Synchronous Activation of ERK and Phosphatidylinositol 3-Kinase Pathways Is Required for Collagen and Extracellular Matrix Production in Keloids*

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A previous study by our group demonstrated increased normal fibroblast (NF) and KK cell proliferation in defined serum-free medium co-culture with KKS compared with that in co-culture with normal keratinocytes, the latter of which was significantly higher than NF or KK cell proliferation in single cell culture (15). It was subsequently demonstrated that NFs and KFs exposed to KK paracrine secretions in co-culture secreted increased amounts of collagens I and III, which were again higher than in NF or KK single cell culture. The transmission electron microscopic morphology of collagen-ECM of NFs co-cultured with KKS approaches that of in vivo keloid tissue sections (13). More recent work from our group investigating the role of the insulin-like growth factor (IGF) system of mitogens in keloids demonstrated heightened activation of ERK and PI3K pathways in KFs co-cultured with KKS, resulting in increased KK proliferation (14). This activation was again greatly enhanced in co-culture compared with single cell culture. In that study, IGF-1 interaction with the IGF-1 receptor appeared to be modulated by the bioavailability of IGF-binding protein-3 (14).

From the above studies, it became clear that fibroproliferation and collagen-ECM production are closely related. We hypothesized that keloid collagen and ECM component production is also linked to a MAPK cascade, specifically the MEK-ERK and PI3K pathways. To test this hypothesis, four samples of KFs were co-cultured in defined serum-free medium with KKS for 2–5 days. KK cell lysate was submitted to Western analysis to assess the activity of these two pathways and their downstream components affecting cell transcription. The conditioned medium from the co-culture was assayed to investigate fibroblast production of collagens I–III, which form the main structural fibers in skin, as well as laminin β2 and fibronectin, which are linking glycoproteins that have roles in cell adhesion and differentiation and which are found predominately in the basal lamina, in the presence or absence of inhibitors of MEK1/2 (U0126) and PI3K (LY294002). Additionally, the activities of the stress-related MAPK pathways JNK and p38 kinase were also investigated using the p38 kinase inhibitor SB203580 and the JNK inhibitor SP600125 to determine their role in keloid collagen and ECM component production.

**EXPERIMENTAL PROCEDURES**

**Reagents**

U0126, LY294002, rabbit anti-phospho-Ser^187/189 MEK1/2, rabbit anti-phospho-Thr^202/Tyr^204 ERK1/2, rabbit anti-Akt, and mouse anti-angiokeratoma of Fordyce disease were purchased from New England Biolabs Inc. (Beverly, MA). SB203580 and SP600125 were supplied by Calbiochem-Novabiochem. Mouse anti-collagen I–III antibody was from Monosan (Am Duen, The Netherlands). Mouse anti-laminin β2 and mouse anti-fibronectin antibodies were supplied by Transduction Laboratories (Lexington, KY). Mouse anti-α-tubulin, rabbit anti-p38 kinase, rabbit anti-phospho-Tyr^182 p38 kinase, rabbit anti-JNK2, and rabbit anti-phospho-Thr^183/Tyr^185 JNK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated donkey anti-mouse and anti-rabbit secondary antibodies were purchased from Pierce. The chemiluminescence detection system (ECL) was supplied by Amersham Biosciences. Tissue culture Petri dishes, 6-well plates, and 96-well plates were purchased from Nunc (Naperville, IL). Medium, fetal bovine serum, and penicillin/streptomycin were from Invitrogen.

U0126, SB203580, and SP600125 are MEK1/2-specific, nonselective PI3K, p38 kinase-specific, and JNK-specific inhibitors, respectively. They were dissolved in dimethyl sulfoxide (with the final concentration never exceeding 0.1%) and stored frozen under light-protected conditions at −20 °C.

**Earlobe KKs and Fibroblast Data Base**

Four strains of keloid-derived fibroblasts (KF16, KF17, KF18, and KF48) and two strains of keloid-derived keratinocytes (KK18 and KK48) were obtained from excised earlobe keloid specimens. No patient had previously received any treatment for the keloids. A full histopathological and clinical examination were supplemented with color slide photo documentation. Written informed consent was obtained before operative excision, and a portion of all excised specimens was sent to the Singapore General Hospital Department of Pathology for histological confirmation of keloid identity.

**Cell Culture**

**Keratinocyte Culture with Earlobe Keloids—Passage 2 keratinocytes** were obtained from excised specimens as previously described (15). Briefly, excised earlobe keloid specimens were washed with Hank’s balanced salt solution containing 150 μg/ml gentamycin and 7.5 μg/ml Fungizone followed by plain phosphate-buffered saline until the solution became clear. The specimens were then cut into 5 × 10-mm pieces, and the epidermis was scored. Dissect (5 mg/ml) in Hank’s balanced salt solution was added, and the specimens were left overnight at 4 °C. The epidermis was carefully scraped off with a scalpel the next day and incubated in a solution of 0.25% trypsin, 0.1% glucose, and 0.02% EDTA for 10 min. Trypsin action was quenched by Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum upon intercellular separation. Suspended cells were transferred to tubes and centrifuged at 1000 rpm for 8 min. The cells were then isolated and seeded in keratinocyte culture medium (80 ml of Dulbecco’s modified Eagle’s medium supplemented with 20 ml of fetal bovine serum, 10 ng/ml epidermal growth factor, 1 × 10⁻³ ml cholera toxin, and 0.4 μg/ml hydrocortisone) at 1 × 10⁵ cells/cm² for 24 h before transfer to keratinocyte growth medium (Life Technologies Corp.). Cell strains were maintained and stored at −150 °C until used. Only cells from the second passage were used in all experiments.

**Fibroblast Culture with Earlobe Keloids—Remnant keloid dermis** was minced and incubated in a solution of collagenase I (0.5 mg/ml) and trypsin (0.2 mg/ml) for 6 h at 37 °C. Cells were pelleted and grown in tissue culture flasks. Cell strains were maintained and stored at −150 °C until used. Only cells from the second passage were used in all experiments.

**Keratinocyte-Fibroblast Co-culture—Keratinocytes** from samples KK18 and KK48 were thawed, centrifuged, and recounted. Cells were seeded at a density of 4 × 10⁵ cells/cm² on Transwell clear polyester membrane inserts (0.4-μm pore size, 0.25-cm² area; Corning-Costar). Cells were maintained for 4 days in serum-free keratinocyte growth medium until 100% confluent in monolayer. The medium was then changed to serum-free defined fibroblast growth medium, and the cells were raised to air-liquid interface for another 3 days, allowing keratinocytes to stratify and to reach terminal differentiation (15). Fresh fibroblasts from samples KF16, KF17, KF18, and KF48 were thawed and seeded on 24-well plates at a density of 5 × 10⁴ cells/well in defined fibroblast growth medium for 3–4 days until 100% confluent. Both the cultured keratinocytes on membrane inserts and the cultured fibroblasts on plates were washed twice with phosphate-buffered saline to remove the old medium before placing the inserts on the plates, complementing the KK-KF co-culture in fresh serum-free defined fibroblast growth medium with or without varying concentrations of U0126, LY294002, SB203580, or SP600125 for 2 or 5 days. At day 2 or 5, membrane inserts with the cultured keratinocytes were removed, and the conditioned medium was collected for collagen, laminin, and fibronectin analysis. Fibroblasts were also harvested for Western blot analysis.

**Western Blotting**

To detect collagen, fibronectin, and laminin in the conditioned medium, 1 ml of conditioned medium was concentrated using a Centricron centrifuge (Millipore Corp., Bedford, Mass.) and then separated by SDS-PAGE under reducing conditions and electroblotted onto nitrocellulose membrane. Blots were incubated with the indicated antibody and horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit secondary antibody (1:1500 dilution). Blots were visualized with the ECL chemiluminescence detection system as recommended by the manufacturer.

To examine the effects of U0126 on the expression of ERK1/2, phospho-Ser^187/189 MEK1/2, and phospho-Thr^202/Tyr^204 ERK1/2, LY294002 on the expression of Akt and phospho-Ser^473 Akt, SB203580 on the expression of p38 kinase and phospho-Tyr^182 p38 kinase, and SP600125 on the expression of JNK and phospho-Thr^183/JNK, NF cells plated at a density of 5 × 10⁶ cells were co-cultured with KKS for the indicated times (2 or 5 days) in defined fibroblast growth medium in the presence or absence of the indicated concentrations of...
**RESULTS**

**Co-culture of NFs or KFs with KKS Results in Increased Fibroproliferation and Collagen I–III Production**—As a confirmatory study on the basal proliferative behavior and collagen production characteristics of three of the KF test samples (KF16, KF17, and KF18), a 5-day co-culture with KFs (KF18) was performed. This was followed by cell proliferation assessment using the MTT assay (Fig. 1A) and Western blotting of the conditioned medium to assess collagen I–III production (Fig. 1B). KFs demonstrated increased cell proliferation and collagen I–III production in co-culture with KKS, and the levels were significantly higher than those obtained with NFs co-cultured with KKS. These results correlate with those of our previous studies (13, 15), establishing their expected behavior.

**Addition of the MEK1/2-specific Inhibitor U0126 Decreases KF Proliferation in Co-culture with KKS**—The proliferative characteristics of KF18 in co-culture with KKK18 over a period of 5 days in the presence or absence of the MEK1/2-specific inhibitor U0126 (10 μM) were assessed using the MTT assay as described above. In the absence of U0126, KF18 cell proliferation was progressive, whereas the addition of U0126 resulted in progressively decreasing cell proliferation (Fig. 3). In vivo, the reduction of cell proliferation was noted to be progressive with time as long as they were co-cultured with KKS, without the need for a 5-day co-culture. KF18 cell proliferation was noted to be progressively reduced by increasing concentrations of U0126, especially between 2 and 6 μM (p < 0.01) (Fig. 4A). Increasing concentrations of U0126 also progressively decreased ERK1/2 phosphorylation, which was synchronous with the falling KF proliferation rates above (Fig. 4C).
Increasing Concentrations of U0126 Decrease Fibronectin Production and Abrogate Collagen I–III and Laminin β2 Production—A 2-day co-culture of KF18 with KK18 in the presence or absence of increasing concentrations of the MEK1/2-specific inhibitor U0126 resulted in complete abrogation of collagen I–III production at 4 μM and above (Fig. 5A). Complete nullification of laminin β2 production was also seen upon the addition of U0126 at 2 μM and above (Fig. 5B). Fibronectin production was decreased by increasing concentrations of U0126, but was never completely abrogated. Of note were the different in-gel migration characteristics, which reflected a change in the molecular mass of the molecule (Fig. 5C). We speculate this to be due to a change in side chain moiety attachment, which might have been altered in the presence of MEK inhibition.

Increasing Concentrations of LY294002 Progressively Decrease Cell Proliferation and Akt-1 Activation—KF18 was co-cultured with KK18 for 2 days in the absence or presence of different concentrations of LY294002 (2, 4, 6, and 8 μM). Similar to the results obtained with U0126 inhibition, KF18 cell proliferation was noted to be progressively reduced by increasing concentrations of LY294002, with a significant difference seen especially between 2 and 4 μM (p < 0.01) (Fig. 6A). Increasing concentrations of LY294002 progressively decreased Akt-1 phosphorylation (Fig. 6C) and abruptly so beyond concentrations of 4 μM. Akt-1 production by itself was unchanged by the treatment (Fig. 4B).

Increasing Concentrations of LY294002 Abrogate Collagen I–III Production and Decrease Laminin β2 and Fibronectin Production—KF18 was co-cultured with KK18 for 2 days in the presence or absence of increasing concentrations of the PI3K-specific inhibitor LY294002. Complete abrogation of collagen I–III production was seen at concentrations of 4 μM and above (Fig. 7A). Laminin β2 production was substantially reduced upon the addition of LY294002 at 2 μM and above, but was not completely nullified (Fig. 7B). Similar to the situation of U0126 inhibition of ERK phosphorylation seen above, a decrease in fibronectin production associated with a change in the migration speed was seen in this instance, but fibronectin production was not abrogated (Fig. 7C).

U0126 Inhibition of MEK1/2 Is as Efficacious as LY294002 Inhibition of PI3K in Decreasing KP Proliferation and Abolishing Collagen-ECM Production in Co-culture with KKS—As a side-by-side comparison study, a 2-day co-culture of KF18 with KK18 was performed in the absence or presence of 8 μM U0126 or LY294002, with cell proliferation assayed using MTT. The concentration of 8 μM was selected for maximal inhibition of KF proliferation and collagen-ECM production based on the data above. The rate of cell proliferation of KF18 in co-culture with KK18 was suppressed in the presence of either inhibitor, with no statistical difference compared with KF18 in single cell culture. This was markedly diminished compared with KF18-KK18 co-culture in the absence of either inhibitor (Fig. 8A). A concurrent assay of KF18 cell lysate showed clear suppression of ERK1/2 and Akt-1 phosphorylation by their respective inhibitors in this comparison (Fig. 8, B–E). The conditioned media from the co-culture groups were assayed for collagens I–III, laminin β2, and fibronectin. Concurrent with earlier findings, collagen I–III and laminin β2 production was totally abolished in the presence of either U0126 or LY294002 (data not shown). Fibronectin production was diminished, but not totally abrogated; and in-gel migration speeds were diminished (data not shown). These data reinforce the suggestion that cross-talk between the MEK-ERK and PI3K pathways exists and that mutual activation of these two pathways is required for ECM secretion.

**Fig. 3.** Effects of U0126 on KK18-induced KF18 cell proliferation. KK18 cells were co-cultured with KF18 cells in the presence or absence of 10 μM U0126 as described under “Experimental Procedures.” KF18 cell proliferation was determined daily for 5 days using the MTT assay. Bars with different letters are significantly different from one another (p < 0.01). Data are expressed as the mean ± S.E. of six samples. Results shown are representative of three independent experiments.

**Fig. 4.** Effects of U0126 on KK18-induced KF18 proliferation and activation of ERK. KK18 cells were co-cultured with KF18 cells in the presence or absence of the indicated concentrations of U0126 for 2 days as described under “Experimental Procedures.” Cell proliferation of the study groups at day 2 of co-culture was determined using the MTT assay (A). Bars with different letters are significantly different from one another (p < 0.01). Data are expressed as the mean ± S.E. of six samples. Results shown are representative of three independent experiments. For analysis of activated ERK1/2, cells were harvested at day 2 and lysed for Western blot analysis as described under “Experimental Procedures.” Blots were incubated with mouse anti-ERK (B) and mouse anti-phospho (p)-Thr202/Tyr204 ERK1/2 (C) antibodies. Experiments were repeated three times with similar results.
Addition of SB203580, a p38 Kinase-specific Inhibitor, Has No Effect on Collagen, Fibronectin, or Laminin β2 Production—To further examine the involvement of the stress-related MAPK cascade p38 kinase in collagen-ECM production, KF48 was co-cultured with KK48 for 2 days in the presence or absence of the p38 kinase-specific inhibitor SB203580 at 10 or 15 μM. SB203580 appeared to reduce the levels of phospho-p38 kinase, but did not completely inhibit p38 phosphorylation at Tyr182 (Fig. 9, E and F). No significant changes were observed in the secreted levels of collagen, laminin β2, or fibronectin compared with the situation of KF48 co-cultured with KK48 in the absence of this inhibitor (Fig. 10, A–C).

Addition of SP600125, a JNK-specific Inhibitor, Has No Effect on Collagen, Fibronectin, or Laminin β2 Production—The role of the stress-related MAPK cascade JNK in collagen-ECM production was also investigated to complement the above study on the role of p38 kinase. To achieve this, KF48 was co-cultured with KK48 for 2 days in the presence or absence of the JNK-specific inhibitor SP600125 at 20 or 50 μM. SP600125 was seen to effectively reduce the levels of phospho-JNK (Fig. 9, G and H). Once again, no significant changes were observed in the secreted levels of collagen, laminin β2, or fibronectin compared with the situation of KF48 co-cultured with KK48 in the absence of this inhibitor (Fig. 10, A–C).

**DISCUSSION**

The hallmark of the keloid is aberrant, excess collagen-ECM deposition and accumulation, which are directly responsible for the gross appearance of this scar tissue. The ECM is a complex structural entity surrounding and supporting mammalian cells comprising collagen, multi-adhesive matrix proteins, proteoglycans, and hyaluronan. These ECM components have complex interactions not just with each other, but also with the cells that they envelop, in the form of cell adhesion, cell-cell signaling, tissue function, and wound repair (21). In the skin, the main structural proteins are collagens I and III, predominantly secreted by fibroblasts and to a small degree by epithelial cells. The laminins and fibronectin are linking glycoproteins that anchor keratinocyte and fibroblast surfaces to the basal lamina in close relationship to collagen IV, which forms the main structural protein for the basement membrane-basal lamina complex responsible for the adherence of the epidermis to the underlying dermis. In addition to cell adhesion, laminins and fibronectin are also important for cell migration and differentiation (22).

Collagen-ECM secretion in fibroblasts has been extensively investigated in osteoblasts, the specialized fibroblasts of bone, and appears to be regulated by a variety of cytokines, including transforming growth factor-β and bone morphogenetic protein-2, via signals through their interaction with specific serine/threonine kinase cell-surface receptors (23, 24), which also control osteoblast cell proliferation and differentiation in vitro. The α1(I) collagen gene appears to be up-regulated by downstream activation of MAPKs (specifically the ERK kinase-ERK pathway) via protein kinase C and phosphorylated tyrosine kinase.
kinase pathways (25). In terms of aberrant fibroproliferation, as seen in liver cirrhosis, acetaldehyde appears to activate the ERK and PI3K pathways via protein kinase C, resulting in the up-regulation of \(\alpha_2(I)\) collagen (26). MAPKs may also be activated by a mechanically coupled transcriptional circuit via intercellular integrins to induce the expression of filamin A in fibroblasts (27). There are four other distinguishable MAPK modules in mammalian cells in addition to ERK that regulate cell growth and differentiation: the JNK and p38 kinase cascades, which are regulated by inflammatory cytokines and cellular stress, and the ERK3 and ERK5 cascades, which are related to serum stress and other as yet undefined stimuli (28). At least one component of aberrant fibroproliferation in scleroderma has recently been found to be p38 kinase-dependent via transforming growth factor-\(\beta\), regulating collagen I mRNA and \(\alpha_2(I)\) collagen promoter activity (29). The ERK and p38 kinase cascades, modulated by HSP27, additionally appear to play important roles in wound contraction in rat fibroblasts (30). Overall, it appears that the primordial MAPK pathways can be used in several permutations, as in the case of ERK, via single or the more recent dual specificity kinases, to achieve the ultimate task of proliferation and differentiation via \(\text{jun} \) and \(\text{fos} \) phosphorylation and attachment to the DNA promoter sequence to allow the binding and production of mRNA by RNA polymerase (31, 32).

The role of PI3K is similarly diverse in the fibroblast. Cell viability regulation by \(\beta_1\) integrin interaction with the ECM in response to mechanical forces in skin fibroblasts appears to be dependent, at least in part, on PI3K-Akt-protein kinase B signaling pathway activation (33). PI3K has also been found to be responsible for stimulation of collagen synthesis by active fibroblast cell spreading on culture plates and platelet-derived growth factor-BB (34). In terms of aberrant fibroproliferation, it has been reported that the PI3K pathway is activated in inflammatory lung conditions after lung fibroblast exposure to serum components or effector substances such as insulin-re-
lated peptides and transforming growth factor-

In keloids, fibroproliferation was recently shown to be regu-

The PI3K-Akt signaling pathway was also found to be activated in synchrony (14). In the case of IGF-1, activation of PI3K results in the formation of phosphatidylinositol 3-phosphate, which can serve as a signal for cell growth via its anti-apoptotic influence (36), complementing the promitogenic MEK-ERK pathway. This combined effector cascade appears to be quite significant in tumor biology (37, 38).

Our results confirm our earlier findings (13–15) that KFs do not produce collagen in isolation, that KFs constitutively produce collagens I–III, and that thisKF collagen production is increased in co-culture with KFs. The interesting finding of abrogation of collagen I–III production by blocking either MEK1/2 or PI3K with its inhibitor points to a yet unexplored element: the potential of cross-talk between these two pathways. Indeed, this interaction between the MEK-ERK and PI3K pathways appears to affect laminin β2 production to a similar degree, with a significant decrease in laminin β2 protein production upon introduction of the PI3K inhibitor LY294002 and complete abrogation upon the addition of the MEK1/2 inhibitor U0126. Further investigation into the role of the cellular stress- and inflammation-induced JNK and p38 kinase cascades by addition of their inhibitors SP600125 and SB203580, respectively, in a similar co-culture system showed that these pathways do not appear to be involved in collagen and ECM component production in this co-culture model.

Not all elements of the ECM are affected in the same way, however. In the case of fibronectin, the in-gel migration speed is changed, and we postulate that this might be attributable to loss of glycosylation or glycophosphorylation of the side chains of the molecule. The implication here is that whereas absolute production may not be related to either the MEK-ERK or PI3K pathway, both pathways may play an important role in the post-translational modifications to fibronectin, ultimately affecting its molecular mass. Supporting this last statement are the results from another study in which acetaldehyde-induced fibronectin production in hepatic stellate cells was seen to be inhibited by the addition of calphostin C (a protein kinase C inhibitor), but not by PD98059 (a MEK inhibitor) or wortman- nin (a PI3K inhibitor), implying a different intracellular pathway branching downstream of protein kinase C (26).

Overall, it can be seen that the discrete MEK-ERK and PI3K pathways appear to play a role in the generation of some (but not all) collagen-ECM components in keloids. To our knowledge, this is the first report establishing a link between MEK-ERK and PI3K activation and collagen-ECM production in this...
fibroproliferative lesion. In addition, it appears that both pathways need to be synchronously activated for collagen I–III and full laminin β2 production. The stress-induced JNK and p38 kinase cascades additionally do not appear to be involved in this fibroproliferative process. A clearer understanding of the intracellular pathways to fibroproliferative scarring will shed more light on this very basic cellular synthetic process and perhaps suggest a new direction for preventing excess collagen-ECM production and deposition in keloids. The identities of the candidate factors triggering keloid fibroblast MEK-ERK and PI3K pathway activation are currently being actively pursued.

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