0023-6837/02/8211-1503$03.00/0
LABORATORY INVESTIGATION
Vol. 82, No. 11, p. 1503, 2002
Printed in U.S.A.


# Wound Healing Involves Induction of Cyclooxygenase-2 Expression in Rat Skin

Ayako Futagami, Masamichi Ishizaki, Yuh Fukuda, Seiji Kawana, and Nobuaki Yamanaka

*Department of Pathology (AF, MI, YF, NY) and Department of Dermatology (AF, SK), Nippon Medical School, Tokyo, Japan*

**SUMMARY:** Cyclooxygenase (COX), an enzyme essential for prostaglandin biosynthesis, has two isoforms, COX-1 and -2. We investigated temporal and spatial changes in localization of these two COX proteins and mRNAs after excisional injury in rat skin. We also quantified the expression of these proteins and studied the effects of a specific COX-2 inhibitor on healing. Immunohistochemistry and in situ hybridization respectively indicated that the COX-2 protein and mRNA were expressed mainly within the basal layer of the epidermis, peripheral cells in the outer root sheath of hair follicles, and fibroblast-like cells and capillaries near epidermal wound edges. Much less intense expression was observed in normal skin than in injured skin. Western analysis demonstrated marked induction of COX-2 protein beginning within 12 hours and peaking 3 days after injury. In contrast, localization of COX-1 protein and mRNA, as well as the amount of protein expression, showed no significant change during wound healing. Administration of the COX-2 inhibitor delayed re-epithelialization in the early phase of wound healing and also inhibited angiogenesis. Thus, COX-2 induction may be important in cutaneous wound healing. (*Lab Invest 2002, 82:1503–1513*).

After skin injury, a complex series of events must proceed for the epidermal and dermal wound recovery. Keratinocytes at the edge of an epidermal wound migrate, proliferate, and differentiate to cover the exposed wound surface, and fibroblasts and capillaries produce a new granulation tissue (Clark, 1993; Martin, 1997). Each process may be regulated by many bioactive substances, including growth factors, extracellular matrix components, and eicosanoids. Eicosanoids such as prostaglandins (PGs), prostacyclins, and thromboxane have been implicated in wound healing in various tissues such as cornea (Joyce and Meklir, 1994), skin (Talwar et al, 1996), gastrointestinal tract (Zushi et al, 1996), and kidney (Cybulsky et al, 1992). In particular, $PGE_2$, which constitutes the major PGs in human and rat skin (Jonsson and Änggård, 1972; Jouvenaz et al, 1970), affects keratinocyte proliferation (Lowe and Stoughton, 1977; Pentland and Needleman, 1986), differentiation (Evans et al, 1993), and angiogenesis in vivo together with $PGE_1$ (Form and Auerbach, 1983; Ziche et al, 1982). Talwar et al (1996) have found that synthetic $PGE_2$ facilitates fibrosis in vivo during healing of wounded rat skin. Furthermore, receptors for $PGE_2$, E-prostanoid $(EP)_2$, and/or $EP_4$ mediate the effect of PGE2 on keratinocyte growth (Konger et al, 1998). Indeed, $EP_4$ receptor mRNA showed up-regulation in a fetal rabbit skin wound (Li et al, 2000). These findings indicate that PGE2 production is essential for cutaneous wound healing. PGs are formed by the combined actions of phospholipase, which releases arachidonic acid (AA) from cell membrane phospholipids, and cyclooxygenase (COX), which converts AA to PGs.

Recently, several investigators have used molecular techniques to confirm the presence of two isoforms of COX, a constitutive form (COX-1) and an inducible form (COX-2) (DeWitt and Smith, 1988; Feng et al, 1993; Merlie et al, 1988; O'Banion et al, 1992). COX-1 is thought to be a "housekeeping enzyme" modulating such physiologic responses as regulation of renal and vascular homeostasis, as well as protection of the gastric mucosa (DeWitt and Smith, 1995; Yang et al, 1998). In contrast, COX-2 is considered to be an "immediate early" gene product that can be synthesized rapidly but transiently in response to tumor promoters, endotoxins, and cytokines (Kujubu et al, 1991; Maier et al, 1990; O'Sullivan et al, 1992). Recently, normal murine epidermis was found to express COX-1 but not COX-2 (Müller-Decker et al, 1998a; Scholz et al, 1995). Mechanical or chemical damage, however, could induce COX-2; such insults included removal of the superficial epidermis by sandpaper or topical application of irritants, such as phorbol 12-myristate 13-acetate (PMA) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA). COX-2 was also induced in fibroblasts and macrophages by interleukin-1 or lipopolysaccharide (LPS) (O'Sullivan et al, 1992; Sato et al, 1997). Consequently, COX-1 is thought to be involved in normal skin homeostasis, whereas COX-2 is important in various responses to insults to the skin such as those to injury. In cell culture studies, COX-2 expression was detected in keratinocytes in a high-calcium

**DOI: 10.1097/01.LAB.0000035024.75914.39**

Received May 31, 2002.
*Address reprint requests to: Dr. Ayako Futagami, Department of Dermatology, Nippon Medical School, 1–1-5 Sendagi, Bunkyo-ku, Tokyo 113–8603, Japan. E-mail: aya-f@nms.ac.jp*

medium or under low-cell-density conditions as a model of activated keratinocytes during wound healing (Leong et al, 1996; Rys-Sikora et al, 2000). However, the specific roles and effects of COX on cutaneous wound healing have not been completely clarified in an in vivo model.

In the present study, we determined changes in localization of the protein and mRNA for the two isoforms of COX during cutaneous wound healing in rats using immunohistochemistry and in situ hybridization. Western analysis was used to quantify expression of the isozymes in protein extracts from normal and injured skin. Furthermore, we histologically evaluated the effects of a nonselective inhibitor of both COX-1 and -2 (indomethacin), as well as those of a selective inhibitor of COX-2 (*N*-2 cyclohexy-4-nitrophenyl methanesulfonamide; NS-398) on wound healing in rat skin. We also measured the effects of these drugs on production of PGE2 at the wound site. NS-398 is a structurally novel sulfonamide nonsteroidal anti-inflammatory drug (NSAID) that specifically inhibits COX-2 activity, showing a 50% inhibitory concentration (IC50) of 0.1 to 3.8 $\mu$M. The drug has no effect on COX-1 activity even at concentrations exceeding 100 $\mu$M (Futaki et al, 1994; Gierse et al, 1995). On the other hand, indomethacin, a well-known prototypical NSAID, inhibits activities of both COX-1 (IC50, 0.1 to 0.74 $\mu$M) and COX-2 (IC50, 0.9 to 0.97 $\mu$M). Daily administration of NS-398 delayed gastric ulcer healing in rats and mice (Mizuno et al, 1997; Shigeta et al, 1998), suggesting an important role of COX-2 in the ulcer healing process. Our results showed characteristic distributions of COX-2 protein and mRNA with marked induction of the former in injured skin. Daily administration of NS-398 delays cutaneous wound healing.

## Results

### *Immunohistochemistry*

In the normal skin, the COX-1 protein was expressed throughout the epidermis, with a slightly greater staining intensity in the suprabasal and granular layers of the interfollicular epidermis and the follicular infundibulum than in other areas (Fig. 1a). The COX-1 was also expressed in hair follicles, particularly in matrix cells, and most sebaceous glands, endothelial cells of small vessels, and fibroblast-like cells (Fig. 1c). On the other hand, we observed only a weak expression of the COX-2 protein in a few isolated basal cells in the interfollicular epidermis (Fig. 1b) and in some endothelial cells of small vessels and fibroblast-like cells (Fig. 1d). However, the COX-2 protein was strongly expressed in hair follicles, particularly in some cells in the midportion of the outer root sheath (ORS) and the medulla of the hair (Fig. 1, d and e), as well as in sebaceous glands (Fig. 1f).

In wound healing before epidermal closure (on Day 3), the COX-1 protein was weakly expressed throughout the epidermal wound edge (Fig. 1, g and h). In the granulation tissue, most endothelial cells of small vessels and fibroblast-like cells were stained for the COX-1 protein (Fig. 1i). In contrast, a markedly strong expression of the COX-2 protein was observed in the head and basal layers of the epidermal wound edge (Fig. 1, j and k). In the granulation tissue adjacent to the wound edges, the COX-2 protein was also strongly expressed in inflammatory cells including fibroblast-like cells (Fig. 1, l and m). As early as 12 hours after injury, some peripheral cells of the ORS in hair follicles near the wound edges were stained strongly for the COX-2 protein (Fig. 1, n and o). Unwounded epidermis distant from the wound edges showed the same staining profile as that of the normal skin for these COX-1 and -2 proteins.

After epidermal closure (on Day 10), the distribution and intensity of staining of these COX proteins returned to the normal pattern in the newly formed epidermis. In the remaining granulation tissue, the COX-1 protein showed the same staining pattern as that of the uninjured dermis, whereas the COX-2 protein was not observed, accompanied by the gradual disappearance of inflammatory cells and small-vessel endothelial cells.

### *Western Blotting*

The expression of the COX-1 protein in both normal skin and wounded skin was detected as a 68-kDa protein band, with the level of expression remaining almost constant during wound healing (Fig. 2, a and b). The weakly expressed the COX-2 protein was identified as a 72-kDa protein in the normal skin. The strong induction of the COX-2 protein was observed after wounding; this became evident within 12 hours after injury, peaked at approximately Day 3, and declined to the basal expression level on Day 10 (Fig. 2, c and d).

### *In Situ Hybridization*

In the normal skin, the COX-1 mRNA was observed throughout the epidermis, with a slightly more intense expression in the suprabasal and granular layers than in others (Fig. 3, a and b). It was also expressed in most hair follicles, sebaceous glands, endothelial cells of small vessels, and fibroblast-like cells (Fig. 3, c and d). On the other hand, the COX-2 mRNA was expressed within only a few isolated basal keratinocytes in the interfollicular epidermis (Fig. 3, e and f). It was less intensely expressed in most hair follicles, sebaceous glands, and fibroblast-like cells (Fig. 3d).

On Day 3, the localization of the COX-1 mRNA ranged from the basal layer to the granular layer in the region of the epidermal wound edge, showing no difference in expression intensity compared with the normal skin (Fig. 3, i and j). However, the COX-2 mRNA expression was intense in the epidermal wound edge with localization in the head and basal layers (Fig. 3, l and m). In the granulation tissue, almost all endothelial cells of small vessels and fibroblast-like cells strongly expressed these COX-1 and -2 mRNAs (Fig. 3, k and n). No reaction products were detected in adjacent sections from the normal skin or wounded

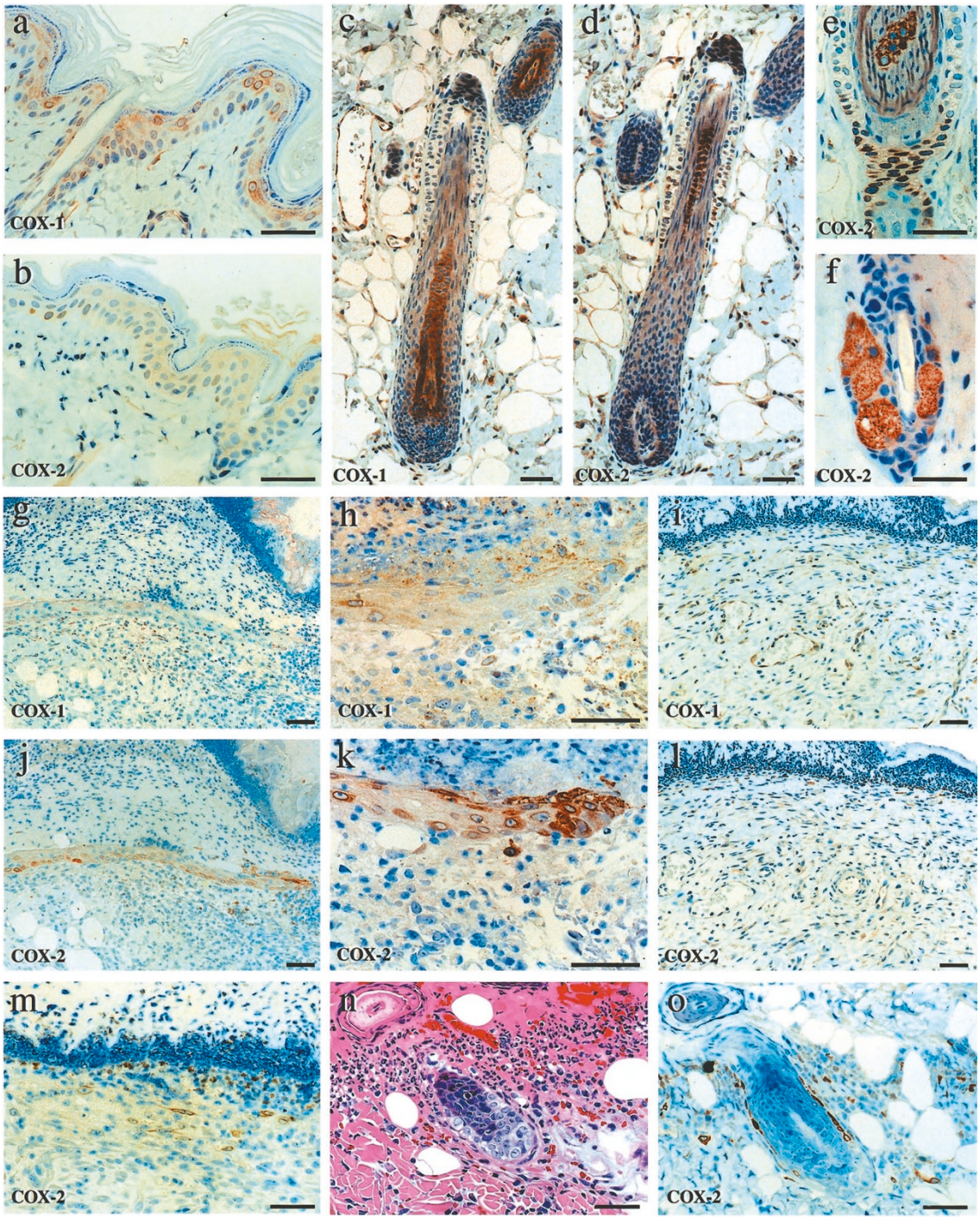


**Figure 1.**

Immunohistochemical distribution of cyclooxygenase (COX) proteins in normal and wounded skin. Staining of normal skin (a to f) is illustrated for epidermis (a and b), a hair follicle and small vessels (c and d), the midportion of a hair follicle (e), and a sebaceous gland (f). Staining of skin 3 days after injury (g to m) is shown for the epidermal wound edge (g and j; and at higher magnification, h and k); and for granulation tissue (i and l; and at higher magnification, m). Staining of skin 12 hours after injury (n to o) is shown for a hair follicle near the epidermal wound edge (o; compared with hematoxylin and eosin staining in n). Scale bars = 100 $\mu$m.

skin using sense probes for COX-1 (data not shown) or COX-2 (Fig. 3, o, p, and q).

### *Effect of NS-398 or Indomethacin on Wound Healing*

*Re-Epithelialization of the Wound.* The percentages of animals showing re-epithelialization in the NS-398-treated group were significantly smaller than those in the control group on Days 3 and 5 after injury. That of the indomethacin-treated group was also significantly smaller than that of the control group on Day 5. However, no significant differences were observed throughout the experiment between animals treated with NS-398 and those treated with indomethacin. On

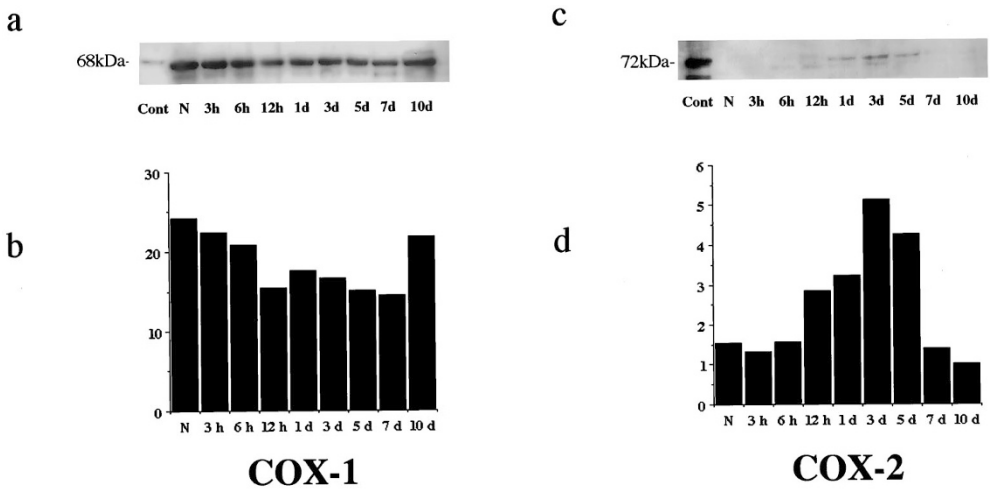


**Figure 2.**

Western blotting and densitometric analysis of COX proteins expression in normal and wounded skin. COX proteins were partially purified as described in "Materials and Methods." Western immunodetection of 68-kDa COX-1 protein is shown in a, with densitometric analysis presented in b. Western immunodetection of 72-kDa COX-2 protein is shown in c, with densitometric analysis in d. Lane designations: *Cont,* sheep COX-1 and COX-2 proteins as positive controls; *N,* samples prepared from unwounded normal skin; *3h, 6h, 12h, 1d, 3d, 5d, 7d, 10d,* samples prepared from wounded skin at the corresponding number of hours or days after injury.

Day 10, wounds in all groups were completely re-epithelialized (Fig. 4a).

*Number of Capillaries.* There were no significant differences in the number of capillaries among all groups on Day 3. However, on Day 5, the NS-398-treated group had significantly fewer capillaries than the control group. On the other hand, on Days 7 and 10, capillaries were significantly more numerous in the former than in the latter. On Day 7, they were also significantly more numerous in the NS-398-treated group than in the indomethacin-treated group. Similarly, fewer capillaries were observed in the indomethacin-treated group on Day 5 and more were observed on Days 7 and 10 than in the control group, although these differences were not significant (Fig. 4b).

*Production of $PGE_2$ at the Site of Wound.* On Day 3, a slight decrease in the production of $PGE_2$ was observed in the NS-398-treated group (1556 $\pm$ 874 pg/mg protein) and the indomethacin-treated group (1166 $\pm$ 328 pg/mg protein) compared with the control group (2428 $\pm$ 411 pg/mg protein), but there were no significant differences among all groups.

## Discussion

In this study, we describe the details of changes in the distribution pattern and expression level of these two COX isoforms during wound healing in rat skin.

We found that in the uninjured rat skin, the COX-1 protein is located in the suprabasal and granular layers of the interfollicular epidermis, matrix cells of hair follicles, sebaceous glands, endothelial cells of small vessels, and fibroblast-like cells. A similar COX-1 distribution pattern has been reported in the normal mouse skin, and its expression in a relatively fewer keratinocytes in the basal and suprabasal layers (Leong et al, 1996; Müller-Decker et al, 1998a, 1998b). Furthermore, by in situ hybridization, we detected the expression of the COX-1 mRNA in the normal rat skin showing a similar localization, including a slightly accentuated intensity of the expression in the same epidermal layers, to that of the protein. Cameron et al (1990) have reported an increased $PGE_2$ synthesis level among the more differentiated cells of the mouse epidermis, whereas Scholz et al (1995) found that the amount of the COX-1 mRNA in isolated mouse keratinocytes increased with terminal differentiation. Our findings are consistent with these findings, suggesting that COX-1 may correlate with differentiation in rat epidermis.

During wound healing, the immunohistochemical localization of COX-1 protein remained unchanged. By Western blot analysis, the expression level of the COX-1 was also found to be constant, in agreement with previous reports (Müller-Decker et al, 1998a; Scholz et al, 1995). Moreover, the localization and expression intensity of the COX-1 mRNA did not differ from findings in the normal skin. Our results indicate that the expression of the COX-1 protein and mRNA is independent of epidermal mitogenic stimulation by skin wounding or irritative treatment with TPA or PMA (Leong et al, 1996; Müller-Decker et al, 1998a, 1998b; Scholz et al, 1995).

The COX-2 protein expression in the normal rat skin was limited to a few isolated basal epidermal cells, the midportion of the ORS of hair follicles, and sebaceous glands. We also observed the COX-2 mRNA in the normal rat skin with a wider distribution than the COX-2 protein but with less intensity. After injury, these COX-2 protein and mRNA were expressed predominantly in the head and basal layers of the epidermal wound edges, which are composed of migratory and proliferative cells (Garlick and Taichman, 1994). Previous reports also demonstrated COX-2 immunostaining in the basal layer of the mouse epidermis after treatment with an irritant, in contrast to the absence of

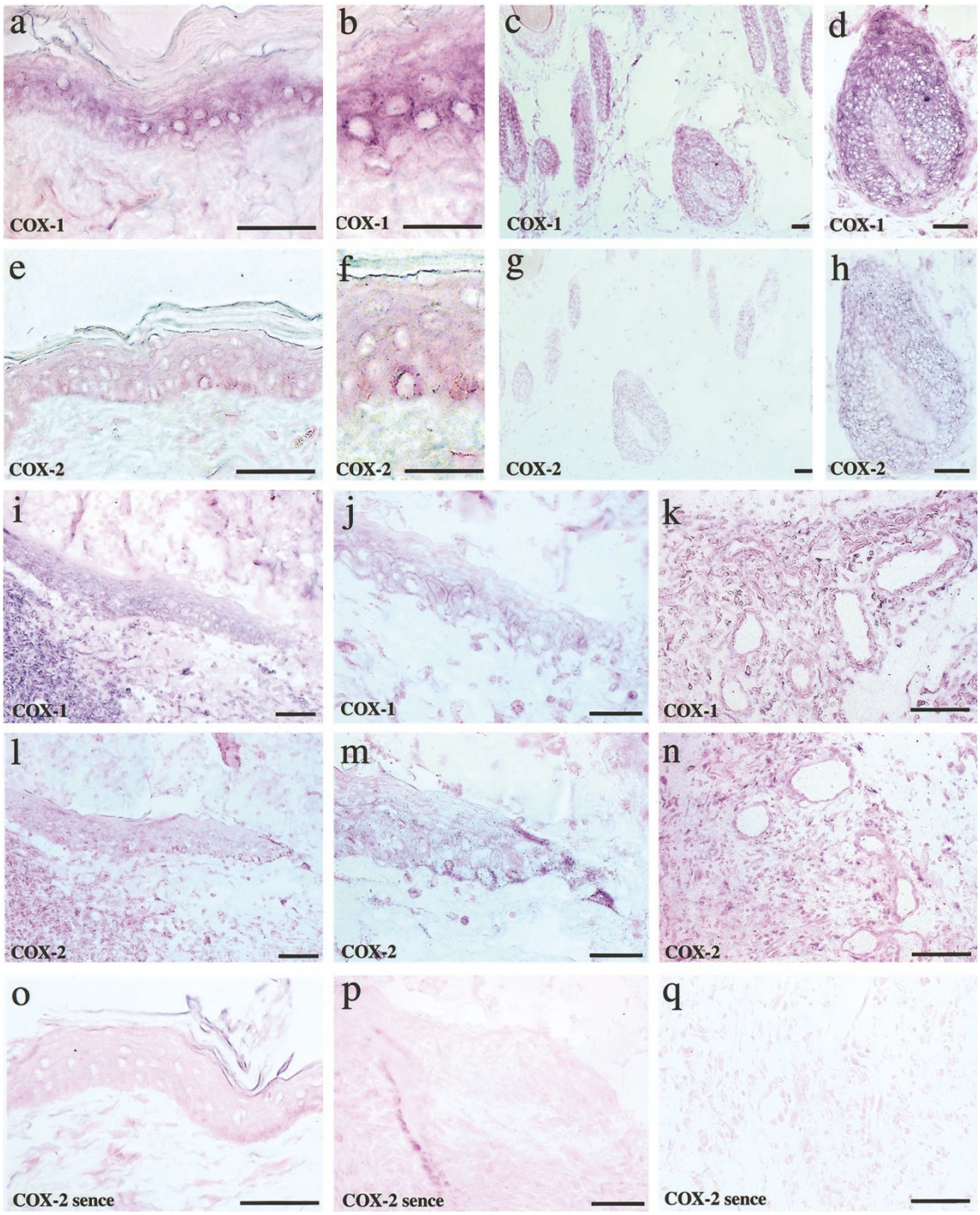


**Figure 3.**

In situ hybridization of COX mRNAs in normal and wounded skin. Normal skin (a to h, and o) is shown for epidermis (a, e, and o; and at higher magnification, b and f); and hair follicles and small vessels (c, d, g, and h). Wounded skin obtained 3 days after injury (i to n) is shown for the epidermal wound edge (i, l, and p; and at higher magnification, j and m); and granulation tissue (k, n, and q). No signal is seen using a sense probe for COX-2 mRNA (o to q). For a, c, d, e, g, h, i, k, l, n, o, and q, scale bars = 100 $\mu$m. For b, f, j, m, and p, scale bars = 50 $\mu$m.

COX-2 immunostaining in the normal mouse skin (Leong et al, 1996; Müller-Decker et al, 1998b). We also observed these COX-2 protein and mRNA in endothelial cells of small vessels and fibroblast-like cells within the granulation tissue. Furthermore, by Western blot analysis, we confirmed a marked induction of COX-2 protein within 12 hours after injury with continuous increase in concentration between Days 1 and 7. This period is characterized by a significant migration and proliferation of epidermal cells at wound edges as well as formation of granulation tissues (Clark, 1993). These findings suggest that an increased expression level of COX-2 after injury, particularly in the early acute phase, may enhance cell

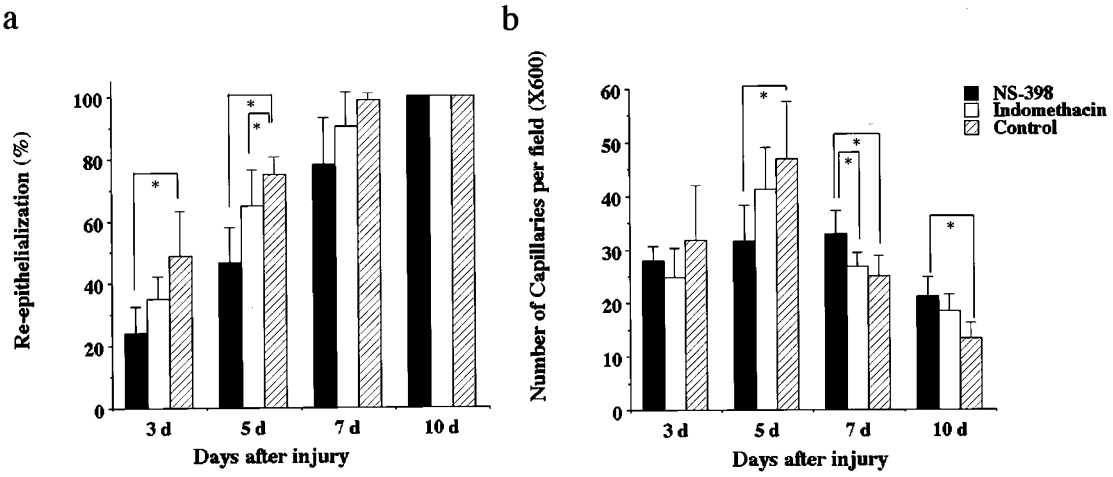


**Figure 4.**

Effect of daily intraperitoneal injection of *N*-2 cyclohexy-4-nitrophenyl methanesulfonamide (NS-398) or indomethacin during wound healing. a, Re-epithelialization is presented as percentages of the wound showing re-epithelialization in groups treated with NS-398 or indomethacin, and also in a vehicle control group, at 3, 5, 7, and 10 days after injury. b, Numbers of capillaries are shown. Numbers of capillaries in groups treated with NS-398 or indomethacin, and in a vehicle control group, are shown at 3, 5, 7, and 10 days after injury. Data are expressed as the mean ± SEM for five separate experiments. $*p < 0.05$.

migration and proliferation that underlie re-epithelialization and angiogenesis.

In addition, we observed a strong expression of the COX-2 protein in some peripheral cells of the ORS near the wound edge. Follicular stem cells necessary for hair growth occupy the bulging portion of the ORS (Cotsarelis et al, 1990). These cells proliferate in response to wounding and may also give rise to migrating epidermal cells. Stem cells are also present within the basal layer of the epidermis; these are responsible for epidermal maintenance and repair (Jones et al, 1995). The characteristic expression pattern of the COX-2 protein may therefore be related to the distribution of follicular and epidermal stem cells. In normal human skin, Müller-Decker et al (1999) reported the COX-2 protein expression in individual keratinocytes of the epidermis and hair follicles. However, according to Michelet et al (1997), COX-1 immunostaining was observed in the dermal papilla from human hair follicles, whereas COX-2 in the dermal papilla was expressed only in the anagen phase of the hair cycle. Although their findings appear to differ from ours, they actually might have observed expression in endothelial cells and fibroblast-like cells within the dermal papilla. Further studies are required to conclusively identify the COX-2-expressing cells in the epidermis and hair follicle.

In this study, we also clearly demonstrated that specific inhibition of COX-2 activity by NS-398 or nonspecific inhibition of COX activity by indomethacin caused a significant delay in wound healing in rat skin. Both re-epithelialization and angiogenesis were delayed by NS-398 treatment. Specifically, re-epithelialization in animals treated with NS-398 was significantly delayed in the early phase, which we found to be the time of greatest induction of the COX-2 expression in wounded rat skin. Savla et al (2001) reported that, in models of wound healing using cat or human airway epithelial cell cultures, addition of indomethacin to the medium for only the first 2 hours of the time course inhibited wound closure to the same extent as addition of indomethacin for the entire experimental time (12 hours). Furthermore, addition of $PGE_2$ for the first 2 hours stimulated wound closure to the same extent as adding $PGE_2$ for the entire time. Their findings suggested that COX-dependent metabolites including $PGE_2$ stimulate cell spreading and migration in the early phase of wound healing, considering that significant cell proliferation was not observed at these initial times. Exogenous $PGE_2$ has been reported to stimulate cell migration in tissue culture models using rabbit corneal endothelial cells (Joyce and Meklir, 1994). We speculate that COX-2-dependent metabolites may be particularly related to these processes in the early phase of wound healing. In the same study (Savla et al, 2001), $PGE_2$ also was added at 2, 4, or 6 hours after wounding, to investigate its effect in the later phase. Although wound closure with addition of $PGE_2$ after a 2-hour delay did not differ from closure with sustained application, longer delays caused failure of wound closure. Likewise, in our study, re-epithelialization was not significantly different between groups in the late phase (Days 7 and 10). These findings suggest that COX-dependent metabolites in the late phase, which may result from COX-1 activity in vivo, had little effect on wound healing. One explanation of these observations may involve differences in expression or function of $PGE_2$ receptors on keratinocytes in healing wounds (Konger et al, 1998). Persistent high concentration of exogenous $PGE_2$ inhibit growth, possibly resulting from down-regulation of $PGE_2$ receptors (Rys-Sikora et al, 2000). Furthermore, previous studies showed that leukotrienes and lipoxins (other AA metabolites whose formation is catalyzed by lipoxygenases) enhance cell proliferation (Kragballe et al, 1985; Leikauf et al, 1990), chemotaxis (Wiggins et al, 1990), and adhesion (Honn et al, 1998). These metabolites also are important for

wound healing, but they were not investigated in our study.

Angiogenesis also was inhibited significantly by NS-398 treatment, and the peak of angiogenesis was clearly delayed compared with control and indomethacin-treated groups. In healing of gastric ulcers in rats, regeneration of the mucosa and angiogenesis in the ulcer base were similarly prevented by subcutaneous injection of NS-398 (Shigeta et al, 1998). Although the dose of NS-398 used in their study (6 mg/kg/day) was higher than that used in our study (5 mg/kg/day), they reported that NS-398 had no effect on basal $PGE_2$ content in rat gastric mucosa and that the drugs significantly suppressed the production of $PGE_2$ in ulcerated gastric tissue. Therefore, we speculated that NS-398 affects mainly $PGE_2$ synthesis mediated by COX-2 during wound healing without affecting basal $PGE_2$ content in normal rat skin. However, whereas NS-398 decreased $PGE_2$ at the wound site in our study, statistical significance was not attained. According to Wallace et al (1998), who used a carrageenan-induced inflammation model in rat, the lowest dose of NS-398 needed to produce a significant reduction of edema and suppression of PG synthesis in the inflammation site, also significantly inhibited COX-1 activity. These results suggest that the dose of NS-398 in our study had little significant effect on either COX-1 activity or PG synthesis at the wound site, so only suppression of COX-2 activity contributed importantly to the significant delay in wound healing. Furthermore, a previous study reported that coupled activation of COX-2 and secretory phospholipase $A_2$ protein were responsible for modulation of $PGE_2$ production in activated keratinocyte cultures during wound healing (Rys-Sikora et al, 2000). As AA release can occur via the action of phospholipase $A_2$, we may need to consider the relationship between $PGE_2$, AA release, and phospholipase $A_2$ during wound healing.

A recent study indicated that oral administration of NS-398 decreased the protein expression level for the basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), in association with the reduced angiogenesis and growth in a COX-2-overexpressing human gastric cancer xenograft in nude mice (Sawaoka et al, 1999). In a coculture model of colon cancer cells with endothelial cells, COX-2-overexpressing cancer cells produce angiogenic factors, including bFGF and VEGF, which stimulate both endothelial migration and tube formation, and the effect is inhibited by NS-398 (Tsuji et al, 1998). Likewise, in skin wound healing, re-epithelialization and angiogenesis are induced by several peptide growth factors, such as bFGF and VEGF. We hypothesized that COX-2 inhibition may suppress the production of these peptide growth factors by COX-2-expressing cells. On the other hand, Tsuji et al (1998) reported that the COX-1 activity in endothelial cells also regulates tube formation, which is inhibited by aspirin but not by NS-398. We demonstrated that COX-1 is expressed constitutively in endothelial cells of rat skin during wound healing. Although the indomethacin-treated group had fewer capillaries than the control group in our study, this difference was not significant. We speculated that the indomethacin dose in our study (0.5 mg/kg/day) may be insufficient for inhibition of the COX-1 activity, because angiogenesis in the healing of gastric ulcers in rats was significantly inhibited in the group treated with indomethacin, the dose (2 mg/kg/day) of which was higher than that used in our study (Shigeta et al, 1998).

We demonstrated the expression patterns of the two isoforms of COX protein and mRNA in rat skin, observing a high COX-2 expression level in the early phase of cutaneous wound healing. COX-2 appears to be important in epidermal and dermal recovery from injury. Local or systemic administration of an NSAID or a more specific COX-2 inhibitor requires caution during the early phase of wound healing.

## Materials and Methods

### *Tissue Samples*

Adult male Wistar rats (6 to 7 weeks old) that were free of known pathogens were used (Saitama animal laboratory, Saitama, Japan). Their average body weight was 200 g. The animal housing room was maintained at a constant temperature (24 $\pm$ 1° C) with 12 hours of light followed by 12 hours of darkness. Rats were provided free access to commercial rat chow and water throughout the experiment. Under inhalation anesthesia with diethyl ether, dorsal hair was removed from each rat using an electric clipper, and 3 days after, eight full-thickness dermal wounds were produced in the dorsal skin with a 6-mm punch. Subsequently, the rats were killed by administration of an anesthetic overdose 3, 6, or 12 hours or 1, 3, 5, 7, or 10 days after injury. Wounded skin was removed for analysis. Similar skin samples were removed from unwounded areas and used as normal control specimens. Each group included three rats. Skin samples from four wounded areas in the upper back were fixed immediately overnight at 4° C in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4). Tissues were bisected perpendicular to the skin surface to include the most extensive wound margin. One-half of these tissues were embedded in paraffin. The other half for use in in situ hybridization were immersed sequentially in 10%, 20%, and 30% sucrose in 0.01 M PBS (pH 7.4) at 4° C for 6 to 12 hours for each sucrose concentration. These tissues were embedded in an optimal-cutting-temperature compound (Sakura, Tokyo, Japan), and then immediately frozen at $-80$° C for storage. Skin samples including the remaining four wounded areas in the lower back were immediately frozen at $-80$° C for Western blot analysis. The experimental procedure was approved by the Animal Experimental Ethical Review Committee of Nippon Medical School, Japan.

### *Immunohistochemistry*

An avidin-biotin-peroxidase complex (ABC) method was used (Hsu et al, 1981). After deparaffinization,

3-μm-thick sections were rinsed for 30 minutes with methyl alcohol containing 0.3% hydrogen peroxide ($H_2O_2$). After washing with PBS, the sections were microwaved for 15 minutes in 10 mM citrate buffer (pH 6.0), and then by incubation with 10% normal rabbit serum in PBS for 1 hour at room temperature. Sections were then incubated for 1 hour at room temperature with polyclonal goat anti-rat primary antibodies in PBS: either anti-COX-1 (M-20; dilution, 1:5000), or anti-COX-2 (M-19; dilution, 1:5000) (Santa Cruz Biotechnology, Heidelberg, Germany). After washing with PBS, sections were treated with biotinylated anti-goat IgG (dilution, 1:300) (DAKO, Glostrup, Denmark) in PBS for 1 hour at 37° C, and then by washing with PBS and incubation with ABC for 30 minutes at room temperature. Peroxidase activity was visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.01% $H_2O_2$. Nuclei were counterstained with Mayer's hematoxylin. To confirm antibody specificity, another section was incubated with the anti-COX-2 antibody preabsorbed with a specific blocking peptide (M-19P; Santa Cruz Biotechnology). This preabsorbed control was then treated with a secondary antibody and stained by the ABC-DAB method.

### *Western Blotting*

The COX protein was partially purified as described by Mizuno et al (1997). Frozen skin samples were homogenized in a solution of 25 mM Tris-HCl (pH 8.0), 0.25 M sucrose, 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 1.0 μM pepstatin A, and 1.0 mM ethylenediaminetetraacetic acid (EDTA). Then, 3-1-propanesulfonate (CHAPS; Sigma Chemical, Steinheim, Germany) was added to yield a final CHAPS concentration of 1% (wt/vol), and the mixture was stirred for 2 hours at 4° C. After centrifugation at 15,000 rpm for 20 minutes at 4° C, the supernatant was concentrated to 50% of the initial volume using an ultrafiltration unit (Advantec, Tokyo, Japan) and loaded onto anion-exchange supports (Macro-prep High Q Supports; Bio-Rad Laboratories, Hercules, California) equilibrated with 20 mM Tris-HCl (pH 8.0) and 0.4% CHAPS. Fractions eluted at 1.0 M NaCl were normalized using a DC protein assay kit (Bio-Rad). Equivalent amounts of protein were applied to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and separated by electrophoresis. The separated proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), and the membranes were blocked overnight at 4° C with a blocking buffer (5% nonfat dried skimmed milk, 1% bovine serum albumin, and 0.1% Tween-20 in Tris-buffered saline (TBS) at pH 7.5). After incubation with primary antibodies, goat anti-rat COX-1 (dilution, 1:2000) and COX-2 (dilution, 1:1000) in the blocking buffer overnight at 4° C, the membranes were washed with TBS (pH 8.0) containing 0.1% Tween 20 and incubated with horseradish-peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology; dilution for COX-1, 1:4000; for COX-2, 1:2000) in the blocking buffer for 2 hours at room temperature. Binding of antibodies was detected with an enhanced chemiluminescence detection system (Chemiluminescence Plus; Amersham Pharmacia Biotech). Protein bands were quantified by densitometry using the public domain of US National Institutes of Health Image software (version 1.60).

### *In Situ Hybridization*

To generate RNA probes for rat COX-1 and COX-2, total RNA was extracted from frozen rat prostate tissues by an acid-guanidine-thiocyanate-phenyl-chloroform extraction method using an RNeasy minikit (Qiagen, Hilden, Germany), and then by reverse transcription using random 9-mers as a reverse primer. The resulting cDNAs were amplified by PCR using an RT-PCR reagent kit (Takara, Osaka, Japan). PCR primer pairs for COX-1 or COX-2 based on published cDNA sequences (Beiche et al, 1996; Feng et al, 1993) were synthesized commercially (Sawady Technology, Tokyo, Japan). Primers specific to COX-1 (5'-forward, CATGGATCCGGATTGGTGGGGGTAG; 3'-reverse, ATCTCGAGGGGCAGGTCTTGGTGTTG) amplified a fragment of 447 bp; primers specific to COX-2 (5'-forward, CTGTATCCCGCCCTGCYGGTG; 3'-reverse, ACTTGCGTTGATGGTGGCTGTCTT) amplified a fragment of 279 bp. The PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, Wisconsin). The sequence of the cloned cDNA was confirmed by nucleic acid sequencing. After linearization of the templates with an appropriate restriction enzyme, antisense and sense probes were synthesized and labeled with digoxigenin (DIG) using SP6 and T7 promoters (Promega), and a DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany).

For hybridization, 6-μm-thick cryostat sections from wounded skin 3 days after injury, as well as similarly prepared normal skin sections were treated with proteinase K (2 μg/ml) in PBS for 15 minutes at 37° C and fixed in 4% PFA in PB for 15 minutes at room temperature. After washing with PBS, the sections were treated with glycine (2 mg/ml) in PBS, acetylated in 0.1 M triethanolamine and 0.25% acetic anhydride, and finally incubated overnight at 45° C with DIG-labeled RNA probes (200 ng/ml) diluted in hybridization buffer (12.5 mM Tris-HCl at pH 7.4, 750 mM NaCl, 1.25 mM EDTA, 50% formamide, 1.25x Denhardt's medium, 312.5 μg/ml tRNA, 156.25 μg/ml salmon-sperm DNA, and 12.5% dextran sulfate). The slides were then washed with 0.5x SSC containing 1 mM EDTA. This was followed by fixation in a mixture of 50% formamide, 5 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.5 mM EDTA for 30 minutes at room temperature; washing with 0.5x SSC; and treatment with RNase A (20 μg/ml) diluted in 10 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 1 mM EDTA for 30 minutes at 37° C. The sections were washed sequentially in 0.5x SSC for 10 minutes at room temperature, 0.5x SSC for 10 minutes at 55° C, 0.2x SSC for 10 minutes at 55° C, 0.2x SSC for 10 minutes at room temperature, and buffer 1 (100 mM Tris-HCl at pH 7.5 and 150 mM NaCl)

for15 minutes at room temperature. The sections were then incubated with a blocking solution (1.5% blocking reagent [Boehringer Mannheim] in buffer 1) for 2 hours at room temperature. Subsequently, the sections were incubated with an alkaline phosphatase-conjugated sheep anti-DIG antibody (dilution, 1:1000; Boehringer Mannheim) in the blocking solution overnight at 4° C. After washing with buffer 1, the sections were rinsed in buffer 3 (100 mM Tris-HCl at pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$). Then, 4-nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indoylphosphate (BCIP) (200 $\mu$l/ml) (Boehringer Mannheim) in buffer 3 was added. The color reaction was stopped by adding 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The sections were fixed for 15 minutes with 4% PFA in PB, rinsed with distilled water, counterstained with nuclear fast red (Merck, Darmstadt, Germany), and coverslipped using glycerol.

### *Drug Treatment*

NS-398 (2.5 mg/kg body weight) (Funacoshi, Tokyo, Japan) or indomethacin (0.25 mg/kg body weight) (Sigma Chemical) was suspended in 0.5 ml of 5% gum arabic (Sigma Chemical) in distilled water and injected into the peritoneal cavity of the rats 3 hours before and after injury. The injection was repeated twice a day (every 12 hours) and continued until the rats were killed 3, 5, 7, or 10 days after injury. The control rats were given intraperitoneal injections of a vehicle (0.5 ml of 5% gum arabic in distilled water). Each treatment group included five rats per time point. After killing by administration of an anesthetic overdose, the skin sample in the area of each wound was fixed overnight in 4% PFA in PB at 4° C, cut perpendicular to the skin surface at the widest wound margin, and embedded in paraffin. Sections (3-$\mu$m thick) were stained with hematoxylin and eosin. The degree of re-epithelialization and the capillary number were evaluated histologically in the section showing the widest original wound margin. To express re-epithelialization as a percentage, the distance between muscle edges as an indicator of wound edges, and the distance that the epithelium had covered across the wound were measured using a standard ocular grid. Then, the following formula was used as described previously (Swift et al, 1999): Percentage of re-epithelialization = (distance covered by epithelium)/(distance between muscle edges) $\times$ 100. Capillaries containing a definite lumen in the plane of a section were counted in 20 optical fields of each cross section of a wound at original magnification $\times$600.

### *Production of $PGE_2$ at the Wound Site*

Wounded skin samples obtained 3 days after injury in each treatment group were immediately frozen at $-80°$ C and homogenized in ethanol. The supernatants were subjected to extraction of PGE2 according to the method of Kawano et al (1987). Supernatants were acidified with 1 N HCl (pH 3.0) and loaded onto octadecylsilyl silica (Wako, Osaka, Japan), suspended in ethanol, and then by centrifugation. The residue was washed with ethanol and centrifuged twice, and then washed with petroleum ether. After centrifugation, fractions were eluted with ethyl acetate and then evaporated. The residue was applied to a silica mini-column (Varian, Walnut Creek, California) for further extraction. $PGE_2$ in the extract was determined using a radioimmunoassay kit (Perkin Elmer Life Science, Boston, Massachusetts).

### *Statistical Analyses*

Results are expressed as means $\pm$ SEM. For statistical evaluations of differences between paired data, Student's *t* test was performed. For multiple comparisons, analysis of variance (ANOVA) was carried out followed by Scheffé's test. Differences were regarded as significant when *p* values were less than 0.05.

## Acknowledgements

The authors thank Drs. Ken Wada, Seiji Futagami, and Atsushi Tatsuguchi (Department of Third Department of Internal Medicine, Nippon Medical School) for their technical assistance.

## References

Beiche F, Scheuerer S, Brune K, Geisslinger G, and Goppelt-Struebe M (1996). Up-regulation of cyclooxygenase 2 mRNA in the rat spinal cord following peripheral inflammation. FEBS Lett 390:165–169.

Cameron GS, Baldwin JK, Jasheway DW, Patrick KE, and Fischer SM (1990). Arachidonic acid metabolism varies with the state of differentiation in density gradient-separated mouse epidermal cells. J Invest Dermatol 94:292–296.

Clark RAF (1993). Mechanisms of cutaneous wound repair. In: Fitzpatrik TB, Eisen AZ, Wolff K, Freedberg IM, and Austen MD, editors. Dermatology in general medicine. New York: McGraw-Hill, 473–486.

Cotsarelis G, Sun T-T, and Lavker RM (1990). Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61:1329–1337.

Cybulsky AV, Goodyer PR, CyrMD, and McTavish AJ (1992). Eicosanoids enhance epidermal growth factor receptor activation and proliferation in glomerular epithelial cells. Am J Physiol Renal Fluid Electrolyte Physiol 262:F639–646.

DeWitt DL and Smith WL (1988). Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. Proc Natl Acad Sci USA 85:1412–1416.

DeWitt DL and Smith WL (1995). Yes, but do they still get headaches? Cell 83:345–348.

Evans CB, Pillai SK, and Goldyne ME (1993). Endogenous prostaglandin $E_2$ modulates calcium-induced differentiation in human skin keratinocytes. Prostaglandins Leukot Essent Fatty Acid 49:777–781.

Feng L, Sun W, Xia Y, Tang WW, Chanmugam P, Soyoola E, Wilson CB, and Hwang D (1993). Cloning of two isoforms of rat cyclooxygenase. Arch Biochem Biophys 307:361–368.

Form DM and Auerbach R (1983). Prostaglandin E2 and angiogenesis. Prog Soc Exp Biol Med 172:214–218.

Futaki N, Takahashi S, and Yokoyama M (1994). NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. Prostaglandins 47:55–59.

Garlick JA and Taichman LB (1994). Fate of human keratinocyte during reepithelialization in an organotypic culture model. Lab Invest 70:916–924.

Gierse JK, Hauser SD, and Creely DP (1995). Expression and selective inhibition of the constitutive and inducible forms of human cyclooxygenase. Biochem J 305:479–484.

Honn KV, Grossi IM, Fitzgerald LA, Umbarger LA, Diglio CA, and Taylor JD (1998). Lipoxygenase products regulate IRG-pIIb/IIIa receptor-mediated adhesion of tumor cells to endothelial cells, subendothelial matrix and fibronectin. Proc Soc Exp Biol Med 189:130–135.

Hsu SM, Raine L, and Fanger H (1981). Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. J Histochem Cytochem 29:557–580.

Jones PHG, Harper S, and Watt FM (1995). Stem cell patterning and fate in human epidermis. Cell 80:83–93.

Jonsson CE and Änggård E (1972). Biosynthesis and metabolism of prostaglandin $E_2$ in human skin. Scand J Clin Lab Invest 29:289–296.

Jouvenaz GH, Nugteren DH, Beerthuis RK, and Van Dorp DA (1970). A sensitive method for the determination of prostaglandins by gas chromatography with electron-capture detection. Biochim Biophys Acta 202:231–234.

Joyce NC and Meklir B (1994). $PGE_2$: A mediator of corneal endothelial wound repair in vitro. Am J Physiol Cell Physiol 266:C269–275.

Kawano K, Sugita M, Oka M, and Tabata N (1987). A simple, rapid and simultaneous extraction of thromboxane $B_2$, 6-keto-prostaglandin $F_1\alpha$ and prostaglandin $E_2$. Jap J Inflammation 7:511–515.

Konger RL, Malaviya R, and Pentland AP (1998). Growth regulation of primary human keratinocytes by PGE receptor $EP_2$ and $EP_3$ subtypes. Biochem Biophys Acta 1401:221–234.

Kragballe K, Desgarlais L, and Voorhees J (1985). Leukotrienes $B_4$, $C_4$ and $D_4$ stimulate DNA synthesis in human epidermal keratinocytes. Br J Dermatol 113:43–52.

Kujubu DA, Fletcher BS, Varum BC, Lim RW, and Herschman HR (1991). TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem 266: 12866–12872.

Leikauf GD, Claesson H-E, Doupnik CA, Hybbinette S, and Grafström RC (1990). Cysteinyl leukotrienes enhance growth of human airway epithelial cells. Am Physiol Lung Cell Mol Physiol 259:L255–261.

Leong J, Hughes-Fulford M, Rakhlin N, Habib A, Maclouf J, and Goldyne ME (1996). Cyclooxygenases in human and mouse skin and cultured human keratinocytes. Exp Cell Res 224:79–87.

Li H, Hebda PA, Kelly LA, Ehrlich GD, Whitcomb DC, and Dohar JE (2000). Up-regulation of prostaglandin $EP_4$ receptor messenger RNA in fetal rabbit skin wound. Arch Otolaryngol Head Neck Surg 126:1337–1343.

Lowe NJ and Stoughton RB (1977). Effects of topical prostaglandin $E_2$ analogue on normal hairless mouse epidermal DNA synthesis. J Invest Dermatol 68:134–137.

Maier JA, Hla T, and Maciag T (1990). Cyclooxygenase is an immediate-early gene induced by interleukin-1 in human endothelial cells. J Biol Chem 265:10805–10808.

Martin P (1997). Wound healing-aiming for perfect skin regeneration. Science 276:75–81.

Merlie JP, Fagan D, Mudd J, and Needleman P (1988). Isolation and characterization of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthase (cyclooxygenase). J Biol Chem 263:3550–3553.

Michelet JF, Commo S, Billoni N, Mahé YF, and Bernard BA (1997). Activation of cytoprotective prostaglandin synthase-1 by Minoxidil as a possible explanation for its hair growth-stimulating effect. J Invest Dermatol 108:205–209.

Mizuno H, Sakamoto C, Matsuda K, Wada K, Uchida T, Noguchi H, Akamatsu T, and Kasuga M (1997). Induction of cyclooxygenase 2 in gastric mucosal lesions and its inhibition by the specific antagonist delays healing in mice. Gastroenterology 112:387–397.

Müller-Decker K, Kopp-Schneider A, Marks F, Seibert K, and Fürstenberger G (1998a). Localization of prostaglandin H synthase isoenzymes in murine epidermal tumors. Mol Carcinog 23:36–44.

Müller-Decker K, Reinerth G, Krieg P, Zimmermann R, Heise H, Bayerl C, Marks F, and Fürstenberger G (1999). Prostaglandin H synthase isozyme expression in normal and neoplastic human skin. Int J Cancer 82:648–656.

Müller-Decker K, Scholz K, Neufang G, Marks F, and Fürstenberger G (1998b). Localization of prostaglandin H synthase-1 and -2 in mouse skin. Exp Cell Res 242:84–91.

O'Banion MK, Winn VD, and Young DA (1992). cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. Proc Natl Acad Sci USA 89:4888–4892.

O'Sullivan MG, Huggins EMJ, Meade EA, DeWitt DL, and McCall CE (1992). Lipopolysaccharide induces prostaglandin H synthase 2 in alveolar macrophages. Biochem Biophys Res Commun 187:1123–1127.

Pentland AP and Needleman P (1986). Modulation of keratinocyte proliferation in vitro by endogenous prostaglandin synthesis. J Clin Invest 77:246–251.

Rys-Sikora KE, Konger RL, Schoggins JW, Malaviya R, and Pentland AP (2000). Coordinate expression of secretory phospholipase $A_2$ and cyclooxygenase-2 in activated human keratinocytes. Am J Physiol 278:C822–833.

Sato T, Kirimura Y, and Mori Y (1997). The co-culture of dermal fibroblasts with human epidermal keratinocytes induces increased prostaglandin $E_2$ production and cyclooxygenase 2 activity in fibroblasts. J Invest Dermatol 109:334–339.

Savla U, Appel HJ, Sporn PHS, and Waters CM (2001). Prostaglandin$E_2$ regulates wound closure in airway epithelium. Am J Physiol Lung Cell Mol Physiol 280:L421–431.

Sawaoka H, Tsuji S, Tsuji M, Gunawan ES, Sasaki Y, Kawano S, and Masatsugu H (1999). Cyclooxygenase inhibitors suppress angiogenesis and reduce tumor growth in vivo. Lab Invest 79:1469–1477.

Scholz K, Fürstenberger G, Müller-Decker K, and Marks F (1995). Differential expression of prostaglandin H synthase isoenzymes in normal and activated keratinocytes in vivo and in vitro. Biochem J 309:263–269.

Shigeta J, Satoru T, and Okabe S (1998). Role of cyclooxygenase-2 in the healing of gastric ulcers in rats. J Pharmacol Exp Therapeutics 286:1383–1390.

Swift ME, Kleinman HK, and Dipietro LA (1999). Impaired wound repair and delayed angiogenesis in aged mice. Lab Invest 79:1479–1487.

Talwar M, Moyana TN, Bharadwaj B, and Tan LK (1996). The effect of synthetic analogue of prostaglandin $E_2$ on wound healing in rats. Ann Clin Lab Sci 26:451–457.

Tsuji M, Kawano S, Tsuji S, Sawaoka H, Hori M, and DuBois RN (1998). Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell 93:705–716.

Wallace JL, Bak A, Mcknight W, Asfata S, Sharkey KA, and Macnaughton WK (1998). Cyclooxygenase-1 contributes to inflammatory responses in rats and mice: Implications for gastrointestinal toxicity. Gastroenterology 115:101–109.

Wiggins RE, Jafri MS, and Proia AD (1990). 12(S)-hydroxy-5, 8, 10, 14-eicosatetraenoic acid is a more potent neutrophil chemoattractant than the 12(R)-epimer in the rat cornea. Prostaglandins 40:131–141.

Yang T, Singh I, Pham H, Sun D, Smart A, Schnermann JB, and Briggs JP (1998). Regulation of cyclooxygenase expression in the kidney by dietary salt intake. Am J Physiol 274:F481–489.

Ziche M, Jones J, and Gullino PM (1982). Role of prostaglandin $E_1$ and copper in angiogenesis. J Natl Cancer Inst 69:475–482.

Zushi S, Shinomura Y, Kiyohara T, Minami T, Sugimachi M, HigashimotoY, Kanayama S, and Matsuzawa Y (1996). Role of prostaglandins in intestinal epithelial restitution stimulated by growth factors. Am J Physiol Liver Gastrointest Liver Physiol 270:G757–762.