Abrogation of TGF-ß by antisense oligonucleotides modulates expression of VEGF and increases angiogenic potential in isolated fibroblasts from radiated skin

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Abstract. The transforming growth factor-ß (TGF-ß) has been identified as an important component of wound healing. Recent developments in molecular therapy offer good prospects for the modulation of wound healing, specifically those targeting TGF-ß. The aim of this study was to analyze the effect of TGF-ß targeting on the expression of angiogenic vascular endothelial growth factor (VEGF), a key regulator of angiogenesis and *in vitro* angiogenic activity in fibroblasts isolated from radiation-induced chronic dermal wounds. The expression of angiogenic VEGF in tissue samples from radiation-induced chronic dermal wounds was investigated by immunohistochemistry and microarray technique. The effect of TGF-ß targeting using antisense oligonucleotides on the expression of VEGF in isolated fibroblasts was analyzed by ELISA and multiplex RT-PCR. Human endothelial cells (ECs) were grown in conditioned medium produced from the treated fibroblasts. EC migration was measured using a modified Boyden chamber; EC tube formation was analyzed under a light microscope. Immunohistochemical investigation and microarray analysis demonstrated a decreased expression of VEGF protein and mRNA in tissue samples from radiation-induced chronic dermal wounds compared to normal human skin. Antisense TGF-ß oligonucleotide treatment significantly up-regulated VEGF secretion *in vitro*. Addition of conditioned medium from TGF-ß antisense-treated fibroblasts resulted in an increase in EC cell migration and tube formation. In conclusion, our results demonstrate that TGF-ß antisense oligonucleotide technology may be a potential therapeutic option for stimulation of angiogenesis in radiation-induced dermal wounds.

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Introduction

Radiotherapy is associated with a wide spectrum of normal tissue reactions, and as the life expectancy of cancer patients increases, normal tissue effects are increasingly of clinical importance (1). The goal of radiotherapy is to precisely target a tumor volume while limiting the volume of normal tissue exposed to radiation. The therapeutic ratio of radiotherapy is achieved by the greater capacity of normal tissue to repair radiation-induced sublethal DNA damage as compared with rapidly proliferating tumor cells. However, normal tissues that also rapidly proliferate, such as skin, are also relatively radiosensitive (2). Tissue toxicity may range from asymptomatic changes in tissue structure and function, to severe cosmetic disfigurement and life-altering changes in organ function (3). The degree of change is related to the biological dose and the volume irradiated (2). Capillaries are the most radiosensitive component of the vasculature; thus, capillary vascular injury may be at the crux of tissue radiosensitivity (4,5). Following the acute skin changes, the skin appears 'normal' for a variable interval of time ranging from a month to years. By definition, late effects of radiation are those that present more than 90 days after the completion of radiotherapy and are associated with injury to the dermis (Fig. 1) (6). Accordingly, the radiation response of the vascular tissue actually occurs in two waves. The acute vascular changes within 24 h are dominated by the radiation-induced apoptotic cell death of endothelial cells. Late vascular effects occur within months after irradiation and include capillary collapse, thickening of basement membrane, scarring of the surrounding tissue as well as telangiectasias and a loss of clonogenic capacity. Radiation-induced late vascular damage may thus contribute to late radiation responses of the skin. In contrast to early reactions, typical late injuries are irreversible and often progressive. The late onset of necrosis and fibrosis in normal tissues is directly related to dermal fibroblast response to radiotherapy (7,8).

Situations in which inflammation does not adaquately resolve, such as radiation injury, appear to involve aberrant cytokine pathways or chronic overproduction of certain cytokines (9,10). Special tissue growth factors such as transforming growth factor-ß (TGF-ß) play a key role in this process (11). TGF- ß is known to be the most potent growth factor involved in wound healing throughout the body (12). Released by degranulating platelets at the site of injury, TGF-ß1 influences the inflammatory response, angiogenesis (13), re-epithelialization, extracellular matrix deposition and remodeling (13,14). TGF-ß1 is the major cytokine responsible for the regulation of fibroblast proliferation and differentiation (15-17). Radiation induces long-term TGF-ß1 overexpression probably owing in part to oxidative stress and an inflammatory response (15,17-19). A comparison of the genotypes of patients with or without late normal tissue injury has been genetically associated with functional polymorphisms in the TGF-ß1 gene (20,21).

In previous studies, targeting of TGF-ß resulted in accelerated wound healing and reduced scarring (12,22). Previously, we were able to demonstrate that the abrogation of TGF-ß1 in normal fibroblasts and keratinocytes results in decreased expression of MMPs (23,24) and increased angiogenic activity (25). As impairment of blood supply may be a contributing factor in radiation-induced nonhealing chronic wounds (26), and vascular endothelial growth factor (VEGF) is thought to be the key regulator of angiogenesis (27). The aim of this study was to investigate the effect of targeting TGF-ß1 expression by antisense oligonucleotides on the expression of pro-angiogenic VEGF and angiogenic activity in fibroblasts isolated from radiated skin.

Materials and methods

Immunohistochemistry. Tissue specimens of radiated skin wounds and normal controls were obtained 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 non-specific 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 was performed using a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany).

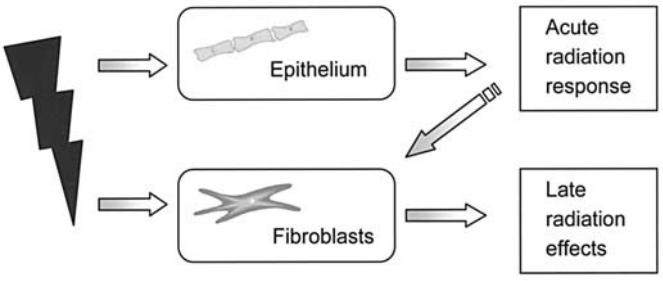

Figure 1. Pathogenesis of late radiation effects. Radiation exposure affects proliferating cells in turnover tissues, such as epithelia. In consequence, a typical acute radiation reaction with tissue hypoplasia and impairment of the barrier function occur. Similarly, irradiation effects are inflicted in connective tissue components (vasculature, fibroblasts). These result in typical generic late radiation sequelae. The impaired barrier function during the acute reaction allows for additional mechanical or chemical trauma, or secondary damage by more subtle changes to the target cells and structures involved in the late response.

Microarray analysis. Extraction of RNA from isolated fibroblasts was performed using RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA concentration was estimated from the absorbance at 260 nm. Approximately 1 μg total RNA was used in each microarray experiment, and for amplification and labeling of mRNA the Smart technique (Smart Fluorescent Probe Amplification Kit; BD Clontech, Heidelberg, Germany) was applied according to the manufacturer's protocol. RNA samples from day 1 were labeled with Cy3, and day 6 or day 21 samples were labeled with Cy5 (Cy™3- and Cy™5-monoreactive dye; Amersham Pharmacia Biotech, Freiburg, Germany). Corresponding Cy3- and Cy5-labeled samples were mixed, vacuum dried and resuspended in 25 μl microarray hybridization buffer (MWG-Biotech, Ebersberg, Germany). Prior to hybridization, the samples were heat denaturated at 95˚C for 5 min. The human 10K (MWG-Biotech) oligo microarray systems on glass slides were used for mRNA profiling. Hybridization of Cy3/Cy5-cDNA was performed using coverslips and a hybridization chamber for 16 h at 42˚C in a water bath. After stringent washing of the glass slides according to the manufacturer's specifications, the hybridization signals of the Cy3 and the Cy5 dyes were measured using a microarray laser scanner (GMS418; Affymetrix, MWG-Biotech).

The ArrayVision (Imaging Research, Inc., St. Catharines, ON, Canada) software was used for evaluation and calculation of signal intensities from the raw data images in 16-bit tagged-image-file (TIF) format. In brief, for evaluation of hybridization results we defined a negative (<3,000), a grey area (3,000-4,999) and a positive range (5,000) of hybridization signal intensities. Signal-to-background (S/B) values were calculated by dividing the signal intensity for each spot with the background signal intensities of the hybridized glass slide. Computer-assisted evaluation of the raw data provided the mean signal intensity and the signal to background ration for each individual gene spot. For statistical evaluation the mean signal intensity and standard deviation (SD) were calculated for each spot from the values obtained

in the 10 individual experiments. Functional grouping of genes was performed on the basis of the database supplied by the array manufacturer.

Cell culture. For *in vitro* analysis, dermal fibroblasts isolated from radiated skin wounds and normal controls were cultured. Cell cultures were carried out in Falcon petri dishes at 37˚C in a 5% CO_2 fully humidified atmosphere. Fibroblasts were cultured in serum-free Fibroblast Growth Medium (PromoCell, Heidelberg, Germany) supplemented with antibiotics. For antisense treatment, the medium from the cultures was aspirated and replaced with Dulbecco's modified minimum essential medium (DMEM) (Fisher Scientific Co., Pittsburgh, PA, USA) containing 5% fetal calf serum (FCS) and antibiotics [Life Technologies, Inc. (Gibco BRL), Gaithersburg, MD, USA] followed by the addition of oligodeoxynucleotides. Human microvascular endothelial cells (HMVEC, PromoCell) 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-ß1 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-ß1 expression at both the mRNA and protein level in human cells has been described previously (28). All experiments were performed with 12.5 μM oligodeoxynucleotides, unless otherwise stated. To determine the effect of oligonucleotides on the expression of MMP mRNA, cell lines were plated at a density of 10^5 cells/microtiter well in 24-well polystyrene plates (Falcon). After 24 h, the cells were rinsed twice with medium and then fresh oligo medium containing antisense or scrambled oligodeoxynucleotides was added, followed by an incubation period of 48 h.

Cytokine immunoassay. To determine the effect of oligonucleotides on VEGF expression, fibroblasts were plated in DMEM at a density of 10^5 cells/microtiter well in 24-well polystyrene plates. After 24 h, the cells were rinsed twice with medium and then fresh oligo medium containing sense or antisense oligodeoxynucleotides was added. Cell culture supernatants were collected after 48 h in sterile test tubes and stored at -20˚C until their use. Then, cytokine (VEGF) concentrations were determined by an ELISA technique (R&D Systems, Wiesbaden, Germany). According to the manufacturer's directions, each ELISA assay measured 100 μl of supernatant. All analyses and calibrations were carried out in duplicate. The calibrations on each microtiter plate included recombinant human cytokine standards provided in the kit. All concentrations were documented as pg/ml. The Student's t-test was used to calculate p-values.

RT-PCR. To isolate RNA from the cells grown in monolayer, cells were directly lysed in the culture dish by the addition of 1 ml RNA-Clean (RNA-Clean System, AGS, Heidelberg, Germany). After addition of 0.2 ml chloroform per 2 ml of homogenate and centrifugation for 15 min at 12,000 x g (4˚C), the aqueous phase was transfered to a fresh tube. After addition of equal volume of isopropanol and centrifugation for 15 min at 12,000 x g (4˚C), the supernatant was removed from the RNA precipitate. The RNA pellet was washed twice with 70% ethanol by vortexing and subsequent centrifugation for 8 min at 7,500 x g (4˚C) was carried out. After drying the RNA pellet, it was dissolved in DEPC water. The RNA was reverse transcribed (StrataScript First-Strand Synthesis System; Stratagene, La Jolla, CA, USA) into cDNA using random oligonucleotide primers. The VEGF mRNA level was measured using a commercially available RT-PCR kit (VEGF-CytoXpress Multiplex PCR Kit, BioSource, San Francisco, CA, USA) according to the manufacturer's instruction manual. To fractionate the MPCR DNA products, MPCR products were mixed with 6X loading buffer and separated on a 2% agarose gel containing 0.5 mg/ml ethidium bromide, visualized with UV light and recorded using a CCD camera. To test the quality of the cDNA, the kit includes primers for GAPDH. Results were obtained in two independent experiments.

Migration assay. Human ECs were grown on gelatin-coated dishes until confluence in Endothel Cell Growth Medium supplemented with 2% FBS. Migration assays were performed in transwell chambers (Corning-Costar Corp., Cambridge, MA, USA). Conditioned medium from the keratinocyte cell line was placed in the lower chambers, which were covered with polycarbonate filters (8-μm pore size). Then, 0.5 ml of $1x10^5$ cells/ml of ECs were placed in the upper chamber. After 4 h of incubation at 37˚C, medium in the upper chamber was aspirated, and cells on the upper surface of the filter were removed with a cotton swab. Cells on the lower surface were fixed, stained with Diff Quick (Dade International Inc., Miami, FL, USA), placed on a microscope slide and covered with a coverslip as previously described (29). The number of stained nuclei was counted in five high-power fields per each chamber. The Student's t-test was used to calculate the p-values.

Results

Immunohistochemistry. The tissue samples obtained from patients with radiation-induced cutaneous wounds and from healthy control skin from the same patients were analysed immunohistochemically for the expression of VEGF. The comparison between negative controls and labeled tissue sections clearly demonstrated the presence of this protein within the investigated samples. The extracellular matrix of the tissue samples showed immunostaining for VEGF within the endothelial cells and the fibroblasts. Comparing normal control skin and tissue samples from radiation-induced chronic dermal wounds, immunohistochemical investigation of VEGF demonstrated a decreased expression of VEGF protein within the radiation-induced wounds. A representative example of VEGF staining is shown in Fig. 2.

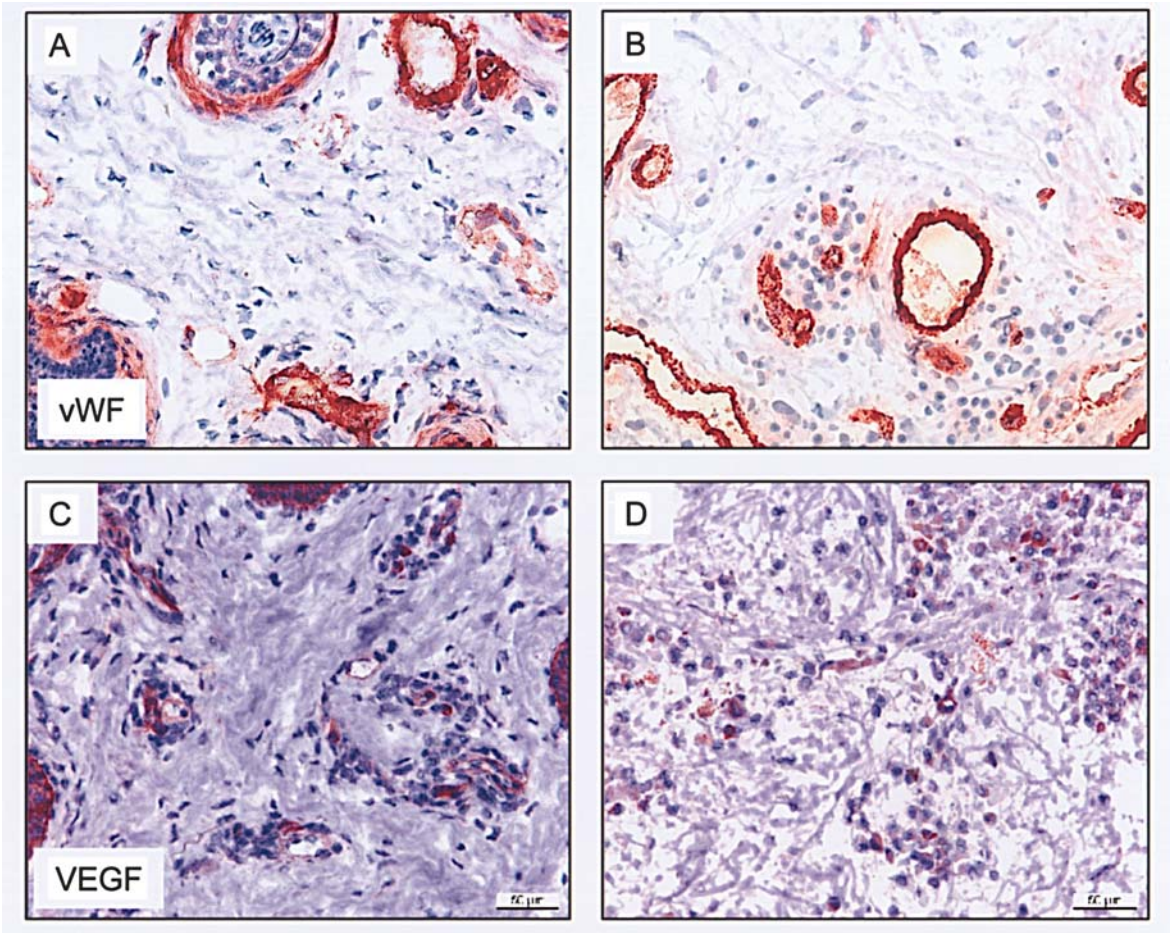

Figure 2. Immunohistochemical investigation of tissue samples from normal human control skin (left) and from chronic radiation-induced dermal wounds (right). (A and B) Expression pattern of endothelial marker vWF; (C and D) expression of angiogenic VEGF.

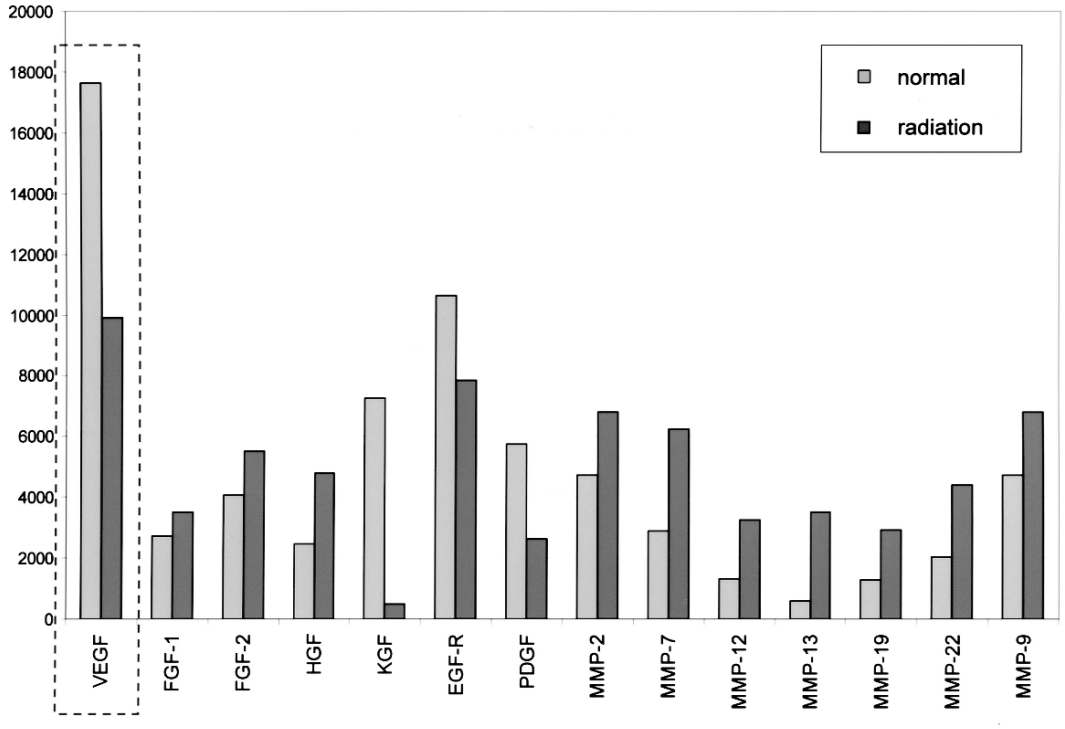

Figure 3. Expression level of the VEGF gene in isolated fibroblasts from dermal radiation wounds and normal control skin (same patients) by microarray hybridization analysis.

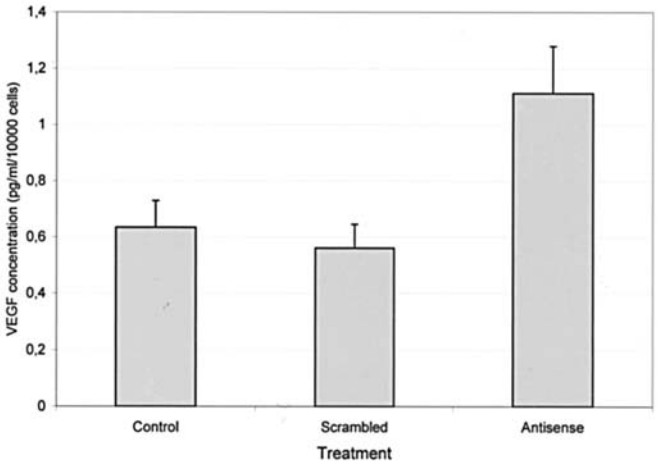

Figure 4. Quantitation of cytokine secretion to the supernatant of human fibroblast isolated from radiation wounds was performed after 48 h after treatment with medium (control) or medium containing TGF-ß antisense or scrambled oligonucleotides. VEGF was significantly increased compared to controls.

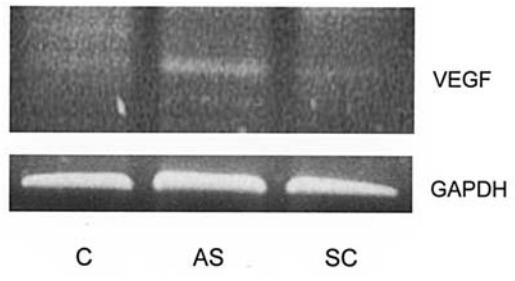

Figure 5. RT-PCR results. The treatment of radiation fibroblasts with TGF-ß antisense oligonucleotides (AS) resulted in a significant increase in expression of mRNA of VEGF compared to the controls (C) [scrambled (SC) or PBS].

Micoarray analysis. Expression levels of genes for different matrix modulators in isolated fibroblasts from dermal radiation wounds and normal control skin (same patients) by microarray hybridization analysis demonstrated increased gene levels for all matrix modulators in fibroblasts from radiation-induced chronic dermal wounds compared to the normal controls (Fig. 3).

In vitro analysis. To analyze the effect of TGF-ß1 antisense oligonucleotides on the expression of VEGF in fibroblasts isolated from radiation wounds, cytokine secretion to the supernatant was quantitated 48 h after treatment with medium (control) or medium containing TGF-ß antisense or scrambled oligonucleotides. The VEGF level was significantly increased by antisense TGF-ß1 oligonucleotide treatment in the cells tested compared to controls ($p<0.01$) (Fig. 4). To analyze the effect of TGF-ß1 antisense oligonucleotides on the expression of mRNA of VEGF in cultured human fibroblasts isolated from radiation skin wounds, a multiplex RT-PCR kit was used. The treatment of fibroblasts with TGF-ß1 antisense oligonucleotides for 48 h resulted in a significant increase in expression of mRNA of VEGF compared to the cells that were treated with either scrambled oligonucleotides or with PBS (Fig. 5).

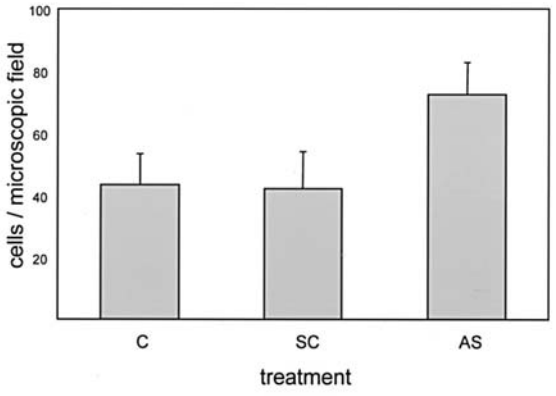

Figure 6. EC migration assay in response to conditioned medium from radiation fibroblasts treated with TGF-ß antisense (AS) or sense (SC) oligonucleotides. *In vitro* migration was measured in Boyden chambers. C, Control.

Effect on endothelial cells. The growth pattern of ECs in response to conditioned medium from fibroblasts isolated from radiation wounds treated with TGF-ß1 antisense or sense oligonucleotides was significantly affected showing an increase in tubule formation in the presence of conditioned medium taken from the TGF-ß1 antisense-treated cells (Fig. 6). The effect of antisense TGF-ß1 oligonucleotides on EC migration was examined using transwell chambers. The addition of non-concentrated conditioned medium from fibroblasts isolated from radiation wounds treated with TGF-ß1 antisense oligonucleotides resulted in a significant increase ($p<0.01$) in HMVEC migration compared with the effect of conditioned medium of the untreated or sense oligonucleotide-treated cells (Fig. 7).

Discussion

Radiation, although a useful modality for cancer therapy, injures surrounding uninvolved tissues, producing a chronic, painful, poorly healing soft tissue ulcer (1,8,9,30). Despite improvements in the techniques of and equipment for radiation, late complications of radiotherapy are becoming an increasingly important concern to both physicians and patients as the number of long-term cancer survivors increases. A better understanding of the molecular events underlying normal tissue injury will permit a more rational approach to the prevention and treatment of normal tissue injury. The prolonged treatment period and high costs of treating radiation-induced ulcers emphasizes the need for a drug or treatment that promotes healing (8,9,30). Over the past several years, major advances in the tools of molecular biology have enabled scientists to move rapidly toward a better understanding of underlying mechanisms responsible for radiation-induced normal tissue injury. More recently, we have learned how these immediate biochemical events rapidly trigger a series of genetic and molecular phenomena leading to clinically and histologically recognizable injury. This process is dynamic and involves a number of pro-

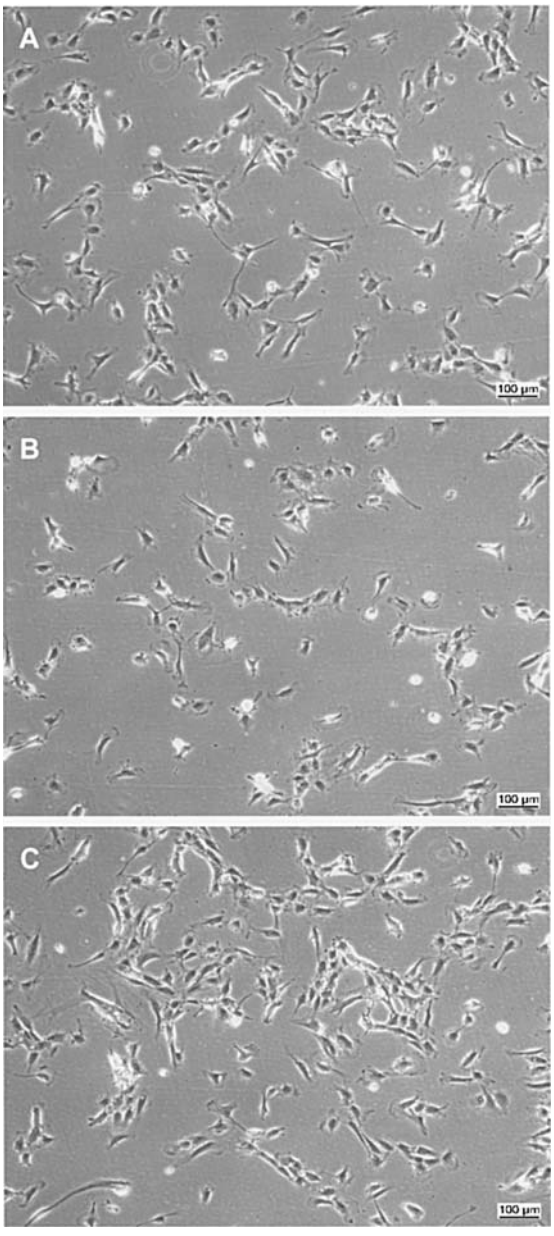

Figure 7. Endothelial cell tube formation in response to conditioned medium from radiation fibroblasts treated with medium (control; A) or medium containing TGF-ß sense (B) or antisense (C) oligonucleotides.

inflammatory cytokines, profibrotic cytokines and chemokines produced by macrophages, epithelial cells and fibroblasts. Furthermore, these events seem to be sustained for months or years beyond the completion of therapy (5,6,8,9,30).

This new information on the pathogenesis of radiation-induced normal tissue injury has created opportunities to identify potential molecular targets for treatment or prevention of side effects. Among the most promising avenues for intervention is the TGF-ß pathway. Irradiation results in increased expression and activation of TGF-ß, which promotes the deposition of fibrous tissue and inhibits epithelial repair (31,32). These changes in the bioavailability of TGF-ß might be reflected as increased circulating levels of the cytokine, which might also contribute to the risk of injury (33). It has previously been demonstrated that disruption of the signal transduction pathway for TGF-ß in transgenic mice deficient in the Smad 3 component of that pathway results in resistance to the development of radiation-induced fibrosis (34). In addition, gene therapy with an adenoviral vector in rats, which results in increased expression of the type 2 TGF-ß receptor, reduces tissue levels of TGF-ß and protects against radiation-induced injury in the lung (35). Although not yet tested in humans, an anti-TGF-ß therapy seems promising as an approach to the prevention and treatment of radiation-induced injury (12). Shah *et al* have previously demonstrated that wounds treated with the addition of neutralizing antibody to TGF-ß1 at the wound site had a lower inflammatory response, less extracellular matrix deposition in the early stages of wound healing and reduced scar formation (36). However, the exact mechanisms are still not well understood. TGF-ß targeting might affect cytokine expression patterns of cells involved in the process of wound healing. This prompted us to investigate the effect of TGF-ß1 antisense oligonucleotides on the expression of VEGF and angiogenic activity in cultured human fibroblasts which were isolated from radiation-induced cutaneous wounds.

This study demonstrated a decreased expression of pro-angiogenic VEGF in tissue samples of chronic radiation wounds compared to normal skin. We also demonstrated decreased levels of VEGF gene expression in fibroblasts isolated from radiation wounds compared to healthy skin. Advances in the understanding of neovascularization have made the process of angiogenesis and angiogenic factor expression prime targets for therapeutic manipulation in wound healing (26,37). Efforts have been made to induce or stimulate new blood vessel formation to reduce the unfavourable tissue effects caused by local ischaemia, or to enhance tissue repair (38). Gene therapy in this environment poses a particular challenge (39). The skin is an ideal candidate for genetic manipulations (40). It is easily accessible, rendering it easy to monitor for adverse reactions and easy to transfect. The epidermis has a high turnover, which is an ideal situation for most gene transfer methods. The predominant cells of the skin, fibroblasts and keratinocytes, are easily harvested and cultured, allowing for testing *in vitro* and for use of skin cells as vehicles in gene transfer. This has led to an increasing interest in the use of gene therapy in skin diseases, especially wound healing (41). The pivotal role of growth factors in the regulation of wound repair has led to the development of multiple molecular genetic approaches that mainly focus on stimulation and enhancement or abrogation of this protein group. In this context, molecular biology has provided synthetic oligonucleotide sequences complementary to target genes, referred to as antisense. Antisense technology has as its basis the selective impairment of protein synthesis in the cytoplasm through the use of antisense oligodeoxynucleotide (ODN) sequences. A small antisense DNA sequence to the initiating AUG codon and slightly beyond hybridize with the 5' end of the mRNA, causing translation arrest (42,43). This provides a major tool for assessing gene expression and the function of the gene product (44,45). Our study demonstrated that the treatment of human fibroblasts isolated from radiation-induced dermal wounds with TGF-ß antisense oligonucleotides *in vitro* efficiently up-regulated pro-

angiogenic VEGF expression. In addition to increased VEGF protein, *in vitro* angiogenic activity was significantly increased. Enhanced angiogenesis due to genetic modulation of genes encoding angiogenic factors may significantly enhance radiation-induced wound healing (39,46).

In summary, we demonstrated that antisense TGF-ß1 oligonucleotide treatment up-regulates VEGF expression in human fibroblasts isolated from radiation wounds. This result should be regarded as a preliminary finding. However, antisense oligonucleotide technology may be a potential therapy for radiation-induced chronic wounds. This may prevent the high prevalence of morbidity associated with this significant health problem.

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