Middle T Antigen-transformed Endothelial Cells Exhibit an Increased Activity of Nitric Oxide Synthase

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Summary

Endothelioma cell lines transformed by polyoma virus middle T antigen (mTa) cause cavernous hemangiomas in syngeneic mice by recruitment of host cells. The production of nitric oxide (NO), as measured by nitrite and citrulline production, was significantly higher in mTa-transformed endothelial cells in comparison with nontransformed control cells. The maximal activity of NO synthase (NOS) was about 200-fold higher in cell lysates from the tEnd.1 endothelioma cell line than in lysates from nontransformed controls, whereas the affinity for arginine did not differ. The biochemical characterization of NOS and the study of mRNA transcripts indicate that tEnd.1 cells express both the inducible and the constitutive isoforms. NOS hyperactivity is not a simple consequence of cell transformation but needs a tissue-specific mTa expression. Since tEnd.1-conditioned medium induces NOS activity in normal endothelial cells, most likely NOS hyperactivity in endothelioma cells is attributable to the release of a soluble factor. This NOS-activating factor, which seems to be an anionic protein, could stimulate tEnd.1 cells to express NOS by an autocrine way. By the same mechanism, tEnd.1 cells could induce NOS in the neighboring endothelial cells, and NO release could play a role in the hemangioma development. Such hypothesis is confirmed by our in vivo experiments, showing that the administration of the NOS inhibitor L-canavanine to endothelioma-bearing mice significantly reduced both the volume and the relapse time of the tumor.

Nitric oxide (NO)[1] is a short-lived free radical gas produced by various cell types, including vascular endothelial cells (1–3). In endothelium, NO is synthesized in response to a large number of stimuli and displays an enlarging spectrum of activities, such as smooth muscle relaxation, inhibition of platelet aggregation and adhesion, and decrease of smooth muscle cells proliferation (4, 5). NO generation is catalyzed by a class of NADPH-dependent NO synthases (NOS), which favor the conversion of L-arginine in L-citrulline and NO with a 1:1 stoichiometry, and are competitively inhibited by N^G-substituted L-arginine analogues (6, 7). At least three different isoenzymes have been characterized (8, 9). Both a constitutive, Ca^{2+}-dependent NOS (cNOS or eNOS) and an inducible, Ca^{2+}-independent enzyme (iNOS) have been detected in endothelial cells (10–13). Because of its short half-life, direct measurement of NO concentration is difficult, and several indirect techniques of detection have been developed. A simple and sensitive procedure monitors the conversion of [^{3}H]arginine to [^{3}H]citrulline, which is stoichiometric with NO (14, 15)

Established cell lines of transformed endothelial cells could represent useful tools to investigate the synthesis and the biological roles of NO. Several endothelioma cell lines have been

[1] *Abbreviations used in this paper* eNOS, endothelial NOS, HUVEC, human umbilical vein endothelial cells, iNOS, inducible NOS, L-ArgMEE, L-arginine monoethylester, L-NAME, N^G-nitro-L-arginine methylester, L-NMMA, N^G-monomethyl-L-arginine hydrochloride, MAEC, murine aorta endothelial cell, mTa, middle T antigen; NAF, NOS-activating factor, NO, nitric oxide, NOS, nitric oxide synthase, Vmax, maximal rate

established from cavernous hemangiomas developed in mice infected with polyoma virus middle T oncogene (16). Middle T antigen (mTa) expression is sufficient to induce malignant transformation in endothelial cells and their establishment in vitro. Murine endothelial cells transformed in vivo (16) or in vitro (17, 18) by polyoma mTa retain functional properties of normal endothelial cells. These cells proliferate at confluence without aspects of overgrowth; uptake acetylated low density lipoproteins; express CD31, vascular cell adhesion molecule 1, E- and P-selectin; respond to IL-1, TNF, and specific endothelial cell growth factors; and produce IL-6 and chemokines (16–19). However, mTa-transformed endothelial cells injected in syngeneic or in immunodeficient mice cause vascular lesions. Histologically, the tumors have the features of a hemangioma with areas resembling Kaposi's sarcoma or with lacunae lined by vascular endothelium (16, 18). The growth of this tumor is characterized by the recruitment of host endothelial cells (18, 20). The mechanisms of in vivo recruitment of host cells are not well known. mTa-transformed endothelial cells release chemokines able to recruit macrophages, which in turn produce angiogenic/chemotactic factors, leading to the progression of the lesion. It has been found that mTa-transformed endothelial cells produce a 40-kD cytokine, distinct from the vascular endothelial growth factor, which induces migration of vascular endothelial cells (21). Furthermore, an unbalance between urokinase-type plasminogen activator and its inhibitor may favor an abnormal degradation of extracellular matrix which permits the migration of host cells (22). These data support a new concept of a tumor sustained by a minute population of transformed cells, which recruit host elements and express malignant behavior in the immunodeficient host.

The present work was designed to study in mTa-transformed endothelioma cells: (*a*) the synthesis of NO; (*b*) the properties of the NOS isoform(s) responsible for NO synthesis, (*c*) the mechanisms regulating NOS activity; and (*d*) the role of NO synthesis in in vivo tumor growth.

Materials and Methods

Reagents Medium 199 (with or without phenol red), Iscove's medium, DMEM, and trypsin/EDTA were from GIBCO-RBC (Paisley, Scotland); FCS was from Irvine Scientific (Santa Ana, CA); plastic for cell culture was from Costar Italia (Milan, Italy), N^G-monomethyl-L-arginine hydrochloride (L-NMMA) was from Bachem Feinchemikalien AG (Bubendorf, Switzerland), collagenase and ionomycin were provided by Boehringer Mannheim (Mannheim, FRG), and L-[2, 3, 4, 5-^{3}H]arginine monohydrochloride (62 Ci/mmol) was from Amersham International (Amersham, Bucks, UK) Other reagents were purchased from Sigma Chemical Co (St Louis, MO). Mouse mAb anti-human endothelial cNOS (eNOS) and anti-murine macrophage iNOS directed against protein fragments of 179 amino acids (eNOS, $W^{1030} \rightarrow L^{1209}$) and 183 amino acids (iNOS, $F^{961} \rightarrow L^{1144}$), respectively, were from Transduction Laboratories (Lexington, KY). Electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA) The composition of Hepes buffer was as follows (in mM). 145 NaCl, 5 KCl, 1 $MgSO_4$, 10 Hepes (sodium salt), 10 glucose, and 1 $CaCl_2$, pH 7 4, at 37°C Ionomycin was dissolved at 1 mM in DMSO and stored at −20°C until use. The protein content of cell monolayers was assessed with the modified micro-Lowry method (kit from Sigma Chemical Co.)

Cell Cultures. Human umbilical vein endothelial cells (HUVEC) and artery (HUAEC) were obtained by treating human umbilical cord veins and arteries with collagenase from *Clostridium histolyticum* (Boehringer Mannheim) (10 mg/ml Iscove's medium), cultured in 75 cm^2 plastic flasks in medium 199 containing 20% FCS and characterized as previously described (23). ras-transformed HUVEC (a gift of Dr M. L. Lanfrancone, Policlinico Monteluce, Perugia, Italy, and Dr. E. Dejana, Istituto Mario Negri) were grown in medium 199 with 20% FCS.

Murine aorta endothelial cells (MAEC) were prepared from male DBA2 mice (Charles River, Calco, Italy). Aortas were washed with buffered saline and then incubated for 30 min at 37°C with collagenase (10 mg/ml). Approximately 10^5 cells were recovered from each aorta and plated on 35-mm-diameter petri dishes coated with collagen (1 mg/ml). Cells were grown in Iscove's medium containing 20% FCS supplemented with 6 mg/liter transferrin, 5 mg/liter insulin, 100 mg/liter soybean lecithin, 6.73 μg/liter sodium selenite, and 400 mg/liter BSA. Cells were characterized by the positivity for factor VIII-related antigen (>87%) and by the uptake of acetylated low density lipoproteins (>90%)

tEnd 1, sEnd.1, eEnd.1 (16), H End.FB (17), and H5V (18) murine endothelial cell lines transformed by mTa of polyoma virus were grown in DMEM supplemented with 10% FCS J774 murine macrophage cell line was grown in DMEM containing 10% FCS

N2A (murine neuroblastoma) cell line (American Type Culture Collection, Rockville, MD) was cultured in 100-mm-diameter petri dishes (~10^6 cells) for 24 h in DMEM with 10% FCS Cells were then exposed overnight to 3 × 10^5 *neo* CFU/ml of the retrovirus vector N-TKmT (16) Medium was changed and cells cultured for 72 h, then selected in the presence of 1 mg/ml G418. Growth of G418-resistant cells was observed after 3 wk and G418 was used for an additional month; cells were then grown without it. mTa insertion was evaluated by Southern blot analysis After electrophoresis, 10 μg of EcoRI-digested genomic DNA was blotted on a nylon filter and hybridized with a specific cDNA probe for mTa. The probe, a 1 5-kb BamHI-EcoRI fragment of pLJ vector (kindly provided by Dr. B. J. Druker, Dana Farber Cancer Institute, Boston, MA) was labeled with α-[^{32}P]dCTP by a random primer labeling method (Amersham International). Posthybridization washes were performed at high stringency and the membrane was exposed on autoradiography. After infection and selection, these N2A cells integrated middle T gene

Measurement of Nitrite. Nitrite production was measured by adding 0.6 ml of cell culture medium to 0.6 ml of Griess reagent (24), and, after 30 min incubation at room temperature in the dark, absorbance was measured at 540 nm in a spectrophotometer (UV/Vis; Perkin Elmer, Norwalk, CT) To rule out measurement interferences by pH indicators, culture medium was prebleached by adding a few milligrams per milliliter charcoal (which did not modify the calibration curve), alternatively, commercially available phenol red–free medium was used.

Measurement of Citrulline Synthesis as a Sensitive Marker of NO Production. Citrulline synthesis was measured both by a radiometric method (detection of intracellular concentration after short time experiments) and by a colorimetric method (measurement of extracellular citrulline accumulation during long-lasting incubations) The former was modified from a previously described technique (14, 15). Cells grown at confluence in 35-mm dishes were washed once with Hepes buffer, and then incubated with 1 ml of the same buffer at 37°C for 20 min 5 μCi L-[^{3}H]arginine and 10 μM

(HUVEC and MAEC) or 100 μM (tEnd.1) L-arginine were added to each dish, and cells were stimulated with 2 μM ionomycin (or DMSO as control); after 15 min, reaction was stopped by washing cells with cold PBS containing 5 mM L-arginine and 4 mM EDTA. After supernatant removal, 0.5 ml ethanol was added to each monolayer and allowed to evaporate; 2 ml of 20 mM Hepes-Na, pH 6, was then added. After 5 min, supernatant was collected and applied to 2-ml columns of Dowex AG50WX-8 (Na^+ form) and eluted with 4 ml of water. The radioactivity corresponding to [^{3}H]citrulline content in 6 ml eluate was measured by liquid scintillation counting. Citrulline synthesis was expressed as pmol citrulline/min/mg cell protein. The colorimetric method for citrulline detection in the extracellular medium was performed as previously described (25). Briefly, cell supernatant was incubated with urease (2 U/ml for 30 min at 37°C). After deproteinization with 5% TCA and centrifugation, 0.1 ml supernatant was allowed to boil for 5 min after mixing with 3 ml chromogenic solution, and absorbance was measured at 530 nm.

Measurement of NOS Activity in Cell Lysates. Cells grown at confluence in 75 cm^2 flasks were detached by trypsin/EDTA (0.05/0.02% vol/vol), the cells from 8 to 12 flasks were pooled and washed with PBS, then resuspended in 1 ml of reaction buffer ([in mM] Hepes 20, EDTA 0.5, dithiothreitol 1, pH 7.2) and sonicated on ice with three 10-s bursts. In each test tube, the following reagents were added to 100 μl lysate at the final concentrations: 2 mM NADPH, 1.5 mM $CaCl_2$, 1–100 μM L-arginine, and 2.5 μCi L-[^{3}H]arginine (0.4 μM) (14, 15). When indicated, NOS inhibitors were added to the other reagents. After a 15 min incubation at 37°C, the reaction was stopped by adding 2 ml Hepes-Na, pH 6, containing 2 mM EDTA. The whole reaction mixture was passed through a Dowex column and [^{3}H]citrulline–related radioactivity was counted, as indicated in the previous paragraph.

mRNA Analysis of iNOS and eNOS. Total RNA was obtained by the guanidine isothiocyanate/cesium chloride method (26). 25 μg of total RNA were electrophoresed on a 1% agarose gel containing 6.3% formaldehyde in 4-morpholinepropanesulfonic acid (MOPS) buffer and blotted on a Nylon Duralon-UV membrane (Stratagene, La Jolla, CA) by the traditional capillary system in 10× SSC (27). Prehybridization and hybridization steps were performed overnight in 50% deionized formamide, 10% dextran sulfate, 1 M NaCl, with 100 μg/ml denaturated salmon sperm DNA at 42°C. The cDNA for endothelial cNOS (11) (a gift from Dr. T. Michel, Brigham and Women's Hospital, Boston, MA) and iNOS (28) (gift from Dr. C. Nathan, Cornell University Medical College, New York) were labeled with α-[^{32}P]dCTP (3,000 Ci/mmol, Amersham International) at 2.2 × 10^8 cpm/μg sp act by the random primer labeling kit (Amersham International) according to the manufacturer's instructions. Posthybridization washes were performed at high stringency (twice in 2× SSC plus 0.1% SDS for 30 min at room temperature, twice in 0.2× SSC plus 0.1% SDS for 30 min at room temperature, and twice in 0.1× SSC plus 0.1% SDS for 30 min at 55°C) and the membrane was exposed on autoradiography with Hyperfilm-MP (Amersham International) and intensifying screens at −80°C for 20 d.

Detection of iNOS and eNOS Proteins. tEnd.1 cells and HUVEC were directly solubilized in boiling Laemmli buffer and proteins separated by SDS-PAGE (8%), transferred to nitrocellulose sheets, and probed with a mAb anti-iNOS (1:500) and detected by enhanced chemiluminescence (ECL; Amersham International). Alternatively, cells (10^7/plate) were lysed in 1 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 50 μg/ml pepstatin, 100 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM PMSF, and 500 μg/ml soybean trypsin inhibitor. After centrifugation at 10,000 *g* for 20 min, supernatants were precleared by 1 h incubation with anti–mouse IgG-agarose and then incubated for 2 h with the anti-eNOS antibodies at a concentration of 3 μg/ml. The immunoprecipitates, recovered on anti–mouse IgG-agarose, were washed four times with lysis buffer, twice with the same buffer without Triton X-100, twice with Tris-buffered saline, and solubilized with Laemmli buffer. Proteins were separated by SDS-PAGE (7%), transferred to nitrocellulose, and probed with anti-eNOS antibody as described above.

Preparation and Characterization of Conditioned Medium. Conditioned medium was prepared from tEnd.1 cells as follows: cells were grown at confluence in 150 cm^2 dishes and then incubated for 48 h with Iscove's medium containing LPS-free 0.45% BSA (GIBCO RBC). The cell viability assessed by trypan blue exclusion was >94%. The medium was centrifuged and used immediately or concentrated by precipitation with 40% ammonium sulphate and stored at 4°C; in this condition activity was stable for 2 wk. The precipitated medium was dialyzed against 20 mM Tris-HCl, pH 7.0, and then applied to a DEAE-Sephacel column (Pharmacia, Piscataway, NJ) equilibrated in 20 mM Tris-HCl, pH 7.0. The bound material was eluted by a stepwise gradient with NaCl (0.05, 0.1, 0.3, 0.5, 0.6, and 1 M). After correction of molarity, fractions were tested for their ability to enhance NOS in HUVEC after 12 h incubation. To assess the effect of enzymatic treatment, conditioned medium was treated with either DNase (10 μg/ml), RNase (50 μg/ml), trypsin (100 μg/ml), or chymotrypsin (100 μg/ml) at 37°C for 1 h. Trypsin and chymotrypsin were blocked by adding PMSF (1 mM). All samples were dialyzed against PBS and tested on HUVEC. Lipids and phospholipids from conditioned medium were extracted as previously described (29) and then tested on HUVEC.

In Vivo Tumor Growth Assay. The H5V endothelioma line was transplanted in syngeneic female C57Bl/6NCrlBR mice (Charles River) as described (18). Animals (eight per group) were inoculated subcutaneously with 10^6 H5V cells on day 0 and checked for tumor appearance and growth three times a week. Tumor growth was measured with calipers and its volume estimated by the formula: length × width^2/2. Animals were treated intraperitoneally with L-canavanine (30 mg/kg; Sigma Chemical Co.) in saline or saline alone from day 0 to day 28.

Statistical Analysis. Each experimental point was performed in duplicate or triplicate per experiment; all data in the text, table, and figures are given as means ± SEM. Statistical analysis was carried out using the Student's *t* test for unpaired data.

Results

NO Synthesis in mTa-transformed Cells

Nitrite Production. After 24-h incubation of cells in culture medium containing 1 mM L-arginine, nitrite concentration was 1.83 ± 1.49 nmol/mg cell protein in the supernatant of HUVEC cultures (n = 5), whereas in tEnd.1 it was about 10-fold higher (18.73 ± 3.84 nmol/mg cell protein, n = 8, p <0.008) (Fig. 1). In both cell types, nitrite concentration was dose dependently correlated to L-arginine level in the culture medium: nitrite formation was not detectable in HUVEC already at 0.1 mM L-arginine, whereas it was still measurable in tEnd.1 in L-arginine–free medium (suggesting the presence of an intracellular pool of arginine for NO synthesis). A similar result was obtained when L-arginine

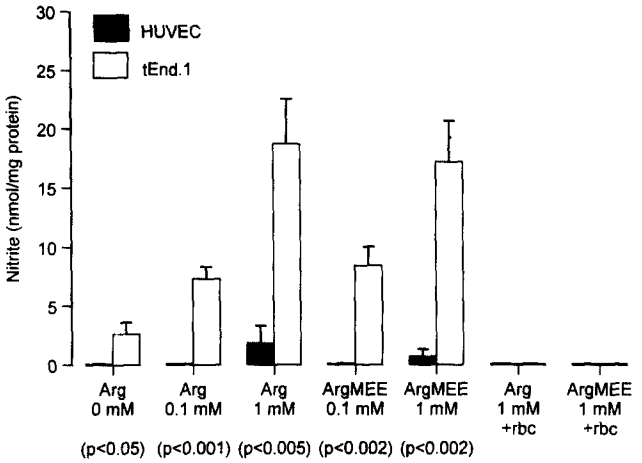

Figure 1. Nitrite accumulation in the supernatant of HUVEC and tEnd.1 cells. After 24-h incubation in fresh medium containing different concentrations of L-arginine or its monoethylester derivative, nitrite concentration was measured in the culture medium with the Griess reagent (see Materials and Methods). To provide hemoglobin as scavenger of NO, 20 μl of packed red blood cells (*rbc*) was added to the culture medium containing the highest arginine concentration. Data are means ± SEM of results obtained from five (*HUVEC*) and eight (*tEnd.1*) experiments performed in duplicate. (*Arg*) L-arginine; (*ArgMEE*) L-arginine monoethylester.

was replaced with L-arginine monoethylester (L-ArgMEE), which permeates plasma membrane better than the standard amino acid. When red blood cells were added to the incubation medium, in order to provide hemoglobin as a scavenger of NO, no production of nitrite in both cell types was observed at the higher substrate concentrations in the medium (Fig. 1).

Citrulline Synthesis (Short-term Incubation). To detect NO synthesis in short time periods, the intracellular content of citrulline was measured in cells incubated in the absence and in the presence of the Ca^{2+} ionophore ionomycin. The synthesis of citrulline after 15 min of incubation was maximal at 10 μM extracellular L-arginine in HUVEC and 100 μM extracellular L-arginine in tEnd.1 cells (data not shown). It was completely inhibited when cells were preincubated for 20 min with 10 mM L-NMMA. Basal citrulline production (expressed as pmol/min/mg protein) was 1.24 ± 0.18 in HUVEC (n = 4) and 10.35 ± 2.85 in tEnd.1 cells (n = 3; p <0.02). After 2 μM ionomycin stimulation, citrulline synthesis (pmol/min/mg protein) was 7.07 ± 1.14 in HUVEC (n = 4) and 38.5 ± 3.32 in tEnd.1 cells (n = 3; p <0.0001).

Citrulline Synthesis (Long-term Incubation). Citrulline production was also measured in the supernatant of cells incubated for 24 h in the absence or in the presence of either L-arginine or L-ArgMEE (0.1–1 mM): extracellular citrulline, measured colorimetrically, was not detectable at any arginine concentration in HUVEC (n = 6), whereas its synthesis was measurable in tEnd.1 (n = 8) and was dependent on substrate availability (although it was detectable even in the absence of extracellular arginine) (Fig. 2).

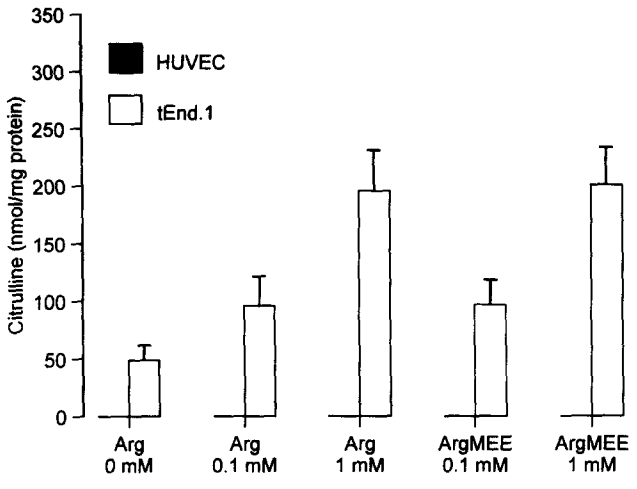

Figure 2. Citrulline accumulation in the supernatant of HUVEC and tEnd.1 cells. After 24-h incubation in fresh medium containing different concentrations of L-arginine or its monoethylester derivative, citrulline concentration was measured in the culture medium with the colorimetric procedure of Boyde and Rahmatullah (25) (see Materials and Methods). Columns related to HUVEC are not visible, since citrulline levels were not detectable at any condition in these cells. Data are means ± SEM of results obtained from six (*HUVEC*) and eight (*tEnd.1*) experiments performed in duplicate.

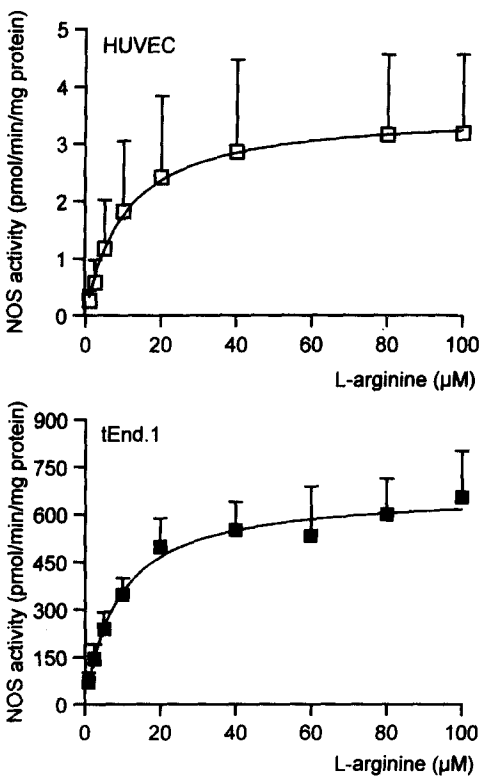

Figure 3. L-arginine dependence of NO synthase (*NOS*) from HUVEC (n = 4) and tEnd.1 (n = 4). Each point is represented as mean value ± SEM; the curves are ideal plots obtained with the Michaelis-Menten equation: $Y = (X \times \text{Vmax})/(X + \text{Km})$, where X = L-arginine concentration (0.4 μM L-[^{3}H]arginine + 1–100 μM L-arginine), and Vmax and Km are the values obtained for each cell type by Lineweaver-Burk transformation.

Characterization of NOS Activity

Kinetics of NO Synthase Activity. When NOS activity was measured in the cell lysates, it exhibited a substrate dependence that followed a Michaelis-Menten pattern (Fig. 3). In HUVEC (n = 4) maximal rate (Vmax) was 3.32 ± 1.68 pmol citrulline/min/mg protein, and half-maximal activity was detectable at 9.3 ± 1.54 μM L-arginine. In tEnd.1 cells (n = 4) the enzyme kinetics showed a Vmax = 702.93 ± 159.85 pmol citrulline/min/mg protein (p = 0.005), and a Km = 9.49 ± 3.53 μM L-arginine (p = NS). When calcium was not present in the test, the [^{3}H]citrulline production was completely abolished in HUVEC, whereas it did not change in tEnd.1 lysate. If cells were preincubated for 24 h with 1 μM dexamethasone, NOS activity was not affected in HUVEC lysate, but it was inhibited by 75 ± 10% (n = 3) in tEnd.1 lysate. D-arginine, ≤1 mM, did not influence the amount of [^{3}H]citrulline produced by normal and transformed endothelial cells, indicating that the D-enantiomer of arginine was not transformed by the lysate, and that NOS in both cell types was stereospecific (data not shown). Furthermore, the two cell types exhibited a different pattern of NOS inhibition by a panel of three NOS inhibitors, L-NMMA, L-NAME (N^G-nitro-L-arginine methylester), and L-canavanine (Fig. 4): IC_{50} values were, respectively, 5.9 μM, 36 μM, and 1.52 mM in HUVEC, and 1.43 mM, 41.1 mM, and 73 μM in tEnd.1.

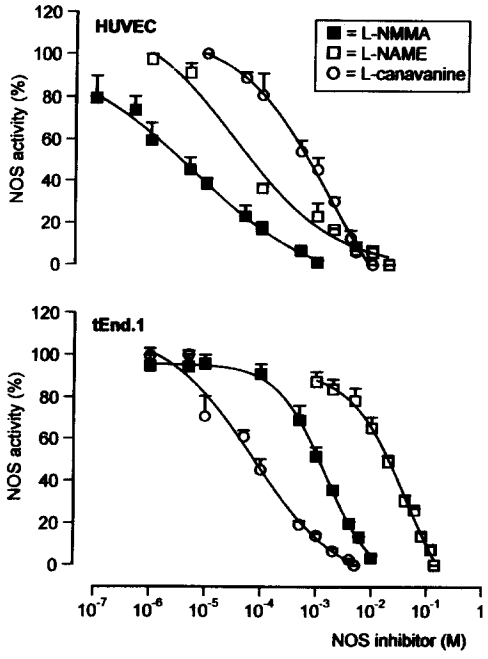

Figure 4. Dose–response curve of NOS activity in the lysate of HUVEC and tEnd.1, in the presence of three different NOS inhibitors. Measurement of enzymatic activity was performed as indicated in Materials and Methods, in the presence of 2.5 μCi (0.4 μM) L-[^{3}H]arginine. Enzyme activity is expressed as percentage of NOS activity (assumed to be 100% in the absence of NOS inhibitors). Each point is a mean ± SEM of three different experiments performed in duplicate.

To exclude the presence of arginase activity, 50 mM L-valine (10) was added to the reaction mixture. No change of the radioactivity in the column eluate was detected, suggesting that arginase activity was virtually absent in HUVEC and tEnd.1 lysate. An alternative source of citrulline in living systems is arginine deiminase, an enzyme converting L-arginine into L-citrulline and ammonia: its activity is detectable in bacteria but not in mammalian cells (8). The existence of such an enzyme in our cells was ruled out as formamidine, a known inhibitor of arginine deiminase (15), did not influence arginine conversion even at 1 mM concentration (data not shown). Moreover benzoylarginine ethylester, a substrate for arginine deiminase (15), did not show any competition with [^{3}H]arginine. Finally, the concentration of extracellular ammonia, as detected by Nessler reagent, was not significantly different in normal and transformed cells after 24 h of incubation (data not shown).

NOS activity was also measured in cell lysates from the murine neuroblastoma cell line N2A, infected with the retrovirus vector N-TKmT. Although these cells expressed mTa, NOS activity was identical to that found in parental nontransfected cells, and in HUVEC and MAEC (Table 1). Similarly, HUVEC transfected with K-ras did not show an increase of enzyme activity. On the contrary, a very high NOS activity was detectable in the lysate of other murine endothelioma cell lines (sEnd.1, H.End.FB) obtained by mTa transfection (16, 17, and Table 1).

Expression of mRNA for eNOS and iNOS. We examined the capacity of murine endothelial cells transformed by mTa to express mRNA of endothelial cNOS and iNOS. RNA was probed with specific cDNA for eNOS and iNOS and analyzed by Northern blot hybridization. Endothelioma

Table 1. *NOS Activity Measured in the Lysates of Different Cell Types*

Cell type	NOS activity	mTa	n
HUVEC	0.34 ± 0.07	–	5
HUAEC	0.25 ± 0.12	–	3
MAEC	0.18 ± 0.10	–	3
tEnd.1	62.2 ± 7.86	+	5
sEnd.1	34.2 ± 13.38	+	3
H5V	52.3 ± 10.1	+	3
H.End.FB	47.4 ± 11.72	+	3
N2A	0.40 ± 0.19	–	3
mTa-N2A*	0.38 ± 0.12	+	3
ras-HUVEC‡	0.55 ± 0.34	–	3

Measurement of enzymatic activity was performed as indicated in Materials and Methods, in the presence of 2.5 μCi (0.4 μM) L-[^{3}H]atginine. NOS activity is expressed as pmol citrulline/min/mg cell protein (mean ± SEM). The expression of mTa in each cell type is indicated.
* N2A transfected with mTa.
‡ HUVEC transfected with K-ras.

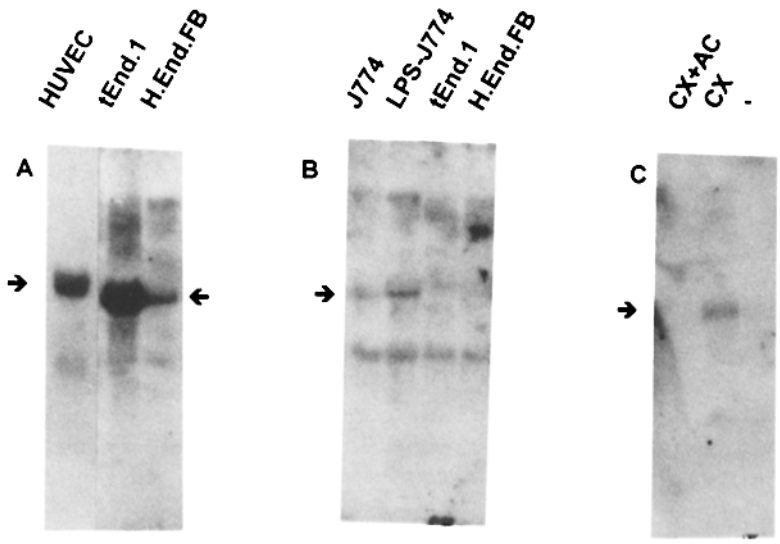

Figure 5. Expression of mRNA for eNOS (*A*) and iNOS (*B* and *C*) in mTa-transformed and nontransformed endothelial cells. (*A*) Northern blot analysis for mRNA levels of eNOS in HUVEC, tEnd.1, and H.End.FB. (*B*) Northern blot analysis for mRNA levels of iNOS in J774 cells under control condition or stimulated for 4 h with LPS (10 μg/ml) before mRNA extraction, and in tEnd.1 and H.End.FB cell lines. (*C*) Northern blot analysis for mRNA levels of iNOS in tEnd.1 cells under control conditions (−) or after 16-h incubation with cycloheximide (*CX*, 10 μg/ml) or cycloheximide and actinomycin D (*CX+AC*, both at 10 μg/ml). Results are from representative experiments and were confirmed in two to three experimental sets each.

(tEnd.1 and H.End.FB) cells expressed a specific eNOS transcript (4.6 kb) with a size different from that observed in HUVEC (4.8 kb) (Fig. 5 *A*). To determine eNOS protein expression, cell lysates from untreated HUVEC and tEnd.1 were immunoprecipitated with a mAb anti-human eNOS, followed by a Western blot analysis using the same antibody. This experiment indicates that eNOS is two- to threefold more abundant in endothelioma cells than in HUVEC (Fig. 6 *A*). Both cell types apparently did not express mRNA for iNOS, in contrast to murine macrophage J774 cells stimulated with LPS (Fig. 5 *B*). After 16 h of preincubation in the presence of 10 μg/ml cycloheximide, iNOS mRNA (4.4 kb) was detectable in tEnd.1 (Fig. 5 *C*), but not in HUVEC (data not shown). Actinomycin D (10 μg/ml) inhibited the effect of cycloheximide (Fig. 5 *C*). To determine whether HUVEC and tEnd.1 cells expressed iNOS proteins, we carried out Western blot analysis of total cellular proteins by using a mAb anti-murine macrophage iNOS. Fig. 6 *B* shows that tEnd.1, but not HUVEC, express a 130-kD protein recognized by antibody anti-murine macrophage iNOS.

Effect of Endothelioma Conditioned Medium on NOS Activity and Expression in HUVEC

Since tEnd.1 NOS activity had features typical of an inducible form (Ca^{2+} independence, inhibition by dexamethasone and L-canavanine), we explored the possibility that endothelioma cells produce soluble factor(s) able to induce NOS synthesis in an autocrine way. HUVEC were incubated for 24 h with medium previously incubated for 48 h with tEnd.1 cells. At the end of incubation the HUVEC monolayer was washed, and NOS activity was measured in the lysate, prepared as previously described, in the presence of 2.5 μCi (0.4 μM) L-[^{3}H]arginine. It is interesting to note that NOS activity increased 300–800-fold (n = 3) and was found to be Ca^{2+} independent; the substrate dependence of enzyme activity followed a typical Michaelis-Menten pattern (Fig. 7 *A*). Similar results were obtained with the conditioned medium from H.End.FB, H5V, and sEnd.1 (data not shown). Conditioned medium from MAEC, N2A cells, mTa-infected N2A cells, and HUVEC transfected with K-ras, did not in-

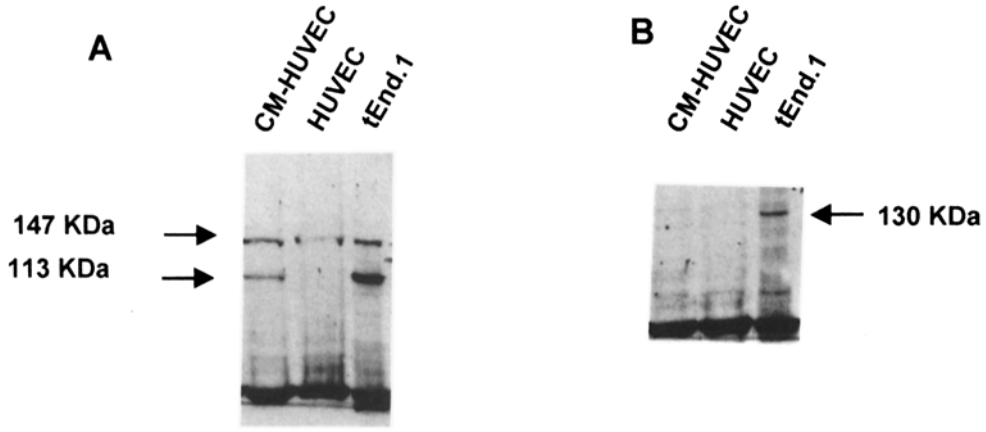

Figure 6. Detection of eNOS and iNOS in HUVEC and tEnd.1 cells. (*A*) immunoprecipitation of cell lysates (1.2 mg protein) from tEnd.1 cells, HUVEC, and HUVEC stimulated for 12 h with conditioned medium of tEnd.1 cells (*CM-HUVEC*) with a mAb anti-human eNOS, followed by a Western blot analysis using the same antibody. (*B*) Western blot analysis of whole cell lysate from tEnd.1 cells, HUVEC, and CM-HUVEC with a mAb anti-murine macrophage iNOS. We did not perform immunoprecipitation experiments because this antibody did not work in such technique using as positive control the murine J774 macrophage cell line.

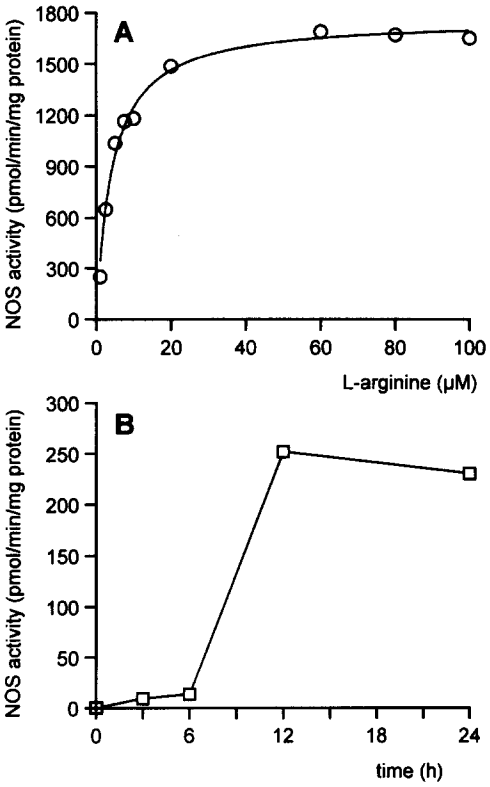

Figure 7. (*A*) L-arginine dependence of NO synthase (*NOS*) from HUVEC after 24-h incubation with 10 ml supernatant of tEnd.1 (dose-dependence curve from an experiment representative of three similar experiments performed in duplicate). (*B*) NOS activity in HUVEC incubated for different time periods with 10 ml supernatant of tEnd.1 (time-dependence curve from an experiment representative of three similar experiments performed in duplicate). In both cases, supernatant was DMEM supplemented with 10% FCS, that after 24-h incubation with tEnd.1 was transferred in 100 mm-diameter dishes containing HUVEC at confluence. At the end of the indicated incubation times, HUVEC were washed three times with PBS and then checked for NOS activity as reported in Materials and Methods. Time dependence was measured by incubating cell lysates with 0.4 μM L-[^{3}H]arginine alone, whereas in dose-dependence experiments 1–100 μM L-arginine was added.

Table 2. *NOS Activity in HUVEC Exposed to Diluted tEnd.1 Conditioned Medium*

Treatment	NOS activity
None	0.41 ± 0.19
Conditioned medium	34.1 ± 3.4
Conditioned medium (1:2)	28.1 ± 2.3
Conditioned medium (1:5)	18.2 ± 0.9
Conditioned medium (1:10)	2.1 ± 0.6

HUVEC were incubated with conditioned medium of tEnd.1 cells supplemented with 10% FCS differently diluted in DMEM containing 10% FCS. Measurement of enzymatic activity was performed as indicated in Materials and Methods, in the presence of 2.5 μCi (0.4 μM) L-[^{3}H]arginine. NOS activity is expressed as pmol citrulline/min/mg cell protein (mean ± SEM of three experiments).

crease NOS activity in HUVEC (data not shown). The increase of NOS activity in HUVEC was already detectable after 3 h of incubation, and reached its maximum at 12–24 h (Fig. 7 *B*): in the presence of cycloheximide, NOS induction at 6 h was inhibited by 72 ± 3% (n = 3). The effect was dose dependent and stimulation was maximal for nondiluted conditioned medium (Table 2).

The stimulation of HUVEC for 12 h with conditioned medium from tEnd.1 cell increased the amount of NOS detected by an antibody recognizing human eNOS (Fig. 6 *A*). Cell lysate from HUVEC was immunoprecipitated with a mAb anti-human eNOS, followed by a Western blot analysis using the same antibody. The treatment with conditioned medium from tEnd.1 cells for 12 h increased the amount of a 147-kD protein and caused the appearance of a 113-kD one, which is also present in tEnd.1 immunoprecipitate (Fig. 6 *A*). On the other hand, the mAb anti-murine macrophage iNOS, tested in Western blot performed on HUVEC cell lysate after stimulation with tEnd.1 conditioned medium, did not immunoreact with a 130-kD protein (Fig. 6 *B*). Moreover, conditioned medium–treated HUVEC did not express mRNA for iNOS, even after preincubation with cycloheximide (data not shown).

We performed a preliminary characterization of the soluble factor, which we named NOS-activating factor (NAF), present in the tEnd.1 conditioned medium and responsible for NOS induction. As NAF was not extracted by organic solvents, we excluded that it was a lipid. It was resistant to treatment with DNase, RNase, and dithiothreitol, but was sensitive to trypsin, chymotrypsin, and heating, thus supporting the hypothesis that it is a protein. Dialytic treatment of conditioned medium suggested a molecular weight higher than 12,000. NAF was retained by anion exchange chromatography and was eluted with NaCl concentrations ranging from 0.5 to 0.7 M. With affinity chromatography NAF did not bind to heparin or Con A at pH 7.4, suggesting the absence of net positive charges and glycosidic moieties containing mannose (not shown).

Inhibition of Hemangioma Growth by the NOS Inhibitor L-Canavanine

It was of interest to obtain preliminary indications as to whether production of NO has relevance in the tumorigenicity of mTa endotheliomas. As recently described, transplantation of H5V cells in syngeneic recipients causes subcutaneous tumors which, after a phase of growth, undergo rejection (18); a proportion of mice (70–90%) experience a second regrowth phase. We focused our attention on the effect of L-canavanine, the most potent in vitro inhibitor of endothelioma cell NOS activity, on tumor take, rejection, and regrowth. L-canavanine caused a significant reduction of tumor volumes throughout the period of first growth (Fig. 8). Preliminary histological analysis on hematoxylin-eosin–stained sections revealed no substantial alteration in tumor texture and composition. Moreover, only 1 of 8 canavanine-treated mice showed a regrowth, compared with 6 of 8 regrowths for control animals on day 60 (Fig. 9).

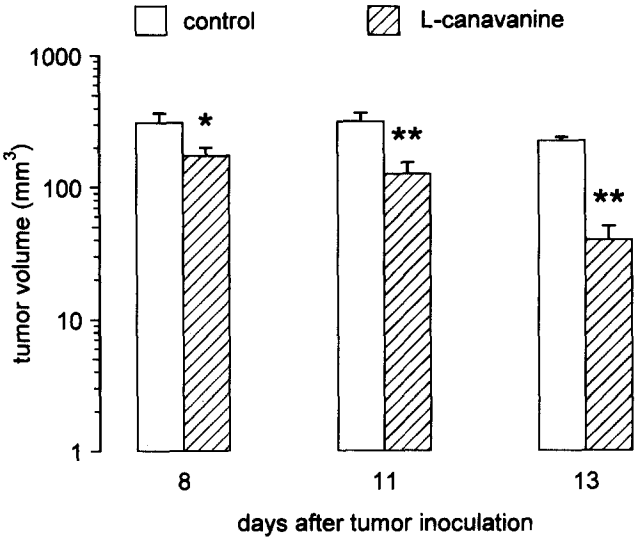

Figure 8. Effect of L-canavanine on H5V tumor growth. Mice (eight per group) were transplanted with 10^6 H5V cells s.c. on day 0, then treated with L-canavanine (30 mg/kg i.p.) or saline from day 0 to day 28. Tumor growth was estimated by calipers at days reported and tumor volume calculated as indicated in Materials and Methods. Owing to a spontaneous regression, after the second week the volume of tumors both in control and treated mice was too small to allow to check differences between the two groups. Data are means ± SEM. (*) $p < 0.05$; (**) $p < 0.01$ (Student's t test).

Discussion

The middle T oncogene of polyoma virus causes the development of endothelial tumors identified as hemangiomas in mice (16, 18). From these lesions endothelioma cell lines could be easily established from different organs. These cells express polyoma mTa, retain functional properties of normal endothelium, and cause hemangiomas in vivo when reinjected in mice (16, 19, 20). It is interesting to note that the in vivo vascular lesions are formed by recruitment of host cells (18, 20). Transformation of endothelial cells by mouse polyoma

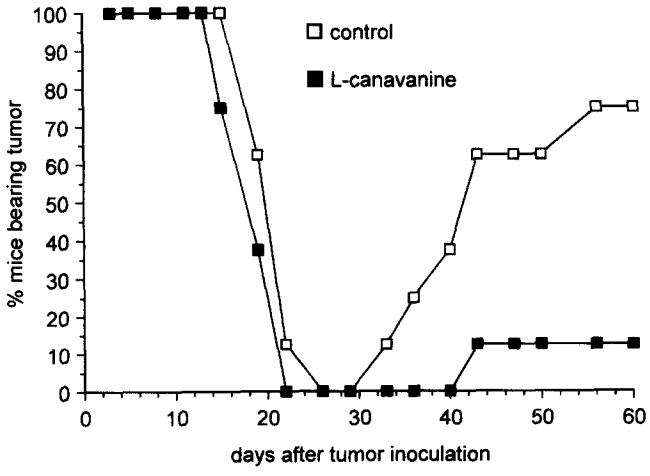

Figure 9. Effect of L-canavanine on H5V cells tumorigenicity. Mice treated as shown were checked for tumor appearance and growth three times a week.

virus requires mTa alone: this protein both regulates and is a substrate for members of *src* tyrosine kinase family (30). Association of mTa with $pp60^{c\text{-}src}$ results in the activation of the tyrosine kinase and then association and phosphorylation of a phosphatidylinositol 3-kinase (30). How these events lead to a mitogenic response is presently not well known.

We had previously used mTa-transformed mouse endothelioma cells as a good tool for analysis of endothelial cell immunobiology: they responded to several cytokines by producing other cytokines, procoagulant activity, and platelet-activating factor (19). In the present work, we aimed to study whether mTa-transformed endothelial cells were able to produce NO, and the mechanisms regulating NOS activity, in order to understand a possible involvement of NO in the endothelioma growth, especially as far as host cell recruitment is concerned. As a first screening, we measured NOS activity in the lysate of several types of endothelial cells (Table 1). A very high NOS activity was shared by endothelioma mouse cells from thymus (tEnd.1), skin (sEnd.1), and midgestation chimeric embryos (eEnd.1, data not shown), as well as by mTa-transformed endothelial cells from mouse heart (H.End.FB, H5V). On the other hand, nontransformed MAEC, HUVEC, and HUAEC exhibited a similar low activity. To establish the role of NO in endothelioma development and the possible linkage of its production with mTa expression, we have chosen to study NO synthesis in tEnd.1. HUVEC were used as a normal, nontransformed control. Indeed, endothelial cells from mouse aorta exhibited the same pattern of NO production as human endothelium (Table 1), but were more difficult to be cultured at the amounts necessary to perform our experiments.

tEnd.1 and HUVEC showed a striking difference in NO production, as measured by nitrite accumulation in the culture medium and cell-associated [^{3}H]citrulline synthesis. In aerobic biological systems, NO is oxidized to the more stable NO_2^-, which is easily detectable in the extracellular medium by the Griess reagent (24). Nitrite accumulation in the medium was 10-fold higher in tEnd.1 than in HUVEC. In both cell types it was arginine dependent and completely blocked by hemoglobin, a well known NO scavenger. These data confirmed that nitrite production was dependent on conversion of arginine into NO. At the contrary of nitrite assay (which requires at least 24 h of incubation to reach detectable amounts of substrate), it is possible to measure short-term (15 min) citrulline production in whole cells, by incubating them with [^{3}H]arginine and detecting the amount of cell-associated [^{3}H]citrulline (14). Basal citrulline production was eightfold higher in tEnd.1 cells than in HUVEC. After ionomycin stimulation, citrulline synthesis increased in both cell types, but was still fivefold higher in endothelioma cells. Citrulline synthesis was again L-arginine dependent, and inhibited by 10 mM L-NMMA in the culture medium. A further approach to citrulline detection is the colorimetric method by Boyde and Rahmatullah (25), which recognizes the ureido group present in the molecule. By this less sensitive technique, after 24 h of incubation the citrulline accumulation was detectable only in tEnd.1 medium but not in the HUVEC super-

natant; in tEnd.1 such accumulation was still dependent on L-arginine concentration.

The radiometric method for citrulline detection is also useful to measure NOS activity in cell lysates (31). NOS kinetics in the two cell types differed significantly as far as maximal catalytic activity was concerned: Vmax was hundredsfold higher in tEnd.1 in comparison with HUVEC, suggesting the presence of a much higher amount of enzyme in mTa-transformed cells. On the other hand, Km was similar in both cases, providing evidence that the enzyme affinity for the substrate was the same. D-arginine was not transformed by the lysate in either cell type, and neither inhibited the enzyme activity, thus confirming the stereospecificity of NOS (8).

The calcium dependence of NOS in HUVEC suggests that these cells contain the constitutive isoform of the enzyme. On the other hand, tEnd.1 NOS activity was not dependent on Ca^{2+} in the reaction buffer, and was strongly inhibited by dexamethasone pretreatment (which did not affect enzyme activity in HUVEC). Such data provide evidence for an inducible form of NOS in tEnd.1, but the ability of ionomycin to enhance citrulline synthesis also in endothelioma cells suggests the existence of a Ca^{2+}-dependent isoform too. It is possible that in tEnd.1 the inducible and constitutive NOS isoforms coexist, as observed in cytokine-stimulated endothelium (24, 32). This hypothesis seems to be confirmed by the finding that, whereas HUVEC express only the transcript for eNOS, in tEnd.1 mRNA for both isoforms is detectable. The iNOS transcript was measurable only after treatment with cycloheximide (an inhibitor of polypeptide chain elongation): this result could be indicative of a rapid degradation of iNOS-mRNA, due to an elevated instability (33). This hypothesis is in agreement with kinetic results (high Vmax), that suggest an increased synthesis of catalytic units in tEnd.1. Interestingly enough, the higher synthesis of citrulline by tEnd.1 cells after ionomycin stimulus fits with the higher expression of cNOS-mRNA in these cells in comparison with HUVEC.

NOS activity in the two cell types studied was different also in terms of sensitivity to several competitive inhibitors. HUVEC NOS was more sensitive to L-NMMA and L-NAME, and highly resistant to L-canavanine inhibition. On the contrary, in tEnd.1 NOS activity was impaired by low concentrations of L-canavanine, whereas efficacy of L-NMMA was lower, and L-NAME was virtually uneffective. A different sensitivity of the inducible and constitutive isoforms of NOS to inhibitors has been already described (24, 34), and L-canavanine has been indicated as a more selective inhibitor of the macrophage inducible form of NOS (15).

Our results show that mTa-transformed endothelioma cells possess an elevated NOS activity, which is probably due to induction of a de novo synthesis of enzyme molecules. As ras-transformed HUVEC did not exhibit NOS hyperactivity, such a feature is not a simple consequence of cell transformation, but requires expression of mTa. On the other hand, expression of mTa in nonendothelial cell lines was not accompanied by an increase of NOS activity, suggesting that mTa-elicited NOS overexpression is tissue specific.

It is difficult to define the possible role of NO in the transformation of murine endothelial cells. NO has been demonstrated to inhibit cell proliferation, probably owing to inhibition of GAPDH, ribonucleotide reductase, and mitochondrial respiration (35). For this reason, it would be hard to suppose the involvement of NO in the mTa-evoked mitogenic stimulus. On the other hand, the cytotoxic activity of NO could impair the functions of the immune system cells, thus favoring the tumor progression: it has been observed that NO production can account for immunosuppression induced by colon adenocarcinoma cells in rat monocytes (36). Our in vivo experiments led us to hypothesize a role for NO in the genesis of hemangiomas: administration of L-canavanine to endothelioma-bearing mice significantly reduced both the volume of subcutaneous angiomas, and (after a transient phase of complete remission) their regrowth, thus suggesting that NO is necessary to the tumor development. An interesting feature of the in vivo vascular lesions is that >95% of endothelial cells from hemangiomas are host derived (20). The continuous presence of endothelioma cells is necessary to maintain the tumor, supporting the hypothesis that endothelioma cells produce an hemangioma-inducing activity. The recruitment of host cells does not depend on the release of mitogenic or angiogenic soluble factors, but requires a tight cell-to-cell contact, since the putative signal is only active over a short distance. Such features could be applied to NO, a free radical that, having a 3–5 s half-life, cannot diffuse beyond the space corresponding to a single cell diameter (34). Thus it can be hypothesized that NO released from endothelioma cells favors the recruitment of normal endothelial cells in the vascular lesions: this suggestion is confirmed by recent data demonstrating that the synthesis of NO is necessary for production of angiogenic activity by LPS-stimulated human monocytes (37). During hemangioma development, the endothelioma cells may first integrate into blood vessels, leading to their rupture: NO could also participate to this process, owing to its characteristics of vasodilator and cytotoxic agent. The effect of L-canavanine on tumor growth in mice is in agreement with this hypothesis.

Finally, our preliminary results with conditioned medium from tEnd.1 support the idea that endothelioma cells release a soluble factor (NAF) able to induce eNOS activity both in endothelioma cells and in normal endothelial cells. An eNOS with a molecular mass of 147 kD was present in resting HUVEC: after treatment with tEnd.1 conditioned medium, the amount of this protein increased consistently, coupled with the appearance of a 113-kD protein. A similar doublet is present in tEnd.1 (Fig. 6 *A*) and in COS-7 cells transfected with eNOS cDNA (38). mRNA for iNOS and iNOS protein are not expressed in HUVEC after stimulation with tEnd.1 conditioned medium. So far, the high NOS activity present in NAF-stimulated HUVEC is attributable to the induction of an enzyme detected by an anti-eNOS mAb (Fig. 6 *A*). On the other hand, the in vitro Ca^{2+} independence of the enzyme and its inducibility by a soluble factor are not criteria sufficient (39) to classify NAF-induced NOS in HUVEC as an iNOS. Indeed, the Ca^{2+}/calmodulin-dependent NOS in

human hepatocyte possesses a 80% homology with macrophage iNOS (40), and the cytokine-induced NOS activity in rabbit chondrocytes is Ca^{2+} dependent (41).

Our preliminary experiments provide evidence that NAF is a thermolabile, anionic protein with a size >12 kD. The release of a NOS-inducing activity has already been reported in different cells: culture medium from rat colon carcinoma cells induces NOS activity in splenic macrophages (36), culture supernatant from activated macrophages evokes NO synthesis in the murine adenocarcinoma cell line EMT-6 (42), transferable products from Kupffer cells elicit conversion of L-arginine to NO in hepatocytes (43), and P1HTR tumor cells stimulate murine macrophages to produce NO (44). Till now, no evidence has been provided about the feature of the soluble factor(s) involved.

Our results suggest the following hypotheses. mTa transformation evokes the synthesis of soluble factor(s) which may be responsible for the high NOS activity in tEnd.1, that is mainly present as the inducible form. It is conceivable that, when few endothelioma cells settle in a vascular district, they recruit the first host cells via NO synthesis. NO could also impair the host immune response against the developing tumor. After few hours, endothelial normal cells are converted by an endothelioma-derived soluble factor into potent NO producers, that cooperate with endothelioma cells in recruiting new host cells. This "cascade" model can account for the need of very low amounts of tumoral cells to evoke the onset of the vascular lesions.

In our laboratory we are currently trying to purify the soluble factor(s) responsible for NOS-inducing activity and present in mTa-endothelioma conditioned medium. It is possible that NAF is related to (or can be identified with) the 40-kD cytokine, derived from mTa-transformed cells, that evokes migration of vascular endothelial cells (18). The characterization of this molecule(s) could provide new information about the genesis and maintenance of vascular tumors.

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