Regulation of 92-kD Gelatinase Release in HL-60 Leukemia Cells: Tumor Necrosis Factor-α as an Autocrine Stimulus for Basal- and Phorbol Ester-Induced Secretion

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Matrix metalloproteinase 9 (MMP-9), also known as 92-kD type IV collagenase/gelatinase, is believed to play a critical role in tumor invasion and metastasis. Here, we report that MMP-9 was constitutively released from the human promyelocytic cell line HL-60 as determined by zymographic analysis. Tumor necrosis factor-α (TNF-α) enhanced the enzyme release threefold to fourfold and the protein kinase C (PKC) activator and differentiation inducer 12-O-tetradecanoylphorbol-13-acetate (TPA) eightfold to ninefold. Gelatinase induction by TNF-α and TPA was inhibited by actinomycin D or cycloheximide, indicating that de novo protein synthesis was required. Neutralizing monoclonal antibodies to TNF-α (anti–TNF-α) decreased the basal MMP-9 release of these cells. In addition, these antibodies also significantly interfered with the TPA-induced enzyme release. Agents that inhibit TNF-α expression in HL-60 cells, such as pentoxifylline and dexamethasone, completely abrogated both the constitutive and TPA-evoked MMP-9 release. Diethyldithiocarbamate, which is known to stimulate TNF-α production in HL-60 cells, exerted a positive effect on MMP-9 release in untreated cells but was inhibitory in TPA-treated HL-60 cells. The PKC inhibitor staurosporine at low concentrations (100 ng/mL) caused a significant augmentation of MMP-9 release in untreated cultures that was blocked by the addition of anti–TNF-α. High concentrations (2 μmol/L) of staurosporine completely abolished the extracellular enzyme activity both in untreated and TPA-stimulated cells. These results suggest, that TNF-α is required for basal and PKC-mediated MMP-9 release in HL-60 leukemia cells. Thus, MMP-9 secretion may be regulated by TNF-α not only in a paracrine but also in an autocrine fashion. This may potentiate the matrix degradative capacity of immature leukemic cells in the processes of bone marrow egress and the evasion of these cells into peripheral tissue.

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ACUTE MYELOID LEUKEMIA is not only characterized by a genetically determined disturbance of proliferation and differentiation of immature progenitor cells but also by an altered egress of these cells from the bone marrow (BM). Acute leukemia can serve as a paradigm for metastasis in general, because for cancer cells to leave primary tumor and to form metastatic colonies they must also be able to cross matrix barriers and penetrate blood vessel walls.[1] These traffics depend on the catalytic modification of extracellular matrix and basement membranes that are mediated by matrix metalloproteinases (MMPs), a family of structurally and functionally related proteolytic enzymes. Among them, two enzymes with molecular weight of 72 kD (MMP-2) and 92 kD (MMP-9) are subclassified as type IV collagenases/gelatinases. They digest denatured collagens (gelatins), intact type IV basement membrane collagen, native collagen type V, and also fibronectin and laminin. Gelatinases are thought to play a critical role in the process of invasion and metastasis, because a strong correlation between type IV collagenolytic/gelatinolytic activity and metastatic potential has been shown for various human tumor cell lines.[2-4] Type IV collagenase is associated with the invading cancer cells of invasive colon, breast, and renal cell carcinomas but is not associated with adjacent normal mucosa.[5-7] Moreover, in plasma of patients with breast and colon cancer, significantly increased levels of 92-kD gelatinase were determined, suggesting this enzyme as a useful additional marker for the dissemination of certain types of cancer.[8]

The regulation of MMPs is complex and occurs at different levels. At the posttranslational level, proteolytic activity is mainly regulated by the extracellular conversion of the inactive proenzyme into the active enzyme and by the interaction with specific tissue inhibitors of matrix metalloproteinases. MMP expression is influenced by a variety of biologic active agents such as growth factors, cytokines, and tumor promoters in various ways at the transcriptional level. Interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), epidermal growth factor (EGF), and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) have been found to be inducers of 92-kD gelatinase in many cell lines.[9-12] Whereas transforming growth factor-β (TGF-β), dexamethasone, and retinoic acid are all known to downregulate the expression of interstitial collagenase (MMP-1) and stromelysin (MMP-3),[13-15] TGF-β can stimulate the secretion of the two gelatinases MMP-2 and MMP-9.[16,17]

In the present report, we investigated the influence of TNF-α, monoclonal antibodies (MoAbs) against TNF-α, TNF-α–expression modifiers, and protein kinase C (PKC) modulators on the release of 92-kD type IV collagenase/gelatinase (MMP-9) in the human promyelocytic cell line HL-60.

MATERIALS AND METHODS

Reagents. All chemical reagents were purchased from Sigma (Munich, Germany). Recombinant human TNF-α (rhTNF-α) and an IgG MoAb to TNF-α were gifts from Knoll AG (Ludwigshafen, Germany); rhIL-1α was provided by Hoffmann La Roche (Grenzach-Wylen, Switzerland); rhIL-3 was from Amgen (Thousand Oaks,

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CA); rhIL-6 was obtained from Boehringer (Mannheim, Germany); native murine IL-9 was a gift from Dr Hültner (GSF, Munich, Germany); rh granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was from Sandoz (Basel, Switzerland); rh granulocyte-CSF (rhG-CSF) was from Genetics Institute (Cambridge, MA); recombinant murine mast cell growth factor (MGF; c-*kit* ligand) was provided by Immunex (Seattle, WA); and rhEGF was obtained from Chiron Co (Emeryville, CA).

Cell culture. HL-60 cells (American Type Culture Collection, Rockville, MD) were cultured under serum-free conditions in Iscove's Modified Dulbecco Medium supplemented with 5 μg/mL transferrin and 0.5 μg/mL insulin. Cells were routinely passaged in 25-cm^2 flasks (Falcon, San Diego, CA) with medium changes once or twice a week. Cultures were evaluated for mycoplasma contamination every 3 months. Viability of cells was determined using the trypan-blue exclusion test and the methylthiotetrazole assay.[18] Cell numbers were either counted under the microscope or electronically with a Coulter Counter (Coulter Electronics Inc, Hialeah, FL). After incubation with TPA, adherent cells had to be detached by treatment with trypsin before counting.

Cell incubation assay. To determine the production of gelatinase, cells were seeded at 1.0 to 1.8 × 10^6/mL in 96- or 24-well microtiter plates (Nunc, Roskilde, Denmark) and treated with different agents. After an incubation period of 24 or 48 hours (as indicated in the text), cells were removed by centrifugation, and culture supernatants (250 μL in 96-well plates and 1 mL in 24-well plates) were subsequently analyzed.

Zymographic analysis and quantification of gelatinase. Gelatinase activity in cell culture supernatants was determined by zymography with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; LKB/Pharmacia, Uppsala, Sweden). This technique was performed on horizontal ultrathin gels (125 × 250 × 0.5 mm) with a 6% to 20% (wt/vol) polyacrylamide gradient. Gels were prepared and polymerized on GelBond PAG film (FMC, Rockland, ME) according to the method of Görg et al[19] and Schickle et al.[20] with the modification that gelatin type I at a final concentration of 1.5 mg/mL was added and copolymerized. Samples were run under nonreducing conditions without prior boiling. After electrophoresis, gels were washed 3 times for 20 minutes in 2% Triton X-100 to remove SDS and to allow proteins to renature. The gels were then immersed in buffer containing 50 mmol/L TRIS pH 7.5, 5 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, and 0.01% (wt/vol) NaN_3 for 24 to 48 hours at 37°C. The zymograms were stained with 0.4% (wt/vol) Coomassie Blue and destained in 35% ethanol/10% acetic acid. Clear zones of gelatin lysis against a blue background stain indicated enzyme activity. Quantitative determination of gelatinase activity was achieved by one-dimensional laser scanning densitometry (Pharmacia/LKB) of the stained zymograms. Activity was expressed in absolute scanning units, representing the integration value of both brightness (in absorption units) and width (in square millimeters) of substrate lysis zones or was converted to relative activity in percent of the control. The values of enzyme activity determined by zymographic analysis actually represent the total amount of secreted MMP-9, because this technique allows the gelatinase to be separated from potentially cosecreted inhibitors during electrophoresis. Subsequent treatment with Triton X-100 leads to an regeneration of proteolytic activity together with an activation of latent proenzymes in the gel.

Cell extraction. After treatment with different drugs for 48 hours, cells were separated from conditioned media by centrifugation. Cell pellets were washed in a buffer containing 50 mmol/L TRIS pH 7.5, 5 mmol/L $CaCl_2$, and 1 μmol/L $ZnCl_2$ and were subsequently treated with this buffer containing 1% Triton X-100 and 1 mmol/L phenylmethylsulfonyl fluoride until more than 95% of the cell plasma membranes were disrupted as monitored by phase microscopy. The supernatants were collected after centrifugation at 12,000*g* and analyzed for gelatinolytic activity by zymography. For control, the corresponding media containing extracellular activity were treated in the same manner.

Determination of TNF-α antigen by enzyme-linked immunosorbant assay (ELISA). TNF-α antigen was measured in cell culture supernatants using a sandwich ELISA with a MoAb to TNF-α provided by Knoll AG that was the same as for the neutralizing experiments. The detection limit was 4 pg/mL.

RESULTS

Influence of various cytokines and differentiation inducers on 92-kD gelatinase release, cell morphology, and cell number. When HL-60 cells were cultured under serum-free conditions, they constitutively released 92-kD type IV collagenase/gelatinase (MMP-9) into the medium as determined by zymographic analysis (Fig 1A). Incubation with TNF-α (100 ng/mL) or TPA (100 ng/mL) resulted in a threefold to fourfold or eightfold to ninefold increase of gelatinase activity per cell in 48-hour culture fluids when compared with untreated control (Fig 1A and B). Cell morphology was changed towards the adherent type by treatment with TPA after 24 to 48 hours, whereas no morphologic changes during the incubation time were observed with TNF-α. Counting of cells indicated that maximal stimulatory doses of TNF-α had no influence on absolute cell number when compared with untreated cultures. Determination of cell numbers in cultures treated with TPA after trypsinization of adherent cells indicated an arrest in cell division, because cell numbers remained unchanged after addition of TPA over an incubation period of 48 hours (data not shown). This shows that TNF-α and TPA increased the yields of gelatinase generated by the individual cell.

In contrast, addition of IL-1α (100 to 500 U/mL), IL-3 (100 to 750 ng/mL), IL-6 (10 to 20 U/mL), IL-9 (10 to 20 U/mL), GM-CSF (100 to 750 U/mL), G-CSF (100 to 750 U/mL), MGF/c-*kit* ligand (100 to 500 ng/mL), EGF (100 to 750 ng/mL), concanavalin A (50 to 250 μg/mL), or bacterial lipopolysaccharide (LPS; 10 to 50 μg/mL) had no or only a small effect (<15% over control) on the secretion of 92-kD gelatinase (data not shown).

Analysis of TNF-α– and TPA-induced 92-kD gelatinase release. TPA was more effective than TNF-α, with a half maximal stimulation at 5 ng/mL or 75 ng/mL, respectively (Fig 2A). At optimal stimulatory concentrations of TNF-α or TPA, cumulative release of 92-kD gelatinase was observed for at least 72 hours, with a maximal production rate between 6 and 24 hours both with TNF-α or TPA (Fig 2B). This stimulation of enzyme secretion was completely abolished on incubation with either actinomycin D (10 μg/mL) or cycloheximide (10 μg/mL), both inhibitors of cellular protein synthesis (Fig 2B). Treatment of HL-60 cells with the nontumor-promoting TPA analogue, 4-α-phorbol 12,13-didecanoate (4-α-TPA; 1 to 100 ng/mL), which is able neither to activate PKC nor to induce cellular differentiation, failed to stimulate MMP-9 release (data not shown).

To compare the level of cell-associated and released gelatinase activity, cells were incubated with TPA (100 ng/mL) or TNF-α (100 ng/mL) for 48 hours. When cell extracts and supernatants were tested for the presence of gelatinolytic activity by zymography, no cell-associated activity could

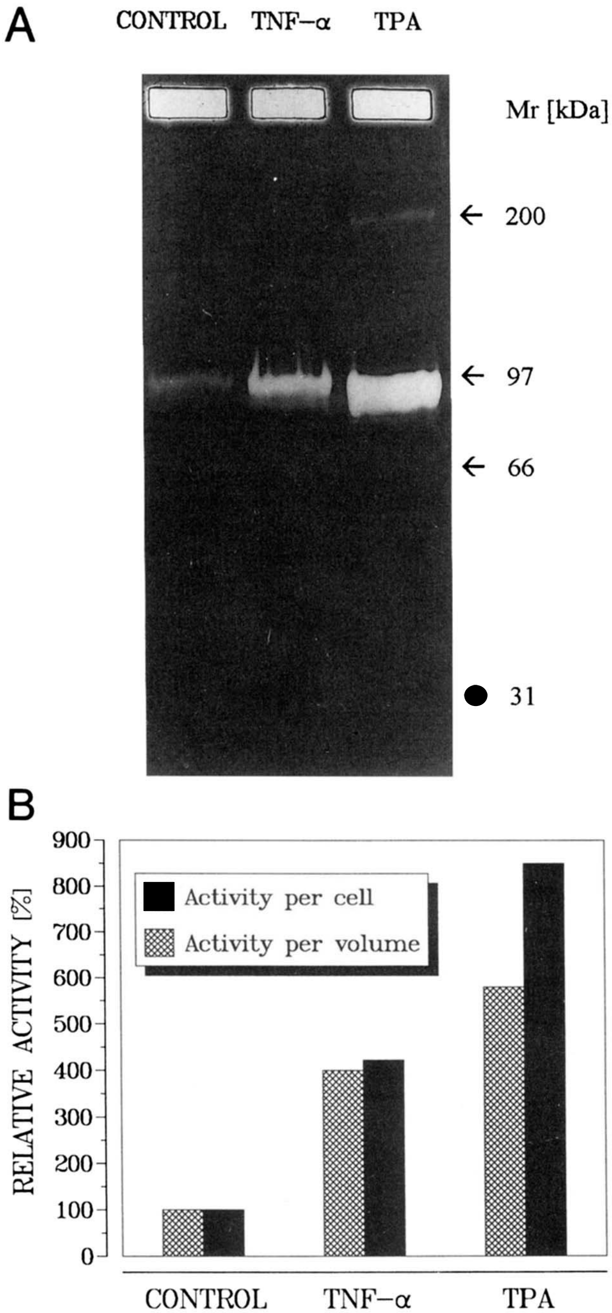

Fig 1. Basal and stimulated release of 92-kD gelatinase from HL-60 cells is shown. After incubation with TNF-α (100 ng/mL) or TPA (100 ng/mL) for 48 hours, cell number was determined, and conditioned media was analyzed by SDS-PAGE zymography and compared with untreated control (A). The light areas that represent zones of lysis in the gelatin gel were quantitated by laser scanning densitometry. Activity is expressed in units of activity per volume and per cell, relative to the basal enzyme release of untreated control (B). Results from a representative determination are shown. Similar results were obtained in four separate experiments.

be detected, whereas the culture supernatants showed 100% MMP-9 activity (data not shown).

Inhibition of basal and TPA-induced release of 92-kD gelatinase by neutralizing TNF-α antibodies. Addition of an MoAb against TNF-α (anti−TNF-α) caused a dose-dependent decrease of the basal 92-kD gelatinase release into the culture supernatant of unstimulated HL-60 cells (Fig 3). Inhibitory concentrations of anti−TNF-α did not alter cell growth and viability (data not shown). This antibody also blocked the TNF-α−induced increase of gelatinase production, showing its neutralizing effect. Moreover, anti−TNF-α almost completely abolished TPA-induced release of 92-kD gelatinase activity (Fig 3).

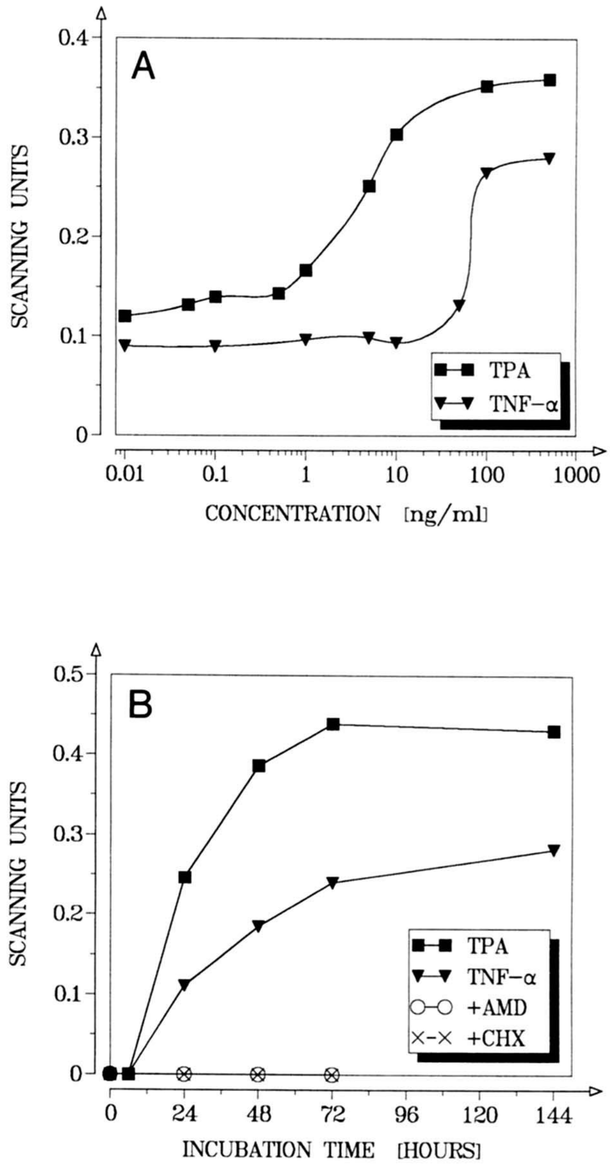

Fig 2. Dose-response curve and time course of stimulated MMP-9 release from HL-60 cells is shown. Cultures were treated for 48 hours with different doses of TPA or TNF-α, and supernatants were analyzed for 92-kD gelatinase by zymography and scanning densitometry (A). HL-60 cells were incubated with a maximal stimulatory concentration of TPA (100 ng/mL) or TNF-α (100 ng/mL) with or without actinomycin D (AMD; 10 μg/mL) or cycloheximide (CHX; 10 μg/mL). Aliquots were taken at different time intervals and analyzed for gelatinase content (B). Results are the mean of duplicate experiments and expressed in absolute scanning units.

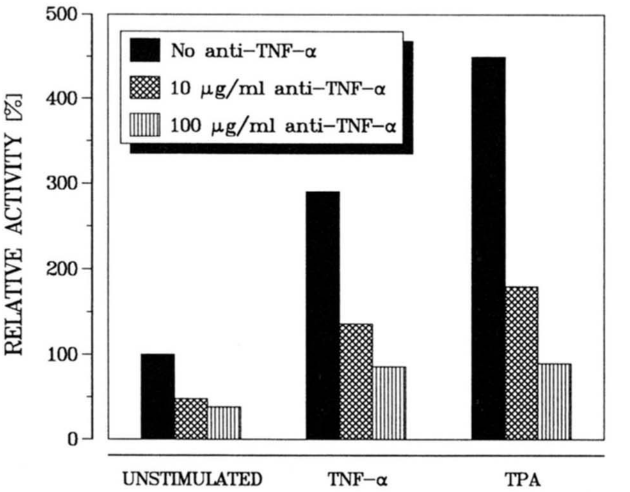

Fig 3. Effect of anti–TNF-α on basal and stimulated release of 92-kD gelatinase is shown. HL-60 cultures, unstimulated or stimulated with TNF-α (100 ng/mL) or TPA (100 ng/mL), were incubated with anti–TNF-α (10 or 100 μg/mL) for 48 hours. Gelatinase release into conditioned media was quantitated by zymographic analysis as described. Results are expressed in percentage of the basal enzyme level of untreated cultures. Mean values of two separate experiments are shown.

Modulation of basal 92-kD gelatinase secretion by TNF-α–expression modifiers and an inhibitor of PKC. Pentoxifylline and dexamethasone, which inhibit TNF-α transcription and translation in HL-60 cells,[21,22] caused a significant dose-dependent decrease in extracellular 92-kD gelatinase activity in HL-60 cells as determined by zymographic analysis. Pentoxifylline (5 mmol/L) or dexamethasone (100 μmol/L) completely blocked the release of this enzyme (Fig 4A and B). Treatment with diethyldithiocarbamate (DDTC) that stimulates TNF-α gene expression in HL-60 cells[23] resulted in a threefold to fourfold increase in 92-kD gelatinase (Fig 4C). Staurosporine, a potent inhibitor of PKC was tested over a concentration range from 0.01 to 1000 nmol/L. Concentrations below 1 nmol/L had only a slight stimulatory effect on 92-kD gelatinase release; higher levels caused a fourfold increase with a maximum at 100 nmol/L. However, at higher concentrations, a dramatic decrease in gelatinolytic activity occurred. Incubation with 2 μmol/L staurosporine completely abolished 92-kD gelatinase production (Fig 4D).

Changes in TNF-α release under these experimental conditions could not be determined, because TNF-α antigen in culture supernatants of noninduced HL-60 cells was below the detection limit (4 pg/mL) of the ELISA (data not shown). However, addition of MoAbs to TNF-α (100 μg/mL) blocked the stimulatory effect of both DDTC (200 μmol/L) or staurosporine (100 ng/mL) on gelatinase release, indicating the involvement of TNF-α in these effects (Fig 5).

Pentoxifylline and dexamethasone at maximal inhibitory concentrations had no significant influence on absolute cell number and cell viability compared with that for untreated cultures after a 48-hour incubation period, whereas staurosporine (1 μmol/L) reduced viability to about 70% of control. DDTC (500 μmol/L) also caused a decrease to about 70% of the control in cell proliferation but simultaneously elevated gelatinase release (data not shown).

To examine potential direct effects on the activity of the 92-kD enzyme itself, zymograms were incubated with incubation buffer in the presence of these agents. No alteration on gelatinolytic activity could be observed with any of the four drugs, indicating that there is neither a direct activating nor inhibiting influence on the enzyme itself (data not shown).

Influence of pentoxifylline, dexamethasone, DDTC, and staurosporine on TNF-α release, gelatinase secretion, and cell number in TPA-stimulated cells. Pentoxifylline or dexamethasone caused a downregulation of TPA-evoked 92-kD activity in a dose-response–dependent fashion as determined by SDS-PAGE zymography (Fig 6A and B). Low concentrations of staurosporine augmented TPA-stimulated gelatinase release slightly, whereas high concentrations showed an inhibitory effect (Fig 6D). In contrast to the findings with nonstimulated HL-60 cells, exposure to DDTC inhibited gelatinolytic activity in TPA-treated cultures (Fig 6C).

Determination of TNF-α antigen in supernatants of cultures treated with TPA (100 ng/mL) alone for 48 hours showed that the release of TNF-α from these cells was dramatically elevated compared with that for untreated control (Table 1). Coincubation with pentoxifylline, dexamethasone, DDTC, or staurosporine in concentrations that inhibited the TPA-stimulated secretion of 92-kD gelatinase also caused a significant decrease in extracellular TNF-α protein. In the presence of the PKC inhibitor, staurosporine, no TNF-α was detectable (Table 1).

In measuring viability and cell number of TPA-treated cells incubated with the different drugs, we found no significant inhibitory influence with pentoxifyllin (5 mmol/L) and dexamethasone (1 mmol/L), but some decrease to 65% in proliferation with DDTC (1 mmol/L) compared with that for the TPA control was found (Table 1). Cell counts in cultures simultaneously treated with TPA and a gelatinase inhibitory concentration of staurosporine (1 μmol/L) were nearly equivalent to those in non-TPA–treated cultures without additive after an incubation period of 48 hours, indicating that staurosporine neutralized the TPA-mediated arrest in cell division (Table 1).

DISCUSSION

In the present study we used the promyelocytic leukemia cell line HL-60 as a model for a disseminating hematologic malignancy. Under serum-free conditions, we found that these cells constitutively release a gelatinase with an apparent molecular weight of 94-kD into the culture supernatant, as determined by gelatin SDS-PAGE. The basal release of this enzyme was markedly augmented by TNF-α and TPA. Davis et al[24] previously described purification and identification of a 94-kD gelatinase from TPA-stimulated HL-60 cells as 92-kD type IV collagenase/gelatinase (MMP-9).

TNF-α– or TPA-evoked secretion of gelatinase was completely abolished by the addition of actinomycin D or cycloheximide, agents that inhibit protein synthesis at the level of transcription or translation. This indicates that continuous synthesis of RNA and protein is required and that the effects

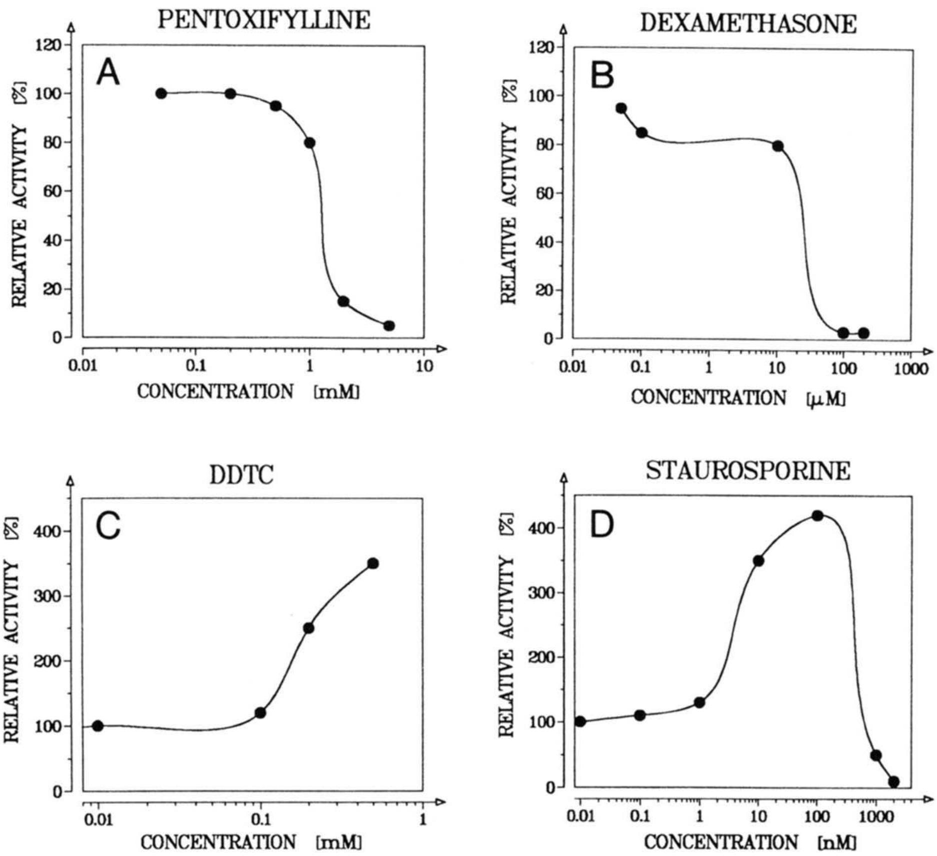

Fig 4. Modulation of basal 92-kD gelatinase production in HL-60 cells by TNF-α–expression modifiers and a PKC inhibitor is shown. Cultures were incubated with or without pentoxifylline (A), dexamethasone (B), DDTC (C), or staurosporine (D) at different concentrations for 48 hours and then analyzed for gelatinase content in culture fluids by zymography and laser scanning densitometry. The graphs show the mean values of two separate experiments performed.

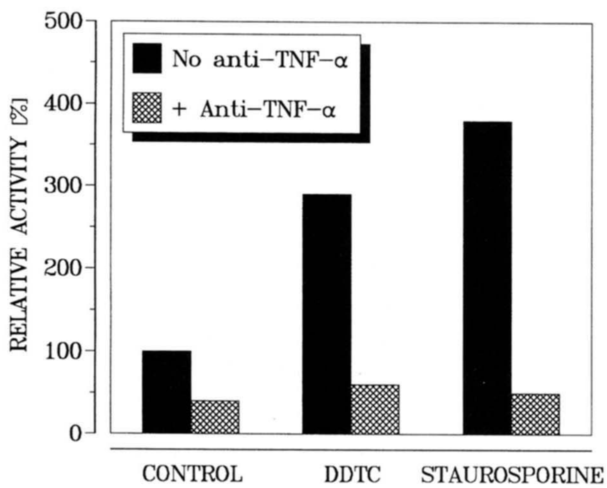

Fig 5. Inhibition of DDTC- and staurosporine-stimulated 92-kD gelatinase secretion by anti–TNF-α is shown. Cells were incubated with stimulatory doses of DDTC (200 μmol/L) or staurosporine (100 ng/mL) alone or together with anti–TNF-α (100 μg/mL) for 48 hours. Supernatants were analyzed by zymography. Enzyme activity was expressed in units of activity relative to the basal gelatinase of the untreated control. Results shown are the data of a single experiment representative of three so performed.

are not because of the release of presynthesized gelatinase from granules. This was confirmed by the observation that no gelatinolytic activity could be found in cell extracts of untreated or stimulated HL-60 cells. Moreover, terminally differentiated human neutrophils that store 92-kD gelatinase in specific granules release gelatinase very rapidly after treatment with TPA or TNF-α.[25,26] Maximal activity is usually observed within 30 to 60 minutes after incubation with TPA, as determined by zymography.[25] On the contrary, in HL-60 cells, enzyme release was only detectable after 6 hours of incubation with TPA or TNF-α. Maximal activity was reached after an incubation period of about 72 hours. This suggests that TPA as well as TNF-α stimulate HL-60 cells to produce gelatinase by de novo synthesis, probably via initiation of cellular maturation. TPA induces monocytic/macrophage-like differentiation and activation of PKC in HL-60 cells.[27,28] Incubation with 4-α-TPA, an analogue that does not activate PKC or induce differentiation, had no stimulatory effect on gelatinase release. In addition, coincubation of TPA-stimulated HL-60 cells with staurosporine, an inhibitor of PKC, neutralized the arrest in cell division and blocked the augmentation of extracellular TNF-α and gelatinase caused by TPA alone. These results indicate that the TPA stimulatory effect on gelatinase production in HL-60 cells is correlated with cellular differentiation and/or PKC activation. Similar to TPA, TNF-α also increases gelatinase production and induces HL-60 cells to differentiate to the monocytic phenotype.[29] However, differentiation induction with TNF-α is not necessarily accompanied by a loss of prolifera-

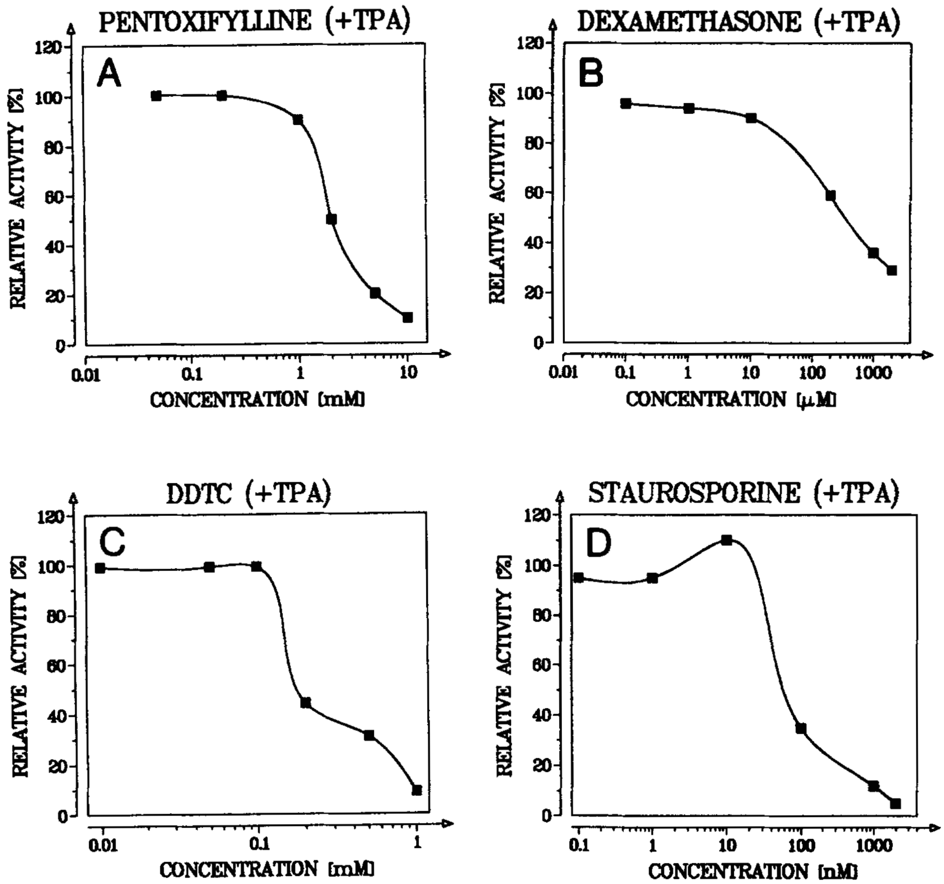

Fig 6. Modulation of TPA-stimulated gelatinase release in HL-60 cells by TNF-α–expression modifiers and a PKC inhibitor is shown. Cultures were incubated with TPA in a maximal stimulatory dosage of 100 ng/mL and simultaneously with or without pentoxifylline (A), dexamethasone (B), DDTC (C), or staurosporine (D) at different concentrations for 24 hours. Gelatinase activity in conditioned media was quantitated by densitometric analysis of the zymograms. Graphs show the mean values of two separate experiments performed.

tive capacity,[30] as supported by our findings. Because a low level of TNF-α gene transcription is present in noninduced HL-60 cells[23] and this basal TNF-α expression is significantly enhanced during monocyte differentiation (for example, through induction with TPA[31] or TNF-α itself[32]), we hypothesized that constitutively secreted TNF-α may be responsible for the basal gelatinase release. Quantification of endogenously produced TNF-α in supernatants of untreated HL-60 cells is difficult because the amounts of TNF-α released by these cells are below the detection limit of the ELISA (<4 pg/mL). But when we used neutralizing MoAbs against TNF-α, we were able to reduce basal MMP-9 activity in culture fluids of unstimulated HL-60 cells to about 35%, when compared with untreated control. This inhibitory effect of anti–TNF-α suggest that the basal MMP-9 release in HL-60 is to a large extent regulated by constitutively secreted TNF-α which acts in an autocrine manner for the maintenance of enzyme release. However, even high excess of neutralizing antibody did not completely abrogate enzyme activity. This may be because of the continuous delivery of TNF-α by the cell, a possible intracrine action of TNF-α, the limited half-life time of the antibody in culture, or, most likely, the other stimulatory contributions to the basal 92-kD gelatinase release. To further analyze the role of TNF-α as an autocrine stimulus for the production of MMP-9 in HL-60 cells, several inhibitors and activators of TNF-α expression were examined. DDTC inhibits proliferation but elevates TNF-α transcription in HL-60 cells.[23] Similarly, DDTC in our system also caused some decrease in the cellular proliferation rate but simultaneously elicited a significant augmentation of the basal gelatinase activity. This indicates again the dependence of MMP-9 secretion on cellular TNF-α expression. Additional evidence is provided by the observation that pentoxifylline and dexamethasone abrogated

Table 1. Effect of Pentoxifylline, Dexamethasone, DDTC, and Staurosporine in TPA-Stimulated HL-60 Cultures on Cell Number and the Release of TNF-α and 92-kD Gelatinase

Treatment	TNF-α (pg/mL)	Gelatinase Activity (%)	Cell No. × 10^6/mL
TPA 100 ng/mL	1070	100	1.4
TPA + pentoxifyllin 5 mmol/L	652	22	1.4
TPA + dexamethasone 1 mmol/L	761	46	1.3
TPA + DDTC 500 µmol/L	470	35	0.9
TPA + staurosporine 1 µmol/L	<4	7	2.2
None	<4	15	2.4

HL-60 cells were seeded at 1 × 10^6/mL and treated with the indicated stimulus. After an incubation period of 48 hours number of viable cells was determined by exclusion of trypan blue. Supernatants were tested for the content of TNF-α by ELISA. Gelatinase activity was determined by zymography and expressed in activity per cell relative to the enzyme level in cultures treated with TPA alone, which was set 100%. The values represent the average of a duplicate experiment.

92-kD gelatinase activity. Both drugs are known to inhibit TNF-α expression in HL-60[21] and other cell systems.

Because the gelatinase inducer TPA acts as an activator of PKC, we further investigated the significance of PKC for the constitutive MMP-9 secretion by incubation with staurosporine, an inhibitor of PKC. Addition of staurosporine to HL-60 cultures showed paradoxical effects on enzyme activity in the supernatants. At low concentrations, staurosporine acted as an potent stimulans of gelatinase release; at higher concentrations, however, it acted as an strong inhibitor. Interestingly, these bifunctional effects of the PKC modulator are consistent with the results of Coffey et al,[33] who found that low levels of staurosporine augmented TNF-α release in LPS-/TPA-stimulated human monocytes, whereas high levels of the PKC inhibitor prevented LPS-/TPA-evoked TNF-α release. The molecular basis of this bifunctional effect remains to be elucidated.

To determine the role of TNF-α in DDTC- as well as staurosporine-enhanced gelatinase release, we analyzed supernatants of HL-60 cells treated with stimulatory concentrations of these drugs. Detection of extracellular TNF-α antigen by ELISA was not successful, obviously because of the low amounts secreted (<4 pg/mL). But indirect evidence for TNF-α as the cause of the DDTC- and staurosporine-mediated effect was achieved by MoAbs to TNF-α. Addition of anti–TNF-α abolished both DDTC- and staurosporine-enhanced gelatinase release in HL-60 (Fig 5). Hence, the influence of staurosporine on 92-kD gelatinase secretion in HL-60 is likely to be caused by a PKC mediated modulation of TNF-α expression.

Anti–TNF-α repressed 92-kD activity in TPA-stimulated cells to about 20% of TPA-control without antibody. Therefore, it is possible that the autocrine secretory TNF-α loop also participates in the TPA-mediated gelatinase augmentation. This assumption is supported by the observation, that pentoxifylline and dexamethasone reduced both the TPA-evoked augmentation in gelatinase and TNF-α release. In contrast to immature HL-60 cells, dithiocarbamates such as DDTC downregulate TNF-α expression in mature monocytes[34] and in other cell systems by inhibiting nuclear factor κB activation.[35] DDTC also caused a strong inhibition of both TNF-α and enzyme secretion in TPA-treated cells. Low concentration of the PKC modulator, staurosporine, did not enhance TPA-stimulated enzyme activity to a great extent (as was observed in untreated cells), whereas high doses completely abolished MMP-9 secretion and TNF-α release. This indicates that TPA, at an optimal stimulatory dosage, leads to an activation of PKC resulting in a stimulation of TNF-α expression that induces gelatinase production, which is downregulated by the inhibition of the kinase at high staurosporine concentrations.

Taken together, our observations show that MMP-9 production in untreated and TPA-induced leukemic HL-60 cells can be regulated by the modulation of TNF-α expression at the transcriptional or posttranscriptional level in both an autocrine and paracrine fashion. Very little is known about the regulatory processes that govern the normal or malignant egress of white blood cells from the BM.[36] Cytokines play a central role in the network of interactions among these cells and their extracellular matrix. There is no doubt that autocrine circuits can be a critical step in myeloid leukemogenesis, because leukemic cells produce their own bioactive factors (eg, TNF-α, IL-1, G-CSF, or GM-CSF) that can modify growth and differentiation.[37-41] But growth stimulation by itself is not sufficient to induce leukemia. Moreover, the autocrine expression of cytokines, as well as factors produced from, for example, BM stromal or endothelial cells, in a paracrine manner, may influence the interaction of cancer cells with their environment during the metastatic process to allow immature cells to leave the BM or to cross vascular barriers.[36,42,43] Recent in vivo studies have shown that TNF-α is ubiquitously and constitutively expressed in different leukemias, with great frequency in samples from patients with acute or chronic monocytic leukemias and chronic lymphocytic leukemia.[39] Moreover, TNF-α levels in serum of patients with acute myeloid leukemia were found to be significantly increased.[44] Furthermore, Ulich et al[45] report that injection of TNF-α in rats induced peripheral neutrophilia accompanied by a significant depletion of BM neutrophils, and that this process was inhibited by the TNF-α–expression inhibitor, dexamethasone. We have identified an autocrine TNF-α loop in the promyelocytic leukemia cell line HL-60 that is responsible for the continuous release of 92-kD type IV collagenase/gelatinase, an matrix-degrading enzyme that is associated with the invasive and metastasizing potential of tumor cells.[2] This loop was also found to be involved in the phorbol ester-induced MMP-9 secretion during monocytic differentiation. Moreover, enzyme release was stimulated by exogenous TNF-α, implicating also an paracrine regulation mechanism. Thus, TNF-α–regulated MMP-9 secretion may be one of the important cytokine-mediated effects, which is possibly involved in the premature egress of leukemic cells from the BM.

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