Differentiated Keratinocyte-Releasable Stratifin (14-3-3 Sigma) Stimulates MMP-1 Expression in Dermal Fibroblasts

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Through the use of a keratinocyte/fibroblast co-culture system, we have recently identified a potent keratinocyte-derived anti-fibrogenic factor (KDAF) for dermal fibroblasts. A sequential chromatography of the active fractions of keratinocyte-conditioned medium (KCM) and peptide mapping of the candidate proteins identified KDAF as being the keratinocyte-releasable 14-3-3 sigma (14-3-3σ) protein, which is also known as stratifin. In this study, we hypothesize that differentiated, but not proliferating, keratinocytes are the primary source of releasable 14-3-3σ in conditioned medium. To address this hypothesis, in a longitudinal study, keratinocyte differentiation was induced by growing these cells in a medium consisting of 50% keratinocyte serum-free medium (KSFM) and 50% Dulbecco's modified eagle's medium without any additives for up to 20 d. When KCM was collected every other day and added to fibroblasts, the level of matrix metalloproteinase (MMP)-1 mRNA expression was markedly increased in fibroblasts receiving KCM and this increase was even greater in cells receiving conditioned media collected at later time points relative to that of controls. The results of a western blot analysis further showed a marked increase in the expression of 14-3-3σ protein in keratinocytes grown in test medium from day 4 to day 10. This finding was consistent with the levels of 14-3-3σ mRNA expression in differentiated keratinocytes. In contrast to a very high level of 14-3-3σ mRNA expression seen in keratinocytes, fibroblasts that are highly responsive to14-3-3σ were unable to express this factor. Interestingly, the level of 14-3-3σ mRNA expression was markedly higher in keratinocytes co-cultured with fibroblasts relative to that of mono-cultured keratinocytes. In conclusion, this study provides evidence that keratinocytes express a high level of 14-3-3σ at the levels of mRNA and protein. But the releasable form of 14-3-3σ protein was only found in conditioned medium derived from differentiated keratinocytes. Further, our recently purified recombinant 14-3-3σ protein mimics the collagenase stimulatory effect of KCM in dermal fibroblasts.

Key words: cell–cell interaction/collagenase/fibroblasts/keratinocyte/protein 14-3-3σ/skin/stratifin
J Invest Dermatol 124:170–177, 2005

The ability to generate or repair injured tissue is essential to the continuity of human life (Scott *et al*, 1994). Epidermal–mesenchymal communication is critical in exchanging the information between keratinocytes and fibroblasts in skin morphogenesis during development and probably in maintaining the integumentary structure in the adult (reviewed in Johnson-wint, 1980). It is also well established that any delay in epithelialization, due to either infection or severity of injury, increases the frequency of developing fibrotic conditions. This is based on the fact that when keratinocytes form epithelialization on the wound within 2–3 wk, only one-third of the anatomically site-matched wounds become hypertrophic, whereas this increases to 78% when a wound is epithelialized later than 21 d. This signifies that in the absence of epithelialization, extracellular matrix (ECM) continues to accumulate until dermal fibroblasts receive signal(s) from epidermal cells to slow down the dynamic process of healing that leads to maturation and remodeling of the healing wound (Machesney *et al*, 1998). We have recently conducted a series of experiments to test our working hypothesis that a keratinocyte-derived anti-fibrogenic factor (s) (KDAF) might function as a wound-healing stopping signal (s) by modulating the expression of key extracellular proteins such as collagenase (matrix metalloproteinase (MMP)-1) and possibly collagen type I and type III in dermal fibroblasts. As previously reported (Ghahary *et al*, 2004), a sequential chromatography of the keratinocyte-conditioned medium (KCM), peptide mapping, and amino acid sequencing of purified KDAF turned out to be a keratinocyte-releasable 14-3-3 sigma (14-3-3σ) protein also known as stratifin.

Stratifin is a member of a large family of highly conserved, acidic dimeric 14-3-3 proteins. Although several members of the protein family appear to be ubiquitously expressed, as reported by Leffers *et al* (1993), stratifin expression seems to be specific to stratified epithelial cells. These investigators used 2D gel electrophoresis

Abbreviations: KCM, keratinocyte-conditioned medium; KDAF, keratinocyte-derived anti-fibrogenic factor; KSFM, keratinocyte serum-free medium; 14-3-3 sigma, 14-3-3σ

and identified several members of this protein family in keratinocytes. Immunofluorescence staining of keratinocytes localized two members of these proteins to the Golgi apparatus, whereas stratifin was distributed diffusely in the cytoplasm. These investigators also found a significant level of stratifin in the medium of cultured human keratinocytes, suggesting a partial secretion of this protein by keratinocytes. Isolation and identification of stratifin as an intracellular keratinocyte-derived stimulator of protein kinase C-mediated phosphorylation has also been reported (Dellambra *et al*, 1995). In another study, Katz and Taichman (1999) also identified epsilon and sigma forms of protein 14-3-3 in KCM.

Although many functional activities have been described for the intracellular members of this protein family, biological roles of the releasable forms of the members of the 14-3-3 protein family in general and 14-3-3σ in particular are completely unknown. Our previous study identified and characterized a KDAF for dermal fibroblasts, which surprisingly turned out to be a releasable form of 14-3-3σ protein. In this study, we have established a keratinocyte/fibroblast co-culture system and showed that our purified recombinant 14-3-3σ protein mimics the collagenase stimulatory effect of KDAF for dermal fiboblasts. We further demonstrated an increase in 14-3-3σ at the levels of mRNA and intracellular protein expression as well as in the releasable form of this protein in differentiated keratinocytes. Interestingly, fibroblasts that were highly responsive to 14-3-3σ failed to express 14-3-3σ mRNA. Thus, fibroblasts might be the main target for the keratinocyte-releasable 14-3-3σ protein.

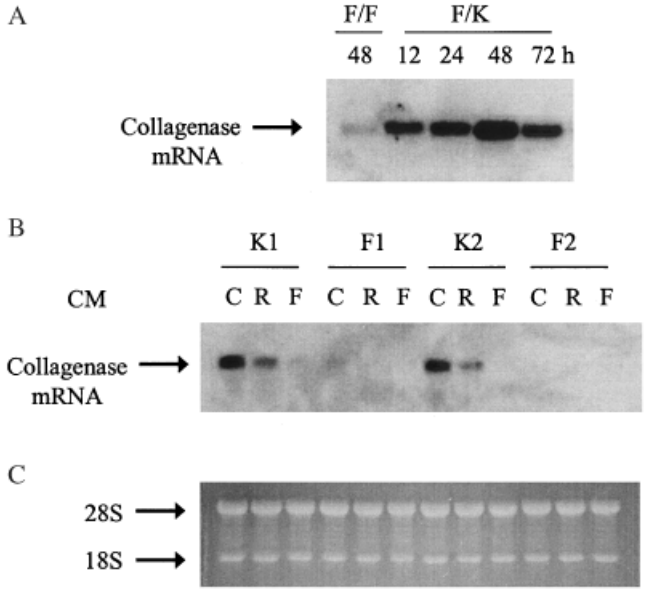

Figure 1
Keratinocyte-conditioned medium (KCM) increases collagenase mRNA expression in dermal fibroblasts. Total RNA from either fibroblasts (F/F) or fibroblasts (F/K) co-cultured with keratinocytes for different time points was extracted and used to evaluate the expression of collagenase mRNA by northern analysis (*panel A*). In panel *B*, two different strains of keratinocytes (K1 and K2) and fibroblasts (FI and F2) were cultured individually and their conditioned media were collected and passed through a Centricon filter with a 30 kDa cut-off. Fibroblasts were then treated with either unfiltrated whole conditioned medium (C), the corresponding filtrate (F), or retentate (R) for 24 h. Cells were then harvested and extracted RNA was used to evaluate the expression of collagenase mRNA by northern analysis. Panel *C* shows the pattern of 18S and 28S ethidium bromide-stained ribosomal RNA used as a loading control of the same blot seen in panel *B*.

Results

Co-culturing keratinocytes with fibroblasts increases the expression of MMP-1 mRNA in fibroblasts In order to identify a KDAF for dermal fibroblasts, we recently established a keratinocyte/fibroblast co-culture system in which keratinocytes and fibroblasts are grown in the upper and lower chambers of this system, respectively. A test medium consisting of 49% keratinocyte serum-free medium (KSFM), 49% Dulbecco's modified Eagle's medium (DMEM), and 2% fetal bovine serum (FBS), which was suitable for both keratinocytes and fibroblasts, was used. As a permeable membrane separates these cells, fibroblasts in the lower chamber can be exposed to any factor released from the keratinocytes. As an index for the anti-fibrogenic effects of keratinocyte-derived factor(s) on dermal fibroblasts, total RNA was extracted from fibroblasts grown in the lower chamber and the expression of MMP-1 mRNA was evaluated by northern analysis. Fibroblasts grown alone and fibroblasts grown in a fibroblast/fibroblast co-culture system were also used as controls. As shown in Fig 1*A*, MMP-1 mRNA expression is significantly increased in fibroblasts (F/K) as early as 12 h post-co-culturing with keratinocytes relative to fibroblasts grown in fibroblast/fibroblast (*lane F/F*) co-culture system. The level of MMP-1 mRNA expression remained high up to 72 h examined. To determine whether this factor is specific to keratinocytes and that it is still releasable from keratinocytes grown in a mono-culture system, conditioned media from two different strains of human keratinocyte (K1 and K2) and fibroblasts (F1 and F2) were collected and fractionated by a 30 kDa cut-off filter of the Centricon Tubes (Millipore, Bedford, Massachusetts). The portions of unfiltered material (C), filtrate (F), and retentate (R) were individually added to fibroblasts for 24 h and the efficacy of any releasable factors on MMP-1 mRNA expression was evaluated by northern analysis. As shown in Fig 1*B*, MMP-1 stimulating activity was only found in the unfiltered KCM (*lane C*) and retentate (*lane R*). But neither the filtrate (*lane F*) of KCM nor any fraction of fibroblast-conditioned medium was able to stimulate the expression of MMP-1 mRNA. MMP-1 stimulatory effect of KDAF was not due to RNA loading as the pattern of 18S ribosomal RNA was relatively the same in all samples (Fig 1*C*). These findings collectively suggest that the size of this protein is about or greater than 30 kDa and the release of this factor is specific to keratinocytes, but not fibroblasts, grown in either a mono- or co-culture system.

14-3-3σ protein mimics the effect of KDAF on collagenase mRNA expression in fibroblasts After having previously demonstrated that KDAF for dermal fibroblasts is in fact the same as stratifin (14-3-3σ protein), here we examined the efficacy and the lasting effect of our human recombinant 14-3-3σ on collagenase mRNA expression in fibroblasts. The recombinant 14-3-3σ protein produced in bacteria was more than 95% pure (Ghahary *et al*, 2004). To evaluate the efficacy of recombinant 14-3-3σ on the

expression of MMP-1 in dermal fibroblasts, cells were treated with either nothing or various concentrations of recombinant 14-3-3σ (0.25, 0.5, 1.0, and 2.5 μg per mL) for 24 h (Fig 2*A*). The results showed a marked increase in the expression of collagenase mRNA in treated fibroblasts. This increase reached its maximum with 0.5–1.0 μg per mL of 14-3-3σ used. In another experiment, the lasting effect of 14-3-3σ in collagenase expression was evaluated. The results again showed a marked increase in expression of collagenase mRNA in treated dermal fibroblasts relative to that of the control. When the 14-3-3σ protein was removed from the culture medium, the level of collagenase mRNA expression remained high up to 6 h and then gradually reduced to its normal value within 36 h of 14-3-3σ removal (Fig 2*B*).

The level of 14-3-3σ protein is greater in differentiated keratinocyte-conditioned medium To address the question of whether differentiated or proliferating keratinocytes release the 14-3-3σ protein, conditioned medium from proliferating keratinocytes grown in KSFM was replaced with test medium in which keratinocytes in the presence of high calcium levels gradually become differentiated. Cells and conditioned medium from day 0, 2, 4, 6, 8, and 10 were then collected and evaluated for intra- and extracellular levels of 14-3-3σ protein by western blot analysis. The level of intracellular 14-3-3σ protein was easily detectable in keratinocytes harvested in all time points. The expression of this protein was, however, markedly increased in keratinocytes grown in test medium for 6, 8, and 10 d (Fig 3*A*). This increase was not due to protein loading as the amount of intracellular β-actin protein was not increased by differentiation (Fig 3*B*). When the levels of 14-3-3σ protein were evaluated in the corresponding conditioned media, the releasable form of this protein was only found in conditioned medium collected on day 6, 8, and 10 (Fig 3*C*). To confirm this finding, in another set of experiments, the level of 14-3-3σ mRNA expression in keratinocytes grown in test medium for 0, 1, 2, 3, 4, 5, 14, 18, and 20 d was evaluated by northern analysis. The results showed a very high level of 14-3-3σ mRNA expression in keratinocytes harvested at all time points. But this expression was higher at early and late time points (Fig 4, *panel A*). To correlate this expression with the differentiation status of keratinocytes, the same blot was rehybridized with cDNA specific for involucrin, which is known as one of the differentiation markers for keratinocytes. The expression of this marker was very high on day 14, 18, and 20 examined. A very faint detectable band of involucrin mRNA was also found in keratinocytes harvested on day 5. In a similar longitudinal experimental setting, keratinocytes conditioned media collected at various time intervals were evaluated for their collagenase stimulatory effects in dermal fibroblasts by northern analysis. The results showed that all samples of KCM added to dermal fibroblasts for 24 h possess a very high collagenase stimulatory effects (Fig 4, *panel B*, day 2–20). Consistent with the levels of 14-3-3σ mRNA expression in differentiated keratinocytes (later time points), the conditioned media collected at later time points showed a higher collagenase stimulatory effect in dermal fibroblasts. The absence of any collagenase stimulatory effect seen in fibroblasts, which received either fresh medium (C) or conditioned medium collected from proliferating keratinocytes grown in KSFM + bovine pituitary extract (BPE) and epidermal growth

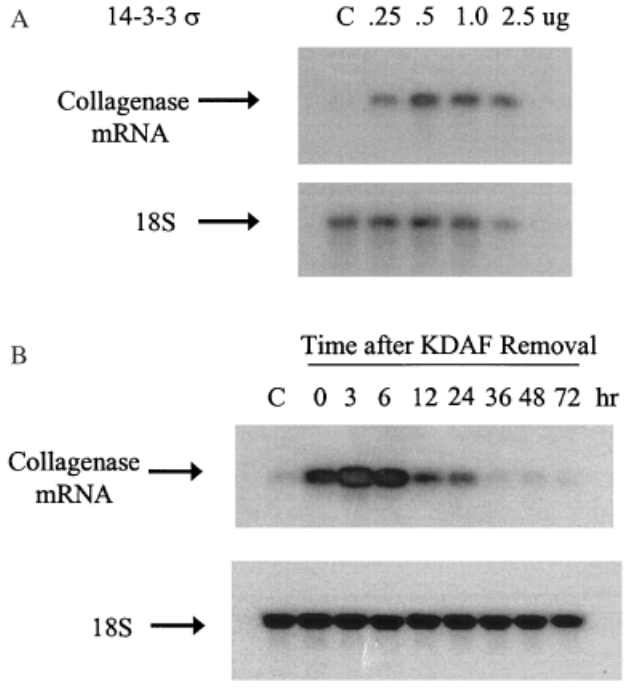

Figure 2
Recombinant 14-3-3 sigma (14-3-3σ) protein increases the expression of collagenase mRNA in dermal fibroblasts. Cells were treated with either nothing (C) or various concentrations (0.25, 0.5, 1.0, and 2.5 μg per mL) of our recombinant 14-3-3σ for 24 h and the expression of collagenase mRNA and 18S ribosomal RNA (loading control) was assessed by Northern analysis (*panel A*). To determine the lasting effect of 14-3-3σ on collagenase expression, fibroblasts were treated with either nothing (C) or 2.5 μg per mL of 14-3-3σ for 24 h. The medium was replaced with fresh medium without 14-3-3σ and cells were then harvested at 0, 3, 6, 12, 24, 36, 48, and 72 h post 14-3-3σ removal. The expression of collagenase mRNA in dermal fibroblasts was then evaluated by northern analysis. The pattern of 18S ribosomal RNA is also shown as a loading control.

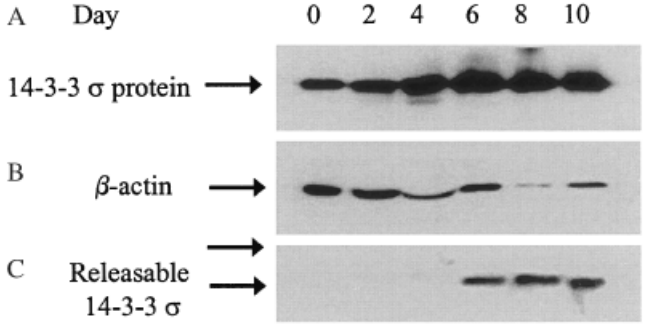

Figure 3
Differentiated keratinocytes release a greater level of 14-3-3 sigma (14-3-3σ) into conditioned medium. To evaluate intra- and extracellular forms of 14-3-3σ protein in differentiated keratinocytes, cells (0.5×10^6 cells per well) were grown in keratinocyte serum-free medium (KSFM) for 48 h and conditioned medium was replaced with test medium. The protein contents of conditioned media collected on day 0, 2, 4, 6, 8, and 10 and corresponding cell extracts were determined by western blot analysis. Panels *A* and *B* show the patterns of intracellular form 14-3-3σ and β-actin, respectively. The pattern of the releasable form of 14-3-3σ is shown in panel *C*.

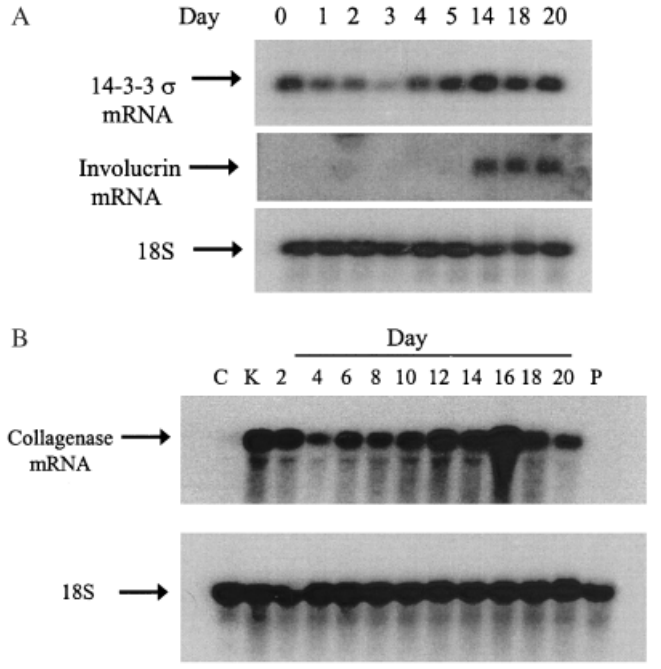

Figure 4
Time-dependent expression of 14-3-3 sigma (14-3-3σ) and involucrin mRNA in keratinocytes. Keratinocytes were cultured in keratinocyte serum-free medium (KSFM) for 48 h and medium was replaced with test medium. Keratinocytes were then harvested on day 0, 1, 2, 3, 4, 5, 14, 18, and 20. Total RNA was extracted and analyzed by northern analysis. The blot was initially hybridized with cDNA specific for 14-3-3σ and subsequently with cDNA for involucrin and 18S ribosomal RNA used as a control for RNA loading (*panel A*). In another set of experiments, keratinocytes were cultured in KSFM for 48 h and medium was replaced with test medium. The conditioned media collected every 48 h up to 20 d were then used to treat fibroblasts for 24 h (day 2–20). Panel *B*, total RNA from cells that received either fresh medium (C), fresh medium plus 1 μg per mL of 14-3-3σ protein (K), keratinocyte-conditioned medium (KCM) collected at different days (day 2–20), or conditioned medium from proliferating keratinocyte grown in KSFM plus additive for 48 h (P). The RNA was extracted and analyzed for collagenase mRNA expression. The blot was subsequently re-hybridized with cDNA specific for 18S ribosomal RNA and used as a RNA loading control.

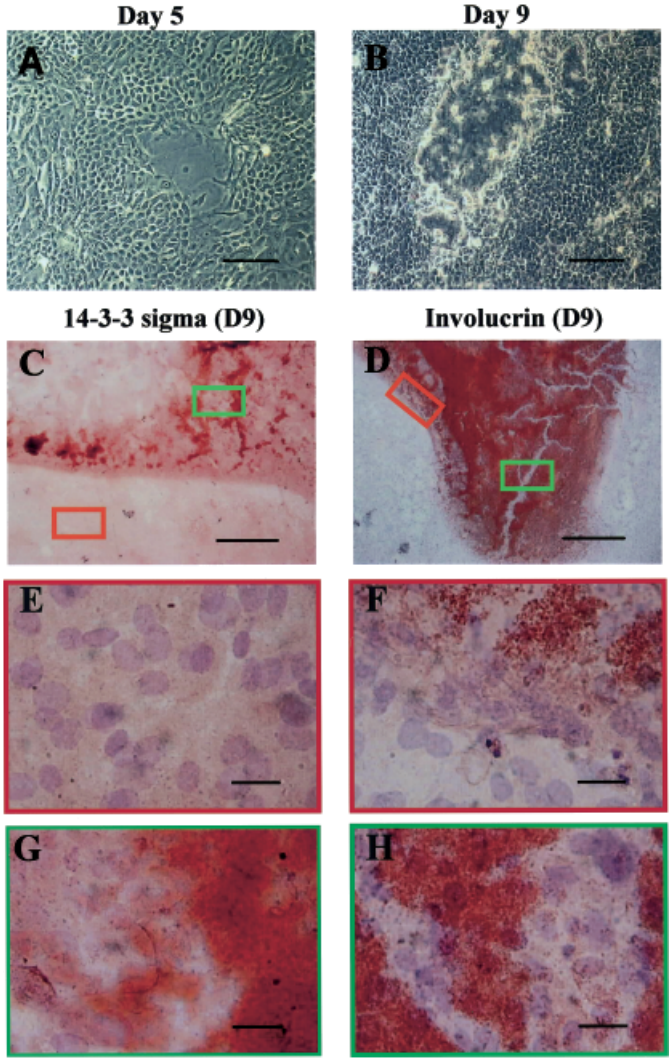

Figure 5
Immunohistochemical detection of 14-3-3 sigma (14-3-3σ) and involucrin in cultured keratinocytes. Panels *A* and *B* show the phase contrast microscopic appearance of keratinocytes grown in test medium to induce differentiation on day 5 (*A*) and day 9 (*B*). *Scale bar* = 200 μm. Panels *C* and *D* show the low magnification of 14-3-3σ (*panel C*) and involucrin (*panel D*) of cultured keratinocytes on day 9. *Scale bar* = 500 μm, whereas panels *E–H* represent a high magnification of corresponding areas related to the red (*panels E* and *F*) and green (*panels G* and *H*) rectangular insets shown in panels *C* and *D*.

factor (EGF) (*lane P*), suggests that proliferating keratinocytes may express 14-3-3σ mRNA (Fig 4*A*, 0 time point) but that it may not be releasable (Fig 3*C*) in sufficient amount to stimulate the collagenase expression in dermal fibroblasts (Fig 4*B*). Further, an addition of 14-3-3σ at a final concentration of 1 μg per mL (*lane K*) into fresh medium added to fibroblasts seems to mimic the collagenase stimulatory effect of KCM in fibroblasts.

To further demonstrate that differentiated keratinocytes express stratifin and that it is correlated with involucrin, a microscopic phase contrast evaluation of keratinocytes as well as an immunohistochemical staining for these proteins were conducted. As shown by the phase contrast appearance of keratinocytes grown in test medium to induce differentiation on day 5 (Fig 5*A*) and 9 (Fig 5*B*), keratinocytes began to form some areas of non-uniformity on day 5 and this changed to form multilayer nodules on day 9. We have found one to three keratinocyte multilayer forming nodules with different sizes in each microscopic field. The results of immunohistochemical staining for stratifin and involucrin showed very high levels of stratifin and involucrin in cultured keratinocytes on day 9 (Fig 5*C* and *D*, respectively), but not day 5 (data not shown), at which keratinocytes become differentiated and form multilayers in culture (Fig 5*G* and *H*). This staining was only seen on the areas that keratinocytes form multilayer nodules.

Dermal fibroblasts co-cultured with keratinocytes fail to express 14-3-3σ mRNA As the level of MMP-1 mRNA expression is markedly increased in response to both KCM and recombinant 14-3-3σ, we investigated whether fibroblasts are merely targets for the keratinocyte-releasable 14-3-3σ protein or whether they have ability to produce this protein. This question was addressed by growing dermal fibroblasts alone or in a keratinocyte co-culture system.

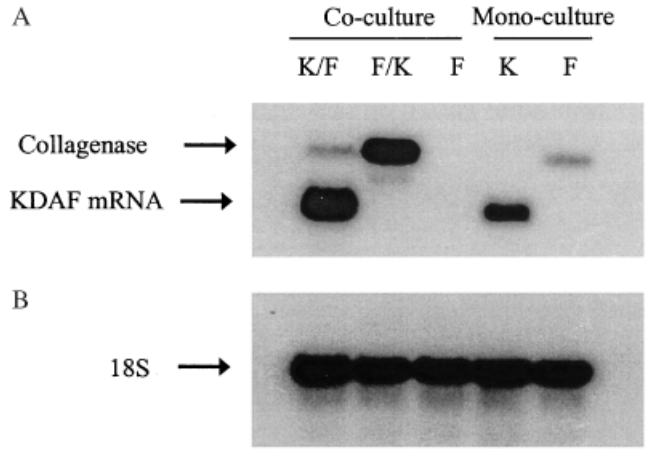

Figure 6
Expression of 14-3-3 sigma (14-3-3σ) mRNA in keratinocytes correlates with collagenase mRNA expression in dermal fibroblasts grown in a co-culture system. To demonstrate the expression of 14-3-3σ mRNA in keratinocytes (K/F) and correlate that with collagenase expression in dermal fibroblasts (F/K), a keratinocyte/fibroblast co-culture system was established. Cells were then individually harvested and the expression of 14-3-3σ and collagenase mRNA detected by northern analysis was compared with those of cells grown in a mono-culture system (*panel A*). The pattern of 18S ribosomal RNA obtained from re-hybridization of the same blot used as an RNA loading control (*panel B*).

Both keratinocytes and fibroblasts were separately harvested and total RNA was extracted and evaluated for 14-3-3σ and collagenase mRNA expression. The results shown in Fig 6 clearly demonstrate that fibroblasts co-cultured with keratinocytes expressed a very high level of collagenase mRNA (*lane F/K*) relative to that of fibroblasts cultured alone (*lane F*). This finding again confirms that keratinocytes release a very potent collagenase-stimulating factor for dermal fibroblasts. When the same blot was re-hybridized with 14-3-3σ cDNA, under both mono- and co-culture conditions, keratinocytes expressed a very high level of 14-3-3σ mRNA. On the other hand, our findings demonstrate that fibroblasts grown alone or in the presence of keratinocytes are unable to express a detectable level of mRNA for 14-3-3σ. Interestingly, keratinocytes co-cultured with dermal fibroblasts seem to express greater levels of 14-3-3σ mRNA relative to those cultured alone. This may indicate that fibroblast-releasable factors stimulate the expression of 14-3-3σ in keratinocytes. We also found that growing keratinocytes on a collagen-coated filter in the top chamber stimulates the expression of collagenase mRNA by these cells relative to those grown on plastic (data not shown**)**. The same blot was finally re-hybridized with cDNA specific for 18S ribosomal RNA and was used as a total RNA loading control **(**Fig 6*B***)**.

Discussion

In this study, we demonstrated that the expression of 14-3-3σ mRNA and intracellular protein is detectable in both proliferating and differentiated keratinocytes; however, Only differentiated keratinocytes were able to release 14-3-3σ protein into conditioned medium. We also demonstrated that our recombinant 14-3-3σ protein mimics the collagenase-stimulating effect of KCM in dermal fibroblasts. This finding was confirmed by another experiment revealing that the presence of 14-3-3σ in culture medium is needed for expression of MMP-1 mRNA in fibroblasts. This is because removal of 14-3-3σ from the fibroblast-conditioned medium reduces the expression of collagenase to its normal value within 36 h.

14-3-3 proteins are a class of highly conserved molecular chaperones. They are a ubiquitous family of acidic eukaryotic proteins with seven known mammalian isoforms, α/β, γ, ε, π, σ, τ(θ), and ζ (Martens *et al*, 1992). Since the discovery of the first 14-3-3 protein (Moore and Perez, 1967) with an unknown function in the brain, the members of the 14-3-3 protein family have been repeatedly re-discovered based on new biological activities, primarily in the context of signal transduction pathways. They have been identified as activators of tryptophan and tyrosine hydroxylase (Ichimura *et al*, 1987; Ichimura *et al*, 1988) and protein kinase C (PKC) inhibitors (Toker *et al*, 1990). Subsequent studies identified the 14-3-3 proteins as molecules that interact with PKC, Raf family members. It is now known that more than 100 other intracellular proteins with critical biological functions including cellular response to DNA damage and cell cycle regulation interact with 14-3-3 proteins (Craparo *et al*, 1997; Hermeking *et al*, 1997; Chan *et al*, 2000; Laronga *et al*, 2000; Yaffe 2002). It should be emphasized that among all members of the protein 14-3-3 family, the σ form is reported to be vital in preventing mitotic catastrophe after DNA damage (Chan *et al*, 1999).

Further, some soluable forms of 14-3-3 protein have been shown to be present in the cerebrospinal fluid (CSF) and has been associated with prion diseases such as Creutzfeldt–Jakob disease and other neurological disorders (Boston *et al*, 1982; Satoh *et al*, 1999). But no biological functions of these soluble forms of 14-3-3 proteins have so far been described. Thus, the collagenase-stimulating activity of keratinocyte-releasable 14-3-3σ for dermal fibroblasts considered to be the first indication of a relevant extracellular biological function of keratinocyte-releasable stratifin.

As mentioned above, 14-3-3 proteins are considered to be intracellular proteins with many critical biological activities. It is therefore assumed that these proteins lack known amino-terminal endoplasmic reticulum (ER) signal peptides and, as such, the mechanism by which these proteins become releasable has yet to be elucidated. There is now supporting evidence to indicate that this is not unique to 14-3-3 proteins as there are several well-known releasable proteins, such as interleukin (IL)-1 (Andree *et al*, 1992; Corradi *et al*, 1995), fibroblast growth factor-2 (Albuquerque *et al*, 1998), and endothelial cell growth factor (Jaye *et al*, 1986), which lack signal peptides and yet are considered to be releasable proteins. In an earlier report, we found a secreted form of annexin II in KCM (Karimi-Busheri *et al*, 2002) and at that time we assayed for the presence of lactate dehydrogenase (LDH) in the medium, as a marker of cell lysis (Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988), and established that the release of this protein into the media was not the result of keratinocyte lysis. Since the same batches of KCM were used in both

studies, we can rule out cell lysis as the cause for release of 14-3-3σ into the KCM. Furthermore, accidental release of 14-3-3 would also be unlikely due to the fact that recombinant KDAF mimics the MMP-1 stimulatory function of KDAF found in KCM.

Since overexpression of MMP has been implicated with non-healing wound conditions, one may speculate that the release of KDAF and the subsequent stimulation of MMP-1 in dermal fibroblasts cause wound healing retardation. The result of our longitudinal experiments revealed that this is unlikely to be the case. This is because only differentiated, but not proliferating, keratinocytes release a sufficient amount of 14-3-3σ protein to stimulate collagenase expression in fibroblasts. Differentiation of keratinocytes was induced by growing these cells in test medium that contained relatively high levels of calcium in which keratinocytes undergo differentiation and expressed a differentiation marker such as involucrin. This finding was further supported by an immunohistochemical staining for involucrin and 14-3-3σ in cultured keratinocytes grown in test medium. The results showed that on day 9, but not day 5, at which keratinocytes become differentiated and form multilayer in culture express high levels of 14-3-3 protein as well as involucrin. This staining was only seen on the areas that keratinocytes form multilayers.

It is of interest to mention that keratinocytes co-cultured with dermal fibroblasts seem to express a greater level of mRNA for 14-3-3σ relative to those grown in a mono-culture condition. This finding may indicate that fibroblasts release a 14-3-3σ stimulating factor, whereas keratinocytes release the 14-3-3σ, which stimulates the expression of collagenase in dermal fibroblasts. Further, it seems that growing keratinocytes on a collagen-coated filter stimulates the expression of collagenase mRNA by these cells relative to those grown on plastic. This seems to be true for fibroblasts as well. Moreover, our unpublished data showed that fibroblasts grown in a 3D collagen gel express greater levels of collagenase relative to those grown on plastic (data not shown). These findings collectively indicate that at early stages of the healing process, proliferating and migrating keratinocytes express a high level of ECM deposition factors (Ghahary *et al*, 2001), whereas at a later stage of healing, when epithelialization is complete, the multi-layer form of differentiated keratinocytes releases some ECM degradation factors such as 14-3-3σ and possibly other members of this protein family. Thus, upon epithelialization and keratinocyte differentiation, this complementary mechanism would then slow down or even terminate the dynamic process of healing.

It is well established that MMP-1 is expressed by several types of malignant cells. This enzyme is one of the few proteolytic enzymes that is able to degrade native fibrillar collagens. As such, an increase in MMP-1 expression is correlated with a prognosis of malignant tumors, including gastric and colon carcinomas (Murray *et al*, 1996; Inoue *et al*, 1999). On the other hand, malignant cells may release some factors influencing the expression of MMP-1 in neighboring cells. Westermarck *et al* (2002) demonstrated that a subgroup of low passage primary tumor cells established from head and neck squamous cell carcinoma (SCC) releases some soluble factors that induce MMP-1 expression in normal and tumor fibroblasts. Although these investigators were not able to identify the nature of these collagenolytic phenotype-inducing factors in conditioned medium, they showed that collagenase-stimulating factors such as tumor necrosis factor-α and IL-1β are not the only factors responsible for these effects. Although it is not known whether stratifin is one of these SCC-releasable factors that increases collagenase expression in fibroblasts, our unpublished data suggest that SCC, at least at the level of mRNA, express stratifin at relatively a high level. We are currently examining whether this factor is releasable and if so, it remains to be seen whether it influences the expression of collagenase in fibroblasts.

In conclusion, the findings of this study collectively provide compelling evidence that differentiated keratinocytes release collagenase-stimulatory 14-3-3σ protein for dermal fibroblasts and that this may in turn control degradation of the major dermal ECM components such as type I and type III procollagen. The keratinocyte secreted 14-3-3 proteins or their fibroblast receptors may provide useful targets for clinical intervention in the control of wound healing and the development of fibrosis.

Materials and Methods

Clinical specimens and cell culture Following informed consent, skin punch biopsies were obtained from patients undergoing elective reconstructive surgery, under local anesthesia, according to a protocol approved by the University of Alberta Hospitals Human Ethics Committee. The study was carried out in accordance with the principles of the Declaration of Helsinki, (World Medical Association of Helsinki, Somerset West, 1996). Biopsies were collected individually and washed three times in sterile DMEM (Gibco, Grand Island, New York) supplemented with antibiotic–antimycotic preparation (100 μg per mL penicillin, 100 μg per mL streptomycin, 0.25 μg per mL amphotericin B) (Gibco). Cultures of fibroblasts were established as previously described (Karimi-Busheri *et al*, 2002). Upon reaching confluence, the cells were released by trypsinization, split for subculture at a ratio of 1:6, and reseeded into 75 cm^2 flasks. Fibroblasts at passages 3–8 were used in either mono- or co-culture system.

To establish cultured keratinocytes, the procedure of Rheinwald and Green (1975) was used for cultivation of human foreskin keratinocytes using KSFM (Gibco) supplemented with BPE (50 μg per mL) and EGF (5 μg per mL). Primary cultured keratinocytes at passages 3–5 were used.

Keratinocyte–fibroblast co-culture system In order to identify a KDAF for dermal fibroblasts, we had previously established (Karimi-Busheri *et al*, 2002; Ghahary *et al*, 2004) a keratinocyte/fibroblast co-culture system in which keratinocytes and fibroblasts are grown in the upper and lower chambers of the system, respectively. For the co-culture experiments, 30 mm Millicell-CM (Millipore) culture plate inserts with 0.4 μm pore size were coated with fetal bovine skin collagen (3 mg per mL). Subsequently, 0.5×10^6 keratinocytes were seeded on the collagen-coated inserts with KSFM supplemented with BPE (50 μg per mL) and EGF (5 μg per mL). In a separate experiment, 0.5×10^6 fibroblasts were seeded in each well of a six-well culture plate containing DMEM with 10% FBS. The cells were incubated in a cell culture incubator for 24 h and the conditioned medium was collected and cells were washed with phosphate-buffered saline (PBS). The co-culture system was then assembled, with the upper chamber being the collagen-coated insert with keratinocytes and the bottom chamber being the fibroblasts grown on a plastic six-well plate. The controls were inserts either alone, with keratinocytes, or with fibroblasts.

Each chamber received 2.5 mL of our test medium consisting of 49% KSFM without additive and 49% DMEM plus 2% FBS. As only a permeable membrane separates these cells, fibroblasts in the lower chamber can be exposed to any soluble factor that may be released from keratinocytes. As an index for the anti-fibrogenic effects of keratinocyte-derived factor(s) on dermal fibroblasts, total RNA was extracted from fibroblasts grown in the lower chamber and the expression of collagenase mRNA was evaluated by northern analysis.

Extraction of cellular RNA and northern analysis Keratinocytes and fibroblasts grown in either a mono- or co-culture system were harvested separately and pelleted by centrifugation at 300 $\times$ g for 10 min. Pellets were then lysed with 500 μL of 4 M guanidium isothiocyanate (GITC) solution and total RNA was isolated by the guanidium isothiocyanate/CsCl procedure of Chomczynski and Sacchi (1987) using phenol:chloroform (1:1), followed by chloroform:isoamyl alcohol (49:1). Total RNA from each individual fibroblast culture was then separated by electrophoresis (10 μg per lane) on a 1% agarose gel and blotted onto nitrocellulose filters. Filters were then baked under vacuum for 2 h at 80°C, pre-hybridized and hybridized according to the procedure described before (Ghahary *et al*, 2004). Hybridization was performed in the same solution at 45°C for 16–20 h using cDNA probes for either human MMP-1, 14-3-3σ, or 18S ribosomal RNA. The probes were labeled with ^{32}P-α-dCTP (DuPont Canada, Streetsville, Mississauga, Ontario, Canada) by nick-translation. Filters were initially washed at room temperature with 2 $\times$ SSC (1 $\times$ = 0.15 M sodium chloride, 0.015 M sodium citrate) and 0.1% SDS for 30 min, and then for 20 min at 65°C in 0.1 $\times$ SSC and 0.1% SDS solution. Autoradiography was performed by exposing a Kodak X-Omat film (Eastman Kodak Company, Rochester, New York) to the nitrocellulose filters at $-$70°C in the presence of an enhancing screen. The cDNA probes for collagenase and 18S ribosomal RNA were obtained from the American Type Culture Collection (Rockville, Maryland). The cDNA for human involucrin was a kind gift from Dr H. Green (Howard Medical School, Department of Cell Biology, Boston, Massachusetts). The cDNA for the 14-3-3σ protein was obtained by extracting keratinocyte total RNA and amplified by RT-PCR by a procedure described in the following sections.

Levels of KDAF in keratinocyte and fibroblast-conditioned media To determine whether the release of KDAF in conditioned media is unique to keratinocytes, fibroblasts, or both, conditioned media from either keratinocytes or fibroblasts were passed through a Centricon filter with a 30 kDa cut-off. The corresponding filtrate and retentate were collected and their volumes were adjusted to the original volume used. Aliquots of whole conditioned medium (C), filtrate (F), and retentate (R) were then used to treat dermal fibroblasts for 24 h. Total RNA was then extracted individually and the expression of collagenase mRNA was evaluated by northern analysis as described in the previous section.

Preparation and efficacy of recombinant 14-3-3σ and KCM on collagenase mRNA expression in dermal fibroblasts The procedure of human recombinanat 14-3-3σ protein preparation was similar to that previously described (Ghahary *et al*, 2004). In general, the expression of collagenase mRNA in dermal fibroblasts in response to either KCM or our purified 14-3-3σ was determined by northern analsyis. To achieve this, three different experiments were conducted. In the first set of experiments, keratinocytes were initially cultured in KSFM (Gibco) supplemented with bovine pituitary extract (50 μg per mL) and EGF (5 μg per mL). KCM was replaced with our test medium and conditioned medium was collected every 48 h up to day 20. These media were then used to treat dermal fibroblasts for another 24 h. Conditioned medium collected from proliferating keratinocytes grown in KSFM + EGF and pituitary extract for 48 h was also used to treat fibroblasts for comparison. Dermal fibroblasts that received fresh test medium with and without 1.0 μg per mL 14-3-3σ for the same duration were also included as positive and negative controls, respectively. In a second set of experiments, the efficacy of recombinant 14-3-3σ on collagenase mRNA expression in fibroblasts was evaluated by treating cells cultured in DMEM supplemented with 2% FBS with either nothing or various concentrations (0.25, 0.5, 1.0, and 2.5 μg per mL) of recombinant 14-3-3σ for 24 h. In the third experimental condition, cells were treated with either nothing or with 2.5 μg per mL of 14-3-3σ for 24 h and conditioned medium was then replaced with fresh medium with no 14-3-3σ. Cells were then harvested at 3, 6, 12, 24, 36, 48, and 72 after 14-3-3σ removal. In these three experimental conditions, cells were then harvested and the expression of collagenase mRNA was evaluated by northern analysis according to the procedure described above. The same blots were subsequently re-hybridized with a cDNA specific for 18S ribosomal RNA and used as an RNA loading control.

Detection of intra- and extracellular levels of 14-3-3σ protein by western Blotting To evaluate the intra- and extracellular forms of 14-3-3σ protein, conditioned medium collected from keratinocytes (0.5×10^6 cells per well) grown in KSFM supplemented with BPE and EGF for 48 h was replaced with test medium without FBS. FBS was removed from test medium because serum proteins interferes with electrophoresis of proteins present in conditioned medium. KCM was then collected on day 0, 2, 4, 6, 8, and 10 and corresponding cells were harvested. Conditioned medium was then passed through a Centricon YM-3 filter device (Millipore) and levels of the protein content of both cell extract and concentrated conditioned medium were determined and an equal amount (50 μg per sample) of total protein was subjected to SDS-PAGE analysis with 12% (wt/vol) acrylamide gel, and electrotransferred onto polyvinylidine difloride membranes (Millipore). Non-specific proteins on membranes were blocked in 5% skim milk powder in PBS–0.1% Tween-20 overnight. Immunoblotting was performed using 2 μg per mL of goat anti-human 14-3-3σ polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California), or 2 μg per mL of mouse anti-human actin monoclonal antibody (Santa Cruz Biotechnology). The membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-goat IgG (Sigma, St Louis, Missouri) or anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) antibodies (1:2500 dilution). Immunoreactive proteins were then visualized using the ECL$^+$ Western blotting detection system (Amersham Biosciences, Buckinghamshire, England).

Immunocytochemistry To perform immunohistochemical staining for stratifin and involucrin in cultured keratinocytes, 5×10^4 cells were grown in a wax circle drawn on glass slides (Snowcoat X-tra, Surgipath, Richmond, Illinois) and left to attach overnight in KSFM + BPE + EGF. The next day the slides were rinsed in PBS, and the medium was switched to test medium to promote differentiation. The medium was changed every 3 d. Cultured keratinocytes were examined for their phase contrast appearance by a Leica DBIRB microscope (Western Opti-Tech, San Francisco, California). Cells were then photographed on day 5 and 9 using a Spot Jr digital camera (Diagnostic Instruments, Heights, Michigan). On each day of harvest, slides were washed with PBS, fixed for 10 min. in acetone, and left to air dry. For immunohistochemical staining, the cultured keratinocytes were stained with a streptavidin–biotin immunoperoxidase procedure. For 14-3-3σ staining, slides were incubated overnight at 4°C in a humidified chamber with goat polyclonal anti-human 14-3-3σ (0.4 μg per mL; Santa Cruz), which is specific for stratifin. Control slides received nonimmune goat IgG (not shown). Biotinylated rabbit anti-goat (1:200, DakoCytomation, Carpintena, California) was used as the secondary antibody. For involucrin staining, mouse monoclonal anti-involucrin (1.6 μg per mL, Sigma) was used overnight using nonimmune mouse IgG (not shown) as a negative control. Biotinylated goat anti-mouse (1:200, Vector Laboratories, Burlingame, California) was the secondary antibody. Detection was performed using StreptABComplex/HRP (DakoCytomation), followed by incubation in DAB. Slides were briefly counterstained in hematoxylin. Stained

slides were examined using a Nikon Optiphot-2 microscope and were photographed using a Nikon Coolpix 990 digital camera (Nikon, Mississauga, ON, Canada). All digital images were analyzed using Quartz PCI, version 4 (Quartz Imaging, Vancouver, BC, Canada). For details, the areas of multilayer and monolayer forming keratinocytes from the low-magnification images were chosen as insert rectangular and then photographed with higher magnification.

This research was supported by the Canadian Institute for Health Research (A. Ghahary, E. E. Tredget, M. Weinfeld, and F. Karimi-Busheri).

DOI: 10.1111/j.0022-202X.2004.23521.x

Manuscript received January 14, 2004; revised June 26, 2004; accepted for publication July 21, 2004

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