

# Direct *in Vitro* Lysis of Metastatic Tumor Cells by Cytokine-activated Murine Vascular Endothelial Cells<sup>1</sup>

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## ABSTRACT

The purpose of these studies was to determine whether vascular endothelial cells can be treated with various cytokines to become cytotoxic against tumorigenic target cells under defined conditions *in vitro*. Microvascular endothelial cells were isolated from immunocompetent mice by perfusion of lungs grown in culture, cloned, and then characterized. The cloned microvascular endothelial cells were activated by incubation with a combination of recombinant tumor necrosis factor  $\alpha$  and recombinant- $\gamma$  interferon (10 units/ml each) for 24 h. Activated endothelial but not control endothelial cells (incubated in medium alone, recombinant tumor necrosis factor  $\alpha$  alone, or recombinant  $\gamma$  interferon alone) produced significant lysis of mouse reticulum cell sarcoma and two different mouse melanomas. Moreover, at the concentrations used here, recombinant tumor necrosis factor  $\alpha$  and recombinant  $\gamma$  interferon did not produce direct target cell lysis. The activated endothelial cell-mediated tumor cell lysis depended on the continuous presence of the cytokines and was not due solely to initial target cell binding. The results demonstrate that, like macrophages, microvessel endothelial cells exposed to low levels of cytokines are capable of lysing tumor cells.

## INTRODUCTION

The majority of malignant cells that enter the blood circulation during the process of metastasis die rapidly (1, 2). For example, in the B16 murine melanoma system, less than 0.1% of circulating cells give rise to metastatic lesions (2). Among the mechanisms that have been proposed to explain the rapid demise of circulating tumor cells are mechanical trauma produced by blood flow (3), inability of tumor cells to withstand deformation (4, 5) and oxygen radicals in the microvascular system (6), and destruction by host defenses, such as T cells (7), natural killer cells (8, 9), and macrophages (10, 11).

The possibility that microvascular endothelial cells *per se* could directly contribute to tumor cell death has not been investigated, yet malignant cells must interact with organ-specific endothelial cells in different ways to produce metastases (12, 13). The adhesion of tumor cells to organ-specific microvascular endothelial cells under *in vitro* (14-18) and *in vivo* (19, 20) conditions correlates with organ-specific metastasis. Furthermore, microvascular endothelial cells constitute an essential part of the blood vascular system, whose function is much more than simply passively separating plasma and interstitial tissue. Endothelial cells actively participate in diverse functions, such as nutrient transport, inflammation, tissue repair, regulation of coagulation, and metabolism of hormones (21-26). Vascular endothelial cells perform various immunological functions,

such as antigen presentation, expression of MHC<sup>3</sup> class I and II antigens and Fc receptors, production of many cytokines (including interleukin 1, interleukin 2, interleukin 6, and transforming growth factor- $\beta$ ), and participation in lymphocyte homing and phagocytosis (27-35).

Many of the biological functions of endothelial cells are similar to those associated with macrophages. Since macrophages can be activated to lyse tumor cells (36-38) and cytokines that affect macrophages (IFN- $\gamma$ , interleukin 1, TNF) can also activate endothelial cells (27, 39), we wished to determine whether endothelial cells treated with cytokines could also become cytotoxic to tumorigenic cells. We isolated microvascular endothelial cells from the lungs of immunocompetent mice, characterized them in detail, incubated the cells with cytokines, and examined their interactions with three mouse tumor cell lines whose potentials to metastasize to the lung differ. We show here that vascular endothelial cells activated by TNF- $\alpha$  and IFN- $\gamma$  can lyse tumor target cells.

## MATERIALS AND METHODS

**Animals.** Specific pathogen-free female C57BL/6  $\times$  C<sub>3</sub>H/HeN F<sub>1</sub> mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The mice were maintained in laminar air-flow cabinets under specific pathogen-free conditions and used at 10 weeks of age.

**Reagents.** Recombinant human tumor necrosis factor ( $2.4 \times 10^7$  units/mg protein) was the gift of Cetus Corporation (Emeryville, CA). Recombinant mouse  $\gamma$ -interferon ( $1.2 \times 10^7$  units/mg protein) was the gift of Genentech, Inc. (South San Francisco, CA). Hybridoma cell lines that produce monoclonal antibodies against MHC class I and II antigens, Mac-1, -2, and -3 antigens, and bovine aorta endothelial cells were purchased from American Type Culture Collection (Rockville, MD). Monoclonal antibody against Thy-1 antigen was purchased from Becton Dickinson (Mountain View, CA). A hybridoma that produces monoclonal antibody, F4/80, against a macrophage-specific antigen was the gift of Dr. D. Hume (M. D. Anderson Cancer Center) (40). Fluorescence microsphere beads with a diameter of 0.85  $\mu$ m were purchased from Polysciences, Inc. (Warrington, PA). Endothelial cell growth supplement was purchased from Sigma Chemical Co. (St. Louis, MO).

**Cell Lines.** The K-1735 melanoma syngeneic to C<sub>3</sub>H/HeN mice was the gift of Dr. M. L. Kripke (M. D. Anderson Cancer Center) (41). The low metastatic clone 19 and high metastatic clone M2 were isolated as described previously (42). The B16-F<sub>1</sub> melanoma syngeneic to C57BL/6 mice was isolated as described previously (2). The K-1735 cells were grown as monolayer cultures on plastic in EMEM (M. A. Bioproducts, Walkersville, MD), supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, and a 2-fold vitamin solution (GIBCO, Grand Island, NY).

<sup>3</sup> The abbreviations used are: MHC, major histocompatibility complex; rTNF- $\alpha$ , recombinant tumor necrosis factor  $\alpha$ ; rIFN- $\gamma$ , recombinant  $\gamma$  interferon; EMEM, Eagle's minimal essential medium; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; BSA, bovine serum albumin; IdUrd, 5-iodo-2'-deoxyuridine; LDL, low density lipoprotein; TNF, tumor necrosis factor; IFN- $\gamma$ ,  $\gamma$  interferon; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; 3LL, Lewis Lung Carcinoma; Dil-Ac-LDL, 1,1-dioctadecyl-1-3,3,3'-tetramethylindocarbocyanine perchlorate-acetylated low density lipoprotein.

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The tumor M-5076 arose spontaneously in the ovary of a C57BL/6 mouse in the laboratory of Dr. W. F. Duning (Papanicolaou Research Institute, Miami, FL) (43), and 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 (44). After i.v., s.c., or i.p. implantation, these tumor cells produce peritoneal organ metastases but no visible lung metastases (44). The M-5076 cells were grown in RPMI 1640 medium supplemented with 15% horse serum, sodium pyruvate, and L-glutamine (M. A. Bioproducts).

**Isolation of Vascular Endothelial Cells from Lungs of Mice.** Vascular endothelial cells were isolated from lungs of mice by a modification of a procedure described by Ryan *et al.* (45). Briefly, mice were given i.v. injections of 1000 units of heparin in 0.5 ml of HBSS. Thirty min later, the mice were killed by cervical dislocation, the rib cage was surgically removed, and the superior and inferior vena cavae were ligated. An incision was made at the right atrium, and a blunt needle attached to a syringe was inserted through the incision and tightened with a ligature of 1-0 silk material. The aorta was then cut with scissors. The lungs were perfused with first 2-4 ml of Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS, followed by 3-5 ml of 0.025% EDTA, and then 5-10 ml digestion medium (200 units/ml collagenase and 270 units/ml DNase). The perfusion suspension was collected via the aorta in different fractions. Each fraction was plated into 24-well tissue culture plates and cultured with medium 199 and endothelial cell growth supplement (Sigma). Different concentrations (1-5%) of tumor-conditioned medium (24-h culture medium of Lewis lung carcinoma cells) were added to some of the culture wells.

**Cloning of Vascular Endothelial Cells.** The procedure for cloning of vascular endothelial cells has been described previously (46). Briefly, when an endothelial cell monolayer formed, it was covered with a thin layer of 0.25% trypsin-0.02% EDTA solution for 1-2 min. The dislodged cells were suspended in medium 199 containing 20% FBS and were gently pipetted to yield a single-cell suspension. The cells were counted in a hemacytometer and diluted to the concentration of 10 cells/ml. One ml of this and 3 ml of medium suspension were added to a 60-mm<sup>2</sup> tissue culture dish. One day later, single adherent cells were identified and marked. Two to 4 weeks later, when colonies developed, all colonies but one were removed with a rubber policeman. Each remaining single colony was expanded to obtain a cell line.

**Immunofluorescence for Marker Antigens and Factor VIII Related Antigen.** Endothelial cells were grown on glass coverslips as monolayers and fixed with 1% glutaraldehyde for 1 h. The fixed monolayer was washed twice with PBS and monoclonal antibodies were added: Mac-1, -2, and -3 and F4/80 as undiluted supernatants from 24- to 48-h cultures of hybridoma cell lines and Thy-1 as purified monoclonal antibody diluted 1:50. After a 1-h incubation, the monolayers were washed 3 times with PBS, and a fluorescent second antibody (goat anti-rabbit IgG) was added at 1:100 dilution. Following a 1-h incubation, the coverslips were washed 3 times with PBS, mounted with a drop of 10% glycerol in PBS, and viewed with an Olympus-BH-2 fluorescence microscope.

**Factor VIII-related antigen staining** was performed as described by Jaffe *et al.* (47). Endothelial cell monolayers were fixed with 3.5% buffered formalin and permeabilized in 0.05% NP-40 detergent (Sigma). The fixed cells were incubated with a 1:5 dilution of rabbit antiserum to human factor VIII:vWF antigen (Accurate Chemical & Scientific Corp., Westbury, NY) for 2-4 h, washed 5 times with PBS containing 1% BSA, and then incubated with a 1:100 dilution of fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) for 45 min. The cells were washed 3 times with PBS, and the coverslips were mounted and examined with a fluorescence microscope (48, 49).

**Angiotensin-converting Enzyme Activity.** The angiotensin-converting enzyme assay kit was purchased from Ventex Laboratories (Proland, ME); it measures the conversion of tritiated tripeptide [<sup>3</sup>H]benzoyl-phenylalanylalanylproline to [<sup>3</sup>H]benzoylphenylalanine and alanylproline. Buffered substrate was added to the endothelial cell monolayer, the cultures were incubated at 37°C for different time intervals (10, 20, and 30 min), and the incubation was terminated by the addition of 0.1 N HCl. [<sup>3</sup>H]Benzoylphenylalanine was separated from unreacted sub-

strate by extraction with Ventex scintillation cocktail 2. An aliquot of the organic phase was counted in a scintillation counter. Enzyme activity was reported as the percentage of substrate utilized (50); 1 unit of angiotensin-converting enzyme activity was defined as that quantity of enzyme required to hydrolyze substrate at an initial rate of 1%/min at 37°C.

**Nonthrombogenic Cell Surface.** Human blood platelets were isolated from a blood buffy coat and washed in PBS containing 5 mM glucose and 5 mg/ml BSA. Monolayers of endothelial cells and fibroblasts were grown in 24-well tissue culture plates, washed, and incubated with EMEM containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 5 mg/ml BSA to reduce serum components bound to the cell surface. Platelets (1 × 10<sup>8</sup>) were added to each culture dish, and incubation continued for 30 min at 37°C. Nonadherent platelets were removed by rinsing the plate 10 times with EMEM. Platelet adherence was determined by phase-contrast microscopy (49).

**Uptake of Acetylated LDL and Phagocytosis.** Endothelial cells were grown on glass coverslips to about 60 to 80% confluency. Cells were incubated with medium containing 10 μg/ml Dil-Ac-LDL at 37°C for 4 h. The medium was removed, and the cells were washed once with Dil-Ac-LDL-free medium for 10 min, rinsed with PBS, and then fixed with 10% buffered formalin for 5 min. Coverslips were inverted over a drop of 10% glycerol prior to fluorescence visualization with standard rhodamine excitation/emission filter combinations. In another assay, cells were labeled with 10 μg/ml Dil-Ac-LDL for 4 h at 37°C. The cells were washed once with PBS and then trypsinized to produce a single-cell suspension. The trypsin was neutralized with medium 199 containing 10% FBS. Cells and collection tubes were kept on ice prior to and during all manipulations. Cell cytofluorographic analysis was performed using an argon laser for excitation, and fluorescence emission above 550 nm was determined. Sample gates were set using stained bovine aorta endothelial cells and mouse skin fibroblasts as positive and negative controls, respectively (51).

**Phagocytosis Assay.** Latex beads (2 × 10<sup>7</sup>) of 0.85-μm diameter were added to endothelial cell monolayer cultures. Twelve to 24 h later, the monolayers were washed 10 times with PBS, fixed in 1% glutaraldehyde, and processed for electron microscopic examination (52).

**In Vivo Growth and Metastatic Potential of Tumor Cell Lines.** K-1735 melanoma, C-19, and M-2 cells in exponential growth phase were incubated at 37°C with 0.25% trypsin-0.02% EDTA in PBS for 1 min. M-5076 cells were treated with 0.02% EDTA in PBS. The cells were then suspended in culture medium (EMEM or RPMI 1640), gently pipetted to obtain a single-cell suspension, counted, and resuspended in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS, at the final concentration of 10<sup>5</sup> cells/0.1 ml for s.c. and intrabronchial injections and 10<sup>5</sup> cells/0.2 ml for i.v. injection. Only tumor cell suspensions of >90% viability were used. For s.c. implantation, tumor cell suspension were injected at the lateral thoracic wall with a 25-gauge needle. For i.v. injection, tumor cells were injected through a 27-gauge needle into the lateral tail vein of unanesthetized mice. The procedure of intrabronchial implantation was described previously (53). Briefly, mice were anesthetized with methoxyflurane and immobilized on a 5 × 7-inch board with rubber bands. The skin around the neck was cleaned with 70% alcohol. A 1-cm ventral midline incision over the trachea was made and the connective tissue was separated to expose the muscular sheath over the trachea, which itself was then exposed. The tissue between two tracheal rings was incised with the bevel of a 25-gauge needle, the mouse's head was elevated, and tumor cells in 0.05 ml HBSS were injected with a blunt-ended 27-gauge needle (bent 60 degrees) inserted down the right trachea to the mainstem bronchus. The skin incision was then closed with metal clips. Mice were allowed to recover under heat lamps and returned to their holding cages for 45 days. At that time, the mice were killed and all organs were examined for the presence of visible tumor deposits.

**Vascular Endothelial Cell-mediated Cytotoxicity.** The cell-mediated cytotoxicity assay was based on the macrophage-mediated cytotoxicity assay described previously (54). Target tumor cells in exponential growth phase were incubated for 24 h in EMEM or RPMI 1640 containing [<sup>125</sup>I]IdUrd (0.2 μCi/ml; specific activity, 2 μCi/mmol; Du Pont-New England Nuclear, Boston, MA). The cells were then washed

1–3 times with HBSS to remove free unbound [<sup>125</sup>I]IdUrd and harvested by a brief trypsinization (K-1735, C-19 and M2) or agitation (M-5076). Viable tumor cells (10<sup>6</sup>) were added to 38-mm<sup>2</sup> wells of a 96-well plate containing confluent endothelial cell monolayers (approximately 5 × 10<sup>4</sup> cells/well). All endothelial cells used in the cytotoxicity assay were obtained from passages 4 to 8. The endothelial cells were preincubated with cytokines (recombinant TNF and rIFN-γ) or medium for different times and washed 2 times prior to the addition of target cells. In some assays, TNF and IFN-γ were present for the duration of the assay (4 to 5 days). At that time, the cultures were washed twice with PBS to remove nonadherent cells, and the remaining viable adherent cells were lysed with 0.1 ml of 0.1 N NaOH. The lysate was absorbed onto cotton swabs, which were placed into 10- × 75-mm glass tubes. Radioactivity was monitored in a LKB gamma counter. The endothelial cell-mediated cytotoxicity was calculated as follows:

$$\% \text{ of cytotoxicity} = \frac{A - B}{A} \times 100$$

where *A* is cpm in target cells cultured with control endothelial cells and *B* is cpm in target cells cultured with activated endothelial cells.

**Fibroblast Cultures.** Fibroblasts isolated from the skin of 3-week-old BALB/c nude mice were the kind gift of Dr. A. Fabra (M. D. Anderson Cancer Center). Cultured fibroblasts at passage 6 or 7 were used here as control cultures for endothelial cells. The fibroblasts were incubated with 10 units/ml recombinant human TNF and 10 units/ml recombinant murine IFN-γ. Target cells labeled with [<sup>125</sup>I]IdUrd were then plated, and the cultures were terminated 5 days later and processed as described above.

**Statistical Analysis.** The significance of the *in vitro* results was determined by the Student's *t* test (two-tailed).

**RESULTS**

**Characterization of Lung Vascular Endothelial Cells.** Endothelial cells isolated from mouse lung were cultivated as described in "Materials and Methods." Two weeks later, some dishes contained 1–3 colonies of endothelial cells that exhibited a cobblestone morphology. The colonies were isolated and expanded to confluent monolayers. One of these cell strains was further cloned by limiting dilution to yield two cloned lines.

The doubling times of the cloned lines were 46–50 h and 24–26 h in medium containing 5 and 20% FBS, respectively. The cloned lines were characterized (Table 1).

When cultured on plastic, the endothelial cells showed contact inhibition of growth and a cobblestone morphology (Fig. 1A). After 24 h of culture on a type IV collagen matrix or with 1% 3LL-conditioned medium, about 80% of the endothelial cells formed capillary-like structures (Fig. 1, B–D). Human platelets did not bind to the mouse lung endothelial cells but did to the control mouse skin fibroblast monolayers. About 70% of the endothelial cells were stained (cytoplasmic distribution) with a rabbit polyclonal antibody raised against human factor VIII:vWF antigen. More than 80% of bovine aorta endothelial cells (positive control) were also stained by the anti-factor VIII:vWF, whereas skin fibroblasts (negative control) were not stained (Fig. 2). About 50% of the mouse lung microvascular endothelial cells selectively bound and internalized Dil-Ac-LDL, as did almost 100% of bovine aorta endothelial cells; mouse K-1735 melanoma cells did not (Fig. 3).

Mouse lung microvascular endothelial cells also exhibited angiotensin-converting enzyme activity, a unique property of lung vascular endothelial cells. The activities of 30–90 units (10 min) were produced by endothelial cell cultures (38-mm<sup>2</sup> monolayer). The endothelial cells also exhibited remarkable phagocytic activity. After 24-h incubation, they phagocytosed 0.85-μm-diameter latex beads at 50 to more than 500 beads/cell (Fig. 4). Finally, almost all of the endothelial cells expressed MHC class I antigen but not macrophage or lymphocyte marker antigens (Table 1). The MHC II expression could be induced by incubation with 10 units/ml rIFN-γ for 24 to 72 h (data not shown).

**Biological Behavior of M-5076 Reticulum Sarcoma and K-1735 C-19 and M2 Cells.** The growth and metastatic potential of the M-5076 reticulum cell sarcoma and two cloned cell lines from a mouse K-1735 melanoma were determined by s.c. and i.v. injection. M-5076 cells were also implanted intrabronchially. M-5076 exhibited peritoneal organ-specific metastasis independent of injection route (Table 2). Although previous reports concluded that M-5076 cells cannot grow in the lung (44),

ENDOTHELIAL CELL-MEDIATED LYSIS

Table 1 Characterization of vascular endothelial cells established from mouse lung

Characteristic	Endothelial cells	Positive control	Negative control
Cobblestone morphology	++ <sup>a,b</sup>	++ (BAE) <sup>c</sup>	– (Fibroblasts)
Contact inhibition of growth	++ <sup>b</sup>	++ (BAE)	– (Fibrosarcoma)
Nonthrombogenic surface	++ <sup>d</sup>	++ (BAE)	– (Fibroblasts)
Factor VIII-associated antigen	+ <sup>e</sup>	+ (BAE)	– (Fibroblasts)
Angiotensin-converting enzyme activity	30–90 units (10 min)	ND <sup>f</sup>	ND
(by [ <sup>3</sup> H]benzoylphenylalanylalanylproline)	15–16 units (20 min)		
	16–17 units (30 min)		
Acetylated-LDL uptake	+ <sup>g</sup>	+++ (BAE)	– (Fibroblasts)
Phagocytosis activity	+++ <sup>h</sup>	+++ (BAE)	– (Fibroblasts)
Angiogenesis activity	+ <sup>i</sup>	+ (BAE)	– (Fibroblasts)
MCH class I antigen	++ <sup>j</sup>	++ (Mouse macrophage)	– (B16-F10 melanoma)
Mac-1, -2, and -3 and F4/80 antigens	– <sup>k</sup>	++ (Mouse macrophage)	– (B16-F10 melanoma)
Thy-1 antigen	+ <sup>l</sup>	++ (T cell lymphoma)	– (B16-F10 melanoma)

<sup>a</sup> –, negative; +, >50% positive cells; ++, >80% positive cells; +++, 100% positive cells.  
<sup>b</sup> Contact inhibition of growth and cobblestone monolayer morphology determined by phase contrast microscopy.  
<sup>c</sup> BAE, Bovine aorta endothelium.  
<sup>d</sup> Nonthrombogenic surface determined by platelet-binding assay, as described in "Materials and Methods."  
<sup>e</sup> Factor VIII-Von Willebrand factor antigen (vWF:Ag) determined by indirect immunofluorescence using rabbit anti-human vWF:Ag antibody and fluorescein-goat anti-rabbit immunoglobulin.  
<sup>f</sup> ND, not done.  
<sup>g</sup> Acetylated-LDL uptake determined by staining with the fluorescence probe (Dil-Ac-LDL).  
<sup>h</sup> Phagocytosis activity determined by the uptake of latex beads of 0.85-μm diameter.  
<sup>i</sup> Formation of capillary structure in tissue culture dish with tumor-conditioned medium.  
<sup>j</sup> Determined by fluorescence cytometry.  
<sup>k</sup> Macrophage marker antigen.  
<sup>l</sup> Lymphocyte marker antigen.

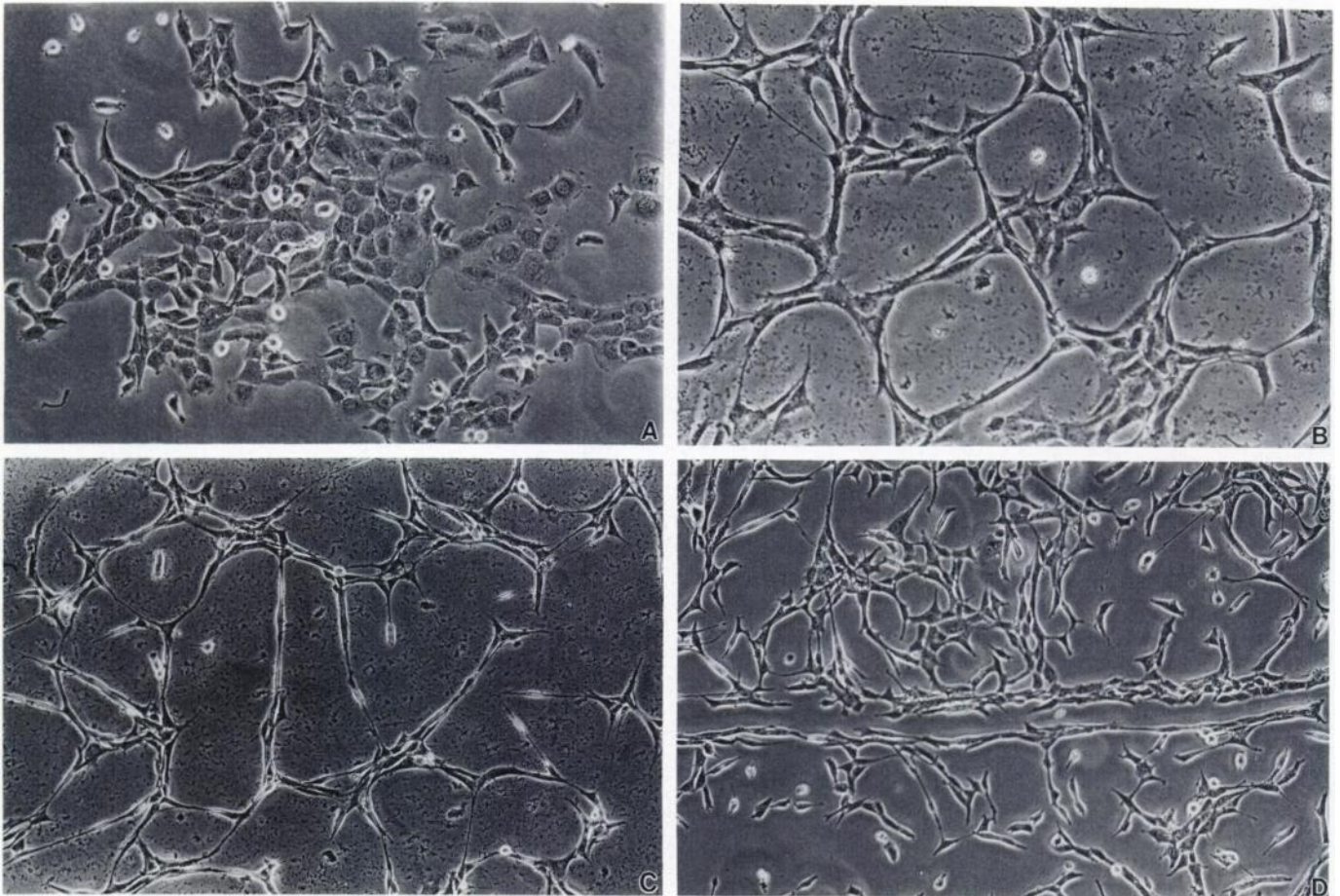


Fig. 1. Mouse lung endothelial cells in tissue culture. *A*, in medium 199 with 10% FBS. *B-D*, in medium 199 with 10% FBS and 5% tumor-conditioned medium (24-h culture medium of Lewis lung carcinoma).  $\times 100$ .

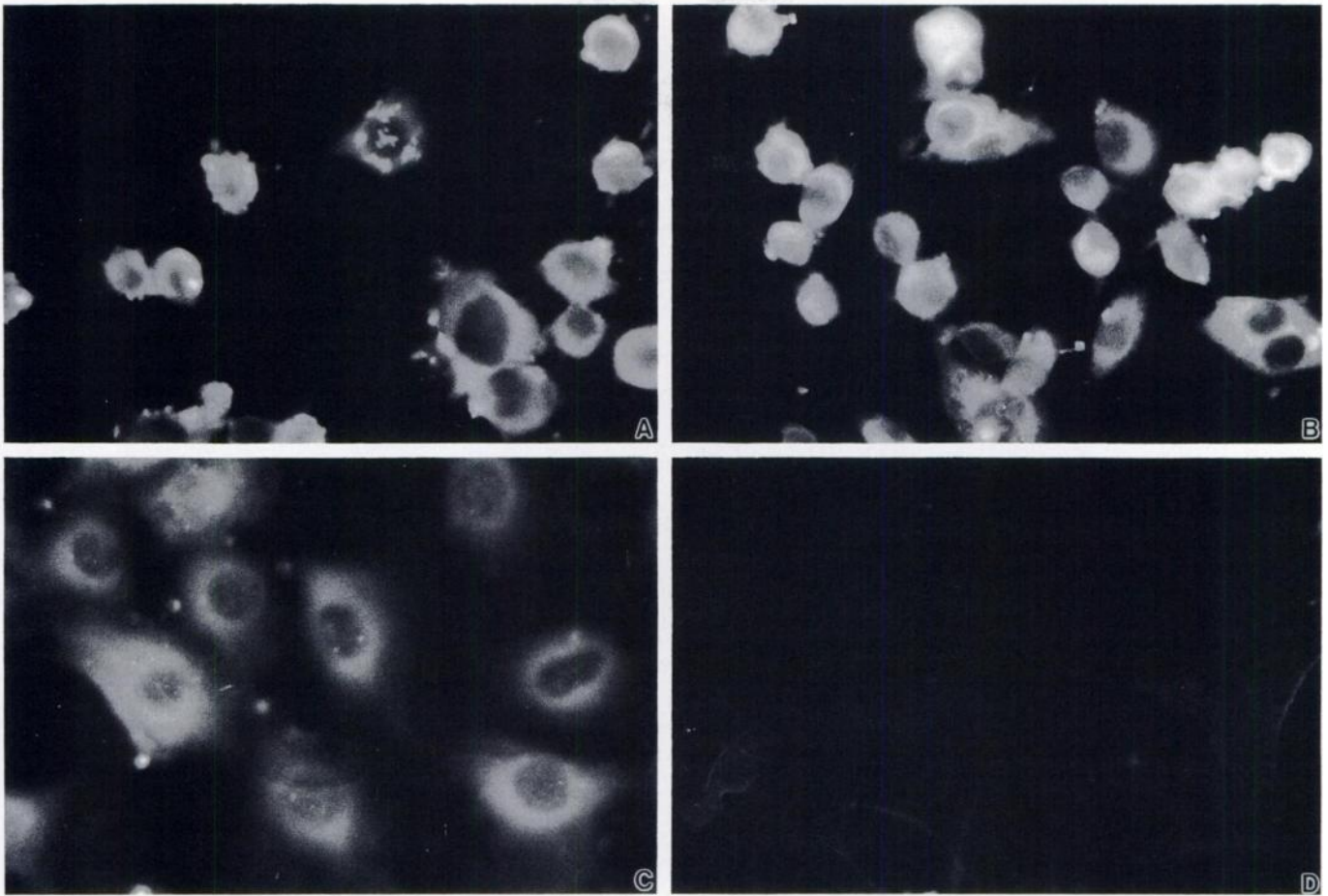
subsequent to intrabronchial implantation M-5076 cells grew in the lung and in the liver as metastases. Both K-1735 C-19 and K-1735 M2 cells grew s.c. in the majority of the mice, but only M2 cells formed lung metastases after i.v. or s.c. injections (Table 2).

**Activation of Mouse Lung Endothelial Cells to Lyse Tumor Cells.** We have determined the *in vitro* conditions necessary to activate microvessel endothelial cells to lyse tumorigenic target cells by plating  $2 \times 10^4$  endothelial cells into each 38-mm<sup>2</sup> well of a flat-bottomed 96-well plate. Twenty-four h later, when endothelial cells formed a confluent monolayer, they were trypsinized and the cell number constituting a confluent monolayer was determined to be about  $5 \times 10^4$  cells. In most assays, target cells were added at a concentration of  $10^4$  cells/well, yielding an endothelial cell to target cell ratio of 5:1. Prior to the addition of target cells, confluent monolayers of endothelial cells were incubated in medium alone or in medium containing rTNF- $\alpha$  at concentrations from 5 to 500 units/ml, rIFN- $\gamma$  at concentrations from 5 to 500 units/ml, or combinations of rTNF- $\alpha$  and rIFN- $\gamma$ . After 24 h, the endothelial cell monolayers were washed, [<sup>125</sup>I]IdUrd-labeled target cells were added, and incubation was continued for 96 h, at which point the assay was terminated. To rule out direct antitumor effects of the cytokines (55–57), tumor cells were incubated with the same concentrations of the cytokines in the absence of endothelial cells.

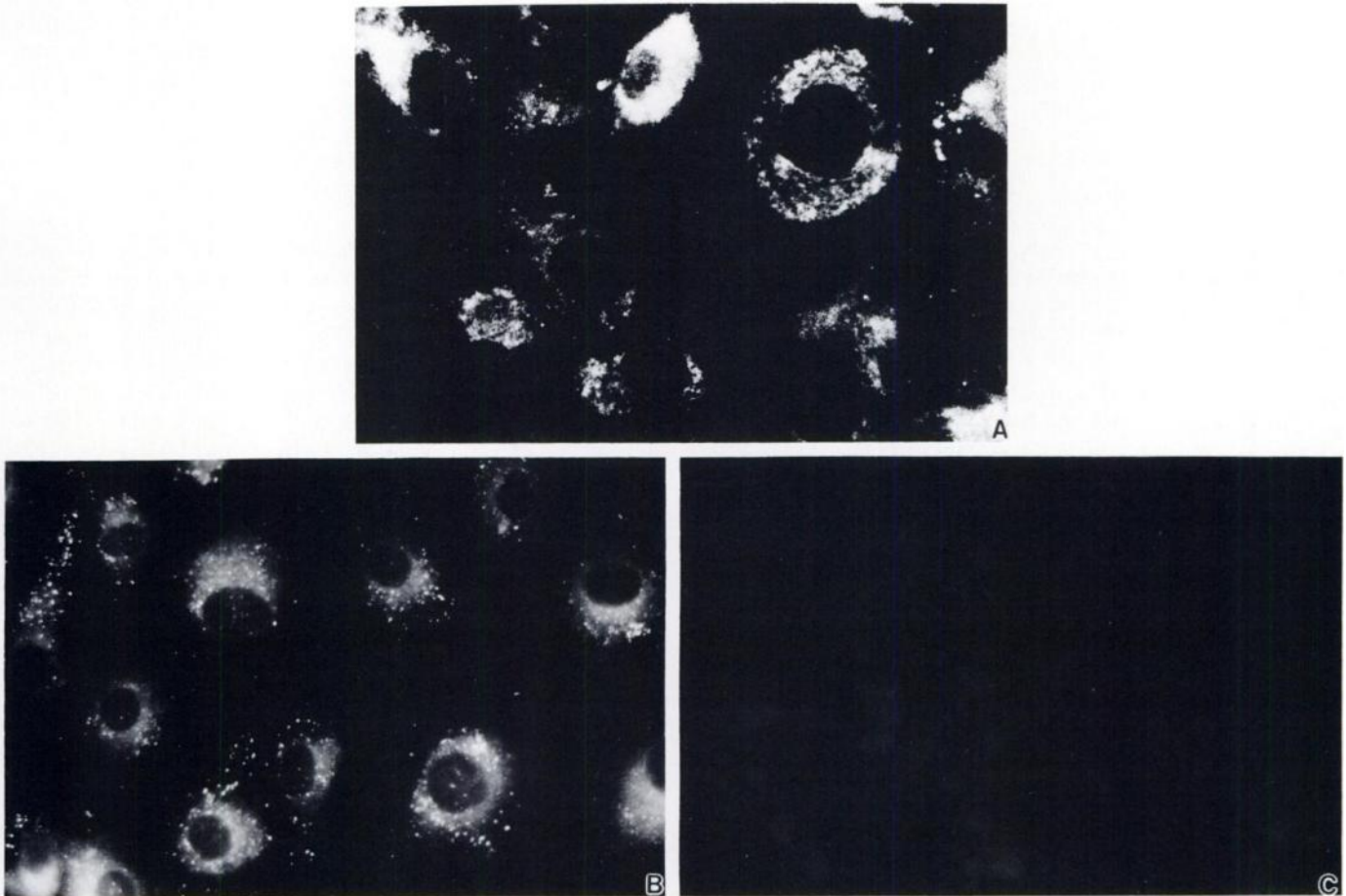
M-5076 cells were resistant to direct cytotoxic effects of rTNF- $\alpha$  and rIFN- $\gamma$  at 500 units/ml each. B16-F<sub>1</sub> cells were

unaffected by 100 units/ml levels of each cytokine. K-1735 C-19 and K-1735 M2 cells incubated with rTNF- $\alpha$  or IFN- $\gamma$  at concentrations exceeding 50 units/ml demonstrated significant lysis ( $P < 0.01$ ). Incubation of these tumor cells with rTNF- $\alpha$ , rIFN- $\gamma$ , or rTNF- $\alpha$  plus rIFN- $\gamma$  at concentrations below 50 units/ml did not result in significant cytotoxicity ( $P < 0.1$ ). In all our studies, we routinely incubated endothelial cells with rTNF- $\alpha$  or rIFN- $\gamma$  at a concentration of 10 units/ml, at which neither rTNF- $\alpha$  nor rIFN- $\gamma$  alone activated endothelial cells to lyse tumor targets. In contrast, incubation of endothelial cells with both rTNF- $\alpha$  and rIFN- $\gamma$  (10 units/ml each) resulted in significant lysis of the tumor cell targets; untreated endothelial cells did not (Table 3). Since M-5076 cells were the most resistant to direct effects of the cytokines and the most sensitive to lysis mediated by endothelial cells, the results suggest that TNF- $\alpha$  and IFN- $\gamma$  do not mediate the lysis of the target cells.

**Control Experiments.** Several control experiments were performed to validate the results of the radioactive release assay. (a) To rule out the possibility that the absence of radioactivity in the adherent cells was due to viable but unattached target cells, supernatants of cytokine-activated endothelial cells and radiolabeled M-5076 cells were collected after 72–96 h and passed through a 0.2- $\mu$ m Millipore filter. More than 99% of the radioactivity was found in the filtrate, suggesting that it was associated with free <sup>125</sup>I and not with viable cells (Table 4). (b) Endothelial cell-mediated cytotoxicity was determined by cell counting. Endothelial cells were incubated with rTNF- $\alpha$  and rIFN- $\gamma$  (10 units/ml each), and unlabeled M-5076 cells ( $10^4$ )



**Fig. 2.** Immunofluorescence staining for antifactor VIII:vWF, as described in "Materials and Methods." *A* and *B*, mouse lung microvessel endothelial cells. *C*, bovine aorta endothelial cells. *D*, normal fibroblast cells.  $\times 100$ .



**Fig. 3.** Uptake of acetylated LDL by immunofluorescence. Cells were labeled with  $10 \mu\text{g/ml}$  Dil-Ac-LDL for 4 h. *A*, mouse lung endothelial cells.  $\times 200$ . *B*, bovine aorta endothelial cells  $\times 200$ . *C*, mouse melanoma K-1735 clone 19 cells.  $\times 100$ .

Fig. 4. Phagocytosis of latex beads (0.85  $\mu\text{m}$  diameter) by mouse lung endothelial cells after 24-h incubation. A,  $\times 1000$ ; B,  $\times 4000$ .

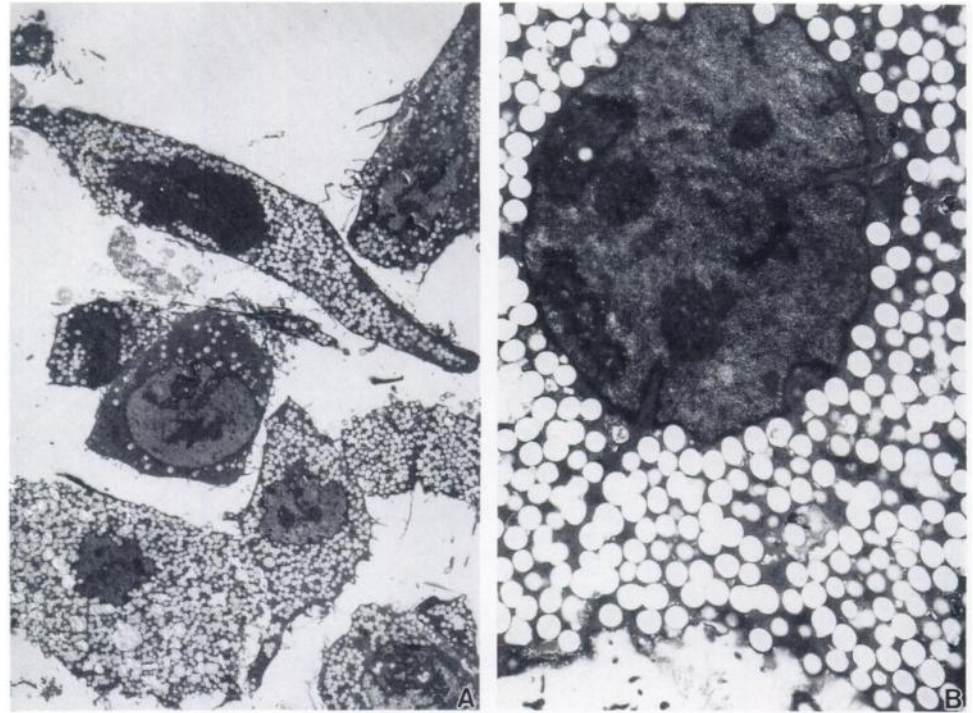


Table 2 Metastasis of M-5076 and two K-1735 cloned cell lines

Route of injection	Tumorigenicity <sup>a</sup>	Incidence (median no.) of visible tumor nodules	
		Lung	Liver
<b>M-5076</b>			
i.v.	None	0/20 <sup>b</sup>	20/20 (>200) <sup>f</sup>
s.c.	20/20	0/20	20/20 (>200)
i.b. <sup>d</sup>	7/10		7/10 (>200)
<b>K-1735 C19</b>			
i.v.	None	0/10	0/10
s.c.	8/10	0/10	0/10
<b>K-1735 M2</b>			
i.v.	None	10/10 (>200) <sup>f</sup>	0/10
s.c.	10/10	4/10 <sup>g</sup> (2-21)	0/10

<sup>a</sup> Mice were given injections of  $10^5$  tumor cells by each route. The tumorigenicity was determined at day 45.

<sup>b</sup> Number of positive mice/number of mice given injection.

<sup>c</sup> Median number of metastases.

<sup>d</sup> i.b., intrabronchial.

were added to each 38-mm<sup>2</sup> well. Four days later, the wells were washed thoroughly, the adherent monolayers were trypsinized, and the cells were harvested. Using a hemacytometer or a Coulter counter, the number of viable cells/well was determined by counting six independent cultures. At the end of the assay, the median numbers of M-5076 cells grown alone, control endothelial cells grown alone, and activated endothelial

cells grown alone were  $1 \times 10^5$ ,  $4.9 \times 10^4$ , and  $4.8 \times 10^4$  cells/well, respectively. The numbers of unactivated endothelial cells and M-5076 cells were  $1.2 \times 10^5$  cells/well, and those of activated endothelial cells and M-5076 cells were  $1.2 \times 10^5$  and  $6 \times 10^4$  cells/well, respectively (50% cytotoxicity) (Table 5).

**Fibroblast Control.** To further rule out the possibility that endothelial cells merely served as carriers for cytokines, which then produced lysis of target cells, we repeated the study with BALB/c nude mouse fibroblasts serving as effector cells. The data summarized in Table 6 demonstrate that, whereas cytokine-treated lung endothelial cells produced significant lysis of M-5076 (60%) and B16-F<sub>1</sub> (51%) cells ( $P < 0.001$ ), skin fibroblasts produced none. Once again, we did not observe any direct effects by the cytokines.

**Initial Binding of M-5076 Tumor Cells to Lung Microvessel Endothelial Cells.** We next sought to rule out the possibility that the activated endothelial cell-mediated lysis could have been due to differences in initial endothelial cell binding of target cells. We therefore examined the binding of radiolabeled M-5076 tumor cells to either activated or control endothelial cells at different time points (Fig. 5). At a tumor cell to endothelial cell ratio of 2:1, the binding of M-5076 tumor cells to both activated and control endothelial cells was very similar. Tumor lysis began after 48 h; thereafter, more M-5076 cells were found bound to normal endothelial than to cytokine-activated endothelial cells (Fig. 5).

Table 3 Lysis of tumor cells by mouse lung endothelial cells

	Radioactivity (cpm $\pm$ SD)		
	M-5076	K-1735 C19	K-1735 M2
Tumor cells <sup>a</sup>	999 $\pm$ 24 <sup>b,c</sup>	3079 $\pm$ 29	4440 $\pm$ 29
Tumor cells with activators <sup>d</sup>	1187 $\pm$ 21 (-19%)	2217 $\pm$ 35 (28%)	2919 $\pm$ 15 (34%)
Tumor cells and control endothelial cells <sup>a</sup>	1076 $\pm$ 31 (1%)	2944 $\pm$ 38 (4%)	4452 $\pm$ 17 (-0.3%)
Tumor cells and activated endothelial cells <sup>d</sup>	169 $\pm$ 41 (84%)	882 $\pm$ 54 (72%)	1474 $\pm$ 38 (67%)

<sup>a</sup> Medium without activators.

<sup>b</sup> A 5-day assay. Tumor cell to endothelial cell ratio was 1:5.

<sup>c</sup> Mean  $\pm$  SD of triplicate cultures (percentage of cytotoxicity).

<sup>d</sup> Medium with activators: recombinant human TNF (10 units/ml) and recombinant mouse IFN- $\gamma$  (10 units/ml).

Table 4 Supernatant analysis of endothelial cell-mediated cytotoxicity against M-5076

Source of supernatant	Radioactivity (cpm)		
	Supernatant <sup>a</sup>	Filter <sup>b</sup>	Filtrate <sup>b</sup>
M-5076	273	37	205
M-5076 and endothelial cells	219	71	178
M-5076 and endothelial cells with cytokines <sup>c</sup>	807	-5	788

<sup>a</sup> Supernatants were collected after centrifugation of tissue culture plates at 1000 rpm for 5 min; the radioactivity in the supernatants was measured.

<sup>b</sup> Supernatants were filtered through a 0.2-μm Millipore filter and the radioactivity in both the filter and filtrate were determined.

<sup>c</sup> rTNF-α (10 units/ml) and rIFN-γ (10 units/ml).

Table 5 Endothelial cell-mediated cytotoxicity against M-5076

	Without cytokines	With cytokines <sup>a</sup>
M-5076	5.4 × 10 <sup>5b</sup>	6.1 × 10 <sup>5</sup>
Endothelial cells	2.9 × 10 <sup>5</sup>	2.8 × 10 <sup>5</sup>
M-5076 and endothelial cells	8 × 10 <sup>5</sup>	4.3 × 10 <sup>5</sup>

<sup>a</sup> rTNF-α (10 units/ml) and rIFN-γ (10 units/ml).

<sup>b</sup> Total cell number in six wells.

**Kinetics of Endothelial Cell-mediated Tumor Cytotoxicity.** To study the kinetics of endothelial cell-mediated tumor cytotoxicity, we pretreated either M-5076 target tumor cells or endothelial cells for different periods of time with rTNF-α and rIFN-γ (10 units/ml each). The target cells or endothelial cells were then washed, admixed, and incubated for 96 h in the following media: (a) fresh medium (Figs. 6A and 7A) or (b) fresh medium containing rTNF-α and rIFN-γ (10 units/ml each) (Figs. 6B and 7B). Pretreatment of M-5076 tumor cells for different time periods with rTNF-α and rIFN-γ did not result in their lysis by medium-treated control endothelial cells (Fig. 6A). However, when rTNF-α and rIFN-γ (10 units/ml) were added to the medium, tumor lysis occurred at expected levels (Fig. 6B). Pretreatment of endothelial cells for 2, 8, or 24 h with 10 units/ml rTNF-α and rIFN-γ produced very low levels of tumor cell lysis when assayed in fresh medium. This low level of tumor cytotoxicity disappeared when endothelial cells were pretreated for 48–72 h (Fig. 7A). When the pretreated endothelial cells were assayed in fresh medium containing rTNF-α and rIFN-γ, high levels of tumor cell lysis were found in the 2- and 8-h pretreatment groups. In endothelial cells pretreated for up to 72 h, the tumor cytotoxicity decreased to a level lower than that found in control cultures.

**DISCUSSION**

Vascular endothelial cells were successfully isolated from mouse lung, grown as monolayer cultures, and expanded to establish cell lines. To exclude possible contamination with fibroblasts, blood monocytes, lymphocytes, or macrophages, the endothelial cells were cloned by a limiting dilution procedure. The cloned cells were characterized as vascular endothelial cells based on the properties of cell morphology (46, 49), contact inhibition of growth (46), nonthrombogenic surface

(49), expression of factor VIII-associated antigen (47–49), angiotensin-converting enzyme activity (50), uptake of acetylated-LDL (51), phagocytic activity (52), and *in vitro* angiogenesis-associated properties (58).

Although the isolated endothelial cells expressed these biological properties, we found the cell populations to be heterogeneous, especially in the expression of factor VIII-related antigen and uptake of acetylated-LDL. Since we used a rabbit polyclonal antibody raised against human factor VIII-related antigen, the detection was based on cross-reactivity between human and mouse factor VIII-related antigens. The antibody reactivity decreased with passage number of the endothelial cells, and the polyclonal antibody produced superior results to those of the monoclonal antibodies.

The uptake of acetylated-LDL, which is mediated by LDL receptors on endothelial cells (51), occurred in about 50% of murine lung endothelial cells. LDL receptors on endothelial cells are sensitive to trypsinization, so a 48-h incubation was necessary for recovery of activity. The endothelial cells did not display marker antigens unique to macrophages and lymphocytes. Angiotensin-converting enzyme activity, a unique property of lung vascular endothelial cells (59), was examined by a radioassay (50). Finally, the endothelial cells showed remarkable phagocytic activity. After 24-h incubation in medium with 0.8-μm-diameter latex beads, the cells could engulf >300 beads/cell, confirming an earlier observation with bovine endothelial cells (51, 52).

We used different mouse tumor lines to study the interaction of tumor cells with control (medium-treated) and activated (cytokine-treated) endothelial cells. We found that these tumor cell lines have different capacities to establish and grow in the lung after s.c. or i.v. injection. The M-5076 reticulum cell sarcoma cells have been shown to preferentially produce peritoneal visceral metastases but not lung metastases, suggesting that these cells are not capable of growing in the lungs (44). We found that M-5076 cells could grow in the lung after intrabronchial but not i.v. injection (53).

Tumor cells that spontaneously metastasize or are injected i.v. reach the lung by the circulatory route and must pass through endothelial cells to reach the organ parenchyma (12, 13). Evidence for the importance of tumor cell/endothelial cell interaction in determining organ-specific metastasis is accumulating (12, 13), and various studies have shown preferential adhesion of metastatic cells to target organ endothelial cells (14, 16–18). The inability of circulating M-5076 cells, known to arrest in the lung (44), to produce lesions could, therefore, be due to cytotoxic interactions with endothelial cells.

Recent studies amply demonstrate that many biological properties of endothelial cells resemble those of macrophages (27, 28, 31, 33, 35), and the term “reticuloendothelial system” denotes such a relationship. These findings prompted us to examine whether, like macrophages, endothelial cells can be activated to become antitumor effector cells by their interaction

Table 6 Lysis of tumor cells by mouse lung endothelial cells or skin fibroblasts

Cells	Cytokines <sup>a</sup>	Lung endothelial cells		Skin fibroblasts	
		M-5076	B16-F1	M-5076	B16-F1
Tumor cells	-	871 ± 8 <sup>b, c</sup>	2897 ± 47	608 ± 12	1450 ± 69
Tumor cells	+	881 ± 13 (-1%)	2884 ± 54 (0%)	694 ± 13 (-12%)	1528 ± 30 (-5%)
Tumor cells and effector cells	-	974 ± 17	3657 ± 104	784 ± 17	2757 ± 68
Tumor cells and effector cells	+	380 ± 25 (61%)	1792 ± 37 (51%)	748 ± 19 (5%)	2880 ± 15 (-4%)

<sup>a</sup> Recombinant human TNF (10 units/ml) and recombinant mouse IFN-γ (10 units/ml).

<sup>b</sup> A 5-day assay. Tumor to effector ratio was 1:5. Endothelial cells were used at passage 5 or 6. Skin fibroblasts were used at passage 6 or 7.

<sup>c</sup> Mean ± SD of triplicate cultures (percentage of cytotoxicity).

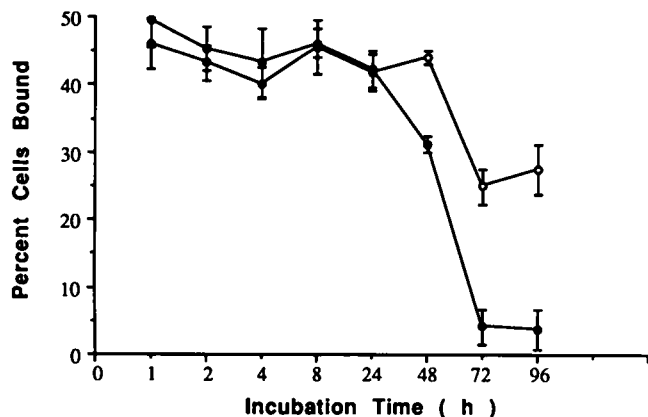


Fig. 5. Binding of M-5076 tumor cells to mouse lung endothelial cell monolayers. Endothelial cells ( $10^5$ ) were plated into each well of 24-well tissue culture plates. When monolayers were formed in about 48 h,  $2 \times 10^5$  [ $^{125}$ I]dUrd-labeled M-5076 cells were added onto the endothelial cell monolayer in each well. At different time points, the tumor-endothelial co-cultures were washed 5 times with PBS, and the cultures were lysed with 0.1% NaOH. Radioactivity in the supernatants was monitored.  $\circ$ , unactivated;  $\bullet$ , cytokine-activated.

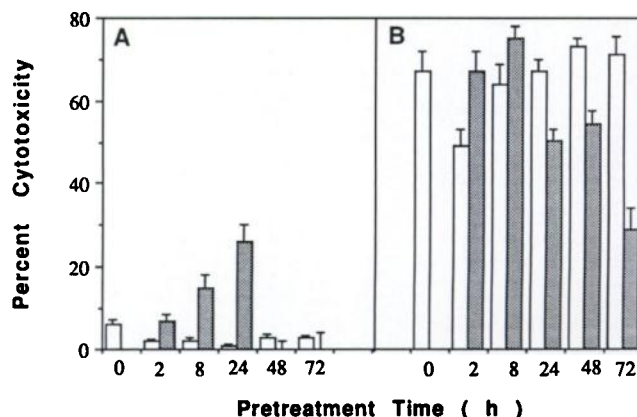


Fig. 7. Kinetics of endothelial cell-mediated tumor lysis: pretreatment of effector cells. Endothelial cell monolayers were pretreated with rTNF- $\alpha$  (10 units/ml) and rIFN- $\gamma$  (10 units/ml). At each time point, the endothelial cell monolayers were washed twice with PBS and [ $^{125}$ I]dUrd-labeled M-5076 cells were added. *A*, assayed in fresh medium. *B*, assayed in fresh medium with rTNF- $\alpha$  (10 units/ml) and rIFN- $\gamma$  (10 units/ml). The endothelial cell-mediated lysis of M-5076 cells was determined at day 5, as described in "Materials and Methods."  $\square$ , unactivated;  $\blacksquare$ , cytokine-activated.

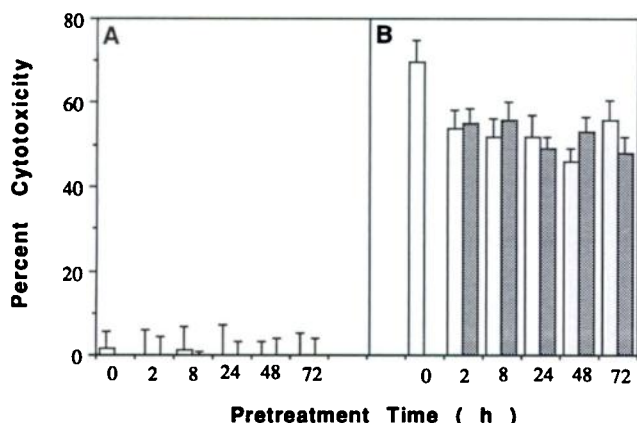


Fig. 6. Kinetics of endothelial cell-mediated tumor lysis: pretreatment of target cells. [ $^{125}$ I]dUrd-labeled M-5076 cells were pretreated with rTNF- $\alpha$  (10 units/ml) and rIFN- $\gamma$  (10 units/ml). At each time point, tumor cells were washed twice with PBS and added to endothelial cell monolayers. *A*, assayed in fresh medium alone. *B*, assayed in fresh medium with rTNF- $\alpha$  (10 units/ml) and rIFN- $\gamma$  (10 units/ml). The endothelial cell-mediated lysis of M-5076 cells was determined at day 5, as described in "Materials and Methods."  $\square$ , unactivated;  $\blacksquare$ , cytokine-activated.

with cytokines. Of several cytokines tested, the combination of 10 units/ml rTNF- $\alpha$  and rIFN- $\gamma$  rendered mouse lung endothelial cells cytotoxic to all tumor cell lines tested here. Endothelial cells incubated with medium, rTNF- $\alpha$ , or rIFN- $\gamma$  alone were not cytotoxic. Although synergism between rTNF- $\alpha$  and rIFN- $\gamma$  has been reported for direct antitumor properties (56, 57), at 10 units/ml this combination did not produce direct lysis of any of the tumor targets (Table 3).

Although the exact mechanism by which cytokine-activated endothelial cells mediate lysis of tumor cells is still unclear, it is unlikely to involve TNF- $\alpha$  or IFN- $\gamma$ . We base this conclusion on the data showing that M-5076 cells most sensitive to lysis by cytokine-activated endothelial cells were resistant to direct cytotoxic effects of the cytokines, even at 500 units/ml each. Moreover, cytokine-treated fibroblasts did not produce lysis of M-5076 cells or B16-F $_1$  cells (Table 6).

The ability of cytokine-activated endothelial cells (in contrast to medium-treated endothelial cells) to kill M-5076 cells was not due to a difference in the initial binding of these cells to endothelial monolayers. Tumor cell lysis began by 48 h of cocultivation and reached a maximum by 96 h. The degree of endothelial cell-mediated tumor cytotoxicity increased with the endothelial cell to tumor cell ratio. Although significant cytotoxicity was achieved with a 1:1 ratio, the optimal ratio was 5:1. Kinetics analysis suggested that, although cytokine pretreatment of endothelial cells for short periods of time can induce low levels of tumor cytotoxicity, the continuous presence of low concentrations of cytokines in the coculture was necessary to produce efficient destruction of tumor cells by activated endothelial cells.

The majority of i.v. injected tumor cells die within hours (2). Endothelial cell-mediated target cell lysis *in vitro* required several days of coincubation. These data suggest that the kinetics of tumor cell lysis by endothelial cells differ between *in vitro* and *in vivo* conditions. It is important to note that, during the process of hematogenous metastasis, tumor cells come in close contact with the vascular endothelial cells of many organs. The passage of tumor cells through a narrow lumen of a capillary could lead to tumor cell death (3-5), although recent studies suggest that poorly and highly metastatic melanoma and lymphoma cells are equally resistant to shear stress (60). In addition, production of oxygen radicals in the lung microvasculature has been proposed as a contributing factor (6). Close contact of tumor cells with endothelial cells (*in vivo* conditions) should provide better exposure to mediators produced by endothelial cells, as compared to the *in vitro* conditions. Preliminary results from our laboratories indeed suggest that direct contact between tumor cells and endothelial cells is necessary, and we are now investigating whether endothelial cells produce and release diffusible cytotoxic molecules (61) that lyse target cells. In summary, our studies show that, under defined conditions, biological substances such as TNF- $\alpha$  and IFN- $\gamma$  can activate endothelial cells to lyse tumor cells. The possibility that this mechanism is operative *in vivo* is under investigation.



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