in vivo *22*: 1-8 (2008)

TGF-beta Antisense Oligonucleotides Modulate Expression of Matrix Metalloproteinases in Isolated Fibroblasts from Radiated Skin

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Abstract. *Background: Transforming growth factor-beta (TGF-beta) 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-beta. The purpose of this study was to analyze the effect of TGF-beta targeting on the expression of matrix metalloproteinases (MMPs) in fibroblasts isolated from radiation-induced chronic dermal wounds. Materials and Methods: The expression of MMPs in tissue samples from radiation-induced chronic dermal wounds was investigated by immunohistochemistry and microarray technique. The effect of TGF-beta targeting using antisense oligonucleotides on the expression of MMPs in isolated fibroblasts was analysed by ELISA and multiplex RT-PCR. Results: Immunohistochemical investigation and microarray analysis demonstrated an increased expression of MMP protein and mRNA in tissue samples from radiation-induced chronic dermal wounds compared to normal human skin. Antisense TGF-beta oligonucleotide treatment significantly down-regulated MMP secretion in vitro. Conclusion: TGF-beta antisense oligonucleotide technology may be a potential therapeutic option for the inhibition of proteolytic tissue destruction in radiation-induced chronic wounds.*

An increasing number of patients survive cancer after having received radiation therapy. Therefore, the occurence of late normal tissue complications among long-term survivors is of particular concern (1). The goal of radiotherapy is to precisely target a tumor 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 to rapidly proliferating tumor cells. However, normal tissues that also rapidly proliferate, such as skin, are also relatively radiosensitive (2). Radiation therefore is associated with a broad spectrum of abnormal tissue reactions. The degree of change is related to the biological dose and the volume irradiated (2). 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. Following the acute skin changes, the skin appears "normal" for a variable interval of time ranging from months to years. In contrast to early reactions, typical late injuries are irreversible and often progressive. The late effects of atrophy and fibrosis are directly related to the dermal fibroblast response to radiotherapy (3). Radiation-induced fibrosis is characterized by progressive induration, edema formation and thickening of the dermis (4).

Normal wound healing involves a complex series of events that ultimately lead to the restoration of injured tissue. This complex process involves a series of overlapping stages, relying on the collaboration of many different extracellular matrix (ECM) components, cell types and soluble mediators. Simplifyied, the process of wound healing is often subdivided into three phases, inflammation, granulation formation and matrix formation and remodeling (5). Situations, in which the inflammation does not adequately resolve, such as radiation injury, appear to involve aberrant cytokine pathways or chronic overproduction of certain cytokines, resulting in uncontrolled matrix degradation and fibrotic sequelae (6, 7). Disturbance in the balance between ECM production and degradation leads to the formation of chronic ulcers with

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Key Words: Fibroblasts, radiation wound, MMP, TGF-beta.

excessive ECM degradation (8). Special tissue growth factors, such as transforming growth factor-beta (TGF-beta), play a key role in this process (9). TGF-beta is known to be the most potent growth factor involved in wound healing throughout the body (10). Released by degranulating platelets at the site of injury, TGF-β1 influences the inflammatory response, angiogenesis, re-epithelialization, ECM deposition and remodeling (11, 12). To increase these processes, TGF-beta modulates expression of matrix metalloproteinases (MMPs), a family of zinc-dependent endopetidases that are collectively capable of cleaving all the components of ECM and basement membrane (5). Expression of these MMPs in healthy tissues is low but their production is markedly increased in situations involving active tissue remodeling and cell migration, such as wound healing and chronic inflammation (13). Han *et al.* have demonstrated that tumor necrosis factor-alpha (TNF-alpha) and TGF-beta synergistically induced pro-MMP-9 in epidermal cells (14). TGF-beta also increased cell migration, as well as both cell-associated and secreted MMP-2 production in wounded cell cultures (15). In recent studies, targeting of TGF-beta resulted in accelerated wound healing and reduced scarring (10, 16). In previous studies we have been able to demonstrate that the abrogation of TGF-beta in normal fibroblast and keratinocytes resulted in decreased expression of MMPs (17, 18) and increased angiogenic activity (19).

The aim of this study was to investigate the effect of targeting TGF-beta expression by antisense oligonucleotides on the expression of matrix metalloproteinases in fibroblasts from radiated skin.

Materials and Methods

Immunohistochemistry. Tissue specimens of radiated skin wounds and normal control skin from the same patient were obtained from excised tissue during surgery and rapidly frozen in liquid nitrogen for later MMP identification. They were cut in 10 μm cryostat sections, transferred onto glass slides, and air-dried overnight at room temperature. The sections were then stored at –20˚C until immunostaining. Immunohistochemistry for MMP-9 and MMP-2 detection was performed by using a streptavidin-biotin complex procedure. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. The 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 over night at 4˚C with the primary antibody. 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, the 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).

Microarray analysis. Extraction of RNA from isolated fibroblasts was performed using RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' 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 manufacturers' 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 minutes. The human 10K (MWG-Biotech) oligo microarray system on glass slides was used for mRNA profiling. Hybridization of Cy3/Cy5-cDNA was performed using cover slips and a hybridization chamber for 16 hours at 42˚C in a water bath. After stringent washing of the glass slides according to the manufacturers specifications the hybridization signals of the Cy3 and the Cy5 dyes were measured using a microarray laser scanner (GMS418; Affymetrix, MWG-Biotech).

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) formate. In brief, for evaluation of hybridization results a negative (<3.000), a grey area (3.000-4.999) and a positive range (≥5.000) of hybridization signal intensities were defined. 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 ratio for each individual gene spot. For statistical evaluation the mean signal intensity and standard deviation (SD) was 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 in Falcon petri (Greiner, Germany) dishes at 37˚C in a 5% CO_2 fully humidified atmosphere 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], Gainthersburg, MD, USA) followed by the addition of oligodeoxynucleotides.

Oligodeoxynucleotides. Phosphorothioated 14-mer oligodeoxy-nucleotides (ODN) were synthesised on an Applied Biosystems 394 DNA synthesiser (Applied Biosystems Inc., Forster City, CA, USA) by means of B-cyanothylphosphoramidite chemistry to minimise 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-beta cDNA. For negative control, the cells were treated by the addition of PBS or oligonucleotides (5'-GTC CCT ATA CGA AC-3') containing the same nucleotides in a scrambled

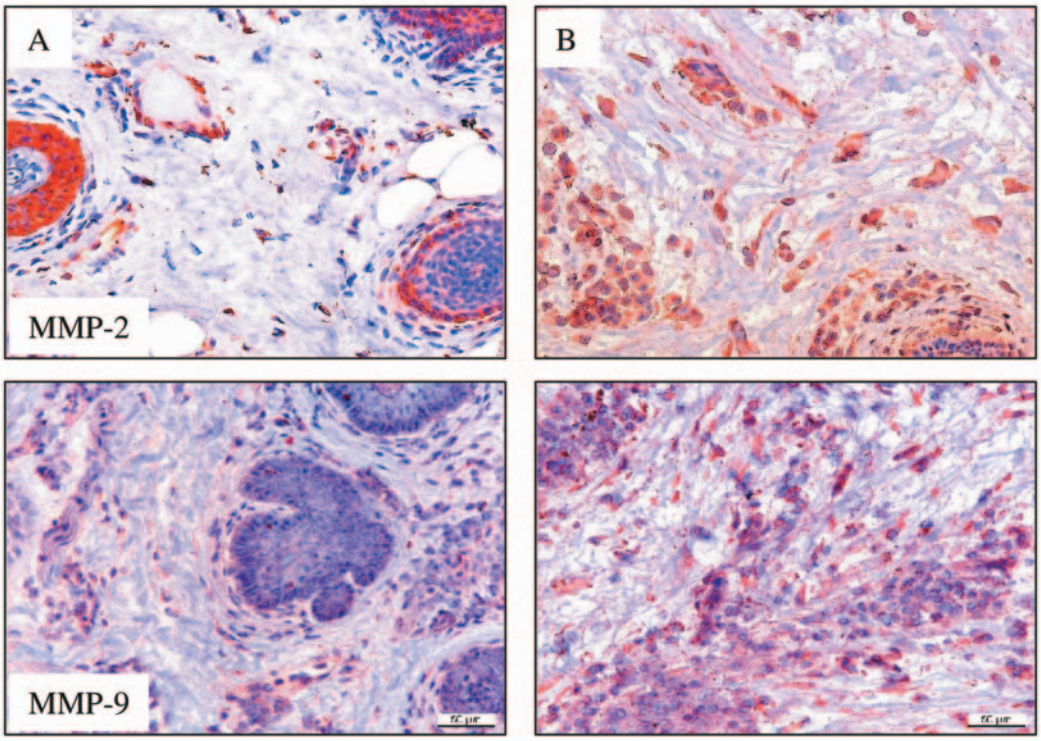

Figure 1. *Immunohistochemical investigation of tissue samples from normal human control skin (left) and from chronic, radiation-induced dermal wounds (right). A+B: expression pattern of MMP-2; C+D: expression of MMP-9.*

order. The *in vitro* inhibitory effect of these antisense ODNs on TGF-beta expression at both the mRNA and protein level in human cells has been described previously (20). All experiments were performed with 12.5 μM ODNs, unless otherwise stated. To determine the effect of oligonucleotides on the expression of MMP mRNA, fibroblasts were plated at a density of 10^5 cells / microtiter well in 24-well polystrene plates (Falcon). After 24 hours the cells were rinsed twice with medium and then fresh oligo medium containing antisense or scrambled ODNs was added, followed by an incubation period of 48 h.

Cytokine immunoassay. Cell culture supernatants were collected in sterile test tubes and stored at –20˚C until used. Then, cytokine (MMP-2, MMP-9) concentrations were determined by an ELISA technique (R&D Systems, Wiesbaden, Germany). The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against recombinant cytokines. 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 kid. All concentrations were documented as pg/ml. Student's *t*-test was used to calculate for *p*-values.

RT-PCR. To isolated RNA from the fibroblasts grown in monolayer, the cells were directly lysed in the culture dish by the addition of 1 ml RNA-Clean (RNA-Clean System, AGS, Heidelberg, Germany). After the addition of 0.2 ml chloroform per 2 ml of homogenate and centrifugation for 15 minutes at 12,000 xg (4˚C), the aqueous phase was transfered to a fresh tube. After the addition of an equal volume of isopropanol and centrifugation for 15 minutes at 12,000 xg (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 minutes at 7,500 xg (4˚C). After drying the RNA pellet, it was dissolved in DEPC water. The RNA was reverse transcribed (StrataScript First-Strand Synthesis Sytem, Stratagene, La Jolla, CA, USA) into cDNA using random-oligonucleotide primers. MMP mRNA levels were measured in all three cell types using RT-PCR (MMP-CytoXpress Multiplex PCR Kit, BioSource, San Francisco, CA, USA) according to the manufacturer's instruction manual. To fractionate the MPCR DNA products, the 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.

Results

The presence of both MMP-2 and MMP-9 proteins was demonstrated within the collagen and the fibroblasts of all the skin samples but increased expression of both proteins was evident within the radiation-induced wounds (Figure 1).

The expression levels of the genes for the different matrix modulators was also increased in the isolated fibroblasts

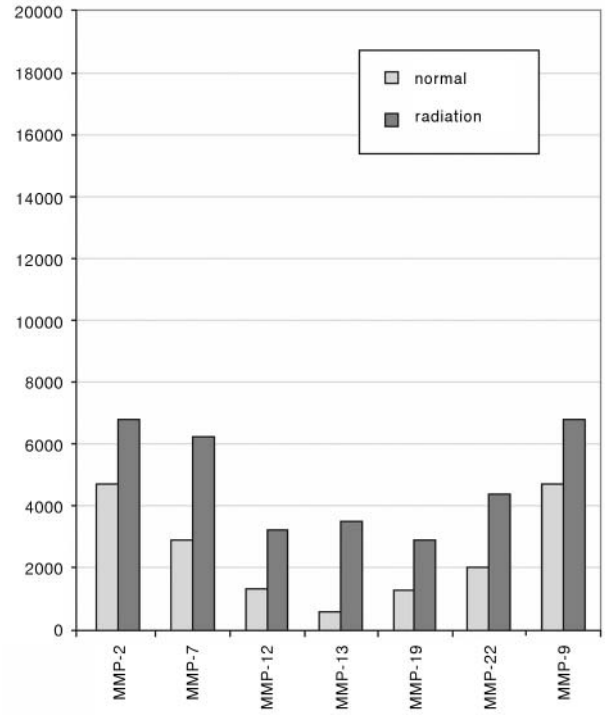

Figure 2. *Expression levels of genes for different matrix modulators in isolated fibroblasts from dermal radiation wounds and normal control skin (same patients) by microarray hybridization analysis.*

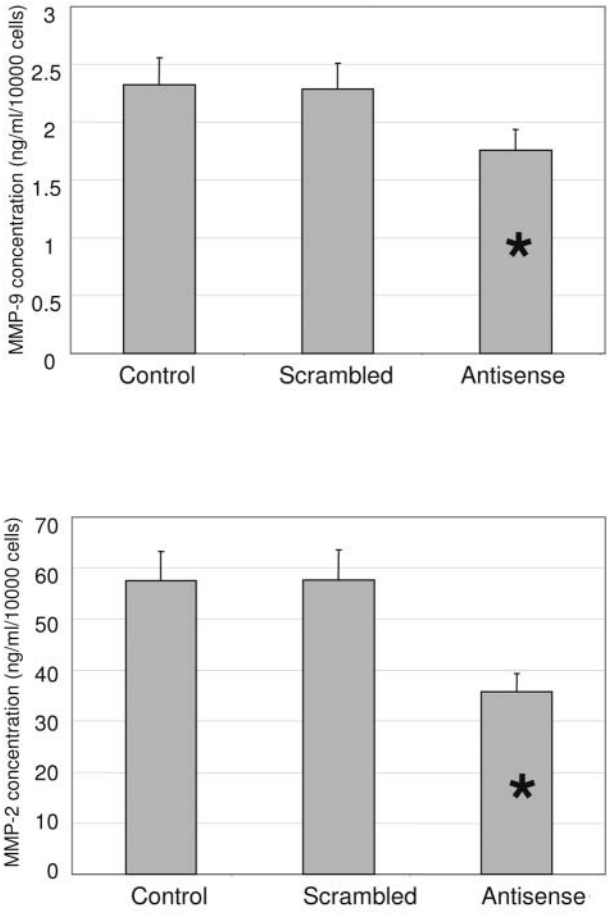

Figure 3. *Cytokine secretion to the supernatant of human fibroblasts isolated from radiation wounds after 48 hours post-treatment with medium (control) or medium containing TGF-beta antisense or scrambled oligonucleotides. MMP-2 and MMP-9 were significantly decreased compared to controls ($p<0.05$).*

from the radiation-induced chronic dermal wounds compared to the normal controls (Figure 2).

The secretion of MMP-2 and MMP-9 from the fibroblasts isolated from radiation-induced wounds was significantly ($p<0.05$) decreased by TGF-beta abrogation compared to controls (Figure 3).

The treatment of fibroblasts with TGF-beta antisense ODNs for 48 h resulted in a decrease of expression of mRNA of MMP-2 and MMP-9 compared to the cells that were treated with either scrambled oligonucleotides or with PBS (Figure 4).

Discussion

Radiation, although an intergral part of modern cancer therapy, injures surrounding uninvolved tissues, often producing a chronic, painful, poorly healing soft tissue ulcer (21). Despite improvement in the techniques of and equipment for radiation, ulcers still develop and continue to be a vexing therapeutic challenge for plastic reconstructive surgeons. Improving basic knowledge of molecular wound healing and pharmaceutical intervention in this area may ultimately help clinicians to identify and proactively intervene in an effort to prevent radiated skin from breakdown and development of a chronic wound. This may prevent the high prevalence of morbidity associated with this significant health problem.

Wound healing requires the remodeling of the ECM, with collagen deposition, in the skin (22). As all chronic wounds begin as acute wounds, it is still not known at what point the healing sequence is interrupted whereby normal acute wound healing fails to occur (23). One of the first major processes in wound healing is inflammation, and this phase is regulated by several pro-inflammatory cytokines which are potent inducers of MMP synthesis in fibroblasts (24). MMPs are essential to the ECM remodeling process as well as the removal of devitalized tissue and the re-epithelialization of cutaneous wounds (25, 26). Spatially and temporally controlled expression of several distinct MMPs is associated with normal wound healing and ulcer repair (27, 28). Several studies have shown this influence

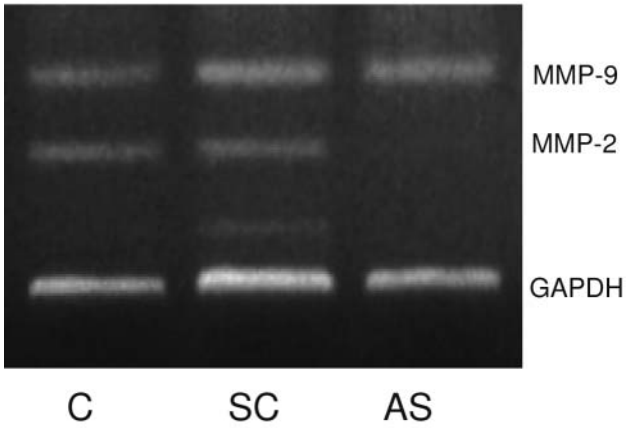

Figure 4. *Expression of mRNA of MMP-2 and MMP-9 in radiation fibroblasts treated with TGF-beta antisense ODNs. C: control, PBS; SC: control, scrambled ODNs.*

in different types of wounds, including acute normally healing human experimental (29), as well as chronic venous stasis and pressure ulcers (30). The present study demonstrated an increased expression of collagenases MMP-2 and MMP-9 in the tissue samples of chronic radiation wounds and increased levels of MMP gene expression in the fibroblasts isolated from radiation wounds compared to healthy skin. Although extracellular proteolysis is necessary for normal wound healing, uncontrolled proteolytic tissue destruction appears to be a pathogenic factor in non-healing wounds. Persistent MMP concentrations could be evidence of early chronification of these lesions indicating a molecular environment in chronic wounds hostile for cell replication after injury. We thus assume a general malfunction of cellular wound healing processes in patients following radiotherapy. The prolonged treatment period and high costs of treating radiation-induced ulcers emphasises the need for a drug or treatment that promotes healing (31).

Cutaneous wound healing is a complicated, multistep process with numerous mediators that act in a network of activation and inhibition processes. Gene therapy in this environment poses a particular challenge (32). Numerous models of gene delivery have been developed, including naked DNA application, viral transfection, high-pressure injection, liposomal delivery, and others. The skin is an ideal candidate for genetic manipulations (33). It is easily accessible, rendering it easy to monitor for adverse reactions and easy to transfect. The epidermis has a high turnover – an ideal situation for most gene transfer methods. The predominant cells of the skin, *i.e.* 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 lead to an increasing interest in the use of gene therapy in skin diseases, especially wound healing (34). 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.

Targeting TGF-beta, a key regulator of wound healing, might affect cytokine expression patterns of cell involved in the process of wound healing (35, 36). 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 ODN sequences. A small antisense DNA sequence to the start codon and slightly beyond hybridize with the 5' end of the mRNA, causing translation arrest (37-39). This provides a major tool for assessing gene expression and the function of the gene product (40, 41). In previous studies, topical application of antisense ODNs targeted to TGF-β mRNA in skin wounds of mice resulted in a marked reduction of fibrosis associated with decreased expression of the TGF-β gene (42). Our study demonstrated that the treatment of human fibroblasts isolated from radiation-induced dermal wounds with TGF-beta antisense ODNs *in vitro* efficiently down-regulated MMP-2 and/or MMP-9 expression. Our report suggests that antisense TGF-β ODNs may have a potential therapeutic role in the treatment of radiation wounds. The potential for modulating gene expression by the use of antisense oligonucelotides has become increasingly important in recent years. This antisense technology has been extensively used *in vitro* and *in vivo* as a tool to study the regulatory mechanisms in biological processes and as potential therapeutic agents in cancer, viral infections and genetic disorders (43). The therapeutic application of antisense has resulted in advances in medicine. However, this potential therapeutic approach is dependent upon the degree of antisense stability, the rate of cellular uptake and accumulation of the antisense molecules, the selection of the proper target gene, the absence of effects on nontargeted genes, minimal toxicity of the antisense ODN, structure and length of the antisense ODN, type and duration of treatment. The specificity of antisense is great since targeted mRNA can discriminate between antisense ODN sequences that differ by one or two bases (37-39). In recent studies, antisense oligonucleotides have been tested for toxicity and clinical activity in a Phase I evaluation in patients with advanced cancer (44).

In summary, we have demonstrated that antisense TGF-beta ODN treatment down-regulates MMP mRNA expression in human fibroblasts in radiation wounds. This result should be regarded as a preliminary finding.

Acknowledgements

The authors would like to thank Petra Prohaska, Department of Otolaryngology Mannheim, for her excellent technical assistance.

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Received July 12, 2007
Revised November 20, 2007
Accepted November 30, 2007