Arrest of B16 Melanoma Cells in the Mouse Pulmonary Microcirculation Induces Endothelial Nitric Oxide Synthase-Dependent Nitric Oxide Release that Is Cytotoxic to the Tumor Cells

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Metastatic cancer cells seed the lung via blood vessels. Because endothelial cells generate nitric oxide (NO) in response to shear stress, we postulated that the arrest of cancer cells in the pulmonary microcirculation causes the release of NO in the lung. After intravenous injection of B16F1 melanoma cells, pulmonary NO increased sevenfold throughout 20 minutes and approached basal levels by 4 hours. NO induction was blocked by *N*^G-nitro-L-arginine methyl ester (L-NAME) and was not observed in endothelial nitric oxide synthase (eNOS)-deficient mice. NO production, visualized *ex vivo* with the fluorescent NO probe diaminofluorescein diacetate, increased rapidly at the site of tumor cell arrest, and continued to increase throughout 20 minutes. Arrested tumor cells underwent apoptosis with apoptotic counts more than threefold over baseline at 8 and 48 hours. Neither the NO signals nor increased apoptosis were seen in eNOS knockout mice or mice pretreated with L-NAME. At 48 hours, 83% of the arrested cells had cleared from the lungs of wild-type mice but only ~55% of the cells cleared from eNOS-deficient or L-NAME pretreated mice. eNOS knockout and L-NAME-treated mice had twofold to fivefold more metastases than wild-type mice, measured by the number of surface nodules or by histomorphometry. We conclude that tumor cell arrest in the pulmonary microcirculation induces eNOS-dependent NO release by the endothelium adjacent to the arrested tumor cells and that NO

is one factor that causes tumor cell apoptosis, clearance from the lung, and inhibition of metastasis. (Am J Pathol 2003; 162:403–412)

In the lung, metastatic neoplasms are the most common malignant tumors. Their formation usually involves hematogenous seeding of metastatic cancer cells into the lung from remote primary tumors. Interactions between intravascular cancer cells and the endothelium are important determinants of metastatic outcome.^{1,2} For example, the expression of constitutive and inducible microvascular adhesion molecules, and the release of reactive oxygen species (NO, O₂⁻, and H₂O₂) by endothelial cells or cancer cells can regulate the mechanisms that govern the metastatic process, including cancer cell adhesion and arrest,³ the production of endothelial matrix metalloproteinases,⁴ and cancer cell apoptosis.⁵

Evidence from *in vitro* and *in vivo* studies has shown that reactive oxygen and nitrogen species can be cytotoxic to neoplastic cells^{6–9} and reduced their adhesion to postcapillary venules.¹⁰ *In vivo*, we have recently demonstrated that the arrest of intravascular B16F1 melanoma cells in the liver induces the rapid local release of nitric oxide (NO) that causes apoptosis of the melanoma cells and inhibits their subsequent development into hepatic metastases.⁵ Because pulmonary endothelial cells generate NO in response to shear stress,¹¹ we have postulated that there is a comparable cytotoxic mechanism in the lung.

Here we provide data showing that the arrest of B16F1 melanoma cells in the pulmonary circulation of wild-type C57B1/6 mice (WT mice) induces the local release of NO that is endothelial nitric oxide synthase (eNOS)-dependent. In turn NO causes the melanoma cells to undergo

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apoptosis and inhibits their development into pulmonary metastatic tumors. The existence of comparable mechanisms in the lung and the liver suggests that NO may be a natural defense against the formation of metastatic tumors in organs with complex microvascular networks.

Materials and Methods

Experimental Design

Fluoresbrite carboxylate YG microsphere-labeled B16F1 melanoma cells were injected into the tail veins of C57Bl/6 mice. The lungs were removed between 0 and 48 hours after cell injection to measure NO production, tumor cell apoptosis, and the clearance of tumor cells from the lung. To study metastasis, unlabeled B16F1 cells were injected and the lungs were collected 7 or 14 days later to count surface metastatic nodules or to quantify metastasis by histomorphometry. All analyses were performed blindly.

Animals and Materials

WT female C57Bl/6 mice, weighing 20 to 22 g, were purchased from Charles River Breeding Laboratories (Montreal, QC, Canada or Kingston, NY) and housed according to University of Manitoba guidelines. eNOS knockout C57Bl/6 mice (eNOS KO mice), age 5 to 6 weeks, were obtained from Jackson Laboratory (Bar Harbor, ME). The murine B16F1 melanoma cell line was obtained from the American Type Culture Collection (Rockville, MD). Fluorescent polystyrene microspheres (15 μm in diameter) were purchased from Bangs Laboratories, Inc. (Fisher, IN). Fluoresbrite carboxylate YG microspheres (0.05 μm) were purchased from Polysciences, Inc. (Warrington, PA) and MitoTracker Red from Molecular Probes (Eugene, OR). α -MEM, Opti-MEM reduced serum medium, penicillin-streptomycin, and trypsin-ethylenediaminetetraacetic acid were purchased from Life Technologies (Burlington, ON, Canada). Avertin (2,2,2-tribromoethanol), diethyldithiocarbamate (DETC), and N^G -nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). 4,5-Diaminofluorescein diacetate (DAF-2 DA) was obtained from Calbiochem (La Jolla, CA). The ApopTag Peroxidase *in Situ* Apoptosis Detection Kit S7100 was from Intergen Company (Purchase, NY).

Cell Culture and Fluorescent Labeling

B16F1 cells were cultured in α -MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution, in a T25 culture flask (Corning Glass, Corning, NY) to 95% confluence. The culture medium was then replaced by Opti-MEM serum-reduced medium and fluoresbrite carboxylate microspheres were added to the flask in a ratio of 100 μl of microspheres per 5 ml of Opti-MEM medium (microsphere stock, 2.5% solids-latex). The flask was gently mixed and put back in the incubator for 2 hours to label the cells with occasional

shaking of the flask. The cells were rinsed three times with Opti-MEM medium to wash off the unincorporated beads and left in α -MEM culture medium overnight to reduce cell aggregation on detaching. The labeled cells were detached on the following day with 0.5% trypsin-ethylenediaminetetraacetic acid at 37°C for 5 minutes. The cell suspension was centrifuged at $170 \times g$ for 3 minutes. The cell pellet was resuspended in pyrogen-free saline and kept on ice before injection. Cells with a viability >95%, measured by trypan blue exclusion, were used in the study.

B16F1 Melanoma Cell Injection

Mice were anesthetized with avertin (30 mg/ml; 0.2 to 0.3 ml/mouse, IP). A fluorescent-labeled cell suspension of 5×10^5 B16F1 melanoma cells in a volume of 150 μl of saline was injected into the tail vein throughout 2 to 3 minutes, using a 30G1/2 needle and 1-ml syringe. The lung was collected under anesthesia at specific times (0 to 48 hours) after injection. For 0-hour samples, the lung tissue was removed immediately at the end of the injection (within 30 seconds to 1 minute). To determine whether the shear stress of tumor cells may also play a role in the induction of NO by lung microvascular endothelial cells, we injected 5×10^5 15- μm fluorescent-labeled polystyrene microspheres in a volume of 150 μl of saline intravenously. The lung tissue was collected 20 minutes afterward for electron paramagnetic resonance (EPR) measurement.

L-NAME Administration

Based on our previous study,⁵ the competitive NO synthase inhibitor L-NAME was administered to inhibit NO production and to test its effects on cell clearance, apoptosis, and metastasis formation. For cell clearance and apoptosis analysis, 5 mg/kg of L-NAME was injected intraperitoneally 1 hour before cell injection and 2.5 mg/kg of L-NAME was co-injected with the cells. For the metastasis assays, 2.5 mg/kg of L-NAME was co-injected with the cells and 5 mg/kg of L-NAME was injected intraperitoneally 20 hours after the cell injection.

EPR Measurement of NO Production

EPR spectroscopy was used to quantitatively measure the production of NO. The NO trapping agents DETC (400 mg/kg in saline, IP) and FeSO_4 /sodium citrate (40 mg/kg and 200 mg/kg, respectively, mixed in water, SC) were given to each mouse 30 minutes before obtaining the lung sample. The mice were anesthetized 10 minutes before sacrifice. The thorax was opened and the lungs were removed (between 30 seconds to 1 minute) and placed onto a precooled Petri dish on ice. The lungs were quickly sliced into smaller pieces, placed into a precooled 1-ml syringe, and transferred into a Suprasil synthetic quartz tube (2.4-mm inner diameter; Heraeus Amersil, Duluth, GA) by pushing the tissue gently through the syringe to fill the tube up to 3 to 4 cm in height. The tube

Figure 1. Induction of NO in the lung after intravenous injection of B16F1 melanoma cells into C57B1/6 mice. B16F1 melanoma cells (5×10^5) in $150 \mu\text{l}$ of saline were injected into the pulmonary microcirculation via the tail vein. The mice were given NO trapping agents 30 minutes before sacrifice. NO was measured by EPR spectroscopy. **A:** The relative EPR signal intensity of the NO-Fe²⁺-(DETC)₂ complex was expressed in arbitrary units. These were calculated by measuring the distance from the peak to the trough of the triplet-hyperfine structure at the $g = 2.04$ location after subtraction of the Cu²⁺-(DETC)₂ signal (495 vertical units = 100 nmol NO/g of wet tissue $\pm 20\%$). ■, B16F1 cell injection into WT mice; ●, 15- μm polystyrene beads injected into WT mice; □, saline injection into WT mice; ○, B16F1 cell injection into L-NAME-treated WT mice; ◇, B16F1 cells injected into eNOS KO mice ($n = 5$ to 10 mice/point). Data are expressed as the mean $\pm$ SE. *, $P < 0.05$ as compared to saline control at 0 minutes; **, $P < 0.01$ as compared to microspheres, L-NAME, saline controls, and eNOS KO mice at 20 minutes; ***, $P < 0.01$ as compared to L-NAME, saline controls, and eNOS KO mice at 20 minutes. **B:** Representative complete EPR spectra of NO-Fe²⁺-(DETC)₂ complex (triplet-hyperfine structure at $g = 2.04$) measured 20 minutes after B16F1 melanoma cell injection [Cu²⁺-(DETC)₂ signal was subtracted].

was then immediately placed into liquid nitrogen until NO was measured by EPR spectroscopy.¹² EPR spectroscopy, used for the measurement of trapped NO-Fe²⁺-(DETC)₂ complex, was performed at 125 K (temperature controller model BVT-3000; Bruker Spectrospin Ltd., Karlsruhe, Germany). The spectra were measured with a Bruker model EMX EPR X-band spectrometer system operating at 9.25 GHz with 100 kHz modulation. The instrument settings were as follows: 1) microwave power, 5 mW; 2) modulation amplitude, 5 G and signal level, 1×10^3 ; and 3) scan range, 500 G. The concentration of the NO-Fe²⁺-(DETC)₂ complex in each sample was assumed to be proportional to the signal amplitude (peak-to-trough) of the triplet-hyperfine structure (hyperfine splitting of 13 G) observed at $g = 2.04$ (see below). Data were expressed as relative EPR signal intensities (arbitrary units) after subtracting the Cu²⁺-(DETC)₂ complex signal observed in all samples. As described in our previous work,⁵ we performed a double-integration calibration to calibrate the conversion from peak-to-peak EPR amplitudes to nmol ON-Fe²⁺-(DETC)₂/gram of wet tissue concentrations (see the caption to Figure 1). The only difference is that we used the Bruker EPR spectrometer for our present work, whereas the Varian E-12 spectrometer was used previously.⁵ We have performed EPR measurements on the same samples at 125 K on both the Varian and Bruker EPR systems to relate the peak height measurements from one instrument to the other. AWIN-EPR software (Bruker Spectrospin Ltd.) was used for nearly all EPR data manipulation on a Compaq Deskpro P500 computer (Compaq Computer Corp., Houston, TX).

In Situ Measurement of NO Generation in Pulmonary Endothelial Cells

NO generation was monitored *in situ* by labeling the pulmonary endothelium with DAF-2 DA that is de-esterified intracellularly to DAF-2. NO and its higher oxides, such as NO_x or nitrous anhydride (N₂O₃), provide the third nitrogen to form a triazo ring from the two amino groups of the nonfluorescent DAF-2 and convert it to diaminotriazolo fluorescein (DAF-2T) that is fluorescent and can be monitored at 490 nm excitation and 530 nm emission.¹³

We used an established intact organ microscopy technique to image microvascular endothelial cells *in situ* in isolated, ventilated, blood-free mouse lungs in real time using an epifluorescence microscope.¹⁴ Briefly, female WT mice, weighing 20 to 22 g were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). A tracheostomy was performed, and artificial ventilation with 95% air and 5% CO₂ (BOC Group, Inc., Murray Hill, NJ) was started through a cannula. The abdomen was opened and the animal was exsanguinated by transection of the major abdominal vessels. A cannula was inserted into the main pulmonary artery via a puncture in the right ventricle, and another was inserted into the left atrium. The lung was cleared of blood by gravity perfusion via the pulmonary artery with an artificial medium (Krebs-Ringer bicarbonate buffer KRB: NaCl, 118.45; KCl, 4.74; MgSO₄/7H₂O, 1.17; CaCl₂/2H₂O, 1.27; KH₂PO₄, 1.18; and NaHCO₃, 24.87, in mmol/L, with 5% dextran and 10 mmol/L glucose at pH 7.4). The flow-through perfusate left the lung via the left atrial cannula.

Once the lung became visibly cleared of blood, the heart-lung preparation was dissected *en bloc* and was placed onto a 48 × 60 × 0.16-mm coverglass window in a specially designed Plexiglas chamber with ports for the tracheal, pulmonary, and left atrial cannulas. The cardiovascular ports were connected to a peristaltic pump that recirculated 30 ml of perfusate at a constant flow rate of 2 ml/min through the pulmonary vascular bed. The chamber was placed on the stage of an epifluorescence microscope fitted with a ×60 objective (Nikon Diaphot TMD) and equipped with an optical filter changer (Lambda 10-2; Sutter Instrument Co., Novato, CA). A local anesthetic (0.05 mg of xylazine, Sigma) was injected subepicardially into the posterior wall of the right atrium to abolish lung movement artifact because of contraction of remaining cardiac muscle. Excitation of the lung surface was accomplished with a mercury lamp fiber optic light source, a fluorescein isothiocyanate filter set for DAF-2T (HQ41001 with 480/40 excitation filter, 505 LP dichroic mirror, and 535/40 emission filter; Chroma Technology Corp., Brattleboro, VT). The integrity of the preparation was continuously monitored by online measurements of intratracheal and pulmonary artery perfusion pressures. Endothelial cells in the subpleural vasculature were positively identified by labeling with Dil-acetylated low-density lipoprotein added to the perfusate (a tetramethylrhodamine isothiocyanate filter set for Dil-acetylated low-density lipoprotein, Chroma Technology Corp.). We used a Nikon Diaphot TMD epifluorescence microscope, a Hamamatsu ORCA-100 digital camera (Hamamatsu Corp., Bridgewater, NJ), and MetaMorph imaging software (Universal Imaging Corp., Downingtown, PA) for imaging. After an equilibration period of 45 minutes with the isolated lung to allow uptake of DAF-2 DA (5 μ mol/L), intravascular dye was removed by perfusion with dye-free medium for 5 minutes to reduce background fluorescence. Mitotracker-Red labeled B16F1 cells (2×10^6) were injected through a pulmonary artery port by peristaltic pump at 2 ml/min. Images of DAF-2T-stained vascular endothelial cells were taken from the same area every 3 seconds for up to 20 minutes during which ventilation was stopped. As a control, after the equilibration period, images were taken during continuous perfusion.

For quantification, each endothelial cell was outlined and its fluorescence intensity was measured throughout time. For each lung, mean fluorescence intensities of four to seven endothelial cells were averaged. Fluorescence intensity of three to four lungs for each condition was averaged. Data are expressed as relative fluorescence intensities (arbitrary units). All results are expressed as mean $\pm$ SE for each condition.

Assessment of B16F1 Cell Apoptosis in Vivo

Lung tissues for *in situ* DNA end-labeling were collected at 0, 4, 8, 24, and 48 hours after tail vein injection of fluorescent-labeled B16F1 cells. No trapping agent was given to these animals. For each time point five animals were used. Frozen sections (12 μ m) were obtained from the upper and median lobes of the right lung and upper

lobe of the left lung. *In situ* DNA end labeling using a digoxigenin-peroxidase detection system (ApopTag S7100 kit, Intergen) was performed on these sections that contained injected fluorescent-labeled melanoma cells. To achieve optimal double labeling, the procedures were performed according to the manufacture's instructions with the following modifications, as previously described.⁵ 1) Proteinase K digestion (20 μ g/ml) was added before postfixing the sections with ethanol/acetic acid (2:1, v/v), to facilitate the unmasking of fragmented DNA in apoptotic tumor cells. 2) Incubation for *in situ* DNA end labeling reaction was performed overnight in a humidified chamber at 37°C, with subsequent 1 hour anti-digoxigenin peroxidase conjugate incubation at room temperature in a humidified chamber and 12 minutes peroxidase substrate color development. 3) The counter stain specimen and mount specimen steps were omitted to prevent the quenching of fluorescence in the microspheres by organic solvents. 4) The slides were mounted with Gel/Mount (Biomedica Corp, Foster City, CA). To generate positive control samples for apoptosis, labeled B16F1 cells were exposed to 2 μ m of NO donor S-nitroso-N-acetyl-penicillamine (SNAP) for 24 hours. Detached and collected cell pellets were resuspended in 1% paraformaldehyde at $1 \times 10^6/150 \mu$ l of saline. Lung tissues were collected immediately after injection and fixed in 1% paraformaldehyde.

The total number of both single fluorescent- and double-labeled cells (fluorescent/ApopTag DNA end-labeling) was scored using a fluorescence microscope with dual illumination of the fluorescence and incandescence ($\times 200$ magnification). More than 100 total cells from both left and right lobes were counted for most samples. The percentage of *in situ* DNA end-labeled melanoma cells was calculated by the formula: *in situ* end-labeled tumor cells (%) = $100 \times [\text{number of double-labeled cells}]/[\text{total number of cells (double labeled + single labeled)}]$.

Analysis of B16F1 Cell Clearance and Fragmentation

Frozen sections (12 μ m) were obtained from the upper and median lobes of the right lung and the upper lobe of the left lung, collected at 0, 4, 8, 24, and 48 hours as described above. Fluorescent-labeled B16F1 cell fragments and the intact fluorescent-labeled B16F1 melanoma cells in 100 microscopic fields ($\times 200$ magnification) were counted from each lobe to determine the number of B16F1 cells in the lung at each time point. The ratio of cell fragments to intact cells at 8 hours and 48 hours was calculated as follows: cell fragments (%) = (cell fragments/cell fragments + intact cells) $\times$ 100. Data were expressed as the mean of five values.

Metastasis Studies

Unlabeled B16F1 cells ($5 \times 10^5/150 \mu$ l saline) were injected into the tail vein of the mice and allowed to grow *in vivo* for 7 or 14 days. All animal surgical procedures were approved by the Bannatyne Campus Protocol Manage-

ment and Review Committee at the University of Manitoba. All animal care was given according to the guidelines of the Central Animal Care Services at the University of Manitoba. The mice were sacrificed under anesthesia and the lungs were fixed in 10% neutral buffered formalin. All metastatic nodules identifiable on the surface of all lung lobes were counted using a dissecting microscope on the day 7 after tumor cell injection. Fourteen days after injection, histomorphometric analysis was performed on histological sections from all five lobes using a Merz Graticule to quantify the percentage of tissue area occupied by the metastases.

Statistical Analysis

Prism software was used for statistical analysis. For EPR, tumor cell apoptosis and clearance, an unpaired *t*-test was used. The Mann-Whitney *U*-test was used for metastasis studies. Comparisons of DAF-2T fluorescence intensity were made using analysis of variance with Bonferroni's test using SigmaPlot 2000 (SPSS Inc., Chicago, IL).

Results

Release of NO in the Lung after B16F1 Cell Injection

To determine whether NO is released in the lung after B16F1 cell arrest, we injected 5×10^5 B16F1 cells, in a volume of 150 μ l of saline, into the lateral tail vein of the WT C57Bl/6 mice. We injected the NO spin trap DETC/FeSO₄ 30 minutes before harvesting the tissue to detect NO generation by EPR spectroscopy. As shown in Figure 1, NO generation was induced as soon as tissue could be harvested after cell injection, increased sevenfold within 20 minutes after cell injection compared to the saline control at 0 minutes, and approached basal levels by 4 hours. Rapid and significant production of NO was also detected in mice injected with 5×10^5 fluorescent microspheres with similar diameter to that of B16F1 cells (16 ± 4 μ m). In these animals, the peak NO level was significantly lower than that induced by B16F1 cell injection ($\sim 66\%$ of peak NO level of B16F1 cells, $P < 0.01$). The injection of saline alone was not associated with significantly increased NO production. Pretreatment of animals with the NO synthase inhibitor L-NAME inhibited NO production at 20 minutes by 64%. NO induction was completely absent in mice defective in the eNOS gene. NO was not detected in B16F1 cells (data not shown).

To identify the cellular origin of NO, 2×10^6 fluorescent-labeled B16F1 cells were introduced into the perfusate of isolated intact WT mouse lungs in which the endothelium had been preloaded with DAF-2 DA. A low level of fluorescence, indicative of NO production, was seen after loading in all groups. Enhanced fluorescence was seen within 2 minutes of attachment to the pulmonary endothelium of WT mice. This fluorescence increased throughout the next 20 minutes at the site of arrest and also in adjacent endothelial cells. Fluorescence did not

change from basal levels after arrest of the tumor cells in eNOS-deficient mice (Figure 2, Table 1).

Apoptosis and Clearance of B16F1 Cells

To determine whether there was a correlation between NO production and apoptosis, lung tissues were harvested at various times after injecting 5×10^5 fluoresbrite microsphere-labeled cells. Cryostat sections of the lung tissue were stained for apoptotic cells with the ApopTag kit. Tumor cells were identified by their fluorescence (Figure 3A) and scored for apoptosis (Figure 3B). Tumor cells became fragmented by 48 hours after injection (Figure 3C). Fragmentation was greater in WT mice than in eNOS KO mice or L-NAME-treated mice (Table 2). As shown in Figure 4A, apoptosis of tumor cells increased with time after injection in WT mice. Enhanced apoptosis could be seen as soon as 4 hours after cell injection, reached a peak value threefold greater than baseline at 8 hours, and remained detectable throughout a 48-hour period. Apoptosis of tumor cells did not increase above baseline values in mice defective in the eNOS gene or in mice pretreated with L-NAME.

Twenty-four hours after cell injection, $\sim 47\%$ of tumor cells had been cleared from the lungs of WT mice; $\sim 83\%$ had been cleared by 48 hours. Clearance was less in mice defective in the eNOS gene or in mice pretreated with L-NAME (Figure 4B).

Metastasis

To evaluate the effect of NO induction on metastasis, we injected 5×10^5 unlabeled B16F1 melanoma cells intravenously into WT mice, eNOS KO mice, or WT mice treated with L-NAME. Lung metastases were measured by counting the number of nodules on the visceral pleural surfaces at 7 days or by histomorphometry at 14 days. Seven days after tumor cell injection eNOS KO mice had 2.3-fold more pleural surface metastases than WT mice ($P < 0.01$) and WT mice pretreated with L-NAME had 2.4-fold more metastases ($P < 0.05$). Fourteen days after B16F1 cell injection there was a twofold greater tumor burden in the lungs of L-NAME-treated WT mice ($P < 0.003$) and ~ 4.9 -fold greater tumor burden in the lungs of eNOS KO mice ($P < 0.001$), compared to the lungs of untreated WT mice. The data are shown in Table 3.

Discussion

This study corroborated our previous observation that the arrest of circulating B16F1 melanoma cells within the microvasculature of a metastatic target organ can trigger the local release of host-derived NO. In turn, NO can induce apoptosis in the neoplastic cells and inhibit their subsequent growth into metastatic tumors. Comparison of data from the studies in the lung and liver showed similarities and differences in the characteristics of this phenomenon between the two metastatic target organs.

Figure 2. Effect of B16F1 melanoma cells on DAF-2T fluorescence in subpleural endothelial cells *in situ* in the intact mouse lung. DAF-2 DA was circulated in the perfusate for 45 minutes of equilibration. The perfusate was then changed to buffer alone to wash out the dye. Tumor cells were added to the perfusate, and images were then acquired every 3 seconds for 20 minutes. Only a few frames from one experiment that is representative of four separate experiments are shown. MitoTracker-Red labeled B16F1 cells arrested in the lumen of the pulmonary capillary as shown in **A**. Endothelial cells are characterized by the elongated nuclear/perinuclear area shapes with the tapered ends pointing toward inflowing perfusate (**B**). DAF-2T fluorescence, indicating increased NO generation, occurred as early as 1 minute after injection and continued to increase at the site of tumor cell arrest throughout time as shown in **B** to **F**. Images are in pseudocolor (intensity scale shown in **G**).

As in the liver, the arrest of tumor cells in the lung microvasculature induced an immediate burst of NO. However, unlike the liver, where increased NO production was detected for less than 5 minutes, the expression of NO in the lung increased throughout a period of 20 minutes and then decreased subsequently throughout a

Table 1. *In Situ* Quantification of NO Production by DAF-2 Fluorescence at Sites of B16F1 Melanoma Cell Arrest

Time (minutes)	WT C57Bl/6 mice (mean $\pm$ SE)	eNOS KO mice (mean $\pm$ SE)
0	0	0
5	45 $\pm$ 10*	13 $\pm$ 6
10	151 $\pm$ 46†	31 $\pm$ 13
15	292 $\pm$ 99†	33 $\pm$ 13
20	347 $\pm$ 119†	43 $\pm$ 17

Pulmonary endothelial cells were preloaded with DAF-2DA as described in Figure 2. Two $\times 10^6$ B16F1 melanoma cells were added to the *ex vivo* perfusate and images were acquired. Endothelial cells adjacent to the arrested tumor cells were outlined and the fluorescence intensity was measured throughout time. For each lung, we determined the mean fluorescence intensities of four to seven endothelial cells and for each condition, three to four lungs were averaged. Data represent the percent increase in DAF-2T fluorescence intensity above baseline values and expressed as relative fluorescence intensities (arbitrary units). All results are expressed as the mean $\pm$ S.E. for each condition.

* $P < 0.05$ as compared to eNOS KO mice at 5 minutes.

† $P < 0.01$ as compared to eNOS KO mice at 10, 15, and 20 minutes.

period of 4 hours. Use of the DAF-2 staining method allowed us to further determine that, in the lung, NO originated from microvascular endothelial cells. Involvement of eNOS was confirmed by the complete absence of a response in eNOS KO mice and by the inhibition of the NO burst in L-NAME-treated mice.

The use of two distinct methods to detect NO production provided consistent evidence that endothelial-derived NO is released after the arrest of tumor cells in the pulmonary microvasculature. NO is a small diatomic-free radical, but it plays complex roles in diverse biological processes in organisms ranging from bacteria to mammals. The main difficulty in measuring NO production is caused by its extremely labile nature and short half-life, which is in the millisecond range. Among the indirect NO detection methods widely accepted in the literature, we chose to use spin trapping of a NO adduct *in vivo* and then measure the trapped NO signal *ex vivo* by EPR spectroscopy. The main advantages of spin trapping are that NO has a high affinity for the spin trapping iron complex and can instantaneously form a much more stable NO-Fe²⁺-(DETC)₂ complex. This complex generates a characteristic three-line structure on the EPR spectrum that can be distinguished from other signals. This technique has been shown to be a reproducible and sensitive NO detection method. We argue that it is the sensitivity and specificity of the EPR spin trapping

Figure 3. Apoptosis and fragmentation of B16F1 melanoma cells in the lung after tail vein injection. Analysis was performed on frozen sections (12 μ m) obtained from the upper and median lobes of the right lung and the upper lobe of the left lung. **A** and **B** show double-labeled B16F1 cell in the lung 8 hours after injection. The ApoptTag assay-positive B16F1 cell in **B** (black arrow) corresponds to the fluorescent-labeled B16F1 cell in **A** (white arrow). **C** shows B16F1 melanoma cell fragmentation 48 hours after injection. Arrows indicate fragmented fluorescent-labeled B16F1 cells.

method that helped us to capture the transient burst of NO release in the lung after the melanoma cell injection. We further quantified the NO peak concentration to be in the range of 50 to 100 nmol/gram of wet tissue using a well-calibrated *in vitro* standard. In addition to the three-line NO-Fe²⁺-(DETC)₂ spectrum, a quartet hyperfine spectrum of Cu²⁺-(DETC)₂ was also detected, which partially overlapped with the NO-Fe²⁺-(DETC)₂ spectrum. Our data were expressed as the relative EPR signal intensity (equivalent to the peak-to-trough amplitude of the three-line spectrum) with the Cu²⁺-(DETC)₂ spectrum subtracted, so that they represent the pure NO-Fe²⁺-(DETC)₂ signal intensity. To our knowledge, this is a more specific approach because most analyses measure the NO concentration on the basis of the uncorrected EPR spectrum alone. The measurements obtained by using DAF-2 DA gave direct morphological evidence for the release of NO by endothelial cells that were in direct contact with arrested tumor cells. Because there was a very low but measurable amount of NO in eNOS knockout

mice by EPR spectroscopy, it is possible that minimal amounts of NO were produced by other NO synthase isoforms.

We postulate that the mechanism responsible for the rapid release of NO from the pulmonary endothelium may be similar to that in the liver, ie, at least partially triggered by local shear forces produced by cell arrest in the microvasculature. In our previous and present studies, the NO burst could be elicited by injection of inert microspheres into the portal⁵ and pulmonary circulation.

Our data support the hypothesis that NO is an effector of B16F1 melanoma cell apoptosis in the lung. There is a large body of evidence that generation of NO, mediated by inducible NOS, can have cytotoxic effects on neoplastic cells and inhibit their potential for growth *in vivo*.^{15–24} In addition to this mechanism, which generates NO throughout a sustained period of time,²⁴ we have shown here that tumor cell arrest can trigger the immediate release of NO via eNOS-dependent mechanisms and subsequently trigger apoptosis in these cells. Previous experiments in the liver have inferred a role for eNOS as an effector of B16 melanoma cytotoxicity on the basis that NO release occurred immediately (<5 minutes) after tumor cell arrest in the hepatic sinusoid.⁵ In a recent study, Carretero and colleagues⁹ have used liver endothelial cells from eNOS^{-/-}, eNOS^{+/+}, and WT mice, treated with cytokines, to observe the release of NO and H₂O₂ and the cytotoxicity of these molecules to B16F1 cells. They found that H₂O₂ was not cytotoxic in the absence of NO, while NO-induced tumor cytotoxicity was increased by H₂O₂ because of the formation of potent oxidants, likely OH⁻ and ⁻OONO. Their work was conducted *in vitro*. In contrast, our work has focused on observations *in vivo* showing that tumor cell arrest in the lung triggers immediate NO release that is eNOS-dependent and cytotoxic. This is consistent with the observation that even a very

Table 2. Tumour Cell Fragmentation after Arrest in the Pulmonary Microvasculature

Group	% Cells that are fragmented (mean $\pm$ SEM)	
	8 hours	48 hours
WT mice	26 $\pm$ 2	56 $\pm$ 2
WT mice + L-NAME	22 $\pm$ 1	35 $\pm$ 2*
eNOS KO mice	22 $\pm$ 1	32 $\pm$ 2*†

Five $\times 10^5$ fluorescent-labeled B16F1 melanoma cells in 150 μ l of saline were injected intravenously into WT C57Bl/6 mice, WT C57Bl/6 mice treated with L-NAME, or eNOS KO mice. Fluorescent-labeled B16F1 cell fragments and intact cells were counted as described in Materials and Methods. Numbers represent the mean and standard error of the five animals per group. The data are expressed as the mean $\pm$ SE of the five lung samples at each time point.

**P* < 0.001 when compared to WT mice at 48 hours.

†*P* > 0.05 when compared to L-NAME-treated WT mice at 48 hours.

Figure 4. Kinetics of apoptosis and cell clearance. Analysis was performed as described above. **A:** The ApopTag assay was used to detect apoptotic cells. The total number of single-labeled and double-labeled cells was scored on all sections using dual illumination with incandescence and fluorescence (original magnification, $\times 200$; FITC UV-2A filter). The percentage of double-labeled apoptotic tumor cells was calculated. The data are expressed as the mean $\pm$ SE of the five lung samples at each time point. ■, B16F1 cell injection into WT mice; ○, B16F1 cell injection into L-NAME-treated WT mice; ◇, B16F1 cells injected into eNOS KO mice. *, $P < 0.01$ (0 versus 8 hours in WT mice, WT versus eNOS KO mice at 8 and 48 hours). **B:** Fluorescent-labeled B16F1 cells were counted to determine the number of B16F1 cells in the lung at each time point. In each animal, labeled B16F1 cells in 100 microscopic fields (original magnification, $\times 200$) were counted. Numbers represent the mean and SE of the five animals per group. *, $P > 0.05$ (WT mice without versus with L-NAME administration at 8 and 48 hours). **, $P < 0.01$ (WT mice without versus with L-NAME treatment and WT mice versus eNOS KO mice at 8 hours); **, $P < 0.01$ (WT mice without versus with L-NAME treatment and WT mice versus eNOS KO mice at 48 hours) of NK cells against tumors.

brief exposure (5 minutes) to NO *in vitro* is capable of triggering apoptosis in the B16F1 cells.⁵ It may be relevant to our observations that, at a clinical level, expression of immunoreactive eNOS in peritumoral microvessels has been identified as a favorable prognostic indicator in premenopausal breast cancer patients.²⁵

In addition to direct DNA damage caused by NO, its cytotoxic effects can also be mediated through generation of peroxynitrite (ONOO⁻) in the presence of superoxide anion (O₂⁻).²⁶ As a potent oxidant, peroxynitrite

can produce tissue damage through nitration of tyrosine residues in protein to form nitrotyrosine.²⁷ We are currently conducting experiments to determine whether nitrotyrosine can be identified in plasma²⁸ or at sites of NO release as a mediator of cytotoxicity, and as a reflection of low level NO production in eNOS knockout mice.

Counts of fluorescent-labeled B16 cells in lung sections, prepared at various times after intravenous injection, confirmed that metastatic cells are cleared from the lungs, as they are in the liver. Tumor cell clearance was regulated in part by NO because fewer cells were cleared from the lungs of L-NAME-treated and eNOS KO mice than from the lungs of WT mice. The mechanisms responsible for this may involve alterations in adhesion between the cancer cells and endothelium because NO has been reported to reduce the attachment of tumor cells to the endothelium.¹⁰ NO-mediated vasodilatation of pulmonary arterioles and venules²⁹ could also diminish cell trapping because of mechanical restriction. NO cytotoxicity to tumor cells may also contribute to the tumor cell clearance because morphologically, cell clearance was associated with fragmentation of the tumor cells and this was also greater in WT mice, compared with L-NAME-treated mice and eNOS KO mice. Previous flow cytometric analysis of B16 cells indicates that necrosis and apoptosis both occur in response to short exposure of these cells to NO.⁵ As in our previous studies, we found that apoptosis and clearance of tumor cells from the lung was associated with fewer metastatic nodules at 7 days and a lesser tumor burden measured by histomorphometry at 14 days.

Extensive experimental and clinical data indicate that the majority of tumor cells die rapidly in the circulation, and only a few of them can survive and proliferate to form distant metastases.^{23,30,31} Wong and colleagues³² demonstrated that this cell death in the lung was because of apoptosis and the observations of our lab in the liver would suggest that the death of these cells by apoptosis is a frequent event.⁵ The importance of apoptosis in the regulation of pulmonary metastasis is evident both because inhibition of apoptosis by overexpression of bcl-2 leads to more metastasis and because metastatic cells undergo markedly less apoptosis than nonmetastatic cells after arrest in the lung.^{32,33} Deformation-induced mechanical trauma and immune-mediated host cell killing have also been implicated in the intravascular death of tumor cells.^{31,34,35} The data that we have presented here demonstrate that the local release of NO at the site of tumor cell arrest contributes to this intravascular cytotoxicity although the limited survival of tumor cells even in mice deficient in eNOS would suggest that other factors must play a role as well.

Although there is evidence that NO may be more cytotoxic to weakly metastatic tumor cells than to highly metastatic cells,⁸ in this study, we have used the B16F1 cell line rather than B16F10 cells because the number of metastatic nodules induced by our protocols was amenable to statistical analysis and because the B16F1 cells are susceptible to NO *in vitro* and *in vivo*.⁵ Preliminary experiments in our laboratories suggest that related cell lines of varying metastatic competence elicit NO release

Table 3. Effects of NO Production on Lung Metastasis of B16 F1 Melanoma Cells in WT Mice 7 and 14 Days after Tumor Cell Injection

	Experiment 1 (no. of macroscopic surface tumor nodules, 7 days)		Experiment 2 (% of total histological area occupied by metastatic melanoma, 14 days)	
	Median	Range	Median	Range
WT mice	125	[12–273]	5.4	[0.3–13.2]
WT mice + L-NAME	304	[60–697]*	10.8	[6.5–29.0]†
eNOS KO mice	286	[106–488]‡	26.2	[8.4–37.9]§

Unlabeled B16F1 cells (5×10^5 cells in 150 μ l saline) were injected into the tail vein of WT mice, into WT mice treated with L-NAME, or eNOS KO mice, as described in Materials and Methods. The metastatic nodules identifiable on the surfaces of all lobes of the lungs were counted under a dissection microscope 7 days after cell injection. Histomorphometric analysis was performed on another group on the 14th day as described in Materials and Methods ($n = 7$ to 11 in each group; all mice had metastatic tumors).

* $P < 0.05$ as compared to WT mice without L-NAME treatment.

† $P < 0.01$ as compared to WT mice.

‡ $P < 0.003$ as compared to WT mice without L-NAME treatment.

§ $P < 0.001$ as compared to WT mice.

of differing magnitude. This is the subject of a current project.

In conclusion, we provide direct evidence showing that the arrest of B16F1 melanoma cells in the pulmonary circulation of C57Bl/6 mice induces the local release of eNOS-dependent NO that is cytotoxic to the cells and inhibits their development into pulmonary metastatic tumors. The existence of comparable mechanisms in the lung and the liver suggests that NO may be a natural defense against the formation of metastatic tumors in organs with complex microvascular networks.

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