

Cyclooxygenase-2 is Involved in the Progression of Thyroid Cancer

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Although the inducible form of cyclooxygenase (COX), COX-2, is highly expressed in various cancers and it is also involved in cancer progression, its role in thyroid cancer is not fully understood. We assessed *in situ* cyclooxygenase expression in normal thyroids (n=6), Graves' thyroids (n=6), thyroid adenomas (n=12), thyroid follicular (n=15) and papillary carcinomas (n=30). In comparison to the constitutive expression of COX-1, COX-2 was highly expressed in thyroid cancers (90.0% of thyroid papillary carcinomas and 73.3% of thyroid follicular carcinomas) and moderately in thyroid adenomas (25.5%), but barely expressed in normal and Graves' thyroid tissues. This quantitative assessment employed immunohistochemical methods. Thereafter we compared the effect of COX-2 inhibition on a human follicular thyroid carcinoma cell line (WRO), and a human papillary carcinoma cell line (NPA), using selective COX-2 inhibitor (NS-398). The treatment with 50 μ M NS-398 suppressed the growth of the COX-2 expressing cells, NPA cells (37.7%; p<0.01) and WRO cells (10.1%; p<0.05). Moreover, at concentrations \geq 100 μ M, NS-398 induced cell death and mitochondrial dysfunction. In addition, 50 μ M of NS-398 inhibited the activation of extracellular signal-regulated kinase in NPA cells after the stimulation with fetal bovine serum. Our results indicate that COX-2 is involved in the progression of thyroid cancer.

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Introduction

In the early 1990s, several epidemiological studies suggested the therapeutic potentials of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) against colorectal neoplasms.¹⁻⁴ Aspirin and NSAID are known to inhibit the production of prostaglandins (PG) via the inactivation of prostaglandin endoperoxide H synthase,⁵ also often called cyclooxygenase (COX). In addition, the inhibitory effect of the drugs has been shown to contribute to their anti-cancer properties. They can suppress the proliferation^{6,7} and migration of cancer cells,⁸ and can also suppress the production of certain angiogenic factors by tumor cells *in vitro*.⁹ Moreover, these studies so provided evidence that linked the anti-cancer properties of drugs to the inhibition of the inducible form of COX, i.e. COX-2, and showed that the anti-cancer properties were not associated with the constitutive form of COX, i.e. (COX-1). In other words, the effects outlined above, including those on malignancies, are now believed to be partially medi-

ated via the aberrantly expressed COX-2. Although these studies were performed mainly in colon cancer both *in vivo* and *in vitro*, the involvement of this enzyme in carcinogenesis or cancer progression other than colon has been reported.¹⁰⁻¹³

In this context, at least certain steps of carcinogenesis or proliferation of thyroid cancer may be enhanced by the overexpression of COX-2. In the present study, we determined the expression of COX-2 in various thyroid tissues and investigated its pathophysiological role(s) *in vitro*.

Materials and methods

Samples

Thyroid cancer, thyroid adenoma, and Graves' disease tissues were obtained from our hospital during surgery from 1985 to 1997. Normal thyroid tissue specimens were obtained from residual nor-

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mal tissues of resected thyroid cancers. A written informed consent was obtained from each patient. Complete pathological reports were available for all the tissue samples.

Materials

Anti-human COX-1 antibody (mouse monoclonal), anti-human COX-2 antibody (goat polyclonal), and NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methane sulfonamide), a selective inhibitor of COX-2, were purchased from Cayman Co. (Ann Arbor, MI, U.S.A.). Etdrac (1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-b] indole-1-acetic acid) was kindly provided by Nippon Shinyaku Co. (Kyoto, Japan). The IC₅₀ values of Etdrac for COX-1 and COX-2 are 15 μ M and 1.4 μ M, respectively.¹⁴ The IC₅₀ values of NS-398 are 220 μ M and 1.77 μ M, for COX-1 and COX-2, respectively.¹⁵ PD98059, a selective MEK inhibitor, was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Anti-extracellular signal-regulated kinase (Anti-ERK) and anti-phosphorylated ERK antibody (rabbit polyclonal) were purchased from Transduction Laboratories (Lexington, KY, U.S.A.) and New England Biolabs, Inc. (Beverly, MA, U.S.A.), respectively.

Cell cultures

NPA cells, a human thyroid papillary carcinoma cell line,^{16,17} and WRO cells, a human thyroid follicular carcinoma cell line,¹⁶ were kindly provided by Dr. G. Juillard (University of California-Los Angeles Medical Center). The cells were maintained in a humidified, 5% CO₂ incubator at 37 °C covered with RPMI medium (for NPA cells) or Ham's F-12 medium (for WRO cells) supplemented with 10% fetal bovine serum (Gibco BRL, Invitrogen Japan K.K., Tokyo, Japan) and antibiotics (5,000 U/ml penicillin, 5mg/ml streptomycin). The primary cultured thyrocytes were obtained surgically from the patients with Graves' disease and maintained as previously described.¹⁸

Immunohistochemistry

The tissues from thyroid glands with various diseases were embedded in paraffin, cut into 4 μ m thick sections and mounted on positively charged The superfrost slides. Sections were deparaffinized, and rehydrated with graded alcohol solutions, and the endogenous peroxidase activity was quenched by the immersion in 3% H₂O₂ in methanol. The nonspecific binding was blocked with 10% mouse (COX-1) or goat (COX-2) serum and the sections were incubated with primary antibody to COX-1 (1:100 dilution) or to COX-2 (1:100 dilution) for 1 hour at room temperature. After washing with PBS, the sections were incubated with a biotinylated secondary anti-mouse (COX-1) or -rabbit (COX-2) antibody for 12 minutes at room temperature. Color development was performed by using an avidin-biotin peroxidase complex solution and 3,3'-diamino benzidine. The counterstaining was briefly performed in methylgreen. The control slides were treated with isotype-matched IgG. The intensity

of COX-2 immunostaining was graded on the following scale: - =no staining, +/- =equivocal staining, + =moderate to intense staining, and ++ =highest intensity staging by T.A and T.U, independently.

Western Blotting

The cells were grown to approximately 80% confluence. They were then washed with PBS, lysed in an ice cold protein lysis buffer [20 mM sodium phosphate (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS at 10mg/ml] with freshly added 20 μ M phenylmethylsulphonyl fluoride. The protein content of the lysate was determined by using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The total protein (30 μ g per lane) was separated by electrophoresis on 10% SDS acrylamide gel, and transferred onto a nitrocellulose membrane. COX-1 or COX-2 signal was detected with the specific antibodies that were used in immunohistochemistry and was visualized by a chemiluminescence method using ECL Western Blotting Detection Systems (Amersham Pharmacia Biotech, Buckinghamshire, England).

Reverse Transcriptase Polymerase Chain Reaction

The total RNA was extracted from the cells by using RNeasy Mini Kit (Qiagen, Tokyo) according to the instructions provided by the manufacturer. The first-strand cDNA was synthesized by reverse transcription at 42 °C for 30 minutes in a 20 μ l reaction mixture containing 500 ng of total RNA and AMV RT (Takara shuzo Co., Shiga, Japan). For PCR reactions, 4 μ l of denatured cDNA was amplified in a 50 μ l final volume with 0.5 U Taq DNA polymerase (Gibco BRL, Invitrogen Japan K.K., Tokyo, Japan), 1 mM of both primers, and Taq buffer containing 1.5 mM MgCl₂ with 1.5 mM of each dNTP. PCR was performed in a thermal cycler using a program of 25 cycles at 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 90 seconds, followed by a 10 min extension at 72 °C. The amplified products were subjected to electrophoresis on 2% agarose gel. The specific primers for the COX-2 PCR were as follows; 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' (forward primer), 5'-AGATCATCTCTGCCTGCCTGAGTATCTT-3' (reverse primer). The predicted size of the fragment was 301bp.

Induction of apoptosis in NPA cells

NPA cells (5×10^5 /well) were cultured in RPMI containing 10% fetal bovine serum with or without NS-398 for 48 hours. After cultivation, DNA fragmentation and disruption of the mitochondrial function were assayed. DNA fragmentation was quantified by the percentage of cells with hypodiploid DNA, as described previously.¹⁹ Disruption of mitochondrial function was estimated by using a decrease in the mitochondrial membrane potential, as previously described.²⁰ In brief, the cells were incubated with a saturating amount of DiOC6 (3,3'-dihexyloxycarbocyanine iodide, Fluoreszenz Technologie, Grottenhofstr, Austria) at 37 °C for 15 minutes, washed, and analyzed by a flow cytometer (Epics XL, Beckman Coulter, Inc.,

Fullerton, CA, U.S.A.).

Phosphorylated ERK assay

NPA cells were maintained until 80% confluency in 60 mm culture dishes. They were then washed once with PBS and pretreated for 12 hours in RPMI containing 0.5% bovine serum albumin with or without NS-398. Thereafter, the cells were stimulated with fetal bovine serum, at a final concentration of 10% (v/v), for 5 minutes and lysed in lysis buffer [150 mM sodium chloride, 20 mM of Tris (pH 7.5), 1% TritonX-100, 5 mM EDTA, 50 mM sodium fluoride, 10% glycerol, and freshly added 1 mM Na_3VO_4 and 20 mM/ml phenylmethylsulphonyl fluoride] after washing twice with ice-cold PBS. Finally, 20 μg of protein was applied to Western blot analysis and the phosphorylated ERK or the total ERK protein signal was analyzed using primary antibodies (1:1000 each).

Results

COX-2 protein expression in vivo

We first examined the expression of in situ COX-1 and COX-2 proteins in the various thyroid tissues. As demonstrated in Figure 1, the immunostaining for COX-1 was detected in the cytoplasm of thyroid follicular epithelial cells in all the tissue samples that were examined. However, some thyroid tissues did not express COX-2. As summarized in Table 1, the proportion of the tissue samples immunohistochemically positive for COX-2 was 27 of 30 (90.0%) for papillary carcinoma, and 11 of 15 (73.3%) for follicular carcinoma, and 3 of 12 (25.0%) for thyroid follicular adenoma. Only a weak COX-2 signal was identified in some of the Graves' disease, and the signal was barely detectable in the follicular epithelial cells of the normal thyroid. The intensity of the COX-2 signal was not uniform in the thyroid cancer samples, but varied from weak to strong. These findings suggested that COX-2 is essentially expressed in the thyroid neoplasm.

COX-2 expression in vitro

In this study the findings of a constitutive expression of COX-1 and an aberrant expression of COX-2 in thyroid cancers are similar

Table 1. COX-2 Immunohistochemistry

Intensity of immunostaining	++	+	+/-	-	% of positive
Papillary carcinoma (n=30)	10	17	3	0	90.0
Follicular carcinoma (n=15)	4	7	4	0	73.3
Follicular adenoma (n=12)	0	3	6	3	25.0
Graves' disease (n=6)	0	0	2	4	0
Normal thyroid (n=6)	0	0	0	6	0

Immunoreactivity for COX-2 was graded as - no staining; +/- equivocal staining; + moderate to intense staining; ++ highest intensity staining.

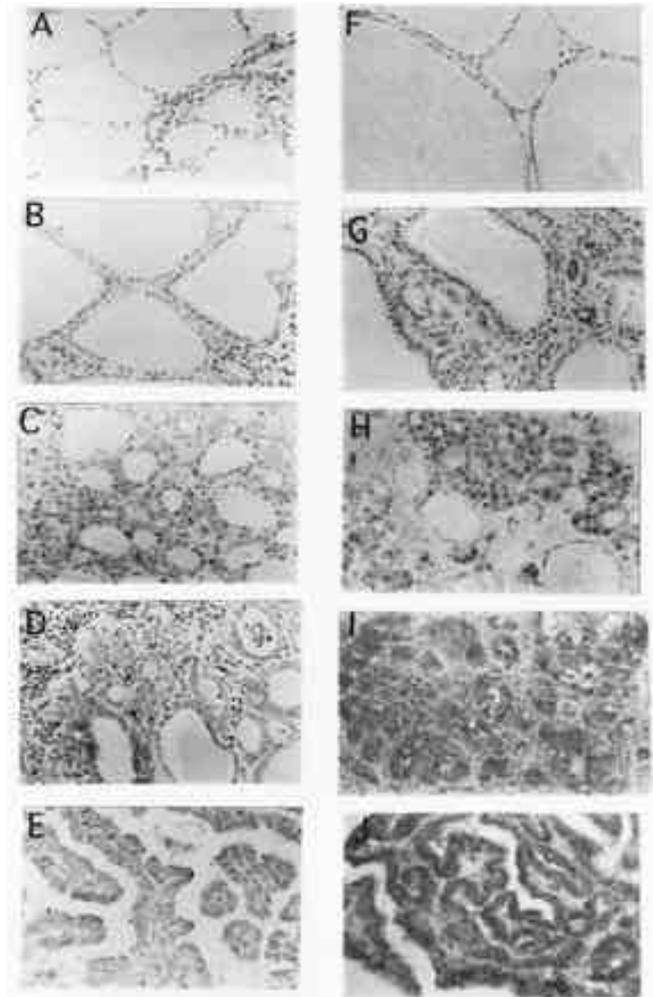


Figure 1. The representative results of the immunohistochemical examination for COX-1(*left*) and COX-2(*right*) in human thyroid tissues. COX-1 was constitutively expressed in the follicular epithelial cytoplasm of normal thyroid (A), Graves' thyroid (B), follicular adenoma (C), follicular carcinoma (D), papillary carcinoma (E). In comparison to COX-1, COX-2 was not detected in the non-neoplastic thyroid tissues [normal thyroid (F) and Graves' thyroid(G)], but easily detected in the follicular epithelial cytoplasm of thyroid neoplasm [follicular adenoma (H), follicular carcinoma (I), papillary carcinoma (J)]. Magnification, $\times 400$.

to those reported in colon cancer.^{21,22} Thereafter we next examined the expression of these cyclooxygenases in the following cells; primary thyrocytes, a human thyroid follicular cancer cell line WRO, and a human thyroid papillary cancer cell line NPA. Western blotting analysis demonstrated the expression of COX-1 protein in these three types, even though the intensity of the signals were not consistent (Figure 2A). In contrast to COX-1, COX-2 was barely expressed in primary thyrocytes, but abundant in NPA and WRO cells (Figure 2A). COX-2 protein expression was almost similar in NPA and WRO cells. These findings were confirmed by the results of the COX-2 mRNA analysis (Figure 2B). These results confirmed that the COX-2 expression was limited to thyroid cancer cells.

COX-2 inhibition and cancer proliferation

We assessed the possible involvement of COX-2 in the proliferation of the thyroid cancer cells. For this purpose, we used two COX-2 inhibitors. The first inhibitor was Etdrac, which selectively inhibits COX-2 at low concentrations, but at high concentrations, it inhibits both COX-1 and COX-2.¹⁴ The second inhibitor was NS-398, a selective inhibitor of COX-2¹⁵ (see Materials and Methods). Both agents showed anti-proliferative effects in a dose-dependent

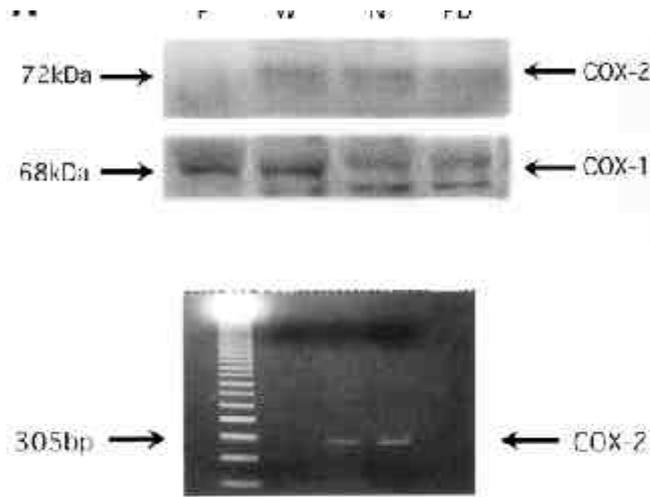


Figure 2. The COX-2 expression in the primary cultured thyrocytes (lane P) and the human thyroid cancer cell lines, WRO (lane W) and NPA (lane N). (A) Western blotting was performed on the three types of thyroid cells. Equal amounts of protein (30µg/lane) were loaded onto a 10% SDS polyacrylamide gel, electrophoresed, and subsequently transferred to nitrocellulose membrane. Western blot was probed with antibody specific for COX-1 (lower panel) or COX-2 (upper panel). In lane PD, NPA cells were treated with PD 98059, a specific MEK inhibitor, for 12 hours. (B) RT-PCR was performed on the using the three types of thyroid cells. The cDNA was synthesized by reverse transcriptase using the total RNA as a template and 4µl of the cDNA was amplified using specific human COX-2 primers.

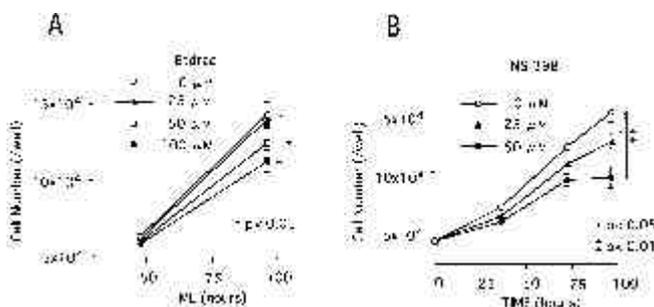


Figure 3. The COX-2 inhibitors suppressed the growth of thyroid cancer cells. NPA cells were grown in the presence of the indicated concentrations of Etdrac (A) or NS-398 (B) for 96 hours and the number of cells was counted at the indicated time. Growth of NPA cells was suppressed by 14.4% (p<0.05) by 100µM of etdrac and 37.7% (p<0.01) by 50µM of NS-398. Value are the mean ± SEM (n=4). Similar results were obtained in three separate experiments.

manner on thyroid cancer cells (Figure 3). This effect was more profound in NS-398 treated NPA cells (37.7% reduction of the cell number at 50µM for 96 h treatment, p<0.01), which expressed higher levels of COX-2, in comparison to the WRO cells (10.9%, at similar conditions, p>0.05, data not shown). When examined microscopically, low concentration of NS-398 (50µM) induced morphological changes in NPA cells which consisted of cytoplasmic elongation (Figure 4B), whereas higher concentrations (100 and 200µM) caused apoptosis of these cells (Figure 4C and D). However, this effect was not seen in the primary thyrocytes (Figure 4E and F). Therefore, the antiproliferative effect of the COX-2 inhibitors was observed only in thyroid cancer cells. These results indicate that COX-2 is involved in thyroid cancer proliferation *in vitro*.

COX-2 inhibitor and apoptosis in thyroid cancer cell

Previous studies have shown that COX-2 inhibitors promote apoptotic cell death in colon cancer cells.^{6,7} In the next series of experiments, we examined whether the cell death that was observed at higher concentrations of NS-398 (Figure 4C and D) was due to apoptosis of the cancer cells. We treated NPA cells with 100µM NS-398 for 48 hours, collected all the floating and dish-attached cells, and then examined the cells for DNA fragmentation and reduced mitochondrial membrane potential. As shown in Figure 5, at the high concentration, NS-398 reduced mitochondrial membrane

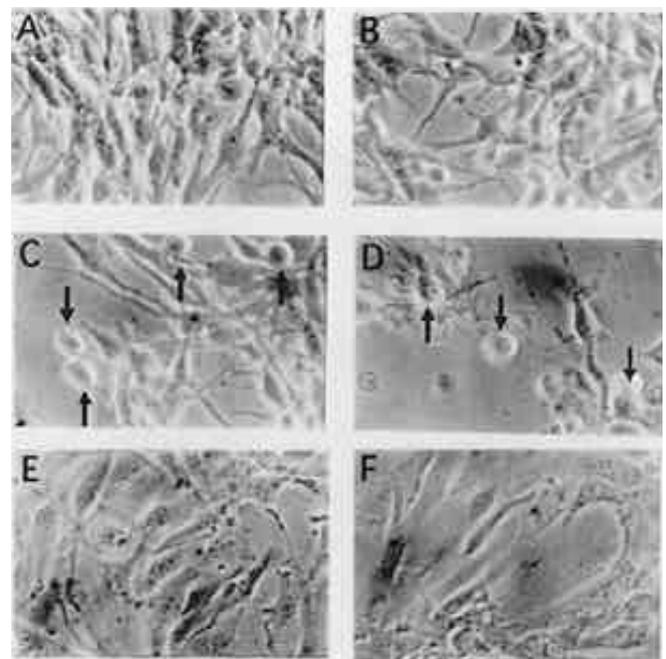


Figure 4. The microscopic findings of NPA cells and primary thyrocytes after 36 or 72 hours treatment with NS-398, respectively. Relative to the control (A), 50 M(B) NS-398 suppressed NPA cell growth. At higher concentrations, the number of NPA cell was lower and condensed nuclei and shrunken cytoplasm were observed (C; 100µM). This effect was more prominent at 200µM (D). In comparison to NPA cells, no significant morphological changes were noted between the control primary thyrocytes (E) and those treated for 72 hours with 200µM NS-398 (F). Magnification, ×200

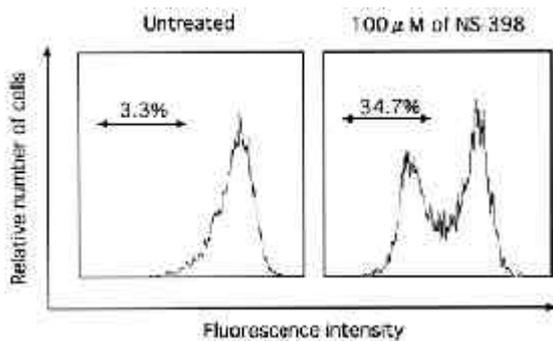


Figure 5. The mitochondrial dysfunction in NPA cells treated with NS-398. NPA cells were treated with 100 μ M of NS-398 for 48 hours. After the treatment, the cultivated cells were incubated with DiOC6 and the reduction of the mitochondrial membrane potential was determined by flow cytometry. The numbers represent the percentage of cells with reduced mitochondrial membrane potential.

potential in 34.7% of NPA cells. However, the same treatment failed to induce a marked change in DNA fragmentation (data not shown).

NS-398 regulates ERK

Our above results showed that NS-398 induced death of the thyroid cancer cell lines when used at high concentrations. However, even at the low concentration, NS-398 suppressed thyroid cancer cell growth without any significant apoptosis. Mitogen-activated protein kinase is known to be activated by various stimuli that are associated with cell growth and differentiation.²³ Recent studies have also shown that NSAIDs disrupt the endothelial cell tube formation through inhibition of ERK activation.²⁴ However, whether or not COX-2 inhibitors including NS-398, are capable of inhibiting ERK activation in thyroid cancer cells, has not been examined. Therefore, we examined whether or not NS-398 can inhibit ERK activation in thyroid cancer cell lines. NPA cells were pretreated without serum for 12 hours, and then stimulated again with serum

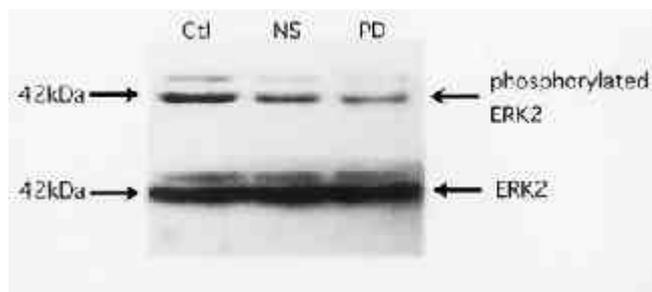


Figure 6. NS-398 inhibited ERK activation. NPA cells were cultured without serum for 12 hours with or without NS-398 and were stimulated with serum for 5 minutes (*lane NS* and *lane Ctl*, respectively). After the lysis, 20 μ g of protein was separated on 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. A western blot was performed with antibodies specific for activated ERK or total ERK. As a positive control (*lane PD*) NPA cells were pretreated with MEK inhibitor, PD98059 (20 μ M for 60 min) and thereafter stimulated with serum for 5 min.

for 5 minutes in order to analyze the activation of ERK. As expected, the activated ERK was increased by the serum stimulation and this response was reduced in the presence of NS-398 (Figure 6). These results indicated that NS-398 influenced the thyroid cancer cell growth via inhibition of ERK activation.

Discussion

Several proteins are involved in the process of carcinogenesis, and some of these proteins may serve as potential targets for novel anti-cancer agents. Among these proteins, COX-2 is one of the most promising candidates because it is expressed in many neoplasms,^{10-13,21,22} is involved in cancer cell growth and progression,⁶⁻⁹ and is weakly or not expressed in normal tissues, with some exceptions.⁵ Williams et al²⁵ showed that when the stromal cells express COX-2, they play an important role in cancer progression by becoming a source of vascular endothelial growth factor. In addition, these results indicate the importance of COX-2 in cancer progression, because COX-2 that is found in stromal cells is essentially induced by mediators origination from cancer cells. In this study, we demonstrated that COX-2 was aberrantly expressed in the thyroid neoplasm *in vivo* and *in vitro*, was similar to those in other studies.²⁶⁻²⁸ In addition, this study found that inhibition of this findings that are enzyme reduced cancer cell growth via the impairment of mitochondrial function and inhibition of ERK activation, as demonstrated *in vitro*.

Several studies have previously demonstrated that the COX-2 inhibitors exert an anti-neoplastic effect through induction of apoptosis.^{5,6} We also showed here that NS-398 induces significant growth suppression of NPA cells. Interestingly, the same agent significantly reduced the mitochondrial membrane potential without changing the hypodiploid DNA fraction (Figure 5). These results do not allow us to make a firm conclusion on whether the cell death observed in our study was apoptosis-related. Since COX-2 inhibitors open mitochondrial pores via a reduction of PGE2 production,²⁹ we assume that NPA cells underwent cell death due to mitochondrial dysfunction.

Mitogen activated-protein kinase cascade is a well-recognized chain of phosphorylase enzymes and is activated when the cells are stimulated with growth signals. Previous studies have shown that ERK is a member of this cascade and in cultured endothelial cells, NSAID including NS-398 are known to disrupt endothelial tube formation and promote cell death by inhibiting ERK activation.²⁴ Our results clearly showed that NS-398 regulated ERK activation in NPA cells (Figure 4). In contrast to these results, Xu et al³⁰ demonstrated that COX-2 was induced through the activation of ERK in rat vascular smooth muscle cells. They also showed that activated ERK enhanced not only the transcription of COX-2 mRNA but also prolonged the half-life of COX-2 mRNA. Other reports^{31,32} have indicated that COX-2 is under the regulation of ERK. These findings are quite the opposite of the hierarchy between COX-2 and ERK if COX-2 regulates ERK. The present results also

showed that ERK regulates COX-2 expression (Figure 2A). Thus, it is possible that MAP kinase pathways and COX-2 may play an important role in cell growth following mutual activation. Since COX-2 null cell can be induced to undergo apoptosis by COX-2 inhibitor,³³ it is possible that NSAID can regulate ERK without COX-2 inactivation.

In summary, we demonstrated in the present study the aberrant expression of COX-2 in thyroid cancer tissues both *in vitro* and *in vivo*, and that inhibition of COX-2 reduced cell growth and induced cell death of NPA cells. Our results also demonstrated that ERK may be regulated by COX-2 and vice versa.

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