Original Article

Characteristic expression of connective tissue components and matrix metalloproteinases (MMPs) during the development of pressure ulcerations

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Chronic ulcerations are caused by pressure, ischemia or attrition and is a serious problem for long term care. However, little is known about biomolecular markers expressed during the development of pressure ulcerations. In the present study, we examined whether various extracellular matrix components correlate with the development of pressure ulcerations. Examination of wound areas and immunohistological staining showed that pressure ulceration develop following excision of the sciatic nerve from the hindlimbs of mice. Our data reveal that the expression of mRNAs encoding type I collagen (collagen α1 (I)), type IV collagen (collagen α1 (IV)), matrix metalloproteinase (MMP)-2, -3 and -9 correlate with the wound area. But laminin-5 (laminin-5 α3) does not correlate with the wound area. These results suggest that the expression of those mRNAs provides specific biomolecular markers for pressure ulcerations. The present study will be useful for developing effective therapeutic agents or prophylactic agents against pressure ulcerations.

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Introduction

Chronic ulcerations of the skin remain a significant health problem especially in an aging population. Pressure ulcerations are injuries to the skin which result from constant pressure due to impaired mobility. The pressure results in reduced blood flow and eventually cause cell death, breakdown of the skin, and the development of an open wound. In addition, pressure ulcers can be caused by various basal diseases e.g. diabetes, spinal impairment, etc. Many patients (4.2 to 9.5%) in long-term health care facilities develop pressure ulcerations in Japan. It is known that open ulcers become sources of pain, disability and/or infection. However, it is still not fully understood the relationship between development of the pressure ulceration and biomolcular.

It is believed that wound healing is series of overlapping events that begins immediately after wounding. Platelet aggregation forms a hemostatic plug and blood coagulation forms the provisional matrix made up of fibrin and fibronectin to prevent excessive blood loss. Platelets release growth factors and adhesive proteins that stimulate the inflammatory response and induce cell migration into the wound using the provisional matrix as a substrate. Wound cleaning is carried out by neutrophils, which solubilize debris, and monocytes, which differentiate into macrophages and phagocytose debris. Macrophages also release growth factors and cytokines to activate reepitherialization and vascularization. In reepithelialization, keratinocytes migrate across the area to re-establish the epithelial barrier. Previous report showed that during re-epithelialization, MMP-1, -3, -9, and -10 are reported to be detected in keratinocytes[1]. Momota et al. showed that laminin-5 α3 LG4 module promotes keratinocyte migration in a MMP-9 dependent manner[2]. Fibroblasts enter the wound site to replace the provisional matrix with granulation tissue composed of fibronectin and collagen. Previous study showed MMP-1, -2, 3, and -14 increase in granulation tissues at a wound site[1]. Angiogenesis after wounding or injury is initiated by the disruption of epidermal and vascular basement membranes, which releases extra cellular matrix (ECM) -bound angiogenic growth factor such as the FGFs[3]. Han et. al. showed that TNF α and TGF β contribute to angiogenesis by inducing expression in dermal fibroblasts and keratinocytes of MMP-9 capable of degrading basement membrane and ECM components[4]. As endothelial cells revascularize the damaged area, fibroblasts differentiate into myofibroblasts and contract the matrix to bring the margins of the wound together. The resident cells then undergo apoptosis, leaving collagen-rich scar tissue that is slowly remodeled in the following months. Thus ECM and MMP have very important role during wound healing. However, it is still unclearly understood the relationship between development of the pressure ulceration and these biomolecular and it is little effective therapeutic medicine.

Presently the evaluation of pressure ulceration is only macroscopic observation. The present study was designed to examine the relationship between the development of wound areas and the expression of mRNAs using a mouse model for pressure ulceration following excision of the sciatic nerve. This study would contribute development of therapeutic medicine.

Materials and Methods

1)Formation of pressure ulcerations on heels of mice

Male ICR mice (7-weeks of age, 30 ± 2 g, Sankyo Laboratory Service Corp, Tokyo, Japan) were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), The pressure ulcerations on heels of mice was made by excision of the sciatic nerve from the hindlimb of mouse according to the method described previous report[5]. The mice were put into cages and were allowed to roam freely in their cages to drink water and feed normally.

The wound area of each mouse was traced on laboratory plastic film (Pechiney Japan Corp, Tokyo, Japan). The film was cut out according to the pattern of the wound area and then was weighed. The wound area was calculated using a calibration curve for standard areas of 1 mm^2, 4 mm^2, and 9 mm^2 of laboratory plastic film. The wounds on the heels of the mice were recorded using a digital camera (Nikon Tokyo, Japan).

2)Light microphotographs of the wound site using HE staining

Tissues sections were obtained from normal skin and from skin with pressure ulcerations in mouse heels. Tissues were fixed in 10% formalin, dehydrated, cleared and embedded in ETP-150C (Sakura Finetek, Koto-ku, Japan). Cross-sections (5 μm) were mounted onto glass slides with DEPC-treated water and were baked overnight at 60℃. The slides were then dewaxed in ethanol, and some were stained with hematoxylin-eosin (HE).

3)Gelatin Zymography

Skin tissues were collected from mouse heels on 0 day, 1st day, 3rd day, 5th day and 7th day after excision of the sciatic nerve and were immediately frozen with liquid nitrogen. After grinding in liquid nitrogen, tissue samples were incubated for 24 hr at 4℃ in extraction buffer (0.01 M cacodylic acid, pH 5.2, 0.15 M $CaCl_2$, 0.15 mM $ZnCl_2$, 2 mM NaCl). Following incubation, each sample was centrifuged for 10 min at 2,000 rpm at 4℃. Soluble proteins were precipitated with acetone and the precipitate was dissolved in nonreducing sample buffer (1% SDS, 5% glycerol, 0.01% bromophenol blue, 0.01 M Tris-HCl buffer, pH 7.4). Soluble proteins were quantified using the BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as a protein

Table 1 Primer sequences

primers		primer sequences	amplified position
collagen α_1 (I)	Sense	5'-GGGCAAGACAGTCATCGAATA-3'	4332-4538
	Antisense	5'- TTTGGGTTGTTCGTCTGTTTC-3'	
collagen α_1 (IV)	Sense	5'- AGCTGCTTTTGTGGAAGTCAT-3'	5698-5947
	Antisense	5'- CTGGTGTTTCACATTCAGTGG-3'	
laminin-5 α_3	Sense	5'- GCAAGTCAATCTGGAGAGCAC-3'	4792-5092
	Antisense	5'- ATGAGTTCCACACAGGGAGTG-3'	
MMP-2	Sense	5'- TTTATGGCTTTCAGCACTCTCA-3'	2584-2807
	Antisense	5'- TGGAGTGGAAAACTGAAAAGGT-3'	
MMP-3	Sense	5'- CCTGCAACCGTGAAGAAGA-3'	1190-1505
	Antisense	5'- CAGGATGCCTTCCTTGGAT-3'	
MMP-9	Sense	5'- TGAATCAGCTGGCTTTTGTG-3'	2532-2773
	Antisense	5'- GTGGATAGCTCGGTGGTGTT-3'	
GAPDH	Sense	5'- CCATGGAGAAGGCCGGGGCCCA-3'	351-582
	Antisense	5'- ATGGCATGGACTGTGGTCATGAG-3'	

standard. Electrophoresis was performed on 7.5% SDS-PAGE gels containing 0.2% gelatin (DIFCO, Sparks, MD, USA). After electrophoresis, the gels were removed and incubated for 60 min in 2.5% Triton X-100 solution. After two washes in water, the gels were incubated for 20 hr at 37℃ in the reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 150 mM NaCl). Staining and destaining of gels were carried out with 0.25% Coomassie brilliant blue and 7.5% acetic acid/5% methanol, respectively.

4)RNA extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Skin tissues were collected from mouse heels on 0 day, 1st day, 3rd day, 5th day and 7th day after excision of the sciatic nerve and were immediately frozen with liquid nitrogen. After grinding in liquid nitrogen, the tissue samples were lysed in 4 M guanidine isothiocyanate containing 25 mM sodium citrate and 0.1 mM β-mercaptoethanol. Total RNA was isolated by extraction in phenol/chloroform. RT-PCR was performed with 1 μg total RNA under the following conditions. First strand cDNA synthesis from total RNA was prepared by oligo-dT priming and reverse transcription using an M-MLV Preamplification System (Gibco, BRL, Gaithersburg, MD, USA). Total RNA was denatured at 70℃ for 10 min and the samples were then incubated with RNA buffer containing 4 μl 5 × First-Strand Buffer, 2 μl 100 mM DTT and 40 units RNaseOUT Recombinant Ribonuclease Inhibitor. The contents were mixed and incubated at 37℃ for 2 min. One μl M-MLV RT was added, and the samples were incubated for 50 min at 37℃. The reactions were terminated by incubation at 70℃ for 15 min. PCR was then performed on 2 μl of the resulting cDNA with the primers listed in Table 1. Thirty cycles of amplification were performed for all constructs using the following conditions: at 95℃ for 1 min; at 60℃ for 30 sec; at 68℃ for 3 min. The PCR products were separated by electrophoresis on 2% agarose gels and were visualized by ethidium bromide staining.

Results

1)Formation of pressure ulcerations on the heels of mice after excision of the sciatic nerve

To evaluate the development of pressure ulcerations, we measured the wound areas after excision of the sciatic nerve. The wound area was about 5 mm^2 and 10 mm^2 on 3rd day and 7th day after excision of the sciatic nerve, respectively, and increased in a time-dependent manner (Fig.1). Further, skin tissues were examined histopathologically in cross-sections before and after excision of the sciatic nerve with HE staining (Fig.2). On 3rd day after excision, less of epidermis and necrosis of dermis in small area of foot pad with edematous changes and moderate infilteration of inflammatory cell in the subcutaneous tissue (data not shown). On 7th day, loss of epidermis was developed and as additional changes, enlarged fibroblast was increased in subcutaneous tissues with moderate to marked infiltrate of inflammatory cells (Fig.2B). Therefore, these results suggest that it is possible to form pressure ulcerations on the heels of mice following excision of the sciatic nerve.

2)Expression of MMP-2 and MMP-9 in pressure ulcerations

To investigate the pathophysiologial roles of MMP-2 and MMP-9 during the development of pressure ulcerations, gelatin zymography of MMP-2 and MMP-9 was performed on tissue extracts from the pressure ulcerations. The expressions of pro MMP-2, active MMP-2 and pro MMP-9 were elevated from 1 day after excision of the sciatic nerve and increased in a time-dependent manner (Fig.3).

3)Expression of collagen α1 (I), collagen α1 (IV), laminin-5 α3, MMP-2, MMP-3, MMP-9, and GAPDH mRNAs in pressure ulcerations

It is known that cutaneous wound healing requires the precise coordination of reepithelialization, dermal repair and angiogenesis. Dermal repair requires the production of extracellular matrix by fibroblasts. We examined the expression of mRNAs encoding matrix components (collagen α1 (I), collagen α1 (IV) and laminin-5 α3) and matrix metalloproteinases (MMP-2, MMP-3 and MMP-9) in pressure ulcerations using RT-PCR. Expression of each mRNA increased in a time-dependent manner after excision of the sciatic nerve, especially the expression

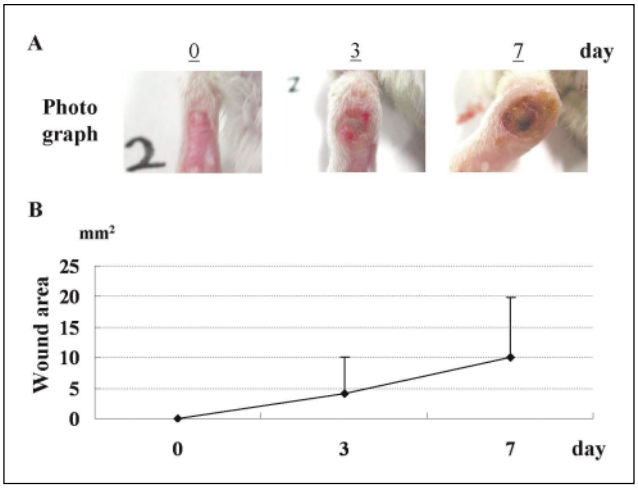


Fig.1 Pressure ulcerations on the heels of mice after excision of the sciatic nerve

Each mouse was bred in a cage without chips after excision of the sciatic nerve. The pressure ulcerations positions were photographed on the indicated day after excision of the sciatic nerve (0 day means before the excision of the sciatic nerve) (A). The graph shows the area of pressure ulceration on the indicated day after excision of the sciatic nerve. The bar indicates means ± S.D., n = 5 (B).

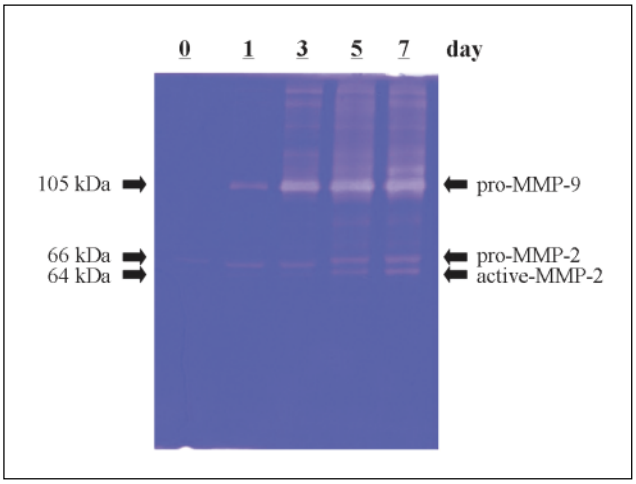


Fig.3 Detection of gelatinase activity by zymography

The samples were obtained from the pressure ulcer positions at the indicated days after the excision of the sciatic nerve and were separated on a 7.5% SDS-PAGE gel containing 0.2% gelatin. The enzyme bands were visualized by incubation at 37 ℃ in the gelatinolytic reaction buffer.

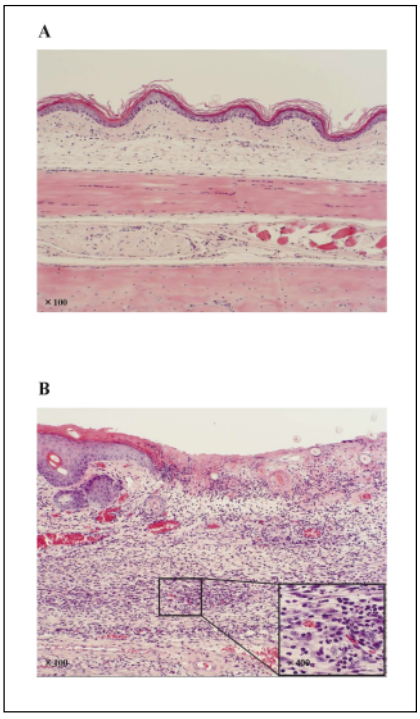


Fig.2 Light microphotographs of the tissues section using HE staining

Tissues sections obtained from normal skin and from skin with pressure ulcerations in mouse heels were stained with HE. A: Normal tissue of heel skin. ; B: Tissue of heel skin on 7th day after excision of sciatic nerves. Note the less of epidermis, necrosis of dermis and infiltration of large number of inflammatory cells in subcutaneous in subcutaneous tissue. Magnification × 100. The inset shows neutrophiles and fibroblasts. Magnification × 400.

of collagen α1 (I), MMP-2 and MMP-3 mRNA expression increased dramatically (Fig.4).

4)Relationship between the formation of pressure ulcerations and expression of mRNAs encoding matrix components and matrix metalloproteinases

Correlation analysis was performed to investigate the relationship between the formation of pressure ulcerations and the expression of mRNAs encoding matrix components and matrix metalloproteinases. A significant correlation between the wound area of pressure ulcerations and the expression of matrix components except laminin-5 α3 and matrix metalloproteinases mRNAs was found (Fig.5). Consequently, our data indicate that the expression of extracellular matrix components and matrix metalloproteinases mRNAs are biological markers expressed during the formation of pressure ulcerations.

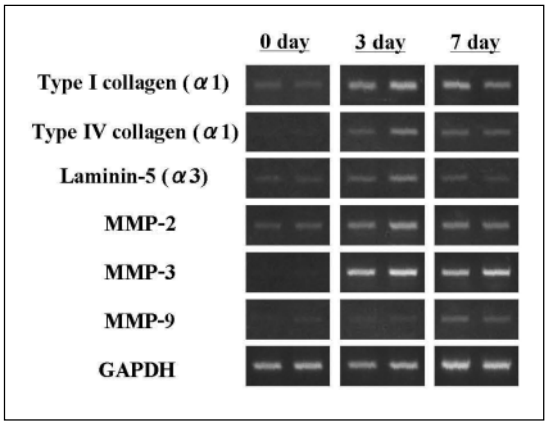
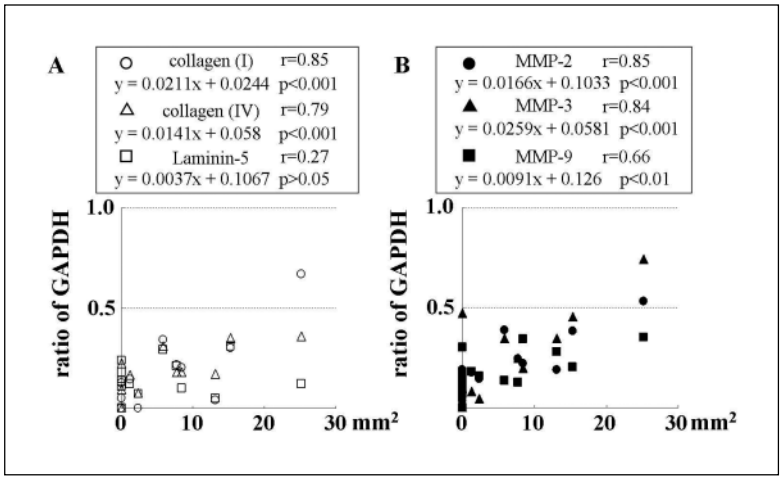


Fig.4 Expression of collagen α1 (I), collagen α1 (IV), laminin-5 α3, MMP-2, MMP-3, MMP-9, and GAPDH mRNA levels in the pressure ulcerations

Total RNA was isolated by extraction in phenol/chloroform from the pressure ulcer at the indicated days after the excision of the sciatic nerve. RT-PCR was demonstrated to determine the expression of collagen α1 (I), collagen α1 (IV), laminin-5 α3, MMP-2, MMP-3, MMP-9, and GAPDH mRNA during pressure ulcerations using two samples obtained from different mice.



Figu.5 Correlation between the area of pressure ulcers and the expression of collagen α1 (I), collagen α1 (IV), laminin-5 α3, MMP-2, MMP-3, and MMP-9 mRNA

Total RNA was isolated by extraction in phenol/chloroform from the pressure ulcer on 0, 3, and 7 day after the excision of the sciatic nerve. RT-PCR was carried out using specific primers for collagen α1 (I), collagen α1 (IV), laminin-5 α3, MMP-2, MMP-3, MMP-9, and GAPDH. Each expression of collagen α1 (I), collagen α1 (IV), laminin-5 α3, MMP-2, MMP-3, and MMP-9 mRNA was represented by the ratio to the expression of GAPDH mRNA parallel to the area of pressure ulcers. (n = 17)

Discussion

In this study we show that denervated mice develop pressure ulcerations. HE staining showed that a lot of nucleuses were stained. These nucleuses may be inflammatory cells that infiltrate in the pressure ulceration. These results are similar to the ischemic and pressure model of Peirce, et. al.[6] and the denervation model of Manley, et. al[7]. Our present data may partly reflect in clinical observations, which have indicated epitherial thickness, inflammatory cells, and blood vessel expansion in pressure ulcerations. We also showed that pressure ulcerations develop in a time-dependent manner using photography and that mRNA expression of several markers increases as the wound area develops. These data suggest that pressure ulcers develop in phases as evaluated by wound area in our model. Our data reveal that mRNA expression of collagen α1 (I), collagen α1 (IV), MMP-2, MMP-3 and MMP-9 increase during development of the wound area. In addition, there was no change in the mRNA expression of other ECM components, including tropoelastin,

fibrillin, and fibronectin.

Wound healing is a dynamic biological process with many complex interactions; i.e. inflammation, reepithelialization, angiogenesis and remodeling[8,9]. The basement membrane zone (BMZ) lies between the epithelial cells, and supports many tissues to act as a scaffold for the structure organization of the epithelium. Type IV collagen and laminin-5 are major components of the BMZ[10]. Laminin-5, which binds to type IV collagen, is a component of the basement membrane and plays important roles in cell bonding, cell differentiation, nerve projection extension, cell proliferation and organization and maintenance of the basement membrane. Earlier studies showed that laminin-5 plays a critical role in wound healing during keratinocyte migration or proliferation[2,11]. However, our study shows that type IV collagen mRNA expression is increased in pressure ulceration. Our result indicates that remodeling of the basement membrane may be related to the development of pressure ulcerations.

It is believed that controlled degradation of extracellular matrix (ECM) proteins, especially components of the basement membrane, is necessary for keratinocyte migration and proliferation during reepithelization and angiogenesis. It has been reported that MMP-1, MMP-2, MMP-3, MMP-9, and MMP-10 are elevated during reepithelization in wound healing[1,12]. Recently, studies have shown that MMPs play an important role during wound healing, but also that excessive MMP activity causes chronic ulceration[1,13,14]. Our observations also indicated that expression of MMP-2 and MMP-9 elevate during the development of pressure ulceration. These results suggest that the expression of MMPs is closely related to inflammation in the development of pressure ulcerations. Although our examinations do not show cytokine expression in this study, the excessive expression of MMPs may contribute to various cytokines, which is able to stimulate the expression of MMPs.

In conclusion, our study shows that mRNAs encoding collagen α1 (I), collagen α1 (IV), MMP-2, MMP-3, and MMP-9 are good candidates as biomolecular markers for the pressure ulcerations. These results will be useful for the development of effective therapeutic agents or prophylactic agent against pressure ulcerations.

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