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Central regions of keloids are severely ischaemic

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Summary We classified scars as keloids, hypertrophic scars and mature scars, and then examined the scars for differences in central and marginal vascularization. We found significant differences in localized hypoxia-induced factor-1α (HIF-1α) expression and vascular density in keloids, but no localized differences in hypertrophic or mature scars. The central areas of keloids exhibited higher HIF-1α expression and lower vascular density than marginal areas, suggesting that the former are severely ischaemic.

Introduction

Wounds that breach the dermis become normal scars through the inflammation, growth and maturation phases of normal wound healing. In keloids and hypertrophic scars, this process is disrupted and fibroblasts derived from the wound actively synthesize immature collagen fibres.[1—3] Keloids and hypertrophic scars contain more cells compared to the normal dermis.[4] We found particularly high adenosine triphosphate (ATP) levels in keloids even after about 10 years,[5] perhaps due to anaerobic glycolysis caused by the reduction in oxygen perfusion from compressed blood vessels. Keloids have an inadequate blood supply,[6] and three-dimensional reconstruction of blood vessels within keloid scars has revealed that the vessels in the central region are crushed and flattened, providing only sparse, inadequate perfusion.[7] Thus, we hypothesized that a significant, localized variation of ischaemic state is a key characteristic of keloids. In this study, we classified scars as keloids, hypertrophic scars and mature scars, and examined ischaemic condition and differences of vascularization in the central and marginal areas by hypoxia-induced factor-1α (HIF-1α) expression, vascular endothelial growth factor (VEGF) expression and vascular density.

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HIF-1, the major transcription factor in response to hypoxia, consists of two subunits, HIF-1α and HIF-1β. Only the HIF-1α subunit is regulated by a reduced oxygen level.[8,9] In addition to hypoxia, growth factors and proinflammatory cytokines, the proteins abundantly released by mast cells such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α have been demonstrated to be critical regulators of HIF-1α stabilization, translocation and activation.[10–15] There is growing evidence that HIF-1α is involved in the inflammatory process by regulating angiogenesis and functions of inflammatory cells.[16–18]

VEGF promotes angiogenesis. Several studies have suggested that angiogenesis and vascular factors play a significant role in keloid progression, due to their prolonged erythematous period and invasive characteristics.[19–21]

Materials and methods

Patients and samples

Eleven samples each from keloid, hypertrophic scar and mature scar tissues in which the central and marginal regions could be clearly distinguished were randomly selected from specimens excised at the Osaka Medical College Hospital between January 2006 and December 2012. Samples were immediately fixed in formalin and embedded in paraffin wax. The central region of each specimen was defined as the central area of the keloid, and the marginal areas were defined as areas in which invasion of healthy tissue and erythema were evident on visual examination. None of the patients had undergone radiotherapy. Patient characteristics and demographics are shown in Table 1. Diagnosis was based on the following clinical and histological criteria: Clinically, keloids extend beyond the borders of the initial injury and are variably pruritic. Hypertrophic scars, in contrast, usually remain within the borders of the initial injury. Histologically, keloids are characterized by the presence of hyalinized collagen bundles, nodular fibroplasia and an accumulation of myofibroblasts expressing α-smooth muscle actin.[22–24] An experienced pathologist performed the histological diagnoses in the present study.

Staining method

All specimens were examined after staining with haematoxylin and eosin (HE) and immunostaining with anti-CD31 antibodies, anti-VEGF antibodies and anti-HIF-1α antibodies. EnVision kits (Dako Cytomation, Kyoto, Japan) were used for immunohistological staining. Anti-human CD31 monoclonal antibodies (Leica Microsystems, Kyoto, Japan) were diluted to 20:1 ratio. Anti-human VEGF monoclonal antibodies (Santa Cruz Biotechnology, Kyoto, Japan) were used at 20:1 dilution. Anti-human HIF-1α polyclonal antibodies (Novus Biologicals, Kyoto, Japan) were diluted to 50:1 ratio.

The tissue specimens embedded in paraffin wax were sliced into 5-μm sections using a microtome. Paraffin sections were dissolved in xylene and alcohol and then treated in a microwave oven with citric acid buffer (pH 6.0). Next, they were treated with 3% hydrogen peroxide in 30% methanol to remove endogenous peroxide, and then incubated with the primary and secondary antibody (anti-mouse immunoglobulin or anti-rabbit immunoglobulin antibody) and subsequently with peroxidase. Colouring of sections was achieved using the EnVision kits. The sections were counterstained with haematoxylin.

Measurement procedure

Five fields of view (400× magnification) were chosen in the central and marginal regions to avoid overlap, and scanned images were printed for visual counts of cells and vascular density. The number of HIF-1α or VEGF-positive fibroblasts was calculated as the percentage of the total number of fibroblasts. In order to measure vascular density, the CD31-positive luminal structures were counted, and the density per unit area was calculated. Representative images of HIF-, VEGF- and CD31-positive cells are shown in Figure 1a–c.

Statistical analysis

Significant differences were determined by two-tailed paired *t*-test and Student's *t*-test. Differences with $P < 0.05$ were considered as significant. Excel 2010 (Microsoft) software was used for all analyses.

Results

We analysed HIF-1α expression, VEGF expression and vascular density in the central and marginal areas of keloids (Figure 2), hypertrophic scars (Figure 3) and mature scars (Figure 4). In keloids, HIF-1α expression was higher in the central areas than in marginal ($P < 0.01$, paired *t*-test), whereas vascular density was higher in the marginal areas than in central ($P < 0.01$, paired *t*-test). No significant differences were observed in VEGF expression between the central and marginal areas. Interestingly, no significant differences were observed in vascular density or in the

Table 1 Specimens.

	No. of scars	Age (years)	Male:Female	Period (months)
Keloid	11	14–78 (57)[a]	6:5	16–42 (27)[a]
Hypertrophic scar	11	1–70 (38)[a]	3:8	8–42 (23)[a]
Mature scar	11	4–60 (38)[a]	3:8	6–720 (223)[a]

The period represents the timing (months) of surgical resection after injury.
[a] Mean (SD).