Proteolytic Release of Latent Transforming Growth Factor-β (TGF-β) Binding Protein-1 (LTBP-1) Fragment in Wound Healing

Koji Mizuno, Hiroshi Wachi, Zenzo Isogai, Masahiko Yoneda, Satoshi Fujii, Ken Watanabe, and Yoshiyuki Seyama

Department of Clinical Chemistry, Hoshi University School of Pharmacy and Pharmaceutical Sciences, 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan, Department of Advanced Medicine, National Center for Geriatrics and Gerontology, 36–3 Gengo, Morioka-cho, Obu, Aichi, 474–8522 Japan, Aichi Prefectural College of Nursing and Health, Tougoku, kamishidami, Moriyama-ku, Nagoya 463–8502, Japan, Department of Molecular and Cellular Pathobiology and Therapeutics, Nagoya City University Graduate School of Pharmaceutical Sciences, 3–1 Tanabe-Dori, Mizuho-ku Nagoya, 467–8603 Japan and Department of Bone and Joint Disease, National Center for Geriatrics and Gerontology, 36–3 Gengo, Morioka-cho, Obu, Aichi 474–8522, Japan

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INTRODUCTION

Wound healing is a dynamic process of tissue remodeling requiring multiple biological responses. Appropriate activation of sequestered growth factors is one of the requirements for wound healing. Several growth factors associate with the extracellular matrix (ECM), and this association may facilitate proper storage and activation in a tissue specific manner.

Transforming growth factor-β (TGF-β) is among the most potent regulators of matrix production, and recently the importance of the extracellular regulation of TGF-β has been appreciated. TGF-β is secreted mostly in an inactive form in what is called the large latent complex (LLC), in which TGF-β polypeptide is non-covalently associated with its N-terminal pro-domain, latency-associated peptide (LAP). In most cases, LAP is covalently linked to latent TGF-β binding protein (LTBP) by disulfide bonds to form the LLC. For the liberation of TGF-β from the LLC, proteolytic cleavage of the LLC may be required. Proteolysis of LLC has been characterized as an initial step in certain latent TGF-β activation reactions. Plasmin is a potential activator of latent TGF-β, and it may be functionally relevant in a number of cell types. Thus, the sequestration and activation of TGF-β are suggested to be regulated by several steps.

The proteolytic cascade initiated by plasmin is a critical step in the wound healing process. Plasmin cleaves several ECM components as well as LTBP-1. Interestingly, impaired activity of the plasmin cascade results in delayed wound healing in mice. Although TGF-β also plays a criti-
cal role in tissue repair and plasmin-dependent activation of latent TGF-β has been reported, the mechanisms underlying these processes are yet to be elucidated.

In the present study, we focused on the proteolytic release of LTBP-1 from dermal connective tissues. We found that an LTBP-1 fragment is released from dermis by plasmin treatment. Furthermore, a similar LTBP-1 fragment and significant plasmin activity were observed in wound surfaces, suggesting that the release of LTBP-1 from matrices by plasmin is physiologically significant in the wound healing process.

**MATERIALS AND METHODS**

**Plasmin Treatment of Skin** —— Normal looking skin samples from excess portions of surgery for benign tumors were used in this study, and the protocol was approved by the ethics committee of the National Center for Geriatrics and Gerontology (Obu, Aichi, Japan). The study was conducted according to the Declaration of Helsinki Principles (Helsinki, Finland). The skin piece was directly digested by plasmin (100 mU/ml: P1) in 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4 Tris buffered saline (TBS) containing 2 mM CaCl2 for 3 hr at 37°C. After enzymatic treatment, the solution was centrifuged at 12000 rpm for 20 min. The extracted solutions obtained by centrifugation were precipitated by adding 95% ethanol containing 1.3% potassium acetate.

The precipitates were resolved in 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted. The blots were blocked with 5% non-fat milk in TBS for 1 hr and then incubated with anti-LTBP-1 monoclonal antibody (mAb 388) purchased from R&D (Morrisville, NC, U.S.A.). After washing with 0.05% Tween 20 in TBS, blots were incubated with peroxidase-conjugated anti-mouse IgG (Dako, Denmark). The blots were developed with chemiluminescent substrate [Amersham (Buckinghamshire, UK)] according to instructions of the manufacturer.

**Detection of LTBP-1 Fragments from Wound Surfaces** —— To investigate the wound surface ECM, we established the following sampling and extraction method. Nineteen samples from different wounds for initial analyses, and an additional sixty-two samples for correlation with clinical features were obtained by gentle swabbing with gauze or a swab (Nihon Menbou, Tokyo, Japan). Samples were obtained from the pressure ulcer wounds with granulation tissue formation, epithelization, or impaired wounds. For simplicity, the wounds with prominent necrotic tissue were excluded. Acute phase of pressure ulcer wounds were also excluded because of heterogeneity such as bacterial infections and debridement. Samples were stored at -20°C until use. This protocol was also approved by the Ethics Committee of the National Center for Geriatrics and Gerontology. All samples were obtained after written informed consent.

The swabbed sample was weighed and extracted with 800 µl of 6 M guanidine hydrochloride, 50 mM Tris-HCl pH 7.4, and 1/100 (v/v) of (St. Louis, MO, USA) protease inhibitor cocktail for 48 hr at 4°C with gentle shaking. The extracts were collected by centrifugation (2 x) and precipitated with 3 fold volume of 95% ethanol containing 1.3% potassium acetate. After adjusting the total protein content, the extracts were resolved by 7.5% (w/v) SDS-PAGE under non-reducing conditions and blotted onto nitrocellulose membranes. The blots were incubated with mAb 388 and the subsequent procedures were performed as described above. The densitometric quantification of each band obtained by anti-LTBP-1 antibody was performed using ImageJ software 1.40 g. Fisher’s Protected Least Significant Difference (PLSD) tests were used to evaluate statistical differences among the three groups.

**Plasmin Activity from Wound Surface** —— Serine protease activities were revealed by casein zymography. Casein (Wako Pure Chemical industries, Ltd., Tokyo, Japan) was incorporated into 10% SDS-PAGE at a final substrate concentration of 0.1% (w/v). Plasmin from human plasma (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) was used for a standard, and protein molecular weight markers were loaded onto the gels and resolved by electrophoresis. The gels were agitated in 2.5% Triton X-100 for 1 hr and subsequently incubated for 16 to 20 hr at 37°C in buffers optimal for proteolysis.

**Statistics** —— Data were analyzed for statistical significance by analysis of variance (ANOVA) with Fisher’s PLSD test. These analyses were performed with the assistance of StatView version 5.0. The results were considered statistically significant when the p value was < 0.05.
RESULTS

LTBP-1 Fragment is Released from Cutaneous Microfibrils by Plasmin

We previously demonstrated that LTBP-1 is located on microfibrils in the dermis. MAb 388, which recognized the carboxyl terminal of LTBP-1 (Fig. 1), was detected as reacting with LTBP-1 secreted from normal skin fibroblasts (Fig. 2, lane NSF). The LTBP-1 fragments migrating at <140 kDa released by plasmin treatment were recognized by mAb 388 (Fig. 2, lane Pl). In addition, a faint band migrating at <200 kDa also observed in plasmin-treated samples. This band was also observed in the guanidine extracts and is similar in size to LTBP-1 in cell culture medium and may represent full-length LTBP-1 molecules. The molecular size of the LTBP-1 fragment generated by plasmin is consistent with the bands observed previously using cell culture.

Since the liberation of TGF-β/LTBP-1 complex from tissues may be important for wound healing, we tried to detect LTBP-1 fragments from the swabbed samples of wound surfaces. LTBP-1 fragments could be observed by immunoblotting using mAb 388 (Fig. 2, lane W1, W2, and W3). The LTBP-1 fragment from the wound surface had a molecular weight similar to the fragment by plasmin digested skin.

Plasmin Activity from Wound Surfaces

Next, we characterized the proteolytic activity from wound surfaces using casein zymography. Caseinolytic signals obtained from samples

Fig. 1. Schematic Presentation of LTBP-1S (Short Form)
A schematic presentation of the domain structure LTBP-1S (short form) is shown.

Fig. 2. LTBP-1 Fragments Obtained from Wound Surfaces
By immunoblotting analyses with mAb 388, LTBP-1 fragments from wound surfaces were similar in size to the plasmin-generated fragments from normal dermis. The clinical features of the wound surface were obtained from three different patients (W1–3). These pressure ulcers contained remodeling granulation tissues. The number of the wound picture corresponds to the lane number. Pl, plasmin digested normal skin; Gu, 6 M guanidine extract from normal skin; NSF, conditioned medium from normal skin fibroblasts.

Fig. 3. The Expression of LTBP-1 Fragments from Wounds Depend on the Plasmin Activity
Samples were obtained from wound surfaces of four randomized patients (lanes 1–4). (a) A proteolytic activity with a molecular mass of approximately 80 kDa was detected by casein zymography. Human plasma plasmin (indicated by Pl) was used as the positive control for casein zymography. (b) LTBP-1 fragments from the corresponding samples using casein zymography were also detected by mAb 388. NSF, conditioned medium from normal skin fibroblasts.
LTBP-1 Fragment was Characteristically Detected in the Wound with Granulation Tissue Formation

LTBP-1 migrating at <140 kDa in wounds were quantified by immunoblotting. (a) The pictures of the ulcerative wounds are shown in the upper panels. (b) Representative blots of the samples from epithelizing wounds (Epithelization) and granulation tissue formation (Granulation) are shown in lower panels. Each number of the upper panels corresponds to the sample number of the blots (lower panels). (c) Denitometric arbitrary values of LTBP-1 bands and relative expression of the LTBP-1 fragment in pressure ulcer are presented by a column graph. The bar indicates standard deviation. E, epithelizing wounds \((n = 19)\); G, wound with granulation tissue formation \((n = 36)\); I, various impaired wounds \((n = 7)\). Data were statistically analyzed using ANOVA with Fisher’s PSLD test. Significant differences among class means are indicated: \(*\), \(p < 0.01\); \(* * *\), \(p < 0.001\) v.s. G (wound with granulation tissue formation).

LTBP-1 Fragments were Preferentially Detected in the Wound with Granulation Tissue Formation

To determine the correlation between LTBP-1 fragment in wound and clinical findings, we further analyzed samples from various wound surfaces. To simplify the study, we selected wounds with proliferative granular tissue formation or epithelizing wounds as shown in Fig. 4a. Impaired healing wounds of various causes were also analyzed. Immunoblotting analyses revealed that the LTBP-1 fragments were dominantly observed in the wound with granulation tissue formation, but not in the wound with epithelization (Fig. 4b). By statistical analyses, LTBP-1 fragments were shown to be a good marker for the wound surface with granulation tissue formation (Fig. 4c).
REFERENCES


