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Roles of nitric oxide in tumor growth

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ABSTRACT **A subclone of the human colon adenocarcinoma cell line DLD-1, which grew reproducibly as subcutaneous tumors in nude mice, was isolated. Such cells, when engineered to generate nitric oxide (NO) continuously, grew more slowly *in vitro* than the wild-type parental cells. This growth retardation was reversed by the addition of *N*-iminoethyl-L-ornithine. In nude mice, however, the tumors from these cells grew faster than those derived from wild-type cells and were markedly more vascularized, suggesting that NO may act as part of a signaling cascade for neovascularization. Recent observations that the generation of NO in human breast and gynecological cancers correlates positively with tumor grade are consistent with this hypothesis. We suggest that NO may have a dual pro- and antitumor action, depending on the local concentration of the molecule.**

Nitric oxide (NO) is an inorganic free radical gas synthesized from L-arginine by a family of isoenzymes called NO synthases (1). Two of these are constitutively expressed and a third is inducible by immunological stimuli. The NO released by the constitutive enzymes acts as an important signaling molecule in the cardiovascular and nervous systems (2) and NO released by the inducible NO synthase (iNOS) is generated for long periods, by cells of the immune system among others, and has been shown to be cytostatic/cytotoxic for tumor cells and a variety of microorganisms (3).

The role of NO in tumor biology is still poorly understood. Growth of solid tumors is regulated by interactions of endothelial cells of the tumor vasculature, tumor-infiltrating immune cells such as T lymphocytes and macrophages, and the tumor cells themselves (4). Most of these cellular components have been shown to generate NO *in vitro* (5–9). More recently, we have shown that NO synthase is present in fresh human tumor tissue, where it is localized to tumor cells in gynecological cancers (10) and to the stroma of breast cancers (11). Furthermore, the enzyme activity in this primary tumor tissue correlated positively with tumor grade (10, 11). Whether NO had any positive or negative effect on the progression of such tumors, however, was not established. To investigate further the effect of endogenous NO on tumors, we decided to engineer a human tumor cell line to generate NO continuously and to compare its growth characteristics *in vitro* and *in vivo* with that of the parental wild-type line that does not generate NO, unless deliberately induced (9).

MATERIALS AND METHODS

Cloning and Engineering of Cell Lines. All lines were derived from the DLD-1 human colon adenocarcinoma cell line obtained from the American Type Culture Collection. All were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum and 1% antibiotics solution containing penicillin and streptomycin (10,000 units/ml). The selection agent, Geneticin sulfate (G418), was added at 1 mg/ml to culture medium of transfected cells. All reagents were obtained from GIBCO/BRL. Cells were incubated in tissue culture grade plastic vessels (Falcon) at 37°C in 5% CO_2/95% air. To isolate and clone sublines of DLD-1, a cell suspension was seeded at low density in 60-mm dishes (1×10^3 cells per ml) in 2 ml of 0.3% agar (Agar Noble; Difco) overlaying a further 2 ml of solid 1.8% agar, both made up in DMEM containing 10% fetal calf serum. Seven days later individual microcolonies were isolated. These grew either as flat, substrate-adherent, cuboidal epithelial colonies or as colonies of smaller more-rounded cells. One of the round sublines, designated clone w/tR, was then expanded *in vitro* and frozen as a master stock at passage 4. Stable expression constructs were made in pBAN2, a 10,865-bp plasmid with a β-actin promoter and a neomycin-resistance gene. J774 murine macrophage iNOS cDNA, which encodes a protein sequence indistinguishable from that reported (12) for the RAW 264.7 (D.W.M., unpublished data), was excised from pBSSKII$^+$ by using *Hin*dIII and ligated into the *Hin*dIII site of the multiple cloning site of pBAN2. Stable transfection of DLD-1 clone w/tR cells was made at passage 5 with this construct (clone iNOS-19). Transfection was achieved by lipofection using 70 μl of Lipofectin (GIBCO/BRL) and 10 μg of plasmid per subconfluent 10-cm-diameter culture plate. As a control, some cells were transfected with the pBAN2 plasmid lacking any insertion (clone pBAN2R). Colonies of each transfected clone were then isolated and grown to confluence in 12-well plates.

Assays for NO Synthase and Nitrites. NO synthase activity was determined by measuring the rate of conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline by soluble cytosolic extracts of cells or disaggregated tumors (13). Concentrations of nitrite (NO_2^-) in the medium used to culture the cells were also determined, by chemiluminescence (14) or by a microplate method based on the Griess reaction (15). In some experiments, nitrate (NO_3^-) in the samples was reduced to NO_2^- by using acid-washed cadmium powder (15) prior to assay for NO_2^-. In other experiments, cells were cultured in the presence of the NO synthase inhibitor, *N*-iminoethyl-L-ornithine (L-NIO) at 250 μM. For assay of murine iNOS mRNA, RNA was extracted from cell pellets or disaggregated tumors by using a FastTrack kit (Invitrogen) as described (16). Reverse transcription–PCR was carried out using a GeneAmp reverse transcription–PCR kit and the murine iNOS-specific primers [sense, bp 580–611 (5′-GCCTCATGCCATTGAGTTCATCAACC-3′) and antisense, bp 940–964 (5′-GAGCTGTGAATTCCAGAGCCTGAAG-3′)], amplifying a 372-bp product. Murine iNOS was amplified in the presence of 1 mM $MgCl_2$ by using 35 cycles of the following sequential steps: 96°C for 35 sec, 62°C for 2 min, and 72°C for 2 min. Amplified fragments were sequenced directly with primers used in the PCR (17).

Studies on Clones in Monolayer Culture. To determine the time course of NO production, cells were plated into 75-cm^2 culture flasks at an initial density of 1.5×10^5 cells per flask

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Abbreviations: NO, nitric oxide; L-NIO, *N*-iminoethyl-L-ornithine; iNOS, inducible NO synthase.

on day 0. On days 2, 4, 6, and 8, samples of culture medium from each cell line used were taken from eight, four, two, and two flasks, respectively, prior to removal of the cells by trypsinization for cell counts. Each cell pool was then pelleted by centrifugation for 5 min at 450 × *g*. Medium samples were assayed for NO_2^- and NO_3^- (NO_x) and the cell pellets were assayed for NO synthase activity. For studies on the comparative growth of clones, cells were seeded at an initial density of 1×10^4 cells per ml into 25-mm^2 tissue culture flasks. At daily intervals, from day 1 to day 6, cells in triplicate flasks were trypsinized and counted by using conventional trypan blue dye-exclusion techniques. Four aliquots from each flask were counted and the mean number of viable cells per ml in each group of three flasks was then calculated for each clone and compared.

Studies on Clones as Tumors in Nude Mice. Young male adult CD1 *nu/nu* mice (5–10 animals per group) were injected subcutaneously with 1×10^6 cells (clones iNOS-19, w/tR, or pBAN2R) suspended in 0.1 ml of DMEM on day 0. Four experiments to determine relative tumor weights on day 28 after injection of cells were carried out. Data obtained from each experiment were considered to be valid provided that the distribution of tumor weights within each group remained normal. In a further experiment (10 mice per group), developing tumors were measured for length (l) and width (w) by calipers on alternate days from day 11 after injection and on day 28 tumor volume was estimated by using the formula—tumor volume = $(l \times w^2)\pi/6$. Tumors obtained from each of the above experiments were either frozen in liquid nitrogen for subsequent iNOS mRNA or enzymic analysis or processed for histology. Fresh frozen sections of clone iNOS-19 and control w/tR tumors were processed for *in situ* detection of iNOS mRNA (18). Hybridization was carried out by using the following oligonucleotides labeled at the 3′ end with adenosine 5′-[α-[^{35}S]thio]triphosphate and terminal deoxynucleotidyltransferase oligonucleotide sequences derived from the murine iNOS cDNA sequence (16): BE39 [antisense, bp 665–630 (5′-CCAGCCTGGCCAGATGTTCCTCTATTTTTGCCTCTT-3′)], BE40 [antisense, bp 1447–1442 (5′-GTGGTGGTCCATGATGGTCACATTCTGCTTCTGGGAA-3′)], and BE41 [antisense, bp 2064–2029 (5′-TGAGGGTACATGCTGGAGCCAAGGCCAAACACAGCA-3′)]. The probes were derived from regions specific for iNOS. For histological studies, 5 age-matched tumors and 5 size-matched tumors from clone iNOS-19 and clone w/tR were studied. These tumors were removed from nude mice either 21 or 28 days after injection of 1×10^6 cells per mouse, fixed in 10% neutral buffered formalin, paraffin-wax-processed, sectioned at 3 μm thickness, and then stained with hematoxylin/eosin or with MSB stain (19). The degree of vascularization, in 5 clone iNOS-19 and 5 clone w/tR size-matched tumors, was estimated by scanning five 100×100 μm^2 tumor sections within a grid as either positive or negative for the presence of erythrocytes. Counts were limited to areas of viable tumor tissue within the capsule of each tumor. Differences between the mean number of blood vessels seen in sections of iNOS-19 and control w/tR tumors were analyzed by using Student's t test.

RESULTS

The concentration of NO_2^- in the growth medium of confluent cultures of DLD-1 clone w/tR cells transfected with iNOS ranged from 8.9 to 14.5 μM, showing that these clones had produced appreciable quantities of NO. No detectable NO_2^- was found in the medium from any of the colonies derived from cells transfected with the plasmid lacking the iNOS insertion or in medium used to culture wild-type clone w/tR cells. The highest NO-producing iNOS sense clone (clone iNOS-19) and a clone transfected with the plasmid lacking any insertion (clone pBAN2R) were then expanded into cell lines and frozen as master stocks at passage 4.

The time course of NO production *in vitro* was determined in cells of clones iNOS-19 and w/tR. The results (Table 1) indicate a marked increase in NO_x concentration with time for clone iNOS-19, with some 90% of the NO_x produced being NO_3^-. No significant concentrations of NO_x were found in medium used to culture the w/tR cells or in medium used to culture either iNOS-19 or w/tR cells in the presence of 250 μM L-NIO. NO synthase activity in clone iNOS-19 cells (Table 2) was evident throughout the duration of the experiment and was maximal during logarithmic cell growth on day 4. No such activity (data not shown) was detected in cells of clone w/tR. PCR amplification and subsequent sequencing of the iNOS PCR fragments from these cells confirmed that the NO produced by clone iNOS-19 was derived from the transfected murine iNOS cDNA and not from the induction of the endogenous human iNOS gene. All sequences obtained (data not shown) were for the murine iNOS mRNA. No iNOS was seen in control w/tR and pBAN2R cells.

Comparative growth rates of clone iNOS-19 and the control clones were studied in three experiments. Fig. 1*A* represents a typical experiment where viable cell counts consistently showed that the clone iNOS-19 cells multiplied at a significantly slower rate than both control lines. The addition of L-NIO in two further experiments effectively inhibited the NO synthase activity in these cells and their growth rate increased so that the cells now behaved like the controls (Fig. 1*B*). L-NIO had no effect on the growth of control w/tR and pBAN2R cells. Samples of medium were also taken daily from the flasks and analyzed for NO_x. NO_x was found only in medium used to grow clone iNOS-19 cells in the absence of L-NIO.

The growth of subcutaneous tumors arising in mice injected 28 days previously with clone iNOS-19 cells was compared with that in mice given clone w/tR cells and, in some experiments, clone pBAN2R transfection-control cells. Tumor weights (Table 3) were consistently higher in mice given the clone iNOS-19 cells, and mean and median values did not differ widely in any one of the four experiments. The rate of growth in terms of mean tumor volumes measured during the course of a fifth experiment was also greater for clone iNOS-19 tumors than for either of the controls (Fig. 2).

Tumors derived from clone iNOS-19 cells were able to generate NO during their growth *in vivo*. Tumors removed from nude mice on day 28 after injection of cells continued to transcribe iNOS mRNA, as observed by *in situ* detection (Fig. 3), and to express active enzyme (20.8 ± 3.9 pmol per min per mg of protein; mean ± SEM of four tumors). Neither iNOS mRNA nor iNOS activity was detected in control tumors derived from clone w/tR or clone pBAN2R cells.

Table 1. Time course of NO_2^- and NO_3^- (NO_x) generation by clone iNOS-19 and w/tR cells in the presence or absence of the NO synthase inhibitor L-NIO (250 μM) *in vitro*

	NO_x, μM			
Day	w/tR	w/tR + L-NIO	iNOS-19	iNOS-19 + L-NIO
1	0	0	0.3	0
2	0	0	0.1	0
3	0	0	6.24	0
4	3.2	1.7	31.8	1.4
5	4.6	3.8	61.7	6.9
6	4.8	0.7	65.6	4.2
7	0.3	0	74.4	2.6
8	3.0	3.8	70.1	8.2

Values given for each time point are the mean of each sample assayed in duplicate, minus background.

Table 2. NO synthase activity in supernatants prepared from cell pellets of clone iNOS-19 collected at intervals during 8 days in culture

Days in culture	Activity, pmol per min per mg of protein
2	20.1 ± 0.98
4	52.2 ± 2.31
6	34.1 ± 0.98
8	28.0 ± 2.19

Each value is the mean ± SEM of one cell pellet assayed in triplicate.

Histological studies (Fig. 4) suggest that clone iNOS-19 tumors were markedly more vascularized than those derived from the parental w/tR cells. Clone iNOS-19 tumors also appeared to be more invasive than the controls, with several of the former having broken through the tumor capsule and infiltrated the subcutaneous musculature. All clone w/tR tumors were surrounded by a discrete fibrous capsule with no signs of breakthrough into surrounding tissues. Confirmation of the increased vascularity of clone iNOS-19 tumors was obtained when the numbers of blood vessels in tumor sections were estimated (Table 4). By using a grid scoring system, significantly more grid squares contained one or more blood vessel(s) in sections of viable clone iNOS-19 tumor tissue as compared with sections of control clone w/tR tumors.

DISCUSSION

These results demonstrate that a subclone of a human colonic adenocarcinoma cell line can be engineered to produce NO endogenously after stable transfection with murine inducible NO synthase cDNA under the control of a constitutive promoter. Our results also show that this generation of NO is continuous when these cells are subsequently grown under *in vitro* conditions and as xenografts in nude mice, thus affording a model in which any effects of endogenous NO production on cell and tumor phenotype can be studied.

The observation that clone iNOS-19 cells proliferated at a significantly slower rate *in vitro* than either of the controls supports previous reports (3) that NO can exert a cytostatic effect on tumor cells. Reversal of this effect by the NO synthase inhibitor L-NIO confirmed that inhibition of proliferation observed was directly related to the production of NO. Of interest was the observation that 90% of the NO_x produced by our

Table 3. Comparative tumor weights of clones iNOS-19, w/tR, and pBAN2R 28 days after subcutaneous injection into nude mice

Clone	Animals, no. per group	Mean tumor weight, g	Median tumor weight, g
iNOS-19	7	0.79 (0.17)	0.59
w/tR	7	0.25 (0.08)	0.19
iNOS-19	7	0.81 (0.27)	0.57
w/tR	7	0.29 (0.06)	0.34
iNOS-19	5	0.98 (0.18)	0.85
w/tR	5	0.44 (0.10)	0.40
pBAN2R	5	0.33 (0.15)	0.26
iNOS-19	10	1.03 (0.16)	0.98
w/tR	6	0.64 (0.12)	0.67
pBAN2R	6	0.45 (0.13)	0.44

Mean and median tumor weights for four experiments are given. Numbers in parentheses are the SEM.

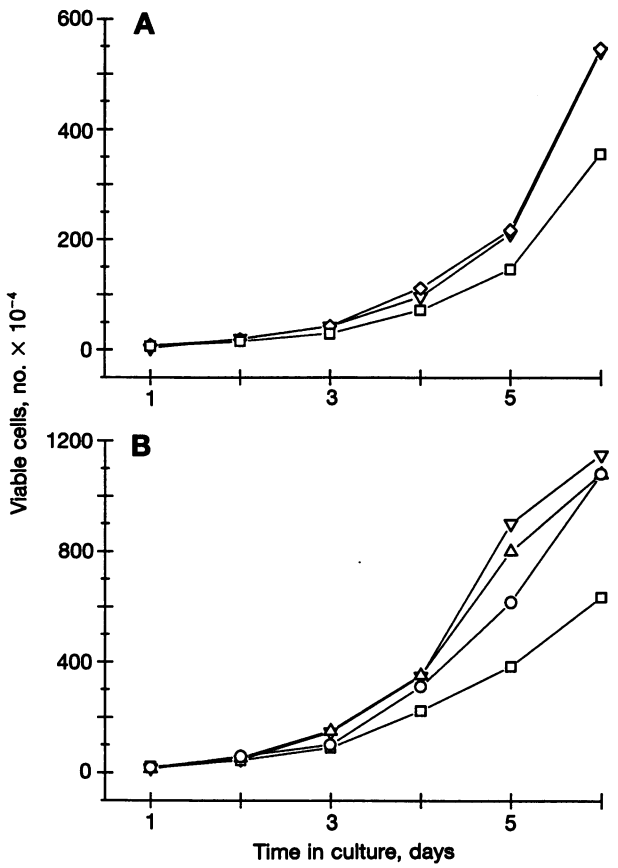

FIG. 1. (*A*) Comparative growth of clones iNOS-19 (□), w/tR (▽), and pBAN2R (◇) as monolayers *in vitro*. (*B*) Comparative growth of clone iNOS-19 in the presence (○) and absence (□) of 250 μM L-NIO, the NO synthase inhibitor, and w/tR clone in the presence (△) and absence (▽) of L-NIO.

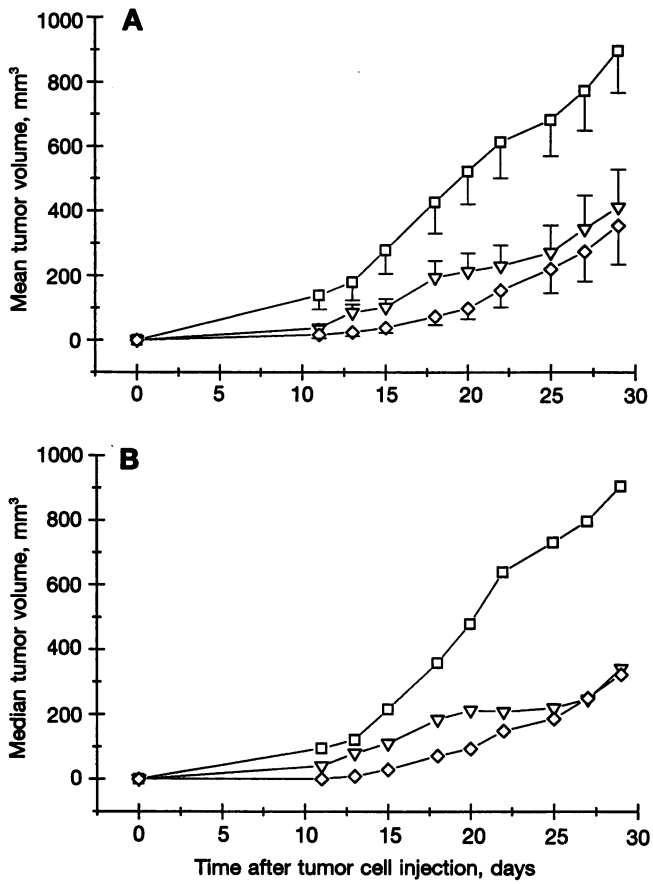

FIG. 2. Comparative outgrowth of subcutaneous clone iNOS-19 (□), w/tR (▽), and pBAN2R (◇) tumors in nude mice. Mean (±SEM) (*A*) and median (*B*) tumor volumes, in groups of 10 mice, are shown.

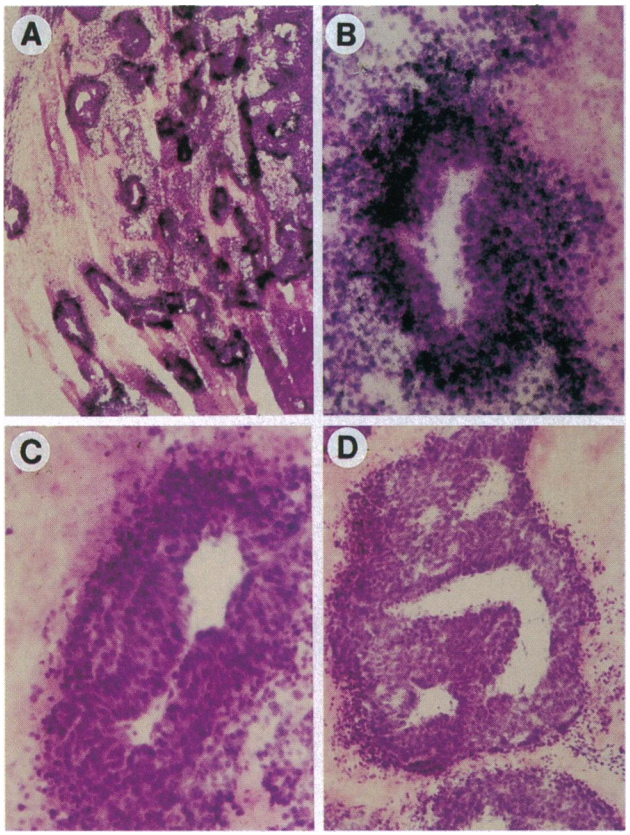

FIG. 3. *In situ* detection of iNOS gene expression. (*A* and *B*) Clone iNOS-19 with widespread distribution throughout the tumor of dense silver grains, specific for iNOS mRNA, localized particularly in viable tissue in areas interfacing with necrotic tissue. (*C*) Control clone iNOS-19 tumor where the labeling of mRNA had been inhibited completely with excess unlabeled oligonucleotide. (*D*) w/tR tumor that was negative for iNOS mRNA. (*A*, ×36; *B–D*, ×180.)

transfected cells *in vitro* was in the form of NO_3^-. This is a situation similar to that found in isolated hepatocytes (R. G. Knowles, personal communication) and contrasts markedly with that seen in other cells such as activated mouse peritoneal macrophages where the ratio of NO_2^- to NO_3^- approximates to 1:1 (3).

Our most significant finding was that clone iNOS-19 tumors in nude mice consistently grew faster than the wild-type and/or transfection controls. Clone iNOS-19 tumors were also significantly more vascularized, as shown by the number of blood vessels seen in histological sections. In addition, they were more invasive than the controls, having penetrated the subcutaneous musculature in some animals. Such observations strongly suggest that the sustained release of NO by the tumor cells had promoted tumor growth.

It has recently been reported that generation of angiogenic activity by human monocytes requires an L-arginine-dependent NO synthase effector mechanism (20) and that NO plays an important positive role in the angiogenesis associated with tissue healing (21). Vascularization is an absolute requirement for sustained growth of solid tumors and its extent correlates positively with tumor metastasis (22, 23). We provide strong evidence that the increased tumor growth associated with clone iNOS-19 cells correlates with increased angiogenesis and propose that this results from the continuous endogenous generation, by these cells, of low concentrations of NO.

The NO synthase activity in our genetically engineered xenografted tumors is similar to that reported (≈20 pmol per min per mg of protein) for human breast cancers (11). This is at least 1 to 2 orders of magnitude lower than the enzyme activity associated

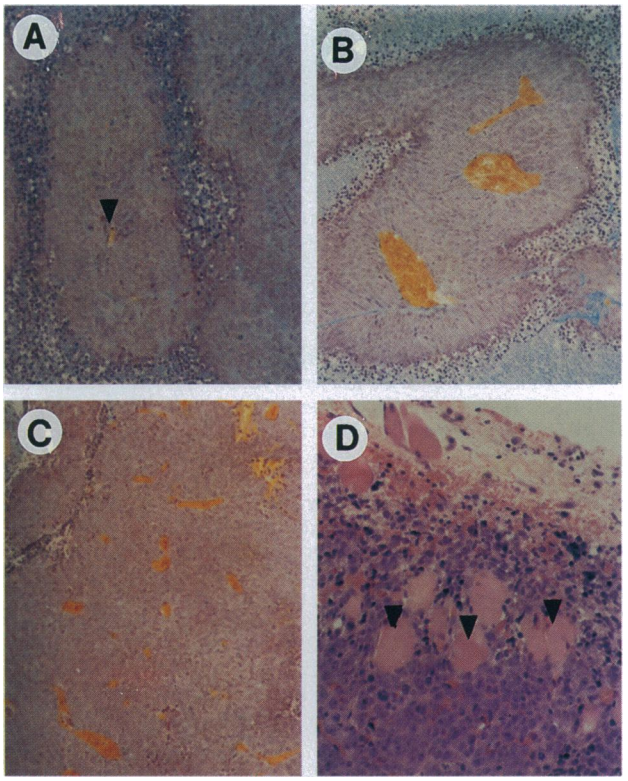

FIG. 4. Histology of clone iNOS-19 and control clone w/tR tumors. (*A* and *B*) MSB-stained cross sections of clone w/tR and iNOS-19 tumors, respectively. Little microvasculature (arrowhead in *A*) is discernible within the areas of viable tumor tissue in the w/tR tumor but in the iNOS-19 tumor (*B*) very obvious large vessels stained bright yellow are located in the center of each main area of viable tumor. (*C*) MSB-stained section of another clone iNOS-19 tumor, again showing numerous blood vessels scattered throughout the viable tissue. (*D*) Hematoxylin/eosin-stained section of a clone iNOS-19 tumor where loss of the capsule is evident and tumor cells have invaded the subcutaneous musculature (arrowheads). Such areas were not seen in any control tumors. (*A–C*, ×100; *D*, ×125.)

with phenomena such as cytotoxicity (3) and apoptosis (24). Thus, with these latter observations, our data support the hypothesis for a dual role for NO in cancer so that when it is produced at high concentrations, NO has antitumor activity, but at lower concentrations, it promotes tumor growth.

The reduced growth rate of NO-generating tumor cells *in vitro* could be reversed by treatment with the NO synthase inhibitor L-NIO. It is possible that the consequences of increased

Table 4. Comparative distribution of blood vessels in subcutaneous clone iNOS-19 and clone w/tR tumors

Tumor type	Tumor no.	Mean % grid squares containing one or more blood vessels	Mean % per tumor type
w/tR	1	4.75 (0.92)	
	2	5.50 (0.93)	
	3	7.75 (2.04)	5.10* (1.40)
	4	3.75 (1.38)	
	5	3.75 (1.33)	
iNOS-19	1	35.25 (5.26)	
	2	22.75 (2.37)	
	3	18.75 (4.33)	25.85* (4.42)
	4	27.5 (4.92)	
	5	25.0 (2.70)	

Values in parentheses are the SEM.
*Difference between the means (Student's *t* test); $P < 0.001$ (iNOS-19 vs. w/tR tumors).

NO production *in vivo* will also be reversed by such treatment. To do so, however, would require an inhibitor with little or no activity against the constitutive NO synthase, whose continuing production of NO in the cardiovascular system is essential for the maintenance of systemic vascular tone. Such a selective inhibitor might prove useful for delaying the growth and progression of cancer in humans.

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1. Knowles, R. G. & Moncada, S. (1994) *Biochem. J.* **298,** 249–258.
2. Moncada, S., Palmer, R. M. J. & Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142.
3. Hibbs, J. B., Taintor, R. R., Vavrin, Z., Granger, D. L., Drapier, J.-C., Amber, I. J. & Lancaster, J. R., Jr. (1990) in *Nitric Oxide from L-Arginine: A Bioregulatory System*, eds. Moncada, S. & Higgs, E. A. (Elsevier, Amsterdam), pp. 189–223.
4. Sutherland, R. M., Rasey, J. S. & Hill, R. P. (1988) *Am. J. Clin. Oncol.* **11,** 253–274.
5. Gross, S. S., Jaffe, E. A., Levi, R. & Kilbourn, R. G. (1991) *Biochem. Biophys. Res. Commun.* **178,** 823–829.
6. Lamas, S., Marsden, P. A., Li, G. K., Tempst, P. & Michel, T. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6348–6352.
7. Stuehr, D. J. & Marletta, M. A. (1987) *Cancer Res.* **47,** 5590–5594.
8. Radomski, M. W., Jenkins, D. C., Holmes, L. S. & Moncada, S. (1991) *Cancer Res.* **51,** 6073–6078.
9. Jenkins, D. C., Charles, I. G., Baylis, S. A., Lelchuk, R., Radomski, M. & Moncada, S. (1994) *Br. J. Cancer* **70,** 847–849.
10. Thomsen, L. L., Lawton, F. G., Knowles, R. G., Beesley, J. E., Riveros-Moreno, V. & Moncada, S. (1994) *Cancer Res.* **54,** 1352–1354.
11. Thomsen, L. L., Miles, D. W., Happerfield, L., Bobrow, L. G., Knowles, R. G. & Moncada, S. (1995) *Br. J. Cancer*, in press.
12. Lyons, C. R., Orloff, G. J. & Cunningham, J. M. (1992) *J. Biol. Chem.* **267,** 6370–6374.
13. Radomski, M. W., Vallance, P., Whitley, S., Foxwell, N. & Moncada, S. (1993) *Cardiovasc. Res.* **27,** 1380–1382.
14. Palmer, R. M. J., Ferrige, A. G. & Moncada, S. (1987) *Nature (London)* **327,** 524–526.
15. Thomsen, L. L., Ching, L. M., Zhuang, L., Gavin, J. B. & Baguley, B. C. (1991) *Cancer Res.* **51,** 77–81.
16. Charles, I. G., Palmer, R. M. J., Hickery, M. S., Bayliss, M. T., Chubb, A. P., Hall, V. S., Moss, D. W. & Moncada, S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11419–11423.
17. Winship, R. S. (1989) *Nucleic Acids Res.* **17,** 1266.
18. Kishimoto, J., Keverne, E. B., Hardwick, J. & Emson, P. C. (1993) *Eur. J. Neurosci.* **5,** 1684–1694.
19. Lendrum, A. C., Frazer, D. S., Slidders, W. & Henderson, R. (1962) *J. Clin. Toxicol.* **15,** 408.
20. Leibovich, S. J., Polverini, P. J., Fong, T. W., Harlow, L. A. & Koch, A. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4190–4194.
21. Konturek, S. J., Brzozowski, T., Majka, J., Pytko-Polonczyk, J. & Stachura, J. (1993) *Eur. J. Pharmacol.* **239,** 215–217.
22. Macchiarini, P., Fontanini, G., Hardin, M. J., Squartini, F. & Angeletti, C. A. (1992) *Lancet* **340,** 145–146.
23. Brawer, M.K., Deering, R. E., Brown, M., Preston, S. D. & Bigler, S. A. (1994) *Cancer* **73,** 678–687.
24. Xie, R. & Fidler, I. J. (1993) *Proc. Am. Assoc. Cancer Res.* **34,** 95 (abstr. no. 564).