棒を用いて、低侵襲でサンプルを採取することができ、条件を検討すれば創傷治癒に関連する細胞外マトリックス分子の検出が可能であった。

D.　考察

この研究によって表面蛋白解析を用いた褥瘡病態の分子診断の枠組みが整備された。病態分類のツールとしての使用が可能であるとともに、適切なマーカーを用いることで褥瘡医療の標準化に用いることも将来可能である。

E.　結論

臨床場面に即した方法で褥瘡創表面の蛋白質を解析する技術的な方法を確立した。

F.　健康危険情報

なし

G.　研究発表

なし

H.　知的財産権の出願・登録状況

なし

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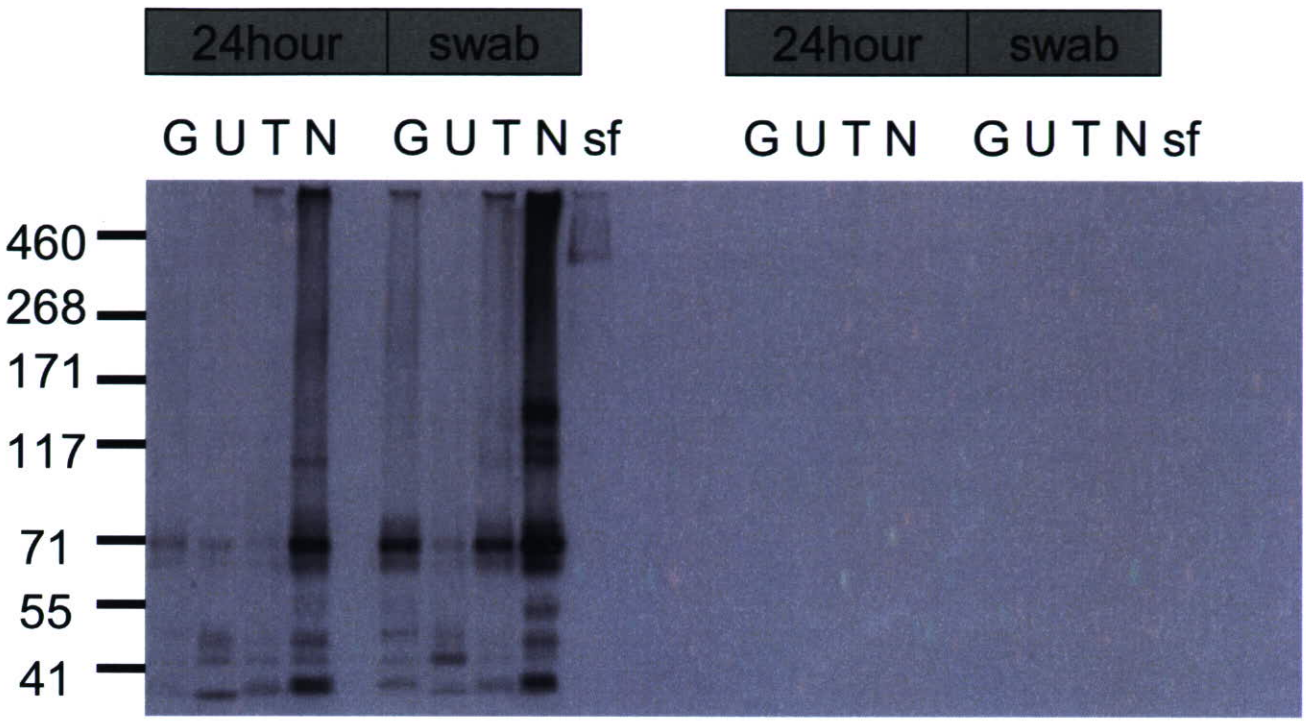
分担研究報告
創面からの蛋白採取法、解析技術の開発
（担当：渡辺　研）



Anti-fibronectin Antibody　　2nd antibody only

G: guanidine HCl
U: urea (8M)
T: Triton-X100 (0.2%)
N: 1M NaCl
sf: skin fibroblast conditioned medium

日常の処置から簡便に蛋白を採取したのち、抽出条件
を検討した。上記に示すような、抽出液を用いた。
ファイブロネクチン抗体によるウエスタンブロットでは塩酸
グアニジン、1M食塩で良好な抽出プロファイルがみられた。
緩衝液は50mM トリス塩酸、中性PH。24時間の滲出液だけでなく、
創面を軽く擦ってもサンプルの採取が可能であった。

厚生労働科学研究費補助金（長寿科学総合研究事業）
分担研究報告書

病理学的検討による記載潰瘍学と創表面蛋白解析の関連性

分担研究者　森　　將晏　岡山県立大学・保健福祉学部・教授

研究要旨

褥瘡における記載潰瘍学と創面蛋白解析の両者の関連を基盤とした創傷皮膚科学では、両者を橋渡しする病理学的な情報は不可欠である。本分担研究では創表面から検出可能な創傷治癒関連蛋白に関して免疫組織学的解析を行うとともに、過去の標本から記載潰瘍学で記述可能な創面の病理組織を解析した。免疫組織化学では創傷関連細胞外マトリックス分子は褥瘡肉芽組織では線維性の分布を失いびまん性に分布しており、創表面から検出される可能性が示唆された。また記載潰瘍学との関連では上皮化しつつある良好な肉芽組織は血管が縦方向に走行する典型的な肉芽組織が発達しており、かつ組織内に線維性のマトリックスが認められたものの、浮腫性肉芽では創の線維性マトリックスが未成熟であった。これらの結果から記載潰瘍学、創面蛋白分析の関連性と妥当性が検証できるとともに、病理学的にも褥瘡肉芽組織の多様性が証明できた。

A.　研究目的

創の評価は褥瘡のように原因が多様である疾患の病態診断と治療の選択に重要なものである。皮膚科医の皮疹の所見はその皮膚病理組織学的所見と密接に関連することは明らかで、皮膚病理組織学は皮膚科診断学の重要な部分を占める。しかしながら、褥瘡などの皮膚潰瘍では疾患の特性から病理学的検査をすることは難しく、経過によって変化しやすい創所見を病理検査で診断する意義も高くないと思われていた。しかしながら、創表面の蛋白解析と臨床所見を機軸とした創傷皮膚科学を考える時に病理と生化学、病理と臨床のように橋渡しとしての病理学は非常に重要であり、本研究の意義は高い。

B.　研究方法

病理検体はすでに報告している岡山県立大学からの検体を使用した。個人情報保護は指針に従った。病理組織切片を用いて以下の２つのアプローチから研究を進めた。

１）創表面から検出可能な創傷治癒関連蛋白に関しての免疫組織学的解析：創面蛋白解析において検出可能であった分子に関して、典型的な創面で免疫組織学的に検討した。

２）臨床写真が保存されている創面に関して臨床所見と病理組織との相関性の検討。過去の臨床写真を記載潰瘍学にて評価し、それと病理学的検討を組み合わせることで臨床・病理の関連を一定数の創面について検討した。

C.　研究結果

蛋白解析で検出したLTBP-1、バーシカン、ヒアルロン酸、ファイブロネクチンを正常部位と肉芽組織の両者で検討した。いずれの分子も正常と肉芽組織では全く異なる局在を示した。つまりLTBP-1、バーシカンなど細胞外マトリックスは肉芽組織中では線維性の分布を消失することが明らかになった。

臨床所見との関連では上皮化しつつある良好な肉芽組織は血管が縦方向に走行する典型的な肉芽組織が発達しており、かつ組織内に線維性のマトリックスが合成されていた（図の左）。一方、我々が記載潰瘍学において浮腫性肉芽(edematous granulation tissue)と記述した創面では肉芽組織の間質の線維形成が不十分であり（図の右）、創のマトリックスが未成熟である可能性が示された。よって創底の線維性マトリックスの成熟は上皮化にとって必要条件であると考えられた。

D.　考察

創表面蛋白解析との関連では検出される創傷治癒関連分子が線維との結合が失われるために褥瘡の創表面から検出されることが示唆された。臨床所見との関連性においては、病理で得にくい所見はあるものの有用であると考えられた。少数例であっても蛋白（生化学）と臨床所見との橋渡しとしての検討が必要である。

E.　結論

肉芽組織のマトリックスの多様性と正常との差異が示された。創表面から検出できる創傷治癒関連分子は肉芽組織では、線維性の分布の消失が認められた。さらに記載潰瘍学での所見の差異を病理的に裏付けることが可能であった。

F.　健康危険情報

なし

G.　研究発表

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・ 遠藤明美、押本由美、道繁祐紀恵、小山恵美子、森　将晏. 臥位における大腿後面臀溝部支持クッションの有効性の検討. 第33回日本看護研究学会学術集会. 2007.7.28-29, 盛岡
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H.　知的財産権の出願・登録状況

なし

研究協力者

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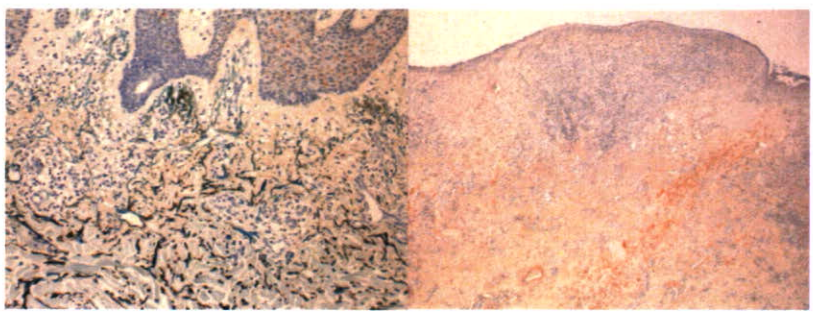
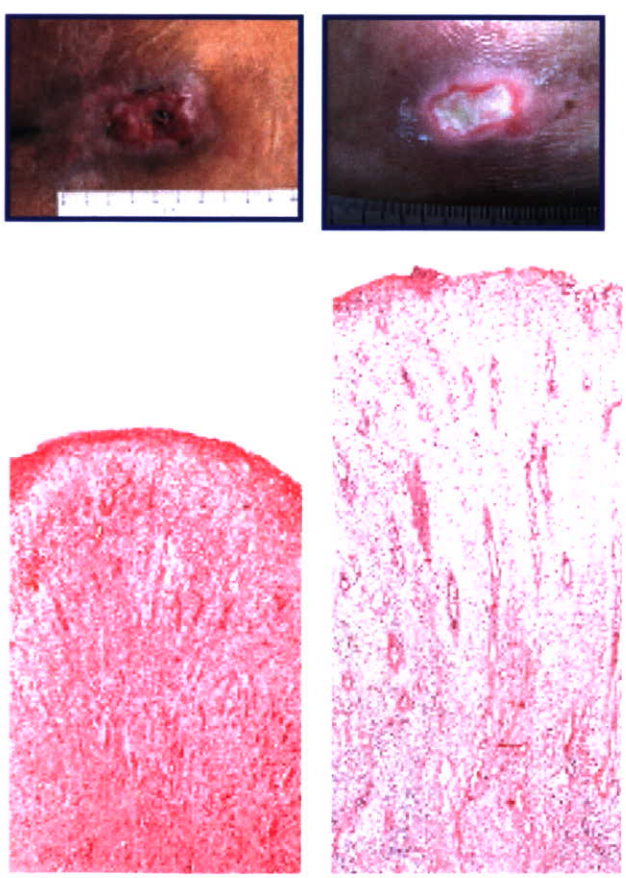
分担研究報告
病理学的検討による記載潰瘍学と創表面蛋白解析の関連性
（担当：森　將晏）



図の説明：左は正常真皮のバーシカン、右は肉芽組織のバーシカンの免疫組織学的染色。正常真皮では弾性線維と同様に分布するが、肉芽組織では全体にびまん性に分布がみられる。 バーシカン肉芽組織の水和性を保つ分子で創表面から検出される。



所見と病理組織との関連を一定数の創面について検討した。上皮化しつつある良好な肉芽組織は血管が縦方向に走行する典型的な肉芽組織が発達しており、かつ組織内に線維性のマトリックスが合成されていた（図の左）。一方、記載潰瘍学において浮腫性肉芽(edematous granulation tissue)と記述した創面では肉芽組織の間質の線維形成が不十分であり、創のマトリックスが未成熟（図の右）である可能性が示された。よって創底の線維性マトリックスの成熟は上皮化にとって必要条件であると考えられた。

III. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻名・ページ・出版年
Hasegawa K, Yoneda M, Kuwabara H, Miyaishi O, Itano N, Ohno A, Zako M, Isogai Z.	Versican, a major hyaluronan-binding component in the dermis, loses its hyaluronan-binding ability in solar elastosis.	J Invest Dermatol	2007;127:1657-63
磯貝善蔵	褥瘡の病態と分類	調剤と情報	2007;13(8):10-14
Abe M, Sogabe Y, Syuto T, Yokoyama Y, Ishikawa O.	Evidence that PI3K, Rac, Rho, and Rho kinase are involved in basic fibroblast growth factor-stimulated fibroblast-Collagen matrix contraction.	J Cell Biochem	2007;102:1290-9
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書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名・出版地	出版年・ページ
磯貝善蔵	高齢者の特徴と高齢期に多い疾病および障害:皮膚科	介護支援専門員テキスト編集委員会	四訂 介護支援専門員基本テキスト 第３巻高齢者保健医療・福祉の基礎知識	長寿社会開発センター	2007; 55-59
石川　治	これだけは知っておこう～皮膚科的疾患　褥瘡,皮膚瘙	遠藤英俊, 田中志子	介護福祉のための医学	弘文堂(東京)	2007;143-157
石川　治	急性期褥瘡の特徴、治療目標は何か、どんな治療法があるか	宮地良樹、真田弘美	褥瘡局所治療ガイドライン	メディカルレビュー社（東京)	2007;117-122

IV. 研究成果の刊行物・別刷

次ページより

Versican, a Major Hyaluronan-Binding Component in the Dermis, Loses its Hyaluronan-Binding Ability in Solar Elastosis

Keiko Hasegawa[1], Masahiko Yoneda[1], Hiroko Kuwabara[2], Osamu Miyaishi[3], Naoki Itano[4], Akiko Ohno[5], Masahiro Zako[5] and Zenzo Isogai[6]

Versican interacts with hyaluronan (HA) at its N-terminus and with fibrillin-1 at its C terminus. As versican in the dermis connects microfibrils to the HA-rich matrix for viscoelasticity, dermal diseases may involve destruction of these complexes. A recombinant versican protein, rVN, covering the HA binding region (HABR) of human versican and a polyclonal antibody, 6084, against rVN were prepared and characterized. Blotting analyses of skin extracts with 6084 and biotin-conjugated HA revealed that versican was a major HA-binding component in the dermis. Matrix metalloprotease-12, which is expressed in areas of solar elastosis, degraded versican and abrogated its HA-binding ability. Immunohistochemical analyses revealed that the elastic materials in solar elastosis lesions were negative for 6084, but positive for 2B1, an antibody recognizing the C-terminus of versican, indicating loss of the HABR in the aggregated elastic fibers. This loss of the HA-binding ability of versican followed by HA exclusion may be responsible for the pathological and phenotypical changes observed in solar elastosis.

Journal of Investigative Dermatology (2007) 127, 1657–1663; doi:10.1038/sj.jid.5700754; published online 15 March 2007

INTRODUCTION

Dermal connective tissue comprises three distinct elements; elastic fibers, collagen fibers, and ground substance. These three elements interact and connect with each other and also play roles in the dermal architecture. Elastic fibers are composed of two distinct elements, elastin and microfibrils. Microfibrils are elastic cross-linked polymers mainly composed of fibrillins and are present throughout elastic fiber elements including elaunin and oxytalan (Sakai *et al.*, 1986). Collagen fibers play important roles in maintaining the structural integrity of the dermis. Ground substance is mainly composed of hyaluronan (HA) and proteoglycans, and contributes to the formation of water-rich matrices. HA, which lacks a core protein, holds huge amounts of water non-covalently and is integrated within the dermal connective tissue through its binding of molecules.

Versican, also called PG-M, is a lectican family proteoglycan that is present in the fetal, neonatal, and adult dermis in humans (Zimmermann *et al.*, 1994; Sorrell *et al.*, 1999). We previously reported that versican interacts with both fibrillin-1 and isolated microfibrils (Isogai *et al.*, 2002). Versican binds fibulin-1 and -2 via its lectin-like domain and localizes to cutaneous microfibrils (Zimmermann *et al.*, 1994; Aspberg *et al.*, 1999; Olin *et al.*, 2001; Isogai *et al.*, 2002). Versican also binds to HA via its N-terminal region (LeBaron *et al.*, 1992) and HA is co-distributed with elastic fibers in the dermis (Bernstein *et al.*, 1996). Therefore, by binding to fibrillin microfibrils and HA, versican can impart viscous properties to cutaneous microfibrils.

Photoaged dermis displays characteristic pathological features, designated as solar elastosis. Both the synthesis and degradation of matrix components have been investigated for the development of solar elastosis (Sellheyer, 2003). In solar elastosis lesions, accumulations of elastin and microfibril components, including fibrillin-1 (Dählback *et al.*, 1990), LTBP-1 (Karonen *et al.*, 1997), versican (Bernstein *et al.*, 1995), fibulin-2 (Hunzelmann *et al.*, 2001), and fibulin-5 (Kadoya *et al.*, 2005) have been reported. However, the structural and functional properties of these accumulated molecules in solar elastosis lesions remain to be elucidated.

Matrix metalloprotease-12 (MMP-12), also known as macrophage metalloelastase, degrades elastin (Shapiro *et al.*, 1993) and plays critical roles in the development of

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Abbreviations: ECM, extracellular matrix; HA, hyaluronan; HABR, hyaluronan-binding region; MMP, matrix metalloproteinase

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emphysema and aortic aneurysms that are caused by elastic fiber degeneration (Hautamaki *et al.*, 1997; Curci *et al.*, 1998). Immunohistochemical studies have revealed that MMP-12 is localized in solar elastosis lesions (Saarialho-Kere et al., 1999) and granulomatous skin disease (Vaalamo *et al.*, 1999). Moreover, ultraviolet irradiation and heat treatment induce MMP-12 at the mRNA level *in vivo* (Chung *et al.*, 2002; Chen *et al.*, 2005). Therefore, MMP-12 is likely to play critical roles in the development of solar elastosis, possibly by modifying the structure and functions of the extracellular matrix (ECM) in the dermis. Thus, the impact of MMP-12 on the structural and functional properties of elastic fibers represent an important issue for a better understanding of the phenotypical and pathological changes of photoaged skin.

In this study, we investigate HA-binding molecules in adult skin and identify versican as a major HA-binding molecule. We also find that the N-terminal fragments of versican are cleaved in adult skin, but are still capable of binding to HA. Moreover, we find MMP-12 abolishes the HA-binding ability of versican *in vitro*, indicating that versican which localizes in solar elastosis lesions, is likely to lose its HA-binding activity.

RESULTS

Recombinant expression and characterization of the HABR of versican

To characterize the HA-binding activity of versican, the HA-binding region (HABR) of versican was recombinantly expressed (Figure 1). An expression construct that spanned the N-terminal half of versican (V3) was created and transfected into 293 human kidney cells. The recombinant polypeptide, designated rVN, was secreted into the medium with a good yield. The purified proteins displayed a doublet of bands on SDS-PAGE (Figure 2, lane 3). Both bands reacted with an anti-hexahistidine antibody and their N-terminal sequences were the same. The binding affinity for HA in the soluble phase was further shown by isopycnic ultracentrifugation (Figure 2b).

Production and characterization of a polyclonal antibody against the HABR of versican

To characterize the HABR of versican, a polyclonal antibody was raised against gel-purified rVN. Western blot analysis of crude conditioned media from normal skin fibroblasts revealed that the antiserum, 6084, specifically recognized the versican core protein (Figure 3). Furthermore, 2B1, a monoclonal antibody specific for human versican (Isogai *et al.*, 1996), showed identical reactivity (Figure 3).

Versican is a major HA-binding component in dermal matrices and its HABR is cleaved in adult skin

To investigate the major components binding to HA in the dermis, adult skin was extensively extracted with 6 M guanidine hydrochloride. The extract was then precipitated, resolved by SDS-PAGE, and blotted onto membranes. Incubations with biotin-conjugated HA and 6084 revealed similar and multiple band patterns, mainly distributed at

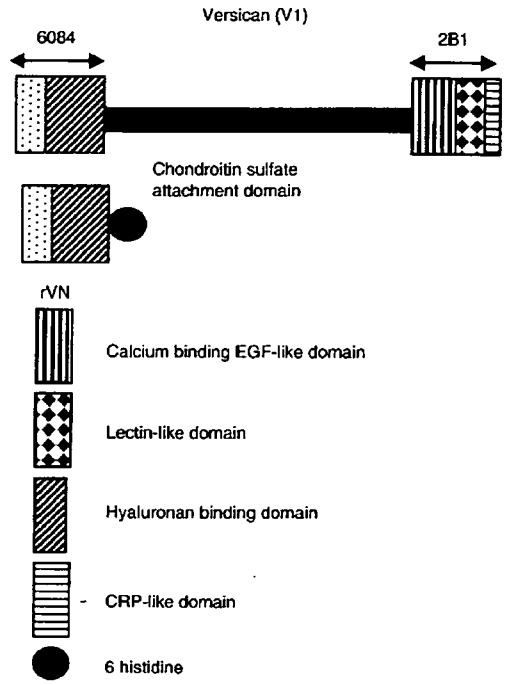

Figure 1. Schematic representation of human versican (V1) and the recombinant protein used in this study. A recombinant versican fragment covering the HABR was designed and designated rVN. The nomenclature of each domain and the antibody recognition sites are indicated.

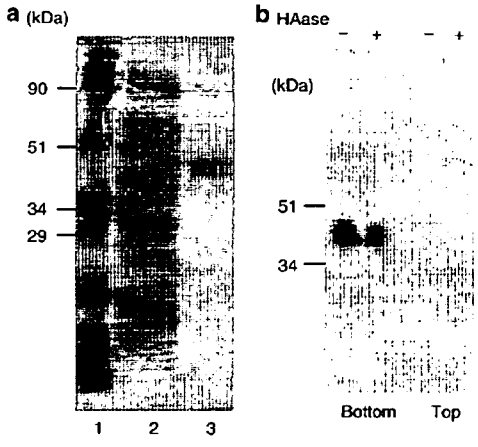

Figure 2. Expression and characterization of the HABR of human versican. (**a**) Conditioned media from 293 cells expressing rVN (lane 2) and the purified rVN polypeptide (lane 3) were resolved by 10% SDS-PAGE under non-reducing conditions. The molecular weights of standard marker proteins are indicated (lane 1). The gel was stained with Coomassie Brilliant Blue R-250. (**b**) Binding assay following cesium chloride density gradient ultracentrifugation. Conditioned medium from transfected cells expressing rVN was incubated with HA and then ultracentrifuged in 0.4 M guanidine hydrochloride containing CsCl. Following the centrifugation, the sample was divided into three fractions. The densities of the bottom and top fractions were 1.44 g/ml and 1.28 g/ml, respectively. Most of the proteins were fractionated at the top. The bottom and top fractions were examined by immunoblotting with an anti-hexahistidine antibody. The rVN detected by the anti-hexahistidine antibody in the bottom fraction shows non-covalent binding to HA in solution. HAase, hyaluronidase.

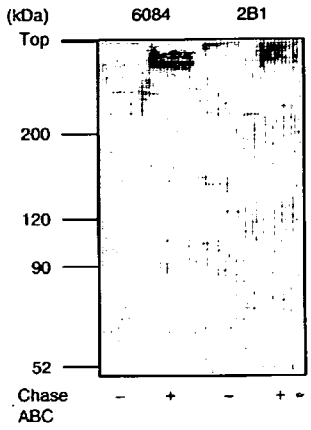

Figure 3. Specificity of polyclonal antibody 6084. Crude conditioned medium from normal skin fibroblasts was incubated with or without chondroitinase ABC (Chase). The samples were resolved by 7.5% SDS-PAGE, blotted onto membranes, and incubated with 2B1 (a mAb against the C-terminus of versican) or 6084 (a polyclonal antibody against the N terminus of versican). The bands in the sample treated with chondroitinase ABC are the versican monomer (arrowheads).

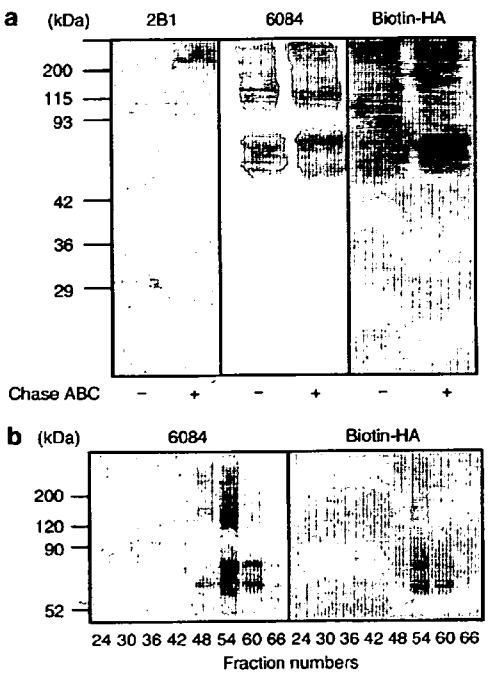

Figure 4. The HABR of versican is a major HA-binding component in skin. (a) Normal skin was extracted with 6 M guanidine hydrochloride and resolved by SDS-PAGE. (b) To compare between the immunoreactivity of 6084 and the HA-binding affinity, the extract was fractionated using a Sepharose CL-2B molecular sieve column. The total volume is at fraction 68 and the void volume is at fraction 23. The representative fractions indicated in the Figure were resolved by SDS-PAGE. The blots in a were incubated with 6084, 2B1, and biotin-HA as described in Materials and Methods. The blots in b were incubated with 6084 and biotin-HA as indicated. The reactivity of 6084 is well correlated with the HA-binding activity in b.

~60 kDa (Figure 4a), indicating the presence of multiple proteolytic sites in the versican core protein. Although most of the bands remained unaffected by chondroitinase ABC digestion, a band at ~500 kDa was generated by the enzyme treatment (Figure 4a). Immunoblotting with 2B1 revealed that the ~500 kDa band in the chondroitinase ABC-treated sample corresponded to the versican monomer (Figure 4a). Immunoblot analyses of the sieved fractions with 6084 demonstrated the presence of the N-terminus of versican that can bind to HA. These results also suggested that the major HA-binding molecules in dermal extracts were N-terminal fragments of versican, probably degraded versican (V1). Since 2B1 does not recognize the HABR of versican. Immunoblot analyses using monoclonal antibodies against stubs obtained following chondroitinase ABC digestion also confirmed that versican was the major large chondroitin sulfate proteoglycan secreted by dermal fibroblasts (data not shown).

Hyaluronidase treatment generated 6084-positive bands at ~50 kDa (Figure 5, arrowhead). However, the yield of 6084-positive bands was approximately one-twentieth the level in the guanidine extract estimated by immunoblotting. Consistent with this result, considerable amounts of versican fragments were detected following re-extraction of hyaluronidase-treated skin with 6 M guanidine hydrochloride (data not shown).

MMP-12 abrogates the HA-binding activity of versican

A blot overlay analysis using biotin-conjugated HA revealed that rVN was capable of binding to HA in solid-phase assays (Figure 6). MMP-12 digested rVN into undetectable small fragments and the HA-binding activity of rVN was completely abrogated as evaluated by HA transblot assays. Reactivity to polyclonal antibody 6084 was also abolished by MMP-12 treatment (Figure 6). Therefore, 6084 was characterized as recognizing a conformational epitope of HABR and its reactivity correlated well with the HA-binding affinity, which was consistent with the results of the extraction studies (Figure 4b). By contrast, MMP-12 cleaved a fibrillin-1 peptide, rF23, but the residual fragments were relatively large (data not shown).

MMP-12 treatment reduces the viscosity of versican-bound microfibrils

To test whether MMP-12 affects the functional properties of versican-bound microfibrils, the viscosity of isolated microfibrils from fetal membranes was measured using a Cannon-Fenske viscometer at 25°C. The statistical difference was determined by two-sided paired-*t*-test with $P<0.0001$. That MMP-12 treatment reduced the viscoelasticity of the isolated microfibrils is seen in Table 1.

Versican in solar elastosis is unable to bind to HA

Immunohistochemistry revealed that the staining patterns of 2B1 and 6084 were similar in normal skin, suggesting that the cleaved N-terminus of versican was still associated with microfibrils in the dermis (Figure 7a–c). In solar elastosis, the staining with 2B1 was heavily positive, whereas that with 6084 was weak or absent (Figure 7d–f). Staining with 2B1 and

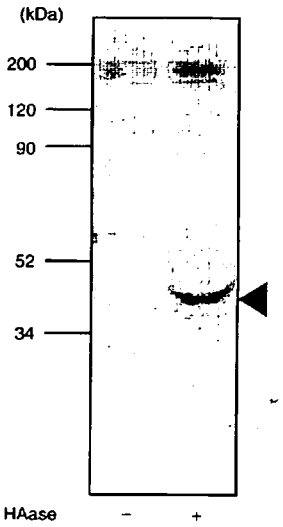

Figure 5. Hyaluronidase treatment releases small amounts of versican fragments. Normal skin pieces were incubated with *Streptomyces* hyaluronidase (+) or buffer alone (–) as described in Materials and Methods. The supernatants were resolved by SDS-PAGE and blotted with polyclonal antibody 6084. 6084-positive bands of ~42 kDa are generated following the enzymatic treatment.

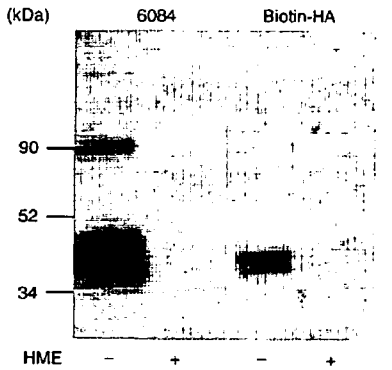

Figure 6. MMP-12 digests rVN and abolishes its reactivity to polyclonal antibody 6084. Purified rVN was incubated with human MMP-12 (+) or buffer alone (–). Aliquots of the samples were resolved by 10% SDS-PAGE, blotted onto membranes, and incubated with 6084 or biotin-conjugated HA. The reactivities toward polyclonal antibody 6084 and biotin-conjugated HA are abolished by MMP-12 treatment.

Table 1. Kinematic viscosity of versican-bound microfibrils

	Non-treated microfibrils[1] (n=40)	MMP-12-treated microfibrils[1] (n=40)
Kinematic viscosity[2]	1.263±0.026 mm²/s	1.177±0.021 mm²/s

[1]Isolated microfibrils were treated with MMP-12 or buffer alone.
[2]Mean±SD.

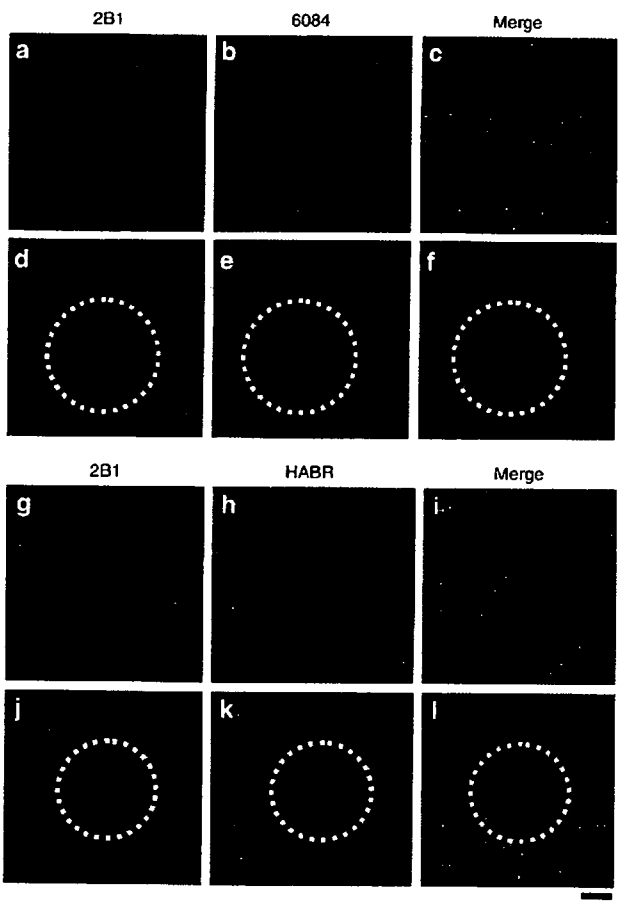

Figure 7. Versican in solar elastosis lesions is unable to bind HA. (**a–f**; **g–l**) Double-immunofluorescence staining of two skin regions with 2B1 (red) and 6084 (green) recognizing the C and N termini of versican, respectively, was performed on (**a–c**; **g–i**) normal skin and (outlined: **d–f**; **j–l**) solar elastosis lesions. Yellow areas in the merged images show coexistence of both termini of versican (**c**, **f**, **i**, and **l**). Colocalization of the immunoreactivities for 6084 and HABR is found in normal skin (**c** and **i**), whereas very little immunoreactivity for 6084 is present in the solar elastosis lesions (**f** and **l**). Bar = 100 μm.

biotin-conjugated HABR in normal skin revealed that versican and HA were colocalized in microfibrils (Figure 7g–i). By contrast, in areas of solar elastosis, HA was rather absent from solar elastosis lesions (Figure 7j–l). To exclude the possibility that the enhanced hyaluronidase activity in solar elastosis leads to the exclusion of HA in the lesions, normal and photoaged skin were incubated with HA and fractionated on a sieve column. However, we did not detect hyaluronidase activity in either normal skin or solar elastosis (Figure 8).

DISCUSSION

Skin texture is largely dependent on the architecture created by its ECM components. For instance, sclerosis, fibrosis, and wrinkle formation show their own characteristic changes in ECM components in the dermis. The pathological features of

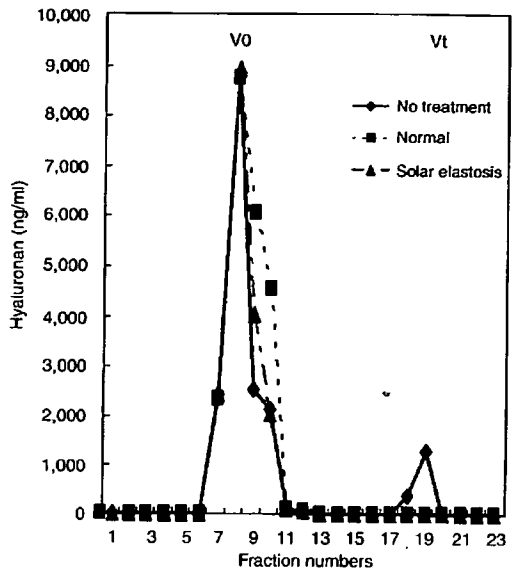

Figure 8. Hyaluronidase activity is not detected in solar elastosis. To exclude the possibility that activated hyaluronidase activity in solar elastosis reduced the accumulation of hyaluronan, skin pieces from normal and solar elastosis skin were minced and incubated with hyaluronan. The treated samples were sieved and the concentrations of HA measured. Incubation with normal skin and solar elastosis skin did not affect the size or amount of HA. Incubation with *Streptomyces* hyaluronidase completely abolished HA (not shown in the graph).

photoaging are characterized as degradation of dermal collagen and accumulation of elastotic material and proteoglycans (Fisher *et al.*, 2002; Sellheyer, 2003). In this study, we focused on the large chondroitin sulfate proteoglycan versican, which links elastic fibers to the ground substance. We have shown, for the first time, that versican is a major HA-binding component and that its HABR is cleaved in adult skin.

Using a mammalian cell expression system, we showed that the G1 domain of versican is sufficient for binding to HA, as previously predicted (LeBaron *et al.*, 1992). The HABR of versican was also able to bind to HA in the soluble phase. A polyclonal antibody, 6084, specifically recognized versican secreted from fibroblasts in both immunoblotting and immunohistochemical analyses, indicating that the recombinant protein, rVN, was properly folded and functional. The observation of a doublet band for rVN may be due to sugar modifications.

The immunoblotting pattern of guanidine hydrochloride extract of skin revealed that the N-terminus of versican is cleaved at multiple sites in adult skin, whereas the versican monomer was detected with 2B1 and 6084 in fibroblast-conditioned medium. The good yield of 6084-positive bands around 60 kDa compared with the yield of 2B1-positive bands suggests a covalent interaction between the C-terminal region of versican and cutaneous microfibrils (Isogai *et al.*, 2002). The cleaved fragments of versican were still capable of binding to HA. The sizes of the versican N-terminal fragments detected by 6084 were larger than rVN, and the extracted fragments were still capable of binding to HA and colocalizing with HA in the normal dermis. Similar fragments of versican have been identified in other studies on fetal skin (Sorrell *et al.*, 1999) and brain (Westling *et al.*, 2004). In addition, ADAMTS 1 and 4 were reported to cleave versican in the brain (Westling *et al.*, 2004) and the proteolytic epitopes of versican generated by ADAMTS 1 and 4 are present in adult arteries (Sandy *et al.*, 2001). This processing of versican and other proteoglycans may be required for maturation of the skin.

Hyaluronidase treatment of skin generated only small amounts of the N-terminal fragment of versican, suggesting that the cleaved N terminus is not simply trapped by HA. It remains unknown how the cleaved N-terminal region of versican interacts with the other ECM components present in the skin.

Accumulation of versican in solar elastosis has been reported (Bernstein *et al.*, 1995; Saarialho-Kere *et al.*, 1999). Accumulation of HA is also observed in dermal connective tissue in the normal dermis and at early stages of solar elastosis (Bernstein *et al.*, 1996). MMP-12 is induced by ultraviolet irradiation *in vivo* (Chung *et al.*, 2002) and is localized to solar elastosis lesions (Saarialho-Kere *et al.*, 1999). Degradation of versican by MMP-12 may affect the architectural role of the ECM in photoaged dermis.

On the basis of our biochemical analyses using recombinant rVN or extracted materials from adult skin, reactivity to 6084 is consistently correlated with the HA-binding activity. Therefore, staining with 6084 could represent HA-binding activity *in situ*. Interestingly, staining with 6084 is absent from areas of massive solar elastosis, whereas 2B1 is positive in such areas, consistent with previous studies (Bernstein *et al.*, 1996; Vaalamo *et al.*, 1999). These observations indicate that the versican accumulated in areas of massive solar elastosis is unable to bind HA. Consistent with the findings, staining of HA is faint in regions of massive solar elastosis. Degradation of versican by MMP-12 could represent a possible mechanism of the aging process of the ECM, As versican and MMP-12 are accumulated during the development of arteriosclerosis and versican degradation is sometimes observed (Halpert *et al.*, 1996). Furthermore, the reduced viscosity of isolated versican-bound microfibrils following MMP-12 treatment may explain these structural-functional correlations. Although other MMPs are possibly involved, the accumulation of MMP-12 and versican may play distinct functional roles in pathological changes through disruption of the viscoelasticity of connective tissues.

MATERIALS AND METHODS

Recombinant expression of the HABR of versican

The HABR of recombinant human versican (rVN) was expressed in mammalian cells as described previously (Reinhardt *et al.*, 1996). To create an expression construct for rVN, the region encoding L^{21} to R^{348} of versican in clone hMV3 (Isogai *et al.*, 1996) was amplified with the sense primer VN1S (5′-AGCTGCTAGCACTACATAAAGTC AAAGTGGGAAAAAG-3′), introducing an *Nhe*I restriction site at the 5′-end, and the antisense primer VN1AS (5′-AGCTCTCGAGTCAAT

GATGATGATGATGATGTCGTTTAAAGCAGTAGGCATCAAATC-3′), introducing a sequence for six histidine residues, a stop codon and a *Xho*I restriction site at the 3′-end. The *Nhe*I–*Xho*I fragment was subcloned into pCEP/γ2III4, containing the sequence for the BM40/SPARC signal peptide. The expressed peptide was purified by chelation chromatography as described previously (Reinhardt *et al.*, 1996). The polypeptide was further purified by sieve chromatography through Sephacryl S300 (Amersham Pharmacia Biotech, Tokyo, Japan) or SDS-PAGE for use in antibody production. The amino-acid sequence of rVN was analyzed using a Model Procise 494 cLC protein sequencing system (Applied Biosystems, Foster City, CA). To test the HA-binding affinity, conditioned medium was incubated with exogenous HA (Seikagaku Kogyo, Tokyo, Japan) and separated by density gradient centrifugation. Briefly, following the incubation with HA, cesium chloride was added to the medium to a final density of 1.36 g/ml and the mixture was centrifuged at 40,000 r.p.m. for 48 hours (Yoneda *et al.*, 1990). Next, the sample was separated into three fractions (top, middle, and bottom) for analyses.

Antibodies

A rabbit antiserum against rVN was produced by Operon Biotechnology (Tokyo, Japan). The antiserum was titrated by ELISA at 1:1000. Monoclonal antibody 2B1 (anti-versican) was characterized previously (Isogai *et al.*, 1996). mAbs against chondroitinase ABC-treated stubs, 1B5 and 2B6, were purchased from Seikagaku Kogyo.

Immunoblotting and transblot assays of skin extracts

Pieces of normal-looking skin were obtained from individuals as unneeded portions after skin surgery at sun-protected sites. Written informed consent was obtained from all patients. The study protocol was approved by the Ethics Committee of the National Center for Geriatrics and Gerontology, and the study was conducted according to the Declaration of Helsinki Principles. Following removal of subcutaneous fatty tissue, skin was weighed, minced into small pieces (~1 mm), and extracted with 10 μl/mg (v/w) of 6 M guanidine hydrochloride, 50 mM Tris–HCl, pH 7.4, 2 mM $CaCl_2$, and 1 mM phenylmethylsulfonyl fluoride for 48 hours at 4°C. The extract was concentrated to 6 ml using a Centriplus 30 (Amicon-Millipore, Billerica, MA) with centrifugation. Next, part of the concentrated extract was fractionated on a CL-2B Sepharose column (total volume, 90 ml; fraction number: 68; Amersham Pharmacia Biotech) in 4 M guanidine hydrochloride and 50 mM Tris-HCl pH 7.5 at a flow rate of 0.1 ml/minute. The fractions were analyzed by dot blotting and Western blotting as described previously (Isogai *et al.*, 2002).

Transblot assays using biotin-conjugated HA were performed as described previously (Zako *et al.*, 2002). Briefly, the blots were sequentially incubated with biotin-conjugated HA (50 μg/ml in Tris-buffered saline) and peroxidase-conjugated streptavidin (1:1,000; Amersham Pharmacia Biotech), and then developed.

Hyaluronidase treatment of skin

Skin pieces (~200 mg) were incubated with 100 TRU (turbidity reducing units) of *Streptomyces* hyaluronidase (Seikagaku Kogyo) in 50 mM acetate buffer, pH 5.0, containing 2 mM PMSF at 37°C for 2 hours. Control samples without enzyme treatment were also prepared. The supernatants were collected by centrifugation at 15,000 r.p.m. at 4°C and precipitated for SDS-PAGE analysis. For some immunohistochemical analyses, skin sections were pretreated with hyaluronidase before HA detection.

Protease treatment of rVN

Purified rVN (~9 μg) and rF23 (~2 μg), a recombinant fibrillin-1 peptide (Reinhardt *et al.*, 1996), were treated with 0.12 μg of MMP-12 (R&D Systems, Minneapolis, MN) in Tris-buffered saline containing 2 mM $CaCl_2$ at 37°C for 6 hours. The digestion was terminated by the addition of one volume of four-fold-concentrated SDS sample buffer to three volumes of the sample, followed by heating at 95°C for 5 minutes. The digested samples were analyzed by Western blotting and HA-binding assays.

Viscosity of versican-bound microfibrils

To study the mechanical properties of hyaluronan–versican–microfibril complexes in solar elastosis, we treated isolated microfibrils with MMP-12, which is expressed in sun-exposed skin.

Briefly, fetal membranes were extracted with 6 M guanidine hydrochloride as described previously (Isogai *et al.*, 2003). Subsequently, the extract was fractionated on a 120 ml CL-2B molecular sieve column (Amersham Biosciences, Uppsala, Sweden), equilibrated with 4 M guanidine hydrochloride/0.1 M Tris–HCl (pH 8.0) at a flow rate of 0.3 ml/minute, and 3.1-ml fractions were collected (12 minutes/tube).

After gel chromatography, the samples were brought to a density of 1.30 g/ml by the addition of CsCl (Yoneda *et al.*, 1990) and a direct gradient was established by centrifugation at 100,000 *g* at 10°C for 45 hours using a P90AT rotor (Hitachi, Tokyo, Japan). The gradients were partitioned into 12 fractions. The 6084-positive fractions were dialyzed against water and incubated with 0.12 μg of activated recombinant human MMP-12 (R&D Systems) in Tris-buffered saline containing 2 mM $CaCl_2$ at 37°C for 24 hours. Viscosity was measured using a Cannon-Fenske viscometer at 25°C. Viscosity was measured 40 times. Statistical difference was determined by two-sided paired-*t*-test with $P<0.0001$ considered to represent any significant difference.

Double-immunofluorescence microscopy

Double-immunofluorescence staining was carried out using formalin-fixed paraffin-embedded specimens chosen from specimens embedded for pathological diagnosis. To simplify the study, we chose specimens showing advanced solar elastosis and normal sun-protected skin without inflammation, as evaluated by hematoxylin–eosin staining. In total, 13 sun-exposed lesion specimens (average patient age 77.2 years; six from the forehead, five from the cheek, and two from the nose) and eight sun-protected specimens (average patient age 73.0 years; three from the abdomen, three from the thigh, one from the back, and one from the chest) were used. Samples were stained with 2B1 (1:50), 6084 (1:50), and biotin-conjugated HABR (Biotin-LP; 1:50; Seikagaku Kogyo). Double immunostaining using combinations of 2B1 and the above antibodies was also performed. The secondary antibodies used were rhodamine-conjugated anti-mouse Ig (1:20: Molecular Probes, Eugene, OR), fluorescein isothiocyanate-conjugated anti-rabbit Ig (1:25; Vector Laboratories, Burlingame, CA), and fluorescein isothiocyanate-conjugated streptavidin (1:20; Vector Laboratories). All samples were examined using an LSM 510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Hyaluronidase activity in skin

One sun-exposed cheek skin sample pathologically confirmed as advanced solar elastosis and one sun-protected abdomen skin sample were minced, ground and extracted with Cell Lytic-MT (Sigma, St Louis, MO) at 4°C for 10 minutes. Supernatants were collected after centrifugation. Next, an extract equivalent to 10 mg of skin was incubated with 1 mg/ml of high molecular weight hyaluronan (Sigma) in a total volume of 1 ml at 37°C for 24 hours. *Streptomyces* hyaluronidase (2 TRU) was used as a positive control. The treated hyaluronan was fractionated on a Superose 6 column (GE Healthcare, Tokyo, Japan) and monitored by its absorbance at 232 nm. The HA contents in the fractions were determined using an HA Assay Kit (IBA method; Seikagaku Corp., Tokyo, Japan) according to the manufacturer's instructions.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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特集 Special Part 1

褥瘡の病態と分類

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はじめに

褥瘡は外力による虚血性皮膚潰瘍と定義されるが，褥瘡の病態は非常に多様で，それらが複雑に絡み合っている。故に，悪化要因と治癒阻害要因を明らかにし，各患者に応じた対策や治療が行われる必要がある。しかし，褥瘡の多様性を解析する診療体系の医学的基盤は十分に確立されていない。

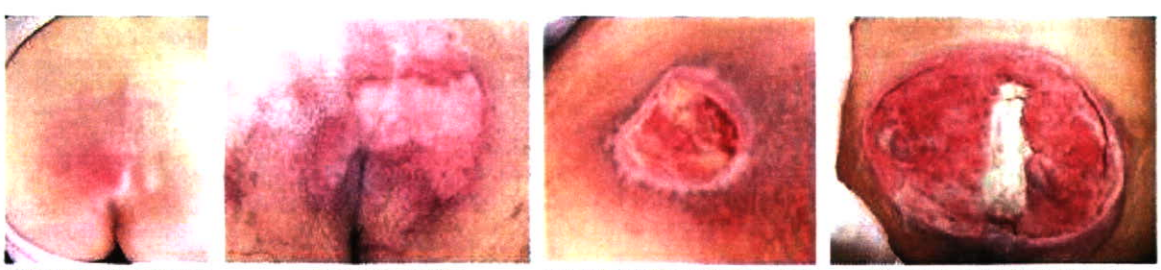
本稿では，褥瘡の多様な病態を概説する。さまざまな観点から病態，分類を挙げるが，それは，褥瘡診療における複眼的な視点が必要だからである。創傷治癒過程では，褥瘡と急性創傷との違いを認識し，それとともに，いったん褥瘡形成されてからの治癒過程では，さまざまな要素が異なることを認識する必要がある。

深さによる分類

褥瘡は，その治癒機転から浅い褥瘡と深い褥瘡に分類することが重要である。「褥瘡の予防・治療ガイドライン」では図1に示すように，Ⅰ～Ⅳ度に分類されている。深さがⅠ度，Ⅱ度の褥瘡，すなわちDESIGN分類（26頁の表1，2参照）でd2までの褥瘡は，いわゆる「浅い褥瘡」であり，Ⅲ度，Ⅳ度の褥瘡は「深い褥瘡」と定義されている。

Ⅱ度褥瘡では通常，皮膚付属器（汗腺，皮脂腺，毛）から表皮細胞が遊走し，瘢痕を残さず治癒する。また，Ⅰ度，Ⅱ度では真皮という剛性を持つ組織が残存することも重要である。治癒後は点状の色素沈着を残すことが特徴である。一方，Ⅲ度，Ⅳ度，すなわちDESIGN分類でのD3，D4ではⅠ度，Ⅱ度の褥瘡と経過を異にする。つまり，肉芽組織を形成した後，治癒する経過をとる。

褥瘡において，これらの治癒機転を理解することはケアに直接関与することであり，重要である。理想的には，褥瘡をこの段階に食い止めるような治療とケアが必要であるが，深部から発症する褥瘡の存在も知ら



Ⅰ度：圧迫を除いても消退しない発赤，紅斑

Ⅱ度：真皮に及ぶ損傷

Ⅲ度：皮膚全層および皮下組織に至る深在性筋膜に及ぶ損傷

Ⅳ度：筋肉・骨支持組織に及ぶ損傷

図1　褥瘡の深さによる違い

れるようになってきている。外用薬による薬物療法は，深い褥瘡に対して不可欠で，基剤の性質を熟知した薬剤師の役割が重要である。

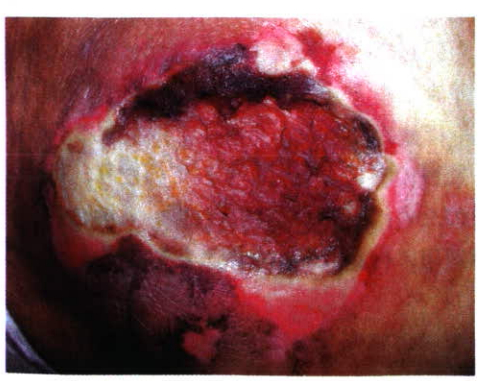
経過による分類

1．急性期褥瘡

褥瘡の急性期（図2）では，紅斑に引き続き水疱や紫斑などの所見がみられる。紫斑は皮膚の出血を意味し，Ⅲ度以上の褥瘡に発展することを示唆する。一般的に，循環障害による組織障害は，虚血の時点から明らかな組織障害の程度が確定するまでに2～3週間の時間経過がある。その間に褥瘡は日を追うごとに変化する。また，この時期には感染の併発の検討が必要とされる。この時点では，刻々と変化する創傷を評価しつつ経過を観察することが重要である。

2．慢性期褥瘡

一概に慢性期褥瘡と称されるものの，病態的には急性期を経過した褥瘡と捉えた方がよいと思われる。慢性期褥瘡では，まず壊死組織除去が必要である。壊死組織が固着した状態は，治癒機転が全く起こらないばかりか，感染症の母地となる。これを取り除くのがデブリードマン（創縁切除）である。通常は健常組織との境界が形成されたころから開始するが，通常，壊死組織は経過を追って明瞭になってくるので，数回のデブリードマンを必要とすることが多い。理論的には，デブリードマンが終了すれば赤色の肉芽組織が増生し，上皮化に向かうはずだが，実際はそうでないことも少なくなく，臨床病態は複雑である。



黒色壊死組織がみられる

図2　急性期仙骨部褥瘡

色調分類

福井基成氏らによって提唱された色調分類は，黒色期，黄色期，赤色期，白色期といった経過による分類で，褥瘡の治癒へ向かう時間経過をよく反映している。今どのような病期であるかを簡便に把握し，褥瘡診療におけるデブリードマンや外用薬の使い方について検討するのに一定の役割を果たした。しかし実際は，このような経過をとるのはⅢ度以上の深い褥瘡に限られる。

DESIGN分類

DESIGN分類については，26頁の表1，2を参照されたい。この分類は，日本褥瘡学会によって作成され，褥瘡診療において注目点を明らかにするとともに，重症度を一定の範囲で規定するものである。さらに「科学的根拠に基づく褥瘡局所治療ガイドライン」では，各指標をそれぞれに改善させるという視点で，統計学的な一定のエビデンスに従って作成された。しかし，実際の臨床現場において，慢性に経過した肉芽組織の改善やポケットの解消などは，経験則によることが多い。そのため，この分類は治療法の選択に使うのは難しいことも多い。

原疾患による分類

褥瘡において，発症の原因となった原疾患を把握することは非常に重要である。すなわち，褥瘡は外力による虚血性皮膚潰瘍であるため，その外力を発生させた，もしくは発生させている原疾患を把握することが重要である。しかし，それは簡単ではなく，患者の実際の生活を観察して初めて原疾患が明らかになること

が多い。特に，歩行が可能な患者の褥瘡には特異的な要因があるため，それを同定し，適切な対策を立てることが重要である。

筆者らは，外力と原疾患との関連において褥瘡を「自動性」と「静止性」に分類している。自動性褥瘡は，褥瘡の原因となる外力を患者自身が生み出せる褥瘡である。全体からすると少数であるが，難治性褥瘡にはこの例も多い。例えば，対マヒのように上半身は動かせるために外力を生じる場合や，精神疾患のために睡眠時に独特の動きや臥位ポジションがある場合，また，パーキンソン病などの神経疾患で，褥瘡部位を支点にして体位を自力で変換している場合などが時にみられる。これらの対策は，自動がほぼ不可能な静止性褥瘡とは異なり，個々の患者に応じた除圧方法を確立することが必要である。

原疾患の経過による分類

褥瘡の発症には通常，基礎疾患の関与があり，筆者らはこれらを原疾患の病期によって分類している（表）。急性期では，臥床によって褥瘡が発症するため，臥位ポジションによって発症する仙骨部，踵部の褥瘡が多い。一方，回復期では，ギャッチアップポジション（上半身を起こした状態）で栄養の注入や食事摂取が開始されたり，車いすが開始されたりするために，尾骨部や坐骨部の褥瘡が多い。病院でのケアが充実してくると，仙骨部，踵部の褥瘡は減少する。また，腸骨部，大転子部の褥瘡は，慢性疾患によるマヒや体の変形によって起こることがしばしばである。

これらは，あくまで脳血管障害や肺炎などの回復可能疾患における褥瘡であり，がん患者などは，これに当てはまらない。ここで重要なのは，原疾患と患者の状況を把握し，褥瘡の悪化要因を適切に見極めて対処することである。また，栄養の注入や車いすなどでのポジショニングに十分留意する必要がある。

表　原疾患の経過による褥瘡の分類

分類	発生部位
疾患急性期褥瘡	仙骨部，踵部
疾患慢性期褥瘡	腸骨部，大転子部
疾患回復期褥瘡	尾骨部，坐骨部

発生部位による分類（凸型褥瘡と凹型褥瘡）

褥瘡の発生部位における特徴については，ほとんど明らかにされていないが，褥瘡ケアの個別化・効率化のためには，発症要因を解析することが重要である。特に，同じ臀部の褥瘡であっても，尾骨部と仙骨部褥瘡ではケア方法が大きく異なる。

理論的には，褥瘡は骨突出部位に発症するはずである。しかし，いったん発症した褥瘡において，圧力が除去されているにもかかわらず治癒しないことは，しばしば経験する。大転子，腸骨部，外顆，踵部のような凸面では，褥瘡は周囲からの皮膚の緊張のため，頂点に発生する。さらに，外顆や踵部では通常，周囲の皮膚のたるみが少ないため，ずれによるポケットが発生しにくいが，肉芽組織も同様に周囲から引っ張られる。

腸骨部，特に腸骨稜上部では通常，褥瘡が骨の上縁に位置し，ここで上下に動くためにポケット形成や壊死が起こりやすい。大転子は通常，緊張が強いうえに，筋肉の動きによるずれが生じて治癒しにくい。一方，尾骨部は坐位のみで圧力を受けるために，臥位では創が凹面に存在する（図3）。創が変形しやすいことによって治癒が妨げられることも多く，注意が必要である。また，坐骨部の褥瘡は，深く潰瘍が進展することが多く，凹面の形を呈することが多い。

これらの知識は，褥瘡の薬物療法に関わる薬剤師にとって非常に重要である。なぜなら，外用薬は期待された部位に使われて初めて効果を発揮するからである。翌日の処置の際にガーゼがずれていたり，創面の物理的因子のために密着が妨げられている場合には，薬物療法が適切に行われていないことを意味する。

褥瘡による感染

褥瘡の診療，特に深い褥瘡の診療で留意すべきことは感染症である。皮膚のバリア機構の破たん，壊死組

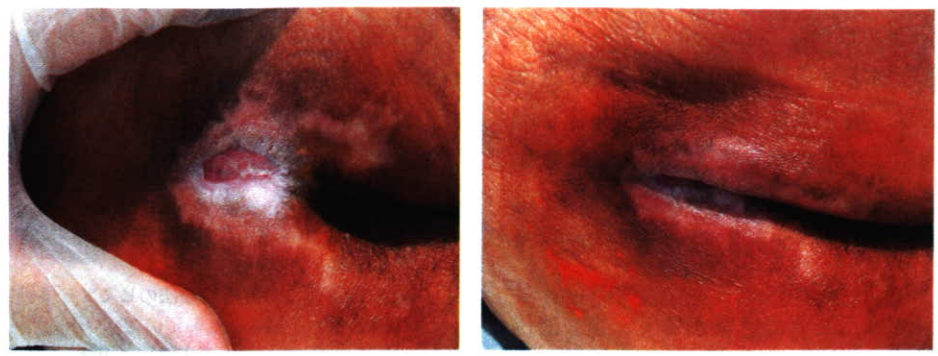


図3　凹面にある尾骨部褥瘡

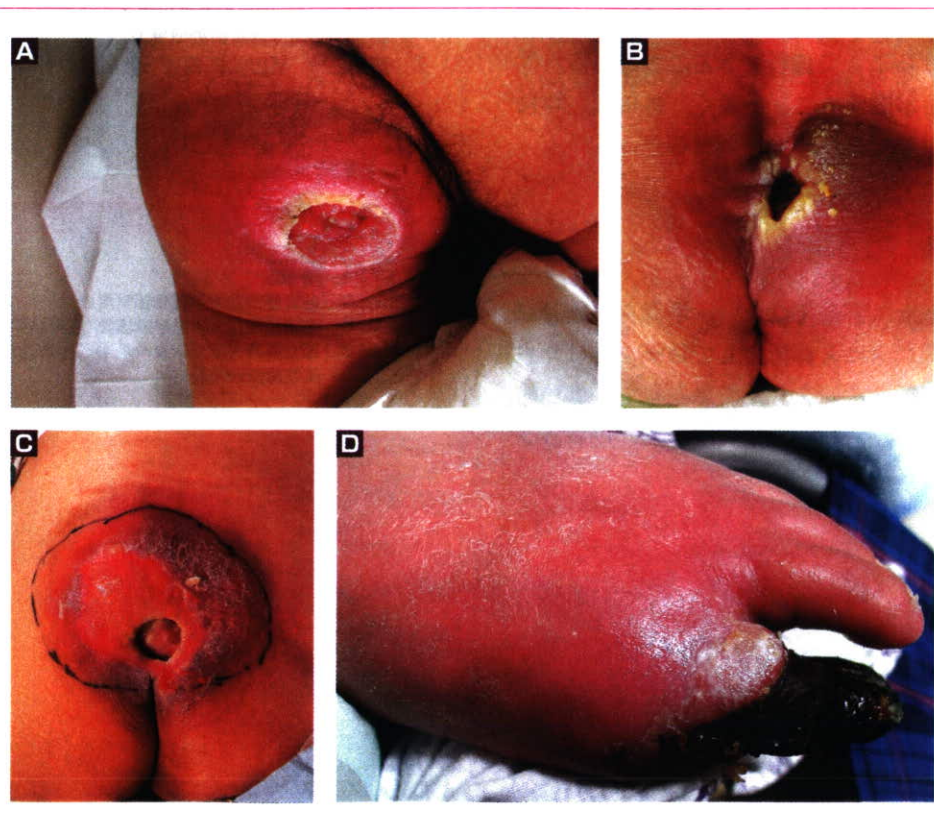


図4　褥瘡に合併した感染症

織の存在，虚血による嫌気的環境，臀部に好発することからなる感染リスクなど，褥瘡の感染の種類を確実に鑑別する必要性がある。細菌感染症は，以下のように考えて分類すると，実地臨床上対応しやすい。

①びまん性の脂肪組織の炎症：蜂窩織炎（図4A）
②組織の壊死を伴う炎症：壊死性筋膜炎と類症（図4B）
③膿瘍形成性炎症（図4C）
④骨髄炎（図4D）

⑤肉芽表面に起こる不顕性感染：いわゆるバイオフィルム感染

このうち，迅速な対応が必要なのは②である。褥瘡に合併した感染では，壊死組織が嫌気的環境を作り出し，さらに嫌気性菌との混合感染によって全身症状を伴って急速に進行する。デブリードマンによる壊死組織の除去と，嫌気性菌をターゲットとした抗菌療法（ペネム系薬剤など）が必要である。

①は通常，ブドウ球菌などの感染が多く，第2世代のセフェム系抗生剤で治療可能である。③も時に遭遇するが，徹底した洗浄が効果的である場合も多い。また，時に切開を必要とする。④に対しては原則的に腐骨除去が必要である。その場合，周囲の腱などの組織を外科的に除去することで施行できることもある。⑤は炎症性の病態を示唆することが多く，外用薬，特に基剤の使い方が重要である。

鑑別診断と周囲の皮膚病変

褥瘡の診断は決して容易ではない。筆者らの施設において，皮膚科医以外から褥瘡として依頼を受けた患者のうち，褥瘡以外の診断をした疾患として，単純疱疹，リウマチ性血管炎，刺激性接触皮膚炎，皮膚カンジダ症，熱傷（Ⅲ度）を経験した。また，周囲にうっ滞性皮膚炎，刺激性接触皮膚炎，カンジダ症をしばしば合併するので，その鑑別も重要である。

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Evidence That PI3K, Rac, Rho, and Rho Kinase Are Involved in Basic Fibroblast Growth Factor-Stimulated Fibroblast–Collagen Matrix Contraction

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Abstract Fibroblast–collagen matrix contraction has been used as a model system to study how cells organize connective tissue. Previous work showed that lysophosphatidic acid (LPA)-stimulated floating collagen matrix contraction is independent of Rho kinase while platelet-derived growth factor (PDGF)-stimulated contraction is Rho kinase-dependent. The current studies were carried out to determine the signaling mechanisms of basic fibroblast growth factor (bFGF)-stimulated fibroblast–collagen matrix contraction. Both bFGF and LPA promoted equally collagen matrix contraction well. Three different inhibitors, LY294002 for phosphatidylinositol-3-kinase (PI3K), C3 exotransferase for Rho and Y27632 for Rho kinase, suppressed the bFGF-stimulated fibroblast–collagen matrix contraction. With bFGF stimulation, fibroblasts spread with prominent stress fiber network formation and focal adhesions. In the presence of Rho kinase inhibitor, focal adhesions and stress fibers were mostly lost. We demonstrated that bFGF stimulation for fibroblast caused transient Rac and Rho activation but did not activate Cdc42. In addition, bFGF enhanced fibroblast migration in wound healing assay. The present study implicates PI3K, Rac, Rho, and Rho kinase as being involved in bFGF-stimulated collagen matrix contraction. The elucidation of bFGF-triggered signal transduction may be an important clue to understand the roles of bFGF in wound healing. J. Cell. Biochem. 102: 1290–1299, 2007.

Key words: wound healing; basic fibroblast growth factor (bFGF); lysophosphatidic acid; Rho; Rac; phosphatidylinositol-3-kinase

Recently, human recombinant basic fibroblast growth factor (bFGF) has been available for the treatment of non-healing skin ulcer [Robson et al., 1992]. Although its significant effects for cutaneous wound healing have been already confirmed in daily clinical practice, little is known about its biological effects on dermal fibroblasts. Fibroblasts synthesize extracellular matrices and organize connective tissues during matrix development and in response to injury. The motile mechanisms by which fibroblasts remodel the extracellular matrices during the morphogenetic processes have been studied using cultured cells in three-dimensional collagen matrices [Tomasek et al., 2002; Grinnell, 2003].

As fibroblasts exert force on and move collagen fibrils of the matrix, collagen concentration can be measured as a decrease in the diameter of free matrices or as a decrease in the height of restrained matrices. During contraction of restrained matrices, collagen fibrils become oriented in the same plane as the restraint, and then mechanical loading develops. In floating matrices, on the other hand, contraction occurs without collagen fibrils developing a particular orientation, and the matrix remains mechanically unloaded [Brown et al., 1998; Tranquillo, 1999; Cukierman et al., 2002; Tomasek et al., 2002; Grinnell, 2003].

The signaling mechanisms in fibroblasts which direct them to regulate collagen matrix contraction depend on whether the cells are mechanically loaded or unloaded at the time that contraction is initiated as well as on the growth factor added to initiate contraction. For example, stimulation of fibroblasts with lysophosphatidic acid (LPA) but not with

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