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ORIGINAL PAPER

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PKC-dependent IL-6 production and inhibition of IL-8 production by PKC activation in normal human skin fibroblasts under extremely high hydrostatic pressure

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Abstract Normal human dermal fibroblasts were found to survive and to be active in producing interleukin (IL)-6 and IL-8 under extremely high hydrostatic pressure, up to 40 MPa (1 atm = 0.101325 MPa = 1.03323 kgf/cm^2), for 20 min. An inhibitor of protein kinase C (PKC) reduced the amount of IL-6 production, whereas IL-8 production was increased following pressure application. The activation of PKC in response to exposure to the pressure stress was detected by using the PKC-specific probe Rim-1. These findings indicate that IL-6 production induced by hydrostatic pressure stresses was dependent on the PKC signaling pathway. In contrast, pressure-induced IL-8 production was inhibited by PKC activity.

Key words Human dermal fibroblast · Hydrostatic pressure · Interleukin-6 · Interleukin-8 · PKC activation

Introduction

It has been known for many years that alteration of the micro- or macro-environment of a cell can trigger a protective action against stress and injury. Varieties of stresses, including elevated or decreased temperatures, heavy metals, viral infections, and alcohol, activate the expression of heat-shock proteins by causing the intracellular accumulation of abnormal or degraded proteins (Lindquist and Craig 1988; Lindquist 1986). As another example, Bi et al. (1995) reported that after they wounded the plasma membrane of sea urchin eggs and embryos by using a laser beam, both the eggs and embryos showed a rapid burst of localized exocytosis. The rate of exocytosis was correlated quantitatively with successful resealing of the membrane (Bi et al. 1995). Recent studies have shown that physical stresses, including electric stimulation (Koyama et al. 1996, 1997; Aizawa et al. 1999), ionizing radiation (Lee et al. 1995), hydrostatic pressure (Takahashi et al. 1997, 1998), and ultraviolet radiation (Wlaschek et al. 1997; Mohamadzadeh et al. 1995; Enk et al. 1996) induce growth factors and immunomodulatory cytokines in mammalian cells. These cellular responses have been recognized as a protective action of cells to adapt a change in their environment.

Skin cells have the capacity to produce a wide range of immunomodulatory cytokines, including interleukin (IL)-1 , IL-6, IL-10, IL-12, and IL-15, after exposure to reactive oxygen species and ultraviolet radiation (Wlaschek et al. 1997; Mohamadzadeh et al. 1995; Enk et al. 1996). These cytokines appear to play a role in maintaining homeostasis in normal skin. Since intense mechanical stress puts the normal skin homeostasis into disorder, the stress-disrupted skin tissue could also trigger the production of immunomodulatory cytokines against the invasion of pathogenic bacteria into the body. To clarify this hypothesis, we investigated whether extremely high hydrostatic pressure stress, up to 40 MPa, induced immunomodulatory cytokine production in normal human dermal fibroblast cells.

We found that normal human dermal fibroblasts survived and enhanced IL-6 and IL-8 production in response to extremely high hydrostatic pressure stresses of up to 40 MPa for 20 min.

Materials and methods

Materials

The human IL-1α assay kit used was purchased from Otsuka Seiyaku (Tokyo, Japan). The human IL-1β and

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human IL-6 ELISA kits were purchased from Boehringer Mannheim (Mannheim, Germany). Predicta IL-8 and IL-15 kits were obtained from Genzyme (MA, USA). Phorbol 12-myristate 13-acetate (PMA), Rim-1, and GF109203X were purchased from Wako Pure Chemical Industries (Osaka, Japan). Calphostin-C was from BIOMOL Research Laboratories (PA, USA). A live/dead viability/cytotoxicity kit was purchased from Molecular Probes (OR, USA).

Cell culture

Normal newborn human dermal fibroblast cells (Kurabo, Osaka, Japan, and Clonetics, CA, USA) were plated at a density of 3×10^4 cells/cm^2 on 35-mm plastic petri dishes (Falcon Plastic, CA, USA) in Dulbecco's modified Eagle medium (DMEM, ICN Biomedicals, OH, USA) containing 10% fetal bovine serum (BioWhittaker, MD, USA), 50 units/ml penicillin and 50 µg/ml streptomycin (BioWhittaker).

The cells were grown at 37°C in a humidified atmosphere of 5% CO_2 to a final density of 3×10^4 cells/cm^2. The cells were then placed in serum-free DMEM containing the same concentrations of penicillin and streptomycin and cultured for another 24 h. Under serum-free conditions, the fibroblasts were confirmed to be in a resting stage, and they did not proliferate. The petri dishes were filled with the serum-free DMEM, and, after the air bubbles had been removed, the dishes were tightly sealed with Parafilm (American National Can, USA). Thereafter, the sealed petri dishes were pressurized.

Application of hydrostatic pressure to the cells

Cultured normal human dermal fibroblasts at a density of 3×10^4 cells/cm^2 on a 35-mm petri dish were placed in a titanium pressurization vessel (inside diameter 62 × 198 mm; Rigo-sha, Tokyo, Japan) connected to a hydrostatic pressure apparatus (Tomita, Tokyo, Japan). Hydrostatic pressure in the range of 5–40 MPa was applied to the cells. Compression and decompression rates were about 3–5 MPa/s and between about –15 and –20 MPa/s, respectively. The temperature was maintained at 37°C during pressurization by means of a water bath. We confirmed that there were no changes in the pH of the culture medium prior to or following exposure to high hydrostatic pressure.

Effects of protein kinase C inhibition on hydrostatic pressure-induced IL-6 and -8 production

Hydrostatic pressure, 40 MPa, was applied to fibroblasts in serum-free DMEM containing a protein kinase C (PKC) inhibitor such as 50 µM GF109203X or 300 nM calphostin C for 20 min. The fibroblasts were then incubated in serum-free DMEM containing 1% bovine serum albumin (BSA, Wako Pure Chemical Industries) and the same concentration of the inhibitor for another 24 h. As a PKC down-regulation technique, normal human dermal fibroblasts were exposed to 100 nM PMA containing serum-free medium for 24 h. After PKC down regulation, the fibroblasts were again placed in the serum-free medium and 40 MPa hydrostatic pressure was applied for 20 min. After the pressure application, the cells were incubated in serum-free DMEM containing 1% BSA for another 24 h, and after this incubation period, cytokine levels in the culture medium were assayed by ELISA.

ELISA for human IL-1α, -1β, -6, -8, and -15

After the application of hydrostatic pressure, the culture medium in each petri dish was changed by adding 1 ml of fresh serum-free DMEM supplemented with 1% BSA, 50 units/ml penicillin, and 50 µg/ml streptomycin (BioWhittaker). The cells were maintained at atmospheric pressure in a 5% CO_2 environment and incubated for another 24 h. After the incubation period, the culture medium was collected and stored at 4°C until the ELISA assay. The fibroblasts were collected and suspended in 1 ml of phosphate buffered saline (PBS) containing 1% BSA. The cell suspension was sonicated for 30 s 15 times at 30-s intervals. The homogenized solutions and culture medium were assayed for IL-1α, -1β, -6, -8, and -15 by using the ELISA assay kit according to the procedure recommended by the manufacturer. Each assay was performed in duplicate, and the data from four independent experiments were averaged.

PKC staining with Rim-1

PKC staining with Rim-1 was performed essentially as described by Chen and Poenie (1993). Briefly, the fibroblasts on coverslips were fixed for 10 min in PBS containing 3.75% paraformaldehyde. After fixation, the fibroblasts were permeabilized in methanol at –20°C for 10 min and washed twice with PBS. The fixed fibroblasts were incubated for 30 min in PBS containing 200 nM Rim-1. After staining, the coverslips were rinsed four times in PBS and mounted on glass slides by using Vectashield mounting medium (Vector Laboratories, CA, USA).

Cell viability test

The fibroblasts were incubated with both 2 µM calcein-AM and 4 µM EthD-1 (live/dead viability/cytotoxicity kit; Molecular Probes, OR, USA) in PBS for 15 min at 37°C. After the incubation period, the fibroblasts were examined under a blue excitation light in accordance with the manufacturer's recommendations.

Statistical analysis

The data were analyzed by using Student's *t*-test.

Results

Hydrostatic pressure-induced IL-6 and -8 production

We found that extremely high hydrostatic pressure stresses triggered IL-6 and IL-8 production in normal human dermal fibroblasts. Normal human dermal fibroblasts remained alive for 60 min at up to 40 MPa, as demonstrated by the calcein-AM and EthD-1 double staining. It is noteworthy that no morphological changes were observed at the high cell density of 3×10^4 cells/cm^2. Therefore, the fibroblasts were subjected to the application of pressure at 5, 10, or 40 MPa for 20 min.

Figure 1 shows the amounts of IL-6 and IL-8 protein produced by the fibroblasts under each of the pressure conditions examined, after 24 h of incubation. The application of pressure resulted in extremely enhanced levels of IL-6 and IL-8 secretion and production ($P < 0.001$, when compared with the control). When pressure was applied at 40 MPa for 20 min, IL-6 secretion was maximally enhanced; the level of secretion was increased about 10-fold compared with that of the control (Fig. 1). Similarly, maximum IL-8 secretion was induced by 10 MPa hydrostatic pressure and represented an increase of about 18-fold over that of the control (Fig. 1). Since the intracellular levels of IL-6 and -8 were increased following the application of pressure, de novo synthesis of IL-6 and IL-8 was also increased by hydrostatic pressure (Fig. 1).

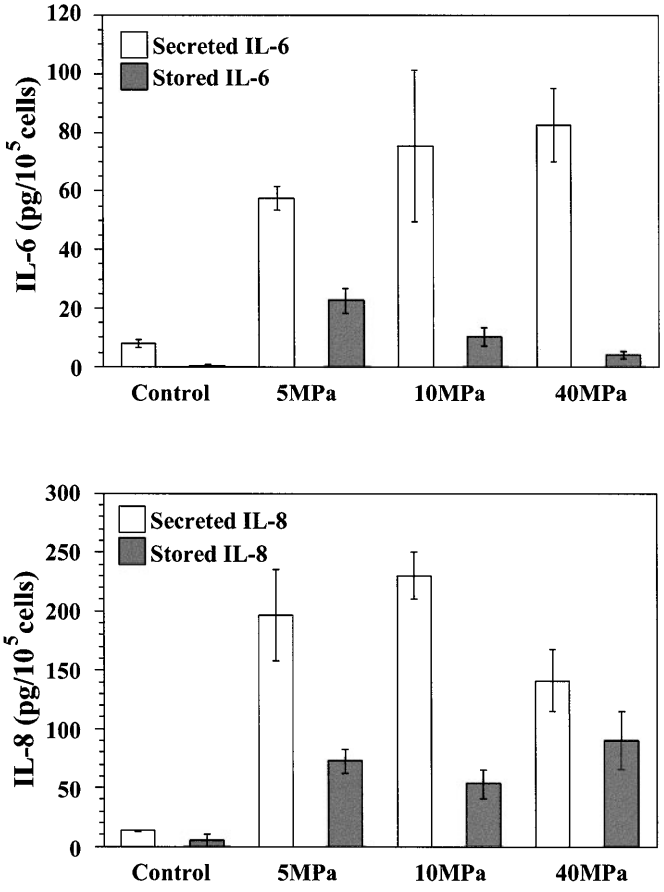

Fig. 1. Hydrostatic pressure-induced interleukin (*IL*)-6 (*top*) and IL-8 (*bottom*) production. There was a statistically significant difference ($P < 0.001$) between pressure-applied cells and the control according to Student's *t*-test

Effects of PKC inhibition on pressure-induced interleukin production

We investigated the intracellular signaling pathways of pressure-induced interleukin production. DNA sequencing studies suggest that both IL-6 and -8 genes contain an AP-1 binding site (Ray et al. 1989; Okamoto et al. 1994). It has been reported that chemically induced IL-6 and -8 secretions may be related to an increase in protein kinase C (PKC) and AP-1 binding activities (Matsuno et al. 1998; Zhang et al. 1992). Therefore, we examined whether the mechanisms of pressure-induced interleukin production involved PKC activity.

A hydrostatic pressure of 40 MPa was applied to the fibroblasts in serum-free medium containing GF109203X, a specific PKC inhibitor, for 20 min. In the presence of the PKC inhibitor GF109203X, pressure-induced IL-6 secretion was reduced to one-fourth that without the inhibitor (Fig. 2). In complementary experiments, calphostin C, another specific inhibitor for PKC, was also examined. Calphostin C suppressed pressure-induced IL-6 secretion by 50%, as shown in Fig. 2. Chronic exposure to phorbol 12-myristate 13-acetate (PMA) causes a drastic depletion in levels of the PMA-sensitive PKC enzymes (Pshenichkin and Wise 1995); therefore, the fibroblasts were exposed to PMA for 24 h and subjected to pressure. In the PKC down-regulated cells, pressure-induced IL-6 secretion was reduced to one-third that of cells without PKC down regulation (Fig. 2). These results indicate that the mechanism of hydrostatic pressure-induced IL-6 secretion involves PKC activity.

Similarly, pressure-induced IL-8 secretion was investigated. In contrast with that of IL-6, pressure-induced IL-8 secretion was enhanced by the addition of PKC inhibitors (Fig. 2). Thus, PKC activation apparently inhibited pressure-induced IL-8 production.

Change in the distribution of PKC in response to pressure stress

To investigate whether hydrostatic pressure stresses activate PKC, the distribution pattern of PKC was examined in fluorescent images obtained by using a specific PKC probe, Rim-1 (Chen and Poenie 1993). Rim-1, a rhodamine conjugate of bisindolylmaleimide, targets the catalytic domain of PKC and is a relatively potent and selective inhibitor of PKC. When the PKCs are activated, Rim-1 binds their catalytic domain. Fibroblasts on coverslips in 35-mm petri dishes were tightly sealed and pressurized at 40 MPa for 20 min. Subsequently, the fibroblasts were fixed and stained

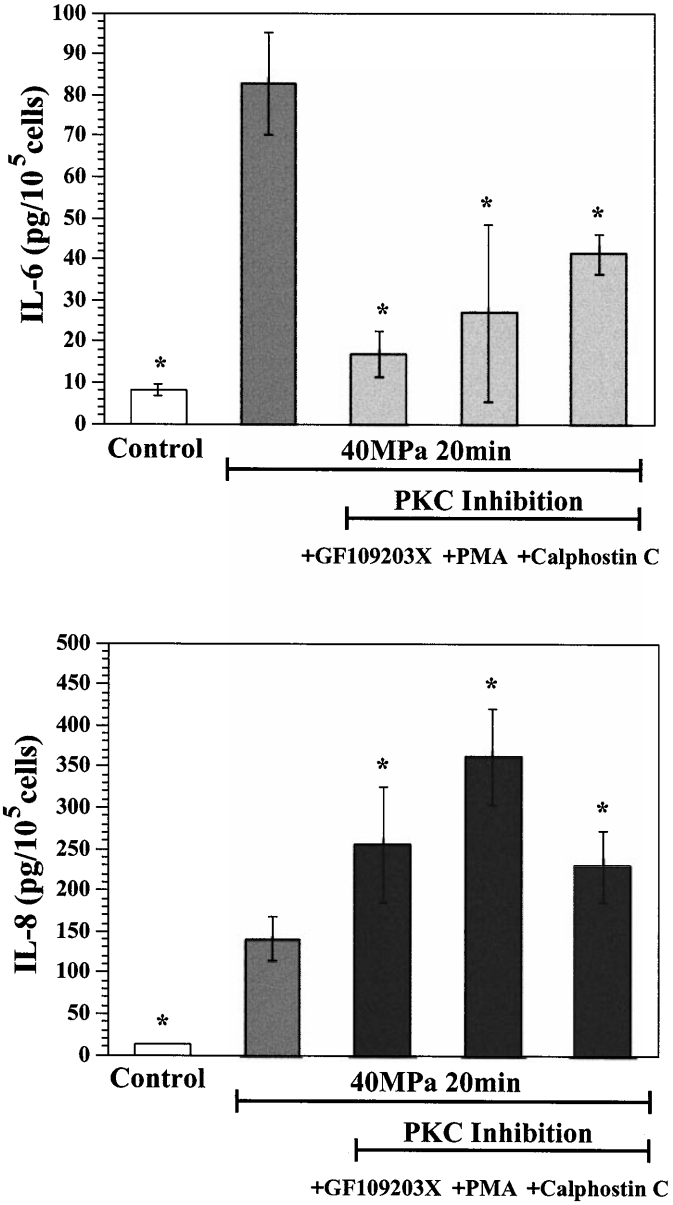

Fig. 2. Effects of inhibitors on hydrostatic pressure-induced IL-6 (*top*) and IL-8 (*bottom*) secretion. Values shown are the means ± SD of four independent experiments, each performed in duplicate (the *asterisks* indicate values of $P < 0.001$ in comparison with the 40 MPa pressure application)

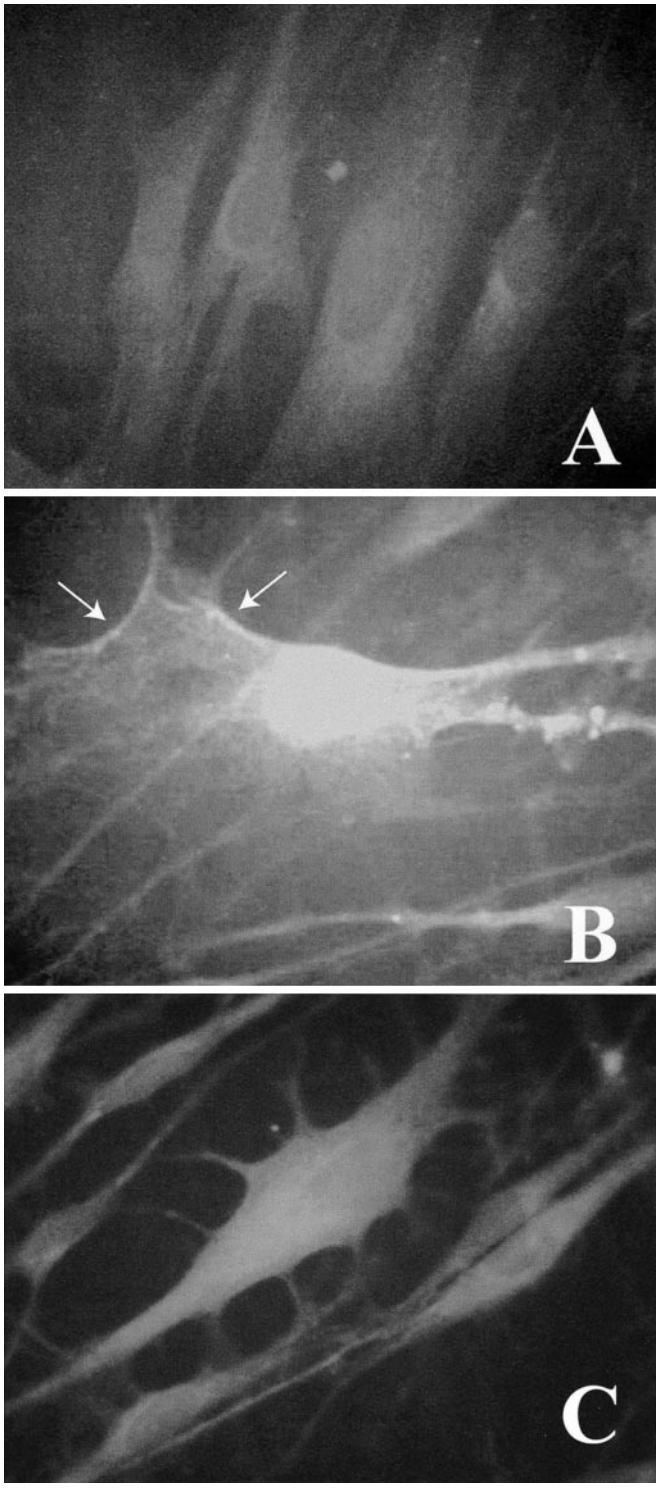

Fig. 3A–C. Activation of protein kinase C (PKC) in response to the application of 40 MPa pressure as visualized by staining with Rim-1. Untreated (**A**), pressure-treated (**B**), and PKC down-regulated (**C**) fibroblasts are shown with Rim-1 staining. The *arrows* (**B**) indicate activated PKC at cell edges

with Rim-1. In the untreated fibroblasts, perinuclear regions of the cytoplasm were slightly stained (Fig. 3A). In the pressure-treated fibroblasts, a marked change in the pattern of staining was observed. Little fluorescence was seen in the cytoplasm, but the perinuclear region and the edges (arrows) of the cells were brightly stained (Fig. 3B). To clarify whether pressure stress induces a change in the distribution of PKC, the PKC in the fibroblasts was down regulated by prolonged exposure to PMA and the fibroblasts were pressurized. A strikingly similar pattern of staining was observed in both the untreated and the PKC down-regulated cells (Fig. 3A and C). These results indicate that hydrostatic pressure stress activates PKC in normal human dermal fibroblasts and changes its distribution.

Discussion

Takahashi and coworkers reported that hydrostatic pressure induced IL-6, tumor necrosis factor α, transforming growth factor-β1, and heat-shock protein 70 mRNAs in a chondrocyte-like cell line (Takahashi et al. 1997, 1998). Our findings presented in this report demonstrated that hydrostatic pressure stresses induce IL-6 and IL-8 production (Fig. 1). The pressure-induced IL-6 secretion involved the PKC signaling pathway, whereas pressure-induced IL-8 production was inhibited by PKC activity. These conclusions are supported by the fact that the selective PKC inhibitors GF109203X and calphostin C and prolonged stimulation with PMA, which causes PKC to be down regulated, inhibit pressure-induced IL-6 secretion but not IL-8 secretion (Fig. 2).

PKC consists of three subspecies, conventional PKCs, novel PKCs, and atypical PKCs, with different distributions and sensitivities to calcium and phorbol esters. The conventional PKCs are calcium- and phospholipid-dependent, whereas the novel PKCs do not require calcium for their activation. Atypical PKCs are both calcium- and phorbol ester-independent and can be activated by phosphatidylserine (Tanaka and Nishizuka 1994; Nishizuka 1992, 1995). In the pressure-treated fibroblasts, the perinuclear region and the peripheral edges of the cells were brightly stained (Fig. 3). Several researchers have reported the chemically induced translocation of PKCs from the cytoplasm not only to the plasma membrane but also to the perinuclear region (Lehel et al. 1995; Shirai et al. 1998). Since pressure-induced PKC activation was inhibited by prolonged stimulation with phorbol ester PMA (Fig. 3), the hydrostatic pressure stress might induce conventional and/or novel PKC activation. Treatment with a fluorescent dye conjugate of bisindolylmaleimide strongly stains activated PKCs and weakly stains untreated PKCs (Dupont et al. 2000). Because the bright staining of the perinuclear region was observed only in cells subjected to hydrostatic pressure stress (Fig. 3B), hydrostatic pressure may also induce PKC production.

Ultraviolet (UV) A (320–400 nm) radiation of human dermal fibroblasts elicits an increase in IL-1α, IL-1β, and IL-6 production (Wlaschek et al. 1997). Singlet oxygen is an early intermediate in the signaling pathway of IL-1 and mediates UVA induction of IL-6 production (Wlaschek et al. 1997). However, we did not detect either IL-1α or IL-1β production (data not shown) from high hydrostatic pressure exposure. Although IL-15 production is induced by UVB (290–320 nm) radiation in human dermal fibroblasts (Mohamadzadeh et al. 1995), we did not detect pressure-mediated IL-15 production (data not shown). Therefore, the high hydrostatic pressure-induced interleukin production process might be different from the UV-induced interleukin mechanism.

What remains unknown in this study is how hydrostatic pressure exposure leads to PKC activation. Several reports have described how mechanical forces are sensed and transduced into biochemical signals via multiple pathways (Lehoux and Tedgui 1998; Chien et al. 1998). In vascular cells, integrins, functioning in the boundary between the extracellular matrix and the cytoskeleton, are likely to be key mechanosensors. In addition, ion channels and other unknown stretch receptors presumably transduce the mechanical signal. Hydrostatic pressure-induced IL-6 and -8 production might also be triggered by physical deformation of the cytoskeleton or the membrane components.

IL-6 is an important inflammatory mediator produced by B-cells, T-cells, hepatocytes, fibroblasts, endothelial cells, and macrophages. The reported biological effects of IL-6 are numerous, and include proliferation and/or differentiation stimuli for myeloma cells, plasmacytomas, hybridomas, T-cells, and hepatocytes. This cytokine also induces thrombocyte production by megakaryocytes and the terminal differentiation of B-cells, and it increases IL-2 production by T-cells (Kishimoto et al. 1992; Kishimoto 1989; Ferry et al. 1997). IL-8 induces chemotaxis of neutrophils, basophils, and T-cells, and activation of neutrophils (Matsushima et al. 1992; Mukaida et al. 1992). Our results suggest that human skin fibroblasts exposed to intense mechanical forces in injured skin regions produce IL-6 and IL-8 against the invasion of pathogenic bacteria into the body.

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