Activation of matrix metalloproteinase-2 in human breast cancer cells overexpressing cyclooxygenase-1 or -2

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Abstract Human breast cancer cell line Hs578T was stably transfected with cDNA for cyclooxygenase-1 or -2. When the cells overexpressing cyclooxygenase-1 or -2 were stimulated with concanavalin A, the processing of matrix metalloproteinase-2 was observed with the aid of gelatin zymography. This processing was not seen in mock-transfected and original cells which did not express detectable cyclooxygenase activity. Furthermore, Northern blotting showed 8–13 fold induction of membrane-type 1 matrix metalloproteinase which processed matrix metalloproteinase-2 in the cells expressing cyclooxygenases. These findings suggest that both isoforms of cyclooxygenase mediate the processing of matrix metalloproteinase-2 through induction of membrane-type 1 metalloproteinase in breast cancer cells.

Key words: Cyclooxygenase-1; Cyclooxygenase-2; Breast cancer cell; Cancer metastasis; Matrix metalloproteinase

1. Introduction

Cyclooxygenase is a rate limiting enzyme for prostaglandin (PG) synthesis from arachidonic acid. It incorporates two molecules of oxygen into arachidonic acid and produces PGG2 which is reduced by the same enzyme to PGH2. Two isoforms of the enzyme are known; cyclooxygenase-1, which is a constitutive enzyme expressed in many tissues, and cyclooxygenase-2, which is an inducible enzyme synthesized at inflammatory lesions by cytokines, growth factors and tumor promoters [1,2]. A number of studies have demonstrated that cyclooxygenases are expressed in tissues obtained from various cancer lesions. The majority of these studies reported an increased expression of cyclooxygenase-2 isoform in cancer tissues [3–10], although the up-regulation of cyclooxygenase-1 was also documented [11,12]. While evidence for the mechanical involvement of cyclooxygenases in the development of colorectal cancer has been accumulating, the role of these enzymes in other cancers has not been fully determined yet [13–16].

The finding that non-steroidal anti-inflammatory drugs reduce the risk of breast cancer [17,18] suggests that cyclooxygenase is involved in the promotion of this type of cancer. In fact, both cyclooxygenase-1 [11] and -2 [4,11] were reported to be elevated in human breast cancer tissues. Furthermore, PG production was reported to be greater in highly metastatic breast cancer cells than in non-metastatic cells [19,20]. However, the involvement of the two cyclooxygenase isoforms in the progress and metastasis of breast cancer is not yet fully understood, although one report mentioned that overexpression of cyclooxygenase-2 in colon cancer CaCo2 cells increased the metastatic potential [21]. The aim of this study was to investigate whether the two cyclooxygenase isoforms could affect the expression of matrix metalloproteinases (MMPs) related to cancer metastasis. Among MMPs, processing by limited proteolysis of MMP-2 by membrane-type 1 MMP produces an active form which plays an important role in metastasis [22]. We introduced the cDNA for each of the two isoforms of cyclooxygenase into human breast cancer Hs578T cells which originally did not show significant cyclooxygenase activity and examined the activation of MMP-2 through the induction of membrane-type 1 MMP.

2. Materials and methods

2.1. Materials

Human breast cancer cells Hs578T were kindly provided by Dr. T. Sasaki (Kanazawa University, Japan). Expression vector pEF-BOS was a generous gift from Dr. S. Nagata (Osaka University, Japan). [1-^{14}C]Arachidonic acid (2.1 GBq/mmol), [α-^{32}P]dCTP (111 TBq/mmol), the Megaprime DNA labeling kit and Hybond-N+ nylon membrane were purchased from Amersham (Buckinghamshire, UK), QuikHyb hybridization solution from Stratagene (La Jolla, CA, USA), restriction enzymes from Toyobo (Osaka, Japan), Isogen from Nippongene (Tokyo, Japan), precoated silica gel plate for thin layer chromatography from Merck (Darmstadt, Germany), concanavalin A, indomethacin and geneticin from Sigma (St. Louis, MO, USA), Iscove's modified Dulbecco's medium and lipofectamine from Gibco BRL (Grand Island, NY, USA) and fetal bovine serum from JRH Bioscience (Lenexa, KS, USA). CT1746 was provided by Celltech (Slough, UK). All the other reagents were of analytical grade.

2.2. Cyclooxygenase assay

Hs578T cells (5×10^5/dish) were cultured in Iscove's modified Dulbecco's medium supplemented with 5% fetal bovine serum, 100 units/ml of penicillin G and 100 μg/ml of streptomycin. The cells were scraped from the dishes, suspended in 100 μl of 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA, and sonicated on ice at 20 kHz three times each for 5 s. The sonicated cells were then incubated at 24°C for 10 min with 25 μM [1-^{14}C]arachidonic acid (1.85 kBq/5 nmol/5 μl ethanol solution) in 200 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 2 μM hematin and 5 mM tryptophan. The reaction was quenched by the addition of ice-cold diethyl ether/methanol/1 M citric acid (30:4:1, v/v) and the product was extracted into an ether layer which was spotted onto a thin layer chromatography plate. The plate was developed with an organic layer of ethyl acetate/water/2,2,4-tri-

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Abbreviations: PG, prostaglandin; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide

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methylpentane/acetic acid (110:100:50:20, v/v) at room temperature. The radioactive products on the plate were visualized and quantified by a Fujix bioimaging analyzer BAS 1000 (Tokyo, Japan). Protein concentration was determined by the method of Lowry et al. with bovine serum albumin as the standard [23].

2.3. Construction of vectors and transfection

The expression vector pEF-BOS with a powerful elongation factor-1α promoter [24,25] was digested with *Sap*I, blunted with a Klenow fragment, and ligated to a neomycin-resistant gene. The resultant vector pBOSNeo was digested with *Xba*I and ligated to cDNAs for human cyclooxygenase-1 [26] or cyclooxygenase-2 [27] with the *Xba*I site attached at both ends. cDNAs for cyclooxygenase-1 and -2 were fully sequenced with the dideoxynucleotide chain termination method [28]. The constructs pBOSNeoCOX-1 and pBOSNeoCOX-2 as well as the parental pBOSNeo vector (mock transfection) were transfected to Hs578T with the aid of lipofectamine according to the manufacturer's instruction. After 3 days the cells were split at a ratio of 1:50 and plated in the medium containing 1 mg/ml of geneticin. Approximately 100 colonies were taken from each transfection and their cyclooxygenase expression was examined with the enzyme activity assay.

2.4. Gelatin zymography

Hs578T cells overexpressing cyclooxygenase-1 or cyclooxygenase-2 were plated in 35-mm dishes at a density of 5×10^4 cells/dish and cultured overnight. The culture medium was changed to 500 μl of the medium without serum and compounds to be tested were added. The cells were incubated for 18–24 h and 10 μl of the medium was subjected to 8% SDS-polyacrylamide gel electrophoresis containing 1 mg/ml of gelatin. The gel was washed twice in 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$, 2.5% Triton X-100, 0.02% NaN_3 and 1 μM $ZnCl_2$, and then incubated at 37°C for 12 h in 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl, 10 mM $CaCl_2$ and 0.02% NaN_3 followed by staining with Coomassie brilliant Blue R-250.

2.5. Northern blotting

Total RNA was isolated from the cells by using Isogen, and 20 μg of the denatured RNA was separated on 1% agarose gel. The RNA was then transferred to Hybond-N+ nylon membrane and UV cross-linked. The membrane was hybridized in QuikHyb hybridization solution at 68°C for 2 h with the membrane-type 1 MMP cDNA [22] probe radiolabeled with the Megaprime DNA labeling kit. The membrane was washed in 0.2×SSC and 0.1% SDS at 65°C for 30 min. The radioactivity on the membrane was detected and quantified with the Fujix bioimaging analyzer BAS 1000.

3. Results

3.1. Overexpression of cyclooxygenase-1 or -2 in the human breast cancer cell line

When the sonicated human breast cancer Hs578T cells were incubated with radiolabeled arachidonic acid, hardly any products were observed on a thin layer chromatogram as shown in Fig. 1. The cDNA for cyclooxygenase-1 or -2 was inserted downstream of the powerful elongation factor-1α promoter in pEF-BOS, which also contained a neomycin resistant gene. The resultant vectors were then transfected to Hs578T cells and geneticin resistant clones were selected. Several clones from each of the transfections exhibited cyclooxygenase activity. Fig. 1 shows thin layer chromatography analysis of the products in clones overexpressing cyclooxygenase-1 or -2. The major product of both of these enzymes was PGE2, although the minor production of 6-keto-PGF1α, a degradation product of unstable prostacyclin, was also observed. The clones with different specific activities were selected as shown in Table 1. The specific activities were 0.06–0.27 nmol/10 min/mg of protein in cyclooxygenase-expressing cells, whereas mock-transfected cells exhibited hardly any enzyme activity.

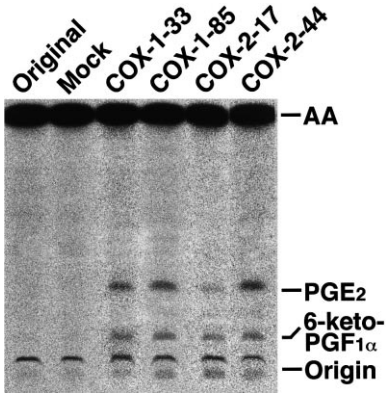

Fig. 1. Thin layer chromatography analysis of arachidonic acid metabolites in Hs578T cells overexpressing cyclooxygenase-1 or -2. The cell extract (500 μg protein) from the cells overexpressing cyclooxygenase-1 (COX-1-33 and -85) or cyclooxygenase-2 (COX-2-17 and -44) was incubated with [1-^{14}C]arachidonic acid under standard conditions. The extract from original and mock-transfected cells was also incubated. AA, arachidonic acid.

3.2. MMP-2 activation in Hs578T cells overexpressing cyclooxygenase-1 or -2

Hs578T cells were incubated with serum-free medium and the harvested medium was subjected to gelatin zymography (Fig. 2A). A band of 68 kDa was observed which corresponded to the inactive form of MMP-2 in terms of its molecular weight. This was in agreement with previously reported results showing that MMP-2 was expressed in Hs578T [29,30]. The addition of concanavalin A to the cells overexpressing either cyclooxygenase-1 or cyclooxygenase-2 resulted in the dramatic appearance of active forms of MMP-2 (64 and 62 kDa) on gelatin zymography (Fig. 2A). Concanavalin A is an inducer of membrane-type 1 MMP which processes MMP-2 to convert it to its active forms [22]. The clone COX-2–44, which showed the highest cyclooxygenase activity (Table 1), produced by far the most densely band of active forms, and the extent of processing appeared to depend on the enzyme activity of the clones rather than on the difference in isoforms. These active forms of MMP-2 were not observed in the original and mock-transfected cells stimulated with concanavalin A. MMP-2 was not significantly processed in cyclooxygenase-expressing cells without concanavalin A treatment.

We also examined the effects of cyclooxygenase inhibitors on MMP-2 activation. The processing of MMP-2 in cyclooxygenase-overexpressing cells was not inhibited in the cells treated with 50 μM indomethacin, a cyclooxygenase inhibitor (Fig. 2B). Furthermore, the following inhibitors were tested with the same results: aspirin at 200 μM, ketoprofen at 50 μM and sulindac at 50 μM (data not shown). Preincubation for up to 48 h of the cells with these inhibitors produced no change in the results (data not shown). Only sulindac sulfide at 50 μM reduced the density of active forms (Fig. 2B). However, the level of the inactive form was also reduced by this compound.

3.3. Induction of membrane-type 1 MMP in Hs578T cells overexpressing cyclooxygenase-1 or -2

To investigate the mechanism of MMP-2 activation, CT1746, a reversible and MMP species non-selective inhibitor was employed. As shown in Fig. 2B, the active forms of MMP-2 (64 kDa and 62 kDa) completely disappeared as a

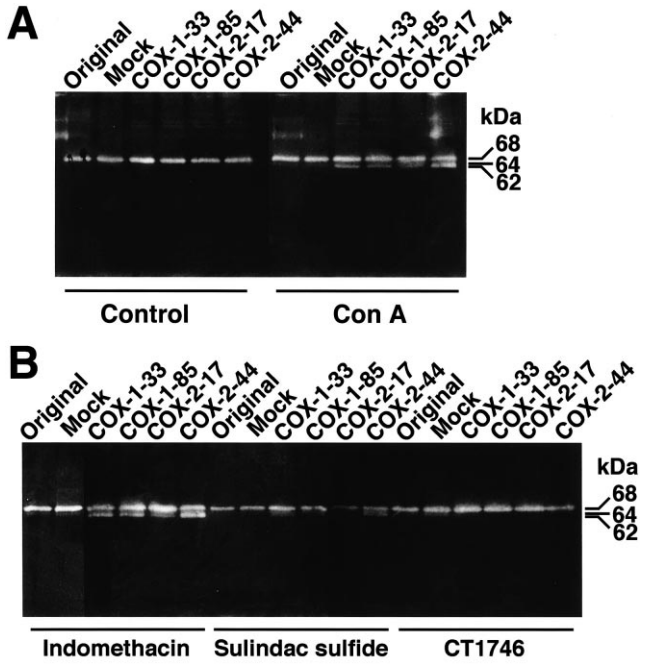

Fig. 2. MMP-2 activation in cyclooxygenase-overexpressing Hs578T cells treated with concanavalin A. A: Cells overexpressing cyclooxygenase-1 (COX-1-33 and -85) or cyclooxygenase-2 (COX-2-17 and -44) as well as original and mock-transfected cells were incubated in the absence (Control) or presence of 20 μg/ml of concanavalin A (Con A). The harvested medium was analyzed by means of gelatin zymography as described in Section 2. B: Effect of various inhibitors on the processing of MMP-2. Indomethacin and sulindac sulfide at 50 μM and CT1746, a reversible and MMP species non-selective inhibitor, at 5 μM were included in addition to concanavalin A.

result of the treatment of cyclooxygenase-expressing cells with CT1746. This result agrees with the previous finding that membrane-type 1 MMP is necessary for the processing of MMP-2 [22]. We further examined by means of Northern blot analysis whether membrane-type 1 MMP mRNA would be increased in the cyclooxygenase-expressing Hs578T cells. As shown in Fig. 3, the expression level of membrane-type 1 MMP was very low in the mock-transfected cells, and the treatment of the cells with concanavalin A showed a four-fold increase of membrane-type 1 MMP mRNA as compared with the non-treated cells. However, when cyclooxygenase-expressing cells were incubated with concanavalin A, a striking induction of membrane-type 1 MMP mRNA was observed (8–13-fold, Fig. 3B). Without concanavalin A treatment, membrane-type 1 MMP mRNA was increased two- to three-fold in the cyclooxygenase-expressing cells compared to the mock-transfected cells.

Table 1
Cyclooxygenase activities in Hs578T cells overexpressing cyclooxygenase-1 or -2

Clone	Cyclooxygenase activity (nmol/10 min/mg of protein)		
	Control	Indomethacin	Sulindac sulfide
Original	< 0.02	ND	ND
Mock-transfected	< 0.02	ND	ND
COX-1-33	0.18	< 0.02	0.03
COX-1-85	0.17	< 0.02	0.06
COX-2-17	0.06	< 0.02	0.05
COX-2-44	0.27	< 0.02	0.22

The cells overexpressing cyclooxygenase-1 (COX-1-33 and -85) or cyclooxygenase-2 (COX-2-17 and -44) as well as original and mock-transfected cells were sonicated and enzyme activities were determined as described in Section 2. Indomethacin or sulindac sulfide was added at a concentration of 5 μM. ND, not determined.

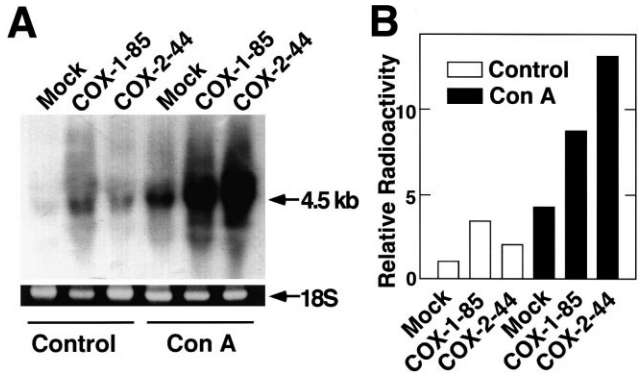

Fig. 3. Membrane-type 1 MMP induction in cyclooxygenase-overexpressing Hs578T cells stimulated by concanavalin A. A: Northern blot analysis of membrane-type 1 MMP. Cells overexpressing cyclooxygenase-1 (COX-1-85) or cyclooxygenase-2 (COX-2-44) as well as mock-transfected cells were incubated for 6 h in the absence (Control) or presence of 20 μg/ml of concanavalin A (Con A). Total RNA was subjected to Northern blot analysis using radiolabeled membrane-type 1 MMP as a probe. Ethidium bromide staining of 18S ribosomal RNA is also shown. B: Radioactivity was quantified and normalized with the intensity of 18S ribosomal RNA, which was determined by NIH image software. Ratios over MMP mRNA of mock cells without concanavalin A are shown.

4. Discussion

We have demonstrated that overexpression of either cyclooxygenase-1 or cyclooxygenase-2 in human breast cancer Hs578T cells brought about the dramatic activation of MMP-2 as a result of treatment with concanavalin A (Fig. 2A). The level of the activation appeared to depend on the enzyme activity of cyclooxygenases rather than on the difference in isoforms. The activation of MMP-2 could be explained by the strikingly increased expression level of membrane-type 1 MMP mRNA in the cyclooxygenase-expressing cells (Fig. 3). Concanavalin A by itself has been shown to elevate membrane-type 1 MMP mRNA both by transcriptional activation and mRNA stabilization but the elevation was not dramatic [31–33]. Under our experimental conditions too, only a slight induction produced by concanavalin A was observed in the mock-transfected cells (Fig. 3). Only a few studies have demonstrated that membrane-type 1 MMP expression and MMP-2 activation are induced from almost undetectable to high levels [34,35]. It is known that the extent of MMP-2 activation is not necessarily proportional to the expression level of membrane-type 1 MMP, which implies that there is a threshold of membrane-type 1 MMP level for MMP-2 activation [32]. At least two molecules of membrane-type 1 MMP are thought to be required for MMP-2 processing, one functioning as substrate presentation complex and the other as a processing enzyme, and these two components should be near each other for efficient MMP-2 processing to take place on the cell surface [36]. Under our experimental conditions, this threshold level of membrane-type 1 MMP

seemed to be at least five times higher than the basal level in the cyclooxygenase-expressing Hs578T cells (Fig. 3B).

As far as we were able to determine, no significant inhibition of MMP-2 activation by cyclooxygenase inhibitors could be observed (Fig. 2B). Even prolonged incubation of the cells up to 48 h with these inhibitors before addition of concanavalin A yielded the same results. Of the various inhibitors tested, only sulindac sulfide caused a slight decrease in the amount of activated forms of MMP-2 (Fig. 2B) but this compound also suppressed the level of the inactive form. This might be explained by the fact that more than 30% of cells had died after 18 h incubation with sulindac sulfide as assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide (MTT) assay (data not shown). Furthermore, sulindac sulfide only partially inhibited cyclooxygenase activity, whereas indomethacin completely inhibited the enzyme activity (Table 1). It was described that MMP-2 activation was inhibited by 50 μM sulindac sulfide in human colon cancer CaCo2 cells overexpressing cyclooxygenase-2 [21]. In any case, however, addition of PGE2, PGF2α or PGD2 could not activate MMP-2 by itself ([21] and F. Kawahara et al., unpublished observations). It has also been reported that the stimulated growth of immortalized ECV endothelial cells which were tumorigenically transformed by cyclooxygenase-1 overexpression was not inhibited by indomethacin [37]. These findings suggest that cyclooxygenases exert their pathophysiological functions not only through PG production but also by means of unknown mechanisms in the growth and metastasis of cancer cells [37]. In this connection, it is of interest that a protein specifically interacting with cyclooxygenase-1 and cyclooxygenase-2 has been identified [38].

In summary, we were able to demonstrate that both cyclooxygenase-1 and -2 play a role in the activation of MMP-2 via membrane-type 1 MMP induction in a human breast cancer cell line. It should be mentioned that not only cyclooxygenase-2 but also cyclooxygenase-1 is up-regulated in breast cancer tissues [4,11]. Our findings, combined with those by other investigators, suggest that both isoforms of cyclooxygenase play a role in the pathophysiology of breast cancer.

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