2．The regulation of STAT-1 and NFKB binding activity in human keratinocytes HaCaT cells. Nakamura K, Oyama N, Kaneko F, Tsunemi Y, Saeki Y, Tamaki K. The 65th Annual Meeting of the SID 2004. April 28- May 1. Rhode Island

H. 知的財産権の出願・登録状況

1．特許取得
なし

2．実用新案登録
なし

3．その他
なし

研究成果の刊行に関する一覧表

雑誌

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Fujita H, et al.	Expression and regulation of RANTES/CCL5, MIP-1α/CCL3, and MIP-1β/CCL4 in mouse Langerhans cells.	J Invest Dermatol	122	1331-3	2004
Yano S, et al.	Mechanical stretching in vitro regulates signal transduction pathways and cellular proliferation in human epidermal keratinocytes.	J Invest Dermatol	122	783-90	2004
Tsunemi Y, et al.	The –431C>T polymorphism of thymus and activation- regulated chemokine increases the promoter activity but is not associated with susceptibility to atopic dermatitis in Japanese patients.	Exp Dermatol	13	715-9	2004
Nakayama T, et al.	Liver-expressed chemokine/CC chemokine ligand 16 attracts eosinophils by interacting with histamine H4 receptor.	J Immunol	173	2078-83	2004
Hieshima K, et al.	CC Chemokine ligands 25 and 28 play essential roles in intestinal extravasation of IgA antibody-secreting cells.	J Immunol	173	3668-75	2004
Hirata T, et al.	Human P-selectin glycoprotein ligand-1 (PSGL-1) interacts with the skin-associated chemokine CCL27 via sulfated tyrosines at the PSGL-1 amino terminus.	J Biol Chem	279	51775-8	2004
Moroi Y, et al.	Effects of MAPK inhibitors on CCR4-mediated chemotaxis against thymus and activation-regulated chemokine (TARC/CCL17).	J Dermatol Sci	36	186-8	2004
Kobayashi J, et al.	Reciprocal regulation of permeability through a cultured keratinocyte sheet by IFN-γ and IL-4.	Cytokine	28	186-9	2004
Furukawa H, et al.	Enhanced TARC production by dust-mite allergens and its modulation by immunosuppressive drugs in PBMCs from patients with atopic dermatitis.	J Dermatol Sci	35	35-42	2004

Furukawa H, et al.	Effect of an antiallergic drug (Olopatadine hydrochloride) on TARC/CCL17 and MDC/CCL22 production by PBMCs from patients with atopic dermatitis.	J Dermatol Sci	36	165-72	2004

（倫理面への配慮）

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Expression and Regulation of RANTES/CCL5, MIP-1α/CCL3, and MIP-1β/CCL4 in Mouse Langerhans Cells

To the Editor:

Chemokines can be divided into two categories in terms of their physiological features, homeostatic and inflammatory (Moser and Loetscher, 2001). Among the inflammatory chemokines, regulated on activation of normal T cell expressed and secreted (RANTES)/CCL5, macrophage inflammatory protein (MIP)-1α/CCL3, and MIP-1β/CCL4 (Moser and Loetscher, 2001) are ligands for CCR5. Recent reports have shown that inflammatory chemokines, such as CCL3, CCL4, and CCL5, can be induced during maturation/activation of human monocyte-derived dendritic cells (DC) and mouse splenic DC (Sallusto *et al*, 1999; Re and Strominger, 2001; Vissers *et al*, 2001). But it remains unclear whether Langerhans cells (LC), a special subset of DC localized in epidermis, are capable of expressing CCL3, CCL4, and CCL5, except for the reports that LC express mRNA for CCL3 both in humans and mice (Heufler *et al*, 1992; Matsue *et al*, 1992; Parkinson *et al*, 1993; Vissers *et al*, 2001). Recently, using a modified panning method, we succeeded in isolating highly purified immature LC (>95%) from mouse skin (Salgado *et al*, 1999; Tada *et al*, 2000). The purified LC in our preparation acquire mature phenotypes during culture even without stimuli (Salgado *et al*, 1999). We used these cells as an *in vitro* system with negligible interference from keratinocytes (KC) or KC-derived cytokines, and examined the expression of CCL3, CCL4, and CCL5 both at the mRNA and at the protein level in freshly isolated (immature) LC and cultured (mature) LC.

First, using specific primer for CCL3, CCL4, and CCL5, we employed RT-PCR to assess the gene expression of these three chemokines in fresh LC and LC cultured for 48 h. The results of RT-PCR showed that CCL5 mRNA expression was not found in fresh LC, but strongly induced after 48 h culture (data not shown). On the other hand, both CCL3 and CCL4 mRNA were expressed equally in fresh LC and cultured LC (data not shown). Our present observation of the expression of CCL3 mRNA by LC was consistent with previous reports (Heufler *et al*, 1992; Matsue *et al*, 1992; Parkinson *et al*, 1993; Vissers *et al*, 2001).

Next, ELISA was carried out to determine the actual amount of protein secreted into culture media at different time points. As shown in Table I, CCL3 and CCL4 were produced relatively early, increasing in amount within the first 6 h of culture. In contrast, CCL5 production was minimal for the first 24 h, but increased remarkably thereafter. We also tested the ability of KC to produce these chemokines. KC were cultured in the same way as LC for 48 h without stimulation. All three chemokines were almost undetectable in the supernatant of 48 h cultured KC (data not shown). We further investigated how the production of these three chemokines is regulated by various stimuli. As shown in Table II, when LC were stimulated with TNF-α, IFN-γ, agonistic anti-CD40 Ab, and *Staphylococcus aureus* Cowen 1 (SAC), the production level of CCL5 in the supernatants was increased significantly. On the other hand, TGF-β, GM-CSF, IL-4, IL-10, and IL-13 had significant inhibitory effects on CCL5 production. The patterns of modulation of CCL3 and CCL4 production were almost identical, except for the cases using anti-CD40 Ab and IL-12. TNF-α, GM-CSF, M-CSF, IL-1β, IL-4, IL-10, IL-13, and lipopolysaccharide (LPS), and SAC significantly enhanced the production of CCL3 and CCL4. Of note, the effect of SAC was particularly prominent among them. Anti-CD40 Ab upregulated CCL3 but not CCL4 production, whereas IL-12 had a slight positive effect on the production of CCL4 but not on that of CCL3.

Finally, we investigated whether the inflammatory chemokines produced by LC are actually chemotactic to T cells. For this purpose, we carried out a chemotaxis assay using mouseCCR5-transfected 2B4 T cells as previously described (Gao *et al*, 2003). Since LC produced much more CCL5 than CCL3/4, we focused on CCL5 in this assay, and used the supernatant of LC cultured for 72 h with or without 100 ng per mL of IFN-γ. LC cultured for 72 h produced CCL5 at 4741 ± 303 pg per mL without stimulation and at 7685 ± 301 pg per mL in the presence of IFN-γ as determined by ELISA (n = 3). As shown in Fig 1, the supernatants of unstimulated and IFN-γ-stimulated LC also induced cell migration, and this migration was partially blocked by neutralizing anti-CCL5 mAb. Judging from this neutralization, the contribution of CCL5 to the migration was more prominent when the supernatant of IFN-γ-stimulated LC was used rather than that of unstimulated LC. In addition, factors other than CCL5 were involved in the cell migration in this experiment.

LC emigrate to the T-cell zone of draining lymph nodes (LN) following maturation to present antigens to T cells, although some of them may stay in the skin (Katou *et al*, 2000, 2003). Therefore, immediate production of CCL3/4 and delayed production of CCL5 should represent distinct roles. Secretion of CCL3/4 can occur easily when LC are still in skin. On the other hand, mature LC are likely to secrete large amounts of CCL5 after their arrival in draining LN. Curiously, LC secreted extremely large amounts of

Abbreviations: KC, keratinocytes; LC, Langerhans cells; SAC, *Staphylococcus aureus* Cowen 1

Table I. Time course of chemokine production by Langerhans cells (LC)

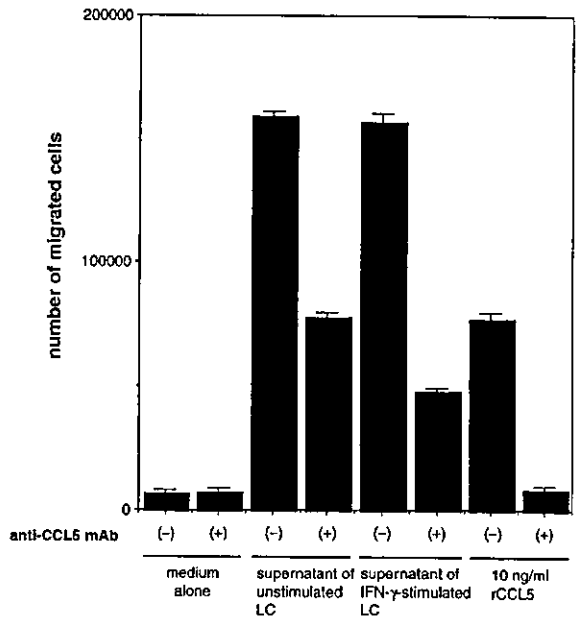
Time	0 h	6 h	12 h	24 h	48 h
CCL3 (pg per mL)	5.2 ± 0.5	37.3 ± 3.8	54.4 ± 9.7	65.0 ± 7.7	111.5 ± 11.1
CCL4 (pg per mL)	8.7 ± 0.5	38.3 ± 4.8	40.4 ± 3.7	41.0 ± 3.4	57.6 ± 10.6
CCL5 (pg per mL)	74.0 ± 2.3	93.0 ± 9.2	100.0 ± 14.8	124.0 ± 4.7	1884.0 ± 107.8

Production of inflammatory chemokines during the maturation of LC. Purified LC (1.5×10^6 per mL) were cultured without stimulation, and the concentration of CCL3, CCL4, and CCL5 was measured at different time points (0, 6, 12, 24, 48 h) in the supernatants by ELISA kit. Results are mean ± (SD) (n = 3). Representative data of three independent experiments.

Table II. Chemokine production by Langerhans cells (LC) cultured for 48 h

Stimuli	None	TGF-β	TNF-α	GM-CSF	M-CSF	IFN-γ	IL-1β	
CCL3 (pg per mL)	111.7 ± 6.3	83.9 ± 4.3[b]	183.8 ± 10.9[a]	334.5 ± 18.8[a]	185.5 ± 34.3[a]	99.9 ± 10.2	369.2 ± 38.7[a]	
CCL4 (pg per mL)	67.9 ± 2.7	53.5 ± 1.4[b]	91.4 ± 1.9[a]	127.1 ± 4.0[a]	100.5 ± 19.7[a]	64.3 ± 4.2	89.1 ± 11.9[a]	
CCL5 (pg per mL)	1183.8 ± 22.7	764.8 ± 75.9[b]	2133.0 ± 230.2[a]	828.8 ± 135.3[b]	1190.0 ± 78.1	3503.5 ± 578.9[a]	1114.8 ± 54.8	
Stimuli	**IL-4**	**IL-10**	**IL-12**	**IL-13**	**IL-18**	**Anti-CD40 Ab**	**LPS**	**SAC**
CCL3 (pg per mL)	248.7 ± 23.7[a]	156.6 ± 7.7[a]	107.3 ± 8.5	186.0 ± 32.1[a]	110.3 ± 15.7	156.7 ± 13.0[a]	183.9 ± 37.7[a]	1761.4 ± 217.9[a]
CCL4 (pg per mL)	114.9 ± 2.7[a]	96.4 ± 4.1[a]	80.0 ± 5.2[a]	96.3 ± 3.9[a]	71.3 ± 3.4	71.0 ± 6.5	95.1 ± 7.5[a]	232.6 ± 17.0[a]
CCL5 (pg per mL)	475.5 ± 50.9[b]	628.3 ± 66.7[b]	1194.8 ± 115.1	649.3 ± 214.9[b]	1174.0 ± 222.4	1756.3 ± 338.1[a]	1247.0 ± 72.5	1436.0 ± 127.0[a]

ELISA of CCL3, CCL4, and CCL5 in culture supernatants of LC. LC (1.5×10^6 per mL) were cultured for 48 h with or without various stimuli. Results are mean ± (SD) (n = 4). Concentration of each stimulator was as follows: TGF-β, 1 ng per mL; TNF-α, 10 ng per mL; GM-CSF, 1 ng per mL; M-CSF, 1 ng per mL; IFN-γ, 100 ng per mL; IL-1β, 10 ng per mL; IL-4, 10 ng per mL; IL-10, 10 ng per mL; IL-12, 2 ng per mL; IL-13, 10 ng per mL; IL-18, 100 ng per mL; anti-CD40 Ab, 20 μg per mL; lipopolysaccharide (LPS), 1 μg per mL; *Staphylococcus aureus* Cowen 1 (SAC), 1:10,000 dilution.
[a]Significant increase ($p < 0.05$) compared with the unstimulated group.
[b]Significant decrease ($p < 0.05$) compared with the unstimulated group. Representative data of three independent experiments.



CCL3/4 when stimulated with SAC. Stimulation with LPS also upregulated CCL3/4 production, although to a lesser extent. In contrast, CCL5 production was enhanced only slightly by SAC and not at all by LPS. The expression of Toll-like receptor (TLR)2 (receptor for SAC) and TLR4 (receptor for LPS) by LC was verified in our previous report (Mitsui *et al*, 2004). Considering the different kinetics between

Figure 1
Chemotaxis assay was carried out using mouse CCR5-transfected 2B4 T cells (Gao *et al*, 2003). Chemotaxis of the transfectant was assessed in 24-well transwells equipped with 5 μM pore polycarbonate membranes. Cells (1×10^6) suspended in 100 μL of medium were transferred to the upper chamber. Culture supernatants of 72 h cultured Langerhans cells (LC) with or without IFN-γ and medium with or without 10 ng per mL of rCCL5 were preincubated with or without 1 μg per mL of anti-CCL5 mAb for 30 min at room temperature. They were then transferred to the lower chamber in 600 μL for each group. After an incubation period of 3 h at 37°C, the upper chambers were removed, and the cells in the lower chamber of each well were collected and counted. Medium alone served as a negative control. Medium containing 10 ng per mL of rCCL5 served as a positive control. Representative data of three independent experiments. Mean ± (SD) (n = 3).

CCL3/4 and CCL5 production, these results overall indicate that CCL3/4 produced by LC play a more important role than CCL5 in early innate immune responses to bacteria through the recruitment of effector cells into the skin. Interestingly, some of the stimulators of CCL3/4 production, such as GM-CSF, IL-4, IL-10, and IL-13, acted in the opposite for CCL5 production. Since CCL5 shares the receptor CCR5 with CCL3/4, the reciprocal regulation of CCL5 production in response to these stimuli may serve as a late negative feedback mechanism against early immune responses elicited by CCL3/4. Although KC are also capable of producing some chemokines, production is often regulated differently between KC and LC (Li *et al*, 1996; Wakugawa *et al*, 2001; Xiao *et al*, 2003a, b), indicating an essential role for LC as the source of chemokines in skin. Understanding the overall mechanism of chemokine production will definitely contribute toward solving the complexity of cutaneous inflammation.

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Mechanical Stretching *In Vitro* Regulates Signal Transduction Pathways and Cellular Proliferation in Human Epidermal Keratinocytes

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Epidermal keratinocytes are continuously exposed to mechanical forces. The human skin surface can be thickened and enlarged by various stresses such as tissue expander or abrasive pressure. To investigate the mechanism of epidermal hyperproliferation by mechanical stress, keratinocytes were plated on flexible silicone dishes, which were continuously stretched by +20%. Stretching of cells for 24 h caused upregulation of 5-bromo-2′-deoxyuridine (BrdU)-positive cells to 200%–220% and activation of extracellular signal-regulated kinases (ERK)1/2. Inhibition of mitogen and ERK with U0126 and phosphoinositide 3-OH kinase attenuated BrdU incorporation and ERK1/2 activation. The EGF receptor kinase inhibitor and the calcium channel inhibitor also inhibited BrdU incorporation and the activation of ERK1/2. Twenty-four hours of stretching stimulated reporter activity driven by activator protein 1 (AP-1), induction of K6, and suppression of K10, which were inhibited by U0126. Our results indicate that mechanical stretching induces proliferative signals on human keratinocytes via induction of calcium influx, phosphorylation of epidermal growth factor receptor (EGFR), and ERK1/2. These mechanisms may contribute to the hyperproliferative nature of the epidermis, which is mechanically stretched by various stimuli.

Key words: ERK/keratinocytes/mechanical stretching/proliferation
J Invest Dermatol 122:783–790, 2004

The skin, especially the epidermal keratinocytes, which protects the human body from the external environment, is continuously exposed to various stimuli. The effects of chemical and UV light stimulation of the skin and the epidermal keratinocytes have been investigated in detail (Hruza and Pentland 1990; Nickoloff *et al*, 1993), and phototherapy has been applied in dermatology as psoralen and ultraviolet A (PUVA) therapy. Little, however, is known about the consequences and mechanisms of action of mechanical stimuli to the skin, especially to the epidermis. The human skin surface can be stretched and enlarged by tissue expander apparatus for skin grafts. The abdominal skin of pregnant women is stretched and extended, which allows the skin to cover a markedly increased area. During wound healing, retracting adjacent tissue may stimulate marginal epidermal cells to cause epithelial spreading into the wound bed. Patients with acanthosis nigricans show skin lesions in intertriginous skin, which is assumed to be induced by continuous mechanical stretching or abrasion. Psoriasis vulgaris tends to affect the skin of rubbed areas such as the elbows and knees. Many dermatological diseases present the Köebner phenomenon, in which mechanically injured skin promotes new eruption. The mechanisms of these phenomena, however, remain to be fully elucidated. Recently, Kippenberger *et al* (2000) reported that ^{3}H-thymidine uptake was induced and extracellular signal-related kinase (ERK)1/2 and c-Jun amino terminal kinase (JNK) were activated in response to keratinocyte stretching. As for fibroblast stretching, fibroblast–collagen matrix contraction causes isometric tension to fibroblasts, ERK and p38 MAP kinases are activated (Lee *et al*, 2000), and the level of c-fos mRNA increases (Rosenfeldt *et al*, 1998), which indicates that these events play a unique role for wound contraction and wound healing (Grinnell, 2000).

Mechanical forces are related to many biological phenomena. Isometric strength training and flexibility training of the human muscles thicken muscle fibers and increase the amount of these muscles (Goldspink *et al*, 1991; Timson, 1991; Booth *et al*, 1998). It is widely accepted that mechanical loading is necessary to construct the architecture of bone and to maintain bone mass (Salter *et al*, 2000; Kurata *et al*, 2001). These observations support the theories of athletic training and rehabilitation. In the cardiovascular field, mechanical forces applied to rat vascular smooth muscle cells (VSMC) were shown to induce the phosphorylation of epidermal growth factor receptor (EGFR) (Iwasaki *et al*, 2000) and mitogen-activated protein kinase (MAPK) (Komuro *et al*, 1996; MacKenna *et al*, 1998; Seko *et al*, 1999; Kushida *et al*, 2001), which then

Abbreviations: AP-1, activator protein 1; BrdU, 5-bromo-2-deoxyuridine; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HaCaT, human keratinocyte cells; HRP, horseradish peroxidase; MEK1/2, mitogen and extracellular signal-regulated kinase; NHK, normal human keratinocytes; PI 3-K, phosphoinositide 3-OH kinase

promoted cell proliferation and hypertrophy of VSMC (Komuro *et al*, 1991; Sadoshima *et al*, 1992; Hishikawa *et al*, 1994). Other groups reported that exposure of human endothelial cells to fluid shear stress stimulated MAPK (Tseng *et al*, 1995). Shear stress was also reported to stimulate the migration and proliferation of endothelial cells (Ando *et al*, 1987) and enhance endothelial cell DNA synthesis during the repair of mechanical denudation (Ando *et al*, 1990). These results may support the mechanism of the alterations in cardiovascular and endothelial morphology and function induced by hypertension.

These phenomena surrounding human skin and other organs prompted us to further investigate the nature of stretched epidermal cells, including the signal transduction pathways induced by mechanical stretching. We demonstrated that mechanical stretching promoted the proliferation of cultured human keratinocytes by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation and that mechanical stretching also inhibited differentiation by the upregulation of keratin K6 and the downregulation of keratin K10.

Results

BrdU incorporation was induced by mechanical stretching To verify observations on the nature of the epidermis surrounding intraepidermal tumors, we examined whether the stretching signal really induced proliferation of cultured keratinocytes on flexible silicone chambers (see Fig 1).

We showed that continuous mechanical stretching of keratinocytes by +20% for 24 h significantly induced BrdU incorporation by 2.2 ± 0.4-fold in NHK (Fig 2) and 2.0 ± 0.5-fold in HaCaT (not shown) compared to non-stretched controls ($p < 0.05$). U0126 (10 μM), wortmannin (1 μM), AG1478 (200 nM), and gadolinium (150 μM), inhibitors of MEK1/2, PI 3-K, EGF receptor, and calcium influx, respectively, strongly inhibited BrdU incorporation induced by mechanical stretching (Fig 2). This suggested that mechanical stretching induced keratinocyte proliferation, i.e., S-phase entry, and that keratinocyte proliferation induced by mechanical stretching was MEK1/2 pathway-, PI 3-K pathway-, EGFR-, and calcium channel-dependent.

Mechanical stretching activates ERK1/2 The above results indicated that mechanical stretching induces cell proliferation through ERK1/2 and PI 3-K pathways, which are also activated by various growth factors and cytokines such as EGF. We examined ERK1/2 phosphorylation by mechanical stretching by harvesting keratinocytes after 1–60 min of stretching and performed western blotting using an antibody to phosphorylated ERK1/2.

Western blotting revealed that phosphorylation of ERK1/2 arose from 2 min of stretching, which reached the maximal level at 5 and 15 min, and then ceased after 30 and 60 min (Fig 3*A*). On the same membrane re-probed with anti-ERK2 antibody, the density of ERK2-positive bands was almost the same among all lanes (Fig 3*B*). The phosphorylation of ERK1/2 by EGF (50 ng per mL) was strongly detected from 1 min and lasted up to 60 min (Fig 3*C*). These results suggested that mechanical stretching activated the ERK1/2 signaling pathway in epidermal keratinocytes *in vitro*.

ERK1/2 activation by mechanical stretching occurs through both MEK1/2 and PI 3-K pathways, while activation by EGF occurs only through MEK1/2 MEK1/2 and PI 3-K are the two major kinases activated by growth stimulatory signals such as EGF. We next investigated whether MEK1/2 and PI 3-K pathways are involved in ERK1/2 activation by mechanical stretching. ERK1/2 activation by mechanical stretching was abolished by pre-treatment with both MEK1/2 inhibitor U0126 (30 min) and PI 3-K inhibitor wortmannin (60 min) in a concentration-dependent manner, while ERK1/2 activation by EGF (50 ng per mL) was affected only by U0126 (10 μM) but not by wortmannin in all doses (Fig 4*A*, *B*). These results indicated that both EGF and

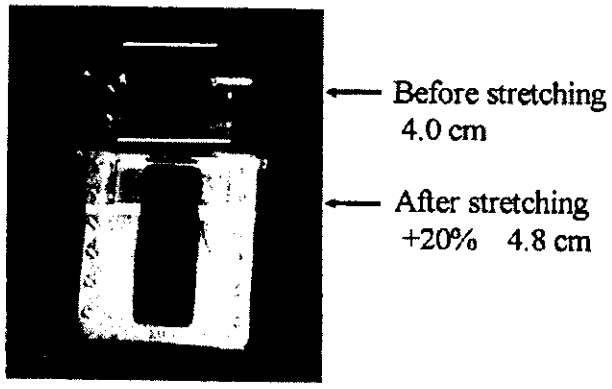

Figure 1
Stretching apparatus. Cells were seeded and grown in transparent and cubic silicone chambers, the bottom of which was covered with collagen type I as described in *Materials and Methods*. The overall view of the flexible silicone chamber before stretching and after stretching to +20% continuously and longitudinally using a stainless-steel stretching device was shown.

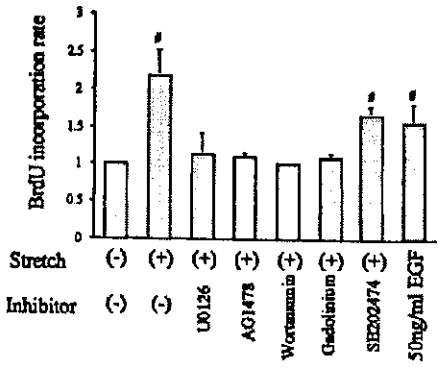

Figure 2
BrdU incorporation was induced by mechanical stretching. Continuous stretching of NHK for 24 h and EGF (50 ng per mL) stimulation caused upregulation of BrdU-positive cells to 200%–220% as detected using a commercial BrdU ELISA kit. This upregulation was inhibited by MEK1/2 inhibitor U0126, PI 3-K inhibitor wortmannin, EGFR phosphorylation inhibitor AG1478, and calcium channel blocker gadolinium (Gd^{3+}). SB202474 was used as a negative control and did not inhibit BrdU upregulation by mechanical stretching (#: $p < 0.01$).

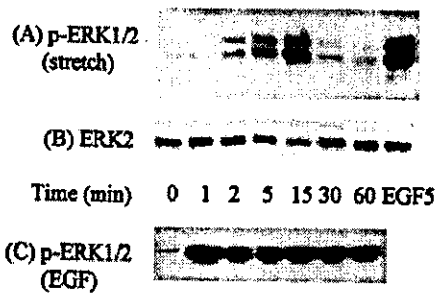

Figure 3
Mechanical stretching causes ERK1/2 phosphorylation. Time courses of ERK1/2 phosphorylation induced by mechanical stretching and EGF treatment of NHK. Equal amounts of protein extracts were immunoblotted with antibodies specific for (*A*) phosphorylated ERK1/2 (p-ERK1/2) and (*B*) ERK2 (after stripping the same membrane). p-ERK1/2-positive bands showing phosphorylation were seen after 5–15 min of stretching and disappeared after 30–60 min of stretching. (*C*) On the other hand, strong phosphorylation of ERK1/2 induced by EGF (50 ng per mL) was maintained for longer periods than that induced by stretching.

mechanical stretching activated ERK1/2. EGF utilized only the MEK1/2 pathway however, while stretching utilized both MEK1/2 and PI 3-K pathways.

Calcium influx is indispensable for activation of ERK1/2 by mechanical stretching, but not for its activation by EGF Calcium metabolism is an important factor in the activation of ERK1/2 in several lines of cells. To investigate the effects of calcium metabolism in the activation of ERK by mechanical stretching, we utilized the calcium influx inhibitor gadolinium (Gd^{3+}). ERK1/2 phosphorylation by mechanical stretching as determined by western blotting was almost completely inhibited by Gd^{3+} pre-treatment (for 60 min, 150 μM), and the inhibition of ERK1/2 phosphorylation by mechanical stretching was concentration-dependent, i.e., 50 μM Gd^{3+} showed moderate inhibition, and 15 μM Gd^{3+} showed only a slight inhibitory effect. On the other hand, activation of ERK1/2 by EGF stimulation (50 ng per mL) was not inhibited by all concentrations of Gd^{3+} (Fig 5). These phenomena, in addition to the experiment with MEK1/2 and PI 3-K inhibitor, delineated the EGF signal transduction pathway and the mechanical stretching-induced signal transduction pathway.

ERK1/2 activation by mechanical stretching requires EGF receptor phosphorylation We inhibited EGF receptor phosphorylation to determine whether it affects the activation of ERK1/2 by mechanical stretching. The EGF receptor kinase inhibitor AG1478, which specifically inhibits EGF receptor autophosphorylation, inhibited the activation of ERK1/2 in NHK by mechanical stretching as well as the activation of ERK1/2 by EGF in a concentration-dependent manner. AG1478 at 200 nM showed almost perfect inhibition, 20 nM AG1478 showed moderate inhibition, and 2 nM AG1478 showed only a slight inhibitory effect on ERK1/2 activation both by mechanical stretching and by EGF (Fig 6). These results suggested that mechanical stretching induced ERK1/2 activation through the activation of EGF receptors probably without stimulation by its ligand.

Mechanical stretching also induced the phosphorylation of EGFR As EGFR autophosphorylation is responsible for the activation of ERK1/2 by mechanical stretching as described above, we next directly investigated EGFR phosphorylation using anti-phospho-EGFR (Tyr845, Tyr992, Tyr1045, Tyr1068) antibodies. Western blotting analysis showed that only Tyr845-EGFR phosphorylation was augmented at 2 and 5 min, as compared to the control (0 min), but decreased thereafter. Tyr992, Tyr1045, or Tyr1068-EGFR phosphorylation was not detected by mechanical stretching, although EGF stimulation strongly phosphorylated all phospho-EGFR antibodies (Fig 7*A–E*). Immunoprecipitation analysis showed that EGFR phosphorylation was augmented at 5–10 min, as compared to the control (0 min), but decreased thereafter (Fig 7*F*). These results suggested that mechanical stretching induced phosphorylation of a part of EGFR prior to ERK1/2 phosphorylation.

AP-1-driven reporter activity is induced by mechanical stretching AP-1, which is a complex of Jun, Fos, or activating transcription factor (ATF), is one of the transcription factors activated by EGF and other cytokines such as TNF and IL-1. We investigated whether the mechanical stretching signal induces AP-1 activation. We transfected keratinocytes with an AP-1 site-driven firefly luciferase construct together with TK promoter-driven renilla luciferase vector, and performed dual luciferase assay. AP-1 luciferase activities were normalized with renilla luciferase activity. Six to forty-eight hours of stretching signal significantly stimulated AP-1 consensus sequence-driven reporter activity in both NHK and HaCaT (not shown) as compared to non-stretched keratinocytes, similar to the effect of EGF (50 ng per mL) (Table I). The activation of AP-1-driven reporter activity by mechanical stretching and EGF stimulation was strongly suppressed by MEK1/2 inhibitor U0126 pre-treatment (30 min, 10 μM) to almost the same level as in

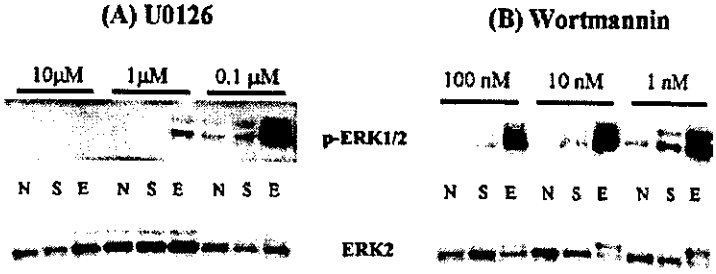

Figure 4
Mechanisms of ERK1/2 activation were different between mechanical stretching and EGF stimulation. Both (*A*) U0126 and (*B*) wortmannin suppressed p-ERK1/2 by mechanical stretching in a concentration-dependent manner in NHK. Only U0126 (10 μM) suppressed p-ERK1/2 in cells stimulated with EGF. The densities of the ERK2 bands in each lane were almost equivalent (N, non-stretched NHK lysate; S, NHK stretched for 15 min; E, NHK stimulated with 50 ng per mL EGF for 15 min).

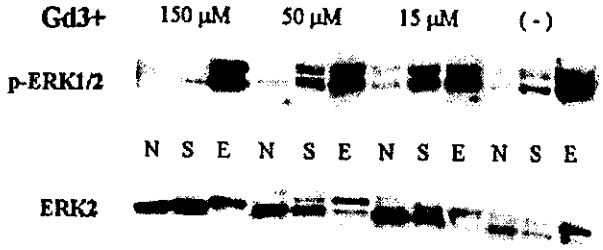

Figure 5
ERK1/2 phosphorylations by mechanical stretching require calcium influx. Gd^{3+} inhibited ERK1/2 phosphorylation by mechanical stretching in a concentration-dependent manner in NHK (15, 50, 150 μM concentration), while it did not inhibit phosphorylation of ERK1/2 induced by EGF stimulation at these concentrations (N, non-stretched NHK lysate; S, NHK stretched for 15 min; E, NHK stimulated with 50 ng per mL EGF for 15 min).

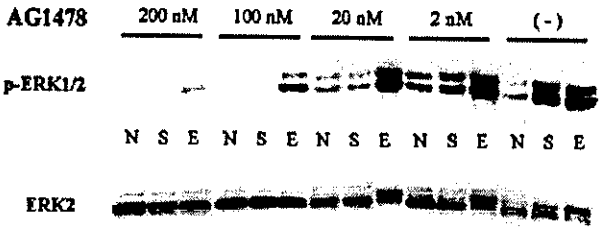

Figure 6
ERK1/2 activation by mechanical stretching requires EGF receptor phosphorylation. AG1478 inhibited ERK1/2 phosphorylation induced by mechanical stretching and EGF stimulation in a concentration-dependent manner in NHK (N, non-stretched NHK lysate; S, NHK stretched for 15 min; E, NHK stimulated with 50 ng per mL EGF for 15 min).

non-stretched controls (Table I). Our results indicated that mechanical stretching induces AP-1 activity via MEK1/2 activation in human keratinocytes.

Mechanical stretching induces epidermal keratinocyte activation with K6 induction and K10 suppression ***In vitro*** Keratins are convenient markers to investigate keratinocyte differentiation. We examined two of the keratins, keratin K6 and keratin K10, which represent activated and differentiated keratinocytes, respectively, in cultured human keratinocytes. Western blotting analysis indicated that 24 h stretched and 50 ng per mL EGF-treated keratinocytes showed strong K6 expression compared to sparse K6 expression in non-stretched controls. Pre-treatment with the MEK1/2 inhibitor U0126 (30 min, 10 μM) abolished these effects (Fig 8*A*, *B*). On the other hand, K10 was not observed in stretched and 50 ng/ml EGF-treated keratinocytes, while non-stretched keratinocytes showed strong K10 expression. U0126 pretreatment (30 min, 10 μM) decreased K10 suppression caused by mechanical stretching and EGF (50 ng per mL) (Fig 8*C*, *D*). These results suggested that mechanical stretching makes quiescent keratinocytes activated rather than differentiated via MEK1/2 activation.

Discussion

We could demonstrate several effects of mechanical stretching *in vitro*. As described in *Materials and Methods*,

Table I. AP-1-driven reporter activity is induced by mechanical stretching

Stretching time (h)	AP-1 activation ratio (against no stretch)
+20% strech	
6	1.52 ± 0.37 times*
12	2.05 ± 0.55 times*
24	1.87 ± 0.24 times*
48	1.41 ± 0.23 times**
EGF 24	2.08 ± 0.71 times*
U0126 pre-treatment	
12	1.04 ± 0.08 times
24	1.08 ± 0.08 times
EGF 24	1.15 ± 0.32 times

Luciferase activity in NHK cell lysates was measured using a luminometer. About 6–24 h of stretching signal stimulated AP-1 consensus sequence-driven luciferase reporter activity, similar to the effect of EGF. The activation of AP-1-driven luciferase reporter activity by mechanical stretching and EGF was suppressed by U0126 pretreatment.
*$p<0.01$,
**$p<0.05$ by using Student's *t* test.

we utilized a unique culturing and stretching system. In this system, basal collagen film itself does not contract or peel off, and it stretches synchronously with a silicon chamber, which enabled attached keratinocytes to be stretched exactly by +20%. First, stretching of keratinocytes seeded on silicone wells for 24 h caused the upregulation of BrdU-positive cells to 200%–220%, and this upregulation was inhibited by the MEK1/2 inhibitor U0126, PI 3-K inhibitor wortmannin, EGFR phosphorylation inhibitor AG1478, and calcium channel blocker Gd^{3+}. These observations indicated that mechanical stretching induces the S-phase entry of keratinocytes via MEK1/2, PI 3-K, EGFR, and calcium channel-dependent pathways. Although there is also a possibility that the effects of stretching are mediated by releasing soluble factors from stretched keratinocytes themselves, this is the first study illustrating the signaling molecules that induce BrdU incorporation by mechanical stretching in human keratinocytes.

ERK1/2 activation in various types of cells is indispensable in cell proliferation and survival (Boulton *et al*, 1991; Guyton *et al*, 1996; Whelchel *et al*, 1997). It is reported that during fibroblast contraction of stressed collagen matrices under isometric tension, both ERK and p38 MAP kinases were activated (Lee *et al*, 2000). We demonstrated that mechanical stretching induced ERK1/2 phosphorylation in a time-dependent manner via a calcium channel and EGFR activation-dependent pathway, thereby leading to the stimulation of AP-1 activity in NHK and HaCaT cells. Moreover, Akt phosphorylation was also induced by mechanical stretching in a time-dependent manner via EGFR activation and calcium channel activation, leading to an anti-apoptotic effect (Yano *et al*, unpublished data). Interestingly, wortmannin and U0126 both inhibited ERK1/2 phosphorylation induced by mechanical stretching. On the other hand, ERK1/2 phosphorylation due to EGF stimulation was not inhibited by wortmannin but was inhibited only by

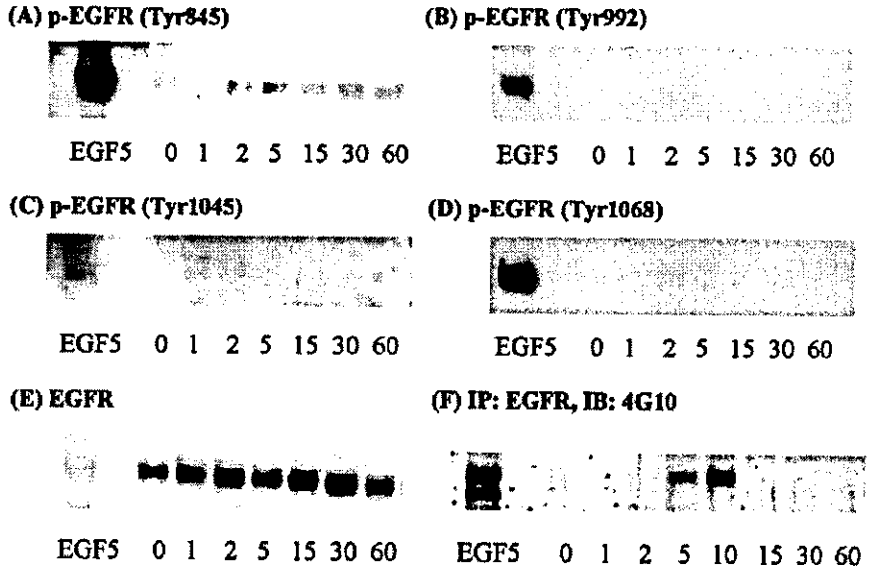

Figure 7
Mechanical stretching induces phosphorylation of EGFR. Time course (0–60 min) of EGFR phosphorylation after stretching NHK. (*A*) EGFR phosphorylation (Tyr845) was seen only after 2–5 min of stretching and disappeared after 15–60 min of stretching. (*B–D*) EGFR phosphorylation (Tyr992, Tyr1045, Tyr1068) was not detected. (*E*) EGFR bands detected on the same membrane were also shown. The densities of bands in all lanes were almost equivalent. (*F*) After immunoprecipitation (IP) with EGFR antibody, EGFR phosphorylation (4G10) was seen only after 5–10 min of stretching and disappeared after 15–60 min of stretching (IP, immunoprecipitation, IB, immunoblot).

U0126. These results suggested that PI 3-K and MEK1/2 pathways may function as activators of ERK in mechanical stretching in these cells, and that both EGF and mechanical stretching activate ERK1/2 through EGFR phosphorylation, while their downstream signal transduction pathways are not exactly the same. The results of the present study represent the first evidence that PI 3-K is responsible for ERK1/2 activation by mechanical stretching in human keratinocytes.

Calcium channel activation on the cell membrane and calcium influx play crucial roles in controlling cell growth (Taylor and Simpson, 1992; Dascalu *et al*, 2000; Wang *et al*, 2000). We showed in this study that the calcium channel blocker gadolinium inhibited the phosphorylation of ERK1/2 induced by mechanical stretching. This result indicated that calcium channels receive mechanical stimuli, and then transmit the signals to downstream pathways. The observation that the calcium channel blocker gadolinium did not affect the phosphorylation of ERK1/2 due to EGF stimulation supports the idea that there are differences in signal transduction between mechanical stretching and EGF stimulation, although both cause EGFR phosphorylation. EGFR exists on the cell membrane, and receives stimulation from EGF and other members of the EGF family. The binding of integrin $\alpha 2\beta 1$ and EGFR is required for EGFR phosphorylation in A431 cells (Yu *et al*, 2000). Therefore, we hypothesized that EGFR received mechanical stimuli and was phosphorylated, and then downstream signals of EGFR such as MEK1/2 and PI 3-K were activated. We showed that EGFR phosphorylation (Tyr845) occurred after 2–5 min of keratinocyte stretching, and that AG1478, an inhibitor of EGFR phosphorylation, blocked the phosphorylation of ERK1/2 induced by mechanical stretching. We also showed EGFR phosphorylation by immunoprecipitation. These results suggested that the mechanical stimulation by stretching of human keratinocytes is received by EGFR, which results in MAPK activation. Similar phenomena were reported in VSMC, which showed the phosphorylation of EGFR (Iwasaki *et al*, 2000), followed by activation of the Ras and Raf pathways (Yamazaki *et al*, 1995; Li *et al*, 1999) upon mechanical stretching. We showed that mechanical stretching phosphorylated only the Tyr845 site compared to EGF stimulation which phosphorylated many sites (Tyr845, Tyr992, Tyr1045, Tyr1068) and that the density of positive bands by mechanical stretching was weaker than that by EGF stimulation (Fig 8*A*, *B*). These suggested that the effect on EGFR by mechanical stretching is neither more various nor stronger than that by EGF stimulation and that their downstream signal transduction pathways are consequently different from each other.

Keratins constitute a large family of cytoskeletal proteins, differentially expressed in various epithelial cells (Schweizer, 1993). K6 is a marker of activated keratinocytes (Komine *et al*, 2000) and is expressed in psoriasis and carcinomas, but not in the healthy interfollicular epidermis. EGF and TNFα induce K6 in keratinocytes (Jiang *et al*, 1993; Komine *et al*, 2000) and there is an AP-1 binding site in the K6 gene promoter (Bernerd *et al*, 1993; Ma *et al*, 1997). We showed that mechanical stretching of keratinocytes for 24 h induced K6 upregulation, which was strongly inhibited by MEK1/2 inhibitor U0126. This result suggests that mechanical stretching induced the signals resulting in keratinocyte activation and proliferation, which were probably mediated via the MEK1/2 activation and AP-1 activation pathway. Although there is a discrepancy between the strong activation of K6 expression and the relatively small AP-1 activation induced by stretching, we suggest that the expression of K6 gene requires not only AP-1 activation but also other signaling or stimulating factors. Unexpectedly, the expression of K10 was clearly abolished when keratinocytes were stretched for 24 h, and K10 downregulation was simultaneously suppressed by U0126. K10 is a marker of differentiated keratinocytes (Ivanyi *et al*, 1989) and is expressed in the differentiating suprabasal layers. These phenomena suggested that mechanical stretching suppresses keratinocyte differentiation probably via the MEK1/2 activation pathway. In the epidermis of normal human palm and sole skin, keratin K9, K6, K16, and K17 were shown to be expressed at the bottom of the deep

primary epidermal ridges possibly due to the greater stress that ridged skin has to withstand (Swensson *et al*, 1998). This study, however, is the first to demonstrate that mechanical forces can alter the expression patterns of keratin, the constituent of intermediate filaments expressed specifically in keratinocytes.

Our investigations elucidated that early activation of signaling pathways such as EGFR, ERK1/2, and PI 3-K in response to mechanical stretching is involved in epidermal cell proliferation, and keratinocyte activation including keratin K6 expression and keratin K10 suppression. Although it shares many features with EGF signaling, substantial parts of the response to mechanical stretching are probably different from those of the response to EGF stimulation. Moreover, we can hypothesize that mechanical stretching on the epidermis or keratinocytes enables epidermal regeneration and establishes new methods of keratinocyte proliferation. Our data and model are useful for explaining cutaneous changes caused by mechanical stress in cases of tissue expander, pregnant women, wound healing, and the Köebner phenomenon, and present additional insights for signaling pathways in human keratinocytes. We are now investigating further functions and clinical application using mechanical stretching systems.

Materials and Methods

Antibodies and materials Anti-phospho-ERK1/2, anti-phospho-EGFR (Tyr845, Tyr992, Tyr1045, Tyr1068), and anti-EGFR were purchased from Cell Signaling (Beverly, Massachusetts). Anti-ERK2, anti-mouse IgG horseradish peroxidase (HRP) conjugate, and anti-rabbit IgG HRP conjugate were from Santa Cruz (Santa Cruz, California). HRP-conjugated anti-phosphotyrosine 4G10 was from Upstate Biotechnology (Lake Placid, New York). Anti-cytokeratin 6 (K6) was from Progen (Heidelberg, Delaware). Anti-keratin 10 (K10) was from Sanbio (San Diego, California). The mitogen and extracellular signal-regulated kinase (MEK1/2) inhibitor U0126 was from Promega (Madison, Wisconsin). The phosphoinositide 3-OH kinase (PI 3-K) inhibitor wortmannin, the EGFR kinase inhibitor AG1478, and negative control SB202474 were from Calbiochem (San Diego, California). The calcium channel blocker gadolinium (Gd^{3+}) was from Sigma (St Louis, Missouri).

Cell culture Normal human keratinocytes (NHK) were purchased from Clonetics (San Diego, California) and were cultured in keratinocyte-SFM (KBM) supplemented with epidermal growth factor and bovine pituitary extract (KGM, Life Technologies, Rockville, Maryland), and used at passage 3 to 5. Spontaneously immortalized human keratinocyte cells (HaCaT) were a generous gift from Dr. Kuroki (Showa University, Japan) with permission from Dr Fusenig (Institute Fur Zell- und Tumourbiologie, Deutsches Kresforschungszentrum, Heidelberg, Germany) and were maintained in minimal essential medium (MEM, Sigma) supplemented with 10% fetal bovine serum, and used at passage 41 to 50. Both types of cells were plated at 1×10^5 cells per cm^2 into flexible and transparent silicone chambers (Taiyo Kogyo, Tokyo, Japan) (Fig 1). Prior to plating, we covered the bottom of chambers with collagen type I (Sigma, 2.5 μg per cm^2) solution, and then dried them overnight. Subsequently, a thin collagen film was formed on the surface of the chamber, which enabled keratinocytes to attach the chambers. Chambers were cultured at 37°C and in a 5% CO_2 incubator. After reaching confluence, the cells were incubated in KBM or MEM for 24 h and then continuously stretched longitudinally by +20% on a stainless-steel stretching device (Towa Kagaku, Tokyo, Japan) (Fig 1). The stretching chambers was achieved by hand carefully, and then we laid them over the stretching device. It took 30 s to stretch and lay them. Finally, chambers and devices were cultured at 37°C and in a 5% CO_2 incubator.

Proliferation assay Confluent NHK or HaCaT cells were stretched continuously for 24 h as described above. For the last 3 h of the 24 h period of stretching, cells were pulsed with 100 μM BrdU. Subsequently, cells were scraped into PBS, and then transferred to 96-well plates and centrifuged. After drying the plates, the incorporation rate of BrdU was determined by Cell Proliferation ELISA with a BrdU colorimetric system (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, cells were fixed with ethanol for 20 min and then incubated with peroxidase-conjugated anti-BrdU antibody (1:100) for 60 min. After washing the cells three times, the reaction with tetramethylbenzidine as a substrate was performed for 15 min. The reaction was stopped by adding 1 N H_2SO_4. Absorbance at 450 nm was measured immediately using a microplate reader (Model 550, BIO-RAD, Hercules, California).

Immunoblotting and immunoprecipitation Cells were stretched for the indicated periods, scraped into lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 μg per mL leupeptin), sonicated, and microcentrifuged for 10 min at 4°C. Supernatants containing equal amounts of protein were boiled for 5 min at 95°C with 2 × SDS sample buffer, separated by SDS-PAGE, transferred onto PVDF membranes, and immunoblotted with anti-phospho-EGFR (1:1000), anti-phospho-ERK1/2 (1:1000), anti-ERK2 (1:500) or anti-EGFR (1:1000) antibody overnight at 4°C. The bound primary antibodies were detected using appropriate secondary antibodies conjugated with HRP (1:1000) for 60 min at room temperature and visualized using an ECL detection kit (Cell Signaling). The membranes were exposed to X-ray film, which was developed and visualized.

Immunoprecipitations were performed as described (Fujimoto *et al*, 2000). Briefly, cell lysates extracted above were incubated with anti-EGFR (1:100) antibody and protein A/G agarose for 3 h at 4°C. After washing, the pellets were mixed with 2 × SDS sample buffer and boiled. After centrifuging, the supernatants were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with HRP-conjugated anti-phosphotyrosine 4G10

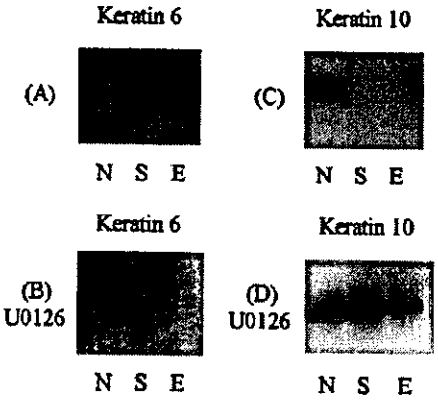

Figure 8
Mechanical stretching induces epidermal keratinocyte activation with keratin K6 induction and K10 suppression *in vitro*. NHK were stretched for 24 h, and then collected and assayed as described in *Materials and Methods*. (*A*, *C*) Keratin K6 induction and keratin K10 suppression by mechanical stretching and EGF stimulation were detected. (*B*, *D*) U0126 abolished these effects on keratin K6 induction and keratin K10 suppression (N, non-stretched NHK lysate; S, NHK stretched for 24 h; E, NHK stimulated with 50 ng per mL EGF for 24 h).

(1:10,000) antibody. All figures present representative results from three independent experiments, all of which yielded similar results.

DNA transfection and luciferase reporter assay The plasmid containing four activator protein 1 (AP-1) binding sites (TGAGTCAGTGAGTCACTGACTCACTGACTCATGAGTCAGCTGACTC) and the firefly luciferase reporter gene (Clonetech, Palo Alto, California), and a control plasmid containing the herpes simplex virus thymidine kinase (TK) promoter region and renilla luciferase reporter were co-transfected into confluent cells using the polybrene-DMSO shock method (Jiang *et al*, 1991). After transfection, NHK or HaCaT were incubated in medium with KGM or MEM with 10% FCS, respectively, for 24 h and then incubated without EGF or FCS for another 24 h, respectively. Then, cells were stretched for the indicated time. Using a Dual Luciferase Reporter Assay system (Promega), cells were collected and lysed, and then the dual intracellular luciferase activity was measured with a luminescencer-PSN (Atto, Tokyo, Japan).

Keratin extraction and immunoblotting After cells reached confluence, they were continuously incubated in KGM and then stretched for 24 h. Cells were collected into PBS with a cell scraper and centrifuged. The pellets were lysed using buffer A (25 mM Tris, 1.5 M KCl, 0.5% Triton X-100, 5 mM EDTA, 1 mM EGTA, 2 mM PMSF), and the keratin was extracted from insoluble pellets using buffer B (25 mM Tris, 9.5 M urea). These extracts were separated by SDS-PAGE, transferred onto PVDF membranes, and immunoblotted with anti-keratin K6 (1:200) and K10 (1:200) antibodies. The bound primary antibodies were detected using anti-mouse-IgG HRP conjugate (1:1000). The membranes were exposed to X-ray films, which were developed and visualized. All figures present representative results from three independent experiments, all of which yielded similar results.

Statistics Proliferation assays and luciferase reporter assays were performed in four independent experiments, which yielded highly comparable results, respectively. Data are presented as mean values ± SD as indicated in the results and Table I. Differences between mean values were analyzed with Student's *t* test, and $p<0.05$ was considered to be statistically significant.

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The −431C>T polymorphism of thymus and activation-regulated chemokine increases the promoter activity but is not associated with susceptibility to atopic dermatitis in Japanese patients

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Background: Thymus and activation-regulated chemokine (TARC) plays an important role in the pathogenesis of atopic dermatitis (AD). We recently detected the single nucleotide polymorphism (SNP) (−431C>T) in the 5′-flanking region of TARC gene.
Objectives: To examine whether the −431C>T SNP of the TARC gene is associated with susceptibility to AD and whether it affects the promoter activity of the TARC gene.
Methods: We investigated the genotype and allele frequencies of the SNP in 193 AD patients and 158 healthy controls by polymerase chain reaction-restriction fragment length polymorphism method. We compared the promoter activities between TARC promoter carrying 431C and that carrying −431T by transient-transfection assay in DJM-1 cell line.
Results: There were no significant differences in genotype or allele frequencies between AD patients and controls (genotype: $P=0.38$, allele: $P=0.22$). Luciferase activity was higher in −431T constructs than in −431C constructs (2.3-fold, $P=9.5\times10^{-6}$).
Conclusion: These results suggest that the −431C>T SNP of the TARC gene enhances the promoter activity of TARC gene but is not associated with susceptibility to AD in Japanese population.

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Key words: atopic dermatitis – gene – promoter – single nucleotide polymorphism – susceptibility – thymus and activation-regulated chemokine

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with elevated serum immunoglobulin E (IgE) levels and tissue and peripheral blood eosinophilia (1). AD is characterized by the predominant infiltration of Th2 cells and the increased secretion of Th2-type cytokines in the lesional skin (1,2).

Thymus and activation-regulated chemokine (TARC)/CC chemokine ligand (CCL) 17 (3,4) is a member of the CC chemokine group. It is a ligand of CC chemokine receptor (CCR) 4 (4–6), which is selectively expressed on Th2 cells (6,7) and serves for the recruitment and migration of cells bearing this receptor (5,6,8,9). TARC is highly implicated in the pathogenesis of allergic disorders, especially AD (10) and bronchial asthma (BA) (11,12). We have shown that TARC protein expression is upregulated in the airway epithelium of BA (11) and the lesional skin of NC/Nga mice,

regarded as a mouse model for human AD (13). The serum TARC levels of patients with AD were significantly elevated and correlated with disease activity, and immunoreactive TARC levels were detected in epidermal keratinocytes (KCs), dermal infiltrating cells, and endothelial cells in the acute and chronic lesional skin (10). These observations strongly suggest that KCs can be a source of TARC in the lesional skin of patients with AD and that KCs producing TARC may be involved the pathogenesis of AD.

Recently, we detected a single nucleotide polymorphism (SNP) in a C-to-T substitution at position −431 (counting from the start codon) (−431C>T) in the 5′-flanking region (accession number: DDBJ; AB088475 and HGV base; SNP001743394) (14), and demonstrated that individuals carrying the −431T allele (−431C/T and −431T/T genotypes) showed significantly increased levels of serum TARC concentration compared with those not carrying the −431T allele (−431C/C genotype), suggesting that the allele may have a dominant effect on TARC production (14).

The TARC gene is located at chromosome 16q13 (15,16), where total serum IgE concentration was reported to be linked (17). Thus, SNP of the TARC gene is a candidate as one of the genetic factors in AD.

In this study, we investigated the association between the −431C>T SNP of TARC gene and AD. Moreover, we attempted to determine the influence of the substitution of T for C at position −431 in the TARC gene 5′-flanking region by transient-transfection assay.

Materials and Methods

Analysis of genotype and allele frequencies in TARC gene SNP

We evaluated 193 unrelated Japanese patients with AD who were diagnosed according to the generally accepted criteria of Hanifin and Rajka (18). The patient group consisted of 137 male and 56 female subjects, aged 11 61 years (mean ± SD, 27.4 ± 7.7) with serum IgE levels in the range of 5 84000 U/ml [median (interquartile range): 7700 (1700 15300)] and peripheral blood eosinophil counts in the range of 0 2446/μl [421 (271 649)]. IgE levels and peripheral blood eosinophil counts were examined prior to therapies. One hundred and fifty-eight Japanese individuals served as control subjects: 89 male and 69 female subjects, aged 18 82 years (24.2 ± 3.12). There was no atopic person in the control group. Previously, we carried out a study similar to this one on AD and asthma (14), and there was no overlap of subjects between these two studies.

Venous blood was drawn from each individual and genomic DNA was extracted from peripheral blood leukocytes using a QIAamp blood kit (Qiagen, Hilden, Germany). Genotyping was carried out by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. We amplified a 5′-flanking region of the TARC gene including a −431C>T SNP by PCR using a set of specific primers; 5′-GGCAGATAAAGCATGGATCTC-3′ (sense: −631 to −611: counting from the start codon) and 5′-GAGAGCATCCTTCATGCATG-3′ (antisense: −391 to −372). PCR cycles consisted of an initial 5-min denaturation at 94°C and 40 cycles of 1-min denaturation at 94°C, 1-min annealing at 55°C, and 1-min extension at 72°C, followed by 7-min extension at 72°C. Genotype was determined by digestion with restriction endonuclease Sal I. The fragments of 199 and 61 bp represented the digested PCR products from −431C allele while PCR products from −431T allele were uncut and remained 260 bp. After the digestion reaction, samples were subjected to electrophoresis and genotypes were determined.

We compared the genotype and allele frequencies between AD patients and controls. Comparison was also made of the genotype and allele frequencies between AD patients with asthma and those without it, between those with allergic rhinitis and those without it, and between those with pollinosis and those without it. In addition, similar comparison was made between these patient subgroups and the control group. Information on the coexistence of asthma, allergic rhinitis, and pollinosis was available in 133, 141, and 71 patients, respectively. There were 55 with asthma of 133 AD patients, 76 with allergic rhinitis of 141, and 25 with pollinosis of 71.

Nucleotide sequence data reported are available in the GenBank database under the accession number AC004382.

All studies were approved by the ethics committee for genome research of the Faculty of Medicine, University of Tokyo. All patients and controls involved gave written informed consent for genetic studies.

Detection of TARC mRNA by reverse transcriptase polymerase chain reaction (RT-PCR)

We used a squamous cell carcinoma cell line (DJM-1) that had been derived from human skin (19). DJM-1 cells were cultured for 72 h in minimum essential medium eagle (MEM) (Sigma, St. Louis, MO, USA) with 10% fetal calf serum (FCS) in humidified 5% CO_2, 95% air at 37°C. Then, total RNA was extracted using a RNeasy Protect Mini kit (Qiagen). Each 2 μg of total RNA was reverse-transcribed with oligo (dT)*n* primer using a SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Transcripts of β-actin, a constitutively expressed gene, served as control. The sequences of the PCR primers were as follows: TARC sense, 5′-ATGGCCCCACTGAAGATGCT-3′, TARC antisense, 5′-TGGAGCAGTCCTCAGATGTCT-3′, and β-actin sense, 5′-AAGCTGTGCTACGTCGCCCT-3′, β-actin antisense, 5′-CAGGGCAGTGATCTCCTTCT-3′. The expected PCR products were 175 bp for TARC and 318 bp for β-actin. For cDNA amplification, PCR was performed. The PCR condition was the same as in PCR-RFLP. PCR products were stained with ethidium bromide and visualized by UV.

Transient-transfection assay

Construction of the reporter gene plasmid. To explore the regulatory effect of the −431C>T SNP on TARC gene expression, we utilized TARC 5′-flanking region/luciferase fusion gene constructs. The reporter gene plasmids were generated by cloning restriction fragments isolated from the 5′-flanking region of the TARC gene. The TARC 5′-flanking regions containing −431C or −431T in genomic DNA of patients with C/C genotype or T/T genotype, respectively, were amplified by PCR using the primers: 5′-GGCTGAGACGCTAGAGTCACA-3′ (sense: −1055 to −1053) and 5′-TGGGAGTAGGTGGG-GTGTAAA-3′ (antisense: +165 to +185). The region contains the putative promoter sequences predicted by PROSCAN version 1.7 (Bioinformatics & Molecular Analysis Section, NIH). The PCR condition was the same as in PCR-RFLP. The 1241 bp PCR products were separated by electrophoresis on agarose gel, subcloned into pUC118 *Hinc*II/BAP vector (Takara Bio Inc, Shiga, Japan) using

a TaKaRa BKL Kit (Blunting Kination Ligation Kit) (Takara Bio Inc). The inserts were prepared from each vector by *KpnI*/*Hind*III restriction digestion, and then cloned into the pGL3-Basic vector (Promega corporation, Madison, WI, USA) containing the firefly luciferase gene. To adjust the reading frame of TARC gene to that of luciferase gene, the region between the ATG start codon of TARC gene and the *Hind*III restriction site was deleted using PCR by TaKaRa Gene Analysis Center (Shiga, Japan). All DNA used in transfection were purified using a Magic Megapreps DNA purification system (Promega).

Cell cultures and transfection studies. DJM-1 cells were cultured in MEM with 10% FCS in humidified 5% CO_2, 95% air at 37°C. Cells were transfected at approximately 80% confluence in six-well plates. Transfection was executed using FuGENE 6 transfection reagent (Roche, Indianapolis, IN, USA). Cells were incubated in 2.0 ml of MEM containing 3 µl/ml of FuGene 6, 0.5 µg/ml of TARC promoter/luciferase fusion gene construct (−431C construct or −431T construct) prepared as described above or pGL3-Basic vector with no promoter, and 0.075 µg/ml of *Renilla* luciferase expression vector, pRL-TK (Promega), as an internal control. Cells were harvested at 12 h after transfection. Cell lysates were prepared and assayed for firefly and *Renilla* luciferase activities using the Dual Luciferase Reporter Assay System (Promega). The variability in transient-transfection efficiency was normalized with the internal control. Firefly luciferase activity, which indicated the promoter activity of the TARC gene, was divided by *Renilla* luciferase activity to calculate the relative luciferase activity and was expressed as a ratio to the value of the pGL3-Basic vector with no promoter (relative luciferase units). All the transfection experiments were performed at least three times. In brief, cells in each well were incubated with 500 µl of cell lysis buffer at 37°C for 15 min, frozen at −80°C and thawed, and centrifuged at 4°C at 20000 × *g* for 10 min. The supernatant containing cellular extract was collected, and the luciferase activities were determined with a luminometer (Luminescencer PSN, Atto, Tokyo, Japan).

Statistical analysis

Statistical significance was determined by Chi-square test for differences of genotype and allele frequencies. All the transfection experiments were repeated at least three times and differences were analyzed for statistical significance using the Student's *t*-test. We employed Bonferroni correction for multiple comparison. We used significance levels as follows: 0.025 (0.05/2) for analysis of genotype and allele frequencies between AD patients and control subjects, 0.016 (0.5/3) for analysis of coexistence of additional atopic disease, and 0.016 (0.05/3) for luciferase assay.

Results

The frequencies of genotypes and alleles at the −431C>T SNP site are summarized in Table 1. There were no significant differences in genotype or allele frequencies between AD patients and controls (genotype $P = 0.38$, allele $P = 0.22$). There was no significant difference in the genotype and allele frequencies between the patients subgroups: between AD patients with asthma and those without it, between those with allergic rhinitis and those without it, and between those with pollinosis and those without it. Nor were there significant differences in the genotype and allele frequencies between these patient subgroups and the control group (data not shown).

Table 1. Genotype and allele frequencies of the −431C>T single-nucleotide polymorphism of TARC gene in atopic dermatitis (AD) patients and controls

		AD ($n = 193$)	Control ($n = 158$)	P[2]
Genotype	C/C	61 (31.6)[1]	61 (38.6)	0.38
	C/T	106 (54.9)	79 (50.0)	
	T/T	26 (13.5)	18 (11.4)	
Allele	C	228 (59.1)	201 (63.6)	0.22
	T	158 (40.9)	115 (36.4)	

Comparisons of genotype and allele distribution showed no significant difference between AD patients and controls.
[1]The numbers in parentheses indicate the percentage.
[2]*P* values were calculated by Chi-square test.

RT-PCR showed that DJM-1 cells expressed mRNA for TARC (data not shown). Thus, we employed this cell line in the transient-transfection assay. The relative luciferase units in transient-transfection assay are shown in Fig. 1. The relative luciferase units of the −431C constructs and the −431T constructs were significantly higher than

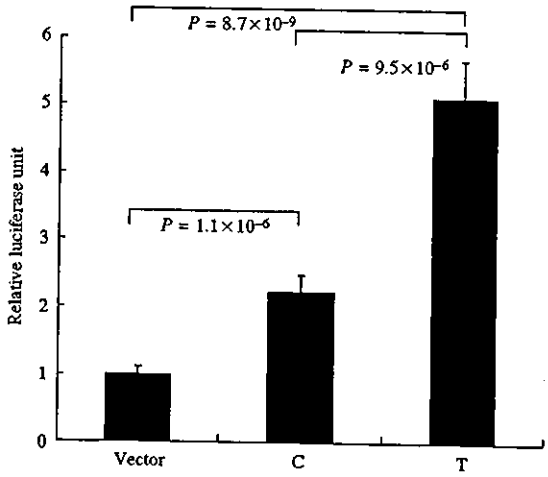

Figure 1. Functional analysis of −431C>T SNP on the promoter activity of thymus and activation-regulated chemokine (TARC) gene. TARC promoter region containing C or T at the position −431 was ligated into the pGL3-Basic promoterless plasmid. DJM-1 cells were transiently cotransfected with test plasmid and pRL-TK internal control plasmid and cultured for 12 h. Luciferase activity was measured by the dual-luciferase reporter assay system. Firefly luciferase activity, which indicated the promoter activity of the TARC gene, was divided by *Renilla* luciferase activity to calculate the relative luciferase activity, and was expressed as a ratio to the value of the pGL3-Basic vector with no promoter (relative luciferase unit). The relative luciferase units of the −431C constructs and the −431T constructs were significantly higher than those of pGL3-Basic vector with no promoter (2.2-fold, $P = 1.1 \times 10^{-6}$ and 5.1-fold, $P = 8.7 \times 10^{-9}$, respectively). In addition, the −431T constructs showed significantly higher luciferase activities than the −431C constructs (2.3-fold, $P = 9.5 \times 10^{-6}$). All values represent the means ± SD. This is a representative from three independent assays. Vector, C, and T indicate pGL3-Basic promoterless vector, −431C construct, and −431T construct, respectively.

those of pGL3-Basic vector with no promoter (2.2-fold, $P = 1.1 \times 10^{-6}$ and 5.1-fold, $P = 8.7 \times 10^{-9}$, respectively). In addition, the −431T constructs showed significantly higher luciferase activities than the −431C constructs (2.3-fold, $P = 9.5 \times 10^{-6}$).

Discussion

The TARC gene is located at chromosome 16q13 (15,16), where total serum IgE concentration was reported to be linked (17), and TARC is functionally important in the pathogenesis of AD (10,13). Thus, SNP of TARC gene is a candidate as a genetic factor in AD. However, between AD patients and controls, there were no significant differences in genotype or allele frequencies in the −431C>T SNP of the TARC gene.

We previously carried out a study similar to this one in AD and asthma (14) and found no association between this TARC SNP and susceptibility to AD or asthma. In this study, we employed another set of DNA samples from AD patients and again found no association. These data suggest that this SNP does not play a role in the pathogenesis of AD, at least in Japanese patients. But, there still remains the possibility that the differences could not be detected because the sample sizes in this study were small. Thus, it is necessary in future to re-examine the results in a greater number of samples. It would be of interest to perform the same study in other populations.

We then attempted to detect the influence of the substitution of T for C at the position −431 in the TARC gene 5′-flanking region by transient-transfection assay. The TARC 5′-flanking region-luciferase construct (−431C and −431T constructs) showed higher luciferase activities than pGL3-Basic vector with no promoter, which suggests that this region has promoter activity. The luciferase activity was more significantly increased in the −431T construct than in the −431C construct, demonstrating that the −431C>T SNP of TARC gene affects the promoter activity of the gene. These are consistent with our recent results that individuals carrying the −431T allele showed significantly increased levels of serum TARC concentration compared with those not carrying the −431T allele (14).

This SNP does not disrupt or create a known transcription factor binding site. Thus, there are two possibilities. One is that this SNP is located in an unknown transcription factor binding site and the other is that it causes a conformational change of DNA and influences the accessibility of transcription factor to DNA. This point needs to be clarified.

Judging from the data in association study and promoter assay, this SNP has an effect on promoter activity but is not associated with susceptibility to AD. This would be because many factors are involved in the pathogenesis in AD and the effect of this TARC SNP would be relatively small. A high concentration of serum TARC is reported in AD patients (10). This elevation of serum TARC would be induced by other cytokines which enhance the TARC production, but not by this TARC SNP in the promoter.

The results of this study, however, should be considered very preliminary because the number of AD patients was small. It is therefore necessary to confirm the results in a larger independent sample in future. The mechanism by which the promoter activity is increased in the promoter containing −431T is unknown. Further study is needed to clarify the mechanism.

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