Expression of insulin-like growth factor-1 receptor in keloid and hypertrophic scar

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Summary

Background. Keloid and hypertrophic scar (HS) are two pathological forms of excessive dermal fibrosis, which are due to aberrant wound-healing responses. Accumulating evidence suggests that aberrant activity of growth factors and increased numbers of growth factor receptors play an important role in the formation of pathological scar.

Aim. We examined the expression level of insulin-like growth factor-1 receptor (IGF-IR) in keloid, HS and normal skin.

Methods. IGF-IR expression was analyzed by immunohistochemistry, real-time PCR and western blotting on tissues and fibroblasts from 30 patients, comprising 10 patients with keloid and 20 with HS (10 with immature and 10 with mature HS), and from 10 age-matched and sex-matched healthy controls.

Results. Immunoreactivity to IGF-IR was found in dermal fibroblasts of keloid (90%), immature HS, (80%) and mature HS (30%), but not in normal skin. There was no statistically significant difference in immunoreactivity scores between keloid and immature HS, but there was a significant difference \( (P < 0.01) \) between mature and immature HS. Real-time PCR and western blot analysis confirmed that there was high expression of IGF-IR in keloid and immature HS fibroblasts, but not in mature HS or normal skin fibroblasts. IGF-IR was expressed in the overlying epidermis, and there was no significant difference between the groups.

Conclusions. IGF-IR may be involved in the pathogenesis of keloid and HS. Given that IGF-IR are predominantly expressed on dermal fibroblasts, targeting of IGF-IR in fibroblasts may be of benefit to prevent scarring.

Introduction

Keloid and hypertrophic scar (HS) are two pathological forms of excessive dermal fibrosis, which are due to aberrant wound-healing responses. Both produce similar uncomfortable signs and symptoms, and both are characterized by excessive proliferation of fibroblasts and overproduction of extracellular matrix (ECM). However, unlike HS, keloid is characterized by invasion, immortality and a high rate of recurrence after surgery. Histopathologically, the presence of distinct collagenized nodules in the dermis is a characteristic feature of HS, whereas the absence of collagenized nodules and the presence of thick collagen bundles characterize keloid.\(^1\) In addition, the major components of HS are \( \alpha \)-smooth muscle actin (SMA)-expressing myofibroblasts, which are generally absent in keloid.\(^2\,3\)

Despite extensive research, the exact pathogenesis of dermal fibrosis remains unclear. Accumulating evidence suggests that aberrant activity of growth factors, such as platelet-derived growth factor,\(^4\) transforming growth factor-\( \beta \)\(^5\) and connective tissue growth factor,\(^6\)
contribute to the pathogenesis of keloid and HS. In addition, increased numbers of growth factor receptors, which result in faster response to their relevant growth factors, also play an important role in the formation of pathological scar.

Insulin-like growth factors (IGF)-1 and IGF-2 are potent mitogens and inhibitors of apoptosis. Activation of the insulin-like growth factor-I receptor (IGF-IR) by binding of IGF-1 and IGF-2 plays important roles in cell proliferation, survival and inhibition of apoptosis. The IGF-1/IGF-IR pathway has been implicated in a number of fibrotic diseases, such as renal fibrosis in IgA nephropathy, fibroproliferative acute respiratory distress syndrome and rat liver fibrogenesis. Moreover, previous studies have demonstrated that IGF and IGF-IR are involved in keloid and HS.

Recently, several studies reported that IGF-IR was overexpressed in keloid fibroblasts, which was shown to enhance the invasive activity of fibroblasts and to make fibroblasts resistant to ceramide-induced apoptosis. However, the expression and role of IGF-IR in HS remains unclear.

To explore the role of IGF-IR in the pathogenesis of HS, we investigated expression levels of IGF-IR in both epidermis and dermis in immature HS, mature HS, keloid and normal skin using immunohistochemistry, real-time PCR and western blotting.

Methods

The study was approved by the Medical and Ethics Committees of the First Affiliated Hospital of Sun Yat-Sen University, and informed consent was obtained from each patient.

Participants

Thirty patients (12 men, 18 women; mean ± SD age 29.10 ± 6.34 years, range 18–44) who had not received any treatment prior to surgical excision were enrolled into the study from March 2010 to June 2012. These 30 cases comprised 10 keloid and 20 HS (10 immature stage and 10 mature stage). Normal skin tissues were obtained from 10 healthy volunteers (5 men, 5 women; mean ± SD 27.30 ± 5.27 years, range 18–36). The patient and control groups had similar distributions with respect to sex, age, ethnicity, and the type, site and cause of scar (Table S1).

Tissue sections stained with haematoxylin and eosin were used for histopathological confirmation of the clinical diagnosis of keloid or HS, according to the histological criteria described by Lee et al. HS was characterized by presence of collagen fibres arranged in nodules (Fig. 1b,c), while keloid had less cellularity and contained thick collagen bundles arranged parallel to epidermis (Fig. 1d). Additionally, we divided the HS into mature and immature stages according to the criteria described by Abdou et al. Immature HS shows numerous capillaries, fibroblasts, inflammatory cells and collagen fibres, and the epidermis may be flattened, ulcerated, or normal (Fig. 1c). Mature HS shows more markedly thickened and hyperesinophilic collagen bundles arranged in whorls or nodular patterns, with minimal inflammation and less vascularity (Fig. 1b).

Cell culture

Primary fibroblast cultures were established as previously described. The specimens were digested in Dulbecco modified Eagle medium (DMEM) with 0.5% dispase overnight at 4 °C, then cultured in DMEM with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 0.1 g/mL streptomycin at 37 °C in a humidified incubator with 5% CO₂. Fibroblasts at passages 3–6 were used in this study.

Immunohistochemistry

Tissue sections on slides were incubated with 3% H2O2 to block endogenous peroxidase, then the slides were incubated with antibodies against IGF-IR (ab39398; Abcam Inc. Boston, MA, USA) overnight at 4 °C. Negative controls were incubated with phosphate-buffered saline. A DAB kit (AR1022; Boster, Wuhang, China) was then used for visualization. The immunostaining results were then evaluated. If the cellular membrane or cytoplasm stained dark-brown, it was considered as positive and evaluated as a percentage (0 was ≤ 5%; 1 was 6–25%; 2 was 26–50%; 3 was 51–75%; and 4 was ≥ 76%). Evaluation of IGF-IR expression was calculated by a double scoring system (multiplying the immunoreactive percentage score by the intensity score). Immunoreactive intensity was graded as 0 = negative, 1 = weakly positive, 2 = moderately positive, and 3 = strongly positive. The immunohistochemistry results were evaluated and scored by two independent observers who were blinded to the clinicopathological information of the patients. The average value from the two observers was used as the final score.
Real-time PCR

Real-time PCR analyses were performed using a real-time PCR system (LightCycler; Roche Molecular Biochemicals, Mannheim, Germany) in accordance with the manufacturer’s instructions. Total RNA was extracted from fibroblasts using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 2 μg of mRNA using reverse transcriptase (Superscript™; Invitrogen) with oligo(dT) as primers. Real-time quantitative (q)PCR was performed using a SYBR Green I Core Kit (Eurogentec, Southampton, UK) and a qPCR thermal cycler (Opticon; Bio-Rad, Hertfordshire, UK). Relative IGF-IR transcript levels were corrected by normalization based on glyceraldehyde 3-phosphate (GAPDH) levels. The sequences of the IGF-IR primers are shown in Table 1.

Western blot analysis

Western blotting was performed as described previously.18 Briefly, the membrane was subjected to immunoblotting with primary antibodies for IGF-IR and GAPDH (#5174, Cell Signaling Technology Inc.).

Statistical analysis

The results of real-time PCR are shown as bar figures. The western blot bands were quantified by densitometry. Results were analysed using SPSS software (version 13.0; SPSS Inc. Chicago, IL, USA). ANOVA and
independent t-tests with Bonferroni correction were used to compare groups of continuous, normally distributed variables, while χ² or Fisher exact test with Bonferroni correction were used to determine the significant difference between groups for qualitative variables. The IGF-1R intensity and immunoreactivity scores were assessed by Kruskal–Wallis analysis, and Bonferroni-corrected Mann–Whitney U-test was used when comparing two groups. Statistical significance was set at \( P < 0.05 \) (\( P < 0.0083 \) for Bonferroni correction).

**Results**

**Immunohistochemistry staining of insulin-like growth factor-1 receptor in keloid and hypertrophic scar**

Fibroblasts in the dermis showed immunoreactivity to IGF-1R in 9 of the 10 (90%) keloid cases (Fig. 1i), 8 of the 10 (80%) immature HS cases (Fig. 1k), and 3 of the 10 (30%) mature HS cases (Fig. 1j), whereas no immunoreactivity was seen in normal skin specimens (Fig. 1i). Of the 10 keloid cases, 8 (80%) had strong staining intensity for IGF-1R, 1 (10%) had moderate staining and 1 (10%) had no staining. Of the 10 immature HS cases, 3/10 (30%) had strong staining, 4/10 (40%) had moderate staining, 1/10 (10%) had weak staining and 2/10 (20%) had no staining. Of the 10 mature HS cases, 3 (30%) had weak staining, while the other 7 (70%) had no staining. There was no significant difference in immunoreactivity scores for the dermis between keloid and immature HS, but there was a significant difference between immature and mature HS (\( P < 0.01 \)) for IGF-1R expression (Table 2). IGF-1R was also found to be expressed in the overlying epidermis of all samples, with no significant difference between them (Fig. 1e–h; Table 1).

**High expression of insulin-like growth factor-1 receptor by fibroblasts in keloid and immature hypertrophic scar**

To confirm IGF-1R expression in keloid and immature HS, we performed western blotting studies using anti-IGF-1R antibody in cultured fibroblasts. No clear staining bands were present for cultured fibroblasts from normal skin or mature HS, and there was no significant difference between them. However, positive IGF-1R bands were evident for cultured fibroblasts from keloid and immature HS, and both were significantly different from normal skin fibroblasts (\( P < 0.001 \) and \( P = 0.001 \), respectively). The IGF-1R band was also significantly stronger (\( P < 0.001 \)) for cultured fibroblasts from keloid than for those from immature HS (Fig. 2).

Real-time PCR also showed that IGF-1R mRNA was overexpressed in primary culture fibroblasts of keloid and immature HS, and was significantly different from that of normal skin and mature HS (keloid vs. normal skin \( P < 0.001; \) keloid vs. mature HS \( P < 0.001; \) immature HS vs. normal skin \( P = 0.002; \) immature HS vs. mature HS \( P = 0.003 \)). The level of IGF-1R mRNA in keloid fibroblasts was higher than that in immature HS fibroblasts (\( P < 0.01 \)). In addition, there

| Table 2 Differences in insulin-like growth factor-1 receptor (IGF-1R) immunohistochemistry expression between normal skin, mature and immature hypertrophic scar (HS), and keloid. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| IGF-1R                          | Normal skin \((n = 10)\) | Mature HS \((n = 10)\) | Immature HS \((n = 10)\) | Keloid \((n = 10)\) |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Dermal expression               | \(\chi^2 = 21.6, \ P < 0.001\) | \(P = 0.07^{\dagger}\) | \(P = 0.10^{\dagger}\) | \(\chi^2 = 11.20, \ P < 0.01\) |
| Positive                        | 9 (90)          | 8 (80)          | 9 (90)          | 9 (90)          |
| Negative                        | 10 (100)        | 7 (70)          | 2 (20)          | 1 (10)          |
| Intensity                       | \(\chi^2 = 11.20, \ P < 0.01\) | \(U = 1.50^{\dagger\dagger}; \ P < 0.1\) | \(U = 17.00^{\dagger\dagger}; \ P = 0.03\) | \(\chi^2 = 25.9^{\dagger\dagger}; \ P < 0.001\) |
| Weak                            | 0 (0)           | 3 (30)          | 1 (10)          | 0 (0)           |
| Moderate                        | 0 (0)           | 0 (0)           | 4 (40)          | 1 (10)          |
| Strong                          | 0 (0)           | 0 (0)           | 3 (30)          | 8 (80)          |
| Scoring                         | 0 (0), 0\(\dagger\dagger\) | 0 (0), 1\(\dagger\dagger\) | 12 (11.25, 12) | 12 (11.25, 12) |
| Epidermal expression            | \(\chi^2 = 0.97; \ P = 1.0\) | \(\chi^2 = 1.50, \ P < 0.1\) | \(\chi^2 = 11.20, \ P < 0.01\) | \(\chi^2 = 25.9^{\dagger\dagger}; \ P < 0.001\) |
| Positive                        | 9 (90)          | 8 (80)          | 9 (90)          | 9 (90)          |
| Negative                        | 1 (10)          | 2 (20)          | 1 (10)          | 1 (10)          |

\(P^1\): comparison between the four groups; \(P^2\): comparison between immature and mature HS; \(P^3\): comparison between immature HS and keloid. \(P < 0.0083\) was considered significant for Bonferroni correction. Data are \(n\ (%)\) unless otherwise stated. \(^{\dagger}\)Fisher exact test; \(^{\dagger\dagger}\)Mann–Whitney \(U\)-test; \(^{\dagger\dagger}\)interquartile range.
was no significant difference in IGF-IR mRNA level between mature HS and normal skin fibroblasts (Fig. 3).

Discussion

In the current study, we found significant upregulation of IGF-IR protein and mRNA in dermal fibroblasts from keloid and immature HS, but only weak expression of both in mature HS dermal fibroblasts, which was almost similar to that of normal skin fibroblasts. In addition, expression of IGF-IR protein and mRNA in keloid fibroblasts was greater than in immature HS fibroblasts. Both Ohtsuru et al.\textsuperscript{12} and Yoshimoto et al.\textsuperscript{13} reported increased expression of IGF-IR in dermal fibroblasts from keloid, but not in those from normal skin. However, in our study, we found overexpression of IGF-IR in dermal fibroblasts from immature HS, but only weak expression in mature HS. Moreover, our study also found expression of IGF-IR in the overlying epidermis of keloid, HS (both stages) and normal skin, with no significant difference between them.

IGF-I and IGF-II are mitogens and differentiation factors, which have been shown to facilitate wound healing by stimulating fibroblast proliferation and enhancing collagen synthesis.\textsuperscript{21} Both receptors exert their effects via IGF-IR, which in turn phosphorylates phosphoinositide 3-kinase and Ras/Raf/mitogen-activated protein kinase (MAPK),\textsuperscript{22} two kinases that play important roles in IGF-IR-induced cellular proliferation and apoptosis inhibition.\textsuperscript{23} Overexpression of IGF-IR was shown to make keloid fibroblasts resistant to ceramide-induced apoptosis,\textsuperscript{14} and targeted disruption of IGF-IR resulted in growth inhibition.\textsuperscript{24} Both keloid and HS occur as the result of a pathological wound-healing process, characterized by excess collagen deposition and hyperproliferation of fibroblasts. Keloid and HS fibroblasts have been shown to possess greater proliferative capacity and to be more resistant to apoptosis than normal dermal fibroblasts.\textsuperscript{25} In the current study, we found that IGF-IR protein and mRNA were overexpressed not only in keloid fibroblasts but also in immature HS fibroblasts. In addition, positive IGF-IR staining and expression intensity in dermal fibroblasts were associated with scar in the immature stages. As the HS matured, expression of IGF-IR nearly disappeared, reaching a level comparable with that of normal skin. Therefore, we speculate that IGF-IR might be involved in the pathogenesis of keloid and HS by inhibiting fibroblast apoptosis and stimulating collagen synthesis by fibroblasts. However, the precise role of IGF-IR in scar formation needs to be explored further.
Epidermal and mesenchymal homeostasis, growth and differentiation are regulated by epidermal–mesenchymal interactions. During wound healing, epidermal keratinocytes play an important regulatory role in the proliferation and apoptosis of the underlying fibroblasts and the production of ECM by cytokines from keratinocytes. Our study found that IGF-IR is expressed in the overlying epidermis of all samples, without any difference between them, indicating that IGF-IR may help in epithelialization and healing of wound by inhibiting keratinocyte apoptosis and promoting keratinocyte renewal. However, the specific role of IGF-I/IGF-IR in epithelial–mesenchymal interactions is unclear, and further studies are needed to answer this question.

Conclusion

We analyzed the expression profile of IGF-IR in keloid, HS and normal skin, and identified significant upregulation of IGF-IR in dermal fibroblasts from keloid and immature HS. Our results indicate that IGF-IR might be involved in the pathogenesis of keloid and HS by inhibiting fibroblast apoptosis and stimulating collagen synthesis, although the specific mechanism remains to be clarified.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
Table S1. Patient demographics and clinical characteristics (Keloids and HS).