

Expression of Cyclooxygenase-2 and Inducible Nitric Oxide Synthase in Human Ovarian Tumors and Tumor-associated Macrophages¹

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ABSTRACT

This study investigates whether and to what extent cyclooxygenase type-2 (COX-2) and inducible nitric oxide-synthase (iNOS), both known to have an immunosuppressive effect, are expressed in human ovarian tumors. Because COX-2 and iNOS can be expressed by activated macrophages, the presence of tumor-associated macrophages and the expression of COX-2 and iNOS by these tumor-associated macrophages were determined. The results obtained may provide insight into the function of COX-2 and iNOS expression by tumors. The expression of COX-2 and iNOS in tumor cells and macrophages was assessed in 18 malignant, 15 borderline, and 14 benign human ovarian tumors by immunohistochemical staining of frozen tissue sections. The intra- and peritumoral macrophages were stained using an anti-CD68 monoclonal antibody. Most of the malignant tumors (15 of 18), 10 of 15 borderline, and 9 of 14 benign tumors showed COX-2 expression in the epithelial cells, a result which indicates that COX-2 expression is not exclusive to malignancy. In addition, COX-2 staining was more intense in the epithelial cells of benign and borderline tumors than in malignant tumors. Weak iNOS staining was observed in 5 of 18 malignant, 4 of 15 borderline, and 5 of 14 benign tumors. The number of tumor-associated macrophages varied widely between the different tumors. The highest number of tumor-associated macrophages ($\geq 20/0.125 \text{ mm}^2$) was observed in malignant tumors, whereas low to moderate intra- and peritumoral macrophage infiltration ($5\text{--}20/0.125 \text{ mm}^2$) was observed in the borderline and benign tumors. COX-2-positive tumor-associated macrophages were found in 3 of 18 malignant tumors, 7 of 15 borderline tumors, and 1 of 14 benign tumors. The number of COX-2-positive tumor-associated macrophages ranged from 3 to 30% of the total macrophage population. Some malignant (4 of 18), borderline (5 of 15), and benign (2 of 14) tumors contained iNOS-positive macrophages. Notable was that COX-2- and iNOS-positive macrophages were predominantly located in the tumor stroma, the regions between tumor and stroma, and in the lumina of the tumor when located in the tumor tissue. These data indicate that not only malignant but also borderline and benign ovarian tumors can exhibit increased levels of COX-2 and iNOS expression. In addition, a small proportion of the tumor-associated macrophages found in malignant, borderline, and benign tumors seems to be in an activated state, judged by their iNOS and COX-2 expression. This subpopulation of tumor-associated macrophages was invariably located in the tumor stroma or in the lumina of the tumor, specifically suggesting that macrophages outside the tumor can be tumor cytotoxic.

INTRODUCTION

The enzyme prostaglandin endoperoxide H synthase type 2 (COX-2)³ is usually expressed in gastrointestinal cancers (1), but also in other types of cancer (2–5). Cyclooxygenases are enzymes that are necessary for converting arachidonic acid into prostaglandin endoper-

oxide (prostaglandin H₂), which exhibits immunosuppressive activity. Two types of cyclooxygenases have been described (reviewed in Ref. 6). COX-1 is constitutively expressed in most mammalian cell types. COX-2 expression is induced in fibroblasts, endothelial cells, monocytes, and some tumor types in response to growth factors, tumor promoters, hormones, endotoxins, or cytokines. Because of the immunosuppressive activity of prostaglandins, COX-2 expression in tumor cells might be a mechanism by which tumors manage to suppress the activation of tumor-associated macrophages (7). This hypothesis is supported by studies where the use of nonsteroidal anti-inflammatory drugs such as aspirin, sulindac, ibuprofen, and meclofenamate are shown to inhibit cyclooxygenases (8, 9) or to inhibit or even prevent tumor growth (10–14).

The expression in tumors of inducible iNOS, the enzyme responsible for the generation of nitric oxide, has also been reported (15–20). Nitric oxide seems, as does PGE₂, to be associated with tumor-related immune suppression when produced in relatively low amounts by tumor cells (21–23). However, when it is secreted in high amounts by either tumor cells or activated macrophages, nitric oxide has been shown to be an important mediator in tumor-cell killing (23–28).

Monocytes recruited into the tumor from the peripheral blood in response to chemotactic cytokines secreted by the tumor (reviewed in Ref. 29) differentiate into tumor-associated macrophages. Activation of these macrophages may result in the secretion of mediators such as nitric oxide and tumor necrosis factor, both of which are capable of lysing tumor cells *in vitro*. Tumors, however, still progress despite a major influx of macrophages with intrinsic antitumor activity that ultimately can represent as much as 50% of the total tumor mass (30, 31). Apparently, the effects of these tumor-associated macrophages and mediators on tumor growth are complex and not yet fully unraveled. Note that we also consider macrophages in nonmalignant tumors to be tumor-associated macrophages.

In this study, the expression of COX-2 and iNOS was immunohistochemically determined in malignant, borderline, and benign human ovarian tumors. In addition, the number of tumor-associated macrophages present in the tumors and the expression of iNOS and COX-2 by these macrophages were semiquantitatively determined.

MATERIALS AND METHODS

Tissue Samples. Tissue samples of 18 adenocarcinomas of the ovary, 15 borderline ovarian tumors, and 14 benign ovarian tumors (cystadenoma) were randomly retrieved from the tissue bank of the Department of Gynecology (University Hospital of Groningen, The Netherlands). At the time of surgery, tumor samples were divided into two groups according to standard procedure. One group was fixed in 8% formalin, embedded in paraffin, and used for standard H&E staining; the other was stored at -180°C .

Immunohistochemistry. Frozen tissue sections cut into $4\text{-}\mu\text{m}$ slices were fixed in acetone. Immunostaining was performed using a goat polyclonal IgG specific for COX-2 (dilution, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA), a mouse monoclonal IgG specific for anti-iNOS (dilution, 1:100; Transduction Laboratories, Lexington, KY), or a mouse monoclonal IgG specific for the CD68 macrophage marker (dilution, 1:50; DAKO, Glostrup, Denmark). The slides were incubated at room temperature for 30 min, and then incubated with a secondary rabbit antimouse or rabbit antigoat antibody conjugated with peroxidase (DAKO).

Received 7/21/00; accepted 7/18/01.

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¹ This work was supported by Grants 94-766 and 98-1660 from the Dutch Cancer Society.

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³ The abbreviations used are: COX, cyclooxygenase; iNOS, inducible nitric oxide-synthase; PGE₂, prostaglandin E₂.

To intensify the staining reaction, the sections were also incubated with a tertiary goat antirabbit peroxidase-conjugated antibody (DAKO). Finally, the antibody-binding sites were visualized by incubating the slides in a 3-amino-9-ethyl-carbazole solution (Sigma Chemical Co., St. Louis, MO), the substrate for peroxidase. The sections were counterstained with H&E staining.

The staining intensity of the areas of COX-2- and iNOS-positive tumors were scored on a scale from 0 (no staining) to 4 (very high staining intensity). Quantification of CD68-positive cells was determined on the basis of at least 10 representative microscope fields of 0.125 mm² (×500). Slides were blindly evaluated twice at different times by two investigators (H. H. and A. H. K.). An adenocarcinoma of the colon known to be positive for COX-2, iNOS and CD68 was used as a positive control. A COX-2-positive sample in which the primary antibody was omitted during the staining procedure served as a negative control.

RESULTS

Tumor Material. Table 1 presents the tumor type and differentiation grade (based on H&E staining) of the analyzed ovarian tumors. All tumors (18 malignant, 15 borderline, and 14 benign) were of epithelial origin.

COX-2 and iNOS Expression in Epithelial Cells of Ovarian Tumors. Sections of all tumors were immunohistochemically analyzed for the expression of COX-2 and iNOS in ovarian epithelial cells. A majority of malignant, borderline, and benign tumors showed COX-2 expression localized in the ovarian epithelial cells (83, 67, and 64% respectively). COX-2 expression in COX-2-positive malignant tumors was moderate (Fig. 1A). Notable was both the high expression of COX-2 in ovarian epithelial cells in 2 of 15 borderline and in 2 of 14 benign tumors (Figs. 1A and 2) as well as that the 2 benign tumors showing strong COX-2 expression in the epithelial cells also showed a more complex pattern of epithelial invaginations as compared with the other benign tumors.

Although most tumors were positive for COX-2 expression in the ovarian epithelial cells, only 28% of the malignant, 27% of the borderline and 36% of the benign tumors showed low staining intensity of the epithelial cells with the anti-iNOS monoclonal antibody (Fig. 1B).

Tumor-associated Macrophages in Ovarian Tumors. Peri- and intratumoral macrophages were visualized using a monoclonal anti-

Table 1 Tumor characteristics and the percentages COX-2 and iNOS expressing intra- and peritumoral macrophages

Tumor type	Grade ^a	COX-2-positive macrophages		iNOS-positive macrophages	
		Intratumoral	Peritumoral	Intratumoral	Peritumoral
Malignant tumors					
Adenocarcinoma	M	— ^b	—	—	—
Adenocarcinoma	M	—	9% ^c	—	—
Adenocarcinoma	M	—	—	9%	—
Adenocarcinoma	M	—	—	—	—
Adenocarcinoma	M	—	14%	—	7%
Serous adenocarcinoma	P	—	—	—	—
Serous adenocarcinoma	P	—	—	—	—
Serous adenocarcinoma	P	—	—	—	—
Serous cystadenocarcinoma	M	—	—	—	—
Serous cystadenocarcinoma	M	28%	20%	—	—
Serous cystadenocarcinoma	M	—	—	—	—
Serous cystadenocarcinoma	M	—	—	—	21%
Serous cystadenocarcinoma	M	—	—	21%	19%
Serous cystadenocarcinoma	P	—	—	—	—
Mucinous cystadenocarcinoma	W	—	—	—	—
Mucinous cystadenocarcinoma	M	—	—	—	—
Mucinous cystadenocarcinoma	W	—	—	—	—
Mucinous cystadenocarcinoma	W	—	—	—	—
Borderline tumors					
Mucinous		—	—	—	—
Mucinous		—	—	—	—
Serous		—	—	—	—
Serous		30%	3%	—	—
Serous		12%	—	—	—
Borderline		—	—	—	—
Mucinous		—	3%	—	5%
Mucinous		—	—	—	—
Mucinous		—	—	—	—
Serous		—	5%	—	14%
Serous		—	5%	—	23%
Mucinous		—	14%	—	—
Mucinous		—	—	—	8%
Mucinous		—	—	—	5%
Serous		9%	—	—	—
Benign tumors					
Mucinous		—	—	—	—
Mucinous		—	—	—	—
Mucinous		—	—	—	—
Mucinous		—	—	—	—
Serous		—	—	—	—
Adenoom		—	—	—	—
Adenoom		—	—	—	—
Serous		—	—	—	—
Serous		—	—	—	—
Serous		—	—	—	7%
Mucinous		—	2%	—	—
Mucinous		—	—	—	—
Serous		—	—	—	4%
Serous		—	—	—	—

^a Tumors were graded according to the degree of differentiation in tissue sections. W, well differentiated; M, moderately differentiated; P, poorly differentiated.

^b —, no COX-2- or iNOS-positive macrophages present.

^c Percentages COX-2- or iNOS-positive tumor-associated macrophages.

Fig. 1. COX-2 (A) and iNOS (B) expression by epithelial cells in ovarian tumor tissue. Sections of malignant, borderline, and benign tumors were immunohistochemically stained with an anti-COX-2 polyclonal antibody (A) or an anti-iNOS monoclonal antibody (B). The data represented here are the levels of staining intensity varying from 0 (no staining) to 4 (very high-intensive staining) in malignant, borderline, and benign tumors.

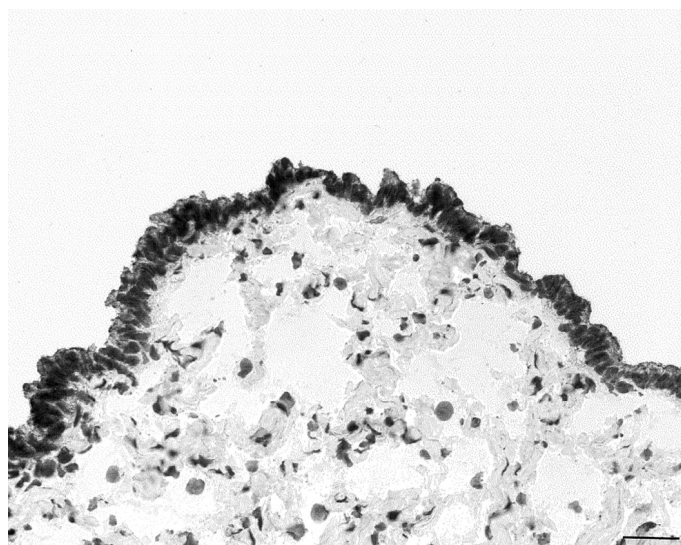
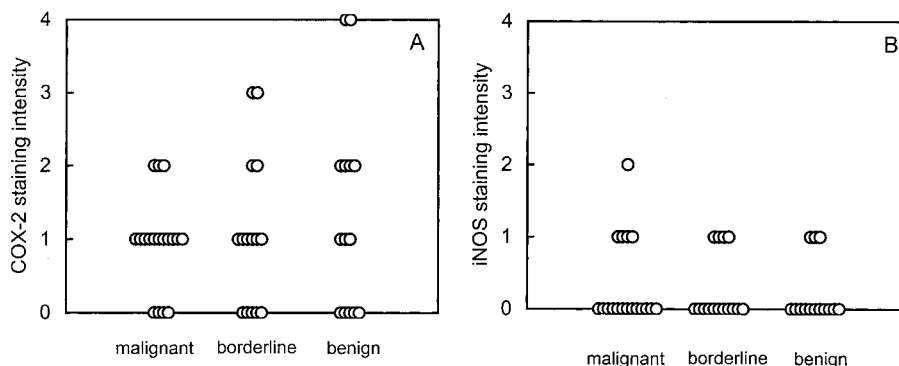


Fig. 2. COX-2 expressed by epithelial cells is present in benign tissue. A benign cyst shows high-intensive staining of the epithelial cell surface surrounding the ovary, whereas stroma is negative for COX-2 staining. Bar, 25 μ m.

DISCUSSION

Our results show that COX-2 expression is not limited to gastrointestinal tract cancer, breast cancer, and lung cancer, but that it can also be found in ovarian tumors. Our results also demonstrate COX-2 expression, normally not constitutively expressed in cells, to be manifest in ovarian epithelial cells of most borderline and benign ovarian tumors. This shows that COX-2 expression is not necessarily correlated to malignancy. Wilson *et al.* (15) described increased COX-2 expression in Barrett's esophagus, demonstrating that COX-2 can also be expressed under premalignant conditions. COX-2 activity in epithelial cells of benign tumors might not necessarily be associated with malignancy but rather with the proliferation of cells, as is suggested by the high epithelial stroma index of some COX-2-positive benign tumors in our study.

The observation that several cancer types express COX-2 could point to a role for COX-2 in tumor progression or carcinogenesis. COX-2 expression might, for example, influence the tumoricidal capacity of tumor-associated macrophages. Several studies have shown that the inhibition of COX-2 by nonsteroidal anti-inflammatory drugs inhibits tumor growth (10, 11, 13, 14, 32). By inhibiting COX-2 expression, which is necessary for the production of PGE₂, nonsteroidal anti-inflammatory drugs will also inhibit PGE₂ production. As a result, PGE₂-induced deactivation of activated macrophages will be

body against CD68 (Fig. 3). As shown in Fig. 4, A and B, 78 and 94% of the malignant tumors, respectively, contained moderate (5–20 macrophages/0.125 mm²) to large numbers (>20 macrophages/0.125 mm²) of intra- and peritumoral macrophages. Intra- and peritumoral macrophages were also found in borderline (40 and 100%, respectively) and benign tumors (43 and 86%, respectively), albeit to a lower extent (Fig. 4). The numbers of peritumoral macrophages was always equal to or greater than the number of intratumoral macrophages.

COX-2 and iNOS Expression in Tumor-associated Macrophages. Because both COX-2 and iNOS are expressed in activated macrophages, we investigated whether macrophages expressing these inducible enzymes were present in the tumors. Tumor-associated macrophages were COX-2-positive in 17% of the malignant, 47% of the borderline, and 7% of the benign tumors (Table 1). COX-2-positive macrophages were predominantly localized in the stroma of the tumor or in the open spaces (lumina) surrounded by tumor cells (Fig. 5). The numbers of COX-2-positive macrophages ranged from 3 to 30% of the total CD68-positive macrophage population (Table 1).

iNOS positive macrophages were observed in 22% of the malignant, 33% of the borderline, and 14% of the benign tumors. Similar to COX-2-positive tumor-associated macrophages, the iNOS-positive tumor-associated macrophages were predominantly found in the stroma or lumen of the tumors.

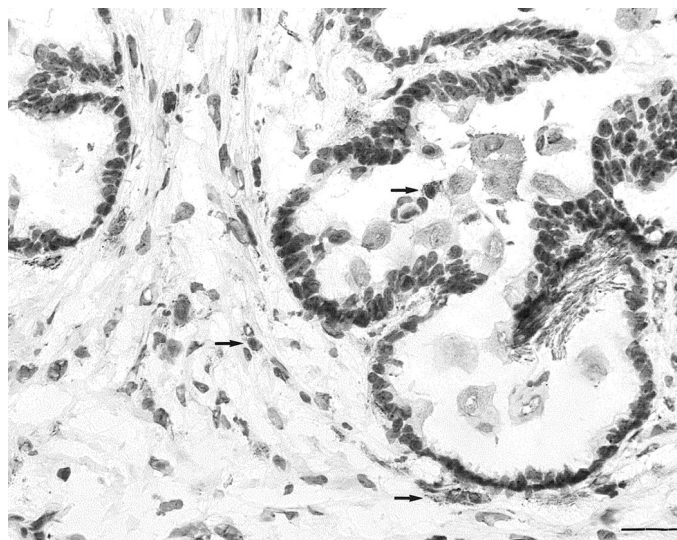
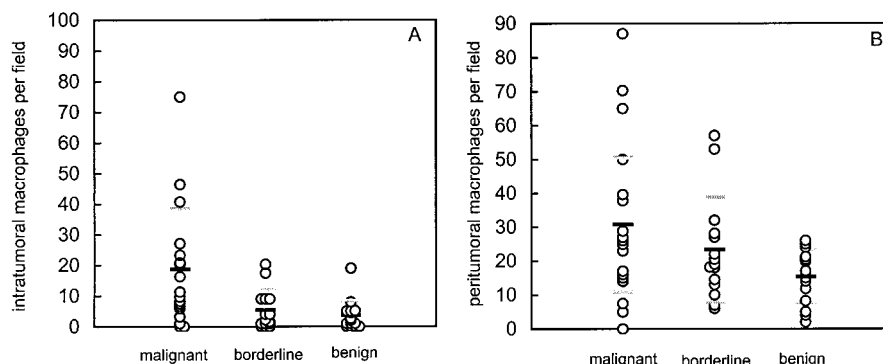


Fig. 3. Tumor-associated macrophages in and around tumor tissue. Macrophages (arrows) were visualized by staining with an anti-CD68 monoclonal antibody. Shown is that the number of peritumoral macrophages is higher than the number of intratumoral macrophages. Most intratumoral macrophages are present in the lumen of the tumor. Bar, 25 μ m.

Fig. 4. The number of intra- (A) and peritumoral (B) macrophages in ovarian tumors. Sections of malignant, borderline, and benign tumors were immunohistochemically stained with an anti-CD68 monoclonal antibody. Expressed are the numbers of tumor-associated macrophages in the tumor itself (A) or in the surrounding stromal tissue (B) of at least 10 representative microscope fields of 0.125 mm² each. The mean values (—) and SDs (—) are also indicated.



suppressed, resulting in a continuously activated tumor-associated macrophage population.

Alternatively, COX-2 expression might protect tumor cells against nitric oxide-mediated apoptosis. Although COX-2 mediated protection against nitric oxide has not been described in tumor cells, elevated COX-2 expression has been proposed as a mechanism to prevent nitric oxide-mediated apoptosis in macrophages that release nitric oxide (33). In addition, it was demonstrated that macrophages with increased COX-2 expression are protected against exogenously supplied nitric oxide (33). This protection was reversed by the addition of the specific COX-2 inhibitor NS-398 or by transfection of a murine peritoneal macrophage cell line (RAW cells) with an antisense COX-2 expression vector.

Similar to COX-2, iNOS expression has been reported in tumors (15–17, 19, 20). Nitric oxide, synthesized by iNOS, is a mediator involved in different processes varying from neurotransmission, to affecting the vasculature, to the killing of tumor cells or microorganisms (34). Thus, the role of nitric oxide in tumor development is ambiguous. When produced by tumor cells, nitric oxide may induce vasodilatation resulting in enhanced permeability in tumor vasculature. In such a case, the blood flow in the tumor will be increased, which could provide a favorable condition for the tumor. On the other hand, when nitric oxide is produced by macrophages, the nitric oxide radical can be converted into peroxynitrites, which are highly cytotoxic. We observed only a weak iNOS expression in the epithelial cells of 5 of 18 malignant ovarian tumors and in 4 of 15 borderline and 5 of 14 benign ovarian tumors. We did not observe a correlation

between the staining intensity with anti-iNOS and the grade of malignancy in the tumors. Thomsen *et al.* (18) also reported iNOS expression in human ovarian tumors. In their study, a positive correlation was observed between the degree of differentiation of the tumors analyzed and the amount of iNOS expression measured quantitatively by performing an *in vitro* enzyme assay.

In the present study, the low to moderate number of tumor-associated macrophages observed in benign and borderline tumors and the moderate to high number of tumor-associated macrophages infiltrating in and around malignant tumors suggest a correlation between the grade of tumor malignancy and the level of recruitment of tumor-associated macrophages. Tumor-associated macrophages express COX-2 and iNOS in some malignant, borderline, and benign tumors. The number of COX-2 or iNOS expressing macrophages in the tumors was always <30% of the tumor-associated macrophages, suggesting the existence of macrophage subpopulations. Interestingly, most positively stained macrophages were present in the stroma surrounding the tumor or in the tumor lumen and not within the tumor itself. This observation, definitely not the result of staining artifacts, inasmuch as the results were compared with negatively stained control slides to specifically prevent such artifacts, may indicate that the tumor effectively suppresses expression of COX-2 and iNOS in the tumor-associated macrophages. Diminished iNOS expression in macrophages, resulting in low nitric oxide production, was also reported by Dinapoli *et al.* (35). They demonstrated, in a mouse model, that progressively growing mammary tumors can suppress the cytolytic activity of host macrophages as a consequence of reduced iNOS expression.

To our knowledge, this is the first study in which COX-2 and iNOS expression were investigated in adenocarcinomas, borderline malignancies, and cystadenomas of the ovary, as well as in tumor-associated macrophages. The results obtained in the present study show that COX-2 and iNOS expression is not limited to malignant disease but can also be expressed in premalignant and benign conditions. Furthermore, based on the results obtained in our study, we hypothesize that tumors can release mediators that can suppress the tumoricidal capacity of tumor-associated macrophages. This hypothesis is endorsed by the observation that COX-2- and iNOS-positive macrophages were predominantly present in the tumor stroma or lumen within the tumor tissue.

ACKNOWLEDGMENTS

We thank Mindert Krans of the Department of Gynecology, University Hospital of Groningen, Groningen, the Netherlands, for his technical assistance.

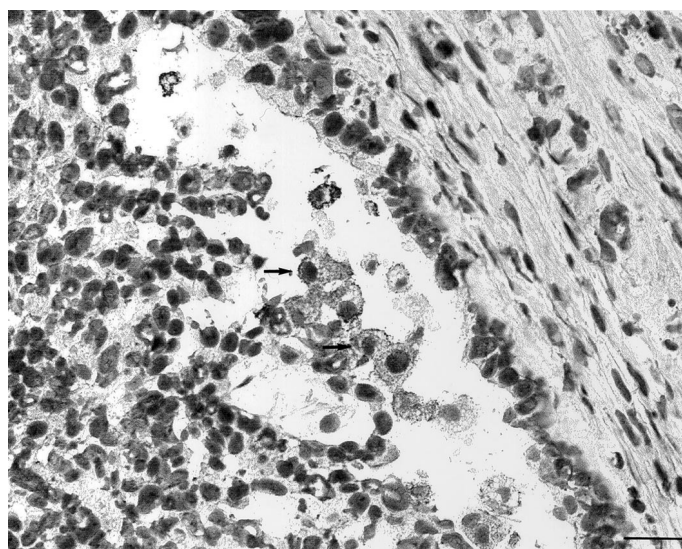


Fig. 5. COX-2-positive macrophages in a malignant tumor. Arrows, COX-positive macrophages in the lumen between tumor tissue and stroma. Bar, 25 μm.

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