Metallothionein: A Protein Confering Resistance in Vitro to Tumor Necrosis Factor

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ABSTRACT

The role of metallothionein (MT) in the cytotoxicity of tumor necrosis factor (TNF) was investigated in vitro. A human epithelial cell line (HEp-2) and a mouse fibroblast line (Cl 1D100) had previously been cultured to become resistant to 100 μM CdCl₂ and were cultured routinely in cadmium-containing medium. The MT content of these cells and the nonresistant parent cell lines (HE and CI 1D) was determined both qualitatively and quantitatively by immunochemical techniques. Immunofluorescence microscopy revealed the cadmium-resistant cell lines to be intensely rich in MT in both the nuclear and cytoplasmic compartments. This finding was confirmed by immuno-electron microscopy which also showed the labeling to be freely distributed and not membrane-bound. In comparison, a very weak labeling of the parent cell lines was observed.

MT concentration, as determined by enzyme-linked immunosorbant assay, was found to be 4.05 ± 1.13% and 3.91 ± 0.7% of the total protein for HEp-2 and Cl 1D100 cells, respectively. We were unable to detect MT in the parent cell lines by this technique.

Dose-survival curves obtained after 3 days treatment of the cells with TNF (0.125–500 ng/ml) revealed that the MT-rich substrains were significantly resistant compared to the parent strains (P < 0.001; t tests). In growth rate studies, where cells were exposed to TNF over a dose range of 0.25–250 ng/ml for 6 days, the resistance of the MT-rich cell lines was confirmed (P < 0.002). These data indicate that MT confers resistance to the cytotoxic effects of TNF in vitro and that sensitivity to TNF may be related to the MT content of the cell.

INTRODUCTION

TNF was first described as a protein present in the serum of mice treated with endotoxin, presensitized with Bacillus Calmette-Guérin (1). It was found to cause the hemorrhagic necrosis of certain tumors and to be cytotoxic to a number of transformed cell lines. Subsequent investigations have shown TNF to be active in many processes other than cell killing such that it is now believed to play an important role in defense against viral, bacterial, and parasitic infections and to be a pleiotropic mediator in many inflammatory reactions (2, 3).

An understanding of the variety of effects exerted by TNF on different cell types is gradually emerging. In common with other cytokines, its action is initiated by the binding of TNF to specific cellular receptors which are then internalized through receptor-mediated endocytosis and finally degraded in the lysosomes (4, 5). An intriguing feature of the cytotoxic action of TNF is that a considerable variation in sensitivity exists between different tumor cell lines (4). This variation in response is apparently not dependent upon the number of cell receptors for TNF nor the affinity for binding since resistant cells may bind the TNF molecule and correspondingly, sensitive cell lines may possess few receptors (4). Thus, although TNF binding is essential for its action, it is not sufficient to lead to lysis of the cell.

The precise mechanism of this target-selective cytotoxicity remains unclear, although it is thought that several strategies may be responsible. Pretreatment of cells with TNF caused increased and prolonged resistance to subsequent killing by TNF, while cells previously resistant to TNF became susceptible to its cytotoxic effects when metabolically crippled by inhibitors of RNA and protein synthesis (9).

Furthermore, dominance of TNF resistance was displayed by heterokaryons formed by the fusion of TNF-resistant and -sensitive cells (10). All of these findings suggest that the induction of a protective protein is responsible, at least in part, for mediating resistance to TNF (8). The nature of this protein was determined by the cytotoxic mechanism of TNF. Much evidence implicates oxygen radicals as mediators of TNF-induced cell injury (11). That TNF requires oxygen for its cytotoxic action was demonstrated by the resistance of previously sensitive L929 murine fibroblasts to TNF under anaerobic conditions (12). Further evidence to support free-radical involvement was provided by reports that TNF cytolyis is inhibited by free-radical scavengers such as glutathione and superoxide dismutase (13). Thus resistance to TNF may be determined by the ability of the cell to scavenge free radicals and repair the resultant cellular injury caused by their release.

MT is a cysteine-rich protein, ubiquitous in animal tissues (14, 15). Its binding and induction by a wide range of metals lead to the generally accepted view that its function is in the homeostatic regulation of essential metals (zinc and copper) and in the detoxification of toxic heavy metals (16). However, recent investigations indicate the protein plays a more central role in mediating host defense. More and more treatments are being demonstrated to induce MT synthesis in situations which do not involve metal administration. Currently, much interest is focused upon the role that MT may play as a scavenger of free radicals. Many of the situations which lead to enhanced MT production can also cause release of oxygen radicals, e.g., administration of interleukin 1 (17) and exposure to X-radiation (18). Furthermore, direct evidence implicating MT as a free-radical scavenger was obtained when Thornalley and Vasak (19) demonstrated the quenching of hydroxyl radicals in the presence of MT.

Recently it has been revealed that TNF enhances levels of MT mRNA in vitro (20) and increases hepatic MT concentration in vivo (21). It is not known, however, whether MT protects against TNF-mediated cytotoxicity. If indeed it does, it should be possible to show that enhanced resistance to TNF is conferred by overexpression of MT. In the present study we propose that MT, functioning as a free-radical scavenger, contributes to the protective strategy of certain cells to the cytotoxic action of TNF. Previously, we have provided evidence to show that cells cultured to overexpress MT exhibit enhanced resistance to ionizing radiation (22) and a number of anticancer agents, e.g., cis-dichlorodiammineplatinum (23), chlorambucil (24), and prednimustine (25). The MT level of these cells was found to be 2–3% of the total cytoplasmic protein, as determined by cadmium-binding assay (26). In the current study immunochemical techniques are used to evaluate the MT status of the cells. The data obtained from several
cell sensitivity assays reveal that the cytotoxicity of TNF is significantly reduced by the presence of MT.

**MATERIALS AND METHODS**

**Cell Lines and Culture Conditions.** The cell lines used in this study were one HE cell line, one fibroblast line (Cl ID), and their substrains adapted to grow in medium containing 100 μM CdCl₂ (HE/HEO and Cl ID/Cl IDO). The cadmium-resistant substrains have a high MT content, as demonstrated by cadmium-binding assay and amino acid analysis. Details of the cells and their culture have been described previously (23). The cadmium-resistant substrains were routinely cultured in medium containing 100 μM CdCl₂.

**Assessment of MT Content.** Cells for immunofluorescence microscopy were suspended in Dulbecco’s modified Eagle’s medium supplemented with serum (as described in Ref. 23) at 10³ cells/ml and plated onto poly-o-lysine coated cover slips in a 16-mm diameter well plate (Costar, Cambridge, MO) in 1-ml volumes. The cells were allowed to adhere for 3 h before CdCl₂ was added to the HE/O and Cl ID/O cells at a final concentration of 100 μM. HE and Cl ID cells were maintained in culture medium without CdCl₂ supplement. The cells were grown for 3 days prior to immunolabeling. After rinsing in 10 mm sodium PBS, pH 7.4, the cells were fixed for 30 min in 3.0% formaldehy. Following a further wash step (3 × PBS; 1 × deionized water) the cells were permeabilized by addition of 0.1% Triton X-100 (Sigma) (v/v) in PBS for 10 min. The wash procedure was repeated and the cells were incubated with a polyclonal antibody, mouse-anti-horse MT, diluted 1:2500 in 10% newborn calf serum. The cells used in this study were incubated with a biotin-conjugated antiserum to mouse IgG (Sigma; diluted 1:100) for 30 min. The wash procedure was repeated and ExtrAvidin-fluorescein isothiocyanate conjugate (Sigma; 1:50) was applied to the cells for 30 min. After a final washing step the cover slips were mounted in glycerol.

The level of unspecified labeling was monitored in each cell line by carrying out the immunolabeling procedure with the omission of primary antibody. A Nikon Photomicroscope with an epi-fluorescence attachment was used for fluorescein isothiocyanate observation.

**Cell Growth Studies.** Experiments were performed with both HE/HEO and Cl ID/Cl IDO cells to obtain growth curves during TNF treatment. Approximately 7,000 HE/HEO and 20,000 Cl ID/Cl IDO cells were plated in 16-mm diameter well plate (Costar) in 1 ml medium and were grown without TNF supplement for 24 h. Cells were then given continuous treatment for 6 days with TNF diluted in ordinary supplemented medium. The TNF was applied in a concentration range of 0.25–250 ng/ml. Controls were maintained without cytokine supplement. Cells (4 replicate wells at each TNF concentration) were harvested by trypsinization, resuspended in normal supplemented medium at 10⁵ cells/ml, and plated onto 96-well microtiter plates (Becton Dickinson Labware, Lincoln, MA) for 72 h. The percentage cytotoxicity was calculated at each dilution of TNF.

**Statistical Analysis.** The paired Student t test was used to determine statistically significant differences between the percentage cytotoxicity values in both pairs of cell lines at each dose level of the crystal violet cytotoxicity assay data. The significance of the cell growth studies was tested by an area under the curve method (38) as follows: cell growth was obtained by calculating the area under the curve (AUC) as a function of time t according to the formula:

\[
AUC = \int_0^t \log \left( \frac{n(t)}{n(O)} \right) dt
\]

where \(n(t)\) is the number of cells at time t, and \(n(O)\) is the number of cells on day 0. The trapezoidal method was used to evaluate the integral. When plotted, AUC was found to be a linear first-order function of log concentration of TNF (log c). The relative sensitivities of the Cd-resistant cells (HE/O and Cl ID/O) and the parent cell lines (HE and Cl ID) to TNF was tested by subjecting the data to a linear first-order regression analysis, with log c as the independent variable. A slope significantly greater than zero was taken as proof that the Cd-resistant cells also show resistance to TNF.
RESULTS

Assessment of MT Content. Fluorescence immunocytochemistry was carried out on all four cell lines (HE100, HE, CI 1D100, CI 1D) to evaluate the MT content and the gross distribution of MT within the cell (Fig. 1). In the control experiment, where primary antibody was omitted, a low intensity labeling of occasional cell nuclei was detected in each cell line (pictures not shown). This probably represents unbound fluorescent conjugate which has gained entry to the nuclei of dead or dying cells and which subsequent washing steps have failed to remove. Immunolabeling of the parental cell lines (HE, CI 1D) revealed a low intensity, background signal comparable to the control experiment; however, strong staining was observed in their cadmium-resistant substrains (HE100, CI 1D100). In these MT-rich cells, labeling was seen to be homogeneous throughout the cell population. Furthermore, the distribution of MT within the cell was also found to be homogeneous, occurring in both nuclear and cytoplasmic compartments.

Electron microscopical immunocytochemistry was carried out on the HE100 and HE cell lines to determine MT distribution on an ultrastructural level. The HE cells were almost totally negative for MT with only the occasional gold particle found in the nucleus and cytoplasm (Fig. 2a). In the HE100 cells, gold particles indicating the presence of MT were abundantly distributed throughout the nucleus and cytoplasm (Fig. 2b). The labeling intensities of nucleus and cytoplasm were judged to be comparable. Within the cytoplasm, gold particles did not seem to adhere to any membranous structure specifically but were freely distributed. The cells were often found to be highly vacuolated and in the HE100 cells, gold particles lined the lumen of the vacuole. In some instances labeling intensity appeared greatest at the periphery of the cell and in the microvilli. Gold particles were also observed attached to the outer membrane surface of microvilli. Examination revealed few morphologically distinct organelles in the cytoplasm of the cells, yet despite this paucity of discernable cytoplasmic organization, the nuclear boundary was clearly evident to allow comparison of the nuclear and cytoplasmic compartments of the cell. Thus the electron microscopy study was able to confirm the findings of the fluorescence immunocytochemistry in that MT appears to be equally distributed between these two compartments in the MT-rich substrains.

Table 1 illustrates the quantitative MT assessment of cell cytosols as determined by ELISA. The high MT content of the cadmium-resistant cells is confirmed; however, no MT was detected in the parental cell lines using this technique.

Cell Sensitivity Studies. In all experiments the MT-rich cells showed considerable resistance to treatment with TNF when compared with the parent cells. The inhibition of cell proliferation caused by TNF was assessed by two methods. In the crystal violet assay the cells were exposed to TNF in the concentration range 0.125–500 ng/ml continuously for 3 days. Fig. 3 shows the effect of this treatment expressed as percentage cytotoxicity. The growth of all cell lines was dose dependently inhibited by TNF but the parent lines were significantly more sensitive to the treatment (P < 0.001; t tests). Three days of exposure to 0.5 mg TNF/ml caused 63.8 and 48.6% cytotoxicity to CI 1D100 and HE100 cells, respectively, compared with values of 88.3 and 77.8% cytotoxicity seen in the corresponding parent lines.

The inhibition of cell proliferation, as expressed as cell counts at certain time intervals, is illustrated in Fig. 4. The data and the subsequent regression analysis (described in the “Statistical Analysis”) confirms the relative resistance of the MT-rich substrains to treatment with TNF (HE/HE100 – slope = 1.51; t5 = 4.42; P < 0.002; CI 1D/Cl...
METALLOTHIONEIN EXPRESSION AND TNF CYTOTOXICITY

Fig. 2. Electron immunocytochemistry of HE (a) and HE100 (b) cells with antibody E9 for metallothionein. The parental HE cells are found to be negative for metallothionein while in the cadmium-resistant HE100 cells an abundance of gold particles is observed throughout the nucleus and cytoplasm (X 40,000). Bar, 0.25 μm.

Table 1. MT content of cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MT (%/total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE</td>
<td>ND*</td>
</tr>
<tr>
<td>HE100</td>
<td>4.05 ± 1.13b</td>
</tr>
<tr>
<td>C1 1D</td>
<td>ND</td>
</tr>
<tr>
<td>Cl1D100</td>
<td>3.91 ± 0.71</td>
</tr>
</tbody>
</table>

*ND, not detected.  
Mean ± SE.

MT concentration of cytosol preparations of the cell lines HE and Cl 1D, and their substrains cultured to be resistant to 100 μM CdCl2, HE100, and Cl 1D100 was measured by means of ELISA. MT concentration is expressed as the percentage of total protein and is the mean value of 4 independent experiments.

1D100 - slope = 1.78; t3 = 10.4; P < 0.002). In the case of the parental HE cell line (Fig. 4a), a value of zero cell number was obtained after 6 days of exposure to TNF over the dose range 10–250 ng/ml. For the purpose of graphical representation only, these points were taken to 1000 cell number such that the logarithmic scale was maintained for ease of comparison with the corresponding data of the other cell lines.

DISCUSSION

The cadmium-resistant cell substrains (HE100 and Cl 1D100) provide an excellent model by which to define the significance of MT as a resistance factor. In a comparison of these cells with their corresponding parent lines (HE and Cl 1D) a remarkable similarity was demonstrated with regards to a wide range of cellular parameters, e.g., growth rate, cell cycle phase distribution, protein maps, and DNA repair activity (23, 24, 39). The only significant difference to emerge was the elevated level of MT of the cadmium resistant substrains. The results of numerous studies have shown the MT-rich cells to be resistant to the anticancer agents cis-dichlorodiammineplatinum, chlorambucil, and prednimustine (23–25). In each case a substantial proportion of the drug or its active metabolite was found associated with MT, indicating that sequestration by MT prevents the interaction of these compounds with susceptible intracellular targets. Using this model system we have also demonstrated resistance to ionizing radiation is conferred by elevated levels of MT (22). In this situation, where an increase in production of reactive oxygen species is implicated as the underlying cytotoxic mechanism, we propose MT functions as a free-radical scavenger. By virtue of the unusual structural feature of MT, i.e., the preponderence of cysteine residues and consequently a high thiol content, MT is believed to function as a sacrificial target for oxidative damage (19).

The data presented in the current study demonstrate that MT confers resistance to cell killing by TNF. In two different cell sensitivity assays, the MT-rich cells consistently exhibited a significant resistance to TNF compared to the corresponding parent lines. Although this...
study has not directly addressed the mechanism of TNF cell killing, the data produced is consistent with the hypothesis that it involves the production of free-radical moieties and that target cells with an adequate buffering capacity, such as the overexpression of the free-radical scavenger MT, are resistant to its effects. Similar conclusions were reached in a recent study when human MCF-7 mammary carcinoma cells acquired resistance to the cytotoxic action of TNF after transfection with the human MT-IIA gene placed under the control of a strong constitutive promoter (40). Surprisingly, the selection procedure for stable transfected cell lines itself resulted in a change in cell response to TNF such that the control-transfected cells (cells transfected with the plasmid vector lacking the MT-II complementary DNA) were also resistant to the cytotoxic effects of TNF. However, the MT-expressing cells were resistant to cadmium toxicity and to the synergistic cytotoxic effects of TNF and cadmium, compared with the control-transfected cells, suggesting that cadmium may sensitise cells to TNF killing. These data indicate that the relationship between overexpression of MT and resistance to the cytotoxic effects of TNF may be more complex than originally perceived and in the present study one should not disregard the possibility that changes other than increased MT expression underlie TNF resistance in the cells after prolonged growth in cadmium.

The thiol-containing protein, glutathione, has also been demonstrated to modify tumor response to TNF. Correlations between high intracellular glutathione levels and tumor resistance to TNF in vivo were found (13). The cell lines used in the present study were analyzed previously for glutathione content and no increase was detected in the MT-rich cells when compared to the parent cell lines (23).

Reactive oxygen species have long been implicated in a wide range of tissue injuries. The classic pattern of events involves free-radical initiated chain reactions of lipid peroxidation, leading to disruption of cellular phospholipids, proteins, and DNA. Mitochondria, which have a high proportion of unsaturated fatty acids, are particularly susceptible to lipid peroxidation with the consequence that energy metabo-
lism is severely disrupted. Correspondingly, the first cellular organs-elles to show signs of damage upon treatment with TNF are the mitochondria (12). Membrane blebs are reported in the mitochondria as little as 2 h after treatment, while within 4 h, evidence of perturbations of the cell nucleus is apparent. Superoxide generation in the mitochondria has been demonstrated to be a key factor in TNF cell killing. Overexpression of the mitochondrial enzyme, MnSOD, an enzyme which deactivates superoxide, conferred resistance to TNF (41). The corresponding depression in MnSOD levels exacerbated TNF cytotoxicity. Since complete protection to TNF cytotoxicity was not afforded by the MnSOD, it is possible that it functions in conjunction with other protective proteins, such as MT, in an orchestrated cell defense strategy.

The immunocytochemical data of the present study show that MT is freely distributed throughout the cytoplasm and nucleus. Its absence from the mitochondria might suggest complementary roles for MT and MnSOD in mediating defense to TNF cytotoxicity. Although generally considered a cytoplasmic protein, nuclear staining for MT has been reported in the liver parenchymal cells and kidney proximal convoluted tubular cells as an early response to cadmium exposure (42). The localization of nuclear MT may have particular relevance in TNF cytotoxicity with respect to the nuclear disruptions reported. Two types of DNA lesions are suggested in the literature. The first involves nonspecific strand breaks of DNA mediated by oxygen radical activity. In such cases the radical quenching reactivity conferred by the thiol groups of MT would enable the protein to limit the extent of damage. MT is even further equipped for repairing free-radical-induced injury by virtue of its high zinc content. Under normal physiological conditions MT occurs as a zinc and copper containing protein. The relationship between MT and zinc metabolism is so close it is suggested MT may serve as a zinc pool to provide zinc to metalloenzymes and the zinc-finger motifs of DNA-binding transcription factors. Previous analysis of MT isolated from the HE100 and Cl 1D100 cells revealed cadmium to be the predominant metal; however, substantial levels of zinc were also recorded (24). Recent investigations show that reactive oxygen species are capable of mobilizing zinc from the thiolate bonds of MT (43). In view of the important role zinc plays in the stabilization of membranes and its participation in a number of DNA and RNA polymerases, MT is strongly suggested as a resistance/repair mechanism against TNF-induced oxidative damage. Stabilization may be provided either directly by the thiol group quenching of free radicals or indirectly by donating zinc for the repair of injuries brought about by free-radical activity.

The second DNA lesion reported in TNF cell killing is DNA fragmentation. The appearance of nucleosome sized DNA pieces (183 base pairs) was inconsistent with the nonspecific DNA strand breaks caused by free-radical activity. Investigations revealed a zinc-sensitive endonuclease was responsible for DNA cleavage (44). Cleavage, which occurred in a dose-dependent manner, was discovered to be inhibited by the presence of zinc. Once again, MT may be implicated in hindering TNF cytotoxicity by virtue of its zinc content.

Whether it is the high zinc or the high thiol content of MT which confers resistance to TNF is not clear. Probably both are involved in making MT such a powerful resistance factor. Previously it has been shown that MT confers resistance to heavy metals, certain alkylating agents, and ionizing radiation. The present study indicates that MT may also give protection to the cytotoxic effects of TNF. MT may thus represent a multiresistance factor. It is important to find out if MT plays a role clinically. If so, it might be of value to inactivate or inhibit the synthesis of MT in neoplastic cells.

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