Keratinocytes Promote Proliferation and Inhibit Apoptosis of the Underlying Fibroblasts: An Important Role in the Pathogenesis of Keloid

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Interactions between epidermal keratinocytes and dermal fibroblasts play an important role in regulating tissue homeostasis and repair. Nevertheless, little is known about the role of keratinocytes in the pathogenesis of keloid. In this study, we investigated the influence of normal skin- and keloid-derived keratinocytes on normal skin- and keloid-derived fibroblasts utilizing a serum-free indirect coculture system. The keloid-derived fibroblasts showed a greater proliferation and minimal apoptosis when cocultured with normal skin- or keloid-derived keratinocytes, and the results were most significant in the latter. This difference was not observed when the fibroblasts were treated with conditioned medium obtained from normal skin- and keloid-derived keratinocytes. Nevertheless, conditioned medium-treated groups showed more proliferation and less apoptosis compared to the nonconditioned medium-treated control groups. We also analyzed the profile of factors involved in cell growth and apoptosis in fibroblasts cocultured with keratinocytes. Extracellular signal-regulated kinase and c-Jun N-terminal kinase phosphorylations and expression of Bcl-2 and transforming growth factor-β1 were all significantly upregulated in the fibroblasts cocultured with keloid-derived keratinocytes. Together, these results strongly suggest that the overlying keratinocytes of the keloid lesion play an important role in keloidogenesis by promoting more proliferation and less apoptosis in the underlying fibroblasts through paracrine and double paracrine effects. ***Key words: interaction/coculture. J Invest Dermatol 121:1326–1331, 2003***

A keloid is often addressed as a benign dermal tumor as it spreads to invade normal skin beyond the boundaries of the original wound and does not regress spontaneously. The pathophysiologic events leading to keloid formation are not yet fully understood. Previously, we showed that a dysregulation in the Fas-mediated apoptosis may be an important mechanism by which keloids arise and that transforming growth factor-β1 (TGF-β1) is an important factor responsible for this resistance to apoptosis observed in keloid-derived fibroblasts (KF; Chodon *et al*, 2000). Most of the studies on keloids have been mainly focused on fibroblasts and little is known about the role of the overlying epidermal keratinocytes. Epidermal homeostasis, growth, and differentiation are controlled by epidermal–mesenchymal interactions (Barker *et al*, 1991; Tuan *et al*, 1994; Boxman *et al*, 1996; Maas-Szabowski *et al*, 1999). Likewise, mesenchymal homeostasis, growth, and differentiation may be controlled by this interaction. It has been reported in the embryologic field that the epithelium regulates the underlying mesenchymal cell apoptosis (Hurle and Ganan, 1986). These indicate a possibility of the epidermal participation in keloid formation. In response to various injurious stimuli, epidermal keratinocytes may release a number of regulatory molecules that can interact with receptors on peripheral cells (McKay and Leigh, 1991; Boyce, 1994). Cytokines from keratinocytes may have regulatory roles in the proliferation and apoptosis of the underlying fibroblasts. Furthermore, KF are also reported to exhibit altered growth responses to various growth factors (Kikuchi *et al*, 1995; Babu *et al*, 1992; Haisa *et al*, 1994).

We designed this study with the hypothesis that abnormalities in epidermal–mesenchymal interaction may have an important role in keloidogenesis, and consistent with this, we show that keloid formation is not an isolated dermal disease and that epidermal–mesenchymal interactions do play a major role in disrupting the balance between fibroblast proliferation and apoptosis leading to the formation of keloids.

MATERIALS AND METHODS

Subjects A total of seven keloid samples were obtained from seven Japanese patients (three women and four men, mean age 29.0 years; range 12–59 years). Keloids were excised from the ear lobe in four patients, the pubic region in two patients, and the shoulder in one patient (mean duration 25 mo; range 8–35 mo). Only typical, clinically clear-cut cases that extend beyond the original boundary of the wound were included in this study. None of the patients had received previous treatment other than pressure therapy. Seven age- and site-matched normal skin specimens (four

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; KCM, conditioned medium from keloid-derived keratinocytes; KF, keloid-derived fibroblast; KK, keloid-derived keratinocyte; MAPK, mitogen-activated protein kinase; NCM, conditioned medium from normal skin-derived keratinocytes; NF, normal skin-derived fibroblast; NK, normal skin-derived keratinocyte; TGF-β1, transforming growth factor-β1.

women and three men, mean age 29.9 years; range 14–54 years) were obtained at the time of other unrelated operations. Informed consent was obtained from all the patients. All procedures were approved by the ethical codes of Hokkaido University School of Medicine in adherence to the Helsinki Principles.

Fibroblast and keratinocyte culture Fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc., Gaithersburg, MD) supplemented with 20% and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) for primary culture and subsequent cultures, respectively, and 5 mg per mL L-glutamine in an atmosphere of 5% CO_2. Fibroblasts in the primary cultures were trypsinized with 0.05% trypsin/0.53 mM EDTA·4Na (Life Technologies, Inc., Carlsbad, CA) and passaged. All experiments were performed with third-passage cells. Primary culture keratinocytes derived from five specimens each of keloid and normal skin were utilized in this study. Split-thickness specimens were rinsed in sterile phosphate-buffered saline and incubated in Dispase II (Roche Molecular Biochemicals, Switzerland) at 37°C for 30 to 40 min depending on the thickness of the specimen. Then, after separation of the epidermis from the dermis, it was incubated in 0.25% trypsin (Life Technologies, Inc.) at 37°C for 30 min followed by neutralization with trypsin neutralizing solution (BioWhittaker, Inc., Walkersville, MD). The obtained epidermis was suspended in DMEM supplemented with 10% FBS and was continuously stirred with a magnetic stirrer for 1 h. Cells were then passed through a 40-μm mesh, collected by centrifugation at $100 \times g$ for 10 min, and cultured in the keratinocyte medium containing Ham's F12 and DMEM (1:3) supplemented with 1.8×10^{-4} mol per L adenine, 10^{-10} mol per L cholera toxin, 10 ng per mL EGF, 0.4 μg per mL hydrocortisone, 5% FBS, and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizome) (Freshney, 1994). Keratinocytes (1×10^4 cells/cm^2) were plated on feeder cells lethally treated with mitomycin C. Instead of murine 3T3 cells, we used normal human skin-derived fibroblasts as the feeder layer (Freshney, 1994).

Coculture of fibroblasts with keratinocytes Six-well plates with inserts (Becton Dickinson & Co., Franklin Lakes, NJ) were used so that there was an upper chamber for the keratinocytes and a lower chamber for the fibroblasts separated by a permeable membrane (pore size 0.4 μm). Keratinocytes were plated over the feeder layer as above and grown until confluency (monolayer). Fibroblasts (1×10^5 cells per well) were plated in six-well plates in DMEM supplemented with 10% FBS and 5 mg/mL L-glutamine. After 48 h, the medium was changed to serum-free medium and incubated for 24 h before being cocultured with the keratinocytes. This coculture system was maintained for 4 d in serum-free medium and then analyzed for fibroblast proliferation and apoptosis. To establish the least possible presence of feeder fibroblasts in the top chamber at the time of transwell coculture, feeder fibroblasts were plated at a low concentration (5×10^3 cells/cm^2) and used for keratinocyte culture only after over 3 wk of incubation alone. It was also checked by the cloning assay technique.

Fibroblast proliferation assay and detection of apoptosis Fibroblasts, after being cocultured with/without keratinocytes for 4 d, were detached with 0.05% trypsin/0.53 mM EDTA·4Na, collected by centrifugation, resuspended, and counted in a Neubauer hemocytometer (Kayagaki Irikha Kougyou Co. Ltd, Japan). For the assessment of nuclear morphology, cells in the bottom chamber were stained with 5 μg per mL Hoechst 33342 dye (Sigma Chemical Co., St. Louis, MO) without trypsinizing. Then 20 to 25 randomly selected fields per well were counted for chromatin condensation or fragmentation at ×200 magnification, using an inverted fluorescence microscope (Olympus, IX-70, Japan).

Measurement of caspase-3 activity Caspase activity assay system (caspase-3/CP32 colorimetric assay kit, BioVision, Inc., Mountain View, CA) was used to determine the enzymatic activity of caspase-3 in the apoptotic cells by colorimetric reaction. Briefly, fibroblasts cocultured with/without keratinocytes for 4 d were collected by centrifugation at $500 \times g$ for 5 min. The supernatant was gently removed, and the cell pellet was lyzed by adding cell lysis buffer. After incubation on ice for 10 min, the cell lysate was centrifuged at $10{,}000 \times g$ for 1 min and the supernatant was transferred to a fresh tube and put on ice. The protein content was measured by Bradford assay using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA). A total of 100 μg of protein was loaded in each individual tube and the volume was adjusted to 50 μL with cell lysis buffer. Fifty microliters of 2× reaction buffer containing 10 mM dithiothreitol was added to each sample. The reaction tube was incubated at 37°C for 90 min after adding 5 μL of caspase-3 colorimetric substrate. Optical density in each tube was determined by a spectrophotometer using a 100-μL cuvet at a wavelength of 405 nm. Background reading from cell lysates was subtracted from the readings of each sample.

Western blot analysis Equal numbers of cells were lyzed with a buffer containing 1% Nonidet P-40, 0.1% sodium deoxycholate, 150 mmol per L NaCl, 50 mmol per L Tris-HCl, pH 7.5, 1 mmol per L phenylmethylsulfonyl fluoride, 0.2 U per mL aprotinin, 10 mmol per L $Na_4P_2O_7$, 10 mmol per L NaF, 4 mmol per L ethylenediaminetetraacetic acid, and 2 mmol per L Na_3VO_4. The protein content was measured by Bradford assay using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA). Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 5% nonfat milk and overnight incubation at 4°C in the presence of anti-human Fas monoclonal antibody (clone CH-11; 1:100; Medical & Biological Laboratories Co., Ltd, Japan), anti-Fas ligand (Fas L) antibody (clone 33; 1:5000; Transduction Laboratories, Lexington, KY), anti-Bcl-2 monoclonal antibody (clone Bcl-2/100; 1:2000; PharMingen, San Diego, CA), anti-phospho-extracellular signal-regulated kinase (ERK) antibody (specific for the phosphorylated forms of ERK1 and ERK2 activated by dual phosphorylation at Thr/Glu/Tyr region; 1:5000; Promega, Madison, WI), anti-phospho-c-Jun N-terminal kinase (JNK) antibody (specific for the phosphorylated forms of JNK1 and JNK2 activated by phosphorylation at Thr/Pro/Tyr region; 1:5000; Promega), anti-phospho-p38 antibody (specific for the phosphorylated form of p38 activated by phosphorylation at Thr/Gly/Tyr region; 1:2000; Promega), or anti-TGF-β1 polyclonal antibody (1:2000; Promega), the membrane was further processed using horseradish peroxidase-conjugated secondary antibody. Proteins were detected by enhanced chemiluminescence system (Amersham, Arlington Heights, IL). Proteins were identified using Jurkat cell lysate for Fas and Bcl-2, human endothelial cell lysate for FasL, and recombinant TGF-β1 (Sigma Chemical Co.) as standards, respectively.

Treatment with conditioned medium Keratinocytes were plated on the feeder layer in 6-cm dishes at a concentration of 1000 cells per cm^2 and cultured in keratinocyte medium. When confluent, after being washed three times with phosphate-buffed saline, the medium was replaced with serum-free DMEM. Then, this medium was collected after 24 h of culture and used as the conditioned medium. KF were plated in six-well plates, incubated for 48 h followed by starvation for 24 h, and then stimulated with the freshly collected conditioned medium for 4 d for the proliferation assay and apoptosis detection as above, for 5 min and 4 d, respectively, for ERK/JNK and Bcl-2 detection by immunoblotting. Cells were lyzed and subjected to western blot analyses for the above-mentioned proteins.

Northern blot analysis for TGF-β1 mRNA The probe of human TGF-β1 cDNA for northern blot analysis was obtained by RT-PCR from a human cDNA library of keloid-derived human dermal fibroblasts. The sense primer (5′-CACGTGGAGCTGTACCAGAA-3′) and antisense primer (5′-TGCAGTGTGTTATCCCTGCT-3′) were designed by an online primer design program (primer3_www.cgi v0.2). The 199-mer PCR product was separated by agarose gel purification and purified by GenePure (Nippon Gene, Japan). After checking the sequence by a sequencing analyzer (377 A, Applied Biosystems Inc., Foster City, CA), the PCR product was used as a probe for northern blot analysis. Total cellular RNA was isolated from fibroblasts cocultured for 24 h using an Isogen RNA extraction kit (Nippon Gene) according to the manufacturer's protocol. RNA was quantitated by spectrophotometry, and equal amounts of RNA (10 μg) were loaded on a formaldehyde-agarose gel. The gel was stained with ethidium bromide to visualize RNA standards, and the RNA was transferred onto a nylon membrane. TGF-β1 cDNA probe was labeled with [α-32P]dCTP using a DNA random primer labeling kit (Takara, Japan), and hybridization was carried out at 42°C for 24 h. Posthybridization washes were performed in 0.1% SDS, 0.2× SSC (1× SSC = 0.15 mol/L NaCl, 0.015 mol/L sodium citrate) at 65°C. The radioactive bands were visualized by autoradiography on Kodak X-AR5 film.

Statistical analysis The data were analyzed using ANOVA followed by Scheffe's *post hoc* analysis.

RESULTS

KF cocultured with keloid-derived keratinocytes (KK) proliferate more than that with normal skin-derived keratinocytes (NK) KF derived from keloid lesions or NF

from normal skin by explant method were cocultured with keratinocytes derived from keloid or normal skin after starvation for 24 h. The proliferation assay carried out after 4 d of coculture showed that NF proliferate more when cocultured with NK or KK compared to culture with DMEM alone control group. In the case of KF, there was significantly more proliferation when they were cocultured with KK in comparison to coculture with NK or the DMEM alone control group (**Fig 1*a***). When we carried out this experiment by treating the fibroblasts with conditioned medium from normal skin-derived or keloid-derived keratinocytes (NCM, KCM) instead of coculturing with KK or NK, KF proliferated more in the presence of conditioned medium compared to the DMEM-alone control group. Nevertheless, no difference was observed between the treatments with KCM and NCM (**Fig 1*b***).

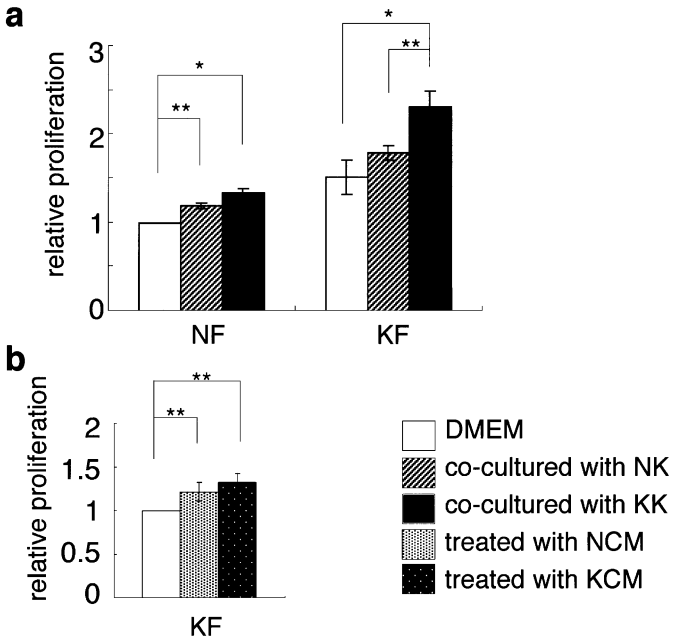

Figure 1. Proliferation assay of fibroblasts after coculture with NK or KK and treatment with KCM or NCM. (*a*) Proliferation assay was carried out after 4 d of coculture or (*b*) treatment with conditioned medium in serum-free medium as described. $^{*}p<0.01$; $^{**}p<0.05$. (ANOVA with Scheffe's *post hoc* tests). Data shown are the mean ± SD of nine independent experiments.

KK make KF more resistant to apoptosis than NK As mentioned above, KF were cocultured with NK and KK. To induce apoptotic stress, cell starvation was maintained for 4 d and then the fibroblasts were stained with the fluorescent chromatin dye Hoechst 33342 to assess nuclear morphology. We found significantly fewer apoptotic cells in the groups cocultured with KK and NK (**Fig 2*a, c, e***). Furthermore, the apoptotic resistance was significantly higher in KF cocultured with KK than with NK. The percentage of apoptotic cells in KF was 20.6% in case of DMEM group whereas it decreased to 10.9 and 4.0% when cocultured with NK and KK, respectively (**Fig 3*a***). A previous study showed a significant increase in caspase-3 proteolytic activity after serum deprivation in KF (Akasaka *et al*, 2000). Here, to confirm the resistance of KF cocultured with KK, we determined the level of active caspase-3. Consistent with the result above, we observed that the least elevated active caspase-3 level was when KF were cocultured with KK (**Fig 3*b***). In case of NF, both NK and KK made NF more resistant to serum-deprivation-induced apoptosis. Nevertheless, there was no significant difference between coculture with NK and KK. We carried out this experiment by treating KF with KCM and NCM instead of coculturing. KF showed a significantly higher resistance in the presence of conditioned medium but no difference was observed between treatment with KCM and NCM (**Fig 3*c***).

KK upregulate the phosphorylation of ERK and JNK in KF ERK cascade is a convergent pathway in the mitogenic action of many growth factors and ERK phosphorylation has antiapoptotic effects. To explore the mechanism of the proliferative and antiapoptotic action of keratinocytes on fibroblasts, we analyzed the expression of phosphorylated ERK in fibroblasts cocultured with NK or KK by immunoblotting using anti-active(phospho)ERK. In KF, the strongest phosphorylated band of ERK was observed when cocultured with KK;

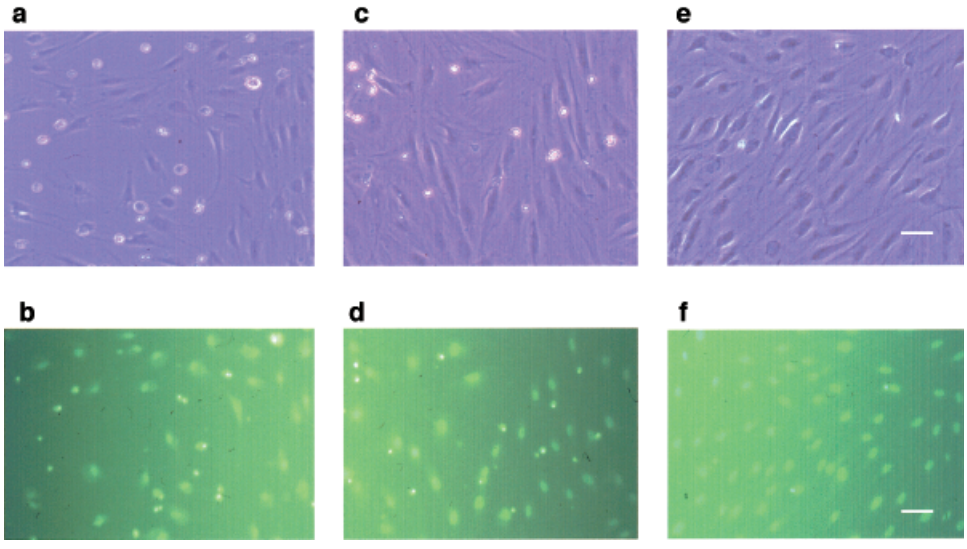

Figure 2. Cell morphology of KF after culturing in DMEM alone or coculturing with NK or KK. KF were grown in DMEM and 10% fetal bovine serum and, after 48 h, starved in serum-free medium for 24 h before being cocultured in double chamber plates with keratinocytes (NK or KK) prepared as described. This coculture system was maintained for 4 d in serum-free medium. (*a,b*) KF grown in DMEM alone without coculture. (*c,d*) KF cocultured with NK. (*e,f*) KF cocultured with KK. Cellular morphologic changes of apoptosis analyzed by phase contrast microscopy (*a,c,e*). The same fields as that of (*a,c,e*) examined by Hoechst 33342 staining (*b,d,f*). Note few apoptotic cells when cocultured with KK (*e,f*) compared to when cocultured with NK (*c,d*) and without coculture (*a,b*). *Bar*, 20 μm.

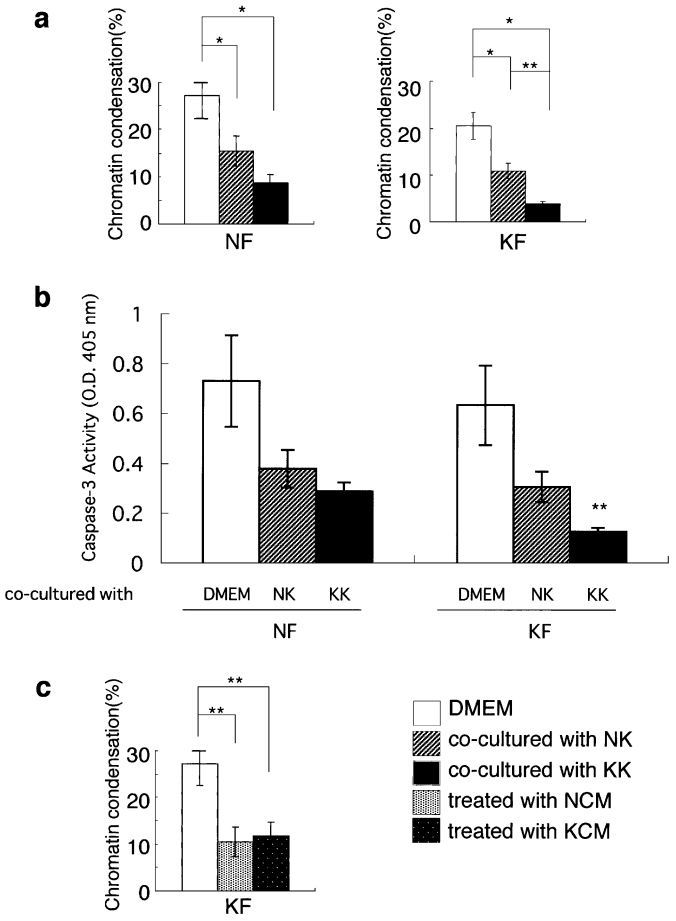

Figure 3. Chromatin condensation (%) of fibroblasts after coculture with NK or KK and treatment with NCM or KCM. Active caspase-3 levels in fibroblasts cocultured with NK or KK. (*a*) Percentages of chromatin condensation of fibroblasts were assessed by Hoechst staining after coculturing with keratinocytes (NK, KK) or (*c*) treatment with conditioned medium (NCM, KCM) for 4 d in serum-free medium. Data shown are the mean ± SD of (*a*) nine independent experiments or (*c*) five independent experiments. *$p < 0.01$; **$p < 0.05$. (ANOVA with Scheffe's *post hoc* tests). (*b*) Active caspase-3 levels in fibroblasts cocultured with NK or KK using caspase activity assay system as described under Materials and Methods. Data shown are the mean ± SD of three independent experiments. **$p < 0.05$ compared to coculture with NK and noncoculture control groups (ANOVA with Scheffe's *post hoc* tests).

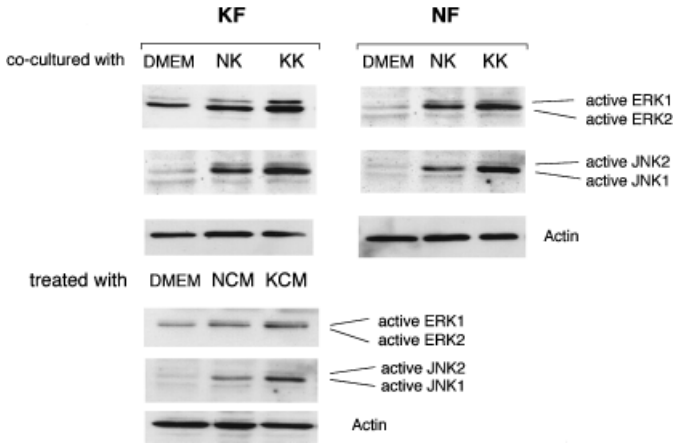

Figure 4. Keratinocytes upregulate the phosphorylation of ERK and JNK in fibroblasts. After 4 d of coculture with NK or KK as described, fibroblasts (KF, NF) were lyzed. Cell lysates were loaded onto 12% polyacrylamide gels and analyzed by western blotting using anti-active(phospho)ERK antibody and anti-active(phospho)JNK antibody. The phosphorylation of ERK and JNK in KF after treating with conditioned medium (NCM, KCM) for 5 min was checked. (Both ERK and JNK were maximally phosphorylated by 5 min of treatment with conditioned medium in the time course study). Each blot was probed for actin to confirm equal loading. *DMEM,* noncoculture control, cultured with DMEM alone.

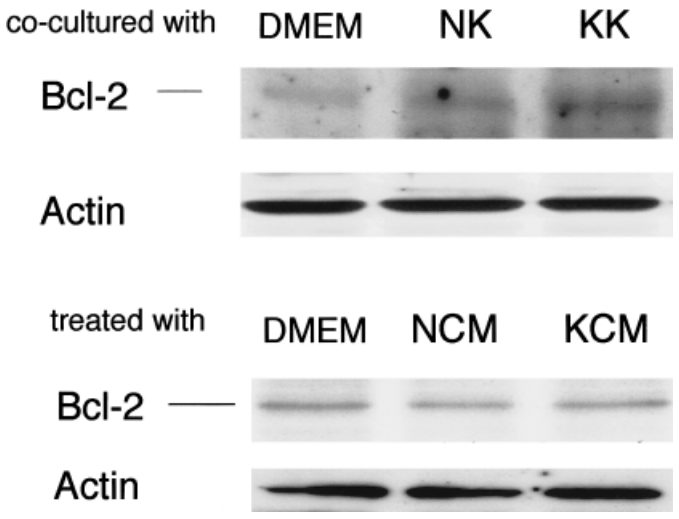

Figure 5. Bcl-2 expression in KF cocultured with keratinocytes (NK, KK) and treated with conditioned medium (NCM, KCM). After 4 d of coculture with keratinocytes (NK, KK) or treatment with conditioned medium (NCM, KCM) as described, KF were lyzed, loaded onto 14% polyacrylamide gels, and analyzed by western blotting using monoclonal anti-Bcl-2 antibody. The results were standardized with actin levels. *DMEM*, noncoculture control, cultured with DMEM alone.

this was less with NK and lowest in the DMEM-alone control group (**Fig 4**). Again, previous studies demonstrated recently that activation of JNK might have antiapoptotic function in fibroblasts. We analyzed the role of JNK in fibroblasts as we did for ERK and we found maximal phosphorylation of JNK in KF when cocultured with KK (**Fig 4**). We also checked the phosphorylation of ERK and JNK after treating KF with conditioned medium (NCM, KCM) instead of coculturing. KCM showed the highest activation of both ERK and JNK (**Fig 4**). Again, in the case of NF cocultured with NK or KK, phosphorylation of both ERK and JNK was strongest when cocultured with KK. p38 activity was also investigated in fibroblasts cocultured with keratinocytes or treated with conditioned medium. Nevertheless, the phosphorylated p38 was not detectable in any of the groups (data not shown).

Bcl-2 expression upregulated in KF cocultured with KK but not when treated with conditioned medium Bcl-2 plays a role in tissue homeostasis as an antiapoptotic gene and has been shown to be dysregulated in diverse pathologic conditions associated with resistance to apoptosis (Hockenbery *et al*, 1990; Nunez *et al*, 1990; Itoh *et al*, 1993; Shinoura *et al*, 1999). We compared the level of expression of Bcl-2 in KF cocultured with NK, KK, or DMEM-alone controls and found significantly strong expression of Bcl-2 when cocultured with KK whereas the expression of Bcl-2 was barely detectable in the DMEM-alone control group (**Fig 5**). Again, we analyzed the level of expression of Bcl-2 in KF after treating them with the conditioned medium for up to 4 d but a result similar to

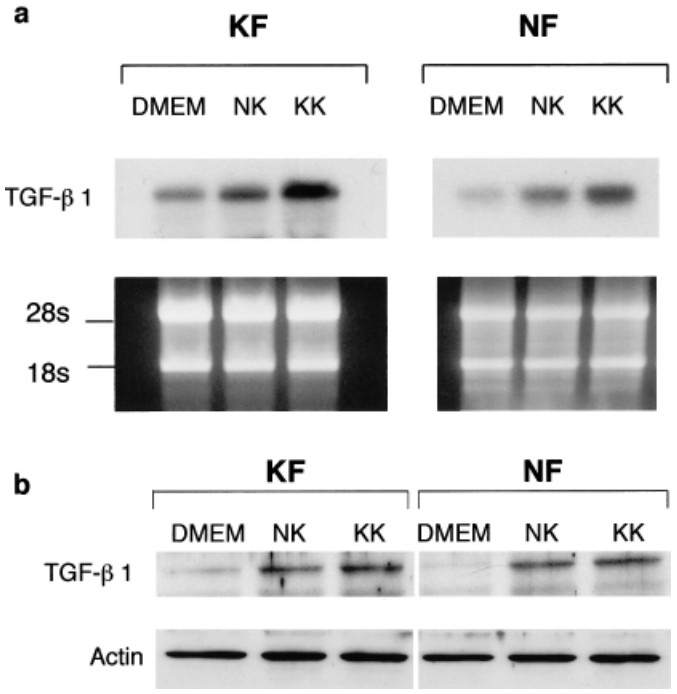

Figure 6. TGF-β1 expression in fibroblasts cocultured with NK or KK. (*a*) After coculturing for 24 h, total RNA was obtained from NF or KF (cocultured with NK, KK, and DMEM alone: noncoculture controls), and TGF-β1 mRNA expression was analyzed by northern blotting. Equivalence of loading is shown by levels of 28S and 18S rRNA. (*b*) Protein extracted after 4 d of coculturing was analyzed for TGF-β1 expression by immunoblotting. Each lane was probed for actin to confirm equal loading.

that obtained in case of coculture system was not observed. There was no difference in the expression among the groups (**Fig 5**).

TGF-β1 expression upregulated in KF cocultured with KK TGF-β1 expression has been shown to be enhanced in keloid fibroblasts (Lee *et al*, 1999; Mikulec *et al*, 2001). In our previous study we showed that TGF-β1 is an important factor responsible for the resistance to apoptosis observed in KF (Chodon *et al*, 2000). Hence we hypothesized that in the coculture system, paracrine or double paracrine effects could have further additive effect on the expression of TGF-β in fibroblasts. After coculturing, we extracted the RNA and protein from the respective fibroblast groups and analyzed the level of expression of TGF-β1 by northern blotting and immunoblotting. In KF, TGF-β1 mRNA (**Fig 6*a***) and TGF-β1 protein levels (**Fig 6*b***) were both significantly higher when cocultured with KK than with NK. This difference was not observed when KF was treated with the conditioned medium (data not shown).

DISCUSSION

In normal wound healing, the scar evolves from granulation tissue rich in cells, and as the wound becomes epithelialized, there is a sharp increase in the number of apoptotic cells, suggesting that apoptosis is the mechanism responsible for the evolution of granulation tissue into a normal scar (Desmouliere *et al*, 1995). Here, we have demonstrated that KF cocultured with KK were significantly more proliferative and resistant to apoptosis than those cocultured with NK. In a similar coculture system as described above, KK cause increased collagen production (Lim *et al*, 2002) as well as increased cell proliferation (Lim *et al*, 2001) in fibroblasts. Taken together, our data support the idea that keloid forms as a result of an abnormal wound healing process with a prolonged proliferative phase and a delayed remodeling phase owing to the proliferative and apoptosis-resistant phenotype of the fibroblasts in a keloid lesion. This phenomenon is further enhanced by the overlying keratinocytes and allows a state of continued production of excessive collagen. TGF-β1 plays an important role in the formation of extracellular matrix proteins. In the remodeling phase, TGF- β1 shows no difference in the dermal staining between hypertrophic scars and normal scars immunohistologically suggesting that TGF- β1 plays a more important role during the earlier phase of wound healing (Niessen *et al*, 2001). It has been also proposed that TGF-β1 might be involved in the early inductive events of epithelial–mesenchymal interaction (Schmid *et al*, 1991). Our results that coculturing KF with KK significantly increased the expression of TGF-β1 support the concept of a prolonged proliferative phase in the presence of KK in keloid.

In KF treated with keratinocyte-derived conditioned medium, there was no difference in proliferation and apoptosis between the NCM and KCM groups although significantly stronger phosphorylated bands of ERK and JNK were observed in case of KCM compared to NCM. This suggests that ERK/JNK are directly activated by some active soluble factor(s) released by the keratinocytes. KCM may contain more active soluble factors than NCM, but the lack of persistent stimulation or an insufficient titer of the factor could account for the lack of difference between KCM and NCM in stimulating proliferation and inhibiting apoptosis. In the coculture system, the interaction between fibroblasts and keratinocytes allows a more dynamic and reciprocal modulation with a resulting amplified effect.

Applying our indirect coculture system, we have investigated here the indirect regulation of Fas/FasL, the mitogen-activated protein kinase (MAPK) family, Bcl-2, and TGF-β1, factors known to play important roles in the modulation of apoptosis and cell survival. We did not find any significant difference in the expression of FasL between NK and KK (data not shown), and as mentioned in our previous study, there was no significant difference in Fas expression between KF and NF (Chodon *et al*, 2000). Four days of coculture with KF or NF also showed no significant differences in the expression of Fas/FasL in fibroblasts (data not shown). Nevertheless, Fas expression solely is not necessarily the cause of biologic responsiveness (Owen-Schaub *et al*, 1994).

Cytokines from keratinocytes may have regulatory roles on dermal fibroblasts regarding proliferation and apoptosis through the MAPK pathway. Consistent with this hypothesis, fibroblasts cocultured with KK, which showed maximum proliferation and minimum apoptosis, displayed the strongest phosphorylated band of ERK and JNK. ERK does not only induce cell proliferation and differentiation, but is also known to be an antiapoptotic signal pathway (Cobb *et al*, 1991; Thomas, 1992; Burgering *et al*, 1993; Gupta *et al*, 1999; Widmann *et al*, 1999) and, recently, phosphorylated JNK has been shown to also indicate antiapoptotic signals (Szabowski *et al*, 2000; Liu *et al*, 1996; Yeh *et al*, 1997; Yujiri *et al*, 1998; Wisdom *et al*, 1999). c-Jun protected fibroblasts from apoptosis and phosphorylation of c-Jun by JNK was required for the antiapoptotic effect in fibroblasts (Wisdom *et al*, 1999).

In keloid tissue, intense Bcl-2 staining was detected in the dermis (Teofoli *et al*, 1999). Consistent with this, we found that the level of expression of Bcl-2 was highest in the fibroblasts cocultured with KK. This was not observed in case of treatment with conditioned medium and thereby indicates that interaction between the keratinocytes and fibroblasts is necessary for the increased Bcl-2 expression, which may be responsible for the antiapoptotic effect in keloid.

Intracellular TGF-β1 in KF is at higher levels compared to NF (Lee *et al*, 1999) and our previous data provided evidence for a pivotal role of TGF-β1 in the resistance of KF to apoptosis (Chodon *et al*, 2000). Here, our data show that coculturing with KK significantly increases the expression of TGF-β1 in KF, suggesting that besides causing an abnormally increased production of

collagen; this may be also responsible for the increase in resistance to apoptosis in the coculture system. Expression of TGF-β receptors (type I and II) and phosphorylated Smad3 are also elevated in KF (Chin *et al*, 2001) and this may be why KF show a greater resistance to apoptosis as compared to NF. A previous study showed that TGF-β1 could inhibit apoptosis through the MAPK pathway (Chin *et al*, 1999) and also by preventing the decline in Bcl-2 during IL-1β-induced apoptosis in myofibroblasts (Zhang and Phan, 1999). Hence, in keloid, TGF- β1 may be inhibiting apoptosis through MAPK and Bcl-2, although at this stage, we cannot rule out an independent antiapoptotic or proliferative mechanism.

Interaction or double paracrine action via soluble factors between the abnormal keratinocytes and fibroblasts with keloid phenotypes, resulting in an increase in the TGF-β1 expression, MAPK phosphorylation, and Bcl-2 expression, promotes fibroblast proliferation and resistance to apoptosis. The resulting disruption of the normal balance between cell death and proliferation could lead to keloid formation. Thus the KK–KF interaction may be responsible for the prevention of the shift to a remodeling phase of wound healing *in vivo*. Here we show that epidermal keratinocytes play an important role in the formation of keloids and hence targeting the dermal fibroblasts along with the overlying keratinocytes may lead to a more effective treating rationale for keloid.

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