Role of Nitric Oxide in Lysis of Tumor Cells by Cytokine-activated Endothelial Cells

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ABSTRACT

The purpose of these studies was to determine whether nitric oxide produced by cytokine-activated murine lung vascular endothelial cells plays a role in their lytic destruction of M-5076 reticulum cell sarcoma. Vascular endothelial cells harvested from perfused lungs of mice were adapted to grow in culture. Cloned lines ascendant to be of endothelial origin were incubated in vitro with interferon-γ and tumor necrosis factor. Lysis of radiolabeled tumor cells and accumulation of nitrite in the culture medium were determined at several time points. The concentration of nitrite in the culture medium directly correlated with endothelial cell-mediated tumor cell lysis. Endothelial cells cultured in L-arginine-free medium did not produce significant tumor cell lysis nor accumulation of nitrite in the medium. Both tumor cell lysis and nitrite accumulation were observed when the deficient medium was reconstituted with L-arginine, suggesting that endothelial cell-mediated tumor lysis was dependent on L-arginine, a precursor of nitric oxide. Moreover, specific inhibition of nitric oxide synthesis by Nω-methyl-L-arginine resulted in complete inhibition of endothelial cell-mediated lysis of the M-5076 reticulum cell sarcoma. Similarly, treatment of cytokine-activated endothelial cells with dexamethasone inhibited both target cell lysis and production of nitrite. Collectively, these results suggest that nitric oxide plays a major role in the lysis of tumor cells mediated by cytokine-activated endothelial cells.

INTRODUCTION

The majority of tumor cells that enter the circulation rapidly die (1-4). For example, quantitative analysis of the distribution and fate of B16 melanoma cells labeled with 125I labeled dUTP demonstrated that less than 0.1% of circulating tumor cells survive to proliferate into secondary lesions (1). Many factors can account for the death of these circulating tumor cells, among them mechanical trauma (5), tumor cell deformability (6), aggregation of tumor cells (7), host defenses such as T-cells (8), natural killer cells (9), macrophages (10), and interaction with vascular endothelial cells (7, 11). In the late case, structural or functional alteration of lung endothelial cells has been shown to alter the incidence of lung metastases (12, 13). Furthermore, the adhesion of tumor cells to organ-specific microvessel endothelial cells has been correlated with preferential metastasis (7, 14-17).

The murine M-5076 reticulum cell sarcoma is an example of a tumor displaying preferential organ metastasis (18). Subsequent to s.c. or i.v. implantations, this tumor produces local growth and metastasis to peritoneal organs. Metastasis to lung tissue is not found, which was initially attributed to the inability of the M-5076 cells to grow in the lung (19). More recently, however, we observed that subsequent to intrabronchial implantation, M-5076 cells produce large local tumors in the lung (20). Since i.v. injection (no lung growth) delivers the cells to the lung capillary bed, whereas intrabronchial injection (growth in the lung) delivers the cells to the airspace, we speculated that the interaction of M-5076 cells with lung endothelial cells may prevent their survival and growth. This would suggest that lung endothelial cells could play a selective-regulative role in the metastasis of M-5076 cells.

Recently, we have demonstrated that murine lung vascular endothelial cells can be activated in vitro by incubation with IFN-γ and TNF-α to directly lyse a variety of tumorigenic cells by a mechanism that was independent of both cytokines (20). We therefore began to search for a mechanism to explain endothelial cell-mediated tumor cell lysis. One possibility, nitric oxide, is an endothelium-derived relaxing factor released by the vascular endothelial cells in response to a number of stimuli, including bradykinin, acetylcholine, and nitravosalidators (21-24). Experiments in dogs have suggested that this potent vasodilator is involved in the pathogenesis of septic shock and cytokine-induced hypotension (25, 26). Nitric oxide has also been implicated as a cytolytic and cytostatic factor in macrophage-mediated tumor cell cytotoxicity under in vitro conditions (27-29), resulting in the inhibition of mitochondria enzyme complexes (I and II) and DNA synthesis in target tumor cells (30-32). These findings prompted us to determine whether nitric oxide released by cytokine-activated endothelial cells is an important mediator of tumor cell cytotoxicity.

MATERIALS AND METHODS

Reagents. Recombinant human TNF-α (2.4 x 10^7 units/mg protein) was the gift of Cetus Corp. (Emeryville, CA). Recombinant murine IFN-γ (1.2 x 10^7 units/mg protein) was the gift of Genentech, Inc. (South San Francisco, CA). Endothelial cell growth factor, NMA, sulfanilamide, and naphthylenediamine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO).

Tumor Cells. The M-5076 tumor arose spontaneously in the ovary of a C57BL/6 mouse in the laboratory of Dr. W. F. Dunning (Papanicolaou Research Institute, Miami, FL) (18); we obtained it from Dr. D. P. Griswold (Southern Research Institute, Birmingham, AL). This tumor has been characterized in our laboratory as a reticulum cell sarcoma (19). After i.v., s.c., or i.p. implantations, the tumor cells produce metastases in peritoneal organs but not lung metastases (19).

M-5076 cells were grown in culture in RPMI 1640 supplemented with endotoxin-free 15% horse serum, sodium pyruvate, and L-glutamine (M. A. Bioproducts, Walkersville, MD).

Endothelial Cells. Vascular endothelial cells were isolated from the lungs of C57BL/6 x C3H HeN mice (B6C,F,) by a pulmonary perfusion method described previously (20). The isolated lung vascular endothelial cells were grown in culture and cloned. The cloned endothelial cells were found to be nonthrombogenic and stained positive for factor VIII-related antigen using an immunofluorescent stain (20). They were also found to be positive for angiotensin-converting enzyme activity and demonstrated phagocytosis of acetylated-low density lipoproteins (20). These cells did not stain positive for macrophage (Mac-1, -2, -3, F4/80) or T-lymphocyte (Thy-1) markers using fluorescein isothiocyanate-labeled immunofluorescent stains (20). The endothelial cells were maintained in medium 199 supplemented with 20% fetal calf serum, 2-mercaptoethanol, 50 μg/ml of penicillin, 50 μg/ml of streptomycin, 2 μg/ml of sodium selenite, and 10% fetal bovine serum (GIBCO, Grand Island, NY).
serum (M. A. Bioproducts) and endothelial cell growth factor supplement (200 ng/ml) and used at passage 5, 6, or 7.

Vascular Endothelial Cell-mediated Tumor Cell Lysis. The endothelial cell-mediated cytotoxicity assay was based on a cytotoxicity assay described previously for macrophages (33). Briefly, M-5076 target cells in exponential growth phase were incubated for 24 h in RPMI 1640 containing [125I]IdUrd (0.2 μCi/ml; specific activity 2200 μCi/mmol; New England Nuclear, Boston, MA). Tumor cells were then washed 3 times with Hanks' balanced salt solution to remove free, unbound radiolabel, and then harvested by a short trypsinization. Viable tumor cells (measured by trypan blue dye exclusion) were added at 1 × 10⁶ to each 38-mm² well of 96-well plates containing confluent monolayers of endothelial cells (approximately 5 × 10⁵ cells/well). IFN-γ (10 units/ml) and TNF-α (10 units/ml) were added to the endothelial cell cultures at the same time as the target tumor cells (20). After 5 days of coculture, the culture medium was aspirated. The remaining viable adherent cells were lysed with 0.1 ml of 0.1 N NaOH. The cell lysate was absorbed onto cotton swabs and placed into 10 x 75-mm glass tubes. Radioactivity was monitored in a gamma counter. Endothelial cell-mediated cytotoxicity was calculated according to the formula:

\[ \text{% cytotoxicity} = \frac{|A - B|}{A} \times 100 \]

where \( A \) is the cpm of target cells cultured with endothelial cells incubated in medium, and \( B \) is the cpm of target cells cultured with cytokine-treated endothelial cells.

Nitrile Assay. Nitrile production was measured using a colorimetric assay as described previously (34). After 5 days of culture, a 50-µl aliquot of culture medium was removed with 50 µl of Greiss reagent (1% sulfanilamide and 0.1% naphthylenediamine dihydrochloride in 2% phosphoric acid). The mixture was incubated for 10 min with shaking at 25°C, and the A₅₅₀ was measured using a microplate reader (model 3550; Bio-Rad Corp., San Francisco, CA). Nitrile concentrations were determined by comparison with a standard solution of sodium nitrite in water. Values of background nitrile levels in cultures that had not been exposed to cytokines were subtracted from experimental values.

Statistical Analysis. The significance of the results was determined by using the Student's \( t \) test (2-tailed).

RESULTS

Lysis of M-5076 Tumor Cells by Cytokine-activated Murine Lung Endothelial Cells. The lysis of M-5076 cells by IFN-γ and TNF-α-activated endothelial cells was determined by the release of radioactivity from target cells prelabeled with [125I] IdUrd. Endothelial cells were incubated with target cells in medium containing IFN-γ (10 units/ml) and TNF-α (10 units/ml). The effector:target ratio was 5:1. Significant lysis of target cells began on day 3 of coculture (37%, \( P < 0.001 \)), and maximal target cell lysis (86%, \( P < 0.001 \)) was obtained after 5 days of cocultivation (Fig. 1A). Control studies demonstrated that the incubation of M-5076 cells in medium with IFN-γ and TNF-α did not lead to cell lysis. These data confirmed our earlier report that IFN-γ and TNF-α activated endothelial cells to lyse the target cells (20).

Correlation between Nitrile Production and Endothelial Cell-mediated Tumor Cell Lysis. We next examined the possibility that nitric oxide, a known mediator of target cell lysis by activated macrophages (27–29), may be associated with or involved in target cell lysis. Since nitric oxide spontaneously oxidizes to nitrates and nitrate in vitro (29), we measured nitrite accumulation.

Nitrile accumulation by cytokine-activated endothelial cells and by cytokine-activated endothelial cells cocultured with M-5076 cells increased within 24 h after the addition of cytokines. Maximal accumulation was found after 96-h incubation (Fig. 1B). Neither M-5076 or endothelial cells alone nor tumor cells treated with the cytokines produced any significant levels of nitrite (\( P > 0.1 \)) (Fig. 1B). The kinetics of nitric oxide production by cytokine-activated endothelial cells at 3 to 4 days was correlated with the level of tumor cell lysis at days 4 and 5 of coculture (Fig. 1, A and B). Specifically, 48 h after the addition of cytokines to endothelial cells, nitrite accumulation reached 80 µM and significant target cell lysis was found 24 h thereafter. In contrast, in medium-treated endothelial cells, nitrite accumulation at 48 h was about 40 µM, a level insufficient to produce target cell lysis. Significant tumor cell lysis was observed only when the level of nitrite accumulation exceeded 80 µM (\( P < 0.01 \)). The production of 60 µM nitrite by endothelial cells cocultured with tumor cells in the absence of cytokines occurred by 96 h of culture, and this did not produce lysis of M-5076 (Fig. 1B).

Inhibition of Endothelial Cell-mediated Tumor Cell Lysis by N⁶-Methyl-L-arginine. To further examine the role of nitric oxide in cytokine-activated endothelial cell-mediated tumor cell lysis, we used NMA, a specific inhibitor of nitric oxide synthesis (35). At a concentration of 0.05 mM, NMA completely inhibited endothelial cell-mediated M-5076 cell lysis and inhibited nitrite production by 48% (Fig. 2). At lower concentrations (0.01 mM), NMA inhibited tumor cell lysis by 89% (\( P < 0.01 \)) and nitrite production by 28% (\( P < 0.05 \)). Below 0.001 mM, NMA had no effect on either tumor cell lysis or nitrite production.

Endothelial Cell-mediated Tumor Cell Lysis is L-Arginine Dependent. Previous reports have demonstrated that L-arginine is a precursor to nitric oxide biosynthesis in cytokine-activated
macrophages (35, 36). Similar data were produced in our study. Endothelial cell-mediated tumor cell lysis in arginine-free medium was inhibited by 88.5% (P < 0.01), and nitrite production was decreased by 80% (P < 0.01). When 1.2 mM L-arginine was added to arginine-free medium, the cytokine-activated endothelial cells produced significant tumor cell lysis (68%, P < 0.01) and nitrite accumulation (78 µM, P < 0.01) (Table 1). Both the lysis of M-5076 cells and nitrite production by endothelial cells cultured in the reconstituted media were inhibited by the addition of 1 mM NMA.

Inhibition of Endothelial Cell-mediated Lysis by Dexamethasone. Previous studies from this laboratory have shown an increased incidence of melanoma metastasis in mice treated with glucocorticoids (37). This was not attributed to alterations in host immunity, but to interaction of tumor cells with capillary endothelium (37). We therefore examined whether dexamethasone would inhibit endothelial cell-mediated lysis of tumor cells. Dexamethasone was added to endothelial cell monolayers before or after (−12 to +24 h) the addition of target cells. The data are shown in Fig. 3A. Indeed, the incubation of cytokine-activated endothelial cells with dexamethasone at concentrations ranging from 0.1 ng to 1000 ng/ml inhibited lysis of M-5076 cells (range, 10 to 79%; P < 0.05). At less than 0.1 ng/ml, dexamethasone had no demonstrable inhibitory effects. The inhibitory effect was observed only when dexamethasone was added 12 to 24 h before the addition of tumor target cells to endothelial cell monolayers. Dexamethasone added 24 h after target cell addition did not significantly inhibit M-5076 lysis (P > 0.1).

We next examined whether dexamethasone inhibits the production of nitrite by cytokine-activated endothelial cells. Dexamethasone was added to endothelial monolayers together with the cytokines and tumor cells. The addition of dexamethasone to cytokine-treated endothelial cells did inhibit the production of nitrite in a dose-dependent manner (Fig. 3B) and in direct correlation with inhibition of tumor cell lysis (Fig. 3A). Although the inhibition of nitrite production was greater in the activated endothelial cells without tumor cells, this difference was not statistically significant (P > 0.1).

DISCUSSION

Our results show that a major mechanism by which cytokine-activated endothelial cells lyse tumor cells (under in vitro conditions) is associated with the production of nitric oxide. The synthesis of nitric oxide by mammalian cells has only been recently demonstrated (38). Although nitrogen oxide-containing vasodilator drugs such as glyceryl trinitrate have been used clinically to decrease systemic resistance and blood pressure for years, their mechanism of action only began to be understood with the discovery that acetylcholine-elicited arterial relaxation is mediated by an endothelium-derived relaxing factor (21) subsequently shown to be identical to nitric oxide (22, 23).

Injection i.p. of lipopolysaccharide into mice is known to activate tumoricidal properties in macrophages and also to increase nitrite concentration in the blood and urine (39). This increase is explained in part by the finding that macrophages are major producers of nitric oxide (27, 29). One possible mechanism for the cytotoxic activity of nitric oxide may involve the formation of an iron-nitrosyl complex, resulting in the inhibition of critical metabolic enzymes with labile iron-sulfur centers (40). Indeed, production of nitric oxide by activated macrophages has been shown to inhibit mitochondrial respiration (30, 31) and DNA synthesis (32).

The role of nitric oxide in macrophage-mediated tumor cytotoxicity prompted us to examine whether its production by cytokine-activated endothelial cells mediated tumor cell lysis. Indeed, nitrite production by cytokine endothelial cells directly correlated with lysis of M-5076 cells. That nitrite production...
and endothelial cell cytotoxicity could be completely inhibited by NMA and also blocked by removal of l-arginine from the medium implies that nitric oxide is a major mediator of M-5076 cytotoxicity. Inhibition of nitrite accumulation by 48–90% completely abrogated endothelial cell-mediated tumor cell lysis. Inhibition of as little as 28% of nitrite accumulation (to less than 60 μM) was sufficient to produce near maximal (89%) inhibition of tumor cell lysis.

Treatment of mice with glucocorticoid steroids can enhance entrapment of tumor cells in the lung microvasculature and reduce cell clearance, hence increasing experimental metastasis (37). A recent study demonstrated that glucocorticoids can inhibit the expression of an inducible nitric oxide synthase in vascular endothelial cells (41). Our data agree with this finding. Treatment of cytokine-activated endothelial cells with low concentrations of dexamethasone significantly inhibited target cell lysis and nitrite production. At the same time, dexamethasone treatment did not alter adhesion of M-5076 to endothelial cells (data not shown). These findings provide some explanation for the in vivo data (37). A reduction in production of nitric oxide may lead to increased tumor cell survival in lung microvasculature. In vivo studies correlating all these parameters are underway.

Although activated macrophages and activated endothelial cells can produce nitric oxide, they respond differently to exposure by cytokines. While endotoxins and IFN-γ are the most potent activating agents for macrophages (42), endothelial cells respond maximally to TNF and IFN-γ (26). Other immunomodulators such as muramyl dipeptide and interleukin-1 can activate macrophages but not endothelial cells (26, 43). This suggests that macrophages and endothelial cells are activated to lyse tumor cells by different pathways. However, since activated macrophages can serve as a major source of TNF (44), they may themselves induce the activation of endothelial cells. The activation of macrophages by endothotoxins could therefore be pleiotropic. The recent finding that endotoxin-activated Kupffer cells can induce production of nitric oxide by hepatocytes (45) supports this theory. Once again, the demonstration that endothelium-derived relaxing factor, a potent vasodilator, is identical to nitric oxide (22, 23) suggests that nitric oxide production may be related to the cardiovascular collapse associated with TNF or endotoxin administration to animals. Consistent with this theory is the indication that nitric oxide synthesis inhibitors can block TNF and endotoxin-induced shock (25, 26). It is important to note that, to date, cytokine-induced nitric oxide release from human cells has not been shown. Therefore, the role of nitric oxide in human pathology is still unclear.

In summary, we have found that nitric oxide produced by cytokine (TNF-α and IFN-γ)-activated lung endothelial cells is a major mediator of tumor cell lysis. Whether cytokine-activated endothelial cells produce additional cytotoxic molecules is under active investigation.

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