Effects of Tumor Necrosis Factor on Sensitive and Multidrug Resistant Human Leukemia and Myeloma Cell Lines

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Two human tumor cell lines exhibiting acquired multidrug resistance (MDR) with increased expression of a cell surface glycoprotein (GP-170) were tested for their sensitivity to human recombinant tumor necrosis factor (rTNF). The drug resistant mutant lines (CEM/V, a T-cell leukemia line resistant to vinblastine, and 8226/D, a multiple myeloma line resistant to doxorubicin), were markedly more sensitive to rTNF in clonogenic assay than were their drug-sensitive parental lines (CEM, 8226). As determined by radioreceptor assay, the number of cell surface receptors for rTNF did not differ on the parental and drug-resistant lines. During the first 24 hours after addition of rTNF, there was a decrease in intracellular ATP content in the CEM/V line but not in the CEM line. No differential effect of rTNF on ATP content was observed between 8226 and 8226/D.

A PLEIOTROPIC TYPE of acquired drug resistance of tumor cell lines to natural product anticancer drugs has been reported in association with increased expression of high-molecular-weight (mol wt) cell surface glycoproteins (GPs). Ling et al have characterized a specific 170-kilodalton (kd) membrane GP (GP-170 P-GP) associated with multidrug resistance. The gene encoding this GP resides on the distal long arm of chromosome 7. Tumor cells expressing the GP-170 often exhibit broad-spectrum resistance to a variety of structurally unrelated alkaloids and antibiotics (eg, vinblastine, doxorubicin), and occasionally, collateral sensitivity to other agents. The cross-resistance phenomenon appears to be due to reduced transmembrane transport and intracellular accumulation or retention of the drugs involved, whereas the mechanism of collateral sensitivity remains obscure. Nonetheless, this pleiotropy apparently is due to alterations in membrane structure and function in the resistant cells. Although induction of this form of drug resistance has been achieved primarily with in vitro techniques, drug-resistant tumor cells from several ovarian cancer patients were recently reported to exhibit the P-GP phenotype immunologically after in vivo treatment with cancer chemotherapy. Increased expression of the RNA message for GP-170 is increased in some tumors from patients who have relapsed after receiving chemotherapy as well as in tumor biopsies obtained from patients before they received any therapy. These findings suggest that this form of drug resistance may prove clinically important. The observation that multidrug resistance may be attributable to specific cellular abnormalities in molecular transport provides the potential that specific pharmacologic or immunologic approaches may be used to overcome multidrug resistance.

Human recombinant tumor necrosis factor (rTNF) is a macrophage-derived cytokine that was recently produced by recombinant DNA technology. In the course of studying the in vitro antitumor effects of rTNF, we found that two GP-170 expressing multidrug-resistant (MDR) human tumor cell lines developed in vitro from leukemia and multiple myeloma patients exhibited markedly enhanced sensitivity to rTNF.

MATERIALS AND METHODS

Tumor cell lines, drugs, and culture methods. The human tumor cell lines used in these studies included the human T-cell leukemia line CEM and its vinblastine-resistant subline CEM/V. CEM and CEM/V were provided for our studies by Dr W. Beck (St Jude's Hospital, Memphis, TN), who previously published characteristics of this line. The 8226 human multiple myeloma line was obtained from the American Type Culture Collection (Rockville, MD). A doxorubicin-resistant subline 8226/D was developed by one of us and previously reported. Both drug-resistant lines were developed by prolonged cultivation in vitro with addition of gradually increasing concentrations of the respective anticancer drug. Doxorubicin (Adria Laboratories, Columbus, OH) and vinblastine (Eli Lilly, Indianapolis) were obtained commercially. rTNF was provided by Genentech (South San Francisco).

Clonal growth and drug sensitivity of the cell lines were assessed by soft agar assay. All drugs were tested by continuous exposure in the agar. Tumor cells were seeded from log-phase suspension cultures at a concentration of 10^3 cells/3 mL Petri dish. Cloning efficiencies of these cell lines in soft agar were from 2% to 5%. Multi-log dose-responses to rTNF were obtained with the rTNF added at the requisite concentrations in a total volume of 0.1 mL per plate. Other drugs (eg, doxorubicin, vinblastine) were also added: 0.1 mL per plate. All experimental points were determined in...
triplicate, and all colony-forming studies were repeated on several occasions to confirm reproducibility of results. Colony counts were
determined with an Omnicon image analyzer, with requirements that all colonies counted be at least 60 μm in diameter and be viable as determined by in situ tetrazolium staining. Sensitivity of the lines to various agents was compared by determining the dose of each agent that reduced colony formation to 50% of control (ID₅₀).

Biochemical methods. Cell-surface receptors for rTNF were determined by radioreceptor assay using the method described by Aggarwal et al. rTNF was radioiodinated with ¹²⁵I using the iodogen reaction, and the radiolabeled protein was separated from free radiolabeled chromatographically on a Sephadex G-25 column (Pharmacia, Piscataway, NJ) and collected in 3% bovine serum albumin. A specific enzyme-linked immunosorbant assay for rTNF was developed (vinblastine and doxorubicin, respectively) than did their respective parent lines. In contrast to the results with the cytotoxic drugs, both CEM/V and 8226/D exhibited markedly increased sensitivity to rTNF as compared with their parental lines. The effects of vinblastine and rTNF on CEM and CEM/V are shown in Fig 1, and the effects of doxorubicin on 8226 and 8226D are shown in Fig 2. Calculations of relative resistance of the cell lines to the cytotoxic agents and to rTNF are summarized in Table 1. At the calculated ID₅₀ values, the MDR cell lines exhibited rTNF sensitivity that was at least two orders of magnitude greater than was observed with the drug-sensitive parental lines. The

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Tumor cell sensitivity in clonogenic assay. Both CEM and CEM/V and 8226 and 8226/D were tested for cellular sensitivity to vinblastine, doxorubicin, and rTNF. Both CEM/V and 8226/D exhibited marked resistance to both vinblastine and doxorubicin as compared with their parental drug sensitive lines. On a quantitative basis, CEM/V and 8226/D exhibited at least two orders of magnitude less sensitivity to the agents against which resistance was developed (vinblastine and doxorubicin, respectively), than did their respective parent lines. In contrast to the results with the cytotoxic drugs, both CEM/V and 8226/D exhibited markedly increased sensitivity to rTNF as compared with their parental lines. The effects of vinblastine and rTNF on CEM and CEM/V are shown in Fig 1, and the effects of doxorubicin on 8226 and 8226D are shown in Fig 2. Calculations of relative resistance of the cell lines to the cytotoxic agents and to rTNF are summarized in Table 1. At the calculated ID₅₀ values, the MDR cell lines exhibited rTNF sensitivity that was at least two orders of magnitude greater than was observed with the drug-sensitive parental lines. The

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\text{Fig 1. (A) Effects of vinblastine on colony-forming cells from the human CEM T-cell leukemia line (---) and the CEM/V vinblastine-resistant subline (-----). (B) Effects of rTNF on these same two lines. The MDR CEM/V cell line is significantly less sensitive to vinblastine and more sensitive to rTNF than is the parental line.}
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CEM and CEM/V and the 8226 and 8226/D lines were also exposed to vinblastine or rTNF with the calcium-channel blocker verapamil added (data not shown). Verapamil (3 \(\mu g/mL\)) had no effect on the intrinsic sensitivity of the parental CEM or 8226 lines to vinblastine; however, at this concentration it partially reversed the vinblastine resistance exhibited by the CEM/V and 8226/D MDR lines. Similar observations were reported previously by Tsuro et al.\(^2\) Verapamil had no effect on the rTNF sensitivity profiles of these tumor cell lines.

Radiolabeled rTNF binding by tumor cell lines. The binding of radiiodinated rTNF to parental and MDR sublines was assessed of both CEM and 8226 and determined at 4\(^\circ\)C. At no time period tested were differences observed in rTNF binding between the drug-resistant cell lines and their parental lines. At 120 minutes, there was approximately twofold greater binding of rTNF to the 8226 than to the CEM cell lines. The CEM and CEM/V were also studied for labeled rTNF binding at 37\(^\circ\)C. At 120 minutes, total binding of labeled rTNF to the CEM and CEM/V was similar at both 4 and 3\(^\circ\)C. At 37\(^\circ\)C, binding of \(^{125}\)I-rTNF to these lines increased substantially with increasing exposure time but did not differ between the parental and MDR sublines.

**Intracellular ATP content.** ATP content was measured in the cell lines to determine whether continuous TNF exposure had any early or persisting effect on cellular energy stores. ATP content decreased by 42% in the first 4 hours after exposure of CEM/V cells to 1,000 U rTNF, and this reduction persisted for at least 24 hours. In contrast, ATP content of the parental CEM cells was not reduced by rTNF exposure. In the myeloma lines, no differential effect was observed in the content of ATP in sensitive or MDR cells. Exposure of 8226 and 8226D cells to 1,000 U rTNF reduced intracellular content of ATP by 66% within 4 hours, with complete recovery within 24 hours despite continued rTNF exposure.

Expression of total cellular RNA for P-GP. The hypothesis that the enhanced cytotoxic effect of rTNF on P-GP-expressing cells was mediated by altering GP-170 expression and was tested by analyzing CEM/V and 8226/D total cellular RNA after various periods of exposure to 1,000 U rTNF. Figure 3 shows the results of RNA dot-blot analysis of 8226/D. High levels of P-GP transcripts were detected in all specimens. After exposure to rTNF, no significant reduction of GP-170 RNA occurred with up to 36 hours of exposure in 8226/D (or in CEM/V, data not shown).

Effects of rTNF on drug/metabolite transport. As an index of the effect of rTNF on cellular transport and accumulation of drugs and metabolites by drug-resistant cells, the accumulation of \(^{14}\)C-doxorubicin by 8226/D cells was studied after a 4-hour preincubation in control medium or with 10, 100, or 1,000 U rTNF added. Labeled doxorubicin \((5 \times 10^{-7} \text{ mol/L at an approximate } ID_{so} \text{ dose})\) was then added for 1 hour. \(^{14}\)C-Doxorubicin accumulation ranged from 880 to 993 cpm in the different aliquots. Thus, rTNF did not have a significant effect on radiolabeled doxorubicin accumulation by the drug-resistant cells.
Fig 3. Dot-blot analysis of 8226/D cells for expression of total cellular RNA for P-GP. High basilar levels of P-GP expression were observed and were not influenced by 1,000 U rTNF for up to 36 hours of exposure. Twofold dilutions of total cellular RNA were spotted on nitrocellulose and hybridized with the P-GP cDNA probe. There was a minor loading error at a dilution of 1:4 at the 0-hour time point, which is slightly exaggerated. Densitometric tracings of the original dot blots demonstrated that within the limits of experimental error for this procedure there were high levels of P-GP transcripts at all time points.

DISCUSSION

In these studies, we documented the marked increase in cytotoxicity of rTNF on clonogenic tumor cells from two MDR human tumor cell lines expressing the P-GP. The degree of collateral sensitivity of these drug-resistant cells was marked, suggesting that the development of drug resistance by the tumor cells and the enhanced rTNF sensitivity may be more than coincidental. Several hypotheses were tested in an attempt to characterize this phenomenon. The first hypothesis was that MDR cells expressed increased numbers of cell surface receptors to rTNF. Radioreceptor analysis established that there was no alteration in rTNF receptor expression by drug-resistant cells. The second hypothesis was that rTNF exposure altered the expression or function of the energy-dependent (ATP) P-GP transport pump in the cell membranes of drug-resistant cells. Cowan et al recently developed the hypothesis that P-GP expression by cells represents a normal stress reaction of mammalian cells to natural product cytotoxins in the environment. One hypothesis we considered was that the cytotoxic action of rTNF on drug-resistant cells might be related to the expression of a functional P-GP. This might be mediated by blocking the expression or function of this transport pump by inhibition of elimination of a normal intracellular cytotoxic metabolite. To test this hypothesis, the drug-resistant human leukemia and myeloma cells were probed to determine whether rTNF had any early effects on either the expression of the total cellular RNA for GP-170 or the function of the pump as reflected by studies of doxorubicin accumulation with rTNF added and by assessment of overall cellular energy stores during the initial 24 hours of exposure as reflected by intracellular stores of adenosine triphosphate. Although the CEM/V line had its cellular ATP level depressed early and for at least 24 hours after TNF exposure, this effect was not observed in 8226/D or the parental lines. We cannot exclude a later effect on subsequent depletion on energy stores on the 8226/D line, as the focus of these studies was on early biochemical events after rTNF exposure and before significant cell death had occurred. The lack of alteration of the degree of TNF sensitivity in the resistant lines by verapamil and the lack of effect of TNF on doxorubicin accumulation suggest that the collateral sensitivity to TNF is not directly related to P-GP function. No significant differences were observed in other parameters in drug-resistant cells as compared with drug-sensitive parental tumor cell lines even though prolonged exposure to rTNF had a significant differential effect on clonogenicity in soft agar of both drug-resistant lines. The precise mechanism responsible for the enhanced sensitivity to rTNF by these drug-resistant cell lines thus remains elusive, and further studies are warranted. Meyers et al reported that several MDR cell lines exhibit increased expression of the cell surface receptor for epidermal growth factor. Recently, Donato et al observed that TNF induces rapid dose-dependent phosphorylation of EGF receptors in cells exhibiting cytotoxicity to TNF. Analysis of EGF receptor expression in the CEM and 8226 cell lines and their MDR sublines would therefore be of interest. We have recently documented the association of increased P-GP expression (immunohistochemically) with in vitro doxorubicin resistance in biopsy samples from patients with myeloma and lymphoma. Because rTNF is currently in clinical trials for cancer therapy, it will also be of interest to determine whether rTNF has enhanced therapeutic activity in patients with leukemia, lymphoma, or myeloma whose tumors have acquired resistance to standard agents such as the vinca alkaloids and doxorubicin, and also exhibit increased expression of P-GP.

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REFERENCES


TNF EFFECTS ON MDR LEUKEMIA AND MYELOMA CELLS


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