Signaling of Mechanical Stretch in Human Keratinocytes via MAP Kinases

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Cells within human skin are permanently exposed to mechanical stretching. Here we present evidence that alterations in cell shape trigger biochemical signaling via MAP kinases in human keratinocytes. In an *in vitro* attempt we demonstrate a fast but transient activation of extracellular signal-regulated kinases 1/2 in response to cell stretch. This activation is reversed by preincubation with functional blocking antibodies directed towards β_1-integrins. As a second member of MAP kinases, stress-activated protein kinase/c-JUN NH_2-terminal kinase was activated in a slower fashion, peaking at 1 h after the initial stimulus. The delay in signal transmission suggests that extracellular signal-regulated kinases 1/2 and stress-activated protein kinase/c-JUN NH_2-terminal kinase do not share the same signaling pathway. p38 was not activated by cell stretching. The contribution of cytoskeletal elements in signal perception and transduction was evaluated by selective disruption of either actin filaments, microtubules, or keratin filaments but showed no clear effect on stretch-induced activation of extracellular signal-regulated kinases 1/2 and stress-activated protein kinase/c-JUN NH_2-terminal kinase. In conclusion we found evidence of a cell-shape-dependent activation of MAP kinases in human keratinocytes disclosing β_1-integrins as putative mechano-transducers. It is likely that alterations of skin mechanics *in vivo* underlying pathogenic processes like wound formation and healing trigger physiologic responses via the MAP kinase pathway.

Key words: MAPK/mechanical/signal transduction/stretch.

J Invest Dermatol 114:408–412, 2000

Mechanical forces applied to human skin are considered to stimulate proliferation and differentiation. By mimicking mechanical pressure in an *in vitro* approach with a keratinocyte line, overexpression of suprabasal keratins and other differentiation markers was found (Görmar *et al*, 1990, 1993). From these observations it follows that epithelial skin cells sense mechanical forces and transform them into differentiation signals. It is therefore assumed that alterations of cell geometry, as induced by stretching and compression, contribute to tissue-specific responses. The importance of cell shape in the control of growth and apoptosis was recently demonstrated in endothelial cells (Chen *et al*, 1997).

In contrast to mechanical pressure the application of stretch seems to trigger growth and proliferation. Stretching of skin as provoked by bending of articulations demands enlarged skin tissue to follow the movement. A more continuous stretching of the skin occurs during embryogenesis. Also in adults continuous stretching of skin occurs, the most prominent example being abdominal stretching during pregnancy. Another example of altered skin mechanics is given by wound formation and wound healing. The disintegration of skin enables the adjacent tissue to retract, leading to a stretch stimulus for the marginal cells next to the wound bed. In addition, these marginal cells are no longer inhibited in their cell shape by neighboring cells and start spreading into the wound bed, providing the basis for re-epithelization together with other mechanisms. Therefore, cell spreading directed towards a cell-free space is itself an example of mechano-sensation (Bereiter-Hahn and Vöth, 1993) and involves the development of mechanical forces by the cells themselves (Bereiter-Hahn and Lüers, 1994; Oliver *et al*, 1995).

In this *in vitro* study cultured keratinocytes were stretched and the activity of members of the MAPK family was followed. Extracellular signal-regulated kinases (ERK1/2) (Boulton *et al*, 1990, 1991) and the stress-activated protein kinase/c-JUN NH_2-terminal kinase (SAPK/JNK) (Kyriakis *et al*, 1994) were activated by a single stretch lasting 5 min, whereas p38/HOG1, which is the mammalian homolog of the osmosensitive *S. cerevisiae* HOG1 kinase (Han *et al*, 1994), did not respond. In addition β_1-integrins took part in mechano-dependent ERK1/2 activation. This further supports the assumption of integrins as mechano-transducers.

MATERIALS AND METHODS

Cell culture and cell treatment Spontaneously immortalized human keratinocyte cell line (HaCaT) (a generous gift from Professor Fusenig, German Cancer Research Institute, Heidelberg, Germany) was cultured in carbonate-buffered Hank's medium with 5% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a 5% CO_2 atmosphere. In addition, experiments were performed with normal human keratinocytes grown in serum-free keratinocyte medium (Gibco) (Stein *et al*, 1997). Twenty-four hours before stretching the medium was replaced for both cell types by

Manuscript received December 14, 1998; revised December 12, 1999; accepted for publication December 13, 1999.

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Abbreviations: ERK1/2, extracellular signal-regulated kinases 1/2; SAPK/JNK, stress-activated protein kinase/c-JUN NH_2-terminal kinase.

serum-free Hank's medium. Consecutively the cells became unidirectionally stretched by 10% for 5 min. After relaxation, protein samples were prepared at the indicated time intervals. Elements of the cytoskeleton became disrupted selectively by treatment with cytochalasin D (0.05, 0.1, 0.2 μg per ml, 2 h before stimulation) for actin fibril disintegration, by nocodazole (5, 10, 20 μg per ml, 5 h before stimulation) to disassemble microtubules (Maniotis *et al*, 1997), and by acrylamide (25, 50, 100 mM, 5 h before stimulation) to modify keratin filaments. Acrylamide induces reversible de-phosphorylation of cytokeratins together with reversible filament aggregation (Eckert and Yeagle, 1990). The effect of the above- mentioned substances on the cytoskeleton was proved by indirect immunofluorescence (data not shown). The functional β_1 blocking antibody was purchased from Gibco (nonpurified, clone P4C10) and applied as described earlier (Gniadecki *et al*, 1997). Briefly, the freeze-dried antibody was reconstituted in 100 μl Hank's medium and added to the culture at a 1:100 dilution according to the manufacturer's instructions. All experiments were done in agreement with the local ethic commission.

Stretching unit Silicone elastomer MED-4011 (Armando Medizin Technik, Düsseldorf, Germany) was mixed, filled into preformed Teflon matrices, and polymerized at 80°C for 3 h. For allowing cell attachment silicone dishes were treated with 5.7% KOH in methanol for 5 min in order to neutralize the polymerization-derived HCl. After washing with double-distilled water, silicone dishes were coated with 2% arginine for 2 h to facilitate keratinocyte attachment (Karasek, 1980) and afterwards rinsed with phosphate-buffered saline. Subsequently the dishes were incubated with fetal calf serum for 2 h. After withdrawal of the serum cells were plated on the silicone rubber. **Figure 1** shows an overall view of the stretching unit and also the extended and relaxed state of the silicone rubber culture dishes.

Proliferation assay HaCaT cells were seeded in silicone chambers (1×10^6 per 7 cm^2) and cultured in Hank's medium with fetal bovine serum. After 24 h cells were incubated in serum-free Hank's medium. After a further 24 h cells were stretched for 24 h. For the last 4 h cells were pulsed with 5-bromo-2′-deoxyuridine (BrdU). Subsequently, the incorporation rate of BrdU was determined using a commercial enzyme-linked immunosorbent assay kit (Roche). Briefly, cells were fixed with ethanol and then incubated with peroxidase-coupled BrdU antibodies (37°C, 80 min). After washing the cells a colorimetric reaction with tetramethylbenzidine as a substrate was performed (room temperature, 15 min, dark). The reaction was stopped by adding 1N H_2SO_4. A hundred microliters were transferred to a microtiter plate and measured at 450 nm in a scanning multiwell spectrophotometer (enzyme-linked immunosorbent assay (ELISA) reader).

Immunoprecipitation and immunoblotting Cells were scraped into lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged. Protein concentration of the supernatant was determined (Biorad DC Protein Assay Kit) and standardized using bovine serum albumin. Supernatants containing 200 μg total protein were immunoprecipitated with the appropriate antibodies at 4°C overnight in the presence of protein A-sepharose (Pharmacia). For sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, samples were mixed with SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue), boiled for 5 min and separated on SDS polyacrylamide gels. For immunoblotting, proteins in the gel were transferred to a PVDF membrane. The membrane was blocked in blocking buffer (Tris-buffered saline (pH 7.6), 0.1% Tween-20, 5% nonfat dry milk) for at least 3 h at 4°C followed by incubation with the primary antibody in Tris-buffered saline (pH 7.6), 0.05% Tween-20, and 5% bovine serum albumin. The bound primary antibodies were detected by using antimouse IgG-horseradish peroxidase conjugate and visualized by the ECL detection system (Amersham).

Kinase activity assays of MAP kinases The activity of ERK1/2, JNK, and p38 was determined by using p42/44 MAP Kinase Assay Kit, SAPK/JNK Assay kit, and p38 MAP Kinase Antibody Kit (New England Biolabs) (De Cesaris *et al*, 1998). Briefly, ERK1/2 was immunoprecipitated by a dual phospho-ERK monoclonal antibody. SAPK/JNK was pulled down by the addition of c-JUN fusion proteins. Precipitates were resuspended in 50 μl kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$). The kinase reaction was carried out in the presence of substrate proteins and 100 μM nonradioactive adenosine-5′-triphosphate (30°C, 30 min). The functional active form of ERK1/2 promotes the phosphorylation of Elk1 (Ser383); SAPK/JNK phosphorylates c-JUN (Ser63). The kinase reaction was stopped by the addition of SDS sample buffer. After protein gel electrophoresis and blotting, the membranes were incubated with antiphospho-Elk1 and antiphospho-c-JUN antibodies, respectively. Signals become visualized by chemoluminescence using horseradish-peroxidase-coupled secondary antibodies. The activity of p38 was determined using a phospho-specific p38 MAP kinase antibody detecting only p38 when activated by dual phosphorylation of Thr180/182. The pull-down of SAPK/JNK was controlled by re-probing the membranes with a SAPK/JNK antibody (New England Biolabs).

RESULTS

Stretch induces DNA synthesis In order to observe a proliferation-relevant effect of mechanical stretching, BrdU incorporation was detected by a BrdU ELISA. **Figure 2** shows the result of 12 independent experiments. Unstretched control cells were set as 100% (SD 25%), whereas a stretching regimen for 24 h caused an upregulation to 239% (SD 29%). These results show a highly statistical significance ($p < 0.0001$). Although cells were held for 24 h under serum-depleted conditions the self-stimulatory properties of HaCaT cells provide residual cell proliferation indicated by BrdU incorporation.

Activation of MAP kinases by changes of cell geometry Both HaCaT cells and normal human keratinocytes respond to mechanical strain by increased MAP kinase activities.

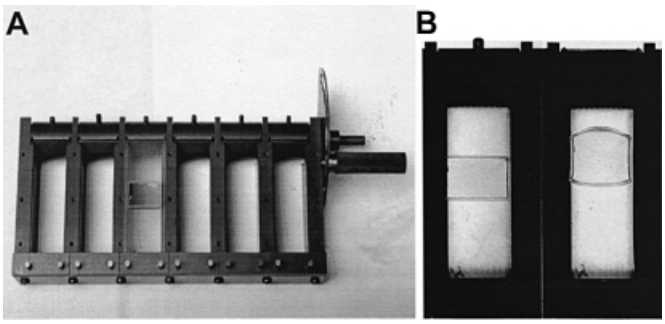

Figure 1. The stretching device. Cells are grown in the rectangular box on top of the silicone sheets. (*A*) An overall view of the stretching device. The silicone chambers can be extended by using the right-hand lever. (*B*) On the left the chamber shows the relaxed state; on the right the chamber is stretched to 10% of the initial length.

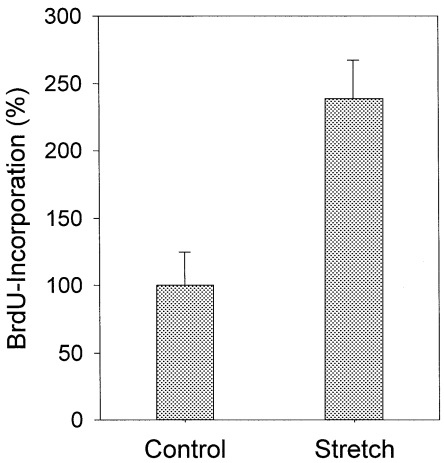

Figure 2. Mechanical stretch induces s-phases. Stretching of HaCaT cells for 24 h caused an upregulation of BrdU-positive cells to 239% as detected by a commercial BrdU ELISA. Each bar represents the mean of 12 independent experiments given as a percentage ± SD. The results show a high statistical significance ($p < 0.0001$).

Immediately after the application of a 5 min single stretch the ERK1/2 activity increased as shown by the phosphorylation of Elk 1 (**Fig 3**). Already 30 min after transient stimulation, the ERK1/2 base level was restored, underlining the transient manner of this activation. In contrast SAPK/JNK activation (detected by c-JUN phosphorylation) showed a slower increase reaching maximum activation 1 h after the stretch stimulus. p38 was not induced by mechanical stretch.

Involvement of cytoskeletal elements in the activation of MAP kinases Selective disruption of cytoskeletal elements was performed in order to test whether MAP kinase signaling is mediated via the cytoskeleton. **Figure 4** summarizes dose-dependent effects of cytochalasin D, nocodazole, and acrylamide on ERK1/2 and SAPK/JNK, respectively, without additional mechano-stimulation. Treatment with cytochalasin D showed a dose-dependent stimulation of ERK1/2, whereas only the highest concentration (0.2 μg per ml) had significant effects on SAPK/JNK activity (**Figs 4*A*, *B***). Incubation with nocodazole caused activation of ERK1/2, peaking around 10 μg per ml. At higher concentrations (20 μg per ml), ERK1/2 activity declined, which may be due to cytotoxic effects (**Fig 4*C***). SAPK/JNK activity was stimulated by nocodazole in a dose-dependent manner (**Fig 4*D***). Acrylamide, which causes de-phosphorylation of cytokeratins, showed no effect on ERK1/2 at 25 and 50 mM. At 100 mM acrylamide ERK1/2 activity decreased (**Fig 4*E***). Low concentrations of acrylamide (25 mM) caused massive SAPK/JNK activation. This stimulation declined with higher concentrations (50 and 100 mM) of acrylamide (**Fig 4*F***). The contribution of the cytoskeleton in mechano-signaling was performed at maximum MAP kinase activation in response to stretch. For the determination of ERK1/2 activation, proteins were extracted immediately after the 5 min stretch stimulus, whereas functional activity of SAPK/JNK was determined 1 h after the stretch stimulus (**Fig 5**). ERK1/2 activation in response to stretch was not altered by treatment with cytochalasin D and acrylamide. Treatment with nocodazole showed stronger activation of ERK1/2 than the stretch-induced ERK1/2 stimulation alone. This effect may be due to an overlapping of the basal ERK1/2 activation induced by nocodazole (see **Fig 4C**). In contrast, the SAPK/JNK pathway

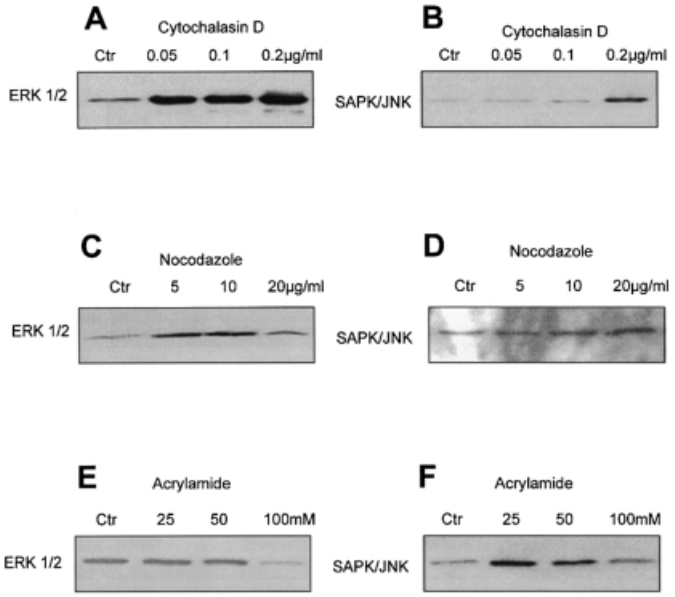

Figure 4. Effect of cytoskeleton-modifying drugs on ERK1/2 and SAPK/JNK activation in HaCaT cells. (*A*) Cells were incubated for 2 h with 0.05, 0.1, and 0.2 μg cytochalasin D per ml and tested for ERK1/2 activity and (*B*) SAPK/JNK activity. (*C*) Cells were incubated for 5 h with 5, 10, and 20 μg nocodazole per ml and tested for ERK1/2 activity and (*D*) SAPK/JNK activity. (*E*) Cells were incubated for 5 h with 25, 50, and 100 mM acrylamide and tested for ERK1/2 activity and (*F*) SAPK/JNK activity. The blot shows representative results (n = 3).

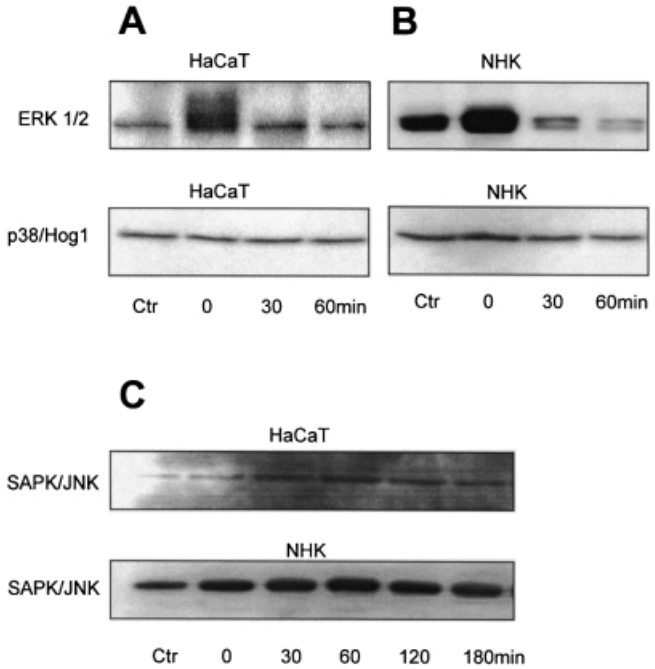

Figure 3. Mechanical stretch activates MAP kinase ERK1/2 and SAPK/JNK. Time course of MAP kinase activation after a single 5 min stretch in HaCaT cell and normal human keratinocytes. (*A*) Activity of ERK1/2, represented by *in vitro* phosphorylation of Elk 1, peaks immediately after stimulation. The basal activity is restored already after 30 min. (*B*) p38 activation observed by a phospho specific p38 antibody (Thr180/182) shows no activity changes. (*C*) SAPK/JNK activity determined by *in vitro* phosphorylation of c-JUN during a time of 180 min. Maximum induction is at 60 min, with a moderate decline of activity. The blot shows representative results (n = 3).

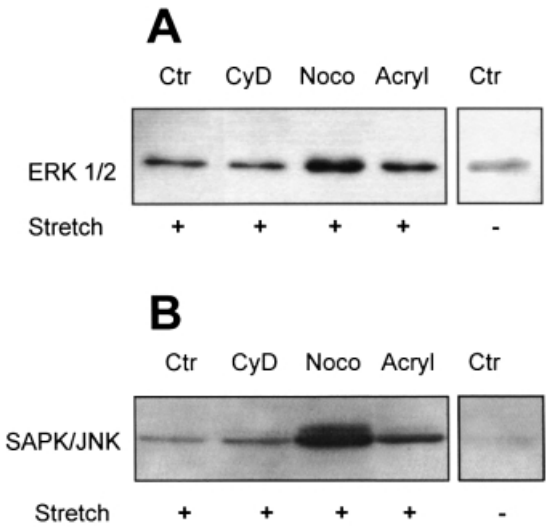

Figure 5. Stretch-induced effects on ERK1/2 and SAPK/JNK activity in HaCaT cells after selective disruption of cytoskeletal elements. The controls are derived from stretched cells without any cytoskeleton manipulation at maximum activation in response to stretch. (*A*) Cytochalasin D (0.1 μg per ml) and acrylamide (50 mM) treatment shows no change of stretch-induced ERK1/2 activation. Nocodazole treatment (10 μg per ml) shows strong ERK1/2 activation (see also **Fig 4*c***). (*B*) No alterations of SAPK/JNK activation in response to stretch are observed on treatment with cytochalasin D and acrylamide. Nocodazole causes SAPK/JNK activity upregulation (see also **Fig 4*d***) (n = 3).

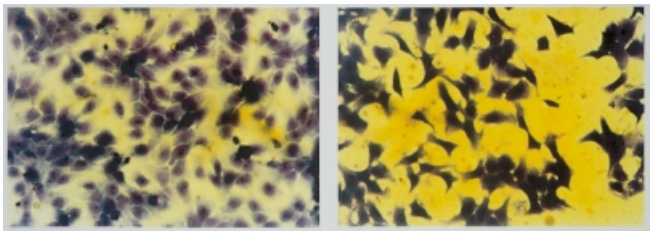

Figure 6. Effects of blocking β_1 antibodies on cell morphology of HaCaT cells grown on silicone dishes. Cells were stained with methylene blue in order to enhance the contrast to the opaque cell support. The left half shows cells in the control state. On the right half the effect of incubation with β_1-integrin antibodies is shown. Colonies become dissociated and cell-cell contacts are diminished.

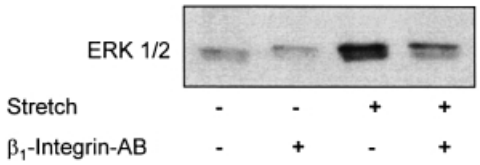

Figure 7. Blocking of β_1-integrins attenuates stretch-induced ERK1/2 activation. HaCaT cells were treated with functional blocking antibody directed towards β_1-integrin subunits for 24 h. The characteristic activation of ERK1/2 induced by a single 5 min stretch was attenuated in cultures treated with β_1-integrin antibodies (n = 3).

was activated by microtubule disintegration but was not further enhanced by cell stretch. The response of SAPK/JNK to mechanical stimulation was insensitive to cytochalasin D and acrylamide treatment.

β_1-integrins mediate stretch-induced ERK1/2 activation
HaCaT cells were incubated for 24 h with blocking antibodies directed towards β_1-integrin. Blocking of β_1-integrin prevented cell-cell contacts, resulting in a characteristic dissociation of the colonies as observed by light microscopy (**Fig 6**). It has been suggested that integrins serve as mechano-transducers (Ingber, 1991). We tested whether functional blocking of β_1-integrin reverses the stretch signaling to MAP kinases and could demonstrate that a lack of β_1-integrin-mediated cell adhesion attenuated the stretch-induced ERK1/2 activation (**Fig 7**).

DISCUSSION

Human skin keratinocytes are highly sensitive towards mechanical stretching and respond with an increased DNA synthesis. These findings support the recent assumption that mechanical stretch supports the proliferative properties of cells (Chen *et al*, 1997; Takei *et al*, 1997). The biologic significance of these *in vitro* findings for the *in vivo* conditions can be found within tissue architecture: the space for a certain cell within a tissue is limited by the surrounding cells. Cells are able to sense their shape and downregulate their proliferation when closely packed. *In vivo* cell stretch can occur during growth or hypertrophy of adjacent tissues, e.g., volume expansion of muscles needs necessarily an enlarged skin surface to cover. Cell stretch can also be induced by disturbance of tissue integrity. Wound formation and wound closure is associated with changes in tissue mechanics, which might trigger the process of tissue repair. In the present *in vitro* attempt, human skin keratinocytes respond to a single mechanical stretch by a rapid and transient activation of ERK1/2, followed by a long-lasting activation of SAPK/JNK. The lack of p38 reaction was not expected because most *in vitro* stresses activate SAPK/JNK and p38 in parallel. A similar separation of the pathways for p38 and SAPK/JNK activation has been described for rat cardiac fibroblasts (MacKenna *et al*, 1998). The activation of ERK1/2 by mechanical stresses seems to be a general response in different cell systems. Stretching of cardiac myocytes and also fluid shear stress in bovine aortic endothelial cells promote the activation of the ERK pathway (Tseng *et al*, 1995; Yamazaki *et al*, 1996). Generally it is assumed that the ERK1/2 cascade promotes proliferation. Recent findings of higher ERK1/2 sensitivity in psoriatic fibroblasts suggest its contribution to the pathogenesis of hyperproliferative diseases (Dimon-Gadal *et al*, 1998). Therefore the increase of s-phases in stretched cells may be due to the ERK1/2 activation. More rarely, mechanical stresses have been found to be associated with a stimulation of SAPK/JNK. Aronson *et al* (1997) found a stimulation of SAPK/JNK in rat muscle cells mediated by muscle contraction. Findings that demonstrate SAPK/JNK activity to be essential in embyogenic morphogenesis support the assumption of its relevance in tissue organization (Sluss *et al*, 1996; Sluss and Davis, 1997).

Structures that transform the mechano-signal into a biologic response are still a matter of debate. There are several lines of evidence that suggest that surface receptors of the integrin family function as mechano-transducers (Ingber, 1991, 1997; Shyy and Chien, 1997). According to this concept integrin β_1 subunits transduce the mechano-signal to the focal adhesion kinase. Focal adhesion kinase autophosphorylation induces binding to Grb2/Sos, which might give rise to consecutive Ras activation (Schlaepfer *et al*, 1994). Ras action is related to a bifurcation of pathways that could lead either to activation of ERK1/2 and SAPK/JNK (for a review see Shyy and Chien, 1997) or, alternatively, to a Ras-independent activation of MAP kinases (Buscher *et al*, 1995; Chen *et al*, 1996). Our findings of stretch-induced MAP kinase activation support the assumption of an integrin-dependent mechano-sensation as blocking of β_1-integrins abrogates the stretch-induced ERK1/2 activation. We therefore assume that stretch is perceived by β_1-integrins and signaled to ERK1/2 independently from intact actin filaments. Disintegration of microtubules by treatment with nocodazole caused an upregulation of ERK1/2 activity peaking around 10 μg per ml. At higher concentrations (20 μg per ml) the ERK1/2 activity declines, indicating cytotoxic effects. The stretch-mediated activation of ERK1/2 could not be differentiated from that of nocodazole. Similarly, SAPK/JNK is activated by nocodazole even without stretch, which could not be separated from stretch-induced SAPK/JNK activation. These findings offer a functional association between MAP kinases and elements of the cytoskeleton. Nagata *et al* (1998) found SAPK/JNK localized along microtubules, which is in agreement with our finding of nocodazole-sensitive SAPK/JNK activity. Klemke *et al* (1997) presented data suggesting that ERK1/2 is involved in cell motility via the actin-myosin cytoskeleton. The high responses of ERK1/2 towards treatment with cytochalasin D support this concept.

In conclusion our findings of stretch-induced MAP kinase activation and the relevance of β_1-integrins as putative mechano-transducers contribute to the molecular understanding of mechano-signaling. It is likely that mechanical stimuli underlying events such as skin growth, wounding, and healing may trigger the various physiologic responses.

We are grateful to Dr. A. Sewell for comments and discussion. We wish to thank Dr. A. Borsche for providing the skin samples (Department of Plastic and Reconstructive Surgery, Diakonie Hospital, Bad Kreuznach, Germany). This work was supported by Deutsche Forschungsgemeinschaft (SFB 474 project B4).

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