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Cyclic stretch induces upregulation of endothelin-1 with keratinocytes in vitro: Possible role in mechanical stress-induced hyperpigmentation

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ABSTRACT

The aim of this study was to investigate the possible pathological relation between mechanical stress and hyperpigmentation. We did this by investigating the influence of cyclic stretch on the expression of keratinocyte- and fibroblast-derived melanogenetic paracrine cytokines in vitro. Using primary human keratinocytes and fibroblasts, alterations of mRNA expression of melanogenetic paracrine cytokines due to cyclic stretch were investigated using a real-time polymerase chain reaction (PCR). The cytokines included basic fibroblast growth factor (bFGF), stem cell factor (SCF), granulocyte/macrophage colony-stimulating factor, interleukin-1α, and endothelin-1 (ET-1) for keratinocytes and bFGF, SCF, and hepatocyte growth factor for fibroblasts. The dose dependence of keratinocyte-derived ET-1 upregulation was further investigated using real-time PCR and an enzyme-linked immunosorbent assay. We also investigated the effects of cyclic stretch on the proliferation and differentiation of keratinocytes. Among the melanogenetic paracrine cytokines investigated, keratinocyte-derived ET-1 was consistently upregulated in all four cell lines. The degree of upregulation increased with the degree of the length and frequency of the stretch; in contrast, cell number and differentiation markers showed no obvious alterations with cyclic stretch. Keratinocyte-derived ET-1 upregulation possibly plays a significant role in the pathogenesis of pigmented disorders, such as friction melanosis, caused by mechanical stress.

1. Introduction

Hyperpigmentation is the most common cosmetic complaint among Asians, and is caused by various stimuli, such as inflammation, aging, and ultraviolet exposure [1–3]. It has been reported that paracrine linkage between keratinocytes, fibroblasts, and melanocytes in the skin plays an important role in these acquired epidermal melanization disorders [1,2]. Human keratinocytes secrete various cytokines, which are known as mitogens or melanogens for human melanocytes. These cytokines include endothelin-1 (ET-1) [1,4,5], granulocyte/macrophage colony-stimulating factor (GM-CSF) [1,6], interleukin (IL)-1α [1,2], stem cell factor (SCF) [7,8], and basic fibroblast growth factor (bFGF) [9,10]. Fibroblasts secrete hepatocyte growth factor (HGF) [1,2,11,12], SCF [1,2,7,8,11], and bFGF [9,10] with the definite potential and concentrations sufficient to stimulate melanocyte proliferation [2].

On the other hand, there have been clinical reports of skin hyperpigmentation disorders in which a mechanical stimulus may have played a significant role in the pathogenesis. These included friction melanosis [3,13–16], pigmentation over a bony prominence [17], nipple–areolar complex pigmentation [3,18], and miscellaneous pigmentation found around intertriginous parts [3]. In these cases, mechanical stresses were considered to be a causative factor. As far as we know, however, there have been no published studies on an etiological association between mechanical stress and hyperpigmentation.

We were intrigued with the idea that mechanical stress may have an effect on cytokine expression by keratinocytes and fibroblasts in vitro. Could, then, mechanical stress on the paracrine cytokine network be a mechanism for epidermal pigmentation? In this study, we first investigated whether there are known melanogenic cytokines upregulated by cyclic stretch of fibroblasts and keratinocytes using a real-time PCR. ET-1 was screened out as a candidate. Second, the influence of the duration, degree, and frequency of cyclic stretch on ET-1 expression was investigated. Third, it is currently being investigated whether several constitutional alterations, such as proliferation and differentiation, are associated with cyclic stretch. This evaluation was done because several previous findings [19–22] supported the possibilities that these alterations are induced by mechanical stress and influence cytokine expression profiles.

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2. Materials and methods

2.1. Cell isolation and culture

Normal skin specimens were obtained from Japanese patients during plastic surgery. Human fibroblasts and keratinocytes were cultured from the specimens, individually, from four donors. Informed consent was obtained from all patients. The internal review board of Kyorin University Hospital approved the study protocol.

Human keratinocytes were isolated using a modification of a method reported previously [2,23–25]. Briefly, the skin specimens were washed three times in phosphate-buffered saline (PBS), were finely shredded with scissors, and were incubated with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in PBS for 16–24 h at 4 °C. The epidermis was separated from the dermis with forceps, and keratinocytes were isolated from the dermis. Keratinocytes were then grown in a serum-free keratinocyte growth medium (KGM) (Keratinocyte SFM; Invitrogen, Carlsbad, CA, USA); the final Ca^{2+} concentration was adjusted to 0.03 mM. Human fibroblasts were isolated for explant after they were separated from the epithelium and grown in fibroblast growth medium (FGM), which consisted of Dulbecco's modified Eagle's medium (DMEM), glutamine 0.6 mg/mL, and 10% fetal calf serum (FCS) [2]. The third generation of cultures of both fibroblasts and keratinocytes were used in the experiments (Table 1).

2.2. Application of uniaxial cell stretching

The cells were plated in deformable silicon-membrane chambers coated with 0.05% porcine type I collagen (AteloCell; Koken, Tokyo, Japan) at an initial density of 2.0×10^4 cells/cm^2 and allowed to attach for 24 h before applying cyclic stretching in FGM (for fibroblasts) or KGM supplemented with 0.5% FCS (for keratinocytes) [2,24,25]. Uniaxial cell stretching was applied using a cell-stretching device (STREX ST-140; STREX, Osaka, Japan). The details about the device and the stretching applied are described elsewhere [26,27]. Two groups of cells were prepared for all the comparative experiments. In one, designated the stretched group, the cells in the chambers were mounted on a cell-stretching device. In the other, designated the control group (unstretched), the cells in the chambers were not subjected to stretching. Rate of cyclic stretch was described with cycles per minute (CPM) unit.

2.3. Quantification of mRNA by real-time polymerase chain reaction

After the designated period of stretching, cells were detached with trypsinization, the number of cells were manually counted using NucleoCounter (Chemometec, Allerød, Denmark), and total RNA was isolated using an RNeasy Mini Kit and QIA shredder (both from Qiagen, Hilden, Germany), followed by reverse transcription using a High Capacity RNA-to-cDNA Kit. We amplified cDNA for 40 cycles with the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA), a TaqMan Fast Universal Master Mix, and the following predesigned primers and fluorescein-labeled probes: human ET-1 (Hs01115919_m1); SCF (Hs00241497_m1); HGF (Hs00900070_m1); bFGF (Hs00960934_m1); IL-1α (Hs99999028_m1); GM-CSF (Hs99999044_m1); keratin 14 (Hs00559328_m1); keratin 10 (Hs00166289_m1); GAPDH (Hs99999905_m1); β-actin (Hs99999903_m1. (All primers were obtained from Applied Biosystems, Foster City, CA, USA.) We calculated expression levels using the comparative CT method with GAPDH and β-actin as endogenous reference genes. These endogenous genes offered identical results; therefore, the data in this article are expressed using GAPDH.

Table 1
Profile of specimens.

Cell name	Abbreviation	Age (years)	Sex	Donor site
Fibroblast 1	F1	47	Female	Groin
Fibroblast 2	F2	25	Female	Thigh
Fibroblast 3	F3	21	Female	Thoracic
Fibroblast 4	F4	52	Female	Face
Keratinocyte 1	K1	19	Female	Abdomen
Keratinocyte 2	K2	28	Female	Thoracic
Keratinocyte 3	K3	23	Female	Thoracic
Keratinocyte 4	K4	60	Female	Face

2.4. Quantifying ET-1 protein by enzyme-linked immunosorbent assay

The conditioned medium for keratinocytes, with or without cyclic stretch, were analyzed by an enzyme-linked immunosorbent assay (ELISA) using an ELISA kit for human ET-1 (QuantiGlo Human ET-1 Chemiluminescent Immunoassay; R&D Systems, Minneapolis, MN, USA). Levels of each factor were measured using a microplate reader (Power Scan HT; Dainippon Pharmaceutical, Osaka, Japan). Data are expressed as the secreted factor per 1.0×10^5 cells.

2.5. Statistical analysis

Results were expressed as the mean ± SD. The nonpaired Student's *t*-test was used to compare the differences between cells with or without cyclic stretch. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Cyclic stretch causes upregulation of ET-1 secretion in keratinocytes

With cyclic stretch, fibroblasts, and keratinocytes are oriented perpendicular to the stretch direction (Fig. 1A). To investigate the influence of cyclic stretching on the melanogenetic paracrine cytokine network in skin, we quantified the expression levels of GM-CSF, ET-1, IL-1α, SCF, and bFGF in keratinocytes and bFGF, HGF, and SCF in fibroblasts (four primary cell lines for each) with or without 24 h of cyclic stretch (60 CPM, 10% of length) using real-time PCR.

The melanogenetic paracrine cytokines in fibroblasts showed no consistent alteration with cyclic stretch, although one cell line did exhibit upregulation of HGF and SCF (Fig. 1B). In contrast, regarding the melanogenetic paracrine cytokines from keratinocytes, expression of ET-1 was significantly upregulated with cyclic stretch in all of the four cell lines investigated (Fig. 1C).

3.2. Upregulation of ET-1 in keratinocytes depends on the degree and frequency of cyclic stretch

ET-1 upregulation of the protein production level was confirmed in culture supernatants of three cell lines using ELISA quantification (Fig. 2A). Assays of ET-1 mRNA expression at different durations of cyclic stretch (12, 24, and 48 h) indicated that ET-1 was upregulated until 24 h, with no further apparent alteration observed between 24 and 48 h (Fig. 2B). Therefore, we designated 24 h as the appropriate duration of cyclic stretch in the following experiments.

To investigate the influence of duration, degree, and frequency of stretch on upregulation of keratinocyte ET-1, the expression and production of ET-1 with or without different degrees of stretch (5%, 10%, and 20% of length) and different frequencies of extension (10 and 60 CPM) were quantified using real-time PCR and ELISA with K1 keratinocytes. Results indicated that ET-1 expression and

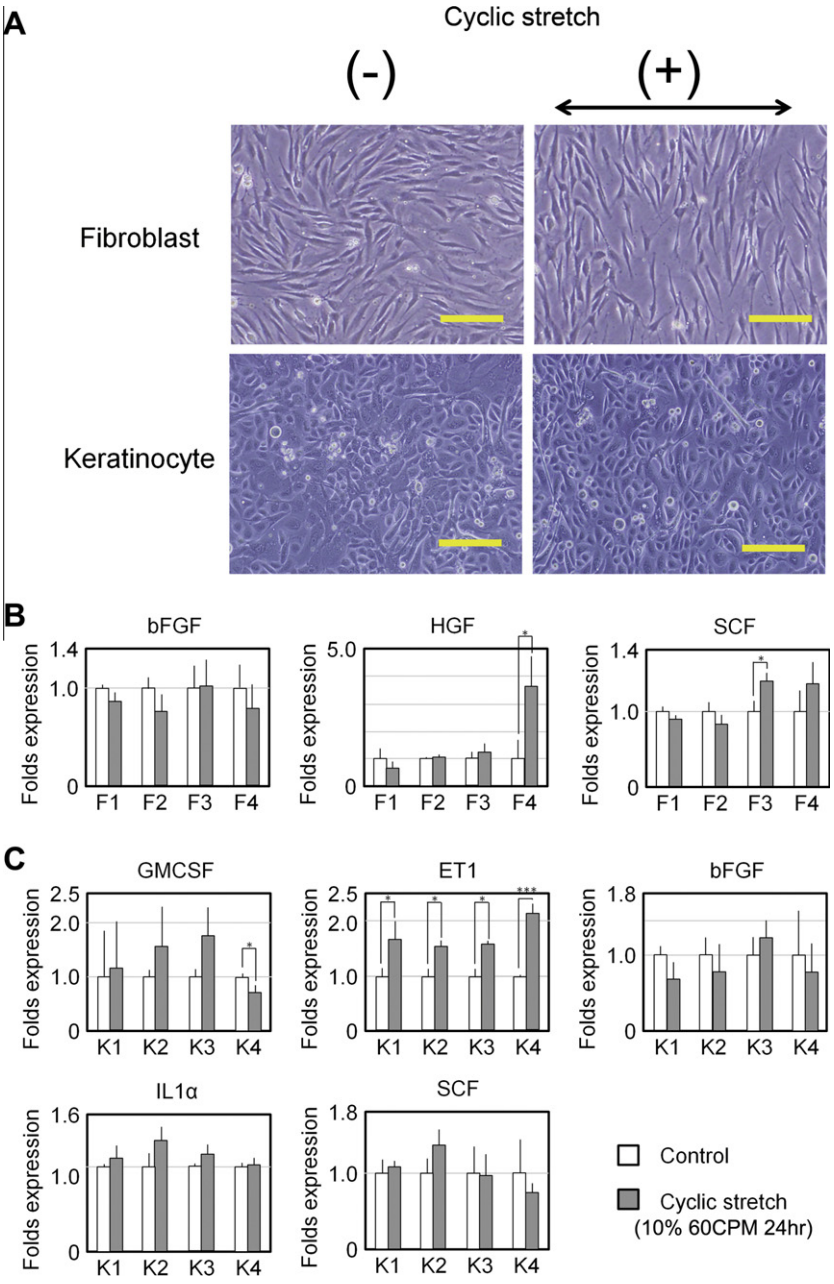

Fig. 1. (A) Phase contrast microscopic findings of fibroblasts and keratinocytes with or without cyclic stretch. Black arrow indicates direction of cyclic stretch. With cyclic stretch, fibroblasts and keratinocytes oriented perpendicular to stretch direction. Scale bar stands for 200 μm. (B) Expression of FGF2, HGF, and SCF mRNA with or without cyclic stretch was investigated for four primary human fibroblast cell lines ($N = 3$ for each). No constant alteration was observed. (C) Expression of GM-CSF, ET-1, IL-1α, SCF, and bFGF mRNA with or without cyclic stretch was investigated for four human primary keratinocyte cell lines ($n = 3$ for each) using realtime PCR. Only ET-1 was consistently upregulated with cyclic stretch in cell lines involved. Error bar indicates standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

production is increasingly upregulated with progression of the degree of stretch in both length and frequency. Thus, upregulation of ET-1 in keratinocytes is thought to depend on the "dose" (degree and frequency) of cyclic stretch (Fig. 3).

3.3. Upregulation of ET-1 with keratinocytes does not result from alteration of cellular proliferation and differentiation of keratinocytes

We explored the possibility that upregulation of ET-1 in keratinocytes is associated with cyclic stretch-induced constitutional alteration of the keratinocytes, such as proliferation and differentiation. To test this possibility, we assessed the cell numbers after 24 h of cyclic stretch. We also assessed the levels of expression of CK14 (undifferentiated keratinocyte marker) and CK10 (differentiated keratinocyte marker) with and without the utmost degree of cyclic stretch (20% of length, 60 CPM) for 48 h. With these settings, cyclic stretch did not cause any apparent alteration in cell number or differentiation (Fig. 4).

4. Discussion

Physicians in cosmetic dermatological clinics sometimes hear complaints about hyperpigmented skin lesions, for which mechanical stimuli are considered to be a causative factor. Among them, the most distinctly established morbidity is friction melanosis [3,13–17]. A representative case appears as asymptomatic, brown-

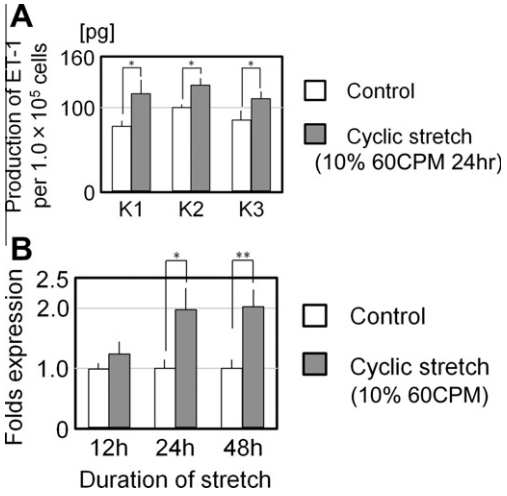

Fig. 2. (A) Production of ET-1 from keratinocyte with or without cyclic stretch. The production of ET-1 in culture supernatant was measured using ELISA. Production of ET-1 is constantly upregulated with cyclic stretch in all of three primary keratinocyte cell lines ($n = 3$ for each). (B) Expression of ET-1 mRNA with or without cyclic stretch for 12, 24, and 48 h ($n = 3$ for each) was quantified using realtime PCR. Error bar indicates standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

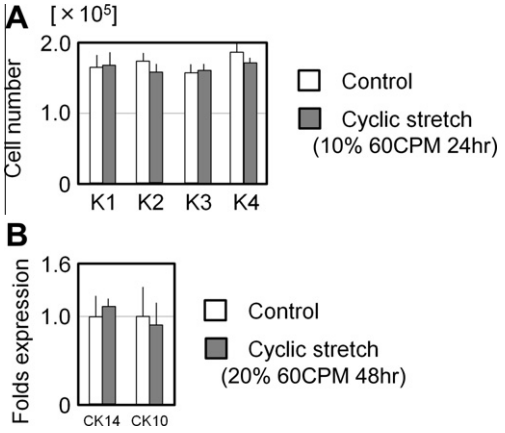

Fig. 4. Alteration of cell number and expression of differentiation marker of keratinocyte with or without cyclic stretch. (A) Cell number with or without cyclic stretch was investigated for four primary keratinocyte cell lines ($n = 3$ for each). The number of cells was not influenced with cyclic stretch. (B) Expression levels of keratinocyte differentiation marker mRNA with or without cyclic stretch ($n = 3$ for each) was investigated. Keratinocyte differentiation was not influenced with cyclic stretch. Error bar indicates standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. CK, cytokeratin.

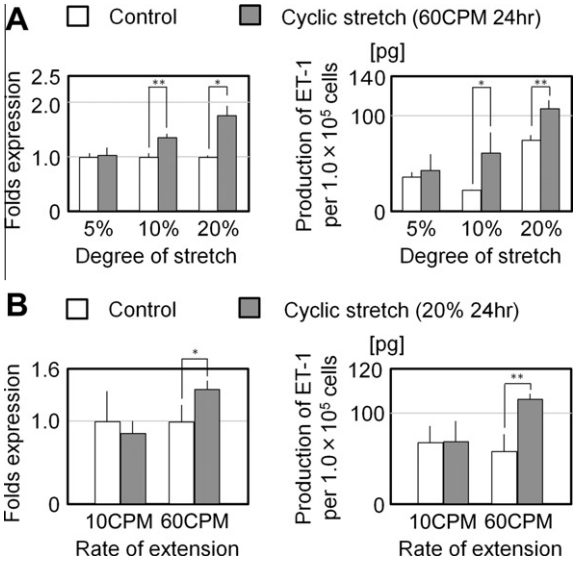

Fig. 3. ET-1 mRNA expression and production in culture supernatant of keratinocyte with different degree in length and frequencies of cyclic stretch. (A) ET-1 mRNA expression and production in culture supernatant with or without 5%, 10%, and 20% cyclic stretch were investigated using realtime PCR and ELISA ($n = 3$ for each). (B) ET-1 expression and production of keratinocyte with 10 and 60 CPM cyclic stretch ($n = 3$ for each) was also investigated. The upregulation of ET-1 increases with increment of stretch length and frequency, respectively. Error bar indicates standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

ish, pigmented patches with undefined margins, usually caused by long-term (many years) use of a nylon towel or brush [3,14,15]. Authors have also reported other types of hyperpigmentation due to repetitive friction as a variant of this disorder [16,17].

Epidermal hypermelanization is apparent in these types of hyperpigmentation, with marked dermal melanin deposition resulting from pigmentary incontinence [3,13–17] and melanin in the cytoplasm of dermal macrophages, with [13,15] or without [14,16,17] deposition of amyloid. We have also experienced patients with similar melanogenetic manifestation at sites of the body where repetitive friction is apparent (e.g., pigmentation of the nipple–areolar complex [3,18], intertriginous parts of the body, and sites where underwear is attached or over a bony prominence [3]). Especially in these cases of daily repetitive friction, the therapeutic interventions have often resulted in limited success, because the friction is unintentionally caused by daily actions. Furthermore, removing the causative factor, friction, is difficult even during the treatment period. We therefore began to investigate the molecular etiology of mechanical stress-associated hyperpigmentation with the aim of developing effective therapeutic strategies in the future.

The results of the present study demonstrated that among paracrine cytokines known to be associated with epidermal pigmentation mechanisms in the skin [1], ET-1 expression in keratinocytes was consistently upregulated with cyclic stretch in all four of the cell lines investigated. Several previous studies described the influence of mechanical strain (hydrostatic pressure or mechanical stretch) on keratinocyte proliferation [19–21], apoptosis [21], and differentiation [23]. We considered that these constitutional alterations (cell density or differentiation status) may be associated with the cytokine secretion pattern of keratinocytes. Therefore, we investigated the cell numbers and markers of keratinocyte differentiation in parallel with alteration of cytokine expression. Our results indicated that cyclic stretch had no apparent influence on cell number or differentiation status, a finding inconsistent with the results of previous studies [19–23].

We think that the differences were predominantly due to the different compositions of the medium (partially caused by the mode of mechanical stress). Our experimental medium contained 0.5% FCS, added for the purpose of stimulating cytokine secretion. It was similar to the medium Okazaki et al. previously employed to assess melanogenetic paracrine cytokines [2,24,25]. In our investigation, FCS addition had an overwhelmingly effect on the influence of mechanical strain, described in previous reports [19–23]. Upregulation of ET-1 in our study is thought to be a functional alteration, rather than a secondary one resulting from constitutional alterations. It has been shown that effects of mechanical loading on cells depends on the magnitude of strain [28,29] and the loading frequency [30]. That the upregulation of ET-1 with cyclic stretch showed dose-dependence supports an intimate association between mechanical stretch and ET-1 upregulation.

It has been shown that ET-1 is an intrinsic melanogen and mitogen for human melanocytes [1,4,5] and that keratinocyte-derived ET-1 causes several hyperpigmented disorders and conditions. In ultraviolet B-induced melanogenesis, keratinocyte-derived ET-1, which is stimulated in an autocrine manner by IL-1α [5] and which works collaboratively with keratinocyte-derived SCF [8,31], induces an increase in tyrosinase activity and stimulates melanocyte proliferation [1,4,5]. In lentigo senilis [32,33] and seborrheic keratosis [34,35], it was also suggested that secretion of ET-1 by keratinocytes and the ET-1-mediated cascade in melanocytes plays an important role in melaninization in clinical immunohistochemical samples. Like these morbidities, cyclic stretch-induced ET-1 upregulation from keratinocytes may play an etiological role in the pathogenesis of mechanical stress-associated hyperpigmentation.

The limitation of our study is that all the procedures were performed in vitro. The pathogenesis of mechanical stress-associated hyperpigmentation is obviously much more complicated, with the influence of concurrent invasion and inflammation signals. Future studies of mechanical stress-associated hyperpigmentation should be oriented to elucidate these in vivo mechanisms, with the assumption that mechanical stress on keratinocytes is associated with upregulation of ET-1 expression.

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