Role of IGF system of mitogens in the induction of fibroblast proliferation by keloid-derived keratinocytes in vitro

Toan-Thang Phan,1 Ivor Jiuin Lim,2 Boon Huat Bay,3 Robert Qi,4 Michael Thornton Longaker,5 Seng-Teik Lee,6 and Hung Huynh7
1National Burns Centre, 2Department of Plastic Surgery, Singapore General Hospital, Singapore 169608; 3Division of Plastic Surgery, Departments of Surgery and 4Anatomy, National University of Singapore, Singapore 119260; 4Institute of Molecular and Cell Biology, Singapore 117609; 7Laboratory of Molecular Endocrinology, Division of Cellular and Molecular Research, National Cancer Centre of Singapore, Singapore 169610; and 5Department of Surgery, Stanford University, Stanford, California 94305-5148

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Phan, Toan-Thang, Ivor Jiuin Lim, Boon Huat Bay, Robert Qi, Michael Thornton Longaker, Seng-Teik Lee, and Hung Huynh. Role of IGF system of mitogens in the induction of fibroblast proliferation by keloid-derived keratinocytes in vitro. Am J Physiol Cell Physiol 284: C860–C869, 2003. First published December 21, 2002; 10.1152/ajpcell.00350.2002.—Keloids are proliferative dermal growths that represent a pathological wound-healing response. We report high proliferation rates in normal (NP) and keloid-derived fibroblasts (KF) cocultured with keloid-derived keratinocytes (KK). IGF binding protein (IGFBP)-3 mRNA and secreted IGFBP-3 in conditioned media were increased in NP cocultured with KK compared with NP but markedly reduced in KF cocultured with KK or normal keratinocytes (NK). IGFBP-2 and IGFBP-4 mRNA levels were elevated, whereas IGFBP-5 mRNA was decreased in KF cocultured with KK or NK. Significant increases in IGFBP-2 and -4 mRNA in KF cocultured with KK did not correlate with protein secretion. Downstream IGF signaling cascade components, phospho-Raf, phospho-MEK1/2, phospho-MAPK, PI-3 kinase, phospho-Akt, and phospho-Erk-1, were elevated in KF cocultured with KK. Addition of recombinant human IGFBP-3 or antibodies against IGF-I or IGF-IR significantly inhibited proliferation of KF. The bioavailability of IGF-I may be related to the levels of IGFBP-3 produced, which in turn influences KF proliferation, suggesting that modulation of IGF-I, IGF-IR, and IGFBP-3, individually or in combination, may represent novel approaches to the treatment of keloids.

Insulin-like growth factor; coculture

Keloids are proliferative dermal growths that represent a pathological wound-healing response to skin injury in susceptible persons. Keloid scars have afflicted humans for centuries (32) and are a disease entity that has, as yet, no animal model. Keloid tissue has long been noted to extend beyond the borders of the original wound, not to spontaneously regress, and, notoriously, to be prone to recurrence (12). The difficulty in treating keloids can be seen by the large numbers of largely empirical modalities of treatment (4, 40). Much has been written about the epidemiology of keloids (4, 45), and much work has been done to elucidate its microstructure (26–28). The biochemical composition of keloids has also been extensively investigated (1, 10). The exact pathophysiological mechanism for keloid formation, however, remains largely unknown, although tension has been suggested to be one factor involved, another being skin pigmentation (45).

Epithelial-mesenchymal interactions between keratinocytes and fibroblasts in the context of normal skin have been the subject of intensive study in the past few decades (33, 34), spurred on by the realization that keratinocyte secretions not only have autocrine and paracrine influences on local growth, inflammation, and metabolism but may also have far-reaching endocrine effects on the systemic immune system (37). The introduction of two-chamber, serum-free coculture techniques has allowed the identity of these protein factors to be elucidated (22, 24). We extrapolated this concept of epithelial-mesenchymal interactions to keloids in two recent in vitro studies examining the possible effects of such interactions on normal fibroblasts (NF) in serum-free, two-chamber coculture with normal or keloid-derived keratinocytes (KK). We demonstrated significantly higher rates of proliferation (31), as well as a significant increase in soluble extracellular-matrix collagen production by NF cocultured with KK (30a).

IGF-I and IGF-II are mitogens and inhibitors of apoptosis for many cell types, including keloid-derived fibroblasts (KF) (9, 19, 25). Acting on IGF receptors, their bioactivity is modulated by the presence of specific IGF binding proteins (IGFBPs), which bind to them with high affinity. At least six IGFBPs have been described (reviewed in Refs. 9, 25), and these are found in many physiological fluids, as well as in the conditioned media of a wide variety of cell types in culture (9). IGFBPs may also have functions unrelated to IGF binding; an example being IGFBP-3, the most abundant binding protein in the serum, which has growth-dant binding protein in the serum, which has growth
inhibitory activity that operates independently of IGF (reviewed in Refs. 9, 18).

Most of the biological effects of IGF-I and -II are mediated by the IGF-I receptor, and IGF-I has been shown to protect cells from apoptosis in vitro and in vivo (9, 19). The IGF-I receptor possesses tyrosine kinase activity and autophosphorylates after binding with IGF-I. The initial postreceptor signal transduction event is the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) (20). This is followed by the initiation of two signaling cascades involving phosphatidylinositol-3 kinase (PI-3K) and Ras/Raf/MAPK (7). Activation of PI-3 kinase results in the formation of phosphatidylinositol-3-phosphate, which can serve as a signal for cell growth (9). The Ras/Raf/MAPK pathway is activated by the tyrosine phosphorylation of IRS-1, with the resultant formation of the IRS-1-growth factor receptor-bound protein-2 (Grb2)-Son of Sevenless (Sos; a guanine nucleotide exchange factor and Ras-activating factor) complex that activates Ras, which in turn binds to and activates Raf, subsequently phosphorylating and activating MAPK. Phosphorylated MAPK, in turn, transmits a signal to the nucleus, with resultant progression of the cell cycle and cell proliferation (9). A wide variety of cells demonstrates this mitogenic response including keratinocytes and fibroblasts (11). It has been suggested that while the MAPK pathway primarily mediates cell proliferative responses to growth factors such as the IGFs, the PI-3K pathway largely mediates the anti-apoptotic effects of the IGFs (30). In the context of skin, IGF-I has been identified as the active paracrine growth-promoting factor secreted by feeder fibroblasts for keratinocyte culture in vitro (3) and was observed to stimulate the nondirectional migration of keratinocytes (2). Treatment of wounds with IGF-I has also been shown to accelerate healing by the stimulation of fibroblast collagen synthesis in addition to its mitogenic effect on keratinocytes and fibroblasts (14, 36). Furthermore, IGF-I receptors have been shown to be involved in the invasiveness of KF and are protective against ceramide-induced apoptosis (19, 43, 55).

On the basis of this information and on the findings of our earlier studies, we hypothesized that the IGF system might play a role as an epithelial-mesenchymal mediator in keloid pathogenesis. We undertook this study using the established in vitro keratinocyte-fibroblast coculture model we used in our previous studies (22, 24, 30a, 31) to examine the possibility that the proliferative effects on NF and KF by KK might be related to changes in the IGF system of mitogens.

MATERIALS AND METHODS

Earlobe KK and fibroblast database. Three lines of keratinocytes (KK4, KK5, KK7) and fibroblasts were randomly selected from a bank of keratinocyte-fibroblast cell strains derived from previously excised earlobe keloid specimens of 23 patients. No keloids had been treated before surgical excision. A full history and examination with color photo documentation and informed consent were taken before the operation. A portion of all specimens was sent to the hospital's Department of Pathology for histological confirmation of keloid identity.

Keratinocyte and fibroblast culture. Passage 2 KK and fibroblasts were isolated from excised earlobe keloid samples as previously described (31). Normal keratinocytes (NK) and NF were randomly derived from a bank of foreskin circumcision specimens of healthy young children, again as previously described (31).

Keratinocyte-fibroblast coculture. Keratinocytes obtained from the three randomly selected keloid strains (KK4, KK5, KK7) were seeded at a density of 4 × 10^5 cells/cm^2 on Transwell clear polyester membrane inserts with 0.4-μm pore size and an area of 0.3 cm^2 (Costar). Cells were maintained for 4 days in serum-free keratinocyte growth medium until 100% confluent in monolayer. The medium was then changed, and the cells were maintained in serum-free defined fibroblast growth medium (DFGM) for a further 3 days, in which time the cells were raised to air-liquid interface to allow keratinocytes to stratify and reach terminal differentiation. NF were seeded in six-well plates at a density of 5 × 10^4 cells/well in DFGM for 3 to 4 days until 100% confluent. One series of NF was seeded on Transwell clear polyester membrane inserts in the manner described above for keratinocytes for the purpose of a fibroblast-fibroblast coculture control.

Cells on both the membrane inserts and the wells were washed twice with PBS to remove the old medium before combination of the inserts and plates for coculture in serum-free DFGM. Controls comprised one series of NF without keratinocyte coculture as a negative control, one series of NF cocultured with NF, and one series of NF cocultured with NK. After 5 days of coculture, inserts with the cultured cells on membrane were removed and the conditioned medium was collected for IGF-I and IGFBP analysis by Western blot. Fibroblasts from the wells were harvested for protein and

Fig. 1. Effects of normal fibroblasts (NF), normal keratinocytes (NK), and keloid-derived keratinocytes (KK) on NF and keloid-derived fibroblast (KF) proliferation. NF and KF in 6-well plates were cocultured, in defined serum-free medium, with NF, NK, and 3 individual KK strains (KK4, KK5, and KK7) grown on permeable membrane inserts for 5 days. At the end of 5 days, the permeable membrane inserts with attached cells were removed, and the lower chamber fibroblasts were assayed for proliferation using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. Bars with different letters are significantly different from one another at P < 0.01. Data are expressed as means of 6 samples ± SE. Results shown are representative of 3 independent experiments.
RNA extraction for Western or Northern blot analysis, respectively.

To determine the effect of exogenous IGFBP-3 and paracrine/autocrine IGF-I production on keloid fibroblast proliferation, KF were cocultured with KK as described above. Anti-IGF-I (1.5 μg/ml), anti-IGF-IR (1.5 μg/ml) (R&D Systems), or 1.2 μg/ml human recombinant IGFBP-3 (hrIGFBP-3) was added to the KK/KF coculture medium, after which cocultured cells were incubated for 5 days and then subjected to [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay.

Cell proliferation assay. MTT assay is a colorimetric assay that tests the metabolic activity of viable cells and is used for indirect cell quantification (38). This was used in a previous study as described (31) and was used in this study to assess fibroblast proliferative response, in coculture conditions, to exogenous hrIGFBP-3, as well as to antibodies against IGF-I and IGF-IR.

Western blotting. To determine changes in the expression of IGF-IR, PI-3K p85, MAPK, Akt, phospho-Akt (Ser473), phospho-Raf (Ser259), phospho-MEK 1/2, phospho-p44/42 MAP kinase (Thr202/Tyr204), and phospho-Elk-1 (Ser383), cocultured NF and KF were lysed in lysis buffer (1 mM CaCl₂, 1 mM MgCl₂, 1% NP-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM PMSF, and 100 μM NaVO₄). Proteins were subjected to Western blot analysis as previously described (16). To detect IGFBPs in conditioned media, 150 μl of conditioned media were 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 (1:7,500). All primary antibodies (except α-tubulin, which was used at a concentration of 0.5 μg/ml) were diluted in TBST at a concentration of 1 μg/ml. Blots were visualized with a chemiluminescent detection system as described by the manufacturer (ECL, Amersham). Rabbit anti-PI-3K p85, rabbit anti-IGF-IR beta, rabbit anti-c-Raf, mouse anti-α-tubulin, anti-phospho-Elk-1 (Ser383), and rabbit anti-IGF-I antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Rabbit anti-human IGFBP-3, -4, and -2 antibodies were obtained from Upstate Biotechnology, Lake Placid, NY. Mouse anti-phospho-specific MAPK (Thr202/Tyr204), rabbit anti-phospho-Raf (Ser259), rabbit anti-phospho-Akt (Ser473), rabbit anti-phospho-MEK 1/2 (Ser217/221), rabbit anti-Akt, and mouse anti-MAPK antibodies were from New England Biolabs, Beverly, MA.

Northern blotting. Total RNA was isolated from cocultured NF and KF, and Northern blotting was performed as described (17). Blots were hybridized with IGF-I (39) and IGFBP-2, -3, -4, and -5 (46) cDNAs. To control for equal RNA loading, blots were rehybridized with GAPDH cDNA (ATCC). Quantitative analysis of gene expression was accomplished by scanning autoradiograms followed by computerized optical densitometry. For each lane, the sum of the density of the bands corresponding to transcripts hybridizing with the probe under study was calculated and normalized to the amount of RNA loaded.

Statistical analysis. All experiments were performed in quadruplicate with the results reflecting means ± SE. For quantitative analysis, the sum of the density of the bands corresponding to the protein from cell lysate blotted with the antibody under study was calculated and normalized to α-tubulin. IGFBP mRNA levels were expressed as a ratio of...
the integrated density of IGFBP mRNA to GAPDH products. Differences in gene expression and cell number were tested using the Mann-Whitney U-test.

RESULTS

Fibroblasts proliferate faster in coculture with KK compared with NK. Figure 1 shows the results of MTT assay of the study and control groups after 5 days of coculture. Controls of NF not in coculture or cocultured with NF did not proliferate significantly in serum-free medium, although the cells remained viable. NF in coculture with NK or KK, however, showed significantly increased cell numbers compared with the previously mentioned controls (P < 0.01). Growth stimulation of NF was more pronounced in coculture with KK than with NK or NF. KF in single-cell culture or cocultured with NF also did not show marked proliferation at day 5 in serum-free media. Significant fibroblast proliferation was, however, seen when KF were cocultured with KK (P < 0.01) compared with NK. Effects of keratinocyte coculture combinations on fibroblast proliferation were ranked as follows: KK/KF coculture showed the fastest proliferation rates, followed by KK/NF, NK/KF and NK/NF [where the numerator represents the keratinocytes in the upper chamber (NK or KK) and the denominator represents the fibroblasts in the lower chamber (NF or KF)].

Fibroblast IGFBP but not IGF-I mRNA levels change when fibroblasts are cocultured with keratinocytes. With the background knowledge that the IGFs are potent mitogens for fibroblasts and keratinocytes (36) and that IGFBPs modulate IGF activity (9), IGF-I and IGFBP expression in NF and KF cocultured with KK were examined. Figure 2 shows that the basal levels of IGFBP-2, IGFBP-4, and IGFBP-5 mRNA in KK were all higher than that observed in NF. IGFBP-5 gene expression was barely detected in NF. The opposite was true for IGFBP-3 mRNA which was lower in KF. Coculture of NF or KF with NK or KK induced IGFBP-2 gene expression in both NF and KF. The origin of the fibroblasts had profound effects on the levels of IGFBP-3 and IGFBP-4 induced by coculture.
IGF-IR secretion changes when fibroblasts are cocultured with keratinocytes. To examine whether IGFBP secretion was altered when fibroblasts were cocultured with keratinocytes, conditioned media were assayed using Western blot analysis (Fig. 3). Single-cell culture NF secreted lower levels of IGFBP-2, -3, and -4 compared with single-cell culture KF. When NF were cocultured with either NK or KK, a significant increase in IGFBP-3, -2, and -4 was observed compared with NF, single-cell culture or NF cocultured with NF (P < 0.01). There were, however, no significant differences in the levels of these three IGFBPs between NK cocultured with NF and KK cocultured with NF.

No significant differences in IGFBP-3, -2, and -4 levels in conditioned media were seen between single-cell culture KF or cocultures of NF/KF and KK/KF (Fig. 3). KK/KF coculture, however, resulted in significantly elevated levels of IGFBP-2 (P < 0.01), but IGFBP-4 was only mildly increased in the conditioned media; IGFBP-3 was, interestingly, significantly reduced in KK cocultured with KF (P < 0.01) (Fig. 3). Correlating this information with the earlier Northern blot, as no IGFBP-3 in the conditioned media came from the NK or KK (Fig. 3B).

Levels of fibroblast IGF-IR and its downstream proteins change in coculture with keratinocytes. As the PI-3 kinase and the signal Ras/Raf/MAPK pathways are important for IGF-induced mitogenesis and anti-apoptosis (30, 53, 54), levels of IGF-IR, PI-3 kinase, Akt, MAPK, phosphorylated Akt, phosphorylated Raf, phosphorylated MEK 1/2, phosphorylated MAPK, and phosphorylated Elk-1 were investigated by Western blot analysis of NF and KF cell lysate. Figures 4B and 5, A and B, show that the levels of IGF-IR in both NF and KF lysates were significantly increased when NF or KF were cocultured with NK or KK (P < 0.01). The IGF-IR levels were significantly higher in the KK/KF groups compared with KK/NF groups (P < 0.01). Coculture of NF with NK yielded IGF-IR levels similar to that of NF single-cell culture; KF from NK/KF coculture, however, showed a significant increase in IGF-IR compared with KF single-cell culture (P < 0.01) (Fig. 4B). The p85 subunits of PI-3 kinase and Akt levels were not significantly altered in NF lysates in all the culture combinations. A slight increase in PI-3 kinase p85 was, however, observed in KF cocultured with NK or KK (Fig. 4C). Total c-raf, MAPK, MEK1, and Elk-1 did not change in coculture conditions.

When NF or KF were cocultured with either NK or KK, a significant increase in basal phosphorylated Akt protein was observed compared with single-cell cultures (P < 0.01) (Figs. 4D and 5, A and B). Coculture of NF with NK or KK resulted in slight increase in phosphorylated Raf levels compared with that of NF single-cell culture (Figs. 5C and 6B). KF from KK/KF coculture, however, showed a significant increase in phosphorylated Raf levels compared with single-cell culture KF, NF, or NK/KF coculture (P < 0.01) (Figs. 5D and 6B). Basal phosphorylation of MEK 1/2 was slightly reduced in NF cocultured with KK (Figs. 5C and 6D), which was not the case in KF cocultured with KK, where a slight increase in phosphorylated MEK 1/2 was seen (Figs. 5D and 6D).

Basal phosphorylation of MAPK was higher in NF than KF single-cell culture (Fig. 6F), which was in-
creased in NF cocultured with KK (P < 0.01) (Figs. 5E and 6F). Phosphorylated MAPK was, however, significantly increased when KF were cocultured with KK compared with KF single-cell culture (P < 0.01) (Figs. 5F and 6F). Mild induction of MAPK phosphorylation was also seen when KK were cocultured with NF or NK. As Elk-1 is the substrate for MAPK, the levels of phosphorylated Elk-1 were also determined. Phosphorylation of Elk-1 was found to be significantly elevated when KF or NF were cocultured with KK (P < 0.01) (Figs. 5, E and F, and 6H). Phosphorylated Elk-1 levels were also significantly elevated when KF but not NF were cocultured with NF or NK (P < 0.01). The levels of phospho-Elk-1 were significantly different between the KK/KF groups and the NF/KF or NK/KF groups (P < 0.01).

Fig. 5. Quantitative analysis of IGF-IR, phosphorylated Akt, phospho Raf, phospho MEK 1/2, phospho MAPK, and phospho Elk-1 in NF and KF. NF and KF were cocultured with NF, NK, and 3 individual KK strains (KK4, KK5, and KK7) for 5 days as described. Lower chamber NF and KF were then harvested, extracted, and cell lysates were subjected to Western blot analysis. Blots were incubated with rabbit anti-human IGF-IRβ (1 μg/ml), rabbit anti-phospho-Akt (Ser473), rabbit anti-phospho-Raf (Ser259) (1 μg/ml), rabbit anti-phospho-MEK 1/2 (Ser217/221) (1 μg/ml), mouse anti-phospho-MAPK (Thr202/Tyr204) (1 μg/ml), and rabbit anti-phospho-Elk-1 (Ser383) (1 μg/ml) antibodies. Densitometric scanning of the IGF-IR and phospho-Akt (A and B), phospho-Raf and phospho-MEK 1/2 (C and D), and phospho-MAPK and phospho-Elk-1 (E and F) bands is shown. Bars with different letters are significantly different from one another at P < 0.01. Data are expressed as means of 4 samples ± SE. Results shown are representative of 3 independent experiments.

Fibroblast proliferation in coculture is attenuated by the addition of anti-IGF-I, anti-IGF-IR, and hrIGFBP-3. To determine if the production of IGF-I and IGFBP-3 by KK and/or KF might play a role in KF proliferation, hrIGFBP-3 and antibodies against IGF-I and IGF-IR were added to the defined serum-free medium of KF cocultured with KK at day 0 and incubated for 5 days. As can be seen in Fig. 7, basal proliferation of KF was significantly attenuated in the presence of either hrIGFBP-3 or anti-IGF-I or anti-IGF-IR antibodies (P < 0.01). These results suggest that the proliferation of KF in coculture with KK may at least, in part, be a consequence of paracrine/autocrine stimulation mediated by IGF-I expression and that IGFBP-3 inhibits KF proliferation by reducing IGF-I bioavailability.
DISCUSSION

After injury, the secretory role of the epidermis undergoes dramatic change to stimulate local and systemic responses (29). These epidermal secreted factors are essential for the proliferation of adjacent mesenchymal cells, including fibroblasts, for the process of repair (6). Ultimately, the function of this epidermal response is to return both skin and body to their uninjured state; in the skin, this results in wound healing and the production of scar tissue. In predisposed individuals, such wound healing becomes pathologically excessive, with resultant keloid scar formation.

Excess deposition of collagen and other extracellular matrix molecules by fibroblasts has been thought to be responsible for the gross appearance of this thick scar tissue that exceeds the boundaries of the original wound (51). This process may start early in the wounding phase when hemostatic fibrin is laid down in the coagulation pathway but is not efficiently lysed by an effete fibroblast population deficient in plasminogen activator but high in inhibitor activator (52). The inflammatory phase itself may be enhanced by neutrophil or macrophage-released profibrotic cytokines, such as platelet-derived growth factor, transforming growth factor-β, and IGF-I, acting on fibroblasts (8, 49). Macrophage release of interleukin-1, important for extracellular matrix degradation, may also be reduced (37). Apart from primary alterations to keloid function, immunological processes in the skin may additionally have a role in keloidogenesis as a result of disordered immune surveillance (21). Interestingly, keloid-prone patients have been shown to have a higher incidence of allergic symptoms and increased numbers of mast cells (44, 47).

Given that the role of the fibroblast is important in the secretion of collagen and other extracellular matrix proteins and that abnormalities in its function may be the result of aberrations in the cytokine milieu, we postulated that the overlying epidermis, in an altered state, might have a role in the modulation of fibroblast activity similar to that of epithelial-mesenchymal in-
interactions in normal skin. In our first study, increased proliferation of NF and KF was seen when these two cell types were cocultured with KK compared with NK. Cohen and Peacock (10) suggested that KF might represent a subgroup of increased collagen-producing fibroblasts, which have either "gone awry" or have been preferentially selected during the process of acute inflammation. KK may, similarly, be the end result of a similar "accident" or selection process in the susceptible patient, with protein growth factor secretion profiles different from that of NF, which in turn affect underlying dermal fibroblast growth and collagen secretion to produce the keloid lesion. This theory was supported by further studies that showed increased secretion of soluble collagen I and III in both NF and KF cocultured with KK. In addition, transmission electron microscopic sections also showed that the pattern of collagen deposition closely resembled that of in vivo keloid tissue (30a).

In this in vitro study, we demonstrated that NF and KF proliferation was increased in coculture with KK compared with coculture with NK, to fibroblast single-cell cultures, and to fibroblasts cocultured with fibroblasts. This was in agreement with our previous report that the regulatory control of fibroblasts by KK is biased toward growth and proliferation (31). This study also showed that the IGF system of mitogens was abnormally regulated when KF were cocultured with KK, which was different to that when NF were cocultured with KK. Induction of KF proliferation by KK was associated with increases in IGF-IR, IGFBP-2, and IGFBP-4 gene expression with inhibition of IGFBP-5 and IGFBP-3. Significant differences were especially noted in IGFBP-3. Gene expression of this protein was upregulated in NF cocultured with KK or NK but was markedly downregulated when KF were cocultured with KK. Western blot analysis of the conditioned media confirmed that IGFBP-3 protein secretion was equivalently affected. IGF-IR was also significantly upregulated in KK/KF coculture compared with KK/NF coculture, in agreement with the findings of Yoshimoto et al. (55). Also interesting was the finding of significantly increased basal phosphorylation of p42/p44 MAPK, phosphorylated Akt, and Elk-1 KK/KF coculture compared with KK/NF coculture. This difference in the expression of the IGF system of mitogens when identical KK were cocultured with the two different subtypes of fibroblasts strongly supports our earlier hypothesis of a feedback system from fibroblasts that are able to change the keratinocyte secretory profile.

As single-cell cultures of fibroblasts have low to undetectable levels of IGF-1 mRNA, it is very likely that the detectable IGF-1 from conditioned media of keratinocyte/fibroblast coculture is from the keratinocyte. It also appears that IGFBP-3 production is induced in fibroblasts by the presence of keratinocytes in addition to its production from an autocrine feedback loop. Our data, which demonstrate a consistent relationship between IGFBP-3 levels and the proliferation of the two fibroblast subtypes when cocultured with KK, might be explained as follows. The significant but weaker stimulatory activity of KK on NF proliferation is associated with the inability of the former to suppress IGFBP-3 expression in the latter in the presence of a constant level of IGF-1, whereas greater proliferation is seen in KF, which downregulates IGFBP-3 in the presence of KK, allowing greater binding of IGF to its receptor, which is itself upregulated in this situation, on the KF cell surface. Given the importance of IGF-1 in cell proliferation and anti-apoptosis (30), increasing IGF-1 bioactivity by the downregulation of IGFBP-3 will thus have consequences on fibroblast proliferation. This theory was supported by the inhibition of KK proliferation in KK/KF coculture by the addition of exogenous hrIGFBP-3, which strongly implies its important role in the regulation of KF cell growth in vivo. It may also be possible that IGFBP-3 inhibits KF proliferation by binding to a putative IGFBP-3 receptor on the KF cell surface, activating growth inhibitory signal transduction pathways (41, 42). However, these mechanisms are not mutually exclusive and both may be relevant in vivo.

The functional significance of IGFBP-4, IGFBP-2, and IGFBP-5 in the skin and specifically in keloids is unknown. In a review by Clemmons (9), both inhibition and potentiation of IGF activity by IGFBP-2 and IGFBP-5 have been reported. In addition, IGFBP-4 has been shown to consistently inhibit IGF-1 action in other experimental systems (9). Our data demonstrated that IGFBP-2 expression is stimulated by keratinocyte/fibroblast coculture and that a positive correlation exists with fibroblast proliferation. Interestingly, IGFBP-2 levels were previously found to be elevated in the sera of patients with cancer (reviewed in Ref. 9), and over-

![Graph](image-url)
expression of IGFBP-2 resulted in the increased tumorigenic potential of adrenocortical cells (15). It is possible that increased IGFBP-2 might have similarly potentiated the response of the fibroblast to IGF-I in this study, which also demonstrated an inverse correlation between IGFBP-5 expression and fibroblast proliferation induced by KK. Although the precise role of IGFBP-5 in keloid formation is unknown, it is interesting to note that IGFBP-5 expression was previously found to be elevated in breast epithelial cells undergoing apoptosis (48). The rates of IGFBP-2 and -4 mRNA expression in the KK cocultured with NK or KK did not correlate with the levels of IGFBP-2 and -4 protein detected in the coculture conditioned medium, suggesting that keratinocytes also have an effect on the rates of IGFBP-2 and -4 biosynthesis in KK.

IGF-I is a mitogenic and anti-apoptotic agent for fibroblasts and keratinocytes and has been shown to accelerate wound healing by the stimulation of collagen synthesis by fibroblasts (14, 36). Our present study demonstrates increased IGF-IR expression in KK cocultured with KK, facilitating activation of the IGF signal cascade and related downstream events, which positively correlates with cell proliferation. This increase in IGF-IR expression in KK as a result of paracrine communication between KK and KK thus facilitates KK proliferation and inhibits apoptosis, a hypothesis that is supported by previous observations showing that overexpression of IGF-IR in KK resulted in increased invasive activity of KK and resistance to ceramide-induced apoptosis (19, 43, 55). Furthermore, MAPK activation by activated IGF-IR results in the nuclear translocation of the enzyme, which in turn phosphorylates transcription factors such as Elk-1 and other kinases, leading to the induction and expression of new genes (5, 35, 50), which have been demonstrated in other studies to be required for the IGF-I-induced proliferation of a variety of cells (13).

In summary, we demonstrated, for the first time, the previously unrecognized role of feedback mechanisms between KK and KK related to the IGF system of mitogens and the basal phosphorylation of its components as one facet of the epithelial-mesenchymal interactions that result in keloid pathogenesis. The observed proliferative effect of KK on KK appears to involve direct growth stimulatory and anti-apoptotic signal transduction pathways (9) and is related to the bioavailability of IGF for cell surface receptor binding. Taken together, our findings suggest that inhibition of IGF-1/IGFIR binding by the transient local expression of anti-sense IGF-I or IGF-IR mRNA, or the administration of exogenous IGFBP-3 to alter IGF bioavailability, either as a single agent or in combination with drugs that block IGF activity, may all represent potential strategies for the treatment or prevention of keloids.

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