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Cyclooxygenase-1 and -2 in human testicular tumours

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Abstract

In this study, we investigated the expression of cyclooxygenase (COX)-1 and -2 in human testicular cancer (TC) and normal testis (NT) tissues, as well as the effects of COX ligands on viability and proliferation. Tumour specimens were obtained from 72 patients with TC and 20 patients with NT. RT-PCR and immunohistochemical methods were used to determine COX expression. While COX expression was not noted in any of the NT tissues, a marked expression was observed in the TC samples. The extent and intensity of immunoreactive COX-1 and -2 polypeptides in the TC tissues was statistically greater than the expression in the NT tissues. The synthetic COX inhibitors inhibited the growth of the TC cells. Both COX-1 and COX-2 are induced in testicular cancer, and these results indicate that both COX-1 and COX-2 are essential for the growth of TC cells.

Keywords: Cyclooxygenase-1 (COX-1); Cyclooxygenase-2 (COX-2); Testicular tumour; Immunohistochemistry; RT-PCR

1. Introduction

Epidemiological studies and animal experiments have demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the incidence of colorectal carcinoma [1–4]. Cyclooxygenase (COX), which produces prostanoids from arachidonic acid, is the principal target of NSAIDs. Prostaglandins (PGs) and the COX enzyme may be involved in the initiation and/or the promotion of carcinogenesis because a major action of NSAIDs is the inhibition of COX. Several reports indicate that the levels of PGs in human colon tumours are higher than those in surrounding normal tissues, and that the major PG is PGE2 [5]. COX is a rate-limiting enzyme in PG synthesis because of its rapid auto-inactivation [6].

COX-1 is present in most tissues and involved in the physiological production of PGs for maintaining normal homeostasis [7]. COX-2, which is induced by mitogens, cytokines, and growth factors, is primarily responsible for PGs produced in inflammatory sites [8,9]. In particular, COX-2 has a well established participation in prostanoid production. COX-2 protein and mRNA are expressed in the synovial tissue of patients with rheumatoid arthritis (RA) and inhibited by glucocorticoids [10]. In human colorectal cancer, immunoreactive COX-2 is found to be elevated in cancer cells, inflammatory cells, vascular endothelial cells, and fibroblasts [11,12]. COX-2 is also expressed in gastric cancer [13], breast cancer [14], hepatocellular carcinoma [15], lung cancer [16], esophageal cancer [17], and pancreatic cancer [18] tissues.

In this study, COX-1 and COX-2 expressions were investigated in human testicular cancer (TC) and normal testis (NT) tissues, and the inhibitory effects of COX-2 inhibitors, NS-398 and Etodolac, and COX-1 inhibitor, Sulindac, on TC-derived cell lines were also investigated.

2. Patients and methods

2.1. Tumour specimens

Tumour specimens were obtained from 72 TC patients, and from 20 NT patients who underwent orchiectomy for prostate cancer.

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2.2. Antibodies

The COX-1-specific antiserum was raised against human COX-1 polypeptide (DHHILHVAVDV). The COX-2-specific antiserum was raised by coupling the unique COOH-terminal insert region of the human COX-2 polypeptide (LDDINPTVLLKER). These antibodies were prepared in goat serum according to standard techniques [19]. The specificity for these antibodies was checked by immunoprecipitation of recombinant COX-1 and -2 polypeptides expressed in transiently-transfected Cos-7 cells as previously described in Ref. [9].

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from TC tissues and NT tissues by acid guanidium thiocyanate-phenol-chloroform method [20]. The primers used were:

(a) human *COX-1*: sense; 5′-TGCCCAGCTCCTG GCCCGCCGCTT-3′ and antisense 5′-GTGCATCAA CACAGGCGCCTCTTC-3′,

(b) human *COX-2*: sense; 5′-TTCAAATGAGATTG TGGGAAAATTGCT-3′ and antisense 5′-AGATCAT CTCTGCCTGAGTATCTT-3′,

(c) human glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*): sense; 5′-CCACCCATGGCAAATTCCAT GGCA-3′ and antisense; 5′-TCTAGAGGGCAGGTC AGGTCCACC-3′.

2.4. Immunohistochemistry

Immunohistochemical staining was performed with the Vectastatin avidin-biotin peroxidase complex kit (Vector Laboratories, Burlingame, CA, USA) [9,21]. Primary antibodies against COX-1, COX-2, or control normal goat serum (Vector Laboratories) were used at 30 μg/ml. Biotinylated rabbit anti-goat IgG (Vector Laboratories) was applied onto the tissue sections, and incubated at room temperature for 30 min. Slides were incubated with avidin DH-biotinylated peroxidase (Vector Laboratories) for 45 min. Finally, colour was developed by immersion of the sections in a peroxidase substrate solution including 0.02% peroxide, 0.1% 3,3′ diaminobenzine tetrahydrochloride (DAB). The sections were counterstained with 0.5% methyl green (Nakarai Tesque, Inc, Kyoto, Japan). Specificity was determined by pre-adsorption of anti-COX-1 or -2 antibodies with COX-1 or -2 synthetic polypeptide (1 mg/ml) before staining.

2.5. Statistical analysis

The extent and intensity of staining with COX-1 and -2 antibodies were graded on a scale of 0 to 4+ by two blind observers on two separate occasions using coded slides, and an average score was calculated [11,20]. A 4+ grade implied that staining was at the maximum intensity throughout the specimen, while 0 implied the staining was absent. Micro-anatomical sites of staining were also recorded. Analyses of data were performed using the analysis of variance (ANOVA) [22].

2.6. Cell cultures

The human TC cell lines NEC-8, was obtained from Health Science Research Resources Bank (HSRRB, Osaka, Japan). Cells were grown in culture flasks (Nunc, Roskilde, Denmark) in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin, in a humidified 5% CO_2 atmosphere at 37 °C. The media were changed every 3 days and the cells were separated via trypsinisation, using trypsis/ethylene diamine tetra acetic acid (EDTA) when they reached subconfluence.

2.7. Cell proliferative studies

Approximately 1.0×10^4 cells placed onto 8×8 mm diameter multichamber slides (Nunc, Copenhagen, Denmark) were treated with specific COX-2 inhibitors (NS-398 and Etodolac) and specific COX-1 inhibitor (Sulindac) dissolved in ethanol. The final concentration in ethanol was $<0.05\%$. Cell viability was measured at day 1 by a microplate reader using a modified 3-[4,5-dimethylthiazol - 2 - thiazolyl] - 2,5 - diphenyltetrazolium bromide (MTT) assay (WST-1 assay; Dojindo, Kumamoto, Japan), and presented as the percentage of control-culture conditions.

3. Results

The 72 patients with TC were aged from 21 to 55 years (mean age 31.0 + 12.3 years) and the 20 patients with normal testis ranged in age from 56 to 74 years with an average of 61.4 + 8.6. The tumour histopathologies were diagnosed by pathologists (Table 1).

3.1. PCR of reverse-transcribed RNA from TC and NT tissues

Amplification of cDNA with the *COX-1*, *COX-2* and *G3PDH* primers used in this study predicted fragments of 303, 304 and 598 base pairs (bp) in length, respectively. We detected specific bands of *COX-1* and *COX-2* mRNA in all samples from TC (*COX-1*: Fig. 1a, lanes 3–7, *COX-2*: Fig. 1b, lanes 3–7), while in the sample from NT no clear band was detected (*COX-1*: Fig. 1a, lane 2, *COX-2*: Fig. 1b, lane 2).

Table 1
T category of 72 testicular tumour patients

Histopathology	Stage	pTis	pT1	pT2	pT3	pT4	Total
Tumours of one histological type							
Seminoma		1	21	4	2	3	31
Embryonal carcinoma		–	3	1	2	2	8
York sac tumour		–	2	1	2	2	7
Choriocarcinoma		–	1	2	1	3	7
Teratoma		1	1	2	–	1	5
Tumours of more than two histological type							
Embryonal carcinoma and teratoma		–	–	1	1	2	4
Choriocarcinoma and others		–	–	1	–	2	3
Other combinations		–	2	1	1	3	7
Total		2	30	13	9	18	72

The tumour having a single histological type was found in 58 patients. Tumours with more than two histological types were found in 14 patients.

3.2. *COX-1, -2 immunostaining of testicular tissues from patients with TC and NT*

To assess the tissue distribution of COX-1 or -2 polypeptides, we stained paraffin-embedded samples with the affinity-purified COX-2 antibody which recognises COX-2, but not COX-1, and with the affinity-purified COX-1 antibody which recognises COX-1, but not COX-2. The specificity of this antibody was proven in previous experiments [11]. Immunoreactive COX-1 was significantly apparent in testicular carcinoma with seminoma tissue (Fig. 2c). A marked expression of COX-2 was also observed in seminoma tissue (Fig. 2d).

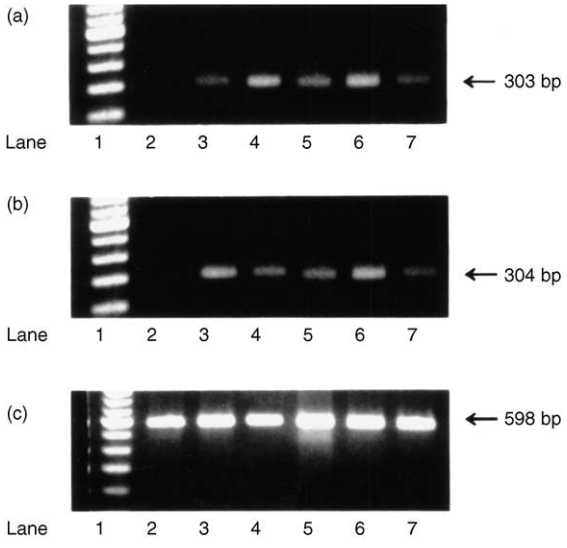

Fig 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *COX-1* and *COX-2* in testicular tissues from patients with testicular tumours (TC) and from normal testis samples (NT). Total RNA was purified from 10 TC (2 seminoma, 2 embryonal carcinoma, 2 yolk sac tumours, 2 choriocarcinoma, 2 teratoma), 5 NT: (a) *COX-1*, (b) *COX-2*, (c) *G3PDH*. Lane 1: markers; 2: normal testis; 3: seminoma; 4: embryonal carcinoma; 5: yolk sac tumour; 6: choriocarcinoma; 7: teratoma.

As shown in a representative cancer tissue section, we found significant expression of immunoreactive COX-1 in cancer cells in all TC groups (Fig. 3a, b, e, g) and also marked expression of immunoreactive COX-2 in cancer cells of all TC groups (Fig. 3b, d, f, h). In contrast, no expression of immunoreactive COX-1 or COX-2 was found in the NT samples (data not shown).

3.3. *Statistical analysis of COX-1 and -2 immunostaining*

In cases where more than two histopathological types were recognised, we consolidated the types. Thus, the total numbers of specimens were greater than the number of patients. Finally, the 14 cases which had more

Table 2
Statistical analysis of Cox-1 and -2 immunostaining

Tumour type	Epithelium Ave±S.D. (*P* value)	Blood vessel Ave±S.D. (*P* value)
COX 1 immunostaining		
Normal tests	0.4±0.4	0.3±0.3
Seminoma	3.4±0.6* (<0.001)	2.3±0.8* (<0.001)
Embryonal carcinoma	3.1±0.6* (<0.001)	2.5±0.9* (0.001)
Yolk sac tumour	2.6±0.6* (<0.001)	2.1±0.8* (<0.001)
Choriocarcinoma	2.7±0.9* (<0.001)	2.0±0.7* (<0.001)
Teratoma	2.9±0.8* (<0.001)	2.3±0.5* (<0.001)
COX-2 immunostaining		
Normal testis	0.4±0.3	0.3±0.3
Seminoma	2.4±0.9* (<0.001)	2.1±0.8* (<0.001)
Embryonal carcinoma	2.5±0.6* (<0.001)	1.7±0.9* (<0.001)
Yolk sac tumour	2.3±0.7* (<0.001)	1.9±0.8* (<0.001)
Choriocarcinoma	2.2±0.9* (<0.001)	2.0±0.6* (<0.001)
Teratoma	2.3±0.8* (<0.001)	2.1±0.7* (<0.001)

Ave, average; S.D., standard deviation. Graded 0–4 on the coded sections by two blind observers: 0, no staining; 4+ maximum intensity Statistical analysis was performed using the analysis of variance (*P* value; ANOVA) COX-1 and COX-2 immunostaining were more intense and diffuse in testicular tumour tissues than in the normal testicular tissues (*$P<0.01$).

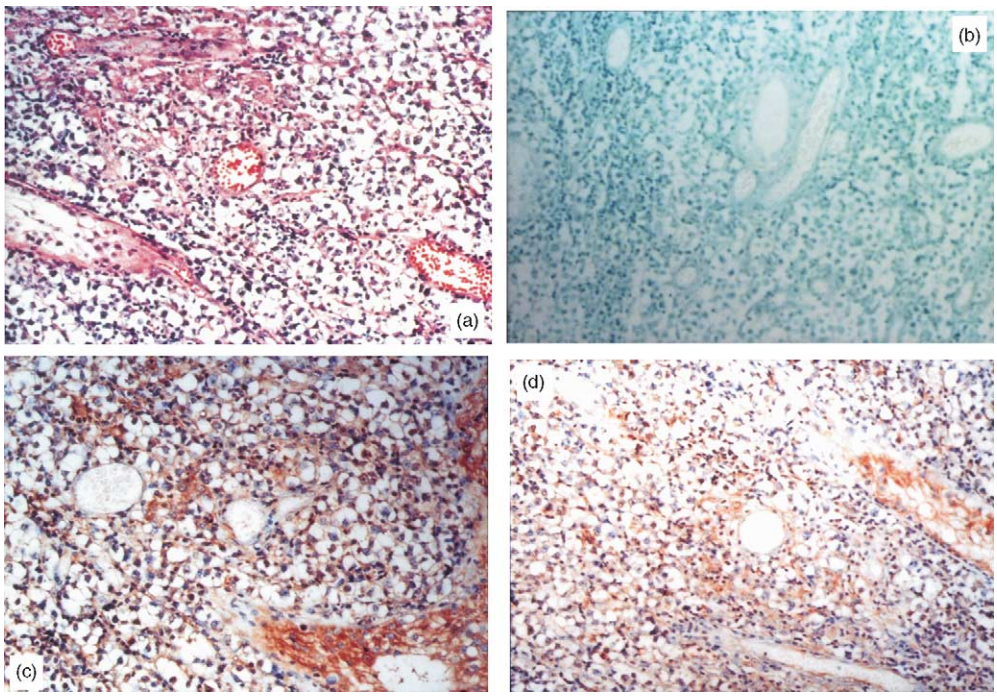

Fig. 2. Representative immunostaining for COX-1 and COX-2 in seminoma tissues: (a) haematoxylin and eosin staining, (b) control staining with normal rabbit immunoglobulin G, (c) COX-1 immunostaining, (d) COX-2 immunostaining (a, b, c, c ×400). Immunoreactive COX-1 was significantly apparent in testicular carcinoma with seminoma tissue (c). A marked expression of COX-2 was also observed in seminoma tissue (d). Control staining with normal rabbit immunoglobulin G was completely negative (b). Haematoxylin and eosin staining were seen in seminoma from testicular carcinoma tissue (b).

than two histopathological types were examined and their type numbers were totalled.

COX-1 immunostaining was significantly more extensive and intense in the tumour cells and in blood vessels than in NT tissues (Table 2). COX-2 immunostaining was also significantly more extensive and intense in the tumour cells and in blood vessels of testicular carcinoma studied than in the NT tissues (Table 2).

3.4. Inhibition of TC proliferation by COX-2 ligands

To investigate the effects of the COX ligands on TC cell proliferation, we analysed cell viability *in vitro* by a modified MTT assay. As shown in Fig. 4a, agents (NS-398, Etodolac and Sulindac) induced a reduction in cell viability with the half-maximal concentration of growth inhibition being in the range of 80 μM. Furthermore, counting cells at days 1, 2 and 3, showed a weak inhibition of cell proliferation following treatment with 80 μM of NS-398, Etodolac and Sulindac (Fig. 4b). The inhibitory effects of both COX-1 and -2 ligands were the same, and were not very strong.

4. Discussion

The molecular study of the relationship between polyunsaturated fatty acids and carcinogenesis reveals novel molecular targets for cancer chemoprevention research. Polyunsaturated fatty acids can enhance tumorigenesis in animal models. Cyclooxygenases (COXs) and lipoxygenases (LOXs) are two important enzyme classes that can metabolise polyunsaturated fatty acids and thereby affect carcinogenesis [23].

It is considered that the angiogenic factor COX is strongly linked to inflammatory diseases and the development of metastasis in several cancers. COX-1 acts to maintain cellular homeostasis. It is present in nearly all tissues and its activity is constantly maintained. COX-2 is induced by several stimuli, such as cytokines and mitogens, and it is involved in the processes of inflammation, cell proliferation and differentiation [24,25]. COX-2 activity is very low in the normal cell state, but appears in macrophages, fibroblasts, vascular endothelial cells, neurones, and other tissues in response to various stimuli, including cytokines such as interleukin 1 (IL-1) and tumour necrosis factor-α (TNF-α) [26,27], carcinogens such as TPA, serum and hormones [11,12]. COX-2 is mainly found in stromal cells, such as macrophages, with no significant expression in epithelial cells. In contrast, COX-2 is markedly expressed in cancer cells, in addition to infiltrative inflammatory cells, vascular endothelial cells and fibroblasts [11]. COX-1 protein is observed in normal gastric mucosa where there is virtually no expression of COX-2, while in gastric cancer cells COX-1 protein is decreased and COX-2

expression is noticeably higher [28]. However, due to a significant increase in prostaglandins in breast cancer tissue, the expression of both COX-1 and COX-2 are more enhanced than in the tissue surrounding the cancer [14].

Our current study of testicular carcinoma also shows results that are similar to those in breast carcinomas. No marked expression of COX-1 and -2 was observed in the normal testis, but they were strongly expressed in the testicular carcinoma tissues.

COX involvement in carcinogenesis and proliferation includes its effects on neovascularisation. An *in vivo* study in the nude mouse revealed that neovascularisation is enhanced in a COX-2-expressing tumour graft, and that a COX-2 selective inhibitor reduces angiogenesis in a dose-dependent manner [29]. Thus, COX-2

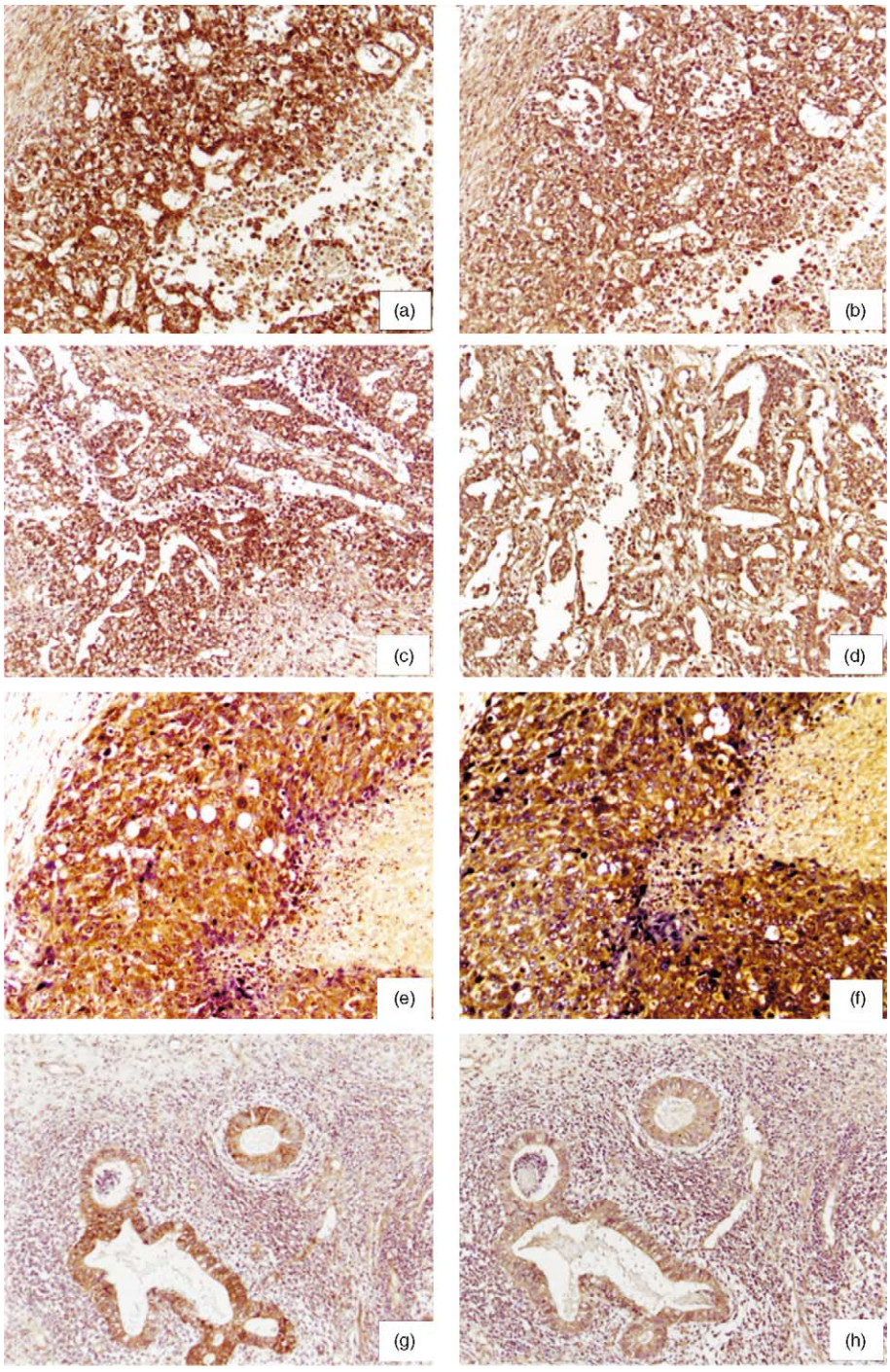

Fig. 3. Representative immunostaining for COX-1 and COX-2 in testicular tumours. Significant expression of immunoreactive COX-1 in cancer cells in all TC groups (a) embryonal carcinoma; (c) yolk sac tumour; (e) choriocarcinoma; (g) teratoma and also marked expression of immunoreactive COX-2 in cancer cells of all TC groups ((b) embryonal carcinoma; (d) yolk sac tumour; (f) choriocarcinoma; (h) teratoma), ×200.

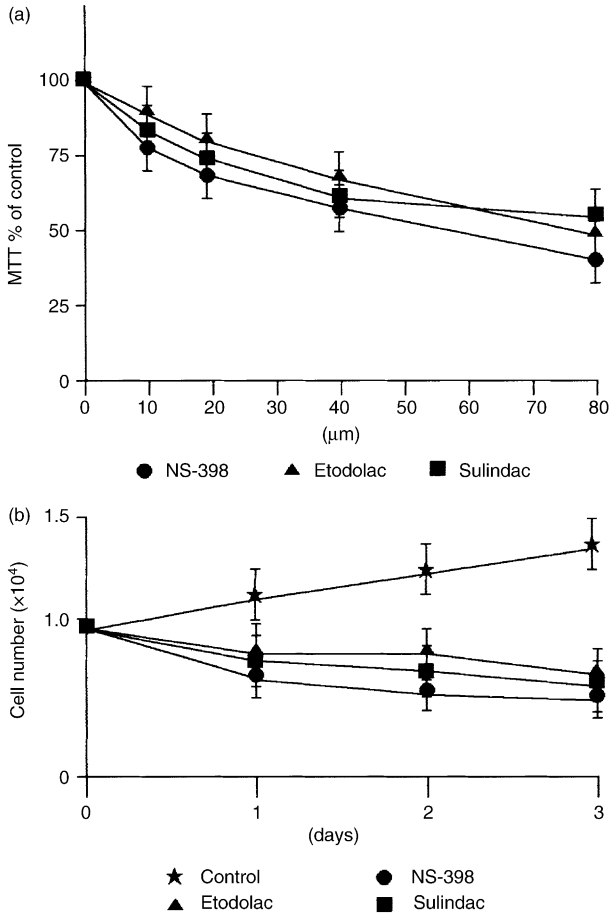

Fig. 4. Effects of COX-1 and COX-2 ligands in human testicular cancer cells. These data are presented as means ±standard deviations (S.D.). Cell numbers were determined at days 1, 2, and 3: (a) modified 5-diphenyltetrazolium (MTT) assay; (b) cell proliferation.

expression enhances angiogenic factors and induces neovascularisation, indicating that COX-2 may be involved in lowering tumour cell apoptosis and the proliferation of tumour cells. However, there have been reports that both COX-1 and COX-2 are induced in vascular endothelial cells during neovascularisation as a result of the stimulation of angiogenic factors. Another study has revealed a marked decrease in vascularisation when COX-1 expression was inhibited by an antisense oligo, whereas no changes were seen by inhibiting COX-2. The *in vivo* study in nude mice with implanted HCT116 (colorectal cancer cells) that showed no expression of COX, revealed that a non-selective inhibitor of COX delayed tumour proliferation and reduced neovascularisation in tumours, while a selective inhibitor of COX-2 did not prevent tumour growth. These results suggest that COX-2 inhibits angiogenesis in tumour cells, and that the expression of COX-1 may be largely responsible for angiogenesis in vascular endothelial cells.

In conclusion, we found immunoreactive COX-1 and -2 were strongly expressed in testicular cancer tissues. The extent and intensity of immunoreactive COX-1 and COX-2 in these cells was greater than in normal testis tissues. We also demonstrated that both COX-1 and -2 inhibitors stopped the growth of TC cells. However, the inhibitory effects of COX-2 inhibitors were the same as the COX-1 inhibitor. Thus, the results of our study strongly indicate that both COX-1 and COX-2 are essential enzymes for propagating the tumour cell growth of testicular cancer cells.

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