Selective Cyclooxygenase-2 Inhibition Does Not Alter Keratinocyte Wound Responses in the Mouse Epidermis after Abrasion

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ABSTRACT

The cyclooxygenase isoforms, COX-1 and COX-2, are the rate limiting enzymes in the biosynthesis of prostaglandin E₂, a major prostaglandin involved in epidermal homeostasis and repair. Epidermal injury results in transient hyperplasia and induction of COX-2 expression. The role of COX-2 in this hyperplasia is unknown, however. In this study, we characterized the epidermal expression of COX isozymes following wounding by abrasion in SKH-1 mice using immunohistochemistry, in situ hybridization, and Western analysis. In addition, we evaluated pivotal keratinocyte functions necessary for the reparative hyperplasia, including proliferation by 5-bromo-2’-deoxy-uridine labeling and differentiation by the expression of involucrin, keratin 1, and keratin 6. Although COX-1 expression in keratinocytes remained unchanged during wound healing, COX-2 expression was induced coincidentally with keratinocyte proliferation and keratin 6 expression, suggesting a role for COX-2 in epidermal repair. The role of COX-2 was also evaluated using the selective COX-2 inhibitor SC-791 and the traditional COX inhibitors indomethacin and diclofenac. Neither inhibitor altered keratinocyte proliferation or differentiation following abrasion, in contrast to dexamethasone, which delayed these responses. Our results indicated that, although COX-2 expression was coincident with transient epidermal hyperplasia and keratinocyte proliferation/differentiation during the healing of epidermal injury, it does not play a pivotal role in this repair process.

The epidermis is a stratified epithelium made up of basal, spinous, granular, and cornified layers. Under physiological situations, a precise balance between keratinocyte proliferation in the basal layer and the loss of corneocytes from the surface of the skin is maintained through a tightly regulated program of proliferation, differentiation, and vertical migration. Keratinocytes undergo distinct morphological changes during this program and initiate the synthesis of proteins that are differentially expressed in each epidermal layer (Eckert et al., 1997). For example, involucrin is expressed by keratinocytes in the late spinous and granular layers. Mechanical disruption of the epidermal cornified layer by tape stripping or mild abrasion results in increased keratinocyte proliferation, transient epidermal hyperplasia, and altered keratinocyte differentiation characterized by the abnormal expression of involucrin and the transient induction of the keratin 6/keratin 16 heterodimer (Bertsch et al., 1976; Eckert et al., 1993; Hatta et al., 1997; Ekanayake-Mudiyanselage et al., 1998). The signals that trigger changes in keratinocyte proliferation and differentiation following wounding are not completely understood. Disruption of the epidermal layer results in keratinocyte proliferation and induction of growth factors and cytokines, such as epidermal growth factor, interleukin-1, and tumor necrosis factor-α, as well as the inducible form of cyclooxygenase (Tsai et al., 1994; Scholz et al., 1995; Maldev and Fischer, 1996; Wood et al., 1996; Hatta et al., 1997)

Cyclooxygenase (COX) enzymes catalyze the rate-limiting step in the production of prostaglandins from arachidonic acid (Smith et al., 1996). Two isoforms of COX enzymes, COX-1 and COX-2, are involved in the biosynthesis of prostaglandins (Williams et al., 1999). COX-1 is a constitutively and ubiquitously expressed protein that is responsible for the physiological concentrations of prostaglandins needed to maintain cellular homeostasis and platelet aggregation. In contrast, COX-2 protein expression is rapidly induced in many tissues or cells in response to injury, inflammation, and/or cellular activation.

ABBREVIATIONS: COX, cyclooxygenase; BrdU, 5-bromo-2’-deoxy-uridine; hCOX, human cyclooxygenase; SC-791, a selective COX-2 inhibitor.
Prostaglandin $E_2$ is the major prostaglandin produced in the skin and is thought to be derived primarily from keratinocytes within the epidermis (Pentland et al., 1990; Ruzicka, 1990). Pentland and Needleman (1986) have demonstrated that endogenous prostaglandin $E_2$ enhanced the proliferative rate of keratinocytes in vitro in subconfluent, but not in confluent cultures, suggesting that this prostaglandin may play a role in epidermal proliferation in vivo (Pentland and Needleman, 1986). Furthermore, prostaglandin $E_2$ has been shown to regulate calcium-induced keratinocyte differentiation resulting in enhanced cornified envelope formation in vitro that was reversed by indomethacin (Evans et al., 1993). Taken together, these data have led some investigators to suggest that prostaglandin $E_2$ modulates keratinocyte proliferation and differentiation (Marks and Furst, 1998). COX-2, the enzyme responsible for the production of prostaglandin $E_2$, has been shown to regulate calcium-induced keratinocyte differentiation in vivo (Marks and Needelman, 1986). Furthermore, the enzyme has been shown to regulate calcium-induced keratinocyte differentiation in vivo (Marks and Furst, 1998). COX-2, the enzyme responsible for the production of prostaglandin $E_2$, has been shown to regulate calcium-induced keratinocyte differentiation in vivo (Marks and Furst, 1998). COX-2, the enzyme responsible for the production of prostaglandin $E_2$, has been shown to regulate calcium-induced keratinocyte differentiation in vivo (Marks and Furst, 1998).

Both COX-1 and COX-2 are expressed in activated or wounded skin and produce prostaglandin $E_2$. The recent finding that COX-2 is induced in skin cancer and that selective COX-2 inhibition attenuates tumor growth has lead many investigators to speculate that COX-2-dependent prostaglandin $E_2$ plays a role in the induction of sustained hyperplasia and tumorigenesis (Fischer et al., 1999; Pentland et al., 1999; Marks et al., 2000). Prostaglandin $E_2$ may also play a role in wound repair, which differs from skin tumorigenesis in that it is characterized by transient, rather than sustained, keratinocyte proliferation. Mild mechanical abrasion results in epidermal injury in the absence of inflammation and consequently allows the dissection of the role of the two COX isoforms in keratinocyte biology in vivo following wound healing. Mild abrasive injury is associated with the induction of both transient hyperplasia and COX-2, suggesting that COX-2-dependent prostaglandin $E_2$ regulates this keratinocyte response following mild epidermal injury (Bertsch et al., 1976; Scholz et al., 1995). The expression and role of COX-2 during mild epidermal wounding has not been carefully investigated, however. In the present study, we investigated COX-1 and COX-2 expression, as well as their role in transient keratinocyte proliferation and differentiation following mild abrasive injury in vivo.

Materials and Methods

Animals. Six- to 7-week-old female SKH-1 hairless mice weighing between 25 and 30 g were obtained from Charles River Laboratories (Wilmington, MA) and housed five per cage under constant humidity/temperature with 12-h light/dark cycles. They were allowed access to water and standard mouse feed ad libitum. All experimental procedures received approval by the Institutional Laboratory Animal Care and Use Committees of Pharmacia. The SKH-1 hairless mouse possesses a splicing defect in the hair root gene resulting in alopecia after the first hair cycle (Jones et al., 1993). No other anomalies have been reported in this mouse strain, and specifically, no immune abnormalities have been reported (Panteleyev et al., 1998).

**In Vivo Selectivity and Comparative Pharmacology of COX Inhibitors.** The potency and selectivities of each COX inhibitor were studied in vitro using recombinant human and murine COX-1 and COX-2. The in vivo selectivities of each inhibitor were studied using the carrageenan-stimulated air pouch model of inflammation adapted to the SKH-1 mouse. Briefly, air pouches were created on the backs of mice by an initial subcutaneous injection of 5 ml of sterile air, followed by reinflation with 2.5 to 5 ml of sterile air on day 2. On day 6, mice were treated orally with varying doses of inhibitors in vehicle (0.5% methylcellulose, 4000 centipoises, 0.025% Tween 20) in a total volume of 0.1 ml/mouse (Sigma-Aldrich, St. Louis, MO) or vehicle alone. At 1 h postdose, the air was removed from the pouch, and inflammation was induced with 2 ml of 1% carrageenan in saline (Carrageenan Lambda type IV; Sigma-Aldrich). After 5 h, 1 ml of 100 µM arachidonic acid (Nu-Chek Prep, Elysian, MN) was used to assess induced COX-2 activity and was calculated as the amount of prostaglandin $E_2$ produced within the air pouch during 15 min. COX-1 activity was determined by cloting whole blood (200 µl, 1 h at 37°C) and measuring the production of thromboxane $A_2$ in the serum. Both thromboxane $A_2$ detected as thromboxane $B_2$, and prostaglandin $E_2$ were measured by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). The efficacious dose yielding 50% inhibition (ED$_{50}$) for COX-1 and COX-2 was determined for each inhibitor in three separate experiments.

**Treatment of Mice with Inhibitors.** Mice were randomly assigned to each of the vehicle control and drug-treatment groups. An in vitro-selective COX-2 inhibitor, SC-791 (Fig. 1; Pharmacia, Peapack, NJ), and two traditional COX inhibitors, indomethacin and diclofenac (Sigma-Aldrich), were administered orally before abrasion to achieve steady-state drug levels at 45, 2, and 10 mg/kg/day, respectively. Dexamethasone (Sigma-Aldrich) was administered at 1 mg/kg/day as a positive control because it has previously been shown to inhibit reepithelialization in mice (Brauchle et al., 1995; Gallucci et al., 2000). Daily doses of each inhibitor were divided and given orally by gavage 12 h apart.

**Abrasion Model.** Disruption of the cornified layer of the epidermis of SKH-1 hairless mice was performed as previously reported (Bertsch et al., 1976; Scholz et al., 1995). Briefly, mice were stroked 20 times across the back, from the hindquarters to the forelimbs, with silicon carbide ultra-fine 600-grit sandpaper (3M, St. Paul, MN). This resulted in the disruption of the cornified layer but did not penetrate the underlying layers of the epidermis, as determined by histological evaluation. Mild transient erythema was the only gross indication of wounding. Mice were sacrificed at various times follow-

Fig. 1. Chemical structure of SC-791.
ing abrasion and the skin removed for histological evaluation. Skin from normal mice served as controls.

**Morphologic and Immunohistochemical Analysis.** For histology, samples were decalcareased, and in situ hybridization, three to six animals per group per time were analyzed. Following sacrifice, skin was dissected, and four 9-mm punch biopsies were cut. Skin samples were fixed in 10% buffered formalin for 24 h, processed routinely, and embedded in paraffin. Paraffin sections (5 μm) were used for the analyses.

For immunohistochemistry, sections were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and then blocked for endogenous peroxidase using 3% H2O2 and avidin/biotin (Vector, Burlingame, CA) where appropriate. Standard immunohistochemical procedures or commercially available assay kits were used for the immunolocalization of COX-2, 5-bromo-2-deoxy-uridine (BrdU), involucrin, and mouse keratin 6 or keratin 1. For COX-2 detection, sections were treated with antigen retrieval solution (Target retrieval solution; DAKO, Carpinteria, CA) before incubation with the primary antibody, a polyclonal rabbit anti-mouse prostaglandin H synthase-2 (Cayman Chemical). Sections were then incubated with a biotinylated anti-rabbit secondary antibody (DAKO), followed by streptavidin-horseradish peroxidase conjugate (DAKO) and amplification with biotinylated tyramide (tyramide signal amplification kit; DAKO). Keratin 6, keratin 1, and involucrin were detected using antigen retrieval (DAKO), polyclonal rabbit anti-mouse antibodies (Covance, Richmond, CA), and an enhanced streptavidin-biotin affinity system (DAKO). For detection of keratinocyte proliferation, mice were injected i.p. with 0.3 ml of a 20 mM solution of BrdU (Boehringer Mannheim, Mannheim, Germany) 3 h before sacrifice. BrdU was detected using a BrdU staining kit (Zymed Laboratories, San Francisco, CA) according to the manufacturer’s recommendations, with the exception that the trypsinization step was omitted. When skin sections were stained for both keratin 6 and BrdU, staining for keratin 6 was performed first, followed by BrdU.

All immunohistochemical markers were detected by incubating the sections in diaminobenzidine (DAKO) or the VIP Chromagen (Vector), and slides were counterstained with Aelican Blue/methyl green (0.25% in sodium citrate buffer; Rowley Biochemical Institute, Danvers, MA) or hematoxylin-1 (Richmond Scientific, Kalamazoo, MI). Appropriate negative and positive controls were used for all immunohistochemical stains. In particular, primary COX-2 antibodies were preincubated with the immunizing peptide before use as a negative control to demonstrate the specificity of the immunohistochemical staining.

Quantitation of BrdU-labeled keratinocytes was blinded, computer assisted, and the BrdU labeling index was expressed as the ratio of BrdU-positive nuclei per micron of epidermal basement membrane. We directly compared the amount of proliferation, which leads to hyperplasia, in the keratin 6-stained regions to that in unstained regions of the same mouse and found that the unstained regions were not different from unabraded controls (p < 0.05). Therefore, only those areas that stained positive for keratin 6 correlated with epidermal injury and the proliferative response, allowing us to eliminate uninjured areas from our evaluation of proliferation. Thus, BrdU-stained skin sections were stained for keratin 6 to identify the epidermal regions that were injured by abrasion, and only keratin 6-positive areas were used to quantitate keratinocyte proliferation. Cells within hair follicles were not counted. Quantitation of epidermal thickness was computer assisted and assessed by measuring the ratio of epidermal thickness to epidermal and dermal thickness using images of H&E-stained slides at 200× magnification. Computer-assisted image analysis was performed with Optimas 6.5 software (Optimas Corporation, Bothell, WA), as previously described (Baron et al., 2000).

**In Situ Hybridization.** RNA probes were generated using rat cDNA based on a sequence for hCOX-1 (GenBank accession no. NM_000962.1) and hCOX-2 (GenBank accession no. NM_000963.1), as previously described (Blomme et al., 2003). Briefly, cDNA fragments of rat COX-1 and rat COX-2 were generated by reverse transcription-polymerase chain reaction, inserted into the pCRRII plasmid, and probes were labeled with a radiolabeled transcription reaction utilizing [35S]UTP (PerkinElmer Life Sciences, Boston, MA) and appropriate RNA polymerases. Sections were deparaffinized, rehydrated, fixed, and then digested with proteinase K. Prehybridization was performed followed by hybridization overnight using hybridization buffer containing tRNA (50 μg/ml) and the appropriate labeled probe at 55°C. Hybridized tissues were dried in a vacuum dissector, coated with photographic emulsion (Kodak, Rochester, NY), and exposed at 4°C for 3 and 5 weeks before development. Developed slides were counterstained with H&E.

**COX-2 and COX-1 Immunoprecipitation and Western Immunoblot Analyses.** Western immunoblot techniques were used to evaluate the expression of COX-1 and COX-2 in the SKH-1 mouse skin. The epidermis was freshly isolated by scraping the skin on dry ice with a scalpel blade, as previously described (Scholz et al., 1995). COX-2 and COX-1 proteins were sequentially immunoprecipitated from 1 mg of total protein, and Western analysis was performed as previously described (Blomme et al., 2003). Recombinant mouse COX-1 and COX-2 proteins (Pharmacia) were used as standards and demonstrated that there was no cross-reaction between the COX-1 and COX-2 antibodies used.

**Statistical Analyses.** A global analysis of variance was performed as a statistical test. Data are expressed as mean ± S.E.M. Statistical analyses were performed by computer assisted two-tailed analysis of variance to compare between group means. The analyses were performed on the raw data values (parametric analysis) using the least significant difference means comparison procedure. The comparisons to the control groups were assessed using two-tailed tests. A p value of 0.05 (p < 0.05) was considered statistically significant.

**Results**

**Characterization of Mild Abrasive Injury as a Model of Transient Epidermal Hyperplasia, Keratinocyte Proliferation, and Differentiation.** Mild abrasion resulted in an injury response limited to the epidermis. Epidermal hyperplasia with hyperkeratosis and multifocal parakeratosis was maximal 48 to 72 h following wounding, and the epidermis returned to its normal morphology by 168 h (Fig. 2). No inflammatory cell infiltrates in the dermis or epidermis were observed at any time point; however, the density of microvessels in the upper dermis was significantly increased (data not shown). Mild abrasion resulted in keratinocyte hyperproliferation, measured by BrdU incorporation (Figs. 3 and 4). BrdU-stained skin sections were stained for keratin 6, a marker of epidermal injury (Ekanayake-Mudiyanselage et al., 1998) to identify the epidermal regions that were injured by abrasion. When BrdU-stained nuclei in keratin 6-positive versus keratin 6-negative regions were compared, only keratin 6-positive regions showed increased proliferation following abrasive injury (not shown). Therefore, only keratin 6-positive areas were used to quantitate keratinocyte proliferation. Abrasion-induced keratinocyte proliferation peaked 24 to 48 h following abrasion, slightly preceding the epidermal hyperplasia (Fig. 3). This increased keratinocyte proliferation was transient, returning to baseline by 96 h postabrasion.

Keratinocyte differentiation was assessed following mild abrasive injury by the evaluation of the structural proteins keratin 1, keratin 6, and involucrin. Keratin 1 expression was found throughout the normal epidermis and was un-
changed following abrasion (data not shown). In contrast, involucrin expression, which is normally confined to the upper spinous and granular layers of the epidermis in normal mouse skin, was expressed in all viable epidermal layers after wounding coincident with hyperplasia at 48 h (Fig. 4). Involucrin expression returned to that observed in the normal epidermis by 120 to 168 h after abrasion (not shown). Keratin 6 was not expressed in the normal mouse interfollicular epidermis (Fig. 4) but was expressed in hair follicles, as previously described (Ekanayake-Mudiyanaselage et al., 1998; Rothnagel et al., 1999). Following abrasion, keratin 6 expression was markedly and transiently induced in the suprabasal layers of the epidermis within 24 to 48 h (Fig. 4). By 120 to 168 h, the healing epidermis no longer expressed keratin 6 (not shown).

Spatial and Temporal Expression of COX-1 and COX-2 following Epidermal Abrasion. As previously reported (Scholz et al., 1995), COX-2 expression was induced following mild abrasive injury. Low levels of COX-2 protein were seen by Western analysis in normal skin, probably reflecting the low constitutive expression of COX-2 in hair follicles (Fig. 5) (Muller-Decker et al., 1998b). COX-2, however, was rapidly induced as early as 2 h after abrasion (not shown) and was maximally expressed 6 to 72 h following abrasive injury. By 96 h, COX-2 protein expression levels had returned to that seen in normal murine skin. In contrast, COX-1 was constitutively expressed in the normal mouse epidermis, and its expression was unaltered during wound healing. Thus, following abrasive injury, epidermal COX-2
induction preceded the changes in keratinocyte proliferation and differentiation and returned to normal levels with a parallel time course.

We evaluated the spatial expression of COX-1 and COX-2 in the skin following abrasive injury by in situ hybridization and immunohistochemistry. In the normal skin, COX-1 mRNA was expressed at similar levels by all viable layers of the epidermis (Fig. 6). Following abrasion, the COX-1 mRNA expression pattern was unchanged (Fig. 6). In contrast, COX-2 mRNA and protein were not expressed in normal interfollicular epidermis and were minimally expressed in follicular keratinocytes (Fig. 6), as previously described (Muller-Decker et al., 1998b). After abrasive injury, COX-2 mRNA and protein were transiently induced in the interfollicular epidermis (Fig. 6). COX-2 protein expression was localized to the basal keratinocytes. Thus, COX-2 expression was induced in basal keratinocytes coincidentally with the onset of their proliferative response following mild abrasive injury.

**Effect of COX Inhibitors and Dexamethasone on Epidermal Repair after Abrasive Injury.** To investigate the role of COX-1 and COX-2 in the reparative keratinocyte responses following abrasion, we compared the effects of an in vitro-selective COX-2 inhibitor, SC-791, with those of two traditional COX inhibitors, diclofenac and indomethacin. The potency and selectivity of SC-791, diclofenac, and indomethacin on recombinant hCOX-1 versus hCOX-2 were 114, 0.04, and 0.1 μM versus 0.004, 0.1, and 0.9 μM, respectively. SC-791 is 1,000 to 10,000-fold more selective for recombinant hCOX-2 than recombinant hCOX-1 enzymes. This COX-2 inhibitor is more selective, in vitro, for COX-2 than previously reported inhibitors and is similar to valdecoxib in structure and potency against recombinant hCOX-2 (Gierse et al., 1996; Talley 1999). Diclofenac and indomethacin inhibited hCOX-1 and hCOX-2 with much less selectivity in vitro. The in vivo selectivity of each COX inhibitor is shown in Table 1. The ED$_{50}$ values for each compound are shown as inhibition of COX-2-dependent prostaglandin E$_2$ production in the carrageenan inflammatory air pouch or of COX-1-dependent thromboxane A$_2$ production from clotted whole blood. Prostaglandin E$_2$ production induced by carrageenan was inhibited 75 to 80% by SC-791 compared with 100% with diclofenac. These data match those reported for the carrageenan air pouch experiments done in COX-1 and COX-2 knockout mice; in COX-1 deficient mice, prostaglandin levels were 75% those of wild-type mice, whereas in COX-2 knockout mice, prostaglandin production was only 25% that of wild-type controls (Langenbach et al., 1999). Thus, COX-2 is the major pathway for prostaglandin production in this inflammation model, regardless of the mouse strain. SC-791 maintained its in vivo selectivity for COX-2 up to 100 mg/kg, beyond which the selectivity was not tested, whereas both indomethacin and diclofenac inhibited the activity of both COX-1- and COX-2-less selectively (Table 1). Although diclofenac has some selectivity for COX-2 compared with COX-1, complete inhibition of COX-2 could not be attained in vivo without some inhibition of COX-1. Furthermore, when stomach prostaglandin E$_2$ was used as a measure of COX-1 activity, the ED$_{50}$ of diclofenac was 2.5 mg/kg, thus confirming a narrow margin of selectivity in vivo. At the doses used in the in vivo wound healing experiments, indomethacin and diclofenac inhibited both COX isoforms, whereas SC-791 completely inhibited COX-2 without inhibiting COX-1. In addition, at similar or lower plasma exposures, both valdecoxib (a structural analog that is biochemically and pharmacologically similar to SC-791) and indomethacin have been shown to maximally inhibit COX-2 and prostaglandin E$_2$ formation in the epidermis following acute incisional wounding or UV exposure (Fischer et al., 1999 and Muller-Decker et al., 2002). Expression of both COX isoforms was unchanged in the presence of these inhibitors (not shown).
TABLE 1
In vivo selectivity of COX inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED50</th>
<th>Air Pouch Prostaglandin E2</th>
<th>Platelet Thromboxane B2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC-791</td>
<td>3.0</td>
<td>50.0</td>
<td>&gt;100.0</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>0.2</td>
<td>9.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.2</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.4</td>
<td>&gt;50.0</td>
<td></td>
</tr>
</tbody>
</table>

The keratinocyte responses were evaluated in the absence and presence of each COX inhibitor at various times following mild abrasive injury (Fig. 7). As expected, the vehicle-treated mice showed a 4-fold induction of keratinocyte proliferation 24 to 48 h after abrasive injury, which returned to baseline by 168 h. This increased keratinocyte proliferation following wounding as well as its resolution was unaltered during treatment with SC-791, indomethacin, and diclofenac. Consistent with these results, the epidermal hyperplasia seen in Fig. 8 was also unaffected by COX inhibition. To obtain confidence in our evaluations, we quantitated the epidermal thickness in hyperplastic regions and keratinocyte proliferation using eight independent keratin 6-stained regions from four histologic sections representing the entire abraded area per mouse using computer-assisted software. The extent of hyperplasia was not significantly different in any of the groups treated with COX inhibitors compared with the control group (p < 0.05) by this evaluation. Thus, inhibition of COX had no effect on keratinocyte proliferation or the transient epidermal hyperplasia induced following mild abrasive injury.

In contrast, dexamethasone treatment delayed the increased keratinocyte proliferation (Fig. 7) and the epidermal hyperplasia (epidermal thickness = 25 ± 2 μm, dexamethasone versus 35 ± 1 μm, vehicle, p < 0.05) occurring 24 h following abrasion. This inhibition by dexamethasone, however, was transient and had recovered by 48 h (Fig. 7; epidermal thickness = 38 ± 4 μm, dexamethasone versus 43 ± 4 μm, vehicle). Dexamethasone treatment also resulted in skin atrophy as expected (full skin thickness at 168 h = 173 ± 28 μm, dexamethasone versus 251 ± 7 μm, vehicle, p < 0.05). The effects of dexamethasone on keratinocyte proliferation and epidermal hyperplasia demonstrated that transient hyperplasia following abrasive injury could be inhibited and may indicate that multiple genes must be altered to perturb this cutaneous wound healing response. Of note, dexamethasone had no effect on either COX isoform expression following abrasion (not shown). Dexamethasone, however, can inhibit COX-2 protein expression at doses higher than 1 mg/kg/day in murine skin (Masferrer et al., 1994; not shown). Thus, the effects of dexamethasone in this study were independent of COX-2 inhibition.

Finally, there was no effect of inhibition of either COX-2 or both COX isoforms on keratinocyte differentiation as evaluated by the pattern of involucrin, keratin 1 (data not shown), and keratin 6 expression at any time following abrasion (Fig. 8). In contrast, mice treated with dexamethasone demonstrated a delay in the up-regulation of involucrin in the basal epidermal layer and of keratin 6 in the suprabasal epidermis that was coincident with the delay in hyperplasia seen with dexamethasone treatment. Thus, the keratinocyte differentiation program occurred normally in the presence of COX-2 inhibition, similar to epidermal hyperplasia and keratinocyte proliferation, but was delayed in the presence of dexamethasone treatment.
Discussion

Nonsteroidal anti-inflammatory drugs are nonselective COX inhibitors and are widely used for the treatment of arthritis, pain, and cardiovascular diseases. Nevertheless, nonsteroidal anti-inflammatory drugs can cause serious side effects due to the inhibition of COX-1. These side effects include gastroduodenal ulceration in about 25% of users, which can often be associated with bleeding and perforation, inhibition of platelet aggregation leading to adverse bleeding events, and in some patients, a decrease in renal blood flow and glomerular filtration rate (Insel, 1996). Because of these side effects, compounds selective against COX-2 with superior safety profiles have recently been developed and have already been approved for several indications, including rheumatoid arthritis, osteoarthritis, and familial adenomatous polyposis (Williams et al., 1999). Induction of COX-2 in both normal as well as proliferating transformed keratinocytes has led to the suggestion that COX-2 inhibitors may inhibit epidermal wound healing by blocking keratinocyte proliferation and differentiation. Thus, we evaluated the role of COX-2 in these pivotal keratinocyte responses in vivo following a mild mechanical skin abrasion.

In this study, we validated that mild abrasive injury is a model of transient epidermal hyperplasia by measuring keratinocyte proliferation. We further characterized this model by evaluating changes in key keratinocyte proteins (involutrin, keratin 1, and keratin 6) that reflect the differentiation program seen after wounding. Altered expression of involucrin, but not keratin 1, and the induction of keratin 6 have been described following the disruption of the cornified layer by tape stripping (Eckert et al., 1993; Ekanayake-Mudiyanselage et al., 1998). Similar to tape stripping, we showed that involucrin was abnormally expressed throughout all layers of the epidermis within 24 h following abrasive injury. After mild abrasive injury, there was also marked induction of keratin 6 expression throughout the suprabasal epidermis by 24 h. Thus, after abrasive wounding, both keratinocyte proliferation and differentiation were transiently altered leading to the transient hyperplasia needed to rapidly repair the epidermis.

Although the induction of COX-2 has been previously described following abrasive epidermal injury, no functional significance was addressed in this study (Scholz et al., 1995). Here, we demonstrated that although COX-2 was transiently up-regulated in proliferating basal keratinocytes following abrasive injury, inhibition of COX-2 or both COX isoforms did not adversely affect either keratinocyte proliferation or differentiation or epidermal hyperplasia. Although we did not measure skin prostaglandins directly, previous studies have shown that valdecoxib, a structural analog of SC-791 that is similar in potency and in vivo pharmacology, and indomethacin can decrease prostaglandin E₂ production in the skin following incisional wounding or acute UV exposure at exposures similar to or lower than those used in this study, indicating that SC-791 and indomethacin inhibit COX-2 and COX activity, respectively, in the epidermis at these doses (Fischer et al., 1999; Muller-Decker et al., 2002). These data indicate that COX-2 is not pivotally required for transient epidermal repair in this simple model of skin injury.

The abrasion model used in this study is associated with mild injury and may not predict the repair process in more severe skin injuries. Indeed, evidence from rodent models suggests that COX-2 expression is up-regulated in gastric mucosal injury and that COX-2 inhibitors may impede gastrointestinal mucosal healing, possibly through inhibition of angiogenesis (Schmassmann, 1998; Takahashi et al., 1998). These observations seen in the gastrointestinal epithelia have been applied globally to all types of epithelial healing, including cutaneous wound healing. Because a potential effect on wound healing is relevant to the use of COX-2-selective drugs as postsurgical analgesics, we and others have studied the effect of COX-2 inhibition on skin wound healing. Using a mouse surgical incisional skin wound model, the chronic pharmacologic inhibition of COX-2 activity was not sufficient to significantly delay wound healing in this cutaneous incisional wound model in two different mouse strains measuring a variety of epidermal parameters (Muller-Decker et al., 2002; Blomme et al., 2003). These results are consistent with postoperative studies of general and orthopedic surgery patients using COX-2-selective drugs, which have not shown any deleterious effects on wound healing (Barton et al., 2002; Rasmussen et al., 2002). Together, these preclinical and clinical data suggest that selective COX-2 inhibitors do not alter the healing process of skin wounds.

Induction of COX-2 expression has been demonstrated to be a consistent feature in diseases of sustained epidermal hyperplasia. For example, COX-2 expression has been characterized in mouse skin induced by tumor promoters, such as the phorbol esters, as well as throughout the epidermis in human squamous cell carcinoma and its precursor actinic keratosis (Muller-Decker et al., 1995, 1998a; Buckman et al., 1998). In contrast, COX-1 typically remained uniformly expressed in both normal human skin and squamous cell carcinoma. From these data, COX-2 induced prostaglandin E₂ has been postulated to play a role in the development of skin cancer (Buckman et al., 1998). Consistent with this hypothesis, suppression of tumor development with a selective COX-2 inhibitor has been observed in mouse models of skin tumorigenesis (Marks and Furstenburger, 1993; Muller-Decker et al., 1998a,b). In contrast to the data obtained in mouse models of skin cancer (Marks and Furstenburger, 1993; Muller-Decker et al., 1998a,b), we demonstrated that keratinocyte proliferation and differentiation following mild epidermal injury by abrasion were unaffected by inhibition of COX-2 or both COX isoforms. Furthermore, the epidermis became hyperplastic and returned to a normal, quiescent state with a similar time course in all treatment groups. A major difference between the normal wound response and skin cancer is that the former results in a transient keratinocyte hyperproliferation, whereas the latter is associated with a sustained keratinocyte hyperproliferation. These data suggest that neither COX-2- nor COX-1- produced prostaglandins are essential for the transient keratinocyte responses that occur following mild abrasive injury. Since COX-2 inhibition can inhibit the development of skin cancer (Muller-Decker et al., 1998a,b; Fischer et al., 1999; Pentland et al., 1999), it is possible that COX-2-dependent prostaglandin E₂ may regulate unique keratinocyte signaling pathways that are present during sustained proliferation but not during transient hyperplasia.

Sustained COX-2 overexpression in the epidermis has been achieved in two transgenic mouse models using either the keratin 5 or the keratin 14 promoters (Neufang et al., 2001;
Bol et al., 2002). Chronic epidermal hyperplasia with aberrant keratinocyte differentiation, as evidenced by decreased expression of involucrin, loricrin, and keratin 1, was observed in the skin of the tail of the model using the keratin 5 promoter (Neufang et al., 2001). Nevertheless, no evidence of epidermal hyperplasia or abnormal keratinocyte differentiation was seen in other skin locations, most notably in the back skin. Prostaglandin E₂ production in the skin of this COX-2 transgenic model was 10-fold higher than that of normal mouse skin (Neufang et al., 2001). Thus, despite a prolonged and aberrant elevation of prostaglandin E₂ production via COX-2 expression in these transgenic mice, only minor skin phenotypic changes in the tail were observed, suggesting that COX-2 overexpression alone may not be sufficient for the sustained keratinocyte responses associated with skin cancer. In contrast, in the transgenic model overexpressing COX-2 using the keratin 14 promoter, a phenotype characterized by alopecia with follicular dysplasia, skin atrophy, and sebaceous gland hypertrophy was described, and this phenotype could be reversed upon administration of a selective COX-2 inhibitor (Bol et al., 2002). These contrasting phenotypes illustrate the complex and still poorly understood role that COX-2-induced prostaglandins play in both normal and dysplastic skin. No studies on wound healing have yet been reported in these transgenic mouse models.

The lack of an effect on epidermal wound healing with selective COX-2 and nonselective COX inhibitors is similar to what has been reported in mice with targeted mutations of genes encoding for pivotal skin growth factors, such as keratinocyte growth factor or transforming growth factor-α (Guo et al., 1996; Scheid et al., 2000). These growth factors are markedly induced during wound healing, and although their spatial and temporal expression suggests a role in the epidermal repair process, they appear to be dispensable for proper healing because of redundant mechanisms. Likewise, the lack of effect of COX-2 inhibition on the epidermal wound healing response probably reflects the presence of redundant pathways with compensatory mechanisms.

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