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ARTICLES

Induction of Interleukin (IL)-1α and β Gene Expression in Human Keratinocytes Exposed to Repetitive Strain: Their Role in Strain-Induced Keratinocyte Proliferation and Morphological Change

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Abstract Recent studies in our laboratory have demonstrated that mechanical strain alters many facets of keratinocyte biology including proliferation, protein synthesis, and morphology. IL-1 is known to play an important role in the autocrine regulation of these basic cellular properties under basal and stimulated conditions. However, it is not known whether IL-1 plays a role in strain-induced alteration of keratinocyte biology. Thus, the objective of this study was to test the hypothesis that cyclic strain stimulates IL-1 expression and that strain-induced changes in keratinocyte function is regulated by IL-1. To test this hypothesis, we examined the effect of cyclic strain (10% average deformation) on keratinocyte IL-1 gene expression and the effect of neutralizing antibodies of IL-1α and IL-1β on strain-induced changes in keratinocyte proliferation, morphology, and orientation. Northern blot analyses demonstrated that steady state levels of IL-1α and β mRNA were elevated by 4 h, peaked at 12 h of cyclic strain (IL-1α, 304 ± 14.2%; IL-1β, 212 ± 5.6% increase vs. static controls) and decreased gradually by 24 h. IL-1 antibodies (IL-1α, 0.01 μg/ml; IL-1β, 0.01 μg/ml) significantly blocked strain-induced keratinocyte proliferation as well as the basal rate of proliferation. In contrast, IL-1 antibodies (IL-1α, 0.01 μg/ml; IL-1β, 0.1 μg/ml) had no effect on strain-induced morphological changes such as elongation and alignment. We conclude that mechanical strain induces IL-1 mRNA expression in keratinocytes. The role of IL-1 in mediating strain-induced changes in keratinocyte biology remains to be determined but appears to be independent of morphological changes. J. Cell. Biochem. 69:95–103, 1998.

Key words: mechanical strain; interleukin (IL)-α and β gene expression; proliferation; protein synthesis; morphology; keratinocyte biology

Keratinocytes are reported to be the major source of IL-1. It is determined that human epidermis contains 100-fold greater levels of IL-1 compared to other tissues [Groves et al., 1996]. IL-1 is a key modulator in skin [Kupper, 1990; Kupper and Groves, 1995] and regulates a wide variety of keratinocyte processes including inflammatory responses, growth, and differentiation [Eller et al., 1995; Ansel et al., 1990; Kupper et al., 1986; Luger et al., 1981; Kang et al., 1996]. In addition to its pleiotropic effects, IL-1 induces multiple secondary cytokines.

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Recent studies have shown that IL-1 increases epidermal wound healing as well as keratinocyte growth [Sauder et al., 1990, Hauser et al., 1986]. This has been confirmed by the observation that intradermal injection of IL-1 results in epidermal hyperplasia [Granstein et al., 1986]. Recent investigations have also revealed that application of mechanical forces on cells increases IL-1 production in periodontal ligament cells [Shimizu et al., 1994] and aortic endothelial cells [Sterpetti et al., 1993]. We previously reported that cyclic strain causes phenotypic alteration of keratinocytes including changes in proliferation and morphology, such that keratinocytes elongate and align perpendicular to the force vector [Takei et al., 1997]. In this study, we hypothesize that IL-1 expression may also be stimulated by repetitive stretch of keratinocytes. Our second objective was to test the hypothesis that IL-1 is involved

in the strain-induced keratinocyte proliferation and morphology.

MATERIALS AND METHODS

Cell Cultures

Human keratinocytes were obtained from neonatal foreskins and maintained in serum-free medium (Keratinocyte SFM, Gibco BRL products, Grand Island, NY) as previously described [Takei et al., 1997; Eisinger, 1985]. The cell culture medium is supplemented with 0.2 ng/ml epidermal growth factor, 30 μg/ml bovine pituitary extract, and 1.02 g/L L-glutamine. Keratinocytes were seeded on flexible membrane culture plates coated with collagen type I (FLEX I, Flexcell Corp., McKeesport, PA), and then incubated for 48 h at 37°C in a 5% CO_2 prior to exposure to strain. Five different keratinocyte cell lines were used to eliminate variations among each cell line and cells from passages 2–4 were used for these experiments.

Strain Protocol

To apply mechanical strain to keratinocytes, the Flexercell Stress Unit (Flexcell Corp.) was used for our studies. This unit consists of a vacuum manifold and vacuum controlling system with solenoid valves. Its mechanism was described in detail previously [Banes et al., 1985, 1990; Sumpio and Banes, 1988]. Briefly, flexible bottom culture plates are deformed by vacuum, the level of which is controlled and monitored by a computer timer program. When flexible membranes were subjected to 150 mmHg of vacuum, the membrane experiences a 10% average strain. For these experiments, keratinocytes were seeded (10,000 cells/cm^2) and then subjected to strain at a frequency of 10 cycles/min (3 s of deformation followed by 3 s of relaxation).

Northern Blot Analysis

Keratinocytes were subjected to cyclic strain for up to 24 h or cultured under stationary condition (control), then total RNA was isolated using the guanidinium isothiocyanate method with TRIzol™ Reagent (Life Technologies Inc., Gaithersburg, MD) as previously described [Chirgwin et al., 1979; Chomczynski and Sacchi, 1987]. RNA (15 μg) was loaded into each lane and separated on 1.1% formaldehyde gels and transferred onto nitrocellulose membrane (Amersham, Arlington Heights, IL), followed by an immobilization by ultraviolet irradiation. IL-1α and β RNA probes (Immunex Corporation, Seattle, WA) and GAPDH probe (ATCC, Rockville, MD) were labeled with [^{32}P] dCTP using a labeling kit (Ready To Go; Pharmacia Biotech, Piscataway, NJ), then purified with a Sephadex G-50 spin column (Boehringer Mannheim, Indianapolis, IN). Nitrocellulose membranes were incubated with a prehybridization solution (1 M NaCl, 5 mM Tris·HCl, 10% Dextran Sulfate, 1% SDS, 100 μl denatured ssDNA) at 42°C for 2 h, followed by hybridization with [^{32}P] labeled probes. Membranes were washed with high stringency solutions (2 x SSC, 0.1% SDS) once, then twice with washing solution (0.1 x SSC, 0.1% SDS). The membranes were exposed to X-OMAT AR film, and developed by using X-OMAT AR processor (Kodak, Rochester, NY). Each band was quantitated by a GS 300 scanning densitometer (Hoeffer Scientific Instruments, San Francisco, CA). Experimental values were normalized based on the data obtained with GAPDH. Five independent experiments were performed and values are presented as the mean ± SEM.

Effects of IL-1 Inhibitors on Cell Proliferation and Morphology

To study the effects of IL-1 antibodies on proliferation, keratinocytes were exposed to the culture medium with or without IL-1 antibodies (IL-1α, 0.01, 0.05, and 0.5 μg/ml; IL-1β, 0.1, 1.0, and 10 μg/ml). IL-1α and β neutralizing antibodies were purchased from R&D systems (Minneapolis, MN). The culture medium containing IL-1 antibody was freshly made and changed every other day. Keratinocytes were subjected to cyclic strain for the indicated time, then the cell number was determined on day 1, 3, 5, and 7, followed by cell counting using Coulter Counter ZM (Coulter Corporation, Miami, FL). Control cells were cultured under stationary conditions in the same incubator. These measurements were obtained in three separate experiments and the data are presented as mean ± SEM.

For morphological assessment, keratinocytes were subjected to strain for 7 days with or without IL-1 antibodies, fixed with 3.7% formalin for 10 min, and stained with 1% crystal violet (Sigma Chemical Co., St. Louis, MO) for 5 min. Keratinocytes from the periphery of the

culture well (high strain area) were observed under a BH-2 photomicroscope (Olympus Optical, Tokyo, Japan).

Statistical Analysis

The values are presented as the mean ± SEM. One-way ANOVA were performed to determine the statistical significance with a multiple comparison method, followed by post-hoc testing (Bonferroni analysis), using a commercially available statistical package (SigmaStat, Jandel Scientific, San Rafael, CA). $P < 0.05$ was considered significant.

RESULTS

Strain-Induced IL-1 Gene Expression

Northern blot analysis showed that steady-state levels of IL-1 gene expression increased in keratinocytes subjected to strain (Fig. 1). IL-1 α expression constantly increased up to 12 h, then decreased at 24 h. This observation was also common to IL-1β. However, the levels of IL-1α mRNA were more pronounced throughout the time course of strain when compared to that of IL-1β. As shown in Figure 2, densitometric analyses revealed an increase in IL-1 mRNA expression by 4 h (i.e., IL-1α, 175 ± 6.6%; IL-1β, 118 ± 3.3% increase vs. static control) and maximum expression of IL-1α and β mRNA at 12 h, (i.e., 304 ± 14.2% and 212 ± 5.6% increase, respectively). Autoradiographic results of IL-1α and β demonstrated that mRNA levels were sustained for up to 24 h as compared to control (no strain), followed by a reduction to the basal levels at 48 h (data not shown).

Cell Proliferation Study With IL-1 Inhibitors

IL-1α and β inhibitors blocked the basal level of keratinocyte proliferation in a dose-dependent manner (Fig. 3A,B, solid lines). Cyclic strain (Fig. 3, broken lines) enhanced keratinocyte growth in the absence of antibodies, which was apparent from day 3 to 4 and enhanced on day 7. The significant stimulation of keratinocyte proliferation after 7 days of cyclic strain confirms the report of an earlier study [Takei et al., 1997]. IL-1α (>0.01 μg/ml) and β (>0.1 μg/ml) antibodies significantly inhibited this strain-induced proliferation as well as the basal rate of cell growth.

IL-1α and β antibodies inhibited keratinocyte growth in a similar fashion. However, the dose of antibody was different. IL-1α (0.01 μg/ml) significantly blocked both strain-induced and basal proliferation, while 0.1 μg/ml of IL-1β caused the same effect on keratinocyte proliferation.

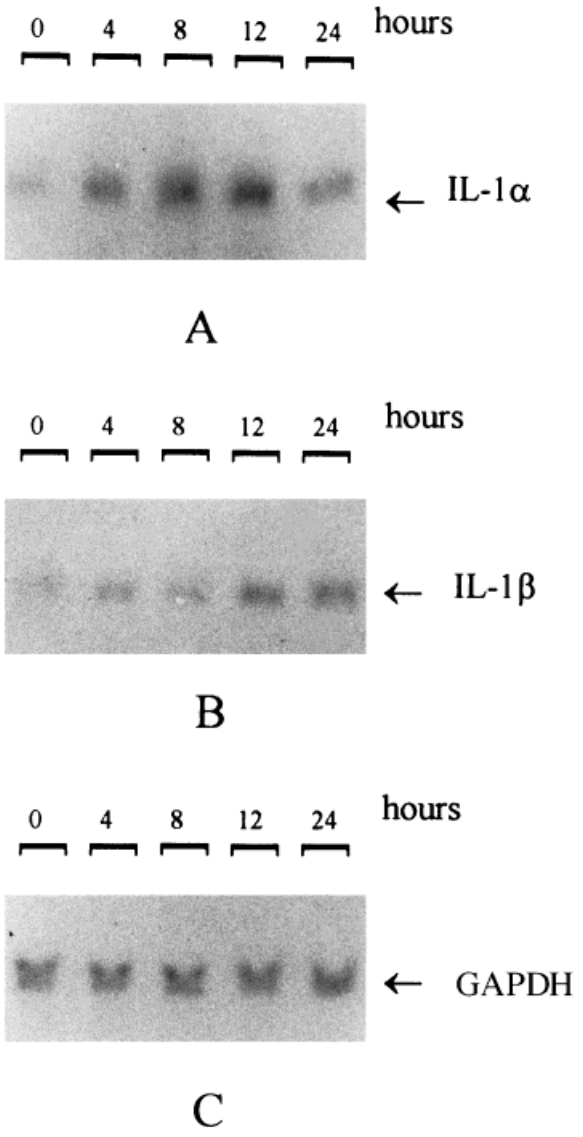

Fig.1. A–C: Effects of cyclic strain on IL-1α and β mRNA expression. **A:** Keratinocytes were subjected to cyclic strain for up to 24 h, then Northern blots were performed as described in Methods. IL-1α gene expression significantly increased at 4 h and peaked at 12 h, followed by a gradual decrease at 24 h. **B:** Keratinocytes were subjected to strain in the same manner as in A, followed by hybridizations with IL-1β probes. There was a significant increase in IL-1β mRNA level at 4 h, which then peaked at 12 h. **C:** Keratinocytes were subjected to strain in the same manner as A and B, then probed with GAPDH. The bands corresponding to GAPDH were unaffected. Similar results were obtained in five independent experiments (n = 5).

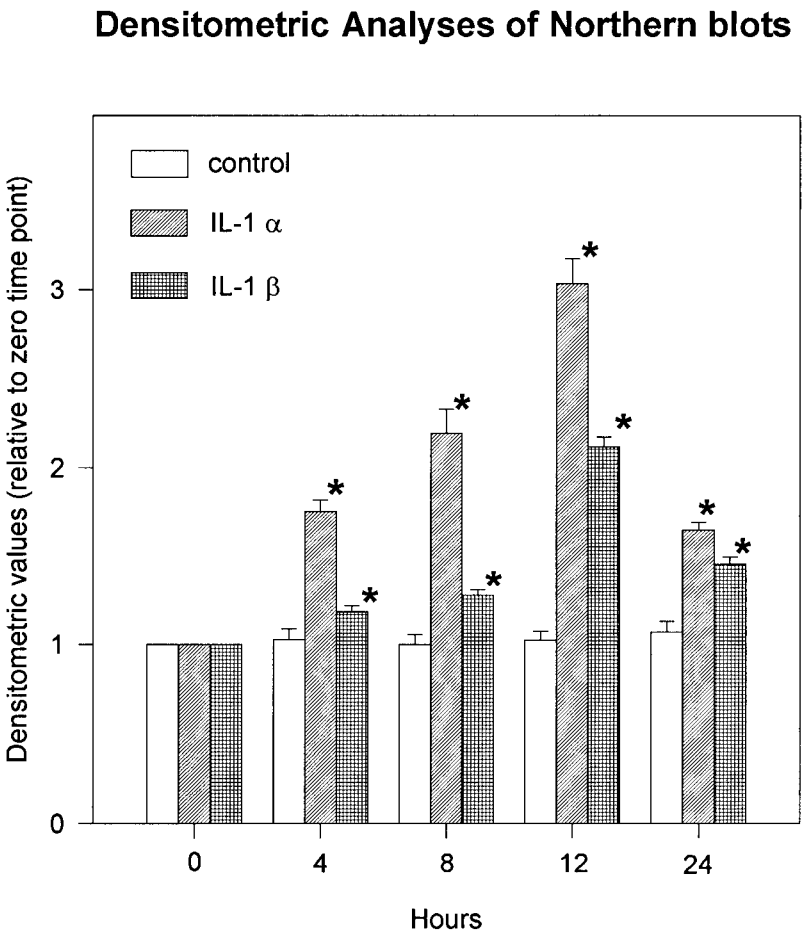

Fig. 2. Densitometric analyses of Northern blots. Densitometric scanning analyses were performed as described in Materials and Methods. Bands corresponding IL-1α and β showed significant increases after 4 h of strain, then peaked at 12 h, followed by decreases at 24 h. Each level of IL-1α and β was normalized by the level of GAPDH, which remained unchanged during the time course of strain. A representative of five independent experiments is shown (n = 5). Values are presented as mean ± SEM. $*P < 0.05$ compared to control.

Effects of IL-1 Inhibitor on Keratinocyte Morphology

Keratinocytes subjected to cyclic strain (10 cycles/min) for 7 days showed significant morphological changes including elongation and alignment. As shown in Figure 4A, keratinocytes demonstrated typical phenotypic changes of elongation, showing that the ratio of the long axis of the cell divided by the short axis was higher under strain condition as compared to stationary control. Strain-induced elongation was unaffected by neutralizing antibodies to either IL-1α (Fig. 4B) or IL-1β (Fig. 4C).

A second strain-induced phenotypic change is alignment; keratinocytes change orientation such that the long axis of cells is oriented perpendicular the force vector. Figure 4A shows the alignment of keratinocytes under cyclic strain; cells became aligned perpendicular to the direction of force, which is demonstrated with an arrow. As shown in Figure 4A and B, antibodies to either IL-1α or IL-1β failed to alter the strain-induced changes in orientation.

DISCUSSION

Our study demonstrates that mechanical strain induces IL-1α and β gene expression in human keratinocytes. Cyclic strain stimulated both IL-1α and β. The levels of IL-1α and β mRNA increase up to 12 h of exposure to strain, then gradually decrease after that time point, reducing to the basal level later (48 h). Although the significance of these increases remains uncertain, strain-induced proliferation appears to be involved in IL-1 related pathways. However, since basal proliferation of keratinocytes was blocked by IL-1α and β neutralizing antibodies, the role of IL-1α and β in mediating strain-induced proliferation remains uncertain. On the other hand, strain-induced morphology was not affected by cyclic strain, thus suggesting that other mechanisms are involved in strain-induced phenotypic alterations.

Mechanical force has been reported to increase IL-1 levels in different cell types, suggesting an important role of strain in regulating IL-1. For example, Sterpetti et al. [1993] re-

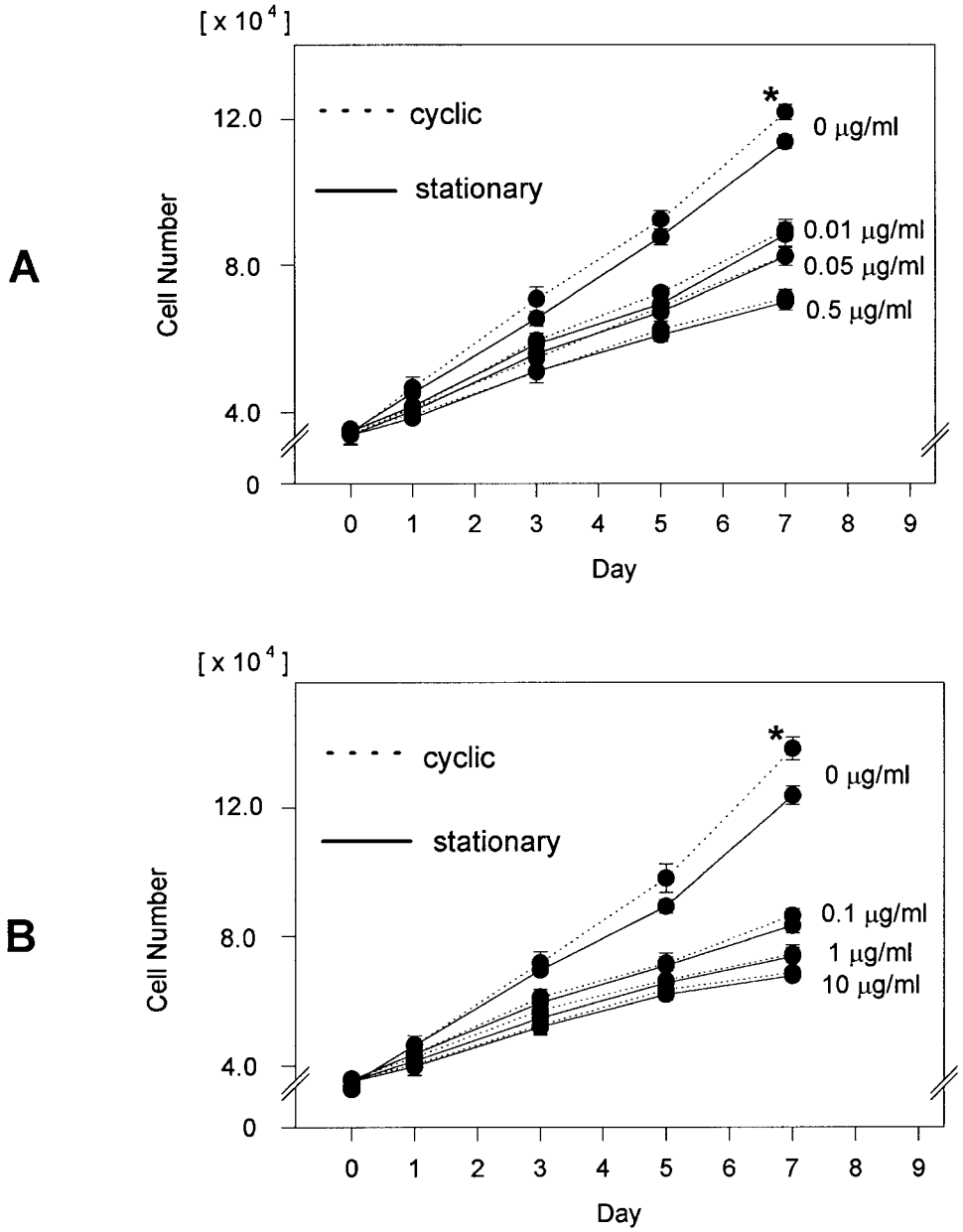

Fig. 3. A,B: Effects of IL-1α and β antibodies on keratinocyte proliferation. **A:** Keratinocytes were subjected to cyclic strain or stationary condition with IL-1α neutralizing antibodies (0, 0.01, 0.05,and 0.5 μg/ml) for up to 7 days. Cell numbers were determined as described in Materials and Methods. Keratinocyte proliferation was downregulated by IL-1α antibodies in a dose-dependent manner. IL-1α antibodies (>0.01 μg/ml) significantly inhibited proliferation under both in cyclic and stationary conditions on day 7. **B:** Keratinocytes were treated with IL-1β neutralizing antibodies (0, 0.1, 1.0, and 10 μg/ml) in the same regimen as A. IL-1β antibodies (>0.1 μg/ml) significantly inhibited proliferation under both in strain and stationary conditions on day 7. Similar results were obtained in three independent experiments, values are presented as mean ± SEM. $*P < 0.05$ compared to control (stationary).

ported that IL-1 was increased in aortic endothelial cells exposed to shear stress. In the present study, we report strain induction of IL-1α and β gene expression in keratinocytes. We detected increases in IL-1 mRNA expressions by 4 h with a maximum activation at 12 h. Kameda and Sato [1994] reported that lipopolysaccharide (LPS) increased IL-1α mRNA in keratinocytes by 6-fold at 2 h. Other investigators have demonstrated that 3-h treatment of cultured keratinocytes with tumor necrosis factor α (TNFα) induced increases in IL-1α (9.2-fold) and IL-1β

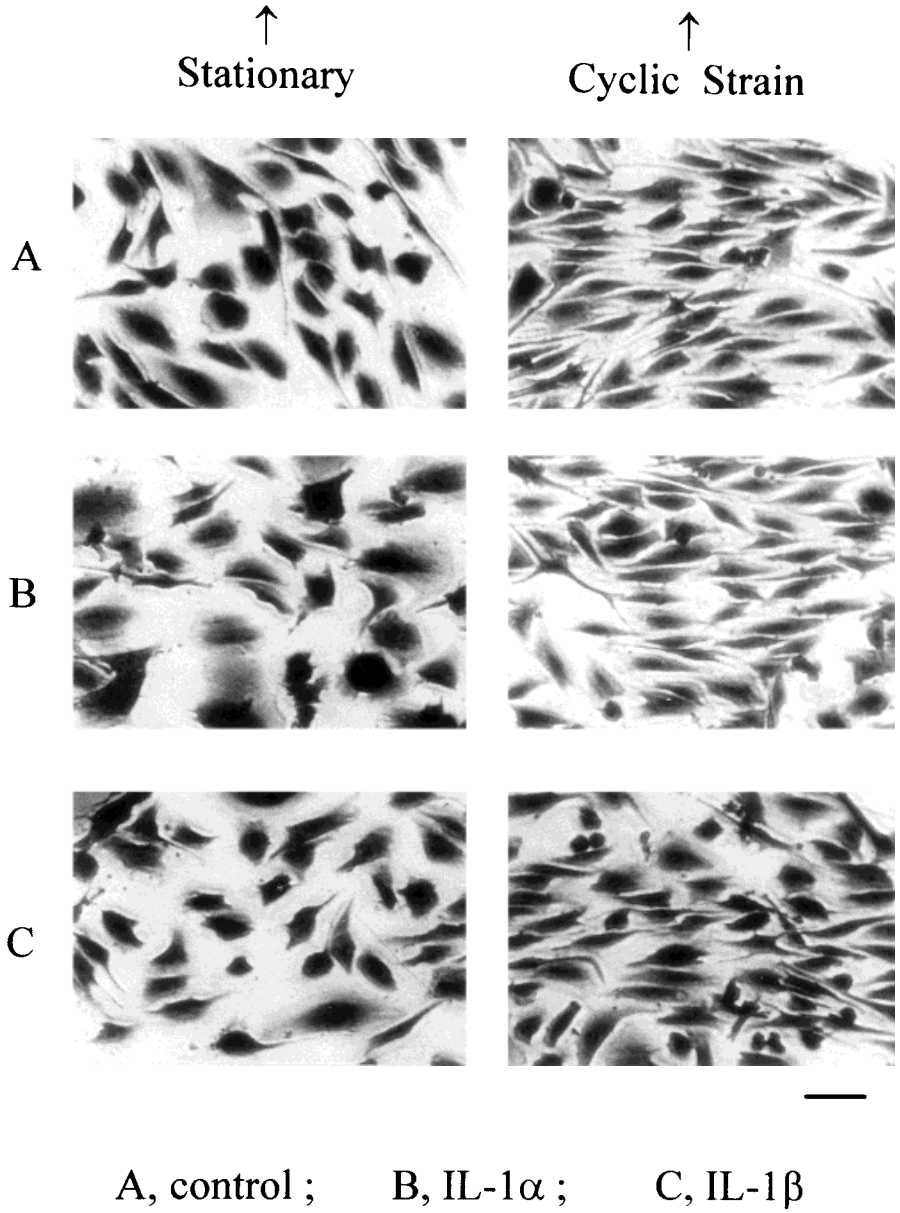

Fig. 4. A–C: Effects of IL-1 antibodies on keratinocyte morphology under strain or stationary condition. **A:** Keratinocytes were cultured without IL-1 antibodies under stationary or strain condition for 7 days. Photographs were taken from the periphery of the culture well under microscopic magnification (x250). Keratinocytes subjected to strain were elongated and aligned perpendicular to the force vector (arrows). The upper side is the center of the well. **B:** Keratinocytes were treated with IL-1α antibodies (0.01 μg/ml) for 7 days in the same manner as A. Keratinocytes in the periphery of the well were still elongated and aligned. IL-1A antibodies did not block the strain-induced morphological features. **C:** Keratinocytes were treated with IL-1β antibodies (0.1 μg/ml) as described in Materials and Methods. Cyclic strain induced cellular alignment as seen in A and B under strain condition. IL-1β antibodies did not inhibit the strain-induced morphological changes. Crystal violet staining, magnification (x250, scale bar = 50 μm).

(2.5-fold) mRNA levels [Kang et al., 1996]. These findings may indicate that chemical stimulation is more potent than cyclic strain in keratinocytes, suggesting that a different mechanism might be involved in IL-1 induction.

The degree of IL-1α gene expression was greater than that of IL-1β throughout the time course of strain (Fig. 2). It has been reported that human keratinocytes express 2 to 4 times higher levels of IL-1α mRNA as compared to blood monocytes while IL-1β mRNA of monocytes is 10 to 20 times higher than that of keratinocytes [Auron et al., 1984; March et al., 1985]. These data suggest that IL-1α appears

to be the primary transcript in keratinocytes. Furthermore, there are diversities among different cell types, which could explain the functional difference between each cell line with regards to IL-1 induction [Kupper, 1990; Kupper and Groves, 1995].

Since Kupper et al. [1986] reported that IL-1 increases keratinocyte growth, numerous observations have supported a role of IL-1 in upregulating keratinocyte proliferation [Blanton et al., 1989; Camp et al., 1990]. Oberyszyn et al. [1993] demonstrated that treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA), also known as a cutaneous carcinogen, induced epidermal hyperplasia. PMA treatment induced both synthesis and release of IL-1 in keratinocytes [Blanton et al., 1989], and stimulated IL-1α mRNA expression [Kameda and Sato, 1994], suggesting a critical role of IL-1 in controlling keratinocyte growth. This finding is supported by reports that intradermal injection of IL-1 caused epidermal hyperplasia [Granstein et al., 1986]. In addition, injection of IL-1α antibodies resulted in a decrease in [^{3}H] incorporation into DNA in keratinocytes [Oberyszyn et al., 1993]. These data confirm that IL-1 is critically important in mediating the proliferative response of keratinocytes.

To assess the effects of IL-1 on proliferation under strain condition, keratinocytes were cultured with or without IL-1 antibodies. Our studies demonstrate that keratinocyte proliferation was blocked by IL-1 antibodies in a dose-dependent manner (Fig. 3). Neutralizing IL-1 antibodies inhibited the basal rate of proliferation as well as strain-induced proliferation as shown in Figure 3. The difference of the antibody concentration is probably due to the difference of 50% Neutralizing Dose (ND_{50}). IL-1α has a ND_{50} of 0.02–0.04 μg/ml, while IL-1β antibody has a ND_{50} of 0.5–1.0 μg/ml as previously described [Symons, 1987]. On the other hand, as shown in Figure 3, the manner in which IL-1 antibodies regulated cell proliferation was similar between α and β isoforms. This observation could be possibly because IL-1α and β were reported to be biologically similar in a wide variety of cells [Kilian et al., 1986].

Two different IL-1 receptors (type I and II) have been identified in keratinocytes [Groves et al., 1994; Grewe et al., 1996]. IL-1 receptor type I (IL-1RI) plays a major role for signal transductions in keratinocytes [Groves et al., 1995], while IL-1 receptor type II (IL-1RII) functions as an endogenous antagonist [Re et al., 1994]. IL-1RII has no significant biological effect as opposed to IL-1RI, even though there is a high affinity of IL-1 to IL-1RII. Therefore, IL-1RII could antagonize the IL-1 mediated responses [Re et al., 1994; Colotta et al., 1993]. However, it remains unknown whether mechanical strain may influence IL-1 receptor type I or II expression in human keratinocytes.

Another interesting aspect of receptor-associated events is that IL-1 receptors have been shown to localize in focal adhesions, which plays an important role in mechanotransduction [Morrison et al., 1989; Meisenhelder et al., 1989]. In addition, growth factors such as PDGF and EGF also colocalize in focal adhesions [Morrison et al., 1989; Meisenhelder et al., 1989], suggesting that the focal adhesion might be a key molecule to mediate growth signals initiated by PDGF, EGF, or IL-1, then lead to keratinocyte proliferation.

Groves et al. [1996] reported that IL-1 could induce hyperproliferative condition in skin and IL-1 appeared to be secreted in an autocrine fashion. Autocrine regulation of cytokines has become clear, as demonstrated by other evidence that addition of IL-1 to cultured human keratinocytes resulted in increases in IL-1α as well as transforming factor α (TGFα) mRNA in an autocrine manner [Kupper, 1990; Kupper and Groves, 1995; Lee et al., 1991]. IL-1 and TGFα have also been shown to stimulate keratinocyte proliferation [Hashimoto and Yoshikawa, 1992; Martin, 1997]. Although strain-induced effects of IL-1 on TGFα need to be investigated, this finding may indicate that strain-induced proliferation might be mediated through IL-1 and TGFα-dependent pathways. Maas-Szabowski and Fusenig [1996] reported that IL-1α and β increased keratinocyte growth factor (KGF), IL-1α, β, and IL-1 receptor type I (IL-1RI) in cultured fibroblasts. These findings may suggest that IL-1 could enhance strain-induced proliferation in an autocrine as well as a paracrine fashion.

Although in vivo studies demonstrated histological characteristics, showing that IL-1 treatment led to an epidermal hyperplasia [Granstein et al., 1986], in vitro effects of IL-1 on keratinocyte morphology have not been described. In the present report, cyclic strain induced keratinocyte elongation and alignment as shown in Figure 4A. Both IL-1α (0.05 μg/ml) and β (0.1 μg/ml) failed to block these pheno-

typic alterations, therefore keratinocytes remained aligned and elongated (Fig.4B and C). It could be postulated that IL-1 may not be a major factor to modulate strain-induced morphology.

In summary, mechanical strain increased steady-state levels of IL-1 mRNA in human keratinocytes, which could be involved in strain-induced proliferation. Neutralizing IL-1α and β antibodies blocked both the strain-induced and basal keratinocyte proliferation. However, these antibodies failed to block the strain-induced morphology changes, suggesting that a different pathway is involved in phenotypic regulation.

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