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Increased expression of cyclooxygenase-2 and nitric oxide synthase-2 in human prostate cancer

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Abstract Cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase-2 (NOS-2) each have an important role in angiogenesis. The expression of these genes was investigated in human prostate cancer by immunohistochemistry, the expression of COX-1 and COX-2 being confirmed by mRNA analysis. Prostate cancer specimens from 12 patients were compared to control prostates from 13 patients operated on for bladder carcinoma. The intensity of COX-2 and NOS-2 immunostaining was significantly stronger in prostate cancer cells than in the non-malignant glandular epithelium of the control prostates. COX-2 and NOS-2 were clearly also expressed in the lesions of prostatic intraepithelial neoplasia (PIN) in control prostates. COX-2 was detected in the muscle fibres of the hyperplastic stroma of some control prostates. No significant difference was detected in COX-1 expression between control and cancer prostates. These results indicate that the expression of COX-2 and NOS-2 is elevated in prostatic adenocarcinoma and in PIN.

Key words Prostate cancer · Prostatic intraepithelial neoplasia · Cyclooxygenase-1 · Cyclooxygenase-2 · Nitric oxide synthase-2

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Introduction

The initial step in the formation of prostaglandins and thromboxanes is catalysed by cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes [23]. COX-1 is constitutively expressed, whereas the low basal activity of COX-2 can readily be stimulated by growth factors and is increased, for example, in colon, gastric, lung and bladder cancers [11, 15, 16, 24]. The increased activity of COX-2 and enhanced formation of prostaglandin E_2 are associated with the stimulated growth of normal and transformed cells [14, 20].

Nitric oxide (NO) is formed from L-arginine by nitric oxide synthase (NOS), which is known to exist in three isoforms. NOS-2 is the inducible isoform; NOS-1 is expressed in neural tissues and NOS-3 in vascular endothelium [6]. NOS-2 is stimulated in head and neck cancers [3]. NO regulates angiogenesis, which is a necessity for the development and continuous growth of tumours [3, 4]. Both cyclooxygenases also seem to have a role in angiogenesis, since COX-1 enhances the angiogenic potential of endothelial cells and the overexpression of COX-2 in colon cancer cells promotes angiogenesis [21]. We, therefore, investigated the expression of COX-1, COX-2 and NOS-2 in human prostatic adenocarcinoma.

Materials and methods

Tissue specimens

Specimens of human prostatic carcinoma were obtained from 12 patients (aged 58–71 years, mean 63 years) undergoing radical prostatectomy. Three carcinomas were of grade I, eight were of grade II and one was of grade III (WHO grade). The pathological T classification of the tumours was T2a in one patient, T2b in three patients, T3a in six patients and T3b in two patients. Three patients had a metastatic regional lymph node but none had bone metastases. The preoperative serum prostate specific antigen (PSA) was, on average, 20.3 ng/ml (range 3.0–60 ng/ml). The mean Gleason score was 5.7 for grade I prostates, 6.3 for grade II prostates and 8 for the single prostate cancer of grade III. Control prostates were

from 13 patients (aged 47–76 years, mean 62 years) undergoing cystoprostatectomy because of bladder carcinoma. These patients did not receive bacille Calmette-Guérin (BCG) or other intravesical chemo- or immunotherapy before surgery. Microscopic hyperplasia was detected in 11 control prostates and prostatic intraepithelial neoplasia (PIN) was detected in 6. These prostates were considered suitable as controls since the prevalence of benign prostatic hyperplasia (BPH) is about 70% among men aged 61–70 years [1]. Use of the tissue specimens was approved by the local ethical committee. Adjacent sections from the same cancer area of the cancerous prostates were used for immunohistochemistry (paraffin blocks) and RNA analysis. Similarly, adjacent slices of the control prostates were used for the corresponding analyses.

Immunohistochemistry

Formalin-fixed and paraffin-embedded prostate specimens were cut into 5-μm sections, which were deparaffinised and blocked for endogenous peroxidase activity by 1% hydrogen peroxide in methanol for 15 min. Rehydrated sections were microwaved in 0.01 M sodium-citrate buffer (pH 6.0) for antigen retrieval. Avidin-biotin immunolabelling was carried out using a Histostain-Plus Kit (Zymed, San Francisco, Calif., USA) and monoclonal primary antibodies against human COX-1 and COX-2 (Cayman Chemical, Ann Arbor, Mich., USA) and against mouse NOS-2 (Transduction Laboratories, Lexington, Ky., USA). The tissue sections were treated with the blocking reagent for 20 min and incubated with the primary antibodies (dilution 1:200) overnight at 4 °C. After subsequent washes with phosphate-buffered saline (PBS; pH 7.5) the sections were treated with biotinylated secondary antibody for 20 min, washed and treated with streptavidin-enzyme conjugate. The specimens were stained with diaminobenzidine and counterstained with Mayers haematoxylin. Negative controls included slides from each prostate specimen, which were treated with 1% normal goat serum in PBS instead of primary antibodies. Finally, the slides were dehydrated and mounted. All slides were examined by a trained pathologist (P.M.). The mean overall intensity of the immunostaining in the cancer cells and stroma of cancer prostates and in the glandular epithelium and stroma of non-malignant control prostates was scored in the analysed tissue sections as follows: 0, absent immunosignal; 1, weak immunosignal (few cells are positive, focal or scattered); 2, moderate immunosignal (marked focal staining or frequent scattered staining); and 3, strong immunosignal (very intense staining in a large focus or intense staining throughout the tumour tissue). When appropriate, values of 0.5, 1.5 and 2.5 were used. No staining was detected in any negative controls without primary antibodies.

Northern blot analysis of the mRNA

The total RNA from 12 prostate specimens was extracted, and the mRNA of COX-1 and COX-2 was analysed by specific cRNA probes as described previously [8, 12, 22]. The mRNA level of glyceraldehyde phosphate dehydrogenase (GAPDH) was analysed with a rat cDNA probe and used as a reference.

Results

Immunohistochemistry of COX-2 and NOS-2 showed significantly stronger staining in prostate cancer cells compared to the normal glandular epithelium of control prostates (Table 1, Fig. 1). The intensity of COX-2 and NOS-2 staining in the stroma of prostate cancer specimens was similar to that in control prostates. In non-malignant control prostates, immunostaining for COX-2 and NOS-2 was weak in both the glandular epithelium and the stroma (Table 1, Fig. 1). No difference was detected in COX-1 staining between the control prostates and prostate cancer samples (Table 1, Fig. 1).

Table 1 The intensity of immunostaining (scale 0–3) in non-malignant control prostates and prostatic adenocarcinomas. Mean and standard errors of the mean are given: $n = 12$–13 for COX-1 and COX-2; $n = 11$–12 for NOS-2. Compared to the control by Student's t-test $^*P < 0.05$

	Control prostates	Prostate cancer
COX-1		
Stroma	1.2 ± 0.2	1.2 ± 0.2
Glandular epithelium/cancer cells	1.3 ± 0.1	1.4 ± 0.2
COX-2		
Stroma	1.0 ± 0.2	1.0 ± 0.2
Glandular epithelium/cancer cells	1.4 ± 0.2	2.0 ± 0.1*
NOS-2		
Stroma	0.4 ± 0.1	0.7 ± 0.1
Glandular epithelium/cancer cells	0.8 ± 0.2	1.4 ± 0.2*

Immunostaining for COX-2 was clearly more intensive in the PIN lesions of both control and cancer prostates than in the normal epithelium of control prostates (Fig. 1B, E). COX-2 staining was mainly seen in the basal epithelial cells of the PIN lesions (Fig. 1E). Also, the expression of NOS-2 protein was higher in the glandular epithelium of the PIN lesions than in the normal epithelium (Fig. 1). However, the intensity of staining for NOS-2 was generally weaker than that of COX-2 (Fig. 1).

In some control prostates clear immunostaining for COX-2 was detected in the muscle fibres of hyperplastic stroma, whereas staining for COX-1 and NOS-2 was usually either weak or negligible in the hyperplastic stroma (Fig. 1A–C). Furthermore, the immunosignal for NOS-2 was weak in the stromal tissues of prostate cancer (Table 1, Fig. 1I).

The expression of genes encoding COX-1 and COX-2 in human prostate tissues was confirmed by Northern blot analysis of COX-1 and COX-2 mRNA (Fig. 2). Two mRNA transcripts of 3 and 5 kb were detected with a probe specific for COX-1, whereas only one mRNA transcript of 5 kb was clearly detected with the COX-2 probe (Fig. 2). The mRNA of both COX-1 and COX-2 was expressed in all analysed samples of control prostates and prostate cancers (Fig. 2). mRNA levels in different specimens varied considerably. The level of COX-2 mRNA was high in the control prostates containing PIN lesions (Fig. 2).

Discussion

The present study indicates that the expression of COX-2 and NOS-2 is stimulated in human prostatic adenocarcinoma and that these inducible genes are clearly also expressed in PIN lesions commonly considered to be precursors for prostate cancer [2]. The smooth muscle fibres in the hyperplastic stroma of some non-malignant prostates were clearly stained for COX-2 (Fig. 1B).

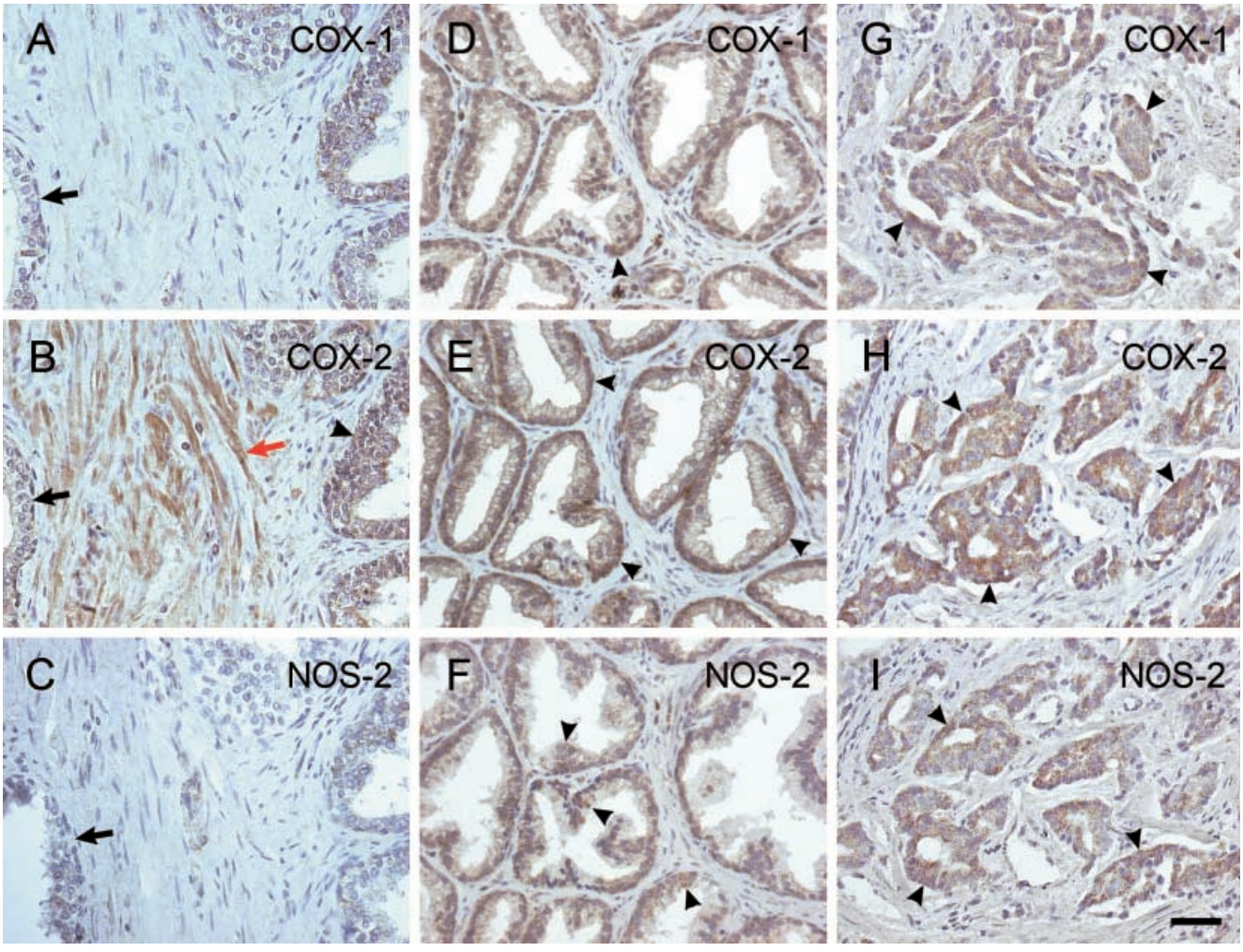

Fig. 1A–I Immunoperoxidase staining (*brown*) of COX-1, COX-2 and NOS-2 proteins in adjacent sections of non-malignant and malignant human prostates. **A–C** Control prostate with benign hyperplasia. COX-2 is stained in the muscle fibres of the hyperplastic stroma (**B**, *red arrow*). COX-1, COX-2 and NOS-2 are not detected in the histologically normal glandular epithelium (*black arrows*), whereas COX-2 is stained in the PIN-type epithelium (**B**, *arrowhead*). **D–F**: Another control prostate with prostatic intraepithelial neoplasia (PIN). COX-2 immunostaining is more intense in the PIN-type epithelium than COX-1 or NOS-2 staining, although NOS-2 is clearly stained in a few epithelial cells (**D–F**, *arrowheads*). **G–I** Adenocarcinoma of prostate (grade II). COX-1, COX-2 and NOS-2 are stained in the cancer cells of a prostatic adenocarcinoma (**H–I**, *arrowheads*). The intensity of COX-2 staining is stronger than that of either COX-1 or NOS-2. *Scale bar* = 40 μm

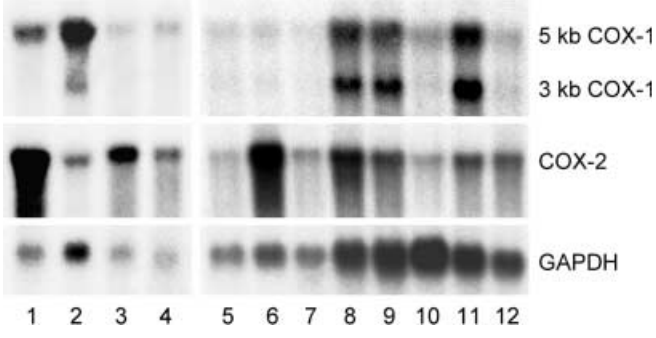

Fig. 2 Northern blot hybridisation of the mRNA of COX-1 and COX-2 in 12 human prostate glands. *Lane 1*: Benign prostatic hyperplasia (BPH) with prostatic intraepithelial neoplasia (PIN); *Lanes 2–4*: BPH; *Lane 5*: normal control prostate; *Lane 6*: BPH and PIN; *Lane 7*: BPH; *Lane 8*: BPH and PIN; and *Lanes 9–12*: adenocarcinoma of prostate (grade II). Ten micrograms of total RNA was analysed in samples 1–4 and 20 μg was analysed in the others. The blots for GAPDH are shown to indicate the loading of the RNA

The increased expression of COX-2 was also recently detected in prostate adenocarcinoma by Gupta et al. [5]. We detected the expression of both cyclooxygenases in prostate cancer and in samples of normal and hyperplastic control prostates, both at the mRNA and the protein level. This is in agreement with the report of O'Neill and Ford-Hutchinson [13], who showed the expression of mRNAs encoding COX-1 and COX-2 in normal human prostate by reverse transcriptase-polymerase chain reaction (RT-PCR). Since both cyclooxygenases are expressed in the prostate and both have a role in angiogenesis and tumour growth [9, 21], further studies are needed to assess the possible role of COX-1 and COX-2 in the growth of human prostate. Moreover, COX-1 and COX-2 may also have other important functions in human prostate.

Klotz et al. [10] demonstrated the expression of NOS-2 in prostate cancer specimens but not in non-malignant prostates with a polyclonal antibody. This is in agreement with our finding of increased immunostaining of NOS-2 in the cancer cells of prostate adenocarcinoma and also in the PIN-type epithelium of both control and cancer prostates. These findings are of special interest since NO is reported to have an important role in angiogenesis and tumour progression, and may also mediate the angiogenic effects of vascular endothelial growth factor [3, 25].

The elevated expression level of COX-2 and NOS-2 in prostatic adenocarcinoma and PIN lesions may have possible therapeutic implications for prevention and treatment since inhibitors of both COX-2 and NOS-2 are reported to inhibit the growth of cancer cells [18, 19]. A specific inhibitor of COX-2 was recently found to induce apoptosis of human prostate cancer cells [7]. The inhibition of COX-1 may also be important since COX-1 is expressed in prostate cancer and the inhibition of COX-1 suppresses the growth of those tumours not expressing COX-2 [17]. This may be related to the inhibition of angiogenesis [17].

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