MECHANISMS OF MICROVASCULAR WOUND REPAIR II. INJURY INDUCES TRANSFORMATION OF ENDOTHELIAL CELLS INTO MYOFIBROBLASTS AND THE SYNTHESIS OF MATRIX PROTEINS

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SUMMARY

Under normal growth conditions, in vitro dermal microvascular endothelial cells (HDMEC) retain an epithelioid morphology and do not synthesize matrix proteins found increased in scar tissue. When injured by a standard scratch, cells at the wound edge and within the culture transform into spindle-shaped, myofibroblast-like cells. To determine if the transformed cells synthesize matrix proteins, expression of type I collagen and alpha smooth muscle actin (α-SMA) was investigated by immunohistochemistry and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Twelve hours following injury, a major upregulation in expression of α-SMA and type I collagen was observed both in cells proximal and distal to the wound edge. Cells with the typical morphology of myofibroblasts and displaying intracellular α-SMA positive fibrils were observed in HDMEC throughout the culture. In contrast, type IV collagen, a basement membrane protein, was not detected in migrating cells. Following completion of wound repair (24–36 h), type I collagen was no longer expressed and type IV collagen synthesis increased to prewound levels. Quantitative RT-PCR confirmed the changes in gene expression for both type I collagen and α-SMA at each time point during repair. These results demonstrate that normal skin microvascular endothelial cells retain an ability to transform into myofibroblast-like cells when injured and to synthesize matrix proteins not expressed in noninjured cells. The synthesis of matrix proteins by injured endothelial cells suggests a direct role for the endothelium in the pathology of scar formation.

Key words: endothelial; reendothelialization; type I collagen; alpha smooth muscle actin; scars.

INTRODUCTION

In skin, abnormal wound healing may result in keloids and hypertrophic scars. Each defect is characterized by an excessive accumulation of type I collagen and alpha smooth muscle actin (α-SMA) (Friedman et al., 1993; Lee et al., 2004). Although several skin cell types have been suggested as the source of matrix proteins, Beranek (1989) was one of the first investigators to propose that a transition of human dermal microvascular endothelial cells (HDMEC) into spindle-shaped cells may be one of the cell types responsible for this synthesis. In vitro the transdifferentiation of HDMEC into spindle-shaped cells following activation with inflammatory mediators was described (Lipton et al., 1991) and the second messengers that control this process reported (Davison and Karasek, 1981; Tuder et al., 1990). In the aorta, the transdifferentiation of aortic endothelial cells into smooth muscle like cells has been described suggesting that endothelial cells may be responsible for the synthesis of matrix proteins in atherosclerosis (Arciniegas et al., 2003).

In vivo an important critical step in skin wound repair is the inflammatory response. The proinflammatory mediators tumour necrosis factor-α (TNF-α) and interleukin-one beta (IL-1β) play pivotal roles in the repair process (Davidson, 1992). In recent studies of the role of proinflammatory cytokines on HDMEC transformation and fibrosis, in vitro spindle-shaped cells induced by proinflammatory cytokines were identified positively as myofibroblast-like cells by cytoplasmic microfilaments with dense bodies and attachment plaques and by the expression of α-SMA, type I collagen, and calponin (Chaudhuri et al., 2000). After short-term exposures to IL-1β or TNF-α (<3 d), the endothelial to myofibroblast transformation was reversible; after long-term exposures (>10 d), the transformation was permanent.

In this study we describe how mechanical injury to the integrity of the skin microvasculature also induces a transformation of HDMEC into myofibroblast-like cells and activates genes responsible for the synthesis of type I collagen and α-SMA. These matrix proteins are not synthesized by resting, noninjured HDMEC and suggest that injury to the vasculature in vivo may play a direct role in the deposition of matrix proteins in the pathology of scar formation.

MATERIALS AND METHODS

Reagents. The following reagents were used in this study: dispase (Collaborative Research, Bedford, MA); Trypsin ethylenediaminetetraacetic acid (EDTA), Iscove's growth medium (Iscove's BRL, Grand Island, NY, US); Alexis extracellular agglutinin 1 (Sigma, St Louis, MO); Dynabeads (Dynal Biotech Inc, NY); endothelial growth media (EGM) (Clonetics, MD). All primary antibodies were purchased from Santa Cruz (Santa Cruz Biotech, CA); AL-
Isolation of skin microvascular endothelial cells. The isolation of HDMEC and the preparation of growth medium and maintenance of cultures were carried out as described previously (Normand and Karasek, 1995). All studies involving human tissue were approved by the Human Subjects Committee of Stanford University School of Medicine, Stanford, CA, and were in accordance with the Declaration of Helsinki Principles. After the first passage, cells were purified twice with Ulex europaeus agglutinin 1 coated on magnetic Dynabeads (Holtboer et al., 1982). Purity of the cell population was confirmed by positive staining for von Willebrand factor and platelet endothelial cell-adhesion molecule-1 (PECAM-1). All experimental studies were performed with Ulex purified passage 3 or passage 4 HDMEC.

Scratch injury. Cells were plated at $5 \times 10^4$ cells/cm$^2$ in 10-mm wells of plastic culture dishes (Falcon) and grown until confluent. Absence of mitotic activity prior to wounding was confirmed by negative staining for Ki-67 in duplicate cultures. Confluent cultures were scratched across the cell surface (either vertically or horizontally) with a 10-μl pipette tip, and detached cells were removed by washing with growth medium.

Immunostaining. For fluorescent immunostaining studies, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. Cells were blocked overnight at 4°C in PBS containing 0.2% bovine serum albumin. After washing with PBS, cells were incubated for 2 h at room temperature with antibodies for α-SMA, type I collagen, and type IV collagen, followed by three 5-min washes with PBS. Fluorescently labeled secondary antibody AlexaFluor 488 was added for 2 h. Stained cultures were washed 3 times with PBS while being shielded from light. Coverslips were mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) nuclear stain (Vector Laboratories, Inc., Burlingame, CA) and stored in the dark. Fluorescent images were recorded digitally with a Zeiss Axiosvert 100 M microscope.

Quantitative RT-PCR. The $2 \times 10^5$ HDMEC (passage 3) were plated in 80-mm culture dishes and grown to confluence. RNA was extracted using the Qiagen RNA extraction kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA quality and yield initially were determined by qualitative RT-PCR. Comparative quantitative RT-PCR was carried out according to the manufacturer's instructions using the Brilliant SYBR green qRT-PCR kit from Stratagene on a magnification X8000 real-time PCR machine (Stratagene) on 3 genes (α-SMA, type I, and type IV collagen) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the normalizing gene to correct baseline values for all samples. Samples from each time point were compared to the 0-h control (referred to as calibrator sample for real-time analysis). The software used computed the mean difference and the standard deviation for 3 independent triplicate wells. The primer sequences for each gene were:

$\text{S-GAPDH-QRT:}$ \[ \text{GAGTCACAGGATTGTCGC} \]
$\text{A-GAPDH-QRT:}$ \[ \text{TGTAGTTGAGGATGTC} \]
$\text{S-QRT-SMA-QRT:}$ \[ \text{TCAGTGTGCAAGGATG} \]
$\text{A-QRT-SMA-QRT:}$ \[ \text{GAAGAATGCAAGGCTCA} \]
$\text{S-QRT-ICOL-QRT:}$ \[ \text{GTGCTAAAGTGGTCGTTG} \]
$\text{A-QRT-ICOL-QRT:}$ \[ \text{CTCTGCGTTTCCTICTCTC} \]
$\text{S-QRT-ICOL-QRT:}$ \[ \text{CTCTGCGTTTCCTICTCTC} \]
$\text{A-QRT-ICOL-QRT:}$ \[ \text{CCTTTCCCTTTGTCACA} \]

RESULTS

Injury induces epithelioid to spindle transformation and increases the size of noninjured cells. As shown in the phase contrast micrograph of Fig. 2b, cells at the wound edge transform quickly (within 2 h) from a classical epithelioid into a more spindle-shaped morphology. Figure 1a illustrates a crystal violet stain of a lower magnification X4. A phase contrast micrograph of HDMEC 2 h following wounding. Cells both at and adjacent to the wound edge convert into a spindle-shaped morphology (arrow). Size of nonwounded cells more proximal to the wound edge increases (arrowhead) when compared to cells more distal from the cut edge (long arrow). Results shown are representative of 3 individual tissue samples. Magnification X320.

Fig. 1. Changes in human dermal microvascular endothelial cell (HDMEC) size and morphology in cells proximal and distal to the wound edge. (a) Low-power magnification of horizontal and vertical wounds illustrating changes in cell size and morphology of HDMEC over a large wound area. Wounds were made in confluent cultures of HDMEC as described in the Materials and Methods section. Cultures were fixed with ethanol 2 h after wounding and stained with crystal violet magnification X64. (b) Phase contrast micrograph of HDMEC 2 h following wounding. Cells both at and adjacent to the wound edge convert into a spindle-shaped morphology (arrow). Size of nonwounded cells more proximal to the wound edge increases (arrowhead) when compared to cells more distal from the cut edge (long arrow). Results shown are representative of 3 individual tissue samples. Magnification X320.