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ORIGINAL ARTICLE

TGF-β Antisense Therapy Increases Angiogenic Potential in Human Keratinocytes *In Vitro**

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Background. Transforming growth factor-beta (TGF-β) has been identified as an important component of wound healing. Recent developments in molecular therapy offer exciting prospects for the modulation of wound healing, specifically those targeting TGF-β. The purpose of this study was to analyze the effect of TGF-β targeting on the expression of angiogenic vascular endothelial growth factor (VEGF), a key regulator of angiogenesis, and *in vitro* angiogenic activity.

Methods. Expression of angiogenic VEGF in tissue samples from chronic dermal wounds was investigated by immunohistochemistry. The effect of TGF-β targeting using antisense oligonucleotides on the expression of VEGF was analyzed by ELISA and RT-PCR in cultured human keratinocytes. Human endothelial cells (EC) were grown in conditioned medium produced from the treated keratinocytes. EC migration was measured using a modified Boyden chamber, EC tube formation was analyzed under the light microscope.

Results. Immunohistochemical investigation demonstrated a decreased expression of VEGF protein in tissue samples from chronic dermal wounds compared to normal human skin. Antisense TGF-β oligonucleotide treatment upregulated VEGF secretion *in vitro*. Addition of conditioned medium from TGF-β antisense-treated keratinocytes resulted in an increase of endothelial cell migration and tube formation.

Conclusions. Our results demonstrate that TGF-β antisense oligonucleotide technology may be a potential therapeutic option for stimulation of angiogenesis in chronic wounds. © 2007 IMSS. Published by Elsevier Inc.

Key Words: Keratinocytes, Chronic wound, VEGF, Angiogenesis, TGF-β.

Introduction

Wound healing is a complex process involving a series of overlapping stages, relying on the collaboration of many different extracellular matrix (ECM) components, cell types and soluble mediators. Simplified, the process of wound healing is often subdivided into three phases: (i) inflammation, (ii) granulation formation, and (iii) matrix formation and remodeling (1). If the normal process of wound healing is disrupted, a chronic non-healing wound can result. Pressure sores, venous ulcers, and diabetic foot ulcers are the three main types of such wounds, accounting for 70% of all chronic wounds (1). Local ischemia is thought to be the major underlying cause for such chronic wounds (1). In addition, other factors contributing to the disrupted healing include alterations in growth factors and protease expression (2–4). Wound repair is initiated with the aggregation of platelets, formation of a fibrin clot, and release of growth factors from the activated coagulation pathways, injured cells, platelets, and ECM, followed by migration of inflammatory cells to the wound site. Thereafter, keratinocytes migrate into the wound and angiogenesis

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is initiated. During the wound healing processes, an abundant blood supply is necessary to meet the enormous local demands of debridement, fibroblast proliferation, ECM synthesis, and epithelialization (5–7). Impairment of blood supply may be a contributing factor in delayed healing, or nonhealing, chronic wounds (8,9). Vascular endothelial growth factor (VEGF) is thought to be a key regulator of angiogenesis (10,11), mainly during the proliferative phase of wound healing (12). VEGF was first isolated as a mediator of vascular permeability in the late 1970s (13). There are now five known isoforms, detectable in different human tissues (14). VEGF acts via two tyrosine kinase receptors located predominantly on endothelial cells (14). During the wound healing process, VEGF is released primarily by keratinocytes but also by macrophages and fibroblasts (12). VEGF levels rise steadily after wounding and serve as a potent angiogenic factor (15). VEGF production is influenced greatly by local tissue conditions including hypoxia, and also by nitric oxide production (16). VEGF causes increased vascular permeability and deposition of a proangiogenic matrix, as well as formation of blood vessels (14). VEGF administration improved granulation-tissue formation in both normal and hypoxic tissues during experimental wounding (17).

Transforming growth factor-beta (TGF-β) is known to be the most potent growth factor involved in wound healing throughout the body (18). Released by degranulating platelets at the site of injury, TGF-β1 influences the inflammatory response, angiogenesis (19), re-epithelialization, ECM deposition, and remodeling (19,20). In recent studies, targeting of TGF-β resulted in accelerated wound healing and reduced scarring (19,21). These effects make TGF-β important in wound repair and have prompted considerable attention in the context (18). However, the molecular mechanisms are still not well understood. In previous studies we were able to show that TGF-β targeting by using antisense oligonucleotides effectively modulated growth factor expression in human wound healing-related cells (22,23). The purpose of this study was to investigate the expression of angiogenic VEGF in tissue samples of chronic wounds and to analyze the effect of TGF-β targeting on the expression of VEGF and their effect on angiogenic potential in human keratinocytes *in vitro*.

Materials and Methods

Immunohistochemistry

Tissue specimens ($n = 5$) of chronic wounds and normal controls were gained from excised tissue during surgery and rapidly frozen in liquid nitrogen for later VEGF identification. They were cut in 10-μm cryostat sections, transferred on glass slides, and air dried overnight at room temperature. The sections were then stored at −20°C until immunostaining. Immunohistochemistry for VEGF detection was performed by using a streptavidin–biotin complex procedure. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. Sections were washed with phosphate-buffered saline (PBS) and incubated with normal rabbit serum in PBS for 30 min at room temperature to block nonspecific antibody reaction. The sections were then incubated overnight at 4°C with the primary antibody (VEGF polyclonal Ab, Cat. #sc-152, Calbiochem, Hamburg, Germany). The slides were washed in several changes of PBS. The sections were then incubated with a peroxidase-conjugated secondary antibody (DAKO, Hamburg, Germany). After being washed twice in PBS, sections were treated with a streptavidin–biotin–peroxidase complex and peroxidase reaction was performed using diaminobenzidine (DAB, DAKO) as chromogen. The different antibodies were diluted to the desired concentrations in PBS. Controls were carried out by omitting the primary antibody. Light microscopy investigation was performed using a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany).

Cell Culture

For *in vitro* analysis a human epidermal keratinocyte (Adult Skin) cell line (Cat. #CC2501, Cell Systems, St. Katharinen, Deutschland) was used. Cell cultures were carried out in Falcon petri dishes at 37°C in a 5% CO_2 fully humidified atmosphere using Dulbecco's modified minimum essential medium (DMEM, Fisher Scientific Co., Pittsburgh, PA) supplemented with 10% fetal calf serum (FCS) and antibiotics (Life Technologies, Inc., Gibco BRL, Gaithersburg, MD). For antisense treatment, the medium from the cultures was aspirated and replaced with DMEM containing 5% FCS and antibiotics followed by the addition of oligodeoxynucleotides. Human microvascular endothelial cells (HMVEC, PromoCell, Heidelberg, Germany) were used for *in vitro* angiogenesis analysis. Cells were grown in Endothel Cell Growth Medium (PromoCell) supplemented with 2% fetal bovine serum (FBS).

Oligodeoxynucleotides

Phosphorothioated 14-mer oligodeoxynucleotides were synthesized on an Applied Biosystem 394 DNA synthesizer by means of B-cyanothylphosphoramidite chemistry to minimize degradation by endogenous nucleases. The antisense oligonucleotide (5′-CGA TAG TCT TGC AG-3′) was directed against the translation start site and surrounding nucleotides of the human TGF-β cDNA. For negative control, cells were treated by the addition of PBS or oligonucleotides (5′-GTC CCT ATA CGA AC-3′) containing the same nucleotides in scrambled order. The *in vitro* inhibitory effect of these antisense oligos on TGF-β expression on both the mRNA and protein level in human cells has been described previously (23,24). All experiments were performed with 12.5 μM oligodeoxynucleotides, unless otherwise stated.