Cyclooxygenases in Human and Mouse Skin and Cultured Human Keratinocytes: Association of COX-2 Expression with Human Keratinocyte Differentiation

JANE LEONG, MILLIE HUGHES-FULFORD, NINA RAKHLIN, AIDA HABIB,* JACQUES MACLOUF,* AND MARC E. GOLDYNE1

Veterans Affairs Medical Center and University of California San Francisco, San Francisco, California 94121; and *Unite 348, INSERM Hopital Lariboisiére, Paris, France

INTRODUCTION

Epidermal expression of the two isoforms of the prostaglandin H-generating cyclooxygenase (COX-1 and COX-2) was evaluated both by immunohistochemistry performed on human and mouse skin biopsy sections and by Western blotting of protein extracts from cultured human neonatal foreskin keratinocytes. In normal human skin, COX-1 immunostaining is observed throughout the epidermis whereas COX-2 immunostaining increases in the more differentiated, suprabasilar keratinocytes. Basal cell carcinomas express little if any COX-1 or COX-2 immunostaining whereas both isozymes are strongly expressed in squamous cell carcinomas deriving from a more differentiated layer of the epidermis. In human keratinocyte cultures, raising the extracellular calcium concentration, a recognized stimulus for keratinocyte differentiation, leads to an increased expression of both COX-2 protein and mRNA; expression of COX-1 protein, however, shows no significant alteration in response to calcium. Because of a recent report that failed to show COX-2 in normal mouse epidermis, we also looked for COX-1 and COX-2 immunostaining in sections of normal and acetone-treated mouse skin. In agreement with a previous report, some COX-1, but no COX-2, immunostaining is seen in normal murine epidermis. However, following acetone treatment, there is a marked increase in COX-1 expression as well as the appearance of significant COX-2 immunostaining in the basal layer. These data suggest that in human epidermis as well as in human keratinocyte cultures, the expression of COX-2 occurs as a part of normal keratinocyte differentiation whereas in murine epidermis, its constitutive expression is absent, but inducible as previously published. © 1996 Academic Press, Inc.

MATERIALS AND METHODS

Two isoforms of the prostaglandin-forming enzyme cyclooxygenase have been cloned and sequenced [1–5]. The isoform designated COX-1 is constitutively expressed in cells whereas the isoform designated COX-2 appears to require specific induction. Studies showing that the ulcerogenic or nephrotoxic effects of drugs that inhibit both COX-1 and COX-2 activity can be prevented by using newer agents (e.g., NS-398) that only inhibit COX-2 activity have led to the concept that COX-1 regulates prostaglandin synthesis associated with cellular homeostasis whereas COX-2 contributes proinflammatory prostaglandins [6].

Human epidermis is a source of prostaglandins [7], and prostaglandin E2 (PGE2) generation can induce cutaneous vasodilation as well as regulate both epidermal cell proliferation and cytokine secretion [8–10]. A previous study from our laboratory further demonstrates that PGE2 influences human keratinocyte differentiation [11]. Recently, normal murine epidermis was found to express COX-1 but not COX-2; however, COX-2 could be induced either by mechanical wounding or by topical application of the phorbol ester TPA [12]. Using specific polyclonal antibodies, the immunohistochemical expression of COX-1 and COX-2 were assessed in specimens of human and murine epidermis and human cutaneous basal cell and squamous cell carcinomas. In addition, Western immunoblotting with specific monoclonal antibodies was used to probe expression of the isozymes in protein extracts from human keratinocytes cultured under conditions favoring either proliferation or differentiation. The results, to be described, suggest that the expression of COX-2 in human keratinocytes is related to differentiation of these cells both in vivo and in culture.
FIG. 1. COX-1 and COX-2 antisera specificity using dot immunoblotting. (a) 1, COX-1 protein + anti-COX-1; 2, COX-2 protein + anti-COX-2; 3, COX-1 protein + anti-COX-1 preabsorbed with COX-1 protein; and 4, COX-2 protein + anti-COX-1 preabsorbed with COX-1 protein. (b) 1, COX-1 protein + anti-COX-2; 2, COX-2 protein + anti-COX-2; 3, COX-1 protein + anti-COX-2 preabsorbed with COX-2 protein; and 4, COX-2 protein + anti-COX-2 preabsorbed with COX-2 protein.

Arbor, MI), the Vectastain ABC-AP kit, levamisole solution, and alkaline phosphatase substrate kit from Vector (Burlingame, CA), the ECL Western blotting analysis system from Amersham (Arlington, IL), PVDF membranes, acrylamide, bis, SDS, Tris, glycine, TEMED, and ammonium sulfate from Bio-Rad (Hercules, CA), the Tri Reagent LS from Molecular Research Center (Cincinnati, OH), the GeneAmp RNA PCR kit from Perkin-Elmer Cetus (Norwalk, CT), and the PMSF from Sigma (St. Louis, MO). The human COX-2 cDNA probe was purchased from Oxford Scientific (Oxford, MI), and the COX-2 oligonucleotides primers were synthesized at the University of California Biomolecular Resource Center (San Francisco, CA).

Antibodies. The monoclonal anti-COX-1 and anti-COX-2 antibodies have been previously characterized as have the polyclonal rabbit antisera against the specific N-terminal sequence for human COX-1 and the polyclonal rabbit antisera against the specific 19-amino acid sequence in the C-terminal portion of human COX-2 [13].

To confirm the specificity of the polyclonal antisera, dot immunoblotting was performed using the Amersham ECL Western blot analysis system. Purified COX-1 and COX-2 proteins were individually applied to an equilibrated PVDF membrane. The membranes were incubated for 1 h at room temperature in blocking buffer (5% nonfat milk+0.1% Tween-TBS, pH 7.5) followed by incubation with either the appropriate anti-COX antisera or preabsorbed antisera for 1 h at room temperature. Following exposure to the primary antisera, the membranes were washed (2×15 min) with blocking buffer, and then exposed to antibody labeled with horseradish peroxidase for 1 h at room temperature. The membranes were again washed (2×15 min) with blocking buffer, exposed to freshly prepared substrate for 1 min, and then evaluated by autoradiography.

Human skin specimens. Formalin-fixed, paraffin-embedded biopsy sections were evaluated for the presence of COX-1 and COX-2 following the protocol enclosed with the Vectastain ABC-AP kit. After rehydration and equilibration in PBS-CMF, specimens were blocked with diluted normal goat serum in buffer for 20 min. The primary antibody, either anti-COX-1 antisera (1:100-1:200 dilution in blocking buffer) or anti-COX-2 antisera (1:200-1:400) was applied for 30 min. After washing (2×5 min) with PBS-CMF, the secondary biotinylated affinity-purified anti-rabbit IgG antibody was applied for 30 min. Following another washing, an avidin-biotinylated alkaline phosphatase complex (ABC-AP complex) was applied to the specimens for 40 min. Vector-red alkaline phosphatase substrate was used to localize the COX enzymes. Controls consisted of samples treated as in the full detection protocol but without the primary antibody. Specificity of immunostaining was assessed by quenching the primary antisera by preabsorbing with the appropriate purified COX protein.

Western immunoblotting. The COX-1 and COX-2 expressed in cultured keratinocytes were studied using Western blotting. Keratinocyte proteins were obtained by resuspending pelleted keratinocytes in extraction buffer (62.5 mM Tris, 0.5 mM DTT, 2% SDS, 1 mM fresh PMSF, pH 6.8), and denaturing the proteins by placing the sample test tubes in a water bath at 95°C for 5 min. Protein concentration was determined using the Bio-Rad DC Protein Assay kit. Electrophoresis of total protein was done using a 5% stacking and 7% running SDS mini-gel under denaturing conditions at a constant 200 V for 50-60 min in cold Tris/glycine SDS buffer, pH 8.3. The proteins were transferred onto equilibrated PVDF membranes in a Mini-Trans-Blot apparatus (Bio-Rad). The membranes were exposed to blocking buffer (5% nonfat milk+0.1% Tween-TBS, pH 7.5) at 4°C overnight. The membranes containing immobilized proteins were then incubated with the appropriate primary monoclonal anti-COX antibodies for 1 h at room temperature, followed by 2×15 min washings with blocking buffer. The membranes were then exposed to antibody labeled with horseradish peroxidase (Amersham ECL kit) for 1 h at room temperature, washed 2×15 min with blocking buffer, and finally exposed to freshly prepared substrate (Amersham ECL kit) for 1 min before performing autoradiography.

FIG. 2. COX-1 and COX-2 immunostaining in normal human skin. (a) Hematoxalin and eosin (H & E) stain, (b) control, (c) anti-COX-1, (d) anti-COX-2, (e) anti-COX-1 preabsorbed with COX-1 protein, and (f) anti-COX-2 preabsorbed with COX-2 protein. Note the increased immunostaining for COX-2 (arrow) in the stratum granulosum (d). Original magnification, 50×.
Antisera Specificity

Specificity of the antisera was confirmed by fluid phase absorption. The N-terminal specific anti-COX-1 antiserum recognized the COX-1 protein but did not cross-react with the COX-2 protein (Fig. 1a). The COX-1 signal could be eliminated by preabsorbing the antisera with COX-1 protein. The C-terminal specific anti-Cox-2 antiserum was likewise specific for COX-2 protein and the signal could be quenched by preabsorbing with COX-2 protein (Fig. 1b).

Immunohistochemical Localization of COX-1 and COX-2 in Human Skin

Figure 2 shows the expression of COX-1 and COX-2 in normal human epidermis. COX-1 immunostaining (Fig. 2c) appeared uniformly distributed throughout the epidermis. Expression of COX-2, on the other hand, appeared primarily suprabasal with less signal in the basal cell layer. Furthermore, COX-2 immunostaining showed increasing intensity in the upper layers of the epidermis with the most intense signal in the stratum granulosum (Fig. 2d). Preabsorption of both antisera with the appropriate COX protein resulted in significant quenching of the immunostaining (Figs. 2e and 2f).
DISCUSSION

The patterns of COX-1 and COX-2 immunostaining in the biopsies of normal human skin suggest that increased expression of COX-2 is part of normal human keratinocyte differentiation. An increasing signal is observed as one moves from the suprabasilar stratum spinosum of the epidermis to the stratum granulosum where the signal is most intense (Fig. 2d). COX-1 expression, on the other hand, appears to be more evenly distributed throughout the epidermis (Fig. 2c) in agreement with previous data documenting its constitutive expression in murine epidermis [12].

The association of increased COX-2 expression with more differentiated keratinocytes is also seen when comparing basal cell and squamous cell carcinomas. The nests of abnormal basal cells, the least differentiated form of keratinocyte in the epidermis, do not show significant COX-2 (or COX-1) immunostaining whereas the islands of abnormal squamous cells show intense reactions for both isoforms. However, even in the squamous cell carcinoma, COX-1 expression is more uniform throughout the islands of abnormal squamous cells whereas COX-2 expression appears to increase from the periphery toward the center of the abnormal islands of squamous cells (Figs. 3d and 3e). These findings further support the concept that in human epidermis, COX-2 expression is associated with differentiation.

In contrast to our finding of COX-2 expression in normal human epidermis, a recent study by Scholz et al. was unable to demonstrate COX-2 protein in unstimulated murine epidermis [12]. We also found that, immunohistochemically, unstimulated murine epidermis failed to express COX-2 (Fig. 4). The possibility that lack of cross-reactivity of our anti-COX-2 antiserum with murine COX-2 is responsible for the observed results is precluded by the fact that stimulation of the epidermis by acetone treatment, which removes the complex lipid barrier of the skin [16], led to the appearance of positive quenchable immunostaining for COX-2 (Figs. 4e and 4f) within the basal cell layer. Thus, human and murine skin appear to differ in normal expression of COX-2.

Whereas Cameron et al. [17] have reported increased PGE2 synthesis among more differentiated, in comparison to basal, mouse epidermal cells, our immunostaining patterns (Fig. 4) for COX-1 and COX-2 suggest that the less differentiated basal cells should be the major source of PGE2. In fact, a previous study by Henke et al. using hairless mice found results opposite to those of Cameron et al.: namely, the basal cells, rather than the more differentiated keratinocytes, generated the most PGE2 [18], a finding consistent with our immunohistochemical observations. It is noteworthy that our studies and those of Henke et al. used hairless mice whereas Cameron et al. studied hair-bearing female inbred SENCAR mice.

We have previously demonstrated that cultured human keratinocytes, induced to differentiate by increasing extracellular calcium, synthesize increased levels of PGE2 within 1 h after the calcium concentration is increased [11]. Whether the increased synthesis is due to increased activity of COX-1 or COX-2 (since some COX-2 is seen in low-calcium medium) is not answered by the current studies. However, using NS398 as a specific COX-2 inhibitor should help to clarify the relative roles of COX-1 and COX-2 in the calcium response. There is also the possibility, suggested by studies in a murine osteoblastic cell line, that PGE2, itself, may feed back to increase the expression of COX-2 [19]. Thus the initial calcium stimulus, possibly operating through the arachidonoyl-specific cytosolic phospholipase A2 which is present in human epidermis [20], may increase COX-1-generated PGE2, which, in turn, induces COX-2 expression as reported in the murine cell line. This possibility is currently under investigation.

The appearance of immunostaining for COX-2 in human epidermis as an accompaniment to differentiation bears relevance to data on the role of PGE2 in cell differentiation. In early studies, Kischer reported the ability of PGE1 to enhance differentiation of chicken skin [21], and subsequently, Schaefer et al. reported the ability of PGE2 to enhance squamous cell development in cultures of chicken skin [22]. In human fetal lung organ
culture, PGE$_2$, but not PGF$_{2\alpha}$, was found to accelerate the process of self-differentiation [23]. In this study, indomethacin retarded self-differentiation, which could be reversed by adding exogenous PGE$_2$ to the indomethacin-treated cultures. In similar experiments, our laboratory found that indomethacin retarded the formation of the cornified envelope, a recognized marker for keratinocyte differentiation; in this model, PGE$_2$ also overrides the indomethacin-induced suppression [11]. Finally, in the U937 human monocytic cell line, differentiation was associated with the induction of COX-2 expression whereas the expression of COX-1 remained unchanged [24].

Whereas the above data support a role for PGE$_2$ in the differentiation of some cell types including keratinocytes, the relative importance of the two cyclooxygenase isoforms remains to be established. In a human monocytic leukemia cell line, it has been reported that phorbol ester-induced differentiation is accompanied by an increase in the levels of COX-1 mRNA and protein with no significant effect on COX-2 mRNA or protein [25]. In complete contrast, exposure of murine epidermal cells to the same phorbol ester results in induction of COX-2 mRNA and protein [12]. Consequently, depending on the cell type, either of the isoforms of cyclooxygenase may be involved in contributing prosta-glandins that may regulate cell differentiation. Further studies are needed to clarify the relative participation of the COX isozymes in human keratinocyte differentiation. However, the data presented implicate the inducible COX-2 as a relevant enzyme in differentiating human keratinocytes and reveal some differences between the expression of COX-1 and COX-2 in murine as compared to human skin.

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REFERENCES


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