Protection from Tumor Necrosis Factor-mediated Cytolysis by Overexpression of Plasminogen Activator Inhibitor Type-2*

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(Received for publication, February 1, 1991)

Pretreatment of HT-1080 fibrosarcoma cells with tumor necrosis factor (TNF) induced resistance to the cytolytic activity of this cytokine in combination with cycloheximide. This resistance correlated with the synthesis of plasminogen activator inhibitor type-2 (PAI-2). HT-1080 cells were transfected with a PAI-2 expression vector in both sense and antisense orientation. The resistance to TNF-mediated cytolysis of transfected cell clones was correlated with the level of PAI-2 expression. Cells expressing antisense PAI-2 RNA showed reduced expression of PAI-2 and increased sensitivity to TNF-mediated cytolysis. Cells expressing constitutively PAI-2 were treated with TNF and cycloheximide to select cells with increased resistance to cytology and enhanced PAI-2 expression. PAI-2 gradually disappeared during a treatment with TNF and cycloheximide. This finding suggested that PAI-2 formed a complex with a target proteinase, which could be involved in mediating the cytolytic activity of TNF.

Bacterial endotoxins stimulate macrophages to secrete a cytokine that causes the necrosis of some murine transplantable sarcomas (1). This cytokine was designated tumor necrosis factor (TNF), but it was subsequently discovered that the major physiological role of TNF is the induction of inflammatory reactions (2, 3). Binding of TNF to high affinity receptors elicits a variety of biological responses (4), including cytotoxicity of cancer cells treated with TNF alone (5-7) and cytolyis of cells treated with TNF and inhibitors of protein or RNA synthesis (8). Furthermore, TNF induces the synthesis of new proteins in various cell types (9-13).

Both normal and cancer cells are lysed when treated with TNF together with inhibitors of RNA or protein synthesis (8). However, a pretreatment with TNF alone protects normal and transformed fibroblasts from the cytolytic activity of TNF and cycloheximide (8, 14). This observation suggests that TNF induces the synthesis of protective protein(s) that prevent or repair its damage (8, 14). The identification and characterization of such protective proteins are relevant for an understanding of the mechanism of action of this cytokine, since the molecular basis of TNF-mediated cytotoxicity and cytolsis is not understood.

One experimental approach to the study of these biological responses is the addition of enzyme inhibitors that protect TNF-treated cells. Inhibitors of phospholipase A2 (15, 16) and of lysosomal enzymes (17) provide some protection from TNF cytotoxicity, but only proteinase inhibitors protect human and murine cells from cytosis (18, 19). This observation led to the suggestion that a proteinase is involved in TNF-mediated cytosis (18, 19).

In a different experimental approach, Wong et al. (20) overexpressed manganous superoxide dismutase (MnSOD) in human embryonic kidney cells and observed only partial resistance to TNF-mediated cytosis. This finding suggests that other proteinase inhibitors, in addition to MnSOD, are induced in cells pretreated with TNF. Plasminogen activator inhibitor type-2 (PAI-2) is a major protein induced by TNF in human fibroblasts (21). PAI-2 is a member of the serpin superfamily of serine proteinase inhibitors (22), which form covalent complexes between inactivated proteinases and serpin fragments (23). The only known target of PAI-2 is urokinase-type plasminogen activator (24).

In the present investigation, we have examined whether PAI-2 protects human cells from TNF-mediated cytosis. This hypothetical activity of PAI-2 is suggested by the observations that small molecular weight proteinase inhibitors block the cytolytic activity of TNF (18), that a serine proteinase is involved in TNF cytosis (19) and that PAI-2 is greatly induced by TNF (21). Furthermore, PAI-2 synthesized by TNF-treated human fibroblasts accumulates in the cytoplasm (21). To assay for its protective activity, PAI-2 was overexpressed in human fibrosarcoma HT-1080 cells. Here, we report a previously unsuspected role of PAI-2 as a protective protein against TNF-mediated cytosis.

MATERIALS AND METHODS

Cell Culture and Transfection—Human fibrosarcoma HT-1080 cells were maintained in Dulbecco's modified Eagle's medium plus 10% heat-inactivated horse serum. 106 cells were plated in a 10-cm dish and transfected after 24 h with 10-20 μg of plasmid DNA with the Trans-affinity kit (Bethesda Research Laboratories) according to the manufacturer's protocol. After 48 h, 0.6 mg/ml G418 (GIBCO) was added to select resistant cells that were cloned by limiting dilution, maintained in 0.3 mg/ml G418, and screened for PAI-2 mRNA and protein.

Construction of Recombinant Expression Vectors—All enzymes were obtained from commercial suppliers and used as directed. The vector pRC/CMV was purchased from Invitrogen; the plasmid pJ7/PAL-2, which contains a full length PAI-2 cDNA (25), was a gift of Dr. Egbert K. O. Kruithof (University of Lausanne, Switzerland). The pRC/CMV DNA was digested with HindIII, and the ends were filled in with the Klenow fragment of DNA polymerase. PAI-2 cDNA was excised with EcoRI, and the ends were filled in. The 1880-bp PAI-2 fragment was then ligated to the pRC/CMV expression vector to create sense and antisense PAI-2 constructs. Restriction digests confirmed the orientation of PAI-2 cDNA in the vector.

Northern Blot Analysis—Total cytoplasmic RNA was isolated from cells either with guanidinium hydrochloride (26) or with a miniprep method (27), separated by formaldehyde-acrylamide (1.2%) gel electro-
porphoresis and transferred to Genescreen Plus membranes (Du Pont-New England Nuclear). These were vacuum-baked for 2 h and hybridized to labeled PAI-2 sense or antisense riboprobes prepared using the transcription kit of Stratagene. Membranes were then washed twice with 0.1% SSC, 0.1% SDS for 15 min and twice with 0.1× SSC, 1% SDS with constant shaking at 50 °C. To check for equal loading, the membranes were stripped by boiling for 10 min in 0.1% SSC, 0.1% SDS and then hybridized to a β-actin probe obtained from Dr. Donald Cleveland (Johns Hopkins University).

Detection of PAI-2—Cell lysate proteins were separated by electrophoresis in 10% polyacrylamide gels and transferred to Immobilon NF membranes (Millipore). These membranes were blocked for 3–5 h with 5% bovine serum albumin, incubated overnight in a 1:1000 dilution of anti PAI-2 monoclonal antibody (National Diagnostics), washed several times with 0.5% NP-40 in PBS, and incubated 1 h with a 1:7000 dilution of goat anti-mouse IgG antibody conjugated with horse radish peroxidase (Cappel). The blots were washed again with buffered saline and stained with 0.05% diaminobenzidine and H2O2 in PBS containing 0.02% NiCl2 to enhance color.

Assay for TNF Cytolysis—106 HT-1080 cells were seeded per well of cluster plates 24 h before addition of TNF and cycloheximide. The cells were treated in triplicate with different doses of TNF and 10 μg/ml cycloheximide, with cycloheximide alone, or kept untreated. The cells were then washed with PBS and stained with 0.2% crystal violet in 10% ethanol. This dye was eluted with 33% acetic acid and the A510 was measured in a Multiscan microdensitometer as described previously (28). Cycloheximide was cytostatic but not cytotoxic at the concentration used in a standard 8-h cytolysis assay. After 8 h, we recovered ~15% fewer cycloheximide-treated cells than growing untreated cells. In longer incubations with cycloheximide alone, the cell number remained approximately constant up to 24 h, but decreased afterwards by ~20% after 48 h.

RESULTS

Induction of Resistance to Cytolysis and Synthesis of PAI-2—To establish whether TNF induced resistance to cytosis, HT-1080 cells were pretreated with this cytokine for up to 6 h and then challenged for an additional 18 h with TNF and cycloheximide. As shown in Fig. 1A, ~50% of the cells were protected after 2 h and ~70% after 6 h of pretreatment. During this time, TNF induced PAI-2 mRNA and protein up to 20-fold over the level of untreated control cells (Fig. 1, B and C). This finding showed that protection from cytotoxicity was correlated with the induction of PAI-2. However, any of the TNF-induced proteins (9–13) could be responsible for this protection.

Cell Transfection and PAI-2 Expression—To show that PAI-2 was specifically responsible for the protection from TNF-mediated cytosis, HT-1080 cells were transfected with a PAI-2 expression vector. A full-length cDNA coding for human PAI-2 was cloned in both orientations into a mammalian expression vector under the control of a cytomegalovirus promoter/enhancer. This vector contained also a neomycin resistance gene driven by the SV40 early promoter/ enhancer that allowed selection with the antibiotic G418 (Fig. 2). The HT-1080 cells were transfected with expression vectors containing PAI-2 cDNA in both sense and antisense orientation; transfected cells were selected on the basis of their resistance to G418. Several G418-resistant clones transfected with the sense vector were isolated and four were analyzed in detail for their expression of PAI-2 mRNA and protein.

Two different pools of cells, A1 and A2, were obtained by transfection with PAI-2 antisense vector. The expression of PAI-2 antisense RNA was analyzed in these cells; A1 cells accumulated much more antisense PAI-2 RNA than A2 cells (Fig. 4A). These cells were also examined for induction of PAI-2 mRNA and protein (Fig. 4, A and B) by TNF. This mRNA was poorly induced in A1 cells, while it was well induced in A2 cells. Therefore, high expression of PAI-2 antisense RNA was effective in blocking the accumulation of PAI-2 mRNA. In accordance with the RNA blot analysis, PAI-2 was inducible in A2 but not in A1 cells.

Resistance to TNF Cytolysis and PAI-2 Expression—All transfected HT-1080 cell clones were analyzed for their sen-

Fig. 1. Induction of resistance to cytolysis (A), expression of PAI-2 mRNA (B), and PAI-2 synthesis (C) in HT-1080 cells. The cells were pretreated with 10 ng/ml TNF for the time indicated in the abscissa, washed, and then challenged with 1 ng/ml TNF and 10 μg/ml cycloheximide for an additional 18 h (A). Cell lysis was measured as described under "Materials and Methods." The surviving cells are shown as a percentage of control cells treated with cycloheximide alone. In this and the following experiments, each point shows the average of three independent assays. The induction of PAI-2 mRNA by a 6-h treatment with 10 ng/ml TNF is shown by Northern blot analysis (B) and that of PAI-2 by Western blot analysis (C).

Fig. 2. PAI-2 expression vectors. Full-length PAI-2 cDNA was cloned into the mammalian expression vector Rc/CMV in both sense and antisense orientation, as described under "Materials and Methods."

Fig. 3. Expression of PAI-2 mRNA (A) and PAI-2 (B). Control HT-1080 cells were either untreated (C) or treated with 10 ng/ml TNF (C+) for 6 h; cloned transfected cells (S1–S4) were untreated. Northern blot analysis was carried out by hybridization to a strand-specific riboprobe (A). The blot was stripped and hybridized to a β-actin probe. Western blot analysis was carried out with an anti-PAI-2 monoclonal antibody (B).
sitivity to TNF-mediated cytolysis. S3 cells were more resistant to cytolysis (Table I) and expressed higher levels of PAI-2 mRNA and protein (Fig. 3) than S2 and S4 cells. S1 cells were as sensitive to cytolysis as control cells and did not express detectable amounts of PAI-2 (Table I). These results suggested that the resistance to cytolysis was correlated with the level of PAI-2 expression.

Cells transfected with the antisense PAI-2 vector were also analyzed for their sensitivity to TNF-mediated cytolysis. Both A1 and A2 cells were sensitive to cytolysis; pretreatment with TNF induced partial resistance (Table II). However, A2 cells pretreated with TNF were as resistant to cytolysis as S3 or control cells, whereas A1 cells were less resistant, presumably because PAI-2 antisense RNA prevented PAI-2 expression (Tables I and II). Therefore, resistance to TNF was correlated with the relative expression of PAI-2.

To further document the relative resistance to TNF-mediated cytolysis, S3, A1, and control cells were examined in dose-response and time-course cytolysis assays (Fig. 5). As expected, S3 cells were more resistant to the treatment with TNF + cycloheximide than either control or A1 cells in both assays. The S3 cells expressing high levels of PAI-2 were completely resistant to 0.1 ng/ml TNF and 10 \( \mu \)g/ml cycloheximide, as compared to ~80% of control cells and 60% of A1 cells expressing antisense PAI-2 RNA (Fig. 5A); 60% of S3 cells survived 48 h of treatment with 1 ng/ml TNF and 10 \( \mu \)g/ml cycloheximide as compared to 39% of control and 20% of A1 cells (Fig. 5B).

Selection of Cells Expressing High Levels of PAI-2 by TNF and Cycloheximide—If PAI-2 provides protection from TNF-mediated cytolysis, it should be possible to select PAI-2 over-expressing cells by treatments with TNF + cycloheximide. For this experiment, we used pooled cells transfected with PAI-2 sense vector that express varying amounts of PAI-2. These cells were treated with 10 ng/ml TNF and 10 \( \mu \)g/ml cycloheximide in two rounds of selection (see Fig. 6, legend). The surviving cells were then examined for PAI-2 expression and resistance to cytolysis. This selection led to increased resistance to cytolysis and PAI-2 expression (Fig. 6).

Fate of PAI-2 in Cells Treated with TNF and Cycloheximide—PAI-2 belongs to the serine proteinase inhibitors (serpin) family (22). Serpins are pseudosubstrates for proteinases that are trapped in stable complexes (29). Therefore, PAI-2 could increase the resistance of HT-1080 cells to TNF-mediated cytolysis by inactivating some proteinase(s). The time course experiment described above (Fig. 5B) showed that the resistance to cytolysis declined with time, suggesting that the protective activity of PAI-2 decreased during the treatment with TNF and cycloheximide. To examine whether the concentration of PAI-2 actually decreased, we treated cells that expressed high levels of PAI-2 with 10 \( \mu \)g/ml cycloheximide either alone or in combination with 1 ng/ml TNF. The cells were treated for various times, as described in Fig. 7, and the PAI-2 level was then examined by Western blot analysis. A 12-h treatment with TNF + cycloheximide greatly reduced the amount of PAI-2 in comparison to cells treated with...

**Table I**

| Cells     | PAI-2 mRNA | Surviving cells % of control |
|-----------|------------|-------------------------------|
| HT-1080   | --         | 25 ± 4                        |
| S1        | --         | 30 ± 3                        |
| S2        | ++         | 51 ± 2                        |
| S3        | +++        | 54 ± 3                        |
| S4        | +++        | 45 ± 4                        |

**Table II**

Effect of antisense PAI-2 RNA on TNF-mediated cytolysis

Control cells and cells transfected with PAI-2 anti-sense vector were either untreated or pretreated for 6 h with 10 ng/ml of TNF. The relative expression of antisense and sense PAI-2 RNA was measured by Northern blot analysis (Fig. 4) and is indicated in an arbitrary scale. All cells were then treated for 8 h with 10 ng/ml TNF and 10 \( \mu \)g/ml cycloheximide. Surviving cells are shown as a percentage of control cells treated with cycloheximide alone. The mean ± S.D. of three independent experiments are shown.

| Cells          | PAI-2 RNA | Surviving cells % of control |
|----------------|-----------|-------------------------------|
| HT-1080 + TNF  | ++        | 37 ± 2                        |
| A1 + TNF       | +         | 20 ± 2                        |
| A2 + TNF       | ++        | 55 ± 3                        |

**Fig. 4.** Northern blot analysis of RNA from control HT-1080 cells (C) and from cells transfected with the antisense PAI-2 vector (A1 and A2). A, antisense PAI-2 RNA was detected with a strand-specific riboprobe. The same blot was stripped and hybridized to a riboprobe for PAI-2 mRNA and then to a \( \beta \)-actin probe. B, Western blot for PAI-2 in the same cells.

**Fig. 5.** Dose response and time course of TNF-mediated cytolysis. A, control HT-1080 cells (C) and cells transfected with sense (S3) or antisense vector (A1) were treated with 10 \( \mu \)g/ml cycloheximide and the indicated TNF concentration for 8 h. B, the cells were treated with 10 \( \mu \)g/ml cycloheximide and 0.1 ng/ml TNF for the indicated time. The surviving cells are shown as a percentage of cells treated with cycloheximide alone, which was equally growth inhibitory for all the cell lines tested (see "Materials and Methods").
FIG. 6. Increased resistance to cytolysis of transfected cells selected with TNF and cycloheximide. Pooled cells transfected with PAI-2 sense vector were treated with 10 ng/ml TNF and 10 μg/ml cycloheximide for 8 h, washed, and cultured in fresh medium. Surviving cells were grown for 2 days, and this selection was repeated once more. Control HT-1080 cells (C), cells transfected with PAI-2 sense vector (S) and transfected cells after two rounds of selection (Se) were treated for 8 h with 1 ng/ml TNF and 10 μg/ml cycloheximide. The surviving cells are shown as a percentage of cells treated with cycloheximide alone. The inset shows the level of PAI-2 in these cells, determined by Western blot analysis.

FIG. 7. Loss of PAI-2 in cells treated with TNF and cycloheximide. Transfected cells overexpressing PAI-2 were treated either with 10 μg/ml cycloheximide alone or with cycloheximide in combination with 1 ng/ml TNF. Cell homogenates prepared at the indicated times were examined by Western blot analysis with anti-PAI-2 monoclonal antibody.

cycloheximide alone; PAI-2 almost disappeared after a 16-h treatment (Fig. 7). We could not detect any PAI-2 degradation product on Western blots with the monoclonal antibody used. The higher level of PAI-2 in cells treated for 16 h with cycloheximide was due to overloading as judged by staining of the blot with Ponceau S dye.

DISCUSSION

Overexpression of PAI-2 increased the resistance of HT-1080 cells to TNF-mediated cytolysis. Conversely, lowered expression of endogenous PAI-2 by means of antisense RNA synthesis increased the sensitivity of these cells to TNF-mediated cytolysis. Therefore, modulation of PAI-2 expression altered the sensitivity of these cells to TNF.

Ruggiero et al. (18) and Suffys et al. (19) have reported that human and murine cells are protected from the cytolytic activity of TNF + cycloheximide by a treatment with low molecular weight synthetic proteinase inhibitors. The inhibitors of chymotrypsin-like proteinases are more active than the inhibitors of trypsin-like enzymes, leading to the speculation that a proteinase with a defined substrate specificity is involved in the cytolytic activity of TNF (18, 19). PAI-2 is the major protein induced in human fibroblasts by TNF and it is not secreted by fibroblasts (21); more than 90% of PAI-2 is found in the cytoplasm of HT-1080 cells and very little is secreted into the medium (12). A cytosolic form of PAI-2 has also been reported by Belin et al. (30). Therefore, PAI-2 may act as an endogenous serpin and protect these cells by inhibiting the hypothetical proteinase involved in the cytolytic activity of TNF. We also measured the expression of urokinase, which is the known target proteinase for PAI-2, and found it to be comparable in both control HT-1080 and transfected cells (data not shown). This apparently excludes that modulation of PAI-2 levels affects urokinase expression.

Other possible explanations for the increased resistance to TNF cytolytic activity of cells transfected with the PAI-2 expression vector are a decrease in the number or affinity of TNF receptors and the production of TNF, since it has been reported that constitutive secretion of TNF down-regulates TNF receptors and confers resistance to its cytolytic activity (31). However, these explanations are unlikely for the following reasons. Although the number and affinity of TNF receptors were not determined directly, the transfected cells responded to TNF with an increase in the expression of the TNF-inducible MnSOD gene (32) similar to that of control cells. Furthermore, TNF mRNA could not be detected by reverse transcription polymerase chain reactions, nor did the transfected cells show a decrease in viability when treated with cycloheximide alone (our unpublished observations).

Treatment with TNF + cycloheximide selected HT-1080 cells characterized by elevated levels of PAI-2 and increased resistance to cytolysis (Fig. 7). These cells did not show an increase in MnSOD expression (data not shown), suggesting that their resistance to TNF-mediated cytolysis corresponds with the level of PAI-2 alone. Furthermore, the disappearance of PAI-2 during the treatment with TNF + cycloheximide suggests that the overexpressed PAI-2 is forming stable complexes with other proteins, possibly with proteinase(s).

Overexpression of PAI-2 does not provide complete protection from TNF-mediated cytolysis. It is thus possible that other protective proteins, like MnSOD (20), may contribute to the resistance to TNF. However, a much greater PAI-2 expression may be needed to completely inactivate a proteinase activity and provide complete protection. This is suggested by the results of experiments with synthetic proteinase inhibitors that protect completely TNF-treated cells from cytolysis when added at sufficiently high concentrations (18). These compounds either inactivate a cellular proteinase activity in the case of irreversible inhibitors or block its activity in the case of competitive reversible inhibitors (18).

Most cells are rapidly lysed when treated with TNF in combination with inhibitors of RNA or protein synthesis, but not when treated with TNF alone (5–8). Therefore, it is likely that most cells are protected by proteins that need to be continuously synthesized. In the presence of inhibitors of RNA or protein synthesis, these proteins cannot be synthesized and the treatment with TNF results in cell lysis. This mechanism of action of TNF may be explained by the hypothesis that TNF activates a cellular proteinase that is directly or indirectly involved in cytolysis. When treated with TNF alone, some cells are induced to synthesize PAI-2 that inhibits this hypothetical proteinase. It should be pointed out that the activation of a proteinase activity is only one of the mechanisms by which TNF may cause cell injury. Overexpression of MnSOD, which carries out dismutation of O₂⁻, provides partial protection, suggesting that reactive oxygen species are also involved in TNF cytotoxicity (20).

Inhibitors of phospholipase A₂ (33) and proteinases (34) block the cytolytic activity of natural killer cells and cytotoxic T lymphocytes. It has thus been proposed that both phospholipase A₂ and a proteinase are involved in cytotoxic T lymphocyte-mediated cytolysis (35–36). TNF activates phospholipase A₂ in some cells (37–39), and a serine proteinase has been implicated in the activation of phospholipase A₂ (40–42). Therefore, a proteinase involved in TNF-mediated cytolysis may possibly participate in natural killer cells and cytotoxic T lymphocyte-mediated cell killing.

2 S. Kumar and C. Baglioni, unpublished observations.
The characterization of such proteinase may shed some light on the function of proteins induced by TNF and may provide further insight into the mechanism of action of TNF.

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