined as described in *Materials and Methods*. The mean ± SD of three independent experiments, each run in triplicate, after normalization with β-galactosidase activity for transfection efficiency is shown. The luciferase activity from the vector control was set at 1 to calculate fold activation. Cytokine treatment did not affect the vector control, pGL2-Basic. **, $p < 0.01$; ***, $p < 0.001$.

Table I. *TNF-α/IFN-γ treatment inhibits invasion of glioma cells*

Cell Treatment	([^{3}H]TdR) of Invaded Cells	% Inhibition
U251-MG		
Control[a]	54,389 ± 2,206[b]	
TNF-α/IFN-γ[c]	10,614 ± 2,334[d]	80[e]
CRT		
Control[a]	8,675 ± 512[b]	
TNF-α/IFN-γ[c]	1,041 ± 85[d]	88[e]

[a] Medium alone for 72 h.
[b] Mean ± SD of triplicate samples; representative of three independent experiments.
[c] TNF-α (50 ng/ml) plus IFN-γ (100 U/ml) for 72 h.
[d] Significantly different from Control ($p < 0.01$).
[e] Compared to Control values.

Influence of TNF-α and IFN-γ on MMP-2 expression in other astroglioma cell lines

Other human astroglioma cell lines were examined to determine whether the inhibitory effect of TNF-α and IFN-γ was restricted to the U251-MG cell line. CRT astroglioma cells are responsive to both TNF-α and IFN-γ; in fact, both cytokines enhance expression of a variety of gene products (class II MHC, ICAM-1, and VCAM-1) in these cells (44, 50, 56). The influence of TNF-α and IFN-γ on MMP-2 mRNA and protein expression in CRT cells was assessed. Constitutive MMP-2 mRNA expression was inhibited by TNF-α, while IFN-γ treatment had a minimal effect. However, pronounced inhibition was observed in the presence of both cytokines (Fig. 9, *A* and *B*). The inhibitory effect of the cytokines was more striking at the protein level (Fig. 9, *C* and *D*). We have also tested two other human astroglioma cell lines (U373-MG, CH235-MG) and have observed inhibition of constitutive MMP-2 gene expression by TNF-α and IFN-γ, and an additive inhibitory effect of both cytokines (data not shown).

Glioma invasion is inhibited by TNF-α and IFN-γ treatment

To determine whether TNF-α/IFN-γ inhibition of MMP-2 expression was functionally relevant, we assessed the ability of TNF-α/IFN-γ treatment to modulate the invasive capacity of the glioma cells. Therefore, the ability of untreated and TNF-α/IFN-γ treated glioma cells to transmigrate a bioactive ECM was tested. Treatment of U251-MG or CRT cells with TNF-α/IFN-γ significantly reduced tumor invasion compared with control cells (Table I). Also, the addition of rTIMP-2 (10 μg/ml) inhibited the invasive

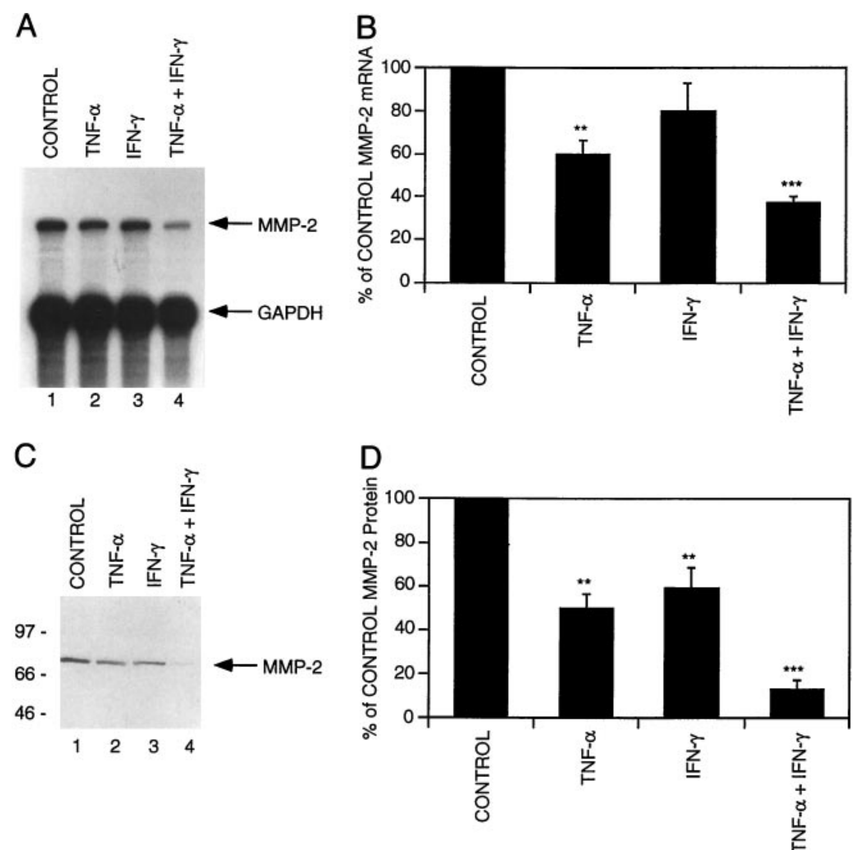

FIGURE 9. MMP-2 gene expression in CRT astroglioma cells: inhibition by TNF-α and IFN-γ. CRT cells were incubated with serum-free medium (*lane 1*), TNF-α (50 ng/ml; *lane 2*), IFN-γ (100 U/ml; *lane 3*), or TNF-α plus IFN-γ (*lane 4*) for 24 h for RNA analysis (*A*) or supernatants were harvested at 48 h and subjected to immunoblotting (*C*). Quantitation of the above experiment and two others is shown in *B* and *D*. Control MMP-2 mRNA/protein expression was set at 100%, and the cytokine treatments represented as the percent of Control (mean ± SD). **, $p < 0.01$; ***, $p < 0.001$.

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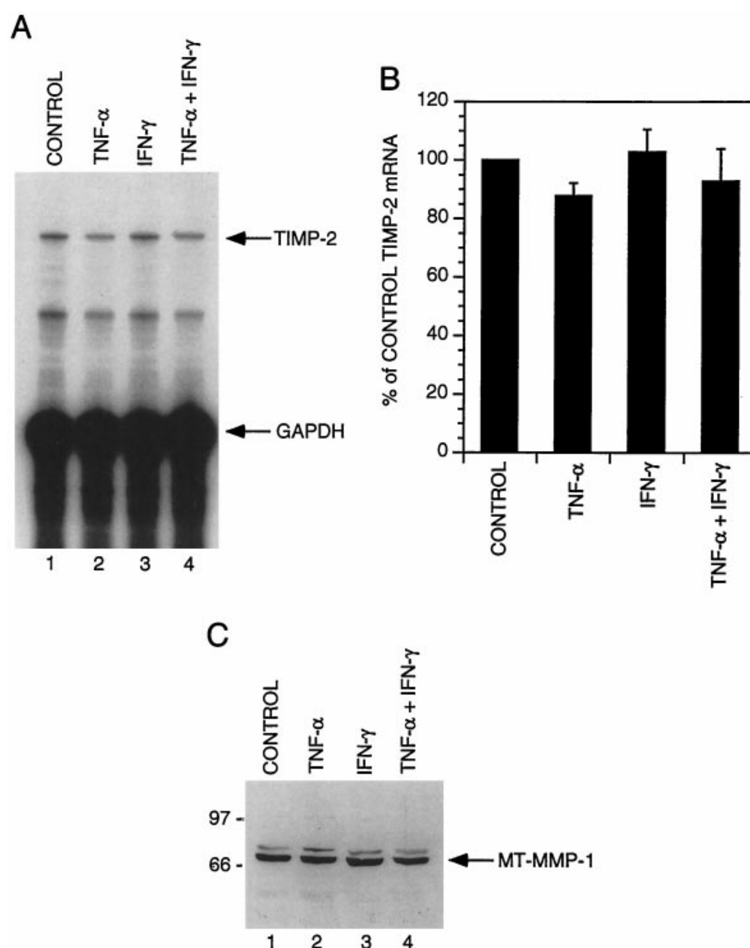

FIGURE 10. TNF-α and IFN-γ do not influence TIMP-2 and MT-MMP-1 expression. U251-MG cells were incubated with serum-free medium (*lane 1*), TNF-α (50 ng/ml; *lane 2*), IFN-γ (100 U/ml; *lane 3*), or TNF-α plus IFN-γ (*lane 4*) for 24 h, then total RNA was isolated and analyzed for TIMP-2 and GAPDH mRNA expression (*A*). We routinely observe a breakdown product when using the TIMP-2 riboprobe. Quantitation of the above experiment and two others is shown in *B*. Control TIMP-2 mRNA expression was set at 100%, and the cytokine treatments represented as the percent of Control (mean ± SD). Cells were stimulated as above for 24 h, then total cell lysates collected and subjected to immunoblotting for MT-MMP-1 expression. Molecular mass markers are indicated on the left (*C*). Representative of two experiments.

activity of U251-MG cells by ~70%, which is comparable to the extent of inhibition observed with TNF-α/IFN-γ treatment. These results indicate that the ability of TNF-α/IFN-γ to inhibit MMP-2 gene expression correlates with the ability of the cytokines to suppress glioma invasion.

TNF-α and IFN-γ treatment does not affect TIMP-2 or MT-MMP-1 expression

The TNF-α/IFN-γ mediated suppression of astroglioma invasion observed in this study correlates with the inhibition of MMP-2 protein expression (both ProMMP-2 and active MMP-2), MMP-2 mRNA expression, and MMP-2 gene transcription. It is well appreciated there are other important participants in MMP-2 regulation, namely TIMP-2 and MT-MMP-1 (for review see Ref. 5). In this regard, we have examined whether TNF-α and IFN-γ affect the expression of TIMP-2 or MT-MMP-1 in human astroglioma cells. As illustrated in Fig. 10*A*, there is constitutive expression of TIMP-2 mRNA in U251-MG cells, which is not significantly affected by TNF-α, IFN-γ, or TNF-α/IFN-γ treatment. Also, MT-MMP-1 is constitutively expressed and is not influenced by TNF-α, IFN-γ, or TNF-α/IFN-γ stimulation (Fig. 10*C*). Identical results were obtained with CRT cells (data not shown).

Discussion

MMP-2 production by tumor cells has been demonstrated to play a fundamental role in ECM degradation and tumor cell invasion (for review see Ref. 5). The recent finding of reduced tumor progression in MMP-2-deficient mice (57) highlights the importance of this molecule. In this study, we wished to determine how MMP-2 gene expression is regulated in human astroglioma cells, as a strong correlation has been observed between astroglioma invasion and MMP-2 expression (17, 19, 20, 22, 23, 27). Our results indicate that two cytokines, TNF-α and IFN-γ, partially inhibit MMP-2 gene expression and can function together in an additive manner for near-complete inhibition of MMP-2 expression in human astroglioma cells. TNF-α/IFN-γ inhibition of MMP-2 expression was observed at several levels: on gelatinolytic activity as determined by zymography, on protein expression (both ProMMP-2 and activated MMP-2) as assessed by immunoblotting, on MMP-2 mRNA expression, and on MMP-2 promoter activity. Our results also indicate that inhibition of MMP-2 mRNA expression by TNF-α and IFN-γ is partially sensitive to the protein synthesis inhibitor CHX, suggesting that de novo protein synthesis is required for optimal inhibition of MMP-2 gene expression. Also, the TNF-α/IFN-γ-mediated suppression of MMP-2 gene expression correlated with decreased invasiveness of astroglioma cells.

MMP-2 gene expression is regulated by numerous mediators in a cell-type and stimulus-specific manner. MMP-2 expression in a variety of tumor cells has been shown to be refractory to IL-1, TNF-α, and phorbol ester treatment (13, 40), while, in glomerular mesangial cells and astrocytes, MMP-2 expression and/or activity is enhanced by these same mediators (42, 55). The literature on IFN effects on MMP-2 expression is conflicting; in KG-2 renal carcinoma cells, IFN-β and IFN-γ, but not IFN-α, inhibit MMP-2 expression and cell migration (58), while in a human salivary gland cell line, IFN-γ alone or in combination with TNF-α enhances MMP-2 expression (41). In melanoma cells, IFN-γ enhances expression of MMP-2 when cells are treated for 3 days, but

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inhibition is seen upon a longer exposure to IFN-γ (7 days) (59). Our results in the human astroglioma cell lines suggest a unique pattern of MMP-2 regulation in that TNF-α alone inhibits expression, IFN-γ alone is moderately inhibitory, and both cytokines exert an additive inhibitory effect that almost completely suppresses MMP-2 expression and astroglioma cell invasion. Previous studies from our laboratory indicate that TNF-α and IFN-γ do not inhibit proliferation of astroglioma cells, and, in fact, TNF-α slightly enhances proliferation (60). TNF-α and IFN-γ have been shown to enhance expression of the adhesion molecules ICAM-1 and VCAM-1 on human astroglioma cells (45, 50), and IFN-γ induces expression of the class II transactivator and class II MHC Ags on a variety of astroglioma cells (44, 46). Thus, the potent suppressive effect of TNF-α/IFN-γ on MMP-2 expression is not a global response of astroglioma cells to these cytokines and suggests a selective inhibitory effect on the MMP-2 gene.

Historically, the MMP-2 gene has been considered refractory to modulation, either inhibition or enhancement, due to a lack of well-characterized regulatory elements in the MMP-2 promoter (for review see Ref. 29). This view is now changing given that the human MMP-2 promoter does, in fact, have a number of potential *cis*-acting regulatory elements including CREB, AP-1, AP-2, PEA3, Sp-1, C/EBP, adenovirus E1A repressor element, and two p53 binding sites (34, 61). Our results clearly demonstrate that the inhibitory effect of TNF-α/IFN-γ is mediated exclusively at the transcriptional level, given the strong inhibition of MMP-2 promoter activity and lack of effect of TNF-α/IFN-γ on TIMP-2 and MT-MMP-1 expression. At present, we do not know if TNF-α/IFN-γ exert their inhibitory response through utilization of any of the known elements of the MMP-2 promoter or if a putative TNF-α/IFN-γ-induced repressor acts at an unknown site within the MMP-2 promoter to mediate suppression of this gene. Studies are underway to map potential TNF-α/IFN-γ inhibitory element(s) in the human MMP-2 promoter.

Another type IV collagenase, MMP-9, has also been shown to be involved in astroglioma invasion (21). In the four astroglioma lines tested in our study, constitutive MMP-9 expression was not detected as assessed by zymography, nor did IFN-γ or TNF-α induce MMP-9 to any appreciable extent (data not shown). Thus, it does not seem that MMP-9 has any involvement in the invasiveness of the U251-MG cell line used in this study, which is in contrast to that observed by Rao et al. (21). Uhm et al. (23) have demonstrated that in vitro, MMP-2 is the primary mediator of glioma invasion, not MMP-9. Our studies also support a role of MMP-2 in glioma invasion, particularly because the addition of TIMP-2 inhibited the invasion activity of U251-MG cells.

TNF-α and IFN-γ are pleiotropic cytokines affecting various parameters of astroglioma functions including proliferation, cytokine/chemokine production, cell motility, expression of adhesion molecules and integrins, expression of complement components, and enhancement of the epidermal growth factor receptor (for review see Ref. 62). Interestingly, TNF-α up-regulation of epidermal growth factor receptor expression on astroglioma cells leads to increased growth inhibition mediated by ^{125}I-labeled mAb 425, which recognizes the epidermal growth factor receptor (63). The authors suggested that immunotherapy with TNF-α in conjunction with other therapeutic modalities may be beneficial in the treatment of brain tumors. Our results demonstrate another beneficial property of TNF-α, that being the inhibition of MMP-2 expression, proteolytic activity, and invasive capacity of glioma cells, which is amplified in the presence of IFN-γ. As local invasiveness is one of the most devastating properties of high-grade gliomas, MMP-2 targeting by cytokine therapy may have implications for arresting glioma invasion.

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