Effect of Cyclooxygenase and Nitric Oxide Synthase Inhibitors on Tumor Growth in Mouse Tumor Models with and without Cancer Cachexia Related to Prostanoids

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ABSTRACT

The potential interaction between cyclooxygenase (Cox) and NO metabolitic pathways in the control of local tumor growth was evaluated. Mice bearing either a sarcoma-derived tumor (C57Bl; MCG 101) or a malignant melanoma (C3H/HeN; K1735-M2) were used. These models were principally different because they demonstrate, in tumor hosts, conditions with and without cancer cachexia, seemingly related to high and low production of prostanoids, respectively. Cox inhibitors (Cox-1 and Cox-2) decreased tumor growth by 35–40% in MCG 101-bearing mice but had no such effect on melanoma-bearing mice, despite the expression of the Cox-2 protein in melanoma cells. Indomethacin reduced prostanoïd production in both tumor (MCG 101) and host tissues and reduced tumor cell proliferation, mainly in vivo. Nitric oxide synthase (NOS) inhibitors (Nω-nitro-l-arginine methyl ester and Nω-nitro-l-arginine) reduced tumor growth in vivo by ~50% in both tumor models. Tumor growth reduction, related to NOS inhibition, was unrelated to prostanoïd production and was an in vivo phenomenon in both tumor models. Specific inhibitors of inducible NOS activity, unexpectedly, had no effect in any tumor model, although inducible NOS protein was present in tumor tissues in large amounts. A combination of Cox and NOS inhibitors had no additive effect on tumor growth (MCG 101). Cox inhibition increased tumor tissue (MCG 101) expression of cNOS mRNA but had no significant effect on tumor tissue expression of the transferrin receptor, vascular endothelial growth factor, or basic fibroblast growth factor. NOS inhibition increased tumor tissue content of cNOS mRNA but showed as well a trend to increase mRNA content of the transferrin receptor and vascular endothelial growth factor. Our results suggest that NOS inhibitors can decrease the local growth of tumors that are either responsive or unresponsive to Cox inhibition. This effect may reflect cross-talk between Cox and NOS pathways within or among tumor cells, or it may represent unrelated pathways within or among tumor cells, or it may represent unrelated pathways within or among tumor cells, or it may represent unrelated pathways within or among tumor cells.

INTRODUCTION

Tumor growth with subsequent cancer cachexia is mediated by complex interactions of cytokines and growth factors, partly communicated by eicosanoids (1). Accordingly, it has been reported that a variety of Cox3 inhibitors attenuate experimental tumor growth (2), tissue invasion (3), and influence on carcinogenesis (4) and prolong survival in experimental animals (5), and probably in clinical cancer (6) as well. Thus, indomethacin, a classic prostaglandin synthase inhibitor, attenuated cancer cachexia, counteracted tumor-related anorexia, and decreased tumor growth in order of magnitudes observed following provision of neutralizing antibodies to cytokines such as IL-1α, IL-1β (7), tumor necrosis factor-α (8), and IL-6 (9). Thus, the improvement of cachexia and depression of tumor growth by Cox inhibition (Cox-1 and Cox-2) may be related to reduced production of prostanoids in either tumor cells or host tissues, or a combination of both.

NOS activities are also related to tumor growth, cellular differentiation, tumor blood flow, and metastatic formation under similar conditions in which Cox inhibitors may act (9–13). Interestingly, it has been recognized that production of NO is related to Cox metabolism both in vitro and in vivo during a variety of experimental conditions (14–16). Therefore, it is possible that both NO and Cox activities are either alternative or common pathways in the regulation of fundamental processes that determine local and systemic progression of cancer (17). The aim of the present study was, therefore, to evaluate to what extent Cox and NO-forming pathways may interact in their influence on tumor growth and subsequent development of cancer cachexia. For this purpose, we have used two different mouse tumor models with and without cancer cachexia related to high and low prostanoid production, respectively.

MATERIALS AND METHODS

Tumor Models

The present in vivo study of tumor growth was undertaken in weight-stable (20–24 g) female mice C57Bl, implanted with MCG 101 tumors, and in C3H/HeN implanted with K1735-M2 melanoma cells (a kind gift from Prof. I. Fidler, The University of Texas M. D. Anderson Cancer Center, Houston, TX). MCG 101 is a nonmetastasizing, undifferentiated epithelial-like solid tumor. This tumor model has been used extensively in our laboratory for the study of cancer cachexia, and it has been grown continuously in vivo since 1972 (18, 19). When implanted s.c., the tumors grow locally with a reproducible growth pattern. The animals die because of cancer cachexia 12–15 days after tumor implantation. The MCG tumor produce PGE2, both in vitro and in vivo, leading to elevated plasma concentrations of PGE2 (1). Cox inhibition by indomethacin, which normalizes systemic levels of PGE2, improves nutritional state and food intake, reduces tumor growth, and prolongs survival (20). In contrast, the K1735-M2 melanoma synthesizes considerably less PGE2 and did not produce overt cachexia during tumor progression. Thus, melanoma-bearing mice with extensive local tumor growth did not die with cachexia. Pulmonary metastases are probably the cause of death beyond 4–5 weeks after tumor implantation (21). Consequently, indomethacin treatment in this Cox-unresponsive model had no influence on either tumor growth, nutritional state, or survival.

All animals were housed in groups of five in plastic cages in a temperature-controlled room (24°C) at increased humidity with a 12-h light/dark cycle and were provided free access to standard chow. Tumor tissue or cultured cell suspensions were inoculated under light i.p. anesthesia (100 μg/kg ketamin and 5 μg/kg xylazin). C57Bl mice were implanted s.c. with 3 mm3 of MCG tumor tissue on both sides of the back to allow unrestrained movement of the animals. The K1735-M2 cell line was suspended in McCoy’s 5A medium in a concentration of 500,000 cells/ml, of which 0.2 ml containing 100,000 cells was inoculated bilaterally in the flanks of the C3H/HeN animals.

Mice were killed by cervical dislocation either 10 days (MCG) or 30 days...
In K1735-M2 tumor models after tumor implantation. The tumors were dissected free for compositional measurements. Food intake, body composition, and tumor dry weight were measured as described elsewhere (22, 23).

Provision of Drugs

Most drugs were provided in the drinking water to minimize the stress reaction by daily injections. Calculation of the dosage of each drug administered was based on measured water consumption corresponding to 3 ml/animal and 24 h (20). Otherwise, a Cox-2 inhibitor (L-745,337) was given as s.c. daily injections in saline, according to recommendation of the provider (24). Two hundred and fifty mice were randomly divided into 16 groups, which received the following treatments with standardized doses.

Experiments in MCG-bearing Mice

For NOS inhibition, the groups were divided as follows: group 1, l-Name (100 mg/kg/day; n = 17); group 2, l-NoArg (100 mg/kg/day; n = 12); group 3, l-Name plus l-NoArg (100 mg/kg/day of each; n = 9); and group 4, t-Name (100 mg/kg/day; n = 6). For iNOS inhibition, the groups were divided as follows: group 5, Amg (100 mg/kg/day; n = 17); and group 6, Smt (50 mg/kg/day; n = 8). For Cox inhibition, there was only one group: group 7, indomethacin sodium (Confortid; 1 mg/kg/day plus 100 mg/kg/day; n = 7); and group 10, indomethacin combined with l-NoArg (1 mg/kg/day plus 100 mg/kg/day; n = 12). There was one control group: group 11 (n = 54). These tumor-bearing mice received normal drinking water only.

Experiments in K1735-M2-bearing Mice

For NOS inhibition, there was only one group: group 12, t-NoArg (n = 10). For iNOS inhibition, the groups were divided as follows: group 13, Amg (n = 10); and group 14, Smt (n = 10). For Cox inhibition, there was only one group: group 15, indomethacin (n = 10). There was one control group: group 16 (n = 20). These tumor-bearing mice received normal drinking water only.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial body weight (g)</th>
<th>Final body weight including tumor (g)</th>
<th>Cumulative food intake (g)</th>
<th>Carcass dry weight (g)</th>
<th>Carcass fat-free weight (g)</th>
<th>Body fat (g)</th>
<th>Tumor wet weight (g)</th>
<th>Tumor dry weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB±indo</td>
<td>10</td>
<td>18.7 ± 0.2</td>
<td>25.8</td>
<td>5.835 ± 0.1</td>
<td>4.264 ± 0.06</td>
<td>1.773 ± 0.08</td>
<td>2.048 ± 0.2</td>
<td>0.364 ± 0.03</td>
</tr>
<tr>
<td>TB-control</td>
<td>8</td>
<td>18.3 ± 0.1</td>
<td>20.1</td>
<td>5.587 ± 0.1</td>
<td>4.079 ± 0.07</td>
<td>1.717 ± 0.1</td>
<td>3.054 ± 0.3</td>
<td>0.545 ± 0.05</td>
</tr>
<tr>
<td>*</td>
<td>10</td>
<td>12.1 ± 0.4</td>
<td>26.1</td>
<td>6.458 ± 0.2</td>
<td>4.631 ± 0.1</td>
<td>1.732 ± 0.2</td>
<td>1.021 ± 0.1</td>
<td>0.172 ± 0.02</td>
</tr>
<tr>
<td>TB-NoArg</td>
<td>12</td>
<td>21.2 ± 0.4</td>
<td>26.4</td>
<td>6.284 ± 0.2</td>
<td>4.365 ± 0.1</td>
<td>1.787 ± 0.1</td>
<td>1.792 ± 0.2</td>
<td>0.311 ± 0.04</td>
</tr>
<tr>
<td>TB-control</td>
<td>12</td>
<td>21.1 ± 0.5</td>
<td>26.4</td>
<td>&lt;0.03</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
</tr>
</tbody>
</table>

* Data on carcass composition and tumor growth in melanoma-bearing mice are not given because these mice were nutritionally unaffected by the melanoma.

† TB, tumor-bearing.

Calculated based on standard variations reported in a previous publication (20).
(10,000 × g for 10 min) after determination of protein concentration in the supernatant (28). The samples were stored at −84°C until analysis. Total protein-equivalents (50 mg each sample) were diluted in SDS sample buffer and were separated on 7.5% SDS polyacrylamide gel (Bio-Rad Laboratories, Solna, Sweden) in the Laemmli buffer system (29). Proteins were transferred to a polyvinylidene difluoride membrane (Amersham Laboratories, Amersham, Buckinghamshire, United Kingdom), which was incubated with polyclonal rabbit anti-iNOS, or monoclonal mouse anti-eNOS antibody (diluted 1:1.500 and 1:250, respectively) overnight at 4°C. The antibodies were purchased from Transduction Laboratories (Lexington, KY; anti-iNOS and anti-eNOS). Bands were detected by chemiluminescence using an alkaline phosphatase-conjugated secondary antibody and CSPD (Western-Light; Tropix, Inc., Bedford, MA; Ref. 30). Membranes were exposed to ECL film (Amersham Laboratories).

Tumor Tissue Expression of NOS and Growth Factors

Specimens of viable tumor tissue from a pool of five mice in each group were used for extraction of total cellular RNA, using guanidinium isothiocyanate, according to standard procedure (31). Poly(A)⁺ RNA was selected, and 5–8 μg were either glyoxal treated and separated in 1.2% agarose gels or run in gels containing formaldehyde and capillary transferred to Hybond N⁺ membranes (Amersham). Murine DNA probes were made by PCR technique using a cDNA library, reversed transcribed from mRNAs from liver or WBCs. Primers were chosen from Mac Vectors database Entrez to give fragments of about 300 bp in length when amplified. These probes were labeled with [α-³²P]dCTP in a random priming reaction using an oligolabeling kit (Roche Diagnostics Scandinavia or Pharmacia-Biotech). Oligonucleotides of 30–40 bp were also used as probes. A mix of four murine antisense oligonucleotides/gene were used to probe VEGF, bFGF, angiogenin, the transferrin receptor, cNOS, and β-actin. These were ³²P-end labeled using [α-³²P]ddATP (Amersham). Prehybridization and hybridization were performed for 24 h each at 42°C (in a Robbins oven) in 50% formamide, 5× SSPE, 0.5% SDS, 10× Denhardt’s solution, and 250 μg/ml denatured low molecular weight DNA, and 50 μg/ml polyadenylic acid. Probe concentration was 5–10 × 10⁵ dpm/ml of hybridization solution. Postwashes were made in 5× SSPE/0.1% SDS with dilution to 0.1× SSPE/0.1% SDS, five concentrations in total for 30 min each at 42°C. Filters were exposed to Hyperfilm MP (Amersham) for 1–11 days. Densitometric analyses of autoradiograms were performed at 550 nm using a Shimadzu CS-930 densitometer.

Tumor tissue expression of Cox-2 and β-actin was measured by semiquantitative PCR. Sixty ng of poly(A)⁺ RNA from tumor tissue were reversed transcribed using GeneAmp RNA PCR kit (Perkin-Elmer). Oligo d(T)₁₆ was
used as a downstream primer. Amplification was done in a reaction volume of 50 μl, using 1:20 volume of the reverse transcriptase-reaction, 2 mM MgCl₂, 200 μM deoxynucleotide triphosphates, 0.15 μM of each primer, and 1.25 units of AmpliTaq. HotStart was performed in HotStart 50 tubes (Molecular Bio-Products, Inc., San Diego, CA). Reaction cycles were: denaturing at 94°C for 1 min, annealing at 58°C for 1.2 min, and extension at 72°C for 1 min for 20–36 cycles, followed by a 5-min extension time at 72°C. Aliquots of the PCR reaction (10 μl) were separated in 4% agarose gels and stained in ethidium bromide.

**Prostaglandin E₂ (¹²⁵I) Assay System**

Blood was collected from tumor-bearing animals on day 10 (MCG) and day 30 (melanoma) after inoculation. Healthy animals were used as normal controls. Sodium citrate was used as an anticoagulant, and indomethacin (10 μg/ml) was used to prevent further breakdown of arachidonic acid. Specimens were kept on ice and centrifuged at 2500 × g for 10 min at 4°C. After acidification, ethanol addition, and centrifugation of plasma, PGE₂ was extracted on Amprep C 18 minicolumns (Amersham RPA 1900), according to the recommendations in the PGE₂ assay system (Amersham RPA 530).

Tumor tissue was homogenized in 0.1 M Tris-HCl buffer (pH 7.4) containing indomethacin as inhibitor. All tubes were kept on ice. After centrifugation, supernatants were treated as mentioned above and applied to C₁₈ minicolumns for extraction of PGE₂. In conditioned media from tumor cell cultures, PGE₂ was extracted by the same procedure. After conversion of extracted PGE₂ by methyl oximation according to the kit instructions, RIA was performed within 6 days. Values were calculated from duplicates.

**Fractionation of Eicosanoids**

**Tissue Preparation.** Tumor tissue from five animals was placed in RPMI 1640 cell culture medium. A 10% homogenate was prepared in a glass tissue grinder, and 2 ml of the homogenate were incubated with 1μCl/μl of [³H]arachidonic acid (Amersham; 2 μCi/ml homogenate) for 3 h at 37°C. Samples were then precipitated with 4 volumes of methanol, vortexed, and refrigerated for 20 min at 4°C. The samples were centrifuged at 1800 × g. The supernatant was collected and evaporated under N₂. Finally, the samples were diluted with methanol to a final concentration of 30% methanol.

**Cell Preparation.** Tumor cells were grown in cell culture flasks and harvested 4 days after splitting. A cell suspension containing approximately 3.4 × 10⁷ cells/ml was made. The cell suspension was homogenized in a glass homogenizer, diluted with 1 volume of McCoy cell culture medium, and incubated with [³H]arachidonic acid (2 μCi/ml homogenate) for 3 h at 37°C. The samples were thereafter treated in the same way as tissue samples.

Tissue and cell extracts (100 μl) were separated on a C₁₈ Pak column (Waters 5 μm; 8-mm inside diameter radius). Eicosanoids were eluted during a series of isocratic elutions with mixtures of methanol and water buffered to pH 5.8, as described (32).

**Statistics**

Results are expressed as mean ± SE. Multiple group comparisons were performed by one-way ANOVA. Scheffe F test was used post hoc. P < 0.05 was considered statistically significant.

The experimental protocol was approved by the Committee for Animal Ethics at Göteborg University.

**RESULTS**

Cyclooxygenase Inhibition and Eicosanoids. The present study confirms that both indomethacin (Cox-1 and Cox-2 inhibition) and more specific Cox-2 inhibitor (L-745,337) decreased tumor growth by ~35–40% (P < 0.01; Fig. 1). Reduced tumor growth was related to attenuated anorexia and improved body composition in MCG-bearing mice (Table 1). Indomethacin had no such beneficial effects in melanoma K1735-M2-bearing mice (results not shown). Thus, melanoma-bearing mice remained nutritionally unaffected by melanoma growth throughout the entire experimental period (30 days), despite having a progressively larger tumor burden than MCG 101-bearing mice close to death. This discrepancy between the two tumor models may be linked to the fact that MCG 101 tumors showed a high production of prostanoids (mainly PGE₂ ~1000 ± 100 pg/10⁶ cells/day), whereas the melanoma cells produced eicosanoids at low amounts (<10 ± 5 pg PGE₂/10⁶ cells/day), evaluated both in vivo and in cultured tumor cells (Fig. 2).

High-performance liquid chromatography fractionation of eicosanoids, produced in either tumor tissue or in cultured tumor cells, confirmed that the main quantitative difference between MCG 101 and K1735-M2 cells was the production of PGE₂. A manifold of arachidonic acid metabolites seemed to be present when eicosanoids in tissue specimens from in vivo growing tumors were fractionated compared with specimens from cultured tumor cells (Fig. 2). This difference may indicate that eicosanoids produced by host cells were also involved.

At the time of sacrifice, MCG 101-bearing mice had elevated plasma concentrations of PGE₂ in the range of 1015 ± 173 pg/ml, which decreased to 90 ± 15 pg/ml by indomethacin treatment and to 234 ± 39 pg/ml by L-744,337. Freely fed non-tumor-bearing control mice had plasma levels of 81 ± 24 pg/ml. The PGE₂ concentrations in tumor tissue were ~1000 ± 150 ng/g in MCG 101 tumors 10 days after tumor implantation in untreated mice. This concentration decreased <20 ng/g by indomethacin treatment, which was comparable with concentrations of PGE₂ in untreated malignant melanoma-bearing mice (10 ± 2 ng/g tumor tissue) having plasma concentration of PGE₂ (<50 pg/ml) close to concentrations in healthy non-tumor-bearing mice. Provision of a Cox-2 inhibitor (L-745,337) decreased PGE₂ concentrations in MCG 101 tumors from 840 ± 57 to 180 ± 60 ng/g (P < 0.01).

**NOS Inhibition.** Inhibitors (L-NOArg and L-Name) of NO production decreased tumor growth in vivo by ~50% in both MCG 101 (Fig. 1) and melanoma-bearing mice (P < 0.01; Fig. 3). L-NOArg and L-Name reduced local tumor growth similarly at concentrations used (100 mg/kg body weight). The stereoisomer D-Name had no effect on tumor growth, demonstrating NOS specificity by L-NOArg and L-Name. NOS inhibition of tumor growth in MCG 101-bearing mice.
was also associated with improved carcass fat-free dry weight, whereas the cumulative food intake was not significantly influenced (Table 1). Selective inhibitors (aminoguanidine and S-methylisothiourea) of iNOS had no beneficial effect on either tumor growth or nutritional state in any of the tumor models. The combination of Cox and NOS inhibition did not show any synergistic effect on tumor growth, food intake, or nutritional state in any of the tumor models. NOS inhibition (L-NAME) had no effect on PGE₂ content in MCG 101 tumor tissue, whereas the host plasma PGE₂ concentrations decreased significantly from 3000 ± 600 pg/ml to 1521 ± 425 (P < 0.05). NOS inhibition by L-NAME (1 mM) reduced only marginally proliferation of cultured MCG 101 (6 ± 3%; P < 0.05) and melanoma cells (7 ± 3%; P < 0.05).

**Immunohistochemistry and Tumor Tissue mRNA.** Cox-1 protein occurred at very low levels in both MCG 101 and melanoma tumors, whereas Cox-2 protein was highly present in MCG tumors and almost not present in melanomas (Fig. 4A). Cox-2 was expressed 500-fold higher in MCG tissue compared with melanoma tissues evaluated by quantitative PCR (Fig. 5). Tumor tissue content of Cox-1 and Cox-2 protein was not changed by indomethacin treatment evaluated in MCG 101-bearing mice (not shown). Both tumors (MCG 101 and K1735-M2) contained substantial amounts of both cNOS and iNOS protein, with even distribution throughout the tumor tissue (Fig. 4B). Particularly, melanoma tumors seemed to have high levels of iNOS, which was confirmed by Western blot analysis (Fig. 6).

Provision of indomethacin to MCG 101-bearing mice increased tumor mRNA expression of cNOS but had no clear-cut effects on tumor expression of the transferrin receptor, angiogenin, VEGF, and bFGF (Table 2). Provision of L-NAME to MCG 101-bearing mice also increased tumor cNOS mRNA. It also showed a trend to increased
tumor expression of the transferrin receptor and VEGF, but L-NoArg provision was without effect on the expression of angiogenin and bFGF. The combination of indomethacin and L-NoArg had no synergistic effect on eNOS mRNA expression in tumor tissue (Table 2).

**DISCUSSION**

We have reported previously that indomethacin prolongs survival and improves food intake and the host nutritional state in both rats and mice bearing methylcholanthrene-induced tumors (5, 20). Similar effects have also been observed in cancer patients (6), although possible underlying mechanisms are not known. The indomethacin-responcing MCG 101 tumor produces high amounts of PGE₂, whereas the malignant melanoma (K1735; Ref. 21), with low prostanoid production, did not respond to indomethacin treatment. We have also reported that indomethacin inhibition of PGE₂ production in MCG 101 tumors leads to decreased net proliferation and prolonged potential doubling time for tumor growth in vivo, whereas indomethacin had no major inhibitory effect on tumor cell DNA synthesis in vitro (1). Increased apoptosis, partly by depression of telomerase activity, may therefore be alternative mechanisms for retarded growth of tumors by Cox inhibition (33, 34). Also, the effect of indomethacin to decrease tumor growth in vivo was not dependent on intact immunity (20) and was not confined to any particular organ or vascular bed in the host, in accordance with reports that indomethacin attenuates tumor angiogenesis (35). On the basis of our previous studies, we have concluded that prostaglandins in tumor tissue compartments promote local tumor growth by stimulation of tumor surrounding cells to produce growth factor(s) for tumor and matrix cell proliferation (1, 36). This broad interpretation has support by observations in more simplified cell models, where Cox activity is related to apoptosis, cell proliferation, cell differentiation, and regulatory peptides (37, 38). Additional information in the present study supports that beneficial effects by indomethacin in vivo may be a Cox-2-dependent phenomenon, because the specific Cox-2 inhibitor (L-745,337; Ref. 24) reduced tumor growth to the same extent as indomethacin (39).

A number of recent reports have indicated that NO activities may either stimulate or retard tumor growth by effects that are superficially similar to mechanisms exerted by Cox pathways (10, 40). Such observations are interesting because NO mediates (14), or is at least related to, Cox activities and tumor growth (10, 41). Therefore, we decided to evaluate whether provision of NO inhibitors at nontoxic doses attenuates cancer cachexia and tumor growth in addition to Cox inhibition (6, 14). For this purpose, we used two different tumor models: one model with cachexia being significantly dependent on Cox, and another model without cachexia, being unresponsive to Cox activity for tumor growth. Our results demonstrate that NO inhibition by oral intake of arginine analogues (L-Name and L-NoArg) inhibited tumor growth in vivo by ~50% in both tumor models independently of eicosanoid formation, whereas cultured tumor cells were only marginally affected by NO inhibition (~10%). These findings agree with conclusions on the role of NO promotion for tumor growth in experimental and clinical cancer (21). However, specific inhibitors of iNOS activity had no effect in any of our tumor models, although iNOS protein was present in both kind of tumors. This phenomenon may indicate a poor correlation between the cellular content of iNOS and NO formation, as reported recently for arachidonic acid-metabolizing enzymes and generation of corresponding metabolites (39). The literature on NO formation and tumor growth is not unanimous (42, 43). Some studies report that low levels of NO promote tumor growth, whereas high levels may be inhibitory. Also, it is not clear whether stimulation of NO production by iNOS induction in tumor cells may promote tumor cell proliferation (44), whereas iNOS induction in surrounding host cells may prevent local tumor growth (45). Dong et al. (13) have reported that iNOS expression in K1735 melanoma cells was inversely related to metastatic growth and progression, findings which agreed with the observation that various clones of melanoma cells expressed iNOS above or below detection levels in tissues (46). Therefore, it is not possible to judge why our tumors, with detectable iNOS, did not respond significantly to iNOS inhibitors. Possible explanations may be insufficient drug concentrations after oral provision, or that iNOS is differentially important for local tumor growth and metastatic spread (39). With this uncertainty in mind, our findings suggest a model where downstream pathways for Cox and NO activation end up into a common effector mechanism(s) for MCG tumors, whereas other mechanisms may be present.

### Table 2. Northern blot analyses of the expression of the transferrin receptor (Tf-R), angiogenin, VEGF, cNOS, bFGF

<table>
<thead>
<tr>
<th></th>
<th>Area in %</th>
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<tbody>
<tr>
<td>Tf-R/β-actin</td>
<td>100</td>
</tr>
<tr>
<td>Angiogenin/β-actin</td>
<td>100</td>
</tr>
<tr>
<td>VEGF/β-actin</td>
<td>105</td>
</tr>
<tr>
<td>cNOS/β-actin</td>
<td>227</td>
</tr>
<tr>
<td>bFGF/β-actin</td>
<td>117</td>
</tr>
</tbody>
</table>

*The drugs were provided in the drinking water for 10 days treatment as described in “Materials and Methods.” There were five animals in each group.

*Indo, indomethacin.

*P < 0.05 versus TB controls with an estimated SD of ± 15%.

"P < 0.10."
in K1735-M2 melanomas. These results both agree and disagree with models suggested by others for normal cellular physiology, where integrated functions between Cox and NOS production seem to be a general phenomenon (47–49).

Previous and present results suggest that the beneficial effects of indomethacin and NO inhibition may in part be host related. This conclusion is based on the observation that in vivo effects were more pronounced than in vitro effects, and that NOS inhibition in MCG-bearing mice did not decrease tumor content of prostaglandins but reduced circulating plasma PGE$_2$, which was associated with improvement in nutritional state (fat-free carcass weight). These observations suggest interaction between Cox and NOS pathways among host tissues and not only within tumor tissue (50–52). Alternatively, tumor growth inhibition by NO may not be independent of prostanoid pathways (53). However, if completely separate pathways occurred, one should expect additive effects by Cox and NOS inhibitors in MCG tumors, which was not observed.

We have earlier reported that provision of indomethacin to MCG 101-bearing mice increased tumor expression of various growth factors (IL-6, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, and transforming growth factor β$_1$) and decreased expression of bFGF and angiogenin, but it left the tumor expression of VEGF, epidermal growth factor, platelet-derived growth factor-A, platelet-derived growth factor-B, IL-1, and the transferrin receptor unchanged (1). The present study adds to previous information that transcriptional expression of cNOS mRNA in tumor tissue was increased 2-fold by either indomethacin or L-NAME treatment (Table 2), confirmed by Western blot analyses. Thus, up-regulation of mRNAs in tumor tissue suggests that both Cox and NOS pathways are related to cellular growth factors in either tumor or in host (endothelial) cells. A decreased flow of prostanoid metabolites and of NO after provision of inhibitors up-regulated cNOS expression by negative feedback to support continuous tumor growth (54). These findings illustrate a phenomenon of cross-talk between Cox and NOS pathways (48). Although the model is not yet defined, our observations may include loops with positive and negative feedback for genes that may in the future be possible to use therapeutically. For example, cancer cells with mutated p53 had accelerated tumor growth associated with increased expression of VEGF for neovascularization related to hypoxia and subsequently assumed increased NO formation (11, 55, 56). It is generally anticipated that both indomethacin and NO influence the tumor vasculature, including effects on tumor blood flow, endothelial cells, and tumor nutrient uptake. However, unpublished work in our MCG 101-bearing mice has, thus far, not confirmed that Cox inhibition reduced angiogenesis in MCG 101 tumors. It now remains to be evaluated whether clinical tumors are sensitive to NOS inhibition in a way that may add to our treatment reported previously of cancer patients with Cox inhibitors alone (indomethacin), particularly in patients with tumors unresponsive to Cox intervention (6, 57).

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