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Nitric Oxide Synthase Activity in Human Gynecological Cancer

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ABSTRACT

Nitric oxide is generated by the NO synthases, a family of isoenzymes expressed in a wide range of mammalian cells. In the vascular and nervous systems distinct isoforms generate NO to act as a signal transduction mechanism. The isoform induced by cytokines, on the other hand, provides a sustained release of NO which mediates some cytotoxic and cytostatic effects of the immune system. Solid tumors are a heterogeneous population of cell types, including tumor, vascular, and infiltrating immune cells. Studies *in vitro* show that NO synthase can be present in many of these cells. However, its presence *in situ* in solid human tumors has not been reported. In this study, we have investigated NO synthase activity and its cellular localization in malignant and nonmalignant human gynecological tissue. Nitric oxide synthase activity was observed in malignant tissue, was highest (≥250 pmol/min/g tissue) in poorly differentiated tumors, and was below detectable levels in normal gynecological tissue. Furthermore, investigations with a polyclonal NO synthase antibody revealed immunoreactivity only in malignant tissue. This was associated with NO synthase activity and localized to tumor cells. Thus NO synthase is present in human gynecological tumors, and its presence seems to correlate inversely with the differentiation of the tumor.

INTRODUCTION

The nitric oxide synthases are a family of isoenzymes which convert L-arginine to L-citrulline and generate NO (1). Cells of the vascular and nervous system possess calcium-dependent NO synthases and produce NO to act as a signal transduction mechanism (2). In contrast, NO synthase induced by cytokines and endotoxin is in most cases calcium independent (1) and provides a sustained release of NO which mediates some cytostatic and cytotoxic effects of the immune system (3).

Solid tumors contain a diverse range of cells including macrophages, T-lymphocytes, endothelial cells of the tumor vasculature, and fibroblasts of the supporting stroma, as well as tumor cells (4). Nitric oxide synthase can be found in many of these cell types in primary cultures or cell lines. The presence of the constitutive calcium-dependent isoform in endothelial cells and the calcium-independent isoform induced by cytokines in macrophages and endothelial cells has been well described (2). For various tumor cell lines, the presence of the calcium-dependent isoform (5, 6) and the cytokine-induced calcium-independent isoform (7, 8) has been documented.

Because of this, we decided to investigate the presence, characteristics, and cellular localization of NO synthase in solid tumors in humans. Here we report our findings from investigations of samples from different types of human gynecological tumors.

MATERIALS AND METHODS

Materials. All chemicals were from Sigma, Boehringer Mannheim, or BDH, unless otherwise indicated. L-[U-^{14}C]Arginine was from Amersham, United Kingdom.

Tissue Collection. Eight cases of gynecological cancer (6 ovarian, 1 mixed mesodermal, 1 endometrial) were entered into the study (Table 1). Malignant tumor tissue from primary and metastatic sites, as well as nonmalignant tissue (either adjacent to tumor tissue or from peritoneal nodules), was obtained from patients undergoing primary laparotomy. Tissue from uterus (2 cases) and cervix (1 case) obtained at hysterectomy from 3 non-cancer patients provided a control group.

Pieces of tissue from each site were divided and either fixed by immersion in neutral buffered formalin for histological diagnosis and immunolabeling studies or freeze-clamped and stored at −70°C for subsequent assay for NO synthase activity and Western blotting.

Histology. Tumor type and grade were determined by histological assessment of hematoxylin and eosin-stained tissue sections (Table 1).

Assay of NO Synthase. Frozen tissue was extracted at 0–4°C by homogenization (with an Ystral homogenizer) in 2.5 volumes of a buffer containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1 mM DL-dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 2 μg/ml aprotinin brought to pH 7.0 at 20°C with HCl. The homogenates were centrifuged at 10,000 × g at 0–4°C for 30 min. Supernatants were stored on ice for up to 2 h before use. Nitric oxide synthase in these supernatants (cytosol plus microsomes) was measured by the conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline as described previously (9). The activity of the calcium-dependent enzyme was determined from the difference between the [U-^{14}C]citrulline generated from control samples and samples containing 1 mM EGTA; the activity of the calcium-independent enzyme was determined from the difference between samples containing 1 mM EGTA[2] and samples containing both 1 mM EGTA and 1 mM N^G-monomethyl-L-arginine. The limit of detection in this assay was 15 pmol/min/g tissue.

Immunohistochemistry of Tissue Sections. Sections (3 μm thick) of paraffin wax-embedded tissue were dewaxed in xylene and brought to PBS through alcohol. They were washed in PBS and endogenous peroxidases were blocked with 1% hydrogen peroxide in methanol for 15 min. After a washing with PBS (5 min), nonspecific immunolabeling was blocked by incubation for 30 min with normal goat serum diluted 1:30. After a further 5 min wash with PBS the sections were ready for immunolabeling.

For NO synthase detection the sections were incubated with rabbit antiserum raised against rat brain NO synthase (10) diluted 1:1000 with PBT. This antibody has proven reactivity with constitutive NO synthase isoforms (10) and across several species including humans (11, 12). After overnight incubation the sections were rinsed with PBS (3 × 5 min) and incubated for 30 min with anti-rabbit biotinylated conjugate diluted 1:20 with PBT. Preimmune serum was used at identical concentrations as a control.

For macrophage detection, adjacent sections were incubated for 1 h with DAKO CD68 PG-M1 mouse monoclonal antibody (DAKO A/S, Glostrup, Denmark) diluted 1:100 with PBT. Another mouse monoclonal antibody raised against an unrelated antigen and used at the same dilution provided the control. After 3 × 5 min rinses with PBS the sections were incubated with anti-mouse biotinylated conjugate 1:20 in PBT.

In all cases the biotinylated conjugate was detected with ExtrAvidin-peroxidase complex diluted 1:20 with PBT. Immunolabeling was developed with diaminobenzidine tetrahydrochloride (10 mg in 15 ml of PBS containing 12 μl of fresh hydrogen peroxide), washed in water, stained briefly with hematoxylin, dehydrated, cleared, and mounted before examination.

Western Blotting of Semipurified NO Synthase. Frozen tissues were defrosted into iced buffer, pH 7.2, containing 50 mM Tris, 5 mM DL-dithiothreitol, 1 μg/ml leupeptin, 10 μg/ml chymostatin, 1 μg/ml pepstatin, 40 μg/ml bestatin, and 50 μg/ml N-α-p-tosyl-L-lysine chloromethyl ketone. Cytosols were extracted by homogenization and ultracentrifugation (100,000 × g for 1

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[2] The abbreviations used are: EGTA, [ethylenebis(oxy-ethylenenitrilo)]tetraacetic acid; PBS, phosphate-buffered saline; PBT, 0.1% Triton X-100 in PBS.

Table 1 *Pathological features and NO synthase activity of malignant tumor specimens*

Case	Tumor type	Grade[a]	Tissue site	NO synthase (pmol/min/g tissue)
1	Ovarian	Moderate	Ovary	<15
2	Ovarian	Moderate	Ovary	<15
3	Ovarian	Moderate	Bowel nodule	54
			Omental nodule	51
4	Ovarian	Poor	Ovary	250
5	Ovarian	Poor	Peritoneum	387
			Omentum	536
6	Ovarian	Poor	Small bowel nodule	666
			Omental nodule	710
			Ovary	970
7	Endometrial	Moderate	Endometrium	27
8	Mixed mesodermal	n/a[b]	Uterus	1950

[a] Tumors were graded according to the degree of differentiation in tissue sections.
[b] n/a, not applicable.

h). ADP-Sepharose eluates were prepared from supernatants as described previously (10). Proteins in ADP eluates were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), and blocked with 5% bovine serum albumin in PBS, and the membrane was incubated with the NO synthase antiserum (see immunohistochemistry studies) diluted 1:100, followed by development with AuroProbe and silver enhancement (Amersham).

RESULTS

Nitric Oxide Synthase Activity. Calcium-dependent NO synthase activity was found in tissue from 10 of the 12 malignant sites investigated (Table 1). The activity varied widely between cases but was highest in poorly differentiated tumors and the mixed mesodermal tumor (range, 250–1950 pmol/min/g tissue) and was ≤54 pmol/min/g tissue for moderately differentiated tumors. For individual cases, where malignant tissue was obtained from multiple sites (primary and/or metastatic), the activity was similar between sites. Nitric oxide synthase activity was <15 pmol/min/g tissue in nonmalignant tissue from the 8 cancer and 3 noncancer patients. Calcium-independent NO synthase activity was below detectable levels for all the tissues investigated.

Immunohistochemistry of Tissue Sections. The NO synthase antiserum immunolabeled tumor cells in all malignant tissues with detectable NO synthase activity (Fig. 1*A*). Immunoreactivity was widespread throughout the tissue sections from the mixed mesodermal tumor and from poorly differentiated ovarian tumors in which the highest NO synthase activities were detected. However, some regions in these tumors showed no immunoreactivity, and others showed positive and negative cells frequently adjacent to each other (Fig. 1*B*). Very few immunoreactive cells were observed in sections from malignant tumors with NO synthase activity below detectable levels or from nonmalignant tissue. Preimmune serum did not react with the malignant or normal tissue sections (Fig. 1*C*). Cells positive for the anti-human macrophage antibody were observed in sections from malignant tissue and these were confined predominantly to the stroma (Fig. 1*D*).

Western Blots. The NO synthase antiserum reacted with proteins present in ADP eluates prepared only from malignant tissue, and protein bands with molecular weights of approximately 160,000 and 135,000 were visible in all these samples (Fig. 2). Weak low molecular weight bands (M_r ~110,000 and ~55,000) were also visible. No

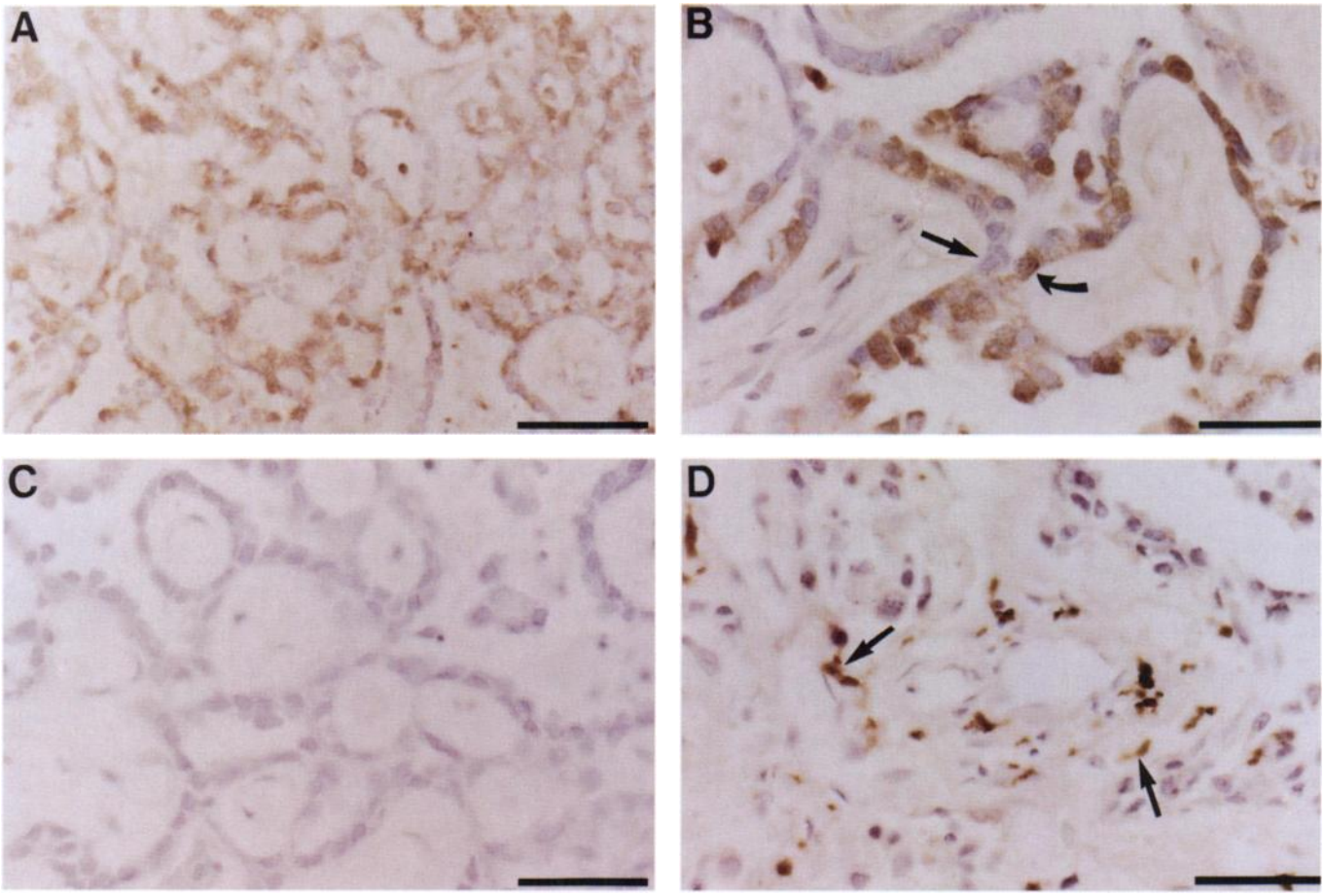

Fig. 1. Tissue from a poorly differentiated ovarian tumor with high NO synthase activity. (*A*) NO synthase immunoreactivity (brown stain) localized to the tumor cells throughout the section (*bar*, 150 μm); (*B*) NO synthase immunoreactive (*curved arrow*) and nonimmunoreactive (*straight arrow*) tumor cells adjacent to each other (*bar*, 40 μm); (*C*) no immunolabeling with preimmune serum (*bar*, 40 μm); (*D*) few cells in the stroma (*arrows*) immunoreactive with the anti-human macrophage marker (*bar*, 90 μm).

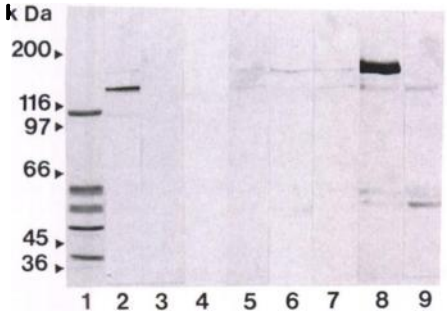

Fig. 2. Western blot of ADP-Sepharose eluates from malignant and nonmalignant tissue from cancer patients and from cervix from a non-cancer patient. *Lane 1*, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of malignant uterine tissue (case 8) stained with Coomassie brilliant blue. Rabbit antiserum diluted 1:100 and reacted with: *Lane 2*, malignant uterine tissue (case 8); *Lane 3*, nonmalignant peritoneal nodule (case 8); *Lane 4*, normal cervix; *Lanes 5–7*, malignant tissue from omental nodule, small bowel nodule, and ovary (case 6) respectively; *Lane 8*, control constitutive calcium-dependent NO synthase from rat brain; *Lane 9*, inducible calcium-independent NO synthase from macrophage J774 cell line.

immunoreactive bands were visible in Western blots of ADP eluates prepared from nonmalignant tissue (Fig. 2).

DISCUSSION

The results of the present study show that high levels of NO synthase activity are present in malignant tissue from gynecological cancers while the enzyme activity is below detectable levels in gynecological tissue from non-cancer patients. This suggests that high NO synthase activity is related to malignancy.

Furthermore, the immunohistochemical and Western blot studies with the NO synthase antiserum revealed immunoreactive proteins in the tissue samples in which NO synthase activity was detected. This immunoreactivity was localized to the tumor cells.

In Western blots the appearance of two protein bands (M_r ~160,000 and ~135,000), with molecular weights which correspond to the known NO synthase isoforms (13–16), suggests that tumor cells may be expressing more than one NO synthase. Alternatively, the lower molecular weight bands detected by this antiserum may correspond to degradation products of the expressed NO synthase (10).

Macrophage infiltration is a common feature of human solid tumors (17). While NO has been proposed to be an important effector molecule of macrophage tumoricidal activity (3), NO generation by cultured human macrophages has not been reproducible. We therefore also examined tumor tissue for the presence of macrophages and their relationship with the localization of NO synthase. The macrophage marker showed that there were few relative to tumor cells and they were predominantly confined to the stroma, where NO synthase immunoreactivity was not observed. Thus, it is unlikely that macrophages in these tumors provide a significant contribution to the detected NO synthase activity.

We found that the NO synthase activity in gynecological tumor tissue was calcium dependent. The classification of NO synthase isoforms has until now been based on biochemical criteria. Thus enzymes have been known as calcium dependent and constitutive or calcium independent and inducible (1). However, it is becoming clear that there are calcium-dependent isoforms which are inducible (18) and calcium-independent isoforms which are apparently constitutive (8). Thus, the present classification based on calcium dependence should be viewed with caution, and the nature of the NO synthase in tumor tissue as well as the role of NO generated remains to be studied in detail.

Nitric oxide is a cytotoxic and cytostatic molecule and at the same time is a very potent vasodilator. This molecule, therefore, may be playing a paradoxical role in tumor development as it does in other systems (19). What remains intriguing and might in the future be the basis for a prognostic diagnosis in some of these tumors is that there seems to be a correlation between the grade of malignancy and the activity of the NO synthase.

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