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Nitric Oxide Synthase Activity in Fresh Cells from Ovarian Tumour Tissue: Relationship of Enzyme Activity with Clinical Parameters of Patients with Ovarian Cancer

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ABSTRACT. Recent studies suggest a dual role for nitric oxide (NO) in tumour biology. High concentrations of NO can mediate tumouricidal activity, whereas lower concentrations have been shown to promote tumour growth. In this study, NO synthase (NOS) activity was investigated in cells that were prepared from tissue from primary and metastatic sites and from malignant effusions in 41 cases of suspected ovarian cancer. NO biosynthesis, determined by nitrite + nitrate (NOx) accumulation in medium from cultured cells prepared from disaggregated tumours or effusions and indicative of the inducible NO synthase isoform, was detected in 37% of the cases investigated (range 10.2–114 μM). There was a significant relationship between NOx and tumour differentiation ($P = 0.014$), with NOx being significantly higher for the more differentiated tumours. NOS activity, determined by the conversion of radiolabelled L-arginine to citrulline by tissue or cell extracts, was detected in 29% of cases (range 0.9–6.9 pmol/min per mg of protein), with all samples tested being moderately or poorly differentiated. Seventy percent of this activity was calcium dependent, indicative of constitutive NOS isoforms. Morphological and immunohistochemical assessment of tumour samples indicated a significant relationship between high macrophage content and NOS activity (as NOx biosynthesis) ($r_s = 0.726$, $N = 16$, $P < 0.01$). The relationship between NOS expression, immune response, and disease progression is complex and not simply dependent on the differentiation status of ovarian cancer. BIOCHEM PHARMACOL **56**;10:1365–1370, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. nitric oxide; cancer; ovarian; human; macrophage

It is a decade since NO§ was discovered as an endogenously generated molecule [1]. Extensive subsequent investigations have revealed a diversity of physiological roles for NO, which fall broadly into three categories: 1) an intracellular signal; 2) a transcellular messenger; or 3) a cytotoxic [2, 3]. Of the three isoforms of NOS, the enzyme responsible for the generation of NO, endothelial NOS and neuronal NOS are constitutive calcium-dependent isoforms producing short bursts of NO for signalling or messenger functions. The inducible NOS isoform (iNOS) is expressed in response to immunologic stimuli, is calcium-independent, and is capable of sustained release of NO, producing higher concentrations with potentially cytostatic/cytotoxic functions [3].

Studies show that NOS is present in human tumours where, in some cases, expression and localisation appears dependent on the type and differentiation status of the tumour [4–8]. Interestingly, the finding that NOS expression in invasive, human breast and gastric cancer tissue appears localised within intratumoural macrophages contrasts with the putative role of NO as an immune defence mechanism released by activated macrophages. The alternative role for NO, as an important signaling or messenger molecule in promoting tumour growth, has been supported by recent studies of the inhibitory effect of a selective iNOS inhibitor on growth of experimental tumour models that constitutively generate iNOS [9].

Our previous study of eight human gynaecological cancers showed a calcium-dependent NOS isoform localized in the tumour cells in poorly differentiated tumours [4]. Because of the small sample size, we were unable to assess whether NOS expression correlated with the differentiation and progression of ovarian cancer. We decided to investigate, in a larger number of human, ovarian cancer cases, the expression of NOS and its relationship with morphological and clinical parameters. Here we report our findings from tumour samples from different sites, type, and grades of 41 cases of suspected ovarian cancer.

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§Abbreviations: iNOS, inducible NOS; NOS, nitric oxide synthase; NOx, nitrite + nitrate.

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MATERIALS AND METHODS

Patients

Thirty-three biopsy samples and 19 ascitic, cyst or pleural fluids were received from 41 patients with ovarian tumours. Thirty-four patients were on first presentation; six patients had recurrent disease after previous cytoreduction and chemotherapy; and one patient was tested twice, on first presentation and on recurrence.

Clinical staging was determined at laparotomy using International Federation of Gynaecologists and Obstetricians (FIGO) guidelines. Histologic classification of the tumours was determined by the histopathologist on the Pembury Hospital site.

Sample Collection

At operation, a 2–3 cm^3 biopsy sample of tumour was collected aseptically into medium (RPMI 1640; Sigma-Aldrich) containing penicillin (100 IU/mI) and streptomycin (100 μg/mL). Up to 1 liter of malignant effusion was collected into a sterile container. All samples were received in the laboratory within 48 hr, and cells prepared immediately for morphological assessment and NO biosynthesis assay. If sufficient material was available, a 1 cm^3 piece of biopsy material and/or cell pellet from the effusion samples was snap frozen in liquid nitrogen and stored for biochemical analysis of NOS activity by the conversion of radiolabelled L-arginine to citrulline.

Cell Preparation

Cells were collected from the fluid samples by centrifugation, and biopsy samples were disaggregated, using crossed scalpels and repeat syringing. Contaminating red blood cells and necrotic cells were removed by density gradient centrifugation. A final suspension containing both single and small clumps of cells was prepared at approximately 1 × 106 cells/mL in culture medium (Dulbecco's modified Eagle's medium from Life Technologies) supplemented with 10% foetal calf serum, penicillin, and streptomycin as above).

Morphological Analysis of Cells

For immunohistochemistry and morphological assessment using May Grunwald Giemsa staining, several cytospin preparations were made of the final cell suspension in culture medium. Samples were assessed morphologically for tumour-cell content by scanning the slide for a representative field and expressing the results as a percentage of positive cells.

For immunohistochemical analysis of macrophage content, cytospin preparations were fixed in acetone/methanol (1:1) for 90 sec. Cytospins were then incubated for 30 min with a primary antibody, raised in mouse, against the human monocyte/macrophage marker CD68 (EBM/11; Dako). Antibody labelling was subsequently visualised using the Alkaline phosphatase anti-alkaline phosphatase (APAAP) technique. Samples were assessed for macrophage content and the results were expressed as a percentage the positive cells.

Determination of NO biosynthesis

Five milliliters of the cell suspension and 5 mL of the culture medium alone (as a control) were incubated for 48 h at 37° in an atmosphere of 95% air/5% carbon dioxide. After incubation, the cell suspension and culture medium control were centrifuged, the supernatants collected, and stored at −20°. The remaining cell pellets were snap frozen in liquid nitrogen for colorimetric determination of total protein by Coomassie Brilliant Blue assay (Bio-Rad), using bovine plasma albumin as a standard. Cells were also incubated as above with 500 μM of either the NO synthase inhibitor L-NMMA, N^G-monomethyl-L-arginine or L-NIO, N-iminoethyl-L-ornithine, and the supernatants collected and stored in the same way.

After the reduction of nitrate to nitrite using cadmium, the concentration of NOx in the supernatants was analysed by the Griess reaction [10]. NO biosynthesis was determined by the difference in NOx concentrations in the culture medium supernatants taken from cell cultures relative to those taken from culture medium alone.

Assay of the Conversion of Radiolabelled L-Arginine to Citrulline

Frozen tissues and cell pellets were thawed in ice-cold 20 mM HEPES buffer (pH 7.2) containing 200 mM sucrose, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), 10 μg/mL of leupeptin, 10 μg/mL of soyabean trypsin inhibitor, and 1 μg/mL of pepstatin A and homogenised (with an Ystral homogeniser) on ice. The homogenates were centrifuged at 10,000 *g* at 2° for 30 min. To remove endogenous arginine, the supernatants were treated with equal volumes of cation-exchange resin (Dowex-50W, sodium form). The NO synthase activity in the supernatants (cytosol + microsomes) was measured by the conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline at 37° for 10 min in 20 mM HEPES buffer (pH 7.2) containing 10 μM tetrahydrobiopterin, 2.5 mM DTT, 400 U/mL of calmodulin, 250 μM $CaCl_2$, 0.5 mg/mL of BSA, 125 μM NADPH, 10 μM arginine, 100 μM citrulline, 60 mM valine (to inhibit arginase), and 0.33 μCi/mL of L-[U-^{14}C]arginine as described previously by Salter *et al.* [11]. The total NO synthase activity was determined from the difference between the [U-^{14}C]citrulline generated in the control samples and samples containing 1 mM L-NMMA; the activity of the calcium-dependent NOS was determined from the difference between the control samples and samples containing 1 mM EGTA; and the activity of calcium-independent NO synthase (indicative of the inducible NO synthase) was determined from the difference between samples containing 1 mM EGTA

TABLE 1. Histological classification of ovarian tumours

Classification	No. of cases
Benign	3
Borderline (epithelial carcinoma of low malignant potential)	2
Malignant	36
Germ cell tumour-dermoid cytst with malignant transformation	1
Epithelial adenocarcinoma	35
Subtype	
Epithelial tumours (malignant + borderline)	37
Serous	13
Mucinous	6
Endometrioid	3
Clear cell	1
Undefined	14
Differentiation	
Malignant tumours	36
Well differentiated	2
Moderately differentiated	12
Poorly differentiated	20
Undefined	2

and those with 1 mM L-NMMA. Total protein content of the tissue supernatants was determined as above (Bio-Rad). The limit of detection in this assay was 0.5 pmol/min per mg of protein.

Statistics

Where results are expressed as mean values, they include SEM. Nonparametric methods were used for statistical evaluation. Spearman's rank correlation coefficient was used to compare the percentage of macrophages with NOx. Fisher's Exact test was used for the contingency table analysis, and the Mann–Whitney *U*-test was used to compare NOx between different groups of patients.

RESULTS

Patients

Of the 41 patients with suspected ovarian cancer, 3 were subsequently found to have benign tumours, 26 had advanced disease (FIGO stage III–IV), 6 were FIGO stage I–II, and for 6 patients, data for clinical staging was not available. Table 1 itemises the histologic classification of these tumours.

NO Biosynthesis

After culture for 48 hr, cells from 14 of the 38 patients (37%) who were tested for NO biosynthesis generated NOx (median 21.2 μM; range 10.2–114.2; Table 2). The addition of NOS inhibitors, L-NMMA or L-NIO, at 500 μM inhibited NO biosynthesis by >62% in all cultures.

Conversion of Radiolabelled L-Arginine to Citrulline

NOS activity, determined by the conversion of radiolabelled L-arginine to citrulline, was detected in tumour samples from 5 of 17 patients (29%) investigated (median 4.9 pmol/min per mg of protein; range 0.9–6.9). Calcium-dependent activity contributed the majority of activity

TABLE 2. Samples in which NO biosynthesis and/or conversion of L-arginine to citrulline was detected

Case	Type	Site	Differentiation[a]	NOS (pmol/min per mg prot.) Ca^{2+} −dep.	NOS (pmol/min per mg prot.) Ca^{2+} −indep	NO_x (μM)
1682	Serous	Ovary	M	—[b]	—	11.8
1721	Mucinous	Ovary	N/A	—	—	16.4
1722	Mucinous	Metastasis	W	—	—	46.8
1733	n/c[c]	Ascites	M	—	—	18.5
1733	n/c	Metastasis	M	—	—	14.3
1734	Dermoid	Ascites	M	—	—	40.4
1734	Dermoid	Ovary	M	—	—	23.9
1749	Serous	Pleural Effusion	P	—	—	33.5
1794	n/c	Ascites	P	—	—	18.1
1796	Mucinous	Ovary	N/A	—	—	114.2
1872	Serous	Ovary	P	0.7	<0.5	<10
1877	Serous	Ascites	P	<0.5	<0.5	10.6
1883	n/c	Ovary	M	4.5	2.2	23.9
1902	n/c	Ovary	P	3.5	1.4	10.2
1903	n/c	Ovary	P	1.2	0.5	<10
1927	Mucinous	Ascites	W	—	—	54.3
1928	Endometrioid	Ovary	P	—	—	10.4
1930	Clear cell	Ascites	M	—	—	26
1964	Serous	Ascites	M	4.8	2.1	—

[a]M = moderate; N/A = not applicable; W = well; P = poor
[b]not done.
[c]not classified.

TABLE 3. Association between NOS activity and tumour differentiation

Tumour histology	NOx (μM) No. tested	No. positive	%	Median of detectable values (range)	Total NOS (pmol/min per mg protein) No. tested	No. positive	%	Median (range)
Benign	2	0	0	—	1	0	0	—
Borderline	2	2	100	65.3 (16.4–114.2)	0	—	—	—
W[a]	2	2	100	50.6 (46.8–54.3)	—	—	—	
M	9	5	55.6	23.9 (11.8–40.4)	6	2	33.3	6.8 (6.7–6.9)
P	21	5	23.8	10.4 (10.2–33.5)	10	3	33.3	1.7 (0.9–4.9)
U	2	0	0	0	—	—	—	—
Total	38	14	36.8	21.2 (21.2–114.2)	17	5	29.4	4.9 (0.9–6.9)

[a]Differentiation status of malignant tumours: W = well; M = moderate; P = poor; U = undefined.

and was detected in all of these cases, while calcium–independent activity was detected in four of the five cases (Table 2).

Cellular Analyses of Samples

Number of tumour cells was assessed on samples from 35 of the 41 patients. As determined by May Grunwald Giemsa staining, there was a variation in the numbers of tumour cells in the final cell preparation. The mean tumour cell percentage was 31% ± 4.5 (range 0–90).

The majority of the remaining cells in the final preparations consisted of macrophages, mesothelial cells, and lymphocytes. Immunocytochemistry, using the EBM/11 antibody against CD68, showed the mean percentage of macrophages in these samples was 38% ± 3.4 (range 2–90). There was a significant correlation between the percentage of macrophages and the detectable level of NOx (r_s = 0.726, N = 16, $P < 0.01$).

Ascitic Compared with Biospy Samples

In 10 cases, ascitic fluid and a solid biopsy from the same patient were tested for NO biosynthesis or the conversion of radiolabelled L-arginine to citrulline. The results were similar in 9 of the 10 comparisons. For seven of these cases, NO biosynthesis and the conversion of radiolabelled L-arginine to citrulline was below the level of detection. Two cases (case numbers 1733 and 1734; Table 2) gave similar levels of NO biosynthesis. In the remaining case (1877), cells from the ascitic fluid generated a NOx concentration of 10.6 μM, just above the level of detection, whereas cells from the biopsy did not generate NOx. Interestingly, cells from either of these samples did not convert radiolabelled L-arginine to citrulline.

Correlation of NOS Activity and Morphology with Clinical Parameters

NOS was not detected, by either NO biosynthesis or conversion of radiolabelled Larginine to citrulline, in cells from tumours that were subsequently histologically judged to be benign. Samples from patients with well differentiated tumours and from those with borderline malignancies all had detectable levels of NOx when compared with 5 of the 9 cases with moderately and 5 of the 21 cases of poorly differentiated tumours (Table 3). Indeed, when patients were grouped as borderline malignant, well, or moderately differentiated, versus poorly differentiated there was a significant association between NOx generated from malignant samples and tumour differentiation status (Fisher's Exact test, $P = 0.014$). Moreover, the level of NOx was significantly higher in the more differentiated group of patients, with an overall median value of 18.5 μM, compared with <10 μM in the group of patients with poorly differentiated tumours (Mann–Whitney *U*-test, $U = 59$, $U' = 214$, $P < 0.02$)

As none of the samples from patients with borderline or well-differentiated tumours were available for testing for the conversion of radiolabelled L-arginine to citrulline, all the samples in which this was detected were from patients with moderately or poorly differentiated tumours.

Data for both clinical staging and for NO synthase activity were available for 15 patients. Two of these patients had early FIGO stage I–II disease, and both had detectable levels of NO synthase activity by the conversion of L-arginine to citrulline. This compared with only 1 of 13 patients with stage III–IV disease (Fisher's exact test, $P = 0.029$). However, there was no significant relationship between the generation of NOx and clinical staging, with detectable NOx in samples from 3 of the 6 FIGO stage I–II patients and 6 of 23 FIGO stage III–IV patients (Fisher's Exact test, $P = 0.339$).

DISCUSSION

In this investigation of 41 cases of suspected ovarian cancer, we found NOS activity present in 17 of the 38 malignant cases, but not in the 3 that were benign. This is consistent with previous reports of NOS activity only in the malignant tissue of gynaecological, breast, and central nervous system cancers [4–6].

Two different biochemical methods have been used to detect NOS enzyme. The conversion of radiolabelled L-arginine to citrulline distinguishes calcium-dependent constitutive isoforms from the calcium-independent inducible isoform. For NO biosynthesis in cultures of cells, the oxidation products of NO (i.e. NOx) have been measured as the end point for the estimation of NOS activity. NOx indicates iNOS activity because this isoform generates a sustained release of NO without the need for a calcium influx into the cell. We have found a significant correlation between iNOS activity and differentiation status, with the highest activity in the well differentiated and borderline malignancies. Limitations of sample availability prevented a similar comparison being made for calcium-dependent constitutive NOS isoforms, although we did show calcium-dependent NOS activity in moderately and poorly differentiated tumour tissue, as has previously been reported [4].

This is the first report of iNOS activity in human ovarian cancer. In our previous study in which a limited number of moderately or poorly differentiated tumours were studied, we were unable to detect iNOS activity. There is evidence for high arginine concentrations in human and murine tumours [9], which, without removal, will compete for radiolabelled arginine in the assay for conversion of L-arginine to citrulline. Here, using a more sensitive method in which the final tissue or cell supernatant was depleted of endogenous arginine before assay, we have detected low level activity of iNOS in the moderately and poorly differentiated tumour. The second assay for iNOS activity, NO biosynthesis by viable cell culture, complements and supports these findings.

The induction of iNOS requires immunomodulatory molecules including cytokines, such as tumor necrosis factor [3]. Indeed, this cytokine has been shown to be expressed in both human ovarian and breast cancer tissue [12–14]. The present study suggests that the intratumoural milieu in borderline and well-differentiated ovarian tumours provides a suitable environment of immunomodulatory molecules for induction of inducible NO synthase. This is in contrast with breast cancer, in which iNOS activity was highest in poorly differentiated tumours. These contrasting observations highlight our lack of understanding of the subtle, regulatory signals for the expression of immunomodulatory cytokines, such as tumor necrosis factor, and inflammatory mediators, such as NO, in the intratumoural environment of solid tumours.

Inducible NOS is normally associated with immune effector cells, such as macrophages, which can be induced to express the enzyme as part of their cytotoxic repertoire. Indeed, using immunohistochemical methods on tissue sections of human breast cancer samples, intratumoural macrophages have previously been shown to express iNOS [5]. Here we report a correlation between macrophage numbers and NO biosynthesis. It is possible that the iNOS detected here is expressed within these host immune cells in ovarian tumour tissue. However, iNOS has also been shown in tumour cell lines [15–18]. Thus, to clarify the cellular localisation of this isoform of NOS in human ovarian cancer tissue, further immunohistochemical studies with whole tissues are required.

At the levels reported in this study, iNOS has been suggested to play an important role in cancer progression. There is increasing evidence for a role for NO in neovascularisation [19, 20]. This angiogenic process is an absolute requirement for the sustained growth of solid tumours, and its extent correlates positively with tumour metastatic potential [21–23]. Here we report expression of iNOS predominantly in borderline or well-differentiated ovarian tumours, which have a better prognosis. In contrast to breast cancer, where it has been suggested that NO may be an important factor in promoting progression of high-grade tumours [5], iNOS may play a significant role in ovarian cancer in the progression of early disease. If this is the case, the relationship between NOS expression, immune response, and disease progression is complex and not simply dependent on the differentiation status of cancer.

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