

EXPRESSION OF MMP¹ AND TIMP¹ IN THE
HEALING PROCESS OF GENERAL AND
RADIATION-COMBINED WOUNDS AND ITS
EFFECTS ON THIS PROCESS

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ABSTRACT A rat model of radiation-combined wound healing was used, and routine light microscopy observation, electron microscopy observation, immuno histochemistry, and in situ hybridization were performed to study MMP¹ and TIMP¹ expression during the healing process. The wound healing process was impaired and delayed. After irradiation for rats receiving 25Gy Gamma ray locally the irradiated wounds healed 6 days later than non-irradiated controls. The following changes of MMP¹ and TIMP¹ expression were found: (1) In the early inflammatory phase and in the period of granulation tissue formation, MMP¹ expression was only slight, if affected at all in the newly formed epidermis of irradiated wounds. Later, the epidermal expression of MMP¹ in radiation wounds was comparatively increased following the delay of the healing process. From the 3rd day to 14th day after wounding, TIMP¹ was weakly positive in the proliferating keratinocytes of control wounds and became negative after epidermal covering, whereas no or only slight epiderma expression was detected in the irradiated wounds before epidermal covering. (2) Compared to controls, MMP¹ and TIMP¹ expression in irradiated wounds was markedly decreased in fibroblasts, endotheliocytes and macrophages. The expression phase was prolonged due to the delay of the healing process. We conclude that the reduced expression of MMP¹ and TIMP¹ in granulation tissue of irradiated wounds retards such important processes as cell migration and angiogenesis, and thus slow the healing process. The expression of MMP¹ in the newly-formed epidermis may help the process of reepithelialization; but in the late healing period, overexpression of MMP¹ and decreased expression of TIMP¹ in the epidermis may hinder the establishment of basal membrane and scar formation.

KEYWORDS Irradiation, Wound healing, MMP¹, TIMP¹

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1 Introduction

Extracellular matrix in wound tissue is a key regulator for cell adhesion, migration, proliferation, and differentiation during cutaneous tissue repair. The amount and organization of extracellular matrix in general wound are determined by a dynamic balance among overall matrix synthesis, deposition, and degradation. Matrix metalloproteinases (MMPs) are one family of structurally related enzymes (zinc-dependent endopeptidases) that have the cooperative ability to degrade nearly all extracellular matrix molecules. The MMPs are generally categorized into collagenases (MMP1), gelatinases, stromelysins, and membrane type MMPs by their substrate specificity. An important mechanism for the regulation of the activity of MMPs is via their binding to a family of homologous proteins referred to as tissue inhibitors of metalloproteinases (TIMP1-4)^[1]. The effects of MMPs and TIMPs in the course of general wound healing and in the formation of some non-healing skin ulcers have been widely reported^[2], but their expression, localization, and effects in the process of radiation-combined wound repair remain unclear. Radiation-combined wound repair is characterized by delayed healing, non-healing or tumorigenesis. The aim of this study was to characterize the changes in MMP1 and TIMP1 expression during the inflammatory, granulation, and early remodeling phases of radiation-combined wound healing, and to analyze the effects of the changed expression on the healing process.

2 Materials and methods

2.1 Animal grouping and animal model establishment

One hundred and twenty adult female Wistar rats (220 ± 20 g, from Animal Center of Academy of Military Medical Sciences) were randomly divided into the non-irradiation group and irradiation group (60 animals per group). All rats were anesthetized with sodium pentobarbital via intraperitoneally administration. The rat dorsal skin was sterilized with 75% ethanol and two round surgical wounds (15 mm in diameter) were made by cutting the dorsal skin deep into the subcutaneous layer. The two wounds were 15 mm apart from each other.

Immediately after wounding, for the irradiation group, a dorsal area 25 mm in diameter including the surgical wound was irradiated with a single dose of 25 Gy ^{60}Co γ rays at a dose rate of 1.67 Gy/min. Shielding against whole body irradiation mimicked the clinical setting of radiotherapy and thus possible depletion of bone marrow-derived elements was prevented. The reason that 25 Gy dosage was selected is that in previous studies of radiation dose-effect relationships, we determined that 25 Gy γ ray irradiation could delay the healing process of cut wounds, but did not cause non-healing skin ulcers^[3].

Each wound area was covered with sterilized vaseline gauze and the rats were indi-

vidually housed to prevent tampering of each other's wounds.

2.2 Observation items

The general condition and local change of the wounds were carefully observed. While the animals were alive, the wound area was traced onto a transparent plastic film. The wound area was measured using the QUANTIMET 970 image analysis system (Leica). Three rats in the same group were sacrificed under sodium pentobarbital narcosis on the 1st, 3rd, 5th, 7th, 10th, 12th, 14th, 17th, 19th, 21st, 24th, 28th, 35th, 45th, and 60th day after wounding and the wound strips harvested. Each strip sample was cut into three segments. The first segment was immediately fixed with fresh 4% paraformaldehyde in phosphate-buffered saline for 5 days, embedded in paraffin, cut into 4 μ m-thick sections and stained with hemotoxylin and eosin. The second segment was snap frozen in liquid nitrogen and 10 μ m-thick sections were cut using a cryostat. These sections were used for immunohistochemical and in situ hybridization studies. The last segment was immediately fixed in 2.5% glutaraldehyde, embedding in EPON 812, and ultrathin sections were cut using an ultramicrotome. Ultrastructural changes were observed using a CM200 transmission electron microscope (Philips).

Sampling: For immunohistochemical and in situ hybridization studies, 3 animals for each time point, 1 block for each animal and 4 sections for each block were studied. Only 1 ultrathin section was studied for each time point.

2.3 Immunohistochemistry

Immunohistochemistry techniques were used to detect the expression of MMP1 and TIMP1. Briefly, goat Anti-MMP1 polyclonal antibody (Santa Cruz Biotechnology, CA) and goat Anti-TIMP1 polyclonal antibody (Santa Cruz Biotechnology, CA) were used at a dilution of 1:200, following the steps indicated in the SP kit (Zymed, San Francisco, CA). Developing was done with 3,3-diaminobenzidine (DAB). Phosphate-buffered saline (PBS) was used as the negative control.

2.4 In situ hybridization

Part-length of TIMP1 cDNA was cloned into pGEM-3Zf(+) and cut by endonuclease EcoR I (625bp), and Part-length of MMP1 cDNA was cloned into pGEM-3Zf(+) and cut by endonuclease EcoR I and BamH I. These were kindly donated by Dr. J-Y Long (Beijing Institute of Basic Medicine). The cDNA probes were labeled with digoxigenin by random primer labeling method (with "DIG DNA Labeling and Detection Kit", Boehringer Mannheim). The plasmid pBR322 was used as a negative control. Hybridization reaction was performed at 42°C for 10h and development with NBT-BCIP for 5h.

2.5 Grading system and statistical analyses

A four level grading system for immunohistochemical and in situ hybridization studies was developed according to the number of the positive cells, and the intensity of the

positive areas. These four levels were defined as: (+) positive cells < 20%, weakly stained; (++) positive cells < 40%, moderately stained; (+++) positive cells < 60%, moderately stained; (+++++) positive cells > 60%, strongly stained. SAS 6.03 was used to perform Repeated Measures Analysis of Variance and student's t-test.

3 Results

3.1 Gross examinations and wound area measurement

No animal died during the observation period of 60d. Wounds in the control animals became smaller by 7d after wounding, and healed completely by 14d after wounding. Wounds in the irradiated animals were larger than that in control animals on the 7th day after wounding, and were healed by 21d after wounding (Fig.1). The average area of the wounds in all the irradiated animals was larger than that in controls ($p < 0.01$).

3.2 Microscopic observations

On the 3rd day after wounding: (1) For the control group there was a large quantity of exudate in the wounds, including many macrophages and neutrophils. Granulation tissue was seen, with many fibroblasts and newly-formed capillaries. At the margin of wounds, epidermal cells were found actively proliferating and migrating toward the wound center. (2) For the irradiation group, the quantity of exudate was reduced in the wounds, especially the number of infiltrating macrophages and neutrophils. Necrotic tissue increased. The vessel occlusion, hemorrhage and shedding of endothelial cells from vessels were also found in the wounds. (3) MMP1 and TIMP1 expression and mRNA transcription were all weakly positive (+) in the epidermal cells, fibroblasts, and endotheliocytes in both the control and irradiated wounds.

On the 5th day after wounding: (1) For the control group, a thick layer of granulation tissue enclosed the wound, and a large number of fibroblasts and newly-formed capillaries were discovered. Epithelial cells replicated and covered part of the wounds. (2) For the irradiation group, the granulation tissue layer in the wounds was thinner than that in the controls and bleeding often occurred near the wound surface. Some deformed fibroblasts (radiation fibroblasts)^[4] were seen in the granulation tissue and irradiated dermis. Compared to the controls, the number of newly-formed capillaries and fibroblasts were decreased. Electron microscope observation showed that some fibroblasts were swelling, and degranulation of endoplasmic reticulum occurred, mitochondrial crista disappeared, and chromatin condensed at the margins of nuclei which was a sign of early apoptosis (Fig.2). There were more apoptotic cells in radiation group than that in controls. Necrotic tissue was also present. (3) MMP1 and MMP1 mRNA were all strongly positive (+++) in the proliferating keratinocytes in both control and irradiated wounds, but were weakly positive (+) in the granulation tissue in both groups. TIMP1 and TIMP1 mRNA were all weakly positive (+) in the newly formed epidermis and

granulation tissue for both groups.

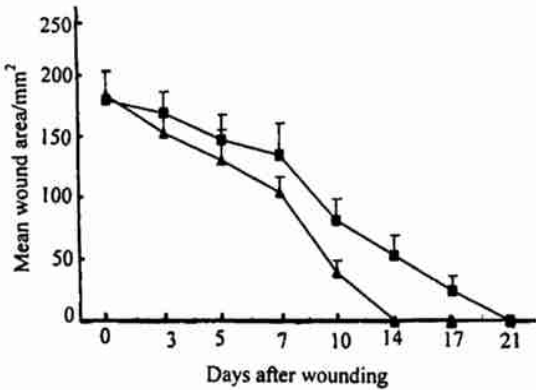


Fig.1 Changes in the mean wound area of the irradiation and non-irradiated (control) groups
▲ Control, ■ Irradiated group

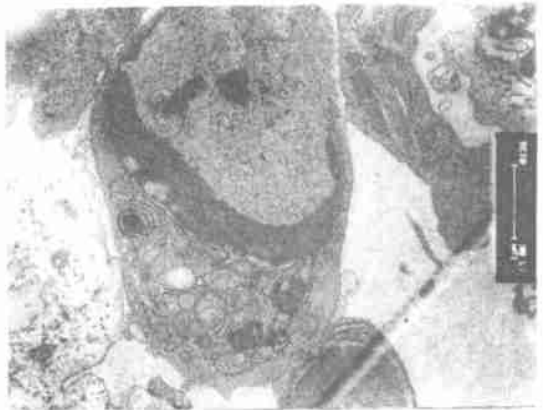


Fig.2 On day 5 after wounding, some fibroblasts in the irradiated wounds showed some signs of early apoptosis, with chromatin condensed at the margin of nuclei. There were more apoptotic cells in radiation wounds than in controls. (by Electronic Microscope)

On the 10th day after wounding: (1) For both groups, the granulation tissue layer was thicker. The epithelial cells proliferated and covered a large part of the control and the irradiated wounds. But the granulation tissue layers in the radiation wounds were thinner than those in the control wounds, and there were fewer capillaries in the irradiation wounds than in the control wounds. Fibroblast and endotheliocyte injuries were noted in the radiation wounds by electron microscope examination. (2) MMP1 was strongly positive (+++) and TIMP1 was weakly positive (+) in the newly-formed epidermis (mainly in the basal cells) of both the control and radiation wounds. In the granulation tissue, MMP1 and TIMP1 were strongly positive (++++) in the control wounds, but comparatively weak (++) in the irradiated wounds, which was mainly localized in fibroblasts, endotheliocytes, and macrophages.

On the 14th day after wounding: (1) The non-irradiated wounds were healed. The number of capillaries and fibroblasts were decreased in the non-irradiated wounds. MMP1 expression in the epidermis and granulation tissue of control wounds were also decreased (++). Meanwhile, the irradiated wounds were not healed, and MMP1 and MMP1 mRNA were still moderately positive (++). (2) TIMP1 and TIMP1 mRNA were weakly positive (+) in the epidermis, and moderately positive (++) in the granulation tissue of both groups.

On the 21st day after wounding: For the non-irradiation group, the number of

fibroblasts and capillaries were greatly diminished, with an increase of extracellular matrix, including collagenous fibers deposited in the control wounds, and MMP1 and TIMP1 were weakly positive (+). The irradiated wounds were healed, and MMP1 and TIMP1 in the wounds were moderately positive (++) .

From the 21st day to the 60th day after wounding: MMP1 and TIMP1 were all weakly positive (+) or negative in both control and irradiated wounds.

3.3 Image analysis of MMP1 and TIMP1 expression (IOD, immunohistochemistry)

In Fig.3, the average IOD of MMP1 expression in the granulation tissue of the two groups is statistically different ($p < 0.01$). In Fig.4, the average IOD of TIMP1 expression in the granulation tissue of the two groups is different ($p < 0.01$).

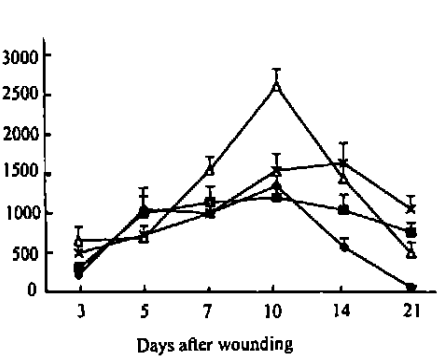


Fig.3 MMP1 expression in the process of general and radiation-combined wound healing
◆ Control epidermis, ■ Irradiation epidermis, △ Control gra-tissue × Irradiation gra-tissue

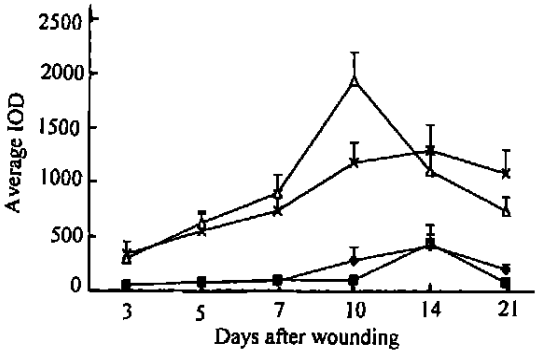


Fig.4 TIMP1 expression in the process of general and radiation-combined wound healing
◆ Control epidermis, ■ Irradiation epidermis, △ Control gra-tissue × Irradiation gra-tissue

4 Discussion

The major effects of MMP1 on the healing process of skin wounds are as follows: (1) The expression of MMP1 in the proliferating keratinocytes may help remove the necrotic tissue, aid the directional cell migration, and thus accelerate the course of reepithelialization. (2) The MMP1 expression in fibroblasts may facilitate cell migration, assist in the degradation of the provisional matrices, and promote the tissue remodeling process. (3) The expression of MMP1 in the endotheliocytes may make valuable contributions to angiogenesis. (4) The MMP1 expression in macrophages may help remove the necrotic and injured tissue, and participate in the tissue remodeling process associated with the granulation and scar tissue formation^[5].

In previous studies, we concluded the main characteristics of the healing process of combined-radiation wounds as follows: (1) The early inflammatory response was severely

inhibited. In particular, the number of infiltrating macrophages and neutrophils was decreased, blood vessels were injured, and hemorrhage was evident. (2) The formation and maturation of granulation tissue were slowed down. Angiogenesis was very difficult. Fibroblasts were damaged, "radiation fibroblasts" were present, and synthesis and secretion of collagen type I and III were also reduced. (3) The reepithelialization process was delayed and the healing time was prolonged^[6,7] In consideration of the important effects of MMP1 in wound repair process, the change of its expression will certainly affect the healing process of radiation-combined wounds, and this study thus gives new insight into the course of general wound healing.

From these results, we concluded that the epidermal expression of MMP1 in radiation wounds was not or only slightly decreased in the early and middle phases of healing process, but was increased in the late period and the expression time was prolonged. MMP1 expression was obviously inhibited in fibroblasts, endotheliocytes and macrophages in the radiation wounds. We think that some epidermal cells might keep their strong proliferating ability after being irradiated, and the synthetic and secreting function of the newly formed cells was not severely harmed too. But the dermal and subcutaneous fibroblasts, endotheliocytes, macrophages and undifferentiated mesenchymal cells were more sensitive to γ rays, and therefore their synthetic and secreting function (including collagen types I, III and MMP1) was obviously affected too.

We also found some large "radiation fibroblasts" in the radiation wounds. Serious injuries were observed in fibroblasts and endotheliocytes by electron microscope examination, and in the radiation group the cell apoptosis rate in the early healing process was higher than that in controls. Radiation-induced apoptosis is related with JNK/SAPK signaling pathway, and the function of ATM and P53^[8,9]. Rudolph R *et al* cultured fibroblasts from the margin of radiation skin ulcer and control skin of patients. Their results showed that fibroblasts from irradiated tissue had a decreased ability to replicate^[10]. Rueger *et al.* and Ferguson *et al.* demonstrated that injection of syngeneic fibroblasts into irradiated wounds may notably enhance wound healing in irradiated skin, as measured the tensile strength of the healing wounds^[11] Gorodetsky R *et al.* confirmed Krueger's observation by using wound strength measurements and detection of collagen types in the dermis and in the granulation tissue^[12] Our results showed that the decreased expression of MMP1 in the irradiated wounds was associated with the harmful effects of γ rays on many kinds of cells including fibroblasts and endotheliocytes. The results also illustrated that malfunctions of fibroblasts, endotheliocytes and undifferentiated mesenchymal cells were the major mechanism of the non-healing of radiation wounds. The epidermal damages had minor effects on the healing process of radiation wounds.

In the course of radiation-combined wound repair, γ ray irradiation inhibited the expression of MMP1 in fibroblasts and endotheliocytes of the granulation tissue, thus affected such process as cell migration and angiogenesis, and contributed much to the delayed healing of the radiation wounds. In the late healing period of radiation-combined wounds, the increased expression of MMP1 might be related to the over degradation of

matrixes and the difficulty in scar formation.

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MMP¹、TIMP¹ 在放射复合伤口愈合过程中的表达 及对伤口愈合过程的影响

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摘要 利用放射复合伤口动物模型,动态观察伤口愈合过程中 MMP¹,TIMP¹,mRNA 转录及蛋白表达的变化及其对伤口愈合和组织改建的影响。结果显示:25Gy(γ 射线)局部照射对伤口愈合有明显的损害作用,照射组伤口较对照组延迟 6d 愈合。在伤口愈合的炎症期和肉芽组织生长期,照射组新生表皮细胞中 MMP¹ 表达与对照组相近,在后期,随着照射组伤口愈合的延迟其表达相对较高。在伤后 3—14 d,TIMP¹ 在对照组新生表皮细胞中呈弱阳性,表皮覆盖后呈阴性,而在照射组表皮覆盖前呈阴性或弱阳性。MMP¹ 与 TIMP¹ 在照射组肉芽组织成纤维细胞、血管内皮细胞、巨噬细胞中表达较对照组明显降低,在愈合后期因辐射所致伤口愈合的延迟其表达时相拖后。结果表明:辐射所致 MMP¹ 与 TIMP¹ 在伤口肉芽组织中表达明显降低,直接影响细胞迁移、血管形成和瘢痕组织形成等病理过程,是辐射影响伤口愈合的重要机制之一。

关键词 放射,伤口愈合。MMP¹,TIMP¹

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