Differential Expression and Regulation of Cyclooxygenase-1 and -2 in Two Human Breast Cancer Cell Lines

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Abstract

Cyclooxygenase (COX), also referred to as prostaglandin endoperoxide synthase, is the rate-limiting enzyme for the metabolic conversion of arachidonic acid to prostaglandins (PGs) and related eicosanoids. Some human breast cancers synthesize large quantities of PGE₂, but the regulatory mechanisms involved are unclear. We have examined the expression of the two isoforms of this enzyme, COX-1 and COX-2, their regulation by tetradecanoyl phorbol acetate (TPA), and the associated PGE₂ production in two human breast cancer cell lines with different biological phenotypes. Estrogen-dependent MCF-7 cells exhibited a relatively high expression of COX-1; COX-2 was barely detectable but was transiently induced by treatment with TPA (10 nM). In contrast, the estrogen-independent, highly invasive, metastatic MDA-MB-231 cell line showed a low expression of COX-1 but a high constitutive level of COX-2. This high COX-2 expression applied to both the protein and mRNA and increased further over a relatively long period of time in the presence of TPA. The extent of PGE₂ production by the two cell lines correlated well with the level of COX-2 protein, suggesting that this isoform is required for both their constitutive and mitogen-induced PGE₂ synthesis. Moreover, overexpression and persistent expression of COX-2 may be influenced by breast tumor hormone status and seem to be a feature of the aggressive, metastatic phenotype.

Introduction

AA³ metabolites, such as PGs, thromboxanes, and various lipoxygenase products, are produced in many tissues and are responsible for a wide variety of biological responses. Among these, the are potent mediators affecting a number of signal transduction pathways that modulate cellular adhesion, growth, and differentiation (1). A major regulatory step occurs at the level of PG endoperoxide synthase, or COX, activity. Two isoforms of COX have been identified; COX-1 is constitutively expressed in most tissues and considered to generate COX, activity. Two isoforms of COX have been identified; COX-1 and COX-2. Elevated levels of PGE₂, a major product of COX-2, have been widely reported in many human breast cancers as well as experimental murine mammary tumor models (7, 8). Several studies with murine mammary tumor cells indicate that PGE₂ may have a multifunctional role in controlling growth, metastasis, and the host immune response in breast cancer (9–11). Furthermore, high levels of PGE₂ are often associated with estrogen receptor-negative tumors that exhibit a high metastatic potential (7, 9). A recent report from our laboratory showed that whereas a high dietary α-6 fatty acid intake stimulates the growth and metastasis of the estrogen-independent MDA-MB-435 human breast cancer cell line in nude mice, these effects are significantly reduced by concurrent treatment with indomethacin, a COX inhibitor (12).

Despite these diverse observations, our understanding of the mechanisms involved in controlling PGE₂ synthesis by breast tumors is limited; the pathological significance of the differential ability of PGE₂ production in breast cancer cell lines is unknown. In the present study, we examined COX-1 and COX-2 expression and their regulation by TPA and PGE₂ production in the estrogen-dependent, poorly invasive, and nonmetastatic MCF-7 cell line (13) and the estrogen-independent, highly invasive, and metastatic MDA-MB-231 cell line (14).

Materials and Methods

Cell Culture. The MCF-7 cell line was provided as a gift by Dr. Robert Clarke (Lombardi Cancer Center, Washington, DC). The MDA-MB-231 human breast cancer cell line was obtained from the American Type Culture Collection (Rockville, MD). Both cell lines were routinely maintained in Iscove's modified Dulbecco's medium (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum, penicillin, and streptomycin, as described previously (14). For treatment with TPA, subconfluent cells were washed and changed to serum-free medium containing 0.1% BSA for 24 h. Incubation was continued with or without TPA for various times.

Preparation of Microsomal Protein. The solubilized microsomal protein was prepared as described by Siros et al. (15) and Müller-Decker et al. (16) with some modification. Subconfluent growth-arrested cells were homogenized on ice in Tris-EDTA-diethylthiocarbamic acid (TED) buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1 mM diethylthiocarbamic acid] containing 2 mM octylglucoside and then centrifuged at 100,000 × g for 1 h at 4°C. The crude pellets were sonicated in TED sonication buffer [20 mM Tris-HCl (pH 8.0), 50 mM EDTA, and 1 mM diethylthiocarbamic acid] containing 45 mM octylglucoside. The sonicates were centrifuged at 13,000 × g at 4°C, and the recovered supernatants were stored at −80°C until the electrophoretic analyses were performed.

Preparation of Cell Lysates. Cells were harvested and lysed as described previously (15). Briefly, cells were rinsed twice and scraped with 1.5 ml of cold Ca²⁺ and Mg²⁺-free PBS containing 4 mM iodoacetate. After centrifugation, the pellets were resuspended in 50 μl CHAPS extraction solution [10 mM CHAPS; 2 mM EDTA (pH 8.0), and 4 mM iodoacetate in PBS] with protease inhibitors. The samples were incubated for 15 min on ice and centrifuged at 15,000 × g for 5 min. The supernatant was processed for electrophoresis. Protein content was measured by using a protein assay kit from Sigma (St. Louis, MO).

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3 The abbreviations used are: AA, arachidonic acid; PG, prostaglandin; COX, cyclooxygenase; TPA, tetradecanoyl phorbol acetate; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]1-propanesulfonate.
Western Blotting. The proteins from microsomes and cell lysates were electrophoresed on a 9% SDS-polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride membrane (DuPont New England Nuclear, Wilmington, DE). Primary antibodies to COX-1 and COX-2 (Oxford BioMed, Oxford, MI) were used at a final dilution of 1:100. Secondary horseradish peroxidase-linked donkey anti-rabbit IgG antibody was used at a dilution of 1:12,000. Filters were developed by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). Ovine recombinant COX-1 and chicken recombinant COX-2 (Oxford BioMed) were used as positive controls.

Isolation of Total RNA and Northern Blotting. Total RNA extraction from cells and the Northern blot analysis procedure have been detailed elsewhere (17). Briefly, cells cultured under the desired conditions were harvested, and total RNA was isolated and fractionated on a 1.2% agarose-formaldehyde gel. After transfer to a nitrocellulose membrane, hybridization was performed with a α-32P-labeled COX-2 cDNA probe (Oxford BioMed). The membranes were subsequently hybridized with a β-actin cDNA probe (a gift from Dr. Z. Ronai, American Health Foundation, Valhalla, NY) to monitor RNA loading.

Assay of COX Enzymatic Activity and PGE2 Measurement. Cells were rinsed twice with ice-cold Ca2+, Mg2+-free PBS containing 4 mM iodoacetate and lysed in CHAPS extraction solution. COX enzymatic activity was assayed by measuring the conversion of added AA (Sigma, St. Louis, MO) to PGE2 as described by Han et al. (5). After addition of AA at a final concentration of 100 μM, the reaction mixture was incubated for 30 min at 37°C. Aliquots of the incubation mixture were assayed for PGE2 by radioimmunoassay using a kit purchased from PerSeptive Diagnostics, Inc. (Cambridge, MA).

Results

Expression of COX-1 in Human Breast Cancer Cell Lines. The constitutive levels of COX-1 and the time course for the effect of TPA on COX-1 expression in both MCF-7 and MDA-MB-231 human breast cancer cell lines were studied by Western blot analysis. Fig. 1 is a representative result and demonstrates that there is a higher basal level of COX-1 in MCF-7 than in MDA-MB-231 cells. A single application of 10 nM TPA to quiescent cells led to a decrease in COX-1 expression in MCF-7 cells, whereas the level in MDA-MB-231 cells was not affected.

Expression of COX-2 and TPA Regulation in Human Breast Cancer Cell Lines. The expression of COX-2 in the MCF-7 and MDA-MB-231 cell lines and its response to TPA were assessed using both Western and Northern blotting techniques. As shown in Fig. 2A, Western blotting with protein samples extracted from microsomes showed no detectable levels of COX-2 in the absence of TPA. With the addition of TPA, COX-2 was induced within 5 h in both cell lines. However, the induction of COX-2 in MCF-7 cells was only transient and had returned to control levels by 24 h despite the continued presence of TPA, whereas COX-2 was persistently expressed in MDA-MB-231 cells, with a band shift that indicated a small change in molecular weight. However, Western blot analysis on cell lysate showed that MDA-MB-231 cells expressed a high constitutive level of COX-2 that increased further over a relatively long period of time in the presence of TPA (Fig. 2B).

Northern blot analysis was performed to determine whether the difference in COX-2 protein basal levels and the induction in response to TPA in two cell lines was due to a different degree of mRNA expression. Fig. 3 shows that there was no detectable basal level of COX-2 mRNA in MCF-7 cells, but that there was a rapid and transient induction by TPA. In contrast, the MDA-MB-231 cells demonstrated a high constitutive expression of COX-2 mRNA, which was greatly and persistently increased in the presence of 10 nM TPA.

COX-2 Enzymatic Activity and the Induction by TPA. To determine the relationship between COX-2 expression and COX enzy-
mic activity in the two breast cancer cell lines, the production of PGE$_2$ was measured in the presence of exogenous substrate. Table 1 shows that MCF-7 cells produced only a low basal PGE$_2$ level when incubated with AA and that treatment with TPA produced a substantial increase after 5 h ($P < 0.01$ compared with unstimulated secretion), but one that showed a decline after 24 h. The MDA-MB-231 cell basal level of PGE$_2$ production from AA was approximately five times higher than that of MCF-7 cells ($P < 0.01$), with an additional increase that was maintained over the 24-h incubation period after TPA exposure.

**Discussion**

There are two critical elements that determine tumor PGE$_2$ production: (a) the regulation of substrate availability, controlled by phospholipase A$_2$; and (b) the capacity of the cell to metabolize free AA to PGs, determined by COX activity. We have previously demonstrated that there is a high level of phospholipase A$_2$ activity in the highly invasive and metastatic MDA-MB-231 cell line (18) and now report that these cells and the MCF-7 cell line, with very different degrees of expression of the malignant phenotype, vary in both constitutive and TPA-induced COX activity.

The present study showed that the biologically aggressive, invasive MDA-MB-231 cell line, in contrast to MCF-7 cells, possesses a high constitutive level of COX-2 and also exhibits a further and prolonged elevation in the activity of the isozyme in response to TPA; moreover, PGE$_2$ production was correlated with the level of COX-2 protein, but not COX-1. This association of COX-2 with invasive capacity is consistent with other reports linking overexpression of the isozyme with changes in cell adhesion and inhibition of apoptosis (5, 6), both of which may result in enhanced local invasion and uncontrolled tumor cell growth.

In clinical studies, high PGE$_2$ concentrations in primary breast cancers have been associated with both high metastatic potential and a lack of estrogen and progesterone receptors (9). Similarly, Fulton and Heppner (10) found in a series of murine mammary tumors that there was a strong positive correlation between PGE$_2$ levels and metastatic capacity. We observed that the progression of dietary linoleic acid-stimulated MDA-MB-435 human breast cancer cells growing in nude mice was suppressed by treatment with indomethacin at a dose that partially blocked the synthesis of several cyclooxygenase but not lipoxygenase products (12). However, indomethacin is primarily an inhibitor of COX-1, and the clinical use of indomethacin is complicated by a high incidence of gastrointestinal side-effects (19). The results reported here suggest that selective COX-2 inhibitors, which are currently undergoing development (19), should be employed in additional studies to evaluate pharmacological inhibition of breast cancer PG production as an approach to chemotherapy or chemoprevention.

In the present study, the degree of COX-2 mRNA induction, enzyme protein production, and enzyme activity as judged by PGE$_2$ synthesis were all closely related in MCF-7 cells. However, in the MDA-MB-231 cell line, scanning densitometry revealed an 8-fold increase in COX-2 mRNA in response to TPA but only a 75% corresponding increase in the isozyme protein (data not shown); PGE$_2$ production correlated well with the level of COX-2 protein but not mRNA. These observations are in agreement with the reports by Lee et al. (20) and Kargman et al. (21) that high COX-2 mRNA expression may occur in human colorectal adenomas without a concomitant elevation in COX-2 protein; it seems likely that COX-2 may undergo complex posttranscriptional and posttranslational modification to yield the active enzyme.

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**References**

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