Tumor necrosis factor induces doxorubicin resistance to lung cancer cells in vitro

Tumor necrosis factor can alter the cell cycle of tumor cells and protect hematopoietic stem cells from cell cycle-specific chemotherapy, but the ability of tumor necrosis factor to protect cancer cells from chemotherapy by manipulation of the cell cycle is unknown. Twenty-four-hour exposure of A549 human lung cancer cells to tumor necrosis factor shifted cells from S phase to G₀/G₁ phase as determined by analysis of isolated cell nuclei with an FACScan Cell Sorter. This effect was not seen in cells exposed to interleukin-1 or interleukin-6. Growth assays demonstrated that tumor necrosis factor slowed the doubling time of A549 cells, confirming that tumor necrosis factor caused G₀/G₁ arrest in these cells. Pretreatment with tumor necrosis factor rendered cells resistant to subsequent 1-hour exposure to doxorubicin, a chemotherapeutic agent active against S phase cells. Tumor necrosis factor did not protect cells against either cisplatin or mitomycin C, drugs not specific for S phase. Measurement of intracellular drug levels indicated that pretreatment with tumor necrosis factor did not affect doxorubicin uptake or efflux. These findings suggest that cells producing tumor necrosis factor within a tumor may render surrounding malignant cells resistant to cell cycle-specific chemotherapy, and this mechanism may explain failure of sequential immunotherapy-chemotherapy protocols. (J THORAC CARDIOVASC SURG 1994;107:43-9)

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The growth of a tumor cell is described by a sequential series of events called the cell cycle.¹⁻³ The cell cycle is composed of four phases called G₁, S, G₂, and M (Fig. 1, A). Cells that are actively synthesizing deoxyribonucleic acid (DNA) in preparation for cell division are in S phase, and M phase refers to dividing cells in mitosis. The "gap phases," which fall between mitosis and DNA synthesis, are called G₁ and G₂. G₂ and M phase cells have gone through DNA synthesis and contain double, or 2N, amounts of DNA, whereas G₁ cells are the products of cell division and contain N amounts of DNA (Fig. 1, B). With certain stimuli, cells can be induced to leave the cell cycle, yet remain capable of reentering the cell cycle and

proceeding through normal cell division.^{2,3} These "resting" or nondividing cells are morphologically the same as G₁ cells and are said to be in G₀ phase (Fig. 1). Because G₀ and G₁ cells contain the same amount of DNA and are difficult to distinguish, resting cells are often referred to as G₀/G₁ cells.

Cell cycle arrest can be achieved by cytokines acting at major control points in G₁,^{4,5} including tumor necrosis factor (TNF).^{6,7} Several studies have shown that cells arrested in G₀/G₁ by TNF are protected against subsequent cytotoxic insults. Cell cycle arrest in G₀/G₁ by TNF can protect hematopoietic stem cells against radiation and cell cycle-specific cytotoxic chemotherapy.^{8,9} Moreover, TNF induces radioresistance in mice, possibly by a similar mechanism.¹⁰⁻¹² Little is known, however, about the potential for TNF to protect cancer cells from chemotherapeutic agents by G₀/G₁ arrest.

Resistance to cytotoxic chemotherapy is a common cause of treatment failure in patients with advanced non-small-cell lung cancer. Although doxorubicin, an anthracycline antibiotic, is active against non-small-cell lung cancer in vitro, overall clinical response rates are less than

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Fig. 1. A, Phases of the mammalian cell cycle. G_1 and G_2 represent the "gap" phases between DNA synthesis (S) and mitosis (M). Cytokines such as TNF can cause cells to enter the "resting" phase (G_0), where they can remain quiescent for an indefinite period, reentering the cell cycle after appropriate stimuli. B, Representation of DNA histogram obtained by analysis with an FACScan Cell Sorter. Staining cell nuclei with propidium iodide allows measurement of the number of events (vertical axis) at a given level of fluorescence (horizontal axis). G_0 and G_1 cells contain N amount of DNA, G_2 and M cells contain $2N$ DNA, and S phase cells contain between N and $2N$ DNA.

20% when doxorubicin is used as single-agent chemotherapy.¹³ In vitro, doxorubicin exerts greater effects on exponentially growing cells than on resting cells.¹⁴ Therefore, because TNF inhibits growth of lung cancer cell lines,¹⁵ we hypothesized that lung cancer cells may become resistant to doxorubicin after cell cycle changes induced by TNF exposure. Because tumors contain a variety of nonmalignant cells that secrete TNF, such as

tumor-associated macrophages,¹⁶ this effect could explain the resistance of some types of cancer to chemotherapy.

Materials and methods

Cell culture. A549 human lung adenocarcinoma cells were obtained from American Tissue Culture Collection (Rockville, Md.). The cell line was passaged and maintained in RPMI medium (Roswell Park Memorial Institute, Buffalo, N.Y.) supplemented with 10% fetal calf serum, 0.03% glutamine, and 2% penicillin-streptomycin solutions (Biofluids, Inc., Rockville, Md.). Cultures were maintained at 37° C in a humidified atmosphere of 5% carbon dioxide and 95% air. Exponentially growing cells were used in all experiments.

Chemicals. Recombinant human TNF was supplied by Genentech (San Francisco, Calif.), and recombinant human interleukin-1 (IL-1) was supplied by Hoffmann-La Roche, Inc. (Nutley, N.J.). Recombinant human IL-6 was purchased from Endogen (Boston, Mass.). Doxorubicin, cisplatin, and mitomycin C were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Flow cytometric DNA analysis. A549 cells were incubated in 100 mm plastic dishes (Costar, Cambridge, Mass.) in various concentrations of TNF, IL-1, or IL-6 for 24 hours, harvested with trypsin-versene solution (Biofluids), and washed in RPMI medium. The pellet was resuspended in 0.2 ml cell lysis citrate buffer containing spermine for nuclear stabilization.^{17, 18} After 10 minutes' incubation at room temperature, 2.0 ml trypsin inhibitor solution containing ribonuclease-A (Sigma) was added to obtain isolated nuclei. The solutions were resuspended, incubated at room temperature, and filtered through a 30 μ m nylon mesh. The nuclei were then stained with propidium iodide solution (Sigma). The samples were analyzed by flow cytometry with an FACScan 3 Cell Sorter, and the cell cycle data were analyzed by CELLFIT program software (Becton-Dickinson, Sunnyvale, Calif.). This experiment was repeated four times.

Growth assays. Cells were plated in T-25 flasks (Nunc, Roskilde, Denmark) at a density of 20,000 cells in 3.0 ml per flask. After overnight incubation, medium was replaced with fresh medium containing TNF at various concentrations. Cells were harvested with trypsin-versene solution on day 3 and counted on a Coulter cell counter (Coulter Electronics, Hialeah, Fla.). This experiment was performed three times with duplicate values for each time point.

Clonogenic assays. A total of 500,000 cells were plated in 100 mm plastic dishes (Costar) and incubated overnight. Medium was then replaced with medium containing various concentrations of TNF. After a 24-hour incubation, the TNF was removed and the cells were washed and then exposed to doxorubicin, mitomycin C, or cisplatin at various concentrations for 1 hour. The drug-containing media were removed, and the cells were washed three times with phosphate-buffered saline solution. The cells were then harvested with trypsin-versene solution, suspended in complete media, centrifuged, resuspended, and counted with a Coulter counter. Cell viability as assessed by trypan blue staining correlated closely with the counts obtained with the Coulter counter. Cells were then serially diluted and plated in 100 mm and 60 mm plastic culture dishes (Costar) at densities of 100,000, 5000, and 250 cells per plate. The plated cells were allowed to develop colonies over an 8-day

Fig. 2. A, Cell cycle effects of TNF. Twenty-four-hour exposure of A549 cells to TNF caused a shift from S phase to G₀/G₁ phase. B, Effect of TNF pretreatment on sensitivity to doxorubicin. A549 cells pretreated with varying concentrations of TNF were less sensitive to subsequent 1-hour treatment with doxorubicin 5 μmol/L (* $p_2 < 0.05$ versus 0 TNF).

interval after which they were fixed in 3:1 methanol/acetic acid solution and stained with crystal violet. Colonies of more than 50 cells were then counted under a microscope, and a plating efficiency was calculated as the number of colonies divided by the number of cells plated. Survival fractions were calculated by dividing the plating efficiency of drug-treated cells by the plating efficiency of untreated control cells. Triplicate values were obtained from each experiment, and each experiment was repeated at least three times.

Measurement of TNF influence on intracellular doxorubicin content. Intracellular doxorubicin levels were measured by fluorescence spectrophotometry. Briefly, 100,000 A549 cells were plated in 100 mm plastic dishes and incubated overnight. Cells were then exposed to TNF 1.0 μg/ml or medium alone for 24 hours. After removal of medium, cells were then exposed to doxorubicin 10 μmol/L for various durations. Cells were washed, harvested with trypsin-versene solution, centrifuged, resuspended in saline solution, and counted on a Coulter counter. Cells were then centrifuged and resuspended in a 1:1 mixture of alcohol/0.3N hydrochloric acid to extract intracellular doxorubicin. After the solution was mixed vigorously in a

vortex, a fluorescence spectrophotometer was used to measure the relative fluorescence of each sample at an excitation wavelength of 479 nm and an emission wavelength of 593 nm. Results were expressed as the relative fluorescence per 100,000 cells. The experiment was repeated three times.

Statistical analysis. Results were expressed as means $\pm$ standard errors. Student's paired *t* test was used to compare mean values.¹⁹ Results were considered statistically significant for $p_2 < 0.05$.

Results

Effects of cytokines on cell cycle and growth of A549. The effect of a 24-hour exposure of various cytokines on A549 cell cycling was evaluated by determination of the DNA content of nuclei isolated from cytokine-exposed cells with an FACScan Cell Sorter. This revealed that TNF caused an increase in the number of cells in G₀/G₁ phase, with a corresponding decrease in cells in S phase (Fig. 2, A). The cell cycle effects of TNF were con-

Fig. 3. Effect of 24-hour pretreatment with TNF 1.0 $\mu\text{g/ml}$ on sensitivity of A549 cells to varying concentrations of doxorubicin, cisplatin, and mitomycin C. TNF caused cells to become relatively resistant to doxorubicin (A). Cells were not protected against cisplatin (B) or mitomycin C (C) (* $p_2 < 0.05$ versus no TNF pretreatment).

firmed by growth assays in which TNF significantly inhibited growth at 72 hours ($25 \pm 7 \times 10^5$ versus $33 \pm 9 \times 10^5$, TNF 1.0 $\mu\text{g/ml}$ versus no TNF, $p_2 = 0.03$). These experiments showed that TNF concentrations that increased the percentage of cells in the G_0/G_1 resting phase of the cell cycle caused a concordant inhibition of growth of A549 cells. Incubation with IL-1 or IL-6 up to 1000 units/ml did not affect the cell cycle of A549 cells, which indicates that the effect was specific for TNF and not generalized to all cytokines (data not shown).

Effects of TNF pretreatment on sensitivity to chemotherapy. After demonstrating that TNF exposure shifts A549 cells to the G_0/G_1 phase of the cell cycle, we investigated the effects of TNF pretreatment on the sensitivity of A549 cells to different chemotherapeutic agents. Twenty-four-hour pretreatment with TNF in varying concentrations resulted in an increase in surviving fraction in A549 cells subsequently exposed to doxorubicin 5000 nmol (Fig. 2, B). We then examined the effect of pretreatment with TNF on chemosensitivity of A549 cells to varying concentrations of doxorubicin, cisplatin, and mitomycin C. Twenty-four-hour pretreatment with TNF 1.0 $\mu\text{g/ml}$ resulted in attenuation of doxorubicin cytotoxicity at doses of 2.5 $\mu\text{mol/L}$ or more (Fig. 3, A). However, TNF pretreatment did not protect cells against two other drugs, cisplatin and mitomycin C, which are not cell cycle specific²⁰ (Fig. 3, B and C).

Effect of TNF on intracellular doxorubicin levels. We examined the effect of TNF on intracellular doxorubicin levels to eliminate decreased drug uptake and increased drug excretion as possible mechanisms for the attenuated sensitivity to doxorubicin seen in TNF-exposed cells. Levels of doxorubicin in TNF-exposed and control cells were similar during uptake as measured at 15, 30, and 60 minutes (Fig. 4, A). Likewise, TNF pre-

treatment did not cause a decrease in drug levels during the efflux phase at 0 and 1 hour (Fig. 4, B).

Discussion

Doxorubicin tumor cytotoxicity may involve several mechanisms, including topoisomerase-II-dependent DNA damage.²⁰ Topo II, a nuclear matrix protein that modulates DNA topology and is involved in cell division, shows increased activity in S phase cells.²¹ Drugs targeted against Topo II are most active against logarithmically growing cells,²¹⁻²³ possibly through cell cycle-dependent mechanisms.²⁴ Our data show that 24-hour pretreatment of A549 cells with TNF shifts cells into the G_0/G_1 resting phase of the cell cycle, subsequently causing these cells to become resistant to doxorubicin (Figs. 2 and 3). Comparison of doxorubicin toxicity with S phase percentage at various TNF doses reveals an inverse relationship (Fig. 2), indicating that as the percentage of dividing cells in a population decreases, doxorubicin resistance increases. These TNF-pretreated cells required a twofold increase in doxorubicin concentration to achieve levels of cytotoxicity equal to those of non-TNF exposed cells (Fig. 3). The fact that TNF did not protect A549 cells against cisplatin, or mitomycin C, two alkylating agents that are active against cells in all phases of the cell cycle,²⁰ gives further evidence that TNF-induced doxorubicin resistance is a cell cycle effect.

Alternative hypotheses for the protective effect of TNF seen in these experiments include increased free radical buffering capacity and decreased intracellular drug levels. Several studies have implicated the involvement of oxygen-derived free radicals in doxorubicin,²⁵⁻²⁷ cisplatin, and mitomycin C cytotoxicity,²⁰ and free radical scavengers, particularly glutathione-dependent enzymes, can render cells resistant to these drugs.²⁸⁻³³ However, where-

Fig. 4. Effect of TNF pretreatment on intracellular doxorubicin levels. After 24-hour pretreatment with TNF 1.0 $\mu\text{g}/\text{ml}$ and subsequent exposure to doxorubicin 10 $\mu\text{mol}/\text{L}$, cells were harvested at various time points, doxorubicin was extracted and measured as described in the *Materials and methods* section, and doxorubicin concentration was determined by measuring the fluorescence of each sample. Relative fluorescence of TNF-pretreated and control cells indicated that TNF did not affect doxorubicin uptake (A) or efflux (B).

as A549 cells produce increased messenger ribonucleic acid transcripts of the free radical scavenger manganous superoxide dismutase in response to TNF, glutathione levels are unchanged.³⁴ In addition, TNF pretreatment did not protect cells against either mitomycin C or cisplatin (Fig. 3), which suggests that increased buffering capacity alone does explain TNF-mediated doxorubicin resistance.

Decreased intracellular drug accumulation is another potential mechanism for TNF-induced doxorubicin resistance. The phenomenon of multidrug resistance as mediated by the P-170 glycoprotein is a common cause of doxorubicin resistance in cancer chemotherapy.¹³ Doxorubicin-resistant cell lines that express the multidrug resistance phenotype can be developed under selective pressure of continuous doxorubicin exposure. Resistant cells will actively pump drug out of the cell, resulting in much lower intracellular drug levels than the parent cell line. Our data indicate that this mechanism is not involved in TNF-induced doxorubicin resistance, because TNF pretreatment did not cause reduced intracellular doxorubicin levels (Fig. 4).

The A549 cell line was used because of its excellent plating efficiency, which lends this line to survival analysis by clonogenic assays. Its inherent *in vitro* chemosensitivity and radiation sensitivity have been explored in the past. Although it is likely that the observations seen here

would apply to other lung cancer cell lines, it is of great interest to see whether cell lines of varying non-small-cell histologic types act in a similar fashion.

In summary, we have shown that TNF pretreatment renders A549 lung cancer cells resistant to the chemotherapeutic agent doxorubicin *in vitro* by shifting cells to the G_0/G_1 resting phase of the cell cycle. This finding implies that inflammatory cells that elaborate TNF, such as tumor-associated macrophages,¹⁶ might confer doxorubicin resistance to surrounding tumor cells. Exposure to doxorubicin while in G_0/G_1 might then promote the multidrug resistance phenotype in tumor cells or increase production of free radical buffering enzymes and enable the cells to retain doxorubicin resistance on reentering the cell cycle. Although another recent report indicates that other factors elaborated by macrophages can render cells resistant to doxorubicin,³⁵ further studies are necessary to determine the role of TNF and other cytokines in drug resistance. The increased resistance of the cell line to subsequent treatment with doxorubicin may have direct clinical applicability, for if patients are treated with innovative trials involving immunotherapy either directly with TNF or with cytokines whose mechanism may be linked to TNF (i.e., IL-2), it may be possible that short-term doxorubicin resistance could be imparted to certain clones. This would be particularly hazardous in combination immunotherapy-chemotherapy trials. Neverthe-

less, the effect may be time limited and may have little importance. The potential to answer clinical relevancy of the in vitro findings can be pursued in vivo with the use of immunodeficient animals.

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Discussion

Dr. Martin F. McKneally (*Toronto, Ontario, Canada*). Dr. Prewitt, is there a way that you can take advantage of this seemingly negative effect, for example, by synchronizing cells and then restarting them once you have stopped the cell cycle?

Dr. Prewitt. Theoretically, TNF could be used to arrest cells arrested in G₀/G₁ and then stimulated to reenter the cell cycle as a synchronized population. Treatment with an S phase-specific drug such as doxorubicin while the synchronized cells are synthesizing DNA could increase the chemotherapeutic effect, because a much larger population of cells would be sensitive to the drug. However, in vivo synchronization of cancer cells has not yet been accomplished and remains an area of active investigation in the field of cell cycle research.