Upregulation of thromboxane synthase in human colorectal carcinoma and the cancer cell proliferation by thromboxane A₂

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Abstract Tumor growth of colorectal cancers accompanies upregulation of cyclooxygenase-2 (COX-2), which catalyzes a conversion step from arachidonic acid to prostaglandin H₂ (PGH₂). Here, we compared the expression levels of thromboxane synthase (TXS), which catalyzes the conversion of PGH₂ to thromboxane A₂ (TXA₂), between human colorectal cancer tissue and its accompanying normal mucosa. It was found that TXS protein was consistently upregulated in the cancer tissues from different patients. TXS was also highly expressed in human colon cancer cell lines. Depletion of TXS protein by the antisense oligonucleotide inhibited proliferation of the cancer cells. This inhibition was rescued by the direct addition of a stable analogue of TXA₂. The present results suggest that overexpression of TXS and subsequent excess production of TXA₂ in the cancer cells may be involved in the tumor growth of human colorectum.

Keywords: Thromboxane; Cancer cells; Carcinoma; Colon; Cell proliferation

1. Introduction

Colorectal cancer is one of the major causes of cancer deaths in the Western world [1,2]. A large number of human colorectal cancers show overexpression of cyclooxygenase-2 (COX-2), which catalyzes a key conversion step from arachidonic acid to PGH₂ [3–8]. Long-term use of the non-steroidal anti-inflammatory drugs, which inhibit COX-2, has been suggested to be associated with a low risk of colorectal cancer although its molecular mechanism of action is at the stage of elucidation [9,10]. COX-2 is proposed as a potential target for cancer prevention [10,11]. However, clinical trial of COX-2 inhibitors was found to be associated with an elevated risk of cardiovascular events [12–14]. The COX-2 inhibitors block the production of a number of biologically active prostanooids such as PGE₂, PGD₂, PGF₂α, PGI₂ and thromboxane A₂ (TXA₂). In a downstream of the COX pathway, thromboxane synthase (TXS) catalyzes the conversion of PGH₂ to TXA₂, which is known as a potent inducer of platelet aggregation and vasoconstriction [15]. In the human colon, the production of TXA₂ is greatly increased in inflammatory bowel diseases [16]. In isolated rat and human distal colon, we found that TXA₂ is a novel secretagogue for Cl⁻ secretion [17,18]. For example, an anti-tumor drug irinotecan stimulates the release of the TXA₂ from the subepithelial layer of rat colonic mucosa, resulting in the enhanced Cl⁻ secretion [17,19]. This TXA₂-elicted pathway may explain the side effect of irinotecan-induced diarrhea.

So far, the expression level of TXS and function of TXA₂ in human colorectal cancer have not been reported. In the present study, we have found that TXS is upregulated in the tissue of human colorectal carcinoma, and that TXA₂ stimulates the cancer cell proliferation.

2. Materials and methods

2.1. Chemicals

9,11-Epithio-11,12-methano-thromboxane A₂ (STA₂; ONO Pharmaceutical Co., Osaka, Japan), sodium 4-{[2-hydroxy-5-(1-imidazolyl)-2-methylbenzyl]-3,5-dimethylbenzoate dihydrate (Y-20811; Yoshitomi Pharmaceutical Industries, Fukuoka, Japan), sodium (E)-3-[4-(3-pyr-idinylmethyl)phenyl]-2-methylacrylate (OKY-1381; Eisai Co., Tsukuba, Japan), sodium (E)-11-[2-(5,6-dimethyl-1-benzimidazolyl)-ethylidene]-6,11-dihydrobenz[b,e] oxepine-2-carboxylic acid monohydrate (KW-3635; Kyowa Hakko Kogyo Co., Shizuoka, Japan) were the generous gifts of their respective manufacturers. Thromboxane B₂ (TXB₂) was obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Human colonic cancer cell lines

HT-29 and T-84 cells were obtained from Dainippon Pharmaceuti
cal Co. (Osaka, Japan) and were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS) and Dulbecco’s modified Eagle medium/nutrient mixture F-12 (1:1 mixture) supplemented with 10% FBS, respectively. WiDr cells were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan) and were maintained in the minimum essential medium (Earle’s) supplemented with non-essential amino acids and 10% FBS. KM12-L4 cells [20] were

Abbreviations: TXA₂, thromboxane A₂; TXB₂, thromboxane B₂; TXS, thromboxane synthase; TP, thromboxane A₂ receptor; STAS, 9,11-epithio-11,12-methano-thromboxane A₂; COX, cyclooxygenase; PECAM-1, platelet-endothelial cell adhesion molecule-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PG, prostaglandin
generous gift from Drs. I. Saiki and J. Murata (University of Toyama) and maintained in minimum essential medium (Earle's) supplemented with 10% FBS.

2.3. Tissue procurement

Human colorectal carcinoma specimens were obtained from surgical resection of Japanese patients at University of Toyama Hospital in accordance with the recommendations of the Declaration of Helsinki and with the ethics committee approval. Informed consent was obtained from all the patients. In all cases, the control specimens were collected from accompanying normal mucosa, which were 5–10 cm apart from the carcinoma. The cancer tissue and the normal epithelial layer were carefully isolated from the resected colon with scissors and forceps. These samples were free from the serosa and muscularis propria. The blood vessels around the tissues were carefully removed. The clinical and histological classifications were carried out by expert pathologists according to the general rules edited by Japanese Research Society for Cancer of the Colon and Rectum and the TNM clinical classification by International Union Against Cancer.

2.4. RNA isolation, Northern blotting and TaqMan assay

Total RNAs from the human tissues and poly A+ RNAs from human colorectal cancer cell lines were prepared as described elsewhere [21], and 10 μg of total RNA or 2.5 μg of poly A+ RNA was separated on 1% agarose/formamide gel and transferred onto a nylon membrane (Zeta-probe GT, Bio-Rad). PCR products of TXS (474 bp; nucleotide positions 333–806), COX-2 (605 bp; nucleotide positions 527–1131), thromboxane A2 receptor (TP) (618 bp; nucleotide positions 322–939) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (493 bp; nucleotide positions 449–941) were used for Northern blotting [21]. Amounts of TXS, COX-2, platelet-endothelial cell adhesion molecule-1 (PECAM-1) and NF-E2 were quantified by real-time PCR (TaqMan assay) using ABI PRISM 7700 sequence detector. In the assay, amount of GAPDH was measured as control.

2.5. Western blotting

Membrane fractions of the human colorectal tissues and human colorectal cancer cell lines were prepared, and Western blotting was performed [21,22]. The blotting was performed with 80 μg of membrane protein. The intensity of the specific band was quantified by using ATTO densitograph 4.0 software. Anti-human TXS polyclonal antibody raised against a peptide corresponding to amino acids 359–377 of the TXS and anti-human COX-2 polyclonal antibody raised against a peptide corresponding to amino acids 567–599 of the COX-2 (Cayman Chemical, Ann Arbor, MI, USA) were used at 1:500 dilution. For negative control, the antibody was pre-incubated with the corresponding blocking peptide (1:1). Anti-ovine COX-1 monoclonal antibody (Cayman Chemical) was used at 1:200 dilution. Horse-radish peroxidase-conjugated anti-rabbit or anti-goat IgG was used as a secondary antibody (1:1000–1:2000 dilution).

2.6. Immunohistochemistry

Fresh-frozen tissues embedded in the Tissue-Tek O.C.T. compound (Sakura Finetecnic Co., Tokyo, Japan) were cut into 6 μm thick sections. The sections fixed in acetone for 5 min were stained by indirect immunoperoxidase technique as previously described [23]. Anti-human TXS monoclonal antibody (Tu-300; Biogenesis, Poole, UK) was used at 1:200 dilution. The sections were counterstained with 5% methyl green.
2.9. DNA fragmentation assay

The KM12-L4 cells were treated with 20 μM of KW-3635 for 2 days. Then, DNA was extracted from the cells by using the SepaGene kit (Sanko Jyunyaku Co., Tokyo, Japan), and 1.5 μg of DNA was separated on 2% agarose gel. The gel was stained with ethidium bromide (0.5 μg/ml). For positive control, the cells were treated with 10 μM of anisomycin for 12 h.

2.10. Statistics

Results are shown as means ± S.E.M. Differences between groups were analyzed by one-way ANOVA, and correction for multiple comparisons was made by using Dunnett’s multiple comparison test or Tukey’s multiple comparison test. Statistically significant differences were assumed at P < 0.05.

3. Results

3.1. Upregulation of TXS in the tissue of human colorectal cancer

In the present study, human colorectal tissues of well or moderately differentiated adenocarcinomas and accompanying normal mucosa from the patients and membrane fractions from each tissue were used. Fig. 1A shows Western blots for detecting TXS (60 kDa) and COX-2 (72 kDa) proteins and Fig. 1B shows the specificity of the antibodies for these bands. Interestingly, an increase in the expression level of TXS protein was observed in 10 out of 10 carcinomas in comparison with that of the accompanying normal tissue (Fig. 1A). Upregulation of COX-2 protein was found as previously reported [4]. In the fresh-frozen tissue sections, the cells in cancer tissues showed clear immunoreactivity for TXS (Fig. 1C, left). Such immunoreactivity was weak in normal epithelial cells in the accompanying colonic mucosa (Fig. 1C, right).

Corresponding to the present finding of TXS protein overexpression, TXS mRNA was also overexpressed in 10 out of 10 carcinomas compared with the level in the normal mucosa (Supplementary material, Fig. S1A). In contrast, no upregulation of TP mRNA was observed in the carcinoma (Fig. S1A). COX-2 mRNA was overexpressed in the carcinoma (Fig. S1A) as previously reported [3]. The overexpression of TXS and COX-2 mRNAs in the present carcinoma samples was not significantly correlated with that of PECAM-1 [24], an endothelial marker, suggesting that the overexpression was not due to angiogenesis-derived increase of blood cells (Supplementary material, Fig. S1B and C).

3.2. Expression of TXS in human colon cancer cell lines

In human colon cancer cell lines such as KM12-L4, T-84, HT-29 and WiDr, significant expression levels of TXS mRNA (Fig. 2A) and TXS protein (Fig. 2B) were observed. In non-colonic cancer cell lines such as HL-60 and HeLa cells, no obvious expression of TXS mRNA was observed (Fig. 2A). Significant expression levels of COX-2 mRNA (Fig. 2A) and COX-2 protein (Fig. 2B) were observed in KM12-L4, HT-29 and WiDr cells.

3.3. Inhibition of the cancer cell proliferation by TXS inhibitors and stimulation of the proliferation by a stable analogue of TXA2

TXS inhibitors such as OKY-1581 [25] and Y-20811 [26] significantly inhibited the proliferation of KM12-L4 colon cancer cells (Fig. 3A–C), suggesting that the product of TXS has a proliferative effect. Thus, effect of STA2, a stable analogue of TXA2, on the cell proliferation was tested. STA2 was previously confirmed to mimic the effect of endogenous TXA2 in rat and human colonic mucosa [18,19]. STA2 significantly accelerated the proliferation of KM12-L4 cells, whereas TXB2 was ineffective (Fig. 3D–E). On the other hand, neither STA2 nor TXB2 stimulated the migration of the cells (data not shown).
3.4. Inhibition of the cancer cell proliferation by the antisense oligonucleotide for TXS

An antisense strategy was employed to deplete endogenous TXS protein in the KM12-L4 colonic cancer cells (Fig. 4A). This strategy decreased TXS protein, while it had no effect on the expression levels of COX-1 and COX-2 (Fig. 4A). Depletion of TXS by the antisense oligonucleotide significantly inhibited the cell proliferation (Fig. 4B), while it had no significant effect on the cell migration (not shown). In the negative control experiments using the 4-mismatch oligonucleotide, both the cell proliferation (Fig. 4B) and the cell migration (not shown) were normal.

Inhibition of the cell proliferation by the antisense oligonucleotide for TXS was rescued by the direct application of STA2 (Fig. 4C). Furthermore, STA2 was effective in the cells treated with the 4-mismatch oligonucleotide (Fig. 4D).

3.5. Inhibition of the cancer cell proliferation by a TP antagonist

The STA2-induced cell proliferation was inhibited by KW-3635 [27], a specific TP antagonist (Fig. 5A). In the absence of STA2, the basal cell proliferation was also inhibited by KW-3635 (Fig. 5B), suggesting that endogenously released TXA2 acts on the receptor in the cancer cells and stimulates the cell proliferation. Similar effects of KW-3635 and STA2 plus KW-3635 were observed in the cells treated with the antisense oligonucleotide for TXS (Fig. 5C) or the 4-mismatch oligonucleotide (Fig. 5D). KW-3635 caused no apoptosis on the cells (Supplementary material, Fig. S2A). No change of the viability was observed in the cells treated with KW-3635 (Supplementary material, Fig. S2B).

4. Discussion

In the present study, we have found that TXS is overexpressed in human colorectal cancer tissues (Figs. 1 and S1A); and that TXS is significantly expressed in the colonic cancer cell lines (Fig. 2). The expression level of TP in the cancer tissues was similar to that in the normal mucosa (Fig. S1A). The cancer cell proliferation was inhibited by TXS inhibitors (Fig. 3A–C),...
antisense oligonucleotide for TXS (Fig. 4) and a TP antagonist (Fig. 5). The inhibition by antisense oligonucleotide was rescued by the addition of a stable analogue of TXA₂ (Fig. 4C). These results suggest that upregulation of TXS in colorectal cancer cells results in increased production of TXA₂ and the released TXA₂ induces proliferation of the cancer cells.

Recently, significant roles of TXS in human cancer cells have been reported in prostate [28] and bladder [29]. In human prostate cancer cell lines, TXS inhibitors reduced the cell migration, and overexpression of TXS accompanied an increase in cell motility [28]. In human bladder cancer cell lines, TXS inhibitors reduced the growth, migration and invasion [29].

The occurrence of upregulation of TXS in the colorectal carcinoma is consistently very high among the samples from different patients (Fig. 1). The upregulation of TXS was not parallel to that of COX-2 in the carcinoma (Figs. 1A and S1A). It is noted that mRNA of nuclear factor E2 (NF-E2), which binds to the NF-E2/AP1 site of the promoter region of TXS [30], was overexpressed in the colorectal cancer tissue, and that this overexpression of NF-E2 was positively and significantly correlated with that of TXS \( (r = 0.647, \ P < 0.05) \) (Supplementary material, Fig. S3A). No significant correlation was observed between the expression levels of NF-E2 and COX-2 \( (r = 0.350, \ P > 0.05) \) (Supplementary material, Fig. S3B). Therefore, the regulatory mechanism of expression of TXS and COX-2 may be independent. In a future study, it is necessary to clarify the mechanism of upregulation of NF-E2 in the colorectal cancer.

Inducible microsomal prostaglandin E synthase (mPGES), which converts PGH₂ to PGE₂, was overexpressed in 83% of human colorectal cancers [31]. An aberrant expression of mPGES-1 in combination with COX-2 can contribute to tumorigenesis [32]. PGE₂ has been suggested to be associated with colorectal carcinoma via two different EP receptors: it stimulates cell proliferation, invasion and migration via the EP4 receptor [33] and angiogenesis via the EP2 receptor [34]. Therefore, both the mPGES-PGE₂ pathway and the present TXS-TXA₂ pathway are involved in colorectal carcinoma. At present, the functional relationship between these two pathways in the colorectal carcinoma is unknown and it would be an interesting subject to be clarified in a future study.

In conclusion, we have found that TXS is overexpressed in human colorectal carcinoma and TXA₂ is involved in the colorectal cancer proliferation. As a novel function of TXA₂ in the colorectal carcinomas, we suggest that TXA₂ stimulates the cancer cell proliferation. For treatment of colorectal cancer, TXS inhibitors which block the production of TXA₂ may be clinically effective.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.felslet.2006.05.007.

References


