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Upregulation of thromboxane synthase in human colorectal carcinoma and the cancer cell proliferation by thromboxane A_2

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Abstract **Tumor growth of colorectal cancers accompanies upregulation of cyclooxygenase-2, which catalyzes a conversion step from arachidonic acid to prostaglandin H_2 (PGH_2). Here, we compared the expression levels of thromboxane synthase (TXS), which catalyzes the conversion of PGH_2 to thromboxane A_2 (TXA_2), 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 colonic 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 TXA_2. The present results suggest that overexpression of TXS and subsequent excess production of TXA_2 in the cancer cells may be involved in the tumor growth of human colorectum.**
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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 PGH_2 [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 prostanoids such as PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (TXA_2). In a downstream of the COX pathway, thromboxane synthase (TXS) catalyzes the conversion of PGH_2 to TXA_2, which is known as a potent inducer of platelet aggregation and vasoconstriction [15]. In the human colon, the production of TXA_2 is greatly increased in inflammatory bowel diseases [16]. In isolated rat and human distal colon, we found that TXA_2 is a novel secretagogue for Cl^- secretion [17,18]. For example, an anti-tumor drug irinotecan stimulates the release of the TXA_2 from the subepithelial layer of rat colonic mucosa, resulting in the enhanced Cl^- secretion [17,19]. This TXA_2-elicited pathway may explain the side effect of irinotecan-induced diarrhea.

So far, the expression level of TXS and function of TXA_2 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 TXA_2 stimulates the cancer cell proliferation.

2. Materials and methods

2.1. Chemicals

9,11-Epithio-11,12-methano-thromboxane A_2 (STA_2; ONO Pharmaceutical Co., Osaka, Japan), sodium 4-[α-hydroxy-5-(1-imidazolyl)-2-methylbenzyl]-3,5-dimethylbenzoate dihydrate (Y-20811; Yoshitomi Pharmaceutical Industries, Fukuoka, Japan), sodium (*E*)-3-[4-(3-pyridylmethyl)phenyl]-2-methylacrylate (OKY-1581; Eisai Co., Tsukuba, Japan), sodium (*E*)-11-[2-(5,6-dimethyl-1-benzimidazolyl)-ethylidene]-6,11-dihydrobenz[*b*,*e*] oxepine-2-carboxylate monohydrate (KW-3635; Kyowa Hakko Kogyo Co., Shizuoka, Japan) were the generous gifts of their respective manufacturers. Thromboxane B_2 (TXB_2) 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 Pharmaceutical 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 the 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

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Abbreviations: TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; TXS, thromboxane synthase; TP, thromboxane A_2 receptor; STA_2, 9,11-epithio-11,12-methano-thromboxane A_2; COX, cyclooxygenase; PECAM-1, platelet-endothelial cell adhesion molecule-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PG, prostaglandin

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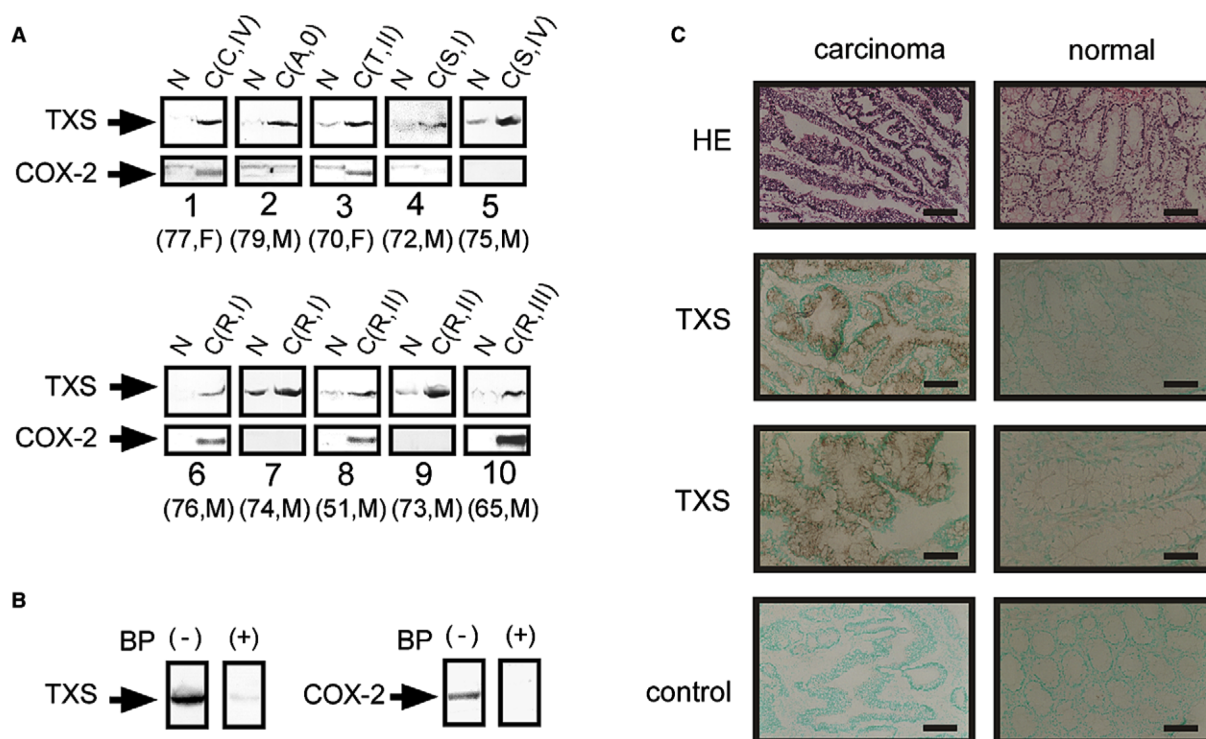

Fig. 1. Upregulation of TXS protein in human colorectal carcinomas. (A) Western blotting for detecting proteins of TXS (60 kDa) and COX-2 (72 kDa) in paired normal mucosa (N) and adenocarcinoma (C). Location of each carcinoma (C, cecum; A, ascending colon; T, transverse colon; S, sigmoid colon; R, rectum) and stage of the carcinoma according to the TNM clinical classification by International Union Against Cancer (0, I, II, III or IV) are indicated in the upper parenthesis. Age and sex (M, male; F, female) of patients are indicated in the lower parenthesis. (B) Specificity of the antibodies for the bands of 60 kDa (TXS) and 72 kDa (COX-2) was confirmed by using the corresponding blocking peptide (BP). (C) Immunostaining for TXS in tissues of the carcinoma and accompanying normal mucosa. Representative pictures of carcinoma (left lane) and normal mucosa (right lane) are shown. HE, histology of the tissue was checked by hematoxylin–eosin staining. Scale bars, 200 μm (left) and 100 μm (right). TXS, tissue sections were immunostained with the anti-TXS monoclonal antibody. Scale bars, 200 μm (upper left), 100 μm (upper right and lower left) and 50 μm (lower right). Control, control staining in the absence of the primary antibody. Scale bars, 200 μm (left) and 100 μm (right).

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 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 A_2 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 colonic 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 Finetechnical 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 (Tü-300; Biogenesis, Poole, UK) was used at 1:200 dilution. The sections were counterstained with 5% methyl green.

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