Fibroblast Collagen Secretion in Co-Culture with Keratinocytes: Normal

Fibroblasts Secrete Collagen in a Keloid-Like Manner

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Running Head Title

Fibroblast Keloid Induction

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Abstract

Keloid scars represent a pathological response to cutaneous injury, reflecting a new set point between synthesis and degradation biased toward ECM collagen accumulation. Using a serum-free two-chamber co-culture model, we recently demonstrated a significant increase in normal fibroblast proliferation when co-cultured with keloid-derived keratinocytes. We hypothesized that similar keratinocyte-fibroblast interactions might influence fibroblast collagen production and examined conditioned media and cell lysate from co-culture for collagen I and III production by Western blot, allied with Northern analysis for procollagen I and III mRNA. Normal fibroblasts co-cultured with keloid keratinocytes produced increased soluble collagen I and III with a corresponding increase in pro-collagen I and III mRNA transcript levels. This was associated with decreased insoluble collagen from cell lysate. When keloid fibroblasts were co-cultured with keloid keratinocytes, both soluble and insoluble collagen were increased with associated procollagen III mRNA upregulation. Transmission electron microscopy of normal fibroblasts co-cultured with keloid keratinocytes showed an ECM appearance similar to in-vivo keloid tissue; an appearance not seen when normal fibroblasts were co-cultured with normal keratinocytes.
Key Words

keloids/ epithelial-mesenchymal interactions/ keratinocyte induction/ serum-free co-culture
Introduction

Keloid scars represent a pathological response to cutaneous injury. They have clinical features and characteristics to differentiate them from hypertrophic scarring, and are characterized by increased proliferation of fibroblasts, especially in the active growing phase (30, 19, 29, 31), as well as an abnormally increased production of collagen up to twenty times that of normal skin \textit{in vitro} (5, 18). Abnormalities in the proportion of collagen subtypes have been shown to exist in these fibroproliferative scars as compared to scarring in normal skin (2, 12), with keloid fibroblasts overproducing type I collagen, while type III collagen expression is unchanged (38). Absolute levels of soluble collagen are also increased in keloids, which may reflect increased collagen synthesis, increased degradation of polymerized collagen or decreased cross-linking (18).

The histological appearance of keloids is largely similar to hypertrophic scars and comprises discrete nodules of collagen organized in whorls, in a dense mesenchyme with rich vasculature, and a thickened epidermal layer (15). Both keloids and hypertrophic scars exhibit features of microvascular occlusion from an excess of endothelial cells, suggesting that a hypoxic microenvironment within a keloid may promote excess collagen production by fibroblasts (16,17). In addition, this hypoxic microenvironment may stimulate an increased production of growth factors such as Vascular Endothelial Growth Factor (VEGF) (34). More accurate differentiation is obtained by electron microscopy, where the ultrastructure of collagen fibrillar organization may separate keloids from hypertrophic scars (18).
Previous studies have focused largely on the role of the fibroblast (termed the ‘keloid fibroblast’ when derived from keloids), as it is primarily responsible for collagen and extracellular matrix production that forms the bulk of keloid tissue. In recent years, however, an increasing body of evidence has shown that autocrine, paracrine and endocrine epithelial-mesenchymal interactions play a major role in normal skin homeostasis, growth and differentiation (10, 25, 24). The secretory role of keratinocytes is now established and known to influence not only the adjacent mesenchyme, but also to have far-reaching systemic effects by modulation of the immune system (4, 14, 11). The corollary that in keloids, certain facets of fibroblast behaviour may also be modulated by the overlying keratinocytes in the epidermis has not been investigated.

Recently, using an in-vitro two-chamber co-culture model, we showed that normal dermal fibroblast proliferation was significantly increased when co-cultured with keloid-derived keratinocytes (which we propose to be called ‘keloid keratinocytes’) in a serum-free medium (23). These data suggested that soluble factors promoting fibroblast proliferation were elaborated by keloid keratinocytes in a manner different from normal skin-derived keratinocytes (termed ‘normal keratinocytes’). Significantly, in the same study, keloid fibroblasts proliferated at a markedly higher rate when co-cultured in identical conditions with keloid keratinocytes as compared to normal keratinocytes. This suggested fundamental differences in the sensitivity of the two fibroblast subtypes to keloid keratinocyte elaborated growth factors.
Based on the above data, we hypothesized that similar paracrine epithelial-mesenchymal interactions which influence fibroblast proliferation, might regulate fibroblast collagen secretory patterns as well. To test this hypothesis, we utilized a similar co-culture model to examine the influence of keloid keratinocytes on collagen production in both normal and keloid fibroblasts. Western blot analyses of conditioned media from co-culture and fibroblast cell lysate (to account for collagen secreted into the immediate pericellular extracellular matrix) were performed to detect collagen type I and III levels when normal and keloid fibroblasts were co-cultured with keloid keratinocytes. Collagen I and III were selected as they represent the two most important collagen types in skin in relation to normal architecture and in wound healing. Northern blot analyses of procollagen I and III mRNA were also performed on fibroblast cell lysate to detect changes in procollagen transcript levels under co-culture conditions. Finally, both transmission and scanning electron microscopy were used to compare the morphological features of the extracellular-matrix collagen secreted by in-vivo keloid tissue fibroblasts and in-vitro normal fibroblasts co-cultured with normal and keloid keratinocytes.
Materials and Methods

Earlobe Keloid Keratinocyte and Fibroblast Database

Four strains of keratinocytes and fibroblasts (samples 2, 4, 7 and 8) were randomly selected from a bank of 24 keratinocyte/ fibroblast strains derived from excised earlobe keloid specimens. All patients (age range, 14 – 21 years) had received no previous treatment for the keloids before surgical excision. Prior to excision, a full history was taken and examination performed, complete with color slide photo-documentation and informed consent. A portion of all specimens was sent to the hospital Department of Pathology for histological confirmation of keloid identity.

Cell Culture

Keratinocyte culture from earlobe keloids

Excised earlobe keloid specimens were washed in Hank’s Balanced Salt Solution (HBSS) containing 150 µg/ml gentamicin and 7.5 µg/ml fungizone followed by plain Phosphate Buffered Saline (PBS) until the washing solution became clear. The specimens were then cut into 5 mm x 10 mm pieces and the epidermis scored. Dispase (5 mg/ml) in HBSS was added to the prepared specimens, which were allowed to sit overnight at 4°C. The epidermis was carefully scraped off with a scalpel the next day and incubated in a solution of trypsin 0.25%, glucose 0.1% and Ethylene Diamine Tetra-acetic Acid (EDTA) 0.02% for 10 min. Trypsin
action was quenched by Dulbecco’s Modified Eagle Medium (DMEM)/10% FCS when intercellular separation was seen. The suspended cells were transferred into tubes and centrifuged at 1000 rpm for 8 min. The cells were then isolated and seeded in Keratinocyte Culture Medium (KCM- 80ml DMEM supplemented with 20 ml FCS, EGF 10ng/ml, cholera toxin $1 \times 10^{-9}$M and hydrocortisone $0.4 \mu g/ml$) at $1 \times 10^5$ cells/cm$^2$ for 24 hours before transfer to Keratinocyte Growth Medium (KGM) (Clonetics, USA). Cell strains were maintained and stored at -150°C until use. Only cells from the second passage were used in all experiments.

*Fibroblast culture from earlobe keloids*

Remnant dermis from the keloids was minced and incubated in a solution of collagenase type-I (0.5 mg/ml) and trypsin (0.2 mg/ml) at 37°C for 6 hours. Cells were pelleted and grown in tissue culture flasks. Cell strains were maintained and stored at -150°C until use. Only cells from the second passage were used for the experiments.

*Normal Human Keratinocytes and Fibroblasts*

Normal keratinocytes were derived from foreskin circumcision specimens of healthy young children (non-neonatal- aged between 5 and 7) using the methods described above. Normal fibroblasts were cultured using the standard explant method. Foreskin cells were selected as they came from an area relatively free of tension and were readily available. Contralateral
normal earlobe skin from the patient was not utilized, as that would be in direct contravention to our Institutional Ethics Committee human subject approval requirements.

**Keratinocyte-Fibroblast Co-Culture**

Keratinocytes obtained from the four randomly selected keloid samples (samples 2, 4, 7 and 8) were thawed, centrifuged and recounted. Cells were seeded at a density of $4 \times 10^5$ cells/cm$^2$ on Transwell clear polyester membrane inserts with 0.4µm pore size and an area 0.3 cm$^2$ (Costar Corp, USA). Cells were maintained for 4 days in serum-free Keratinocyte Growth Medium (KGM) until 100% confluent in monolayer. The medium was then changed to serum-free Defined Fibroblast Growth Medium (DFGM) and raised to air-liquid interface for another 3 days to allow keratinocytes to stratify and reach terminal differentiation (39).

Normal and keloid dermal fibroblasts were thawed from frozen stock and seeded in 6 or 12-well plates at a density of $1 \times 10^5$ or $5 \times 10^4$ cells/well in DFGM for 3 to 4 days until 100% confluent.

One series of normal fibroblasts was seeded on the Transwell clear polyester membrane inserts, in the manner described above for keratinocytes, for the purposes of a fibroblast-fibroblast co-culture control.

Both the cultured keratinocyte layer on membrane inserts and the cultured fibroblasts in plates were washed 2 times with PBS to remove the old medium before combination of
the inserts and plates for co-culture in fresh serum media (for electron microscopic analysis) or serum-free DFGM (for western and northern blot analysis). Study groups thus comprised 1 strain each of normal or keloid fibroblasts co-cultured with 3 samples of keloid keratinocytes, and the same strains of normal or keloid fibroblasts co-cultured with normal keratinocytes. Controls comprised normal or keloid fibroblasts without keratinocyte co-culture as a negative control, with positive controls of normal or keloid fibroblasts co-cultured with normal fibroblasts. At day 5, inserts with the cultured keratinocytes on membrane were removed and the conditioned medium collected and stored at -150°C for later analysis. Fibroblasts were also stored at -150°C for later protein or RNA extraction for western or northern blot analysis, respectively.

**Cell Lysis**

Co-cultured fibroblasts (n=5) and non-co-culture fibroblasts (n=1) were washed twice with phosphate buffered saline solution and lysed in homogenisation buffer containing 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 100 mM NaCl, 0.5% Nonidet P-40, 1 mg/ml protease inhibitor cocktail (Boehringer Mannheim, Germany). The lysate was clarified by centrifugation at 13,000 rpm for 15 min and immediately subjected to western blot analysis.
Western Blot

Immunoblot analysis was carried out on both conditioned media and cell lysates. Protein concentrations were first determined and equal amounts of protein (50 µg of cell lysate or 100 µl of conditioned media) was electrophoresed on 8% sodium dodecyl sulfate-polyacrylamide gels using the Protein II system (Bio-Rad) and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad) in 25 mM Tris base, 190 mM glycine and 20% methanol for electroblotting.

Blotted nitrocellulose was washed twice with de-ionised water and then blocked in freshly prepared Tris-buffered saline solution (TBST) containing 0.1% Tween-20 and 7% skimmed milk for 2 hours at room temperature. The nitrocellulose membrane filters were then incubated with either mouse monoclonal antibodies against collagen I and III (Monosan, Sanbio, Netherlands) or the housekeeping protein α-tubulin (Santa Cruz, Santa Cruz, CA) for 18 h at 4 °C. The filters were washed 6 times in TBST, and were incubated for 1 hour in horseradish peroxidase-conjugated anti-mouse antiserum (1:2500 dilution, Amersham, Oakville, Ontario). Filters were washed a further 3 times with TBST and once with TBS before Western blots were visualized by a chemiluminescence-based photoblot system (ECL; Amersham).

Quantitative analysis of collagen I and III production was accomplished by computerized optical densitometry (Gel Doc 2000 Quantity 1 program- Bio Rad, USA) of the blotted filters.
Northern Blot

Total RNA was isolated from fibroblast cell lysate and Northern blotting was performed as previously described (13). Briefly, blots were hybridized with procollagen I and III cDNAs (a kind gift from Dr Ziv Peled, Laboratory of Developmental Biology and Repair, New York University). 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 assessment (Gel Doc 2000 Quantity 1 program- Bio Rad, USA).

Computerised Gel Densitometry

A Bio-Rad Gel scanner and densitometer with the Gel Doc 2000 Quantity 1 program was utilised to assess concentrations of the bands as obtained by both western and northern blots. These were measured as Arbitrary Density Units (ADU).

Statistical Analysis

The Mann Whitney U-Test was used to determine differences in band density after calculating the sum of the densities of the bands and normalization with $\alpha$-tubulin for cell lysate protein, and GAPDH for mRNA.
Electron Microscopy

**Transmission Electron Microscopy**

Normal fibroblasts in co-culture with normal or keloid (sample 8) keratinocytes in serum media were grown on glass slides at the bottom of the wells of 24-well plates. Serum media was utilized to mimic the *in-vivo* interstitial environment, approximating that of the *in-vivo* keloid sample, which was to be used for comparison. At day 5, the plates were carefully lifted from the bottom of the wells and fixed in 3% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer for 30 minutes (3). The fibroblasts were post-fixed in 1% osmium tetroxide and dehydrated in an ascending series of alcohol. Specimens were then embedded in araldite. Ultrathin sections were cut and doubly stained with uranyl acetate and lead citrate before viewing in a Philips CM120 BioTwin electron microscope.

**Scanning Electron Microscopy**

Normal fibroblasts co-cultured on glass slides with normal or keloid (sample 8) keratinocytes as described above were fixed, at day 5, in 3% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate for 30 minutes, followed by 2% osmium tetroxide in buffer solution. After fixation, the cells were dehydrated in increasing concentrations of methanol until absolute levels were reached. The cells were then transferred to acetone before drying in a Balzers critical point dryer (model CPD 030), using liquefied carbon
dioxide as the transition fluid (33). All cells were coated with 20 nm gold in a Balzers sputter coater (model SCD 004) before examination in a Philips XL-30 field emission gun scanning electron microscope.
Results

Western Blot Analysis of Soluble Collagen

After 5 days of co-culture, conditioned media was subjected to western blot analysis to examine soluble collagen I and III secreted by normal fibroblasts (NF) in 4 study groups (NK/NF, KK2/NF, KK4/NF, KK7/NF), 1 positive control group (NF/NF) and 1 negative control group (0/NF). Soluble collagen I and III produced by keloid fibroblasts (KF) was assayed on the same gel with 4 study groups (NK/KF, KK2/KF, KK4/KF, KK7/KF), 1 positive control group (KF/KF) and 1 negative control group (0/KF). Gels were scanned and subjected to optical densitometry, yielding the following results (Fig. 2).

*Keratinocytes- especially keloid keratinocytes- increase soluble collagen I and III production by Normal and Keloid Fibroblasts.*

Soluble collagen I production by NF and KF was doubled when co-cultured with NK or KK compared to fibroblasts not in co-culture (0/NF and 0/KF respectively). In the NF set, co-culture with all 3 KK samples produced significantly higher levels of collagen I compared to co-culture with NK. (*p <0.01). In the KF set, compared to co-culture with NK, 2 of the 3 KK samples induced a significantly higher level of soluble collagen I production (*p <0.01).
Soluble collagen III production by both NF and KF were even more markedly elevated in co-culture with keratinocytes, with a ten-fold increase seen in the NF set, and up to eight-fold increase seen in the KF set. Comparing the effects of KK with NK in co-culture, it could be seen that in the NF set, 1 KK sample induced significantly more NF collagen III production compared to NK (*p <0.01), whereas in the KF set, all 3 KK samples induced greater KF collagen III secretion compared to NK (*p <0.01).

_Co-culture with keratinocytes inverts the soluble collagen I to collagen III secretion ratio by fibroblasts._

NF or KF not in co-culture (0/NF and 0/KF respectively) produced 3 times as much soluble collagen I as collagen III. This ratio was not markedly altered by co-culture with equivalent fibroblasts (NF/NF ad KF/KF respectively). Co-culture with keratinocytes, however, inverted this ratio in all study groups for both NF and KF sets.

**Western blot analysis of Insoluble collagen I and III from cell lysate.**

After extraction of the conditioned media, fibroblasts remaining in the lower chamber were subjected to cell lysis as earlier described. Insoluble collagen I and III was assayed on the same gel for both NF and KF sets in the same groups as outlined above from which the following observations were made (**Fig. 3**).
Insoluble collagen I and III production in keloid fibroblasts is increased by co-culture with keloid keratinocytes

The presence of KK in co-culture significantly increased KF production of insoluble collagen I and III for all 3 KK samples compared to KF not in co-culture (0/KF). Co-culture with NK, however, did not significantly change KF insoluble collagen I and III production compared to 0/KF. Comparing NK/KF to the 3 KK/KF groups, KF production of insoluble collagen I was significantly increased in co-culture with 2 of 3 KK samples (*p <0.01), whereas insoluble collagen III production was increased by co-culture with all 3 KK samples (*p <0.01).

Insoluble collagen III production in keloid fibroblasts, but not normal fibroblasts, is suppressed by co-culture with keloid keratinocytes

NF production of insoluble collagen I and III was not significantly changed in the presence of normal or keloid keratinocytes. However, normal fibroblasts in co-culture with all 3 KK samples produced significantly less insoluble collagen III (‡p<0.01) compared to co-culture with normal keratinocytes. Insoluble collagen I levels were not statistically different in this comparison group.
Northern blot analysis of procollagen I and III mRNA from cell lysate.

Procollagen I and III mRNA was hybridized with procollagen I and III cDNA with blot rehybridization with GAPDH cDNA to control for equal loading as earlier described. Both NF and KF sets was assayed on the same gel in the same groups as outlined above. (Fig. 4)

Whereas procollagen I and III mRNA expression largely parallels insoluble collagen production by non co-cultured and co-cultured fibroblasts, co-culture with keloid keratinocytes results in normal fibroblast expression of procollagen I and III mRNA which parallels soluble collagen production.

Procollagen I and III mRNA expression by NF and KF not in co-culture paralleled insoluble collagen I and III production, where collagen III > collagen I. A similar picture was seen in NF/NF and KF/KF co-culture. Where NF or KF were co-cultured with NK, procollagen I mRNA was significantly higher than procollagen III mRNA expression but this was not reflected in the levels of insoluble (no significant difference) or soluble collagen I and III (collagen III > collagen I) production. In co-culture with KK, however, NF procollagen I and III mRNA expression paralleled soluble collagen I and III production, with increased production of both collagen types in all 3 KK/NF groups.
Summary of Results

Cross analysis of the above data reveals a few overall points.

*Normal fibroblasts and keloid fibroblasts represent two distinct fibroblast subtypes.*

That the 2 populations of fibroblasts, NF and KF, represent distinct cellular subtypes can be seen by their *in-vitro* response to co-culture. Resting state collagen production, when not in co-culture (0/NF, 0/KF), is similar for both NF and KF in terms of the ratio of collagen I to III produced, both for soluble and insoluble collagen. Procollagen I and III mRNA expression relates more to insoluble collagen levels in this group. Co-culture with equivalent fibroblasts (NF/NF, KF/KF) similarly upregulate procollagen I mRNA in both, but downstream collagen production, both soluble and insoluble, begins to show differences, with NF appearing to behave more responsively than KF. This strongly suggests the important role of post-translational modification by KF changing the cellular gene response. When NF or KF are co-cultured with keratinocytes, be they NK or KK, significant increases in soluble collagen I and III output is seen in both subtypes, which is paralleled by increases in procollagen I and III mRNA expression, especially for KK/NF groups. Interestingly, the ratio of soluble collagen I to III produced by NF and KF in co-culture with keratinocytes is inverted compared to the resting state.
Keloid keratinocytes exert a different response on fibroblasts compared to normal keratinocytes

Compared to NK, exposure of NF and KF to KK in co-culture results in intriguing differences in collagen I and III production, both soluble and insoluble. NF respond in co-culture with KK by producing more soluble collagen I and III (*p<0.01, Fig 2), apparently at the expense of insoluble collagen III which appears to be suppressed (+p<0.01, Fig 3), compared to co-culture with NK. NK/NF co-culture results in procollagen I expression which is higher than that of procollagen III, this is not reflected by a similar ratio of soluble or insoluble collagen I and III production by NF. The KF response is similar for soluble collagen I and III (*p<0.01, Fig 2), but insoluble collagen I and III are increased at the same time (*p<0.01, Fig 3) compared to co-culture with NK. As is the case for the NK/NF group, procollagen I mRNA expression is higher than procollagen III mRNA expression in the NK/KF group, which again is not reflected in KF soluble or insoluble collagen I and III production. Compared to the negative control (0/NF, 0/KF), NF procollagen I and III mRNA expression more closely parallels soluble collagen production when co-cultured with KK, whereas KF procollagen expression more closely parallels insoluble collagen production.

Electron Microscopy

Normal Fibroblasts Co-Cultured with Keloid Keratinocytes are Induced to Secrete Collagen in a Pattern Similar to that of In-Vivo Keloid Tissue.
Due to the finite number of cells available for northern and western blot analysis, a further randomly selected keloid specimen, sample 8 (S8) was used for electron microscopic study.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed on an *in-vivo* glutaraldehyde fixed earlobe keloid specimen sample 8 (S8), NF in serum co-culture with S8 KK at day 5, and NF in serum co-culture with NK at day 5.

Distinct differences were seen in the collagen/extracellular matrices of NF co-cultured with NK compared to those co-cultured with KK. NK/NF fibroblasts showed sparse collagen fibrils in a homogenous extracellular matrix, with a regular, largely parallel alignment and minimal interlocking (Fig. 5A). At higher magnifications, straight fibrils of similar diameters with varying lengths with occasional short, curved fibrils were observed (Fig. 5B).

In contrast, KK/NF fibroblasts showed markedly higher numbers of fibrils in a homogenous extracellular matrix (Fig. 5C). A more random and disorganized whorled appearance was seen with greater degrees of criss-crossing and possible interlocking. Numerous curved fibrils of similar diameters with side-to side coalescence suggestive of early fascicle formation were observed at higher magnification (Fig. 5D). The curved
fibrils were interspersed with straight, more regular fibrils. There was no obvious interfibrillar bridging.

The extracellular matrix collagen of KK/NF fibroblasts exhibited many similarities to keloid tissue in-vivo. The random, whorled organization of the fibrils was once again seen in a homogenous extracellular matrix, in spite of minor obscuration by cross and oblique sections of fibrils resulting from the sectioning of keloid tissue (Fig. 5E). At higher magnification, an appearance very similar to that of KK/NF fibroblasts (Fig. 5D), with many curved fibrils interspersed between straight fibrils was observed (Fig. 5F). Some differences in fibrillar thickness could be seen, but fascicle formation was not as obvious.

**Scanning Electron Microscopy**

Scanning electron microscopy (SEM) was performed on NF in serum co-culture with NK at day 5, and NF in serum co-culture with S8 KK at day 5.

The collagen/extracellular matrix of NF co-cultured with NK compared to those co-cultured with KK correlated with the morphology seen on TEM. Lower magnifications of NK/NF fibroblasts showed fewer, straighter intercellular collagen strands (Fig. 6A) with thin fascicles. In marked contrast, KK/NF fibroblasts at the same magnification revealed a much denser and more random collagen meshwork with obvious fascicle formation (Fig. 6C). At very high magnifications, NK/NF fibroblast collagen was largely straight
and seen to have noticeably regular intervals between knobbed protrusions on the fibril surface suggestive of more regular polymerization. The collagen fascicles comprised 2 to 4 fibrils (Fig. 6B).

KK/NF fibroblast collagen, however, showed a denser picture with a decreased interval between the surfaces protrusions, which now appeared to encrust the fibril. Both straight and curved fibrils were seen amidst what appeared to be seemingly random cross-linking between fibrils orientated in different directions. The resultant, thicker, fascicles comprised 5 or more fibrils, with coalescence of the fibrils making counting from the electron micrographs difficult (Fig. 6D).

Overall, both TEM and SEM micrographs showed that NF co-cultured with KK were induced to secrete collagen in a pattern similar to that of in-vivo keloid tissue.
**Discussion**

Pathological over-healing in susceptible individuals leads to the development of keloids, which are characterized by an abnormal, abundant deposition of collagen and extracellular matrix components. Studies have shown that the predominant collagen subtypes in keloids are types I and III with small amounts of type V and VI (1). Alpha-1 (I) procollagen and its mRNA levels in human keloid fibroblast lines cultured in serum media are both elevated, with concurrent increase of the Type I/Type III collagen ratio when compared to normal skin or hypertrophic scars (7, 9). Whereas normal skin fibroblasts in a three-dimensional culture system showed a gradual decline in alpha-1 (I) and alpha-1 (III) procollagen mRNA levels with time, keloid fibroblasts did not (32), suggesting a feedback mechanism defect. Such collagen produced is abnormal with a cross-linkage pattern dissimilar from normal skin as assessed by the presence of mercaptoethanol reducible peptides (7).

Excess collagen deposition may be the result of increased collagen production by up-regulation of one or more growth factors as TGF-β, or increased sensitivity of the keloid fibroblasts to these factors (29, 40, 22). Autocrine positive feedback stimulation of keloid fibroblasts by TGF-β1 to produce more TGF-β1, together with type-I and type-VI collagen by the process of gene activation, is another mechanism that has been described (28). Yet other studies have shown that growth factors as Fibroblast Growth Factor (FGF), and Endothelial Cell Growth Factor (ECGF) (37; 36) down-regulate collagen gene expression by keloid fibroblasts at the pretranslational level. Thus, decreased levels
of these growth factors that suppress collagen gene expression in the keloid microenvironment may be another explanation for excess pro-fibrotic gene expression and activation with a concomitant increase in collagen production. However, these data are currently not known. Investigations into immunoregulatory cytokine profiles of keloid-predisposed individuals have shown alterations in quantities of IL-6, TNF-α and IFN-β from peripheral blood mononuclear-cell fractions as compared to normal controls (26).

Collagen accumulation within a developing keloid reflects the balance between synthesis and degradation. Specifically, degradation is regulated by the net activity of proteases and their inhibitors. As such, decreased collagen degradation has also been put forth as a possible cause for the collagen build-up seen in keloids, either by increased levels of collagenase inhibitor (8) or by decreased levels of collagenase (27).

Overall, recent research has thrown new light onto the molecular biological mechanisms that regulate keloid fibroblast behaviour and their role in the pathogenesis of keloid scars. The suggestion is that keloid fibroblasts are independent of normal control mechanisms, either in terms of feedback or sensitivity to growth factors, with elevated transcriptional and translational activity for collagen production. The ‘keloid fibroblast’ may also represent a fibroblast subtype (21), originally derived from normal dermis, which has gone awry or has been preferentially selected during wound healing. Theories as to why this process occurs are plentiful, but the actual regulatory stimuli remain elusive (5).
Interestingly, keloids do not develop spontaneously, but only in response to cutaneous injury.

Recent studies have shown that autocrine, paracrine and endocrine epithelial-mesenchymal interactions play important roles in skin homeostasis, growth and differentiation (10, 25, 24). The secretory role of keratinocytes is now known to not only influence the adjacent mesenchyme, but also to have far-reaching systemic effects by modulation of the immune system (4, 14, 11). Thus, there exists the possibility that certain facets of keloid fibroblast behaviour may be modulated by the overlying keratinocytes in the epidermis.

For this study, the experimental material uniformly comprised previously untreated earlobe keloid samples obtained from individuals with a narrow age range. To date, a library of 24 such keloids has been established as part of a research data and sample collection. Using keratinocyte and fibroblast cell strains from these samples, we have demonstrated, for the first time, differences between normal and keloid fibroblast collagen response to co-culture with normal or keloid keratinocytes. Normal and keloid keratinocytes, like normal and keloid fibroblasts, also appear to be distinct subtypes from these observed differences, which reinforces earlier work (23) suggesting the role of epithelial-mesenchymal interactions in keloid pathogenesis.

The most marked response to co-culture with keratinocytes can be seen in soluble collagen I and III production, which is similarly elevated both in normal and keloid
fibroblasts. Where this is reflected in procollagen mRNA production by NF, the same cannot be said for KF, suggesting more active post translational modification processes in the latter fibroblast cell type. NF production of soluble collagen I and III is associated with suppression of insoluble collagen III production which is not seen in KF, where insoluble collagen I and III production is also increased. We hypothesize that this suggests a fundamental difference between NF and KF cell types- whereas NF tend to produce a finite amount of collagen which is portioned between soluble and insoluble types and has to divert its synthetic machinery to produce more of one at the expense of the other, KF are not similarly constrained in its pattern of collagen production, which is likely to be a reflection of its autonomous nature.

Interestingly, the normal ratios of soluble collagen I to III are reversed in the presence of keratinocytes but not fibroblasts. This higher collagen III production approximates the early wounding state, which is probably replicated in this in-vitro environment, where fibroblasts are acutely exposed to keratinocytes. We are currently looking at the changes in NF and KF response with prolonged exposure to investigate if this higher collagen III production persists or changes with time.

Under electron microscopic examination, the collagen produced by NF co-cultured with KK in-vitro has an appearance similar to that of in-vivo keloid collagen extracellular matrix, with marked disorganization of collagen fibrils as well as morphological irregularities in the strands, which might suggest deficient or abnormal polymerization.
We hypothesize that this finding is due to the excess of soluble secreted collagen forms accounting for the structural instability of the keloid scar, which may be the inciting factor leading to further collagen production by the fibroblasts in an effort to produce a more stable extracellular matrix milieu.

The overall picture emerging from our data is that NF can be induced to take on some features of KF (as determined by collagen production and organization) by exposure to KK. This implies a significant contribution of an abnormal epidermis to the pathogenesis of keloid tissue. In addition, KF, whilst also sensitive to keratinocyte gene products, may possess an inherent abnormality leading to autonomous excess collagen production.

It is the skin that is first exposed to trauma, sometimes minor, that starts off the sequence of events leading to keloid formation. This study suggests that the role of the epidermal keratinocyte may be more important in fibrogenesis than previously realised. The data presented in this study, taken together with previous studies, supports the theory of a fundamental difference in cell biology, not only in fibroblasts but also in keratinocytes, as the basic abnormality leading to the predilection to keloid scar formation. The observed differences in NF or KF response to co-culture with KK reflects a strong paracrine element which ultimately implies that the release by keratinocytes of genetically determined growth factors in predisposed individuals results in paracrine stimulation of underlying fibroblasts in the dermal mesenchyme to produce collagen and extracellular matrix in a disorganised manner. The end product is the keloid scar with all its described morphological characteristics.
Whether all the fibroblasts, or just a particular subpopulation of the fibroblasts in the co-culture system are influenced to behave in this way remains to be evaluated. Similarly, the question as to why keloids tend to occur in some (for example the earlobe, face and neck) in a reported 75% of cases (20, 35) but not all parts of the body deserves further investigation.

In summary, normal fibroblasts co-cultured with keloid-derived keratinocytes produced increased soluble collagen types I and III, when compared with equivalent normal fibroblasts co-cultured with normal keratinocytes or normal fibroblasts. Normal fibroblast production of insoluble collagen III is correspondingly suppressed. Keloid fibroblasts co-cultured with keloid keratinocytes produce increased amounts of both soluble and insoluble collagen I and III when compared to co-culture with normal keratinocytes. Electron microscopic appearances of the collagen-ECM produced by normal fibroblasts co-cultured with keloid keratinocytes show an *in-vitro* morphological appearance similar to that seen *in-vivo* in keloid scar tissue. This study has shown, for the first time, that the overlying epidermis of keloid tissue has profound effects on the production and organization of collagen by fibroblasts in the mesenchyme. This is likely to comprise paracrine signalling by growth factor release or growth factor receptor activation and/or sensitization. Further studies are underway to assess keratinocyte influence on fibroblast production of other extracellular matrix components, as well as assays of possible growth factors and receptors responsible for this epithelial-mesenchymal interaction.
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Figure Legends

1. Summary time-point graph illustrating the steps taken in co-culture experiments.

2. Western blot for secreted soluble collagen I and III from day 5 co-culture conditioned media. NF co-cultured with the 3 KK samples (KK/NF) and 1 NK sample (NK/NF) with controls of NF not in co-culture (0/NF) and NF co-cultured with NF (NF/NF) are depicted on the left of the solid bar of the x-axis. KF co-cultured with the 3 KK samples (KK/KF) and 1 NK sample (NK/KF) with controls of KF not in co-culture (0/KF) and KF co-cultured with KF (KF/KF) are depicted on the right of the solid bar of the x-axis. Optical densitometry readings are depicted on the y-axis (Arbitrary Density Units). Significant increases in collagen I or III levels (p< 0.05) between NK/NF vs. KK/NF groups, and NK/KF vs. KK/KF groups are marked with an asterisk *.

3. Western blot for total insoluble collagen I and III from day 5 co-culture cell lysates. NF co-cultured with the 3 KK samples (KK/NF) and 1 NK sample (NK/NF) with controls of NF not in co-culture (0/NF) and NF co-cultured with NF (NF/NF) are depicted on the left of the solid bar of the x-axis. KF co-cultured with the 3 KK samples (KK/KF) and 1 NK sample (NK/KF) with controls of KF not in co-culture (0/KF) and KF co-cultured with KF (KF/KF) are depicted on the right of the solid bar of the x-axis. Optical densitometry readings are depicted on the y-axis (Arbitrary Density Units). Significant increases in collagen I or III
levels (p< 0.05) between NK/NF vs. KK/NF groups, and NK/KF vs. KK/KF groups are marked with an asterisk *, whereas significant decreases in the same groups are marked with a cross †.

4. Northern blot of collagen I and III mRNA from day 5 co-culture fibroblast cell lysates. NF co-cultured with the 3 KK samples (KK/NF) and 1 NK sample (NK/NF) with controls of NF not in co-culture (0/NF) and NF co-cultured with NF (NF/NF) are depicted on the left of the solid bar of the x-axis. KF co-cultured with the 3 KK samples (KK/KF) and 1 NK sample (NK/KF) with controls of KF not in co-culture (0/KF) and KF co-cultured with KF (KF/KF) are depicted on the right of the solid bar of the x-axis. Optical densitometry readings are depicted on the y-axis (Arbitrary Density Units). Significant increases in collagen I or III levels (p< 0.05) between NK/NF vs. KK/NF groups, and NK/KF vs. KK/KF groups are marked with an asterisk *, whereas significant decreases in the same groups are marked with a cross †.

5. Transmission electron microscopy (TEM):
An increased number of fibrils in a more random organization is seen in KK/NF co-culture (Figs 5C and 5D) compared to that of NK/NF co-culture at day 5 (Figs 5A and 5B). KK/NF extracellular matrix appearance is similar to that of in-vivo keloid tissue (Figs 5E and 5F). For a full description please refer to the text.
6. Scanning electron microscopy (SEM)

A denser, more random fibrillar organization with thicker fascicle formation is seen in KK/NF co-culture (Figs 6C and 6D) compared to that of NK/NF co-culture at day 5 (Fig 6A and 6B). These appearances correspond to those described for transmission electron microscopy (Fig 5). For a full description please refer to the text.

A. Collagen ECM from NK/NF co-culture (Magnification, x 5,000. Bar = 5µm)
B. Collagen ECM from NK/NF co-culture (Magnification, x 50,000. Bar = 500nm)

C. Collagen ECM from KK/NF co-culture. Keloid keratinocytes derived from sample S8 (Magnification, x 5,000. Bar = 5µm)

D. Collagen ECM from KK/NF co-culture. Keloid keratinocytes derived from sample S8 (Magnification, x 50,000. Bar = 500nm)
Keratinocytes seeded on permeable inserts

Inserts placed into wells commencing Co-Culture

Fibroblasts seeded in culture plates

Collection of Conditioned Media and Cells for Lysis

Time (days)

-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5
Soluble Collagen I and III in Conditioned Medium

A

B

Collagen I

Collagen III

ADU (Arbitrary Density Units)

Groups

0/NF NF/NF NK/NF KK2/NF KK4/NF KK7/NF 0/KF KF/KF NK/KF KK2/KF KK4/KF KK7/KF

C

Collagen I

Collagen III

* * *
Procollagen I and III mRNA from Cell Lysate

Collagen I
Collagen III
GAPDH

Procollagen I and III mRNA from Cell Lysate

ADU (Arbitrary Density Units)

Groups

Collagen I
Collagen III