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

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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.

INTRODUCTION

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 E_2 (PGE_2) 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 PGE_2 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.

MATERIALS AND METHODS

Reagents. Purified Cox-1 (ram seminal vesicles) and Cox-2 (sheep placenta) proteins were purchased from Cayman Chemical (Ann

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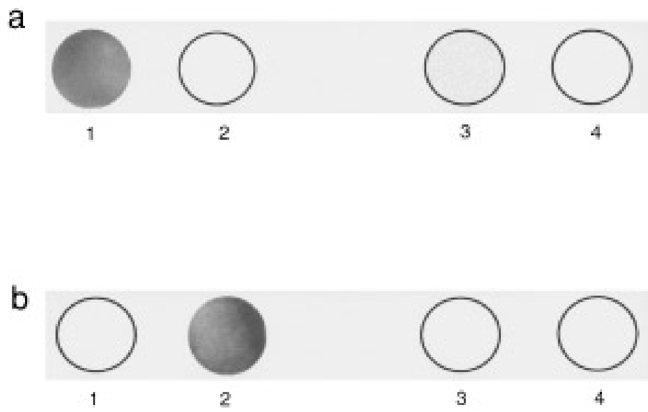


FIG. 1. COX-1 and COX-2 antisera specificity using dot immunoblotting. (a) 1, COX-1 protein + anti-COX-1; 2, COX-2 protein + anti-COX-1; 3, COX-1 protein + anti-COX-1 preabsorbed with COX-1 protein; and 4, COX-2 protein + anti-COX-1 preabsorbed with COX-1 protein. (b) 1, COX-1 protein + anti-COX-2; 2, 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 antiserum against the specific N-terminal sequence for human COX-1 and the polyclonal rabbit antiserum 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 biopsies (obtained for clinical diagnostic purposes) from normal human skin and from basal and squamous cell carcinomas were kindly supplied by Dr. Timothy McCalmont of the Department of Pathology at the University of California San Francisco. Five-micrometer tissue sections were deparaffinized in xylene (2× 10 min), rehydrated with sequential washes in 95 and 70% ethanol, and equilibrated in phosphate-buffered saline (PBS–CMF) before being evaluated for expression of COX-1 and COX-2 using immunohistochemistry.

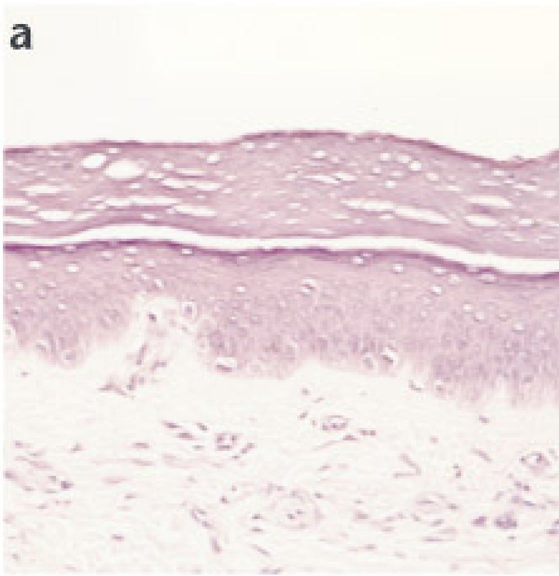
Mouse skin specimens. Karnosky-fixed, paraffin-embedded biopsies from hairless mouse skin were kindly supplied by the laboratory of Dr. Peter Elias of the Section of Dermatology at the San Francisco VA Medical Center. The 5-μm sections were treated in the same way as human skin for immunohistochemistry. Biopsies were provided from mice whose skin was either untreated or acetone treated prior to sampling.

Human keratinocyte cultures. Human keratinocytes were isolated from neonatal foreskins by treatment with 50% dispase for 24 h at 4°C. Primary cultures were established in serum-free keratinocyte growth medium KGM containing 0.07 m*M* calcium and 70 μg/ml of bovine pituitary extract. Cells were utilized after the second passage. For biochemical studies, keratinocytes were cultured in T150 flasks (Nunc, Inc.) containing either low (0.07 m*M*) or high (1.2 m*M*) calcium. The cells were harvested by trypsinization and washed in PBS–CMF before protein, DNA, or RNA extraction.

Immunohistochemistry. 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 antiserum (1:100–1:200 dilution in blocking buffer) or anti-COX-2 antiserum (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 m*M* Tris, 0.5 m*M* DTT, 2% SDS, 1 m*M* 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 transfered onto equilibrated PVDF membranes in ice-cold electrophoresis buffer with 10% methanol at a constant 300 mA using 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×.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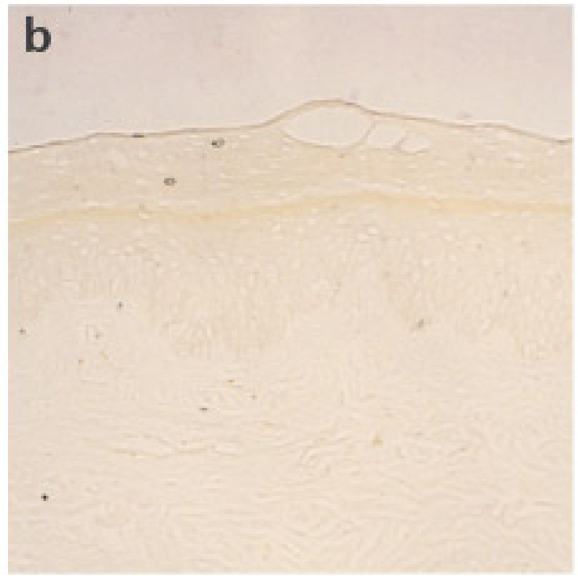
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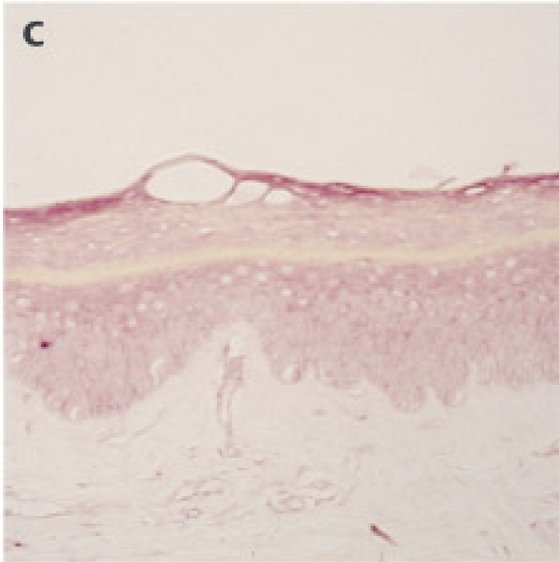
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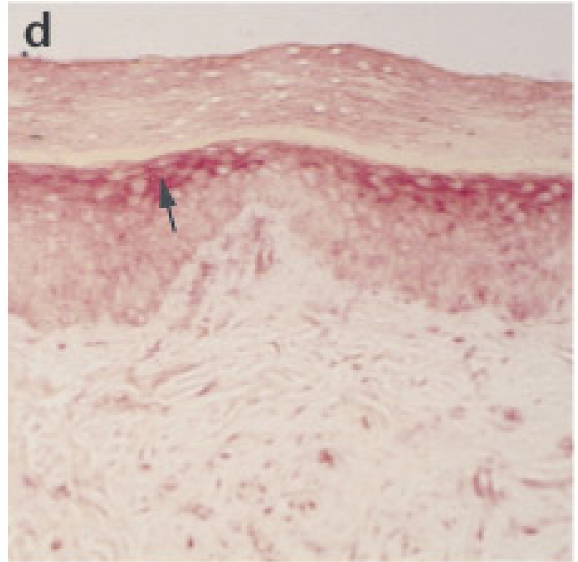
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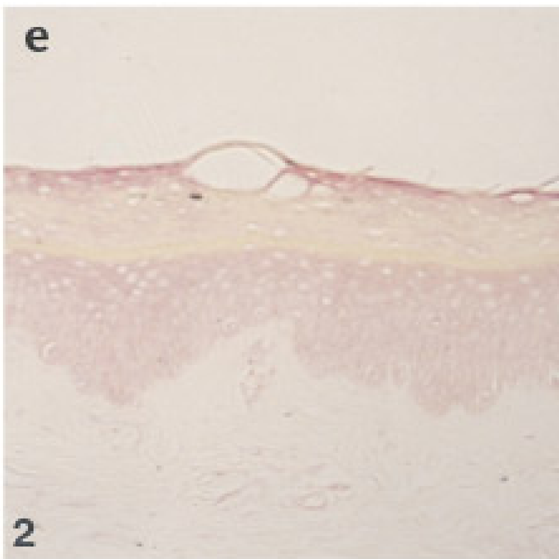
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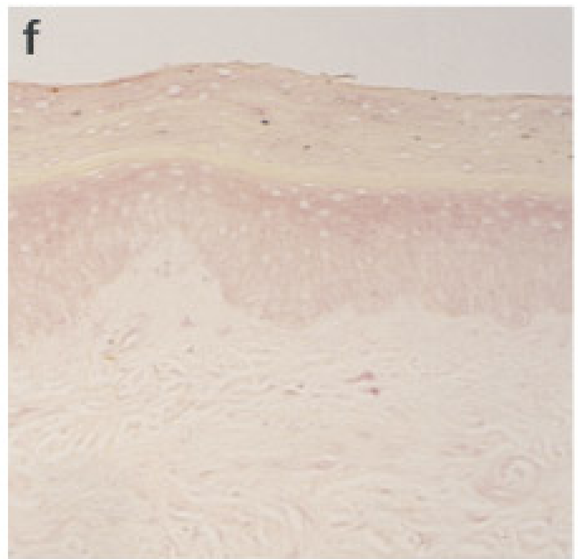
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RNA isolation. Total RNA was obtained from cultured keratinocytes using the Tri Reagent LS protocol. Briefly, keratinocytes were harvested by trypsinization and the pellets were collected. Tri Reagent (1 ml) was added to each pellet sample and allowed to react for 5 min at room temperature. Chloroform (300 μl) was then added to each sample and the samples were mixed throughly. After 10 min at room temperature, the mixtures were centrifuged for 15 min at 4°C. The aqueous layer which contained the RNA was removed and the RNA was precipitated with isopropanol at −20°C. The RNA pellet was washed with cold 70% ethanol twice and resuspended in DEPC-treated water. Total RNA concentration was determined using the A_{260}/A_{280} ratio. The mRNA was purified using an oligo-dT column prepared according to the Invitrogen protocol and its purity was checked by running an RNA formaldehyde gel (1% agarose).

Reverse transcription (RT) reactions and cDNA amplification by PCR. RT reactions of purified keratinocyte mRNA were run according to the GeneAMP RNA PCR kit protocol. Copies (2.5×10^4) of pAW109, which contains an IL-1a RNA template, were added as an internal control for the reverse transcriptase reactions. Aliquots of the RT reaction were then used for PCR amplification of COX-2 and cyclophilin cDNAs. Cyclophilin was used as an internal control for the PCRs. All PCRs were run with the RT cDNA, AmpliTag, PCR buffer, primers, MGC12, and dNTP, according to the GeneAMP RNA PCR kit protocol. The PCRs were optimized for the particular primers to be used. COX-2 primers were designed and tested for specificity based on published GenBank human sequences. The COX-2 primers produced the predicted 724-bp product and it was identified by Southern blot using a human COX-2 cDNA probe from Oxford. The COX-2 and cyclophilin PCRs were run for 30 cycles. PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide to visualize DNA bands. Photographs were then taken using Polaroid 667 film with a Polaroid DS-34 camera.

RESULTS

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 antiserum 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).

Immunohistochemical Localization of COX-1 and COX-2 in Basal and Squamous Cell Carcinomas

Figure 3 shows the immunostaining patterns for COX-1 and COX-2 in basal and squamous cell carcinomas. In the basal cell carcinoma little immunostaining was observed for either COX-1 or COX-2 (Figs. 3c and 3e). In contrast, the squamous cell carcinoma showed strong staining for both isozymes (Figs. 3d and 3f). However, COX-2 expression appeared strongest near the center rather than at the periphery of the islands of transformed squamous cells whereas the immunostaining for COX-1 was more uniform throughout the islands.

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

Figure 4 shows the localization of COX-1 and COX-2 expression in hairless mouse skin. In untreated mouse skin, there was a faint, scattered COX-1 signal among the basal cells but no evidence of COX-2 immunostaining (Figs. 4c and 4e). However, with acetone-treated skin, induction of both COX-1 and COX-2 proteins became clearly evident along the basal cell layer (Figs. 4d and 4f). Strong expression of COX-2 was also observed in the sebaceous glands of both untreated and treated mice. The signals were quenched when the antisera were preabsorbed with the respective COX proteins (data not shown).

COX-1 and COX-2 Expression in Cultured Human Keratinocytes

Because the immunostaining pattern for COX-2 in human epidermis showed an increased association with the more differentiated suprabasal keratinocytes, we studied the expression of COX-1 and COX-2 in keratinocyte cultures under conditions that respectively favor either proliferation or differentiation [14, 15]. Protein extracts from keratinocytes maintained in low calcium-containing medium (0.07 m*M*) for 72 h were compared to extracts taken from keratinocytes induced to differentiate by switching the calcium concentration to 1.2 m*M* for 72 h. The Western blots probed with the monoclonal antibody for COX-1 (Fig. 5a) or for COX-2 (Fig. 5b) showed that the COX-1 protein was equally expressed in both low and high calcium cultures whereas expression of COX-2 protein was significantly upregulated by increasing the calcium concentration in the medium.

Expression of COX-2 mRNA in Cultured Keratinocytes

Because of the increased expression of COX-2 protein under culture conditions favoring differentiation, the expression of COX-2 mRNA was next evaluated in a

separate experiment. An RT–PCR was performed on extracted keratinocyte RNA using the COX-2 primer developed in the laboratory of one of the authors (M.H.-F.). The results reflect an increase in the expression of COX-2 mRNA by keratinocytes cultured for 96 h in high calcium whereas COX-2 mRNA expression in keratinocytes cultured for an equal time in low calcium was barely detectable.

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.

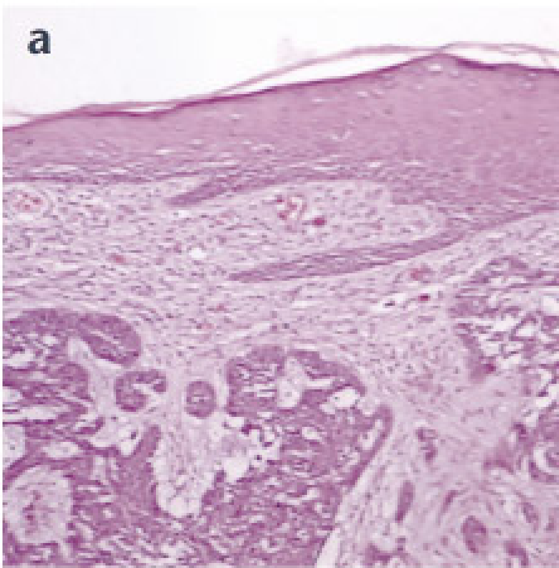
Whereas Cameron *et al.* [17] have reported increased PGE_2 synthesis among more differentiated, in comparison to basal, mouse epidermal cells, our immunostaining patterns (Fig. 4) for COX-1 and COX-2 suggest that the less differentiated basal cells should be the major source of PGE_2. In fact, a previous 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 PGE_2 [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 PGE_2 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 PGE_2, 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 A_2 which is present in human epidermis [20], may increase COX-1-generated PGE_2, 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 PGE_2 in cell differentiation. In early studies, Kischer reported the ability of PGE_1 to enhance differentiation of chicken skin [21], and subsequently, Schaefer *et al.* reported the ability of PGE_2 to enhance squamous cell development in cultures of chicken skin [22]. In human fetal lung organ

FIG. 3. COX-1 and COX-2 immunostaining in basal and squamous cell carcinomas. Basal cell carcinoma: (a) H & E, (c) anti-COX-1, (e) anti-COX-2. Squamous cell carcinoma: (b) H & E, (d) anti-COX-1, (e) anti-COX-2. Note the characteristic immunostaining with anti-COX-2 in the epidermis overlying the basal cell carcinoma (e) but a lack of significant staining of the nests of transformed basal cells for either COX-1 (c) or COX-2 (e). Original magnification, 50×.

FIG. 4. COX-1 and COX-2 immunostaining in mouse skin. Normal skin: (a) H & E, (c) anti-COX-1, (e) anti-COX-2. Acetone-treated skin: (b) H & E, (d) anti-COX-1, (f) anti-COX-2. Note staining of sebaceous glands (s) for both COX-1 and COX-2. Acetone treatment induces stronger immunostaining for COX-1 and COX-2 among the basal cells of the epidermis (arrows). Original magnification, 50×.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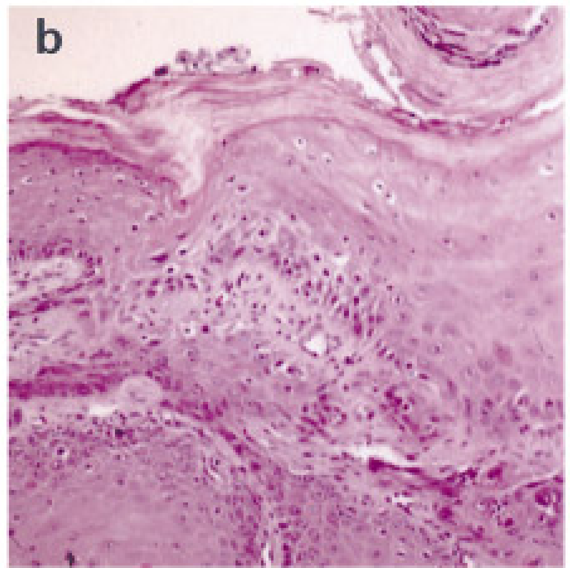
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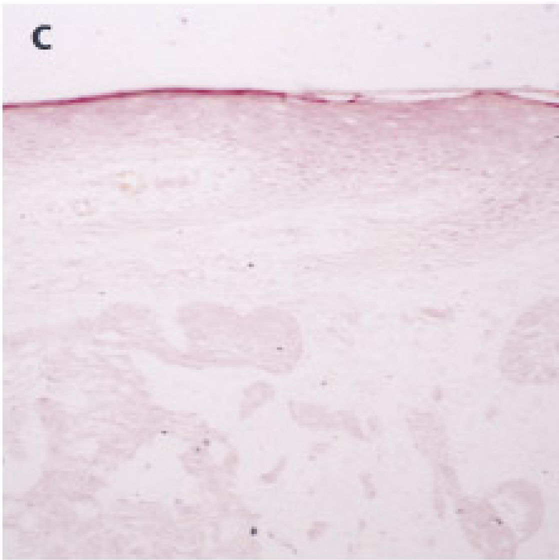
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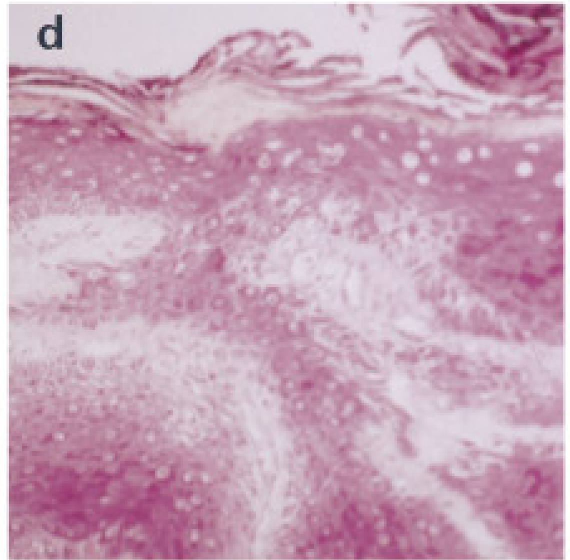
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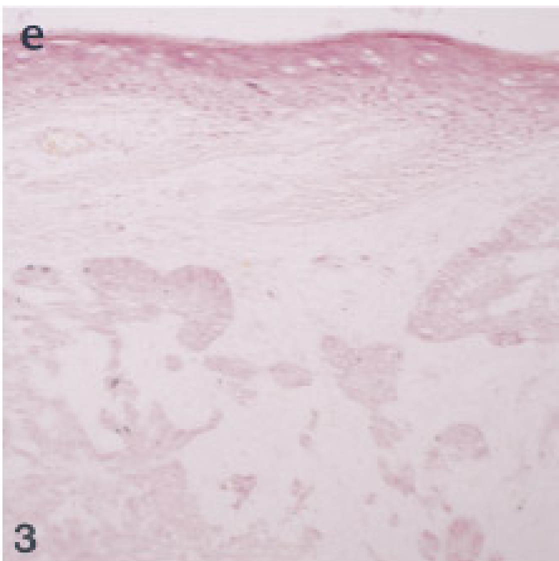
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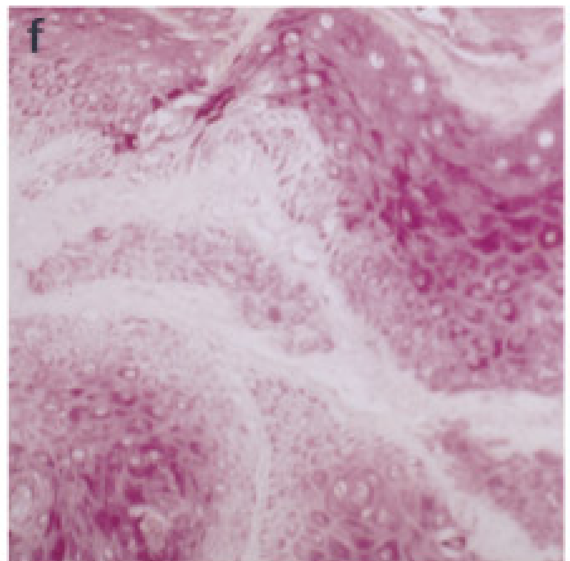
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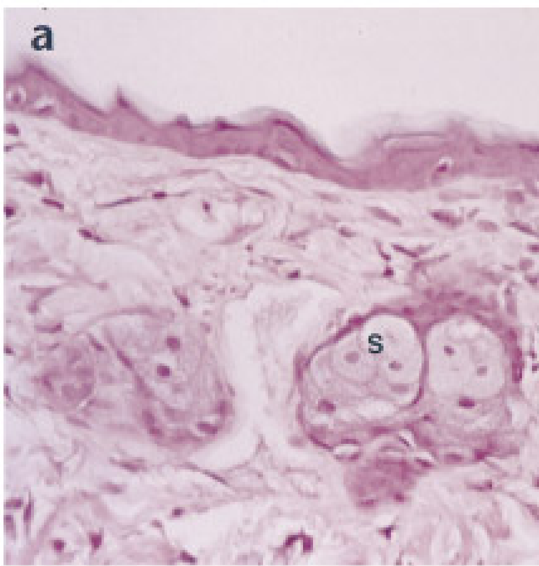
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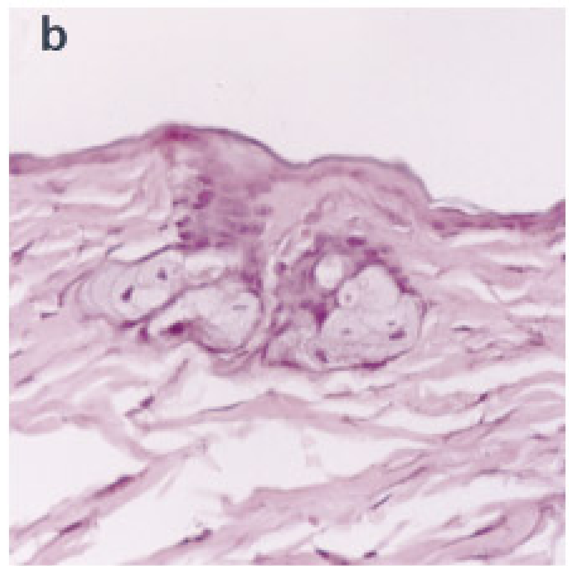
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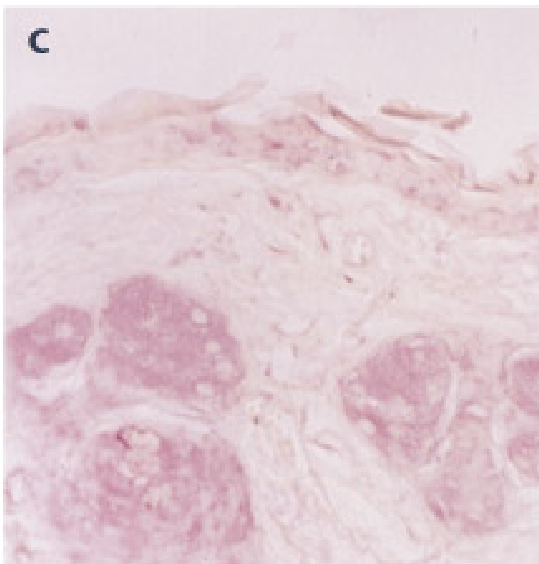
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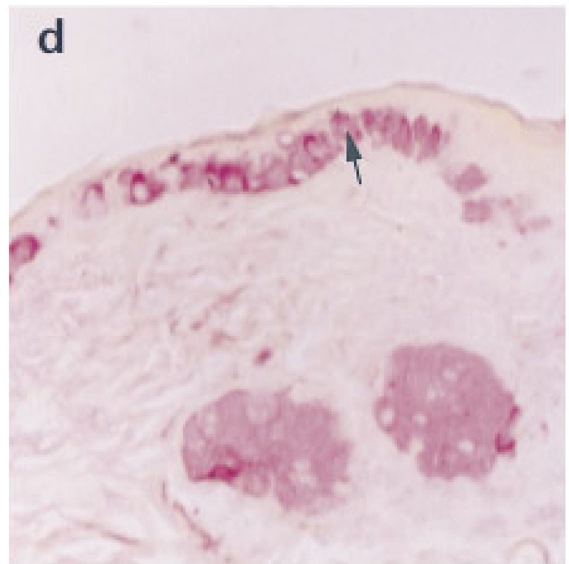
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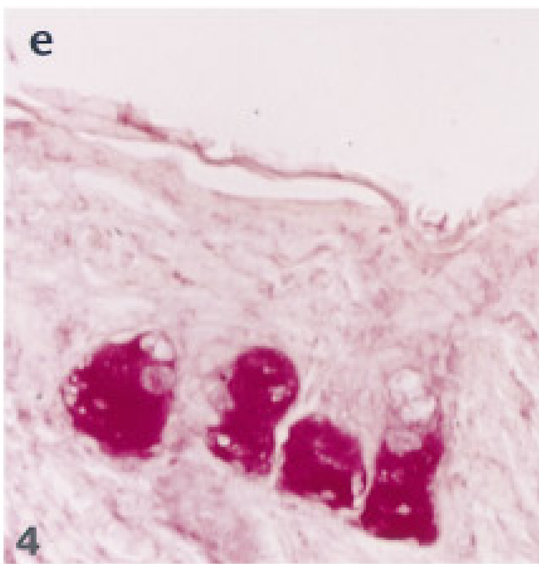
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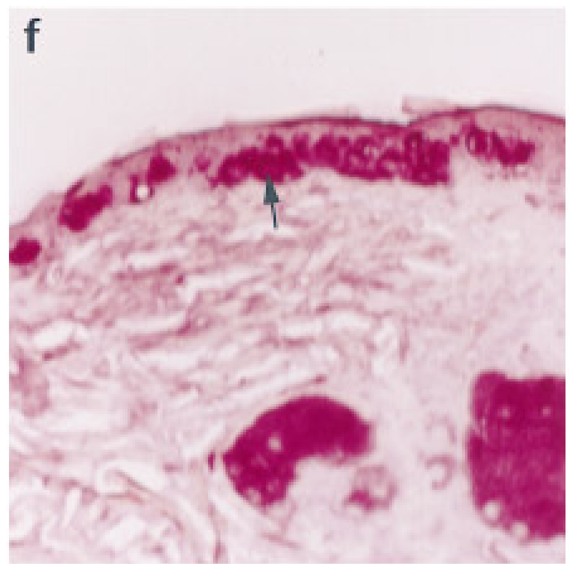
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culture, PGE_2, but not PGF_{2a}, was found to accelerate the process of self-differentiation [23]. In this study, indomethacin retarded self-differentiation, which could be reversed by adding exogenous PGE_2 to the indomethacin-treated cultures. In 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 prostaglandins 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.

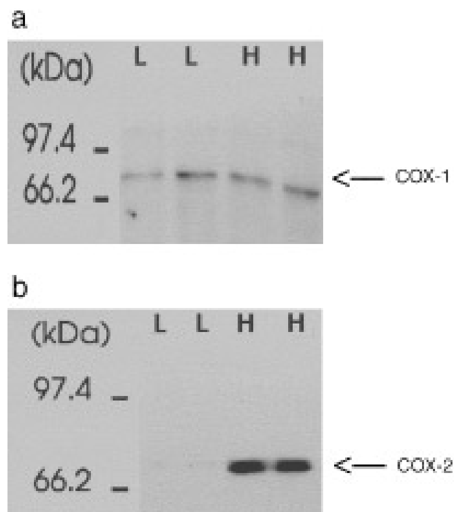

FIG. 5. Western immunoblot of extracted proteins from cultured human keratinocytes using anti-COX-1 (a) and anti-COX-2 (b) monoclonal antibodies. L, cells cultured for 72 h in 0.07 m*M* calcium; H, cells cultured for 72 h in 1.2 m*M* calcium. Each lane represents a separate culture flask.

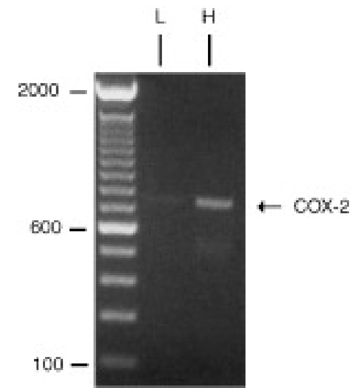

FIG. 6. Expression of COX-2 mRNA in human keratinocytes cultured for 96 h as detected by RT–PCR. L, culture medium containing 0.07 m*M* calcium; H, culture medium containing 1.2 m*M* calcium. Cells were confluent at the time of RNA extraction.

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