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Immunohistochemical Analysis of Radiation-induced Non-healing Dermal Wounds of the Head and Neck

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Abstract. *Persistent, poorly healing wounds are a significant clinical problem in patients who have had previous irradiation. The pathology of chronic dermal ulcers is characterised by excessive proteolytic activity which degrades the extracellular matrix (required for cell migration) and growth factors and their receptors. Interestingly, the molecular basis of radiation-induced dermal wounds is poorly understood. The aim of this study was to investigate, by immunohistochemistry, the expression of the endothelial marker vWF, of angiogenic bFGF, VEGF and IL-8, of collagenases MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2, in tissue samples from radiation-induced chronic dermal wounds and healthy control skin. Performing immunohistochemical detection of microvessels, an equivalent density of microvessels was observed within tissue samples from normal healthy skin and from radiation-induced non-healing cutaneous wounds. Investigation of angiogenic bFGF and VEGF demonstrated a decreased expression of both factors in the radiation-induced dermal wounds. The expression of angiogenic IL-8 was weak in both the healthy skin samples and the radiation-induced wounds. In addition, an increased expression of collagenases MMP-2 and MMP-9 protein within the radiation-induced wounds was demonstrated. While the expression of TIMP-1 showed no difference of expression between normal control skin and tissue samples from radiation-induced wounds, TIMP-2 expression was slightly increased compared to healthy controls. Our data suggest that radiation-induced dermal injuries often fail to heal because of decreased angiogenesis and persistently high concentrations of MMPs with an imbalance of their tissue inhibitors. The basic mechanisms of wound healing in radiation-induced dermal wounds at the molecular level need to be understood further for the development of innovative treatment strategies.*

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In head and neck surgery, persistent, poorly healing wounds remain a significant clinical problem in patients who have had previous irradiation. Any localised trauma, surgery, or infection to irradiated skin can precipitate a major non-healing wound or incisional rupture. This impaired healing from irradiation is a source of prolonged patient morbidity, resulting in fistula formation, fibrosis, carotid artery exposure (Figure 1) or rupture, or even death. Postoperative complications after radiation exposure have been reported to occur in as many as 67% of patients (1). Well-vascularized flaps are sometimes required for coverage of these poorly healing wounds; however, their flap incisions can also break down in a previously irradiated field. Key tenets in managing previously irradiated skin are to prevent its breakdown and to promptly institute conservative therapy whenever skin injury is present (2). Several studies have focused on characterising the molecular environments of acute and chronic wounds in an attempt to understand the processes that change an acute healing wound into a chronic one (3,4). However, the molecular basis of radiation-induced wounds is still poorly understood.

In general, wound healing is a complex process involving a series of overlapping stages, relying on the collaboration of many different extracellular matrix 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 remodelling (5). If the normal process of wound healing is disrupted, a chronic non-healing wound can result.

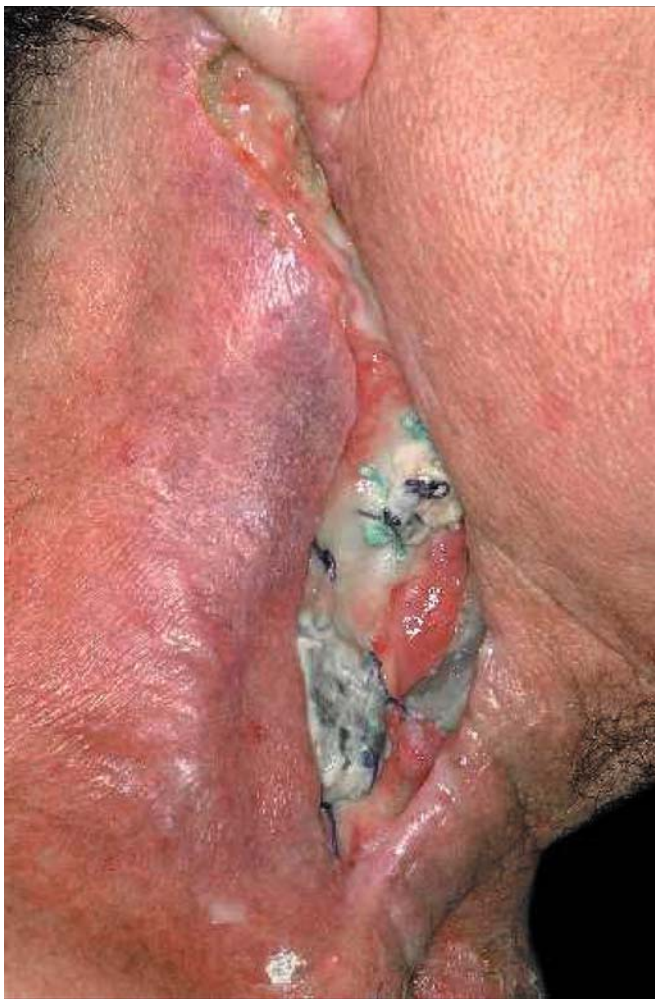

Figure 1. *Typical example of a cervical radiation-induced chronic dermal wound in a patient who received laser treatment and neck dissection with adjuvant radiotherapy because of a squamous cell carcinoma of the hypopharynx. The dermal wound is complicated by a pharyngocutaneous fistula with carotic artery exposure.*

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 extracellular matrix (ECM), followed by migration of inflammatory cells to the wound site. Thereafter, keratinocytes migrate into the wound and angiogenesis is initiated. During the wound healing processes, an abundant blood supply is necessary to meet the enormous local demands of debridement, fibroblast proliferation, extracellular matrix synthesis and epithelialisation (6,7). Impairment of the blood supply may be a contributing factor in delayed healing, or non-healing, chronic wounds (8,9). Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and Interleukin-8 (IL-8) are important regulators of angiogenesis (10,11), mainly during the proliferative phase of wound healing (12).

During the process of angiogenesis, fibroblasts deposit and remodel the granulation tissue. Cell migration, angiogenesis, degradation of provisional matrix and remodelling of newly formed granulation tissue all require controlled degradation of the ECM. Disturbance in the balance between ECM production and degradation leads to the formation of chronic ulcers with excessive ECM degradation (13). Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopetidases that collectively degrade nearly all extracellular matrix and basement membrane proteins (5). MMPs are involved in numerous biological and pathological processes, with their involvement in wound healing having been first shown in guinea pigs (14,15). In the first phases of wound repair, MMP activities are necessary for the removal of devitalised

tissue, for angiogenesis, for contraction of wound matrix, for migration of fibroblasts, and for keratinocyte migration and epithelialisation (16). During the final phase of wound healing, MMPs participate in the remodelling of newly synthesised connective tissue (17,18). Two of them, MMP-2 (gelatinase A or 72-kD type IV collagenase) and MMP-9 (gelatinase B or 92-kD type IV collagenase), have the capacity to degrade type IV collagen, an important component of epithelial and subendothelial basement membranes (19).

Matrix metalloproteinases activity is further modulated *via* binding to their natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (20). In general, TIMPs can inhibit the activity of all MMPs *in vitro* (21). However, various TIMPs have preferential inhibitory capabilities against different pro-MMPs. For instance, TIMP-1 preferentially forms a complex with pro-MMP-9, while TIMP-2 reduces pro-MMP-2 activation (20). The mechanism involves forming non-covalent stoichiometric complexes with the zinc-binding site of the active form of MMPs (22). Except for TIMP-3, which is bound to ECM, TIMPs are present in a soluble form in most tissues and body fluids but show differences in tissue distribution (20). The expression of TIMP-1 is up-regulated by various growth factors, cytokines, retinoids and glucocorticoids, while the expression of TIMP-2 is mainly constitutive. An imbalance between TIMP and MMP activities is considered to result in excessive degradation of matrix components (21).

The aim of this study was to investigate the expression of the endothelial marker von Willebrand-Factor (vWF), angiogenic VEGF, bFGF and IL-8, the collagenases MMP-2 and MMP-9 and their tissue inhibitors TIMP-1 and TIMP-2, in tissue samples of radiation-induced chronic wounds.

Materials and Methods

We examined tissue samples from patients who had previously received surgery followed by adjuvant radiotherapy because of head and neck squamous cell carcinoma. They now presented with a severe non-healing dermal wound of the neck which had been in the radiation field. Tissue samples from healthy skin served as control tissue. All patients received well-vascularized flaps for coverage of these poorly healing wounds. The tissue specimens of chronic wounds and normal skin controls were gained from excised tissue during surgery and rapidly frozen in liquid nitrogen skin for later vWF, VEGF, bFGF, IL-8, MMP-2, MMP-9, TIMP-1 and TIMP-2 identification. They were cut in 10-µm cryostat sections, transferred to glass slides, and air-dried overnight at room temperature. The sections were then stored at -20˚C until immunostaining. Immunohistochemistry for vWF, VEGF, bFGF, IL-8, MMP-2, MMP-9, TIMP-1 and TIMP-2 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 (vWF polyclonal Ab, DAKO, Hamburg, Germany; VEGF polyclonal Ab, cat-# sc-152, Santa Cruz Biotechnologies, Heidelberg, Germany; bFGF polyclonal Ab, cat-# sc-7911, Santa Cruz; IL-8 monoclonal Ab, cat-# sc-8427, Santa Cruz; MMP-9 polyclonal Ab, cat-# sc-6841, Santa Cruz; MMP-2 polyclonal Ab, cat-# sc-6838, Santa Cruz; TIMP-1 monoclonal Ab, cat-# MAB970, R&D System, Wiesbaden, Germany; TIMP-2 monoclonal Ab, cat-# MAB971, R&D System). 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 Amino-Ethyl-Carbazol (AEC, cat-#: K3464, DAKO) as chromogen. The different antibodies were diluted to the desired concentrations in PBS. Controls were carried out by omitting the primary antibody. Light microscopic investigation was performed using a Zeiss Axiophot microscope. A sample of all tissue specimens was sent to the Department of Pathology for routine examination to exclude recurrent malignancies within the skin.

Results

The tissue samples obtained from patients with radiation-induced cutaneous wound healing and from healthy control skin were analysed immunohistochemically for the expression of vWF, VEGF, bFGF, IL-8, MMP-2, MMP-9, TIMP-1 and TIMP-2. The comparison between negative controls and labelled tissue sections clearly demonstrated the presence of all proteins within the investigated samples.

Performing immunohistochemical detection of the microvessels on cryostat sections, the von Willebrand Factor antibody gave an intense staining of the endothelial cells of the blood vessels, but did not cross-react with the lymphatic endothelium. The background was very low. We found a substantial heterogeneity in microvessel labelling when comparing different tissue samples and also within different areas of the same tissue sample. Overall, the immunohistochemical investigation using antibodies directed against the endothelial marker von Willebrand Factor (vWF) demonstrated an equivalent number and density of microvessels within tissue samples from normal healthy skin and from radiation-induced non-healing cutaneous wounds. Obliteration of microvessels within the wound sections was not observed. A representative example of vWF immunostaining is given in Figures 2 A-B.

Under light microscopy, it appeared that angiogenic VEGF and bFGF labelling was mainly localised within the keratinocytes rather than in the extracellular matrix. No significant labelling of VEGF or bFGF could be observed within the fibroblastic cells of the stroma. Therefore, the distribution pattern of both the VEGF and bFGF staining reaction in the tissue over the whole section was heterogeneous. The staining reaction was predominantly localised in the cytosol of each cell. No staining of the nucleus of the keratinocytes was observed. Investigation of bFGF and VEGF demonstrated a decreased expression of both bFGF

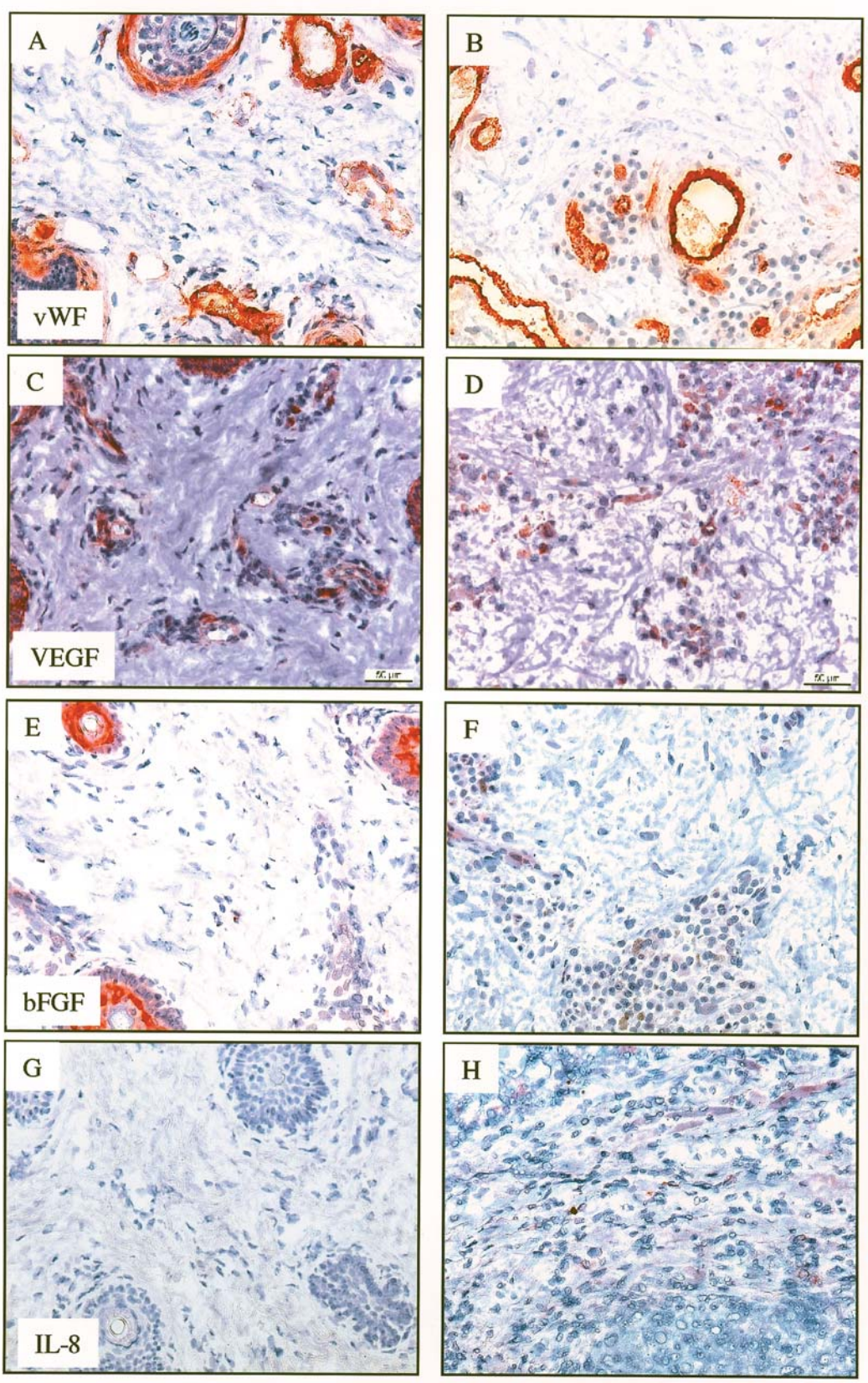

Figure 2. *Immunohistochemical investigation of tissue samples from normal human control skin (left) and from chronic, radiation-induced dermal wounds (right). A+B: Expression pattern of vWF; C+D: Expression of VEGF; E+F: Expression of bFGF; G+H: Expression of IL-8.*

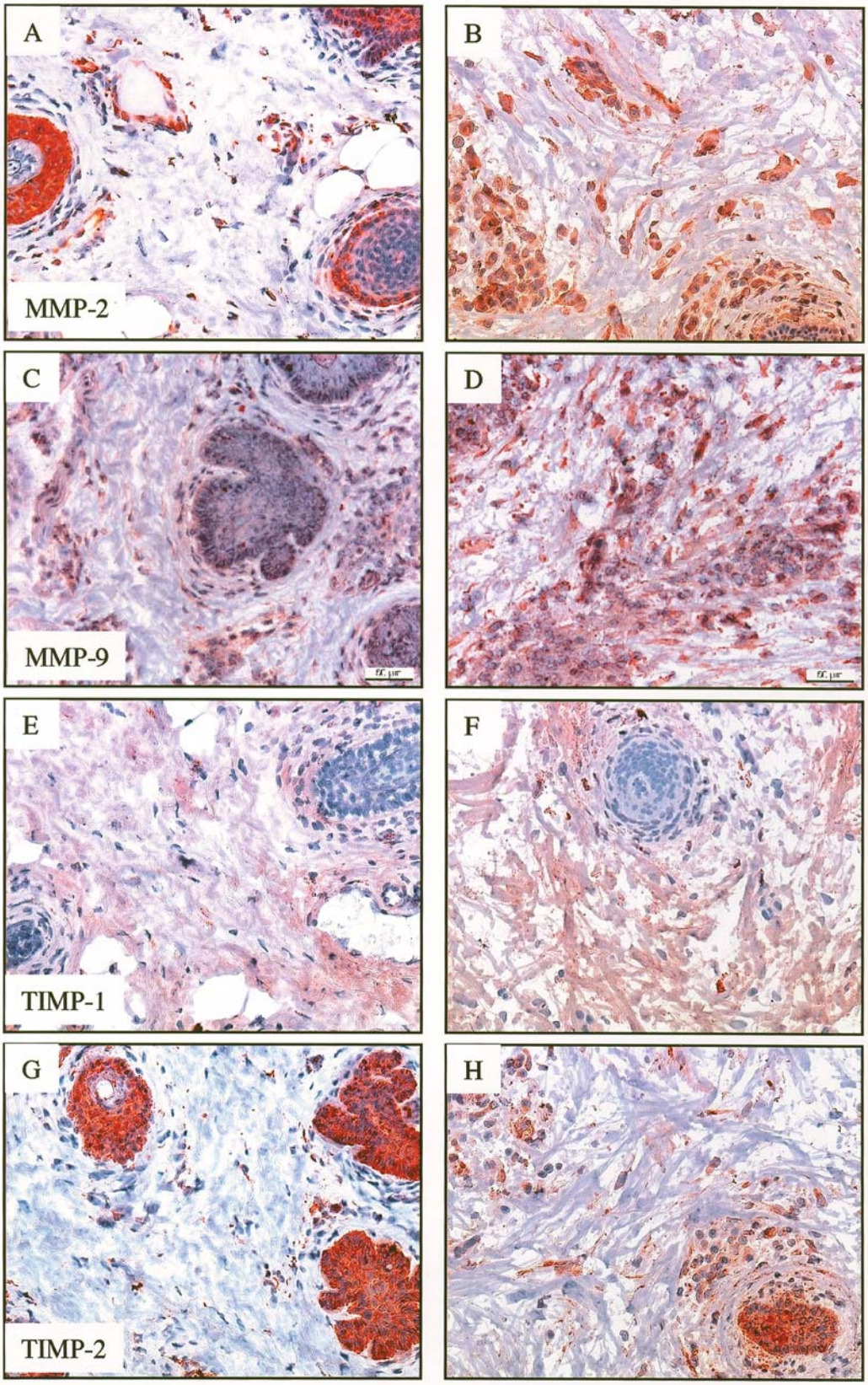

Figure 3. *Immunohistochemical investigation of tissue samples from normal human control skin (left) and from chronic, radiation-induced dermal wounds (right). A+B: Expression pattern of MMP-2; C+D: Expression of MMP-9; E+F: Expression of TIMP-1; G+H: Expression of TIMP-2.*

and VEGF protein in tissue samples from chronic, radiation-induced dermal wounds compared to tissue samples from normal human control skin. Representative examples of VEGF and bFGF immunostaining are shown in Figures 2 C-F. The expression of angiogenic IL-8 was weak in both the healthy skin samples and the radiation-induced non-healing wounds. No difference of expression pattern was notable (Figures 2 G-H).

The immunohistochemical investigation of collagenases MMP-2 and MMP-9 and their tissue inhibitors TIMP-1 and TIMP-2 demonstrated strong labelling of keratinocytes for MMP-2 and TIMP-2. The extracellular matrix of the tissue samples showed immunostaining for all factors within the collagen and the fibroblasts. Comparing normal control skin and tissue samples from radiation-induced chronic dermal wounds, the immunohistochemical investigation of collagenases MMP-2 and MMP-9 demonstrated an increased expression of both MMP-2 and MMP-9 protein within the radiation-induced wounds. Representative examples of MMP-2 and MMP-9 staining are shown in Figures 3 A-D. While there was no difference of expression pattern of TIMP-1 between normal control skin and tissue samples from radiation-induced chronic dermal wounds, TIMP-2 expression was slightly increased within the tissue samples of radiation-induced wounds (Figures 3 E-H).

Discussion

Radiation, although a useful modality for cancer therapy, injures surrounding uninvolved tissues, producing a chronic, painful, poorly healing soft tissue ulcer (23). Despite improvements in the techniques of, and equipment for, radiation, ulcers still develop and continue to be a vexing therapeutic challenge for plastic reconstructive surgeons. Improving the basic knowledge of molecular wound healing and pharmaceutical intervention in this area ultimately may 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.

During the initial phase of radiation therapy, an acute inflammatory reaction occurs. Generally, these changes resolve, and within 6 months chronic epithelial and dermal changes consistent with radiation injury may be apparent clinically and microscopically (23). The skin may be atrophic, oedematous, firm, hyper- or hypopigmented, teleangiectatic, and scaly, brittle or fissured (23). In some patients, in response to minor trauma and or spontaneously, the skin may break down and an ulcer may develop. The ulcer may widen to encompass the area of radiation damage. It is often painful with raised red irregular edges. The base is dirty and shaggy, granulation tissue is sparse and unhealthy. The wound shows little propensity to either epithelialize or contract (23).

Ulceration and poor or non-healing of skin injured by ionising radiation have been primarily attributed to tissue ischemia caused by progressive obliterative endarteritis of the microvasculature. This theory has been based on finding stasis within and occlusion of small vessels shortly after radiation therapy (24). Although microvascular occlusion and obliterative endarteritis do occur in chronic radiation injury, they are not uniformly evident throughout the affected tissues. Recent experimental evidence in rabbit hind limbs has confirmed that tissue hypoxia, and by implication ischemia, occurred in subcutaneous tissues during the acute phase of radiation injury. However, 11 weeks after the irradiation, subcutaneous tissue hypoxia was much less evident and at all times was above the level (20 mmHg) necessary for collagen synthesis and accumulation (25).

Angiogenesis is a complex and multistage process, which is controlled by a variety of factors. VEGF, bFGF and IL-8 are potent direct angiogenic factors that stimulate *in vitro* endothelial cell migration and activation, and *in vivo* angiogenesis (10,11,26). This study demonstrated a decreased expression of angiogenic bFGF and VEGF in tissue samples of radiation-induced dermal wounds compared to normal skin. Recent advances in the understanding of neovascularisation have made the process of angiogenesis and angiogenic factor expression prime targets for therapeutic manipulation in wound healing (9,27). Efforts have been made to induce or stimulate new blood vessel formation to reduce the unfavourable tissue effects caused by local ischemia, or to enhance tissue repair (28). Consequently, intense interest is now focused on the pharmacological application of angiogenic growth factors in the compromised wound (29,30). Recent evidence suggests that recombinant growth factor therapy may provide the added stimulus to the healing of certain types of chronic wounds (9,27,29,30).

Proteolytic degradation of ECM is essential for repair and remodelling of cutaneous wounds (31). As all chronic wounds begin as acute wounds, it is still not known at what point the healing sequence is interrupted and normal acute wound healing fails to occur (4). 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 and inflammatory cells (32). Spatially and temporally controlled expression of several distinct MMPs seems to be associated with normal wound healing and ulcer repair (33,34). Several studies have shown this influence in different types of wounds, including acute normally healing human experimental (35) as well as chronic venous stasis and pressure ulcers (36). This study demonstrated an increased expression of collagenases MMP-2 and MMP-9 in tissue samples of chronic wounds compared to normal skin. Although extracellular proteolysis is necessary for normal wound healing, uncontrolled proteolytic tissue destruction appears to be a pathogenic factor in non-healing wounds.

It is likely that the balance between protease and inhibitor concentrations plays a crucial part in successful wound healing. Therefore, it is important to compare the expression of MMPs to the expression of their tissue inhibitors. In the present study, the expression of TIMP-1 showed no difference of expression pattern between normal control skin and tissue samples from radiation-induced chronic dermal wounds, while TIMP-2 expression was slightly increased within the tissue samples of radiation-induced wounds. The persistence of increased concentrations of proteases during the wound healing process seems to contribute to the failure of the acute wound to heal. Possible, high concentrations of pro-inflammatory cytokines and proteases act as a positive feedback loop involving inflammatory cells releasing cytokines, which stimulate wound cells to secrete proteases that destroy tissue and prevent the wound from closing (37,38). Compared with acute wounds, fluid from non-healing ulcers contains high concentrations of activated gelatinases and low concentrations of MMP inhibitors (36). Accordingly, the expression of TIMPs by keratinocytes was found to be reduced in chronic wounds (34). Nevertheless, it has been suggested that decreased inhibitors of MMP and increased proteases (MMP) leading to excessive proteolysis retards the healing of venous ulcers (39). Our data are in line with the aforementioned findings, indicating that the physiological balance between MMPs and their endogenous antagonists is disturbed in patients with radiation-induced dermal wounds.

Our data should be regarded as a preliminary finding. However, they concur with other studies on chronic dermal ulcers (40,41) and suggest that radiation-induced dermal injuries often fail to heal because of decreased angiogenesis and persistently high concentrations of MMPs. Persistent MMP concentrations and an imbalance of their inhibitors could be evidence for an 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 (42). The basic mechanisms of wound healing on the molecular level need to be understood further to develop innovative treatment strategies, such as inhibiting (43) or applying growth factors (27,44,45) or proteinase inhibitors (46) to the non-healing ulceration.

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