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特集 Special Part 2

外用薬の特性に基づいた選択と使い方

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はじめに

医療保険制度や介護保険制度の見直しにより，入院期間の短縮，在宅介護へのシフトなど医療・介護環境が大きく様変わりしている。2007年4月からは，保険薬局が新たに医療機関として認められた。すでに在宅医療では医師や看護師，ヘルパーなどの業務の確立が進んでおり，新規参入となる薬剤師がどのように足場を築いていくかが大きな課題である。薬剤師としての職能や役割が他職種に認知されるような試みが重要となる。

高齢化が進み始めた時期に浮上してきたのが「褥瘡」という病気であり，これまで医療者側の認識が不足していた分野だけに，適切な対応が求められる。しかし褥瘡は，病院のみならず在宅においても，医師や看護師だけでは決して十分な対応ができていない実情にある。在宅医療の普及に伴い，褥瘡はますます大きな問題としてクローズアップされていくことが予想され，患者・家族のためのチーム医療に，薬局・薬剤師の関わりが重要となってくる。本稿では「科学的根拠に基づく褥瘡局所治療ガイドライン」（日本褥瘡学会）に採用された筆者の理論を基に，外用療法の考え方を述べる。

外用薬の剤形

褥瘡に用いられる外用薬にはさまざまな種類があり，病態による使い分けが基本となる。剤形には粉末や軟膏，噴霧剤があり，粉末製剤では，粉末を創部に直接散布するタイプや，粉末を溶解液に溶解し，その外用液をガーゼに浸して創部に当てるタイプがある。ほかには，新しい噴霧式のスプレー剤や，古くから使われている軟膏がある。外用薬の多くは軟膏の剤形をとっており，それぞれの持つ特性を正しく理解することが必要である。粉末製剤や噴霧剤では，浸出液の量など適切な湿潤環境を保持できるように，ガーゼやフィルム材の併用が必要となる。どの外用薬を使用する場合でも，創の湿潤状態を把握し適切に保持する点は共通している。

軟膏基剤の成分

外用薬に関して，薬剤師として知っておくべきことがある。それは，軟膏基剤（以下，基剤）についてである。

軟膏は微量の薬効成分が含有され，その薬効成分の薬理作用が治療目的に利用されている。しかし，軟膏の約99％が基剤で占められていることを忘れてはならない。つまり，薬理作用に基剤の効果が大きく影響することは無視できないのである[1]。軟膏の選択指標としては，薬効成分だけでなく，使用されている基剤の特性を理解することも，"創面の湿潤状態に合わせて外用療法を行う"うえで重要である。実際，薬効成分から選んでも，その基剤の特性が創の湿潤状態に合うとは限らないため，基剤の特性も選択肢に含まれる。基剤は表1のように分類され，基本的な構成成分は水や油である。この基剤が創の保湿，水分の補給，浸出液の吸収に関係し，結果的には湿潤環境に影響を与える。

すなわち，油性基剤や水分含有量の少ない乳剤性基剤では保湿性，水溶性基剤では浸出液の吸収性，水分含有量の多い乳剤性基剤では水分の補給などの特性を

表1 外用薬の基剤による分類

創の浸出液	分類		基剤の種類	外用薬(代表的な製品)	水分含有率	水分吸収率
少ない(水分補給・保湿が必要)	乳剤性基剤	水中油型(O/W)	親水軟膏，バニシングクリーム	オルセノン軟膏	73%	—
				ゲーベンクリーム	67%	—
		油中水型(W/O)	吸水軟膏，コールドクリーム，親水ワセリン，ラノリン	ソルコセリル軟膏	25%	—
				リフラップ軟膏	21%	—
	油脂性基剤	鉱物性	白色ワセリン，プラスチベース	プロスタンディン軟膏	—	—
		動植物性	単軟膏，亜鉛華軟膏	亜鉛華軟膏	—	—
	懸濁性基剤		ハイドロゲル基剤	ソフレットゲル	—	—
			FAPG基剤		—	—
	水溶性基剤(吸水性)		マクロゴール軟膏	アクトシン軟膏	—	—
				ブロメライン軟膏	—	—
				テラジアパスタ	—	—
			マクロゴール軟膏(+白糖)	ユーパスタ	—	76%
			マクロゴール軟膏(+ビーズ)	カデックス軟膏	—	370%
多い(浸出液の吸収が必要)			マクロゴール400(+ビーズ)	デブリサン(ペースト)	—	300%

〔2)より〕

持つため，創の湿潤状態を考慮する必要がある。創の湿潤状態に合わない基剤を選択した場合，薬効が得られないばかりか，悪化することさえある。軟膏はその薬効成分の種類や薬理作用のみが注目されがちであるが，選択する際にはどの基剤が使われているかを知ることが大切である。

例えば，壊死組織の付着した浸出液の少ない創に対してブロメライン軟膏を使用した場合には，基剤の浸出液の吸収性が災いし，ほとんど効果が得られない。それは，ブロメラインが酵素であり，水分のないところでは酵素が活性化しないからである。また，浸出液の多い清浄化された肉芽組織に対してオルセノン軟膏を使用することは，水分量の多い基剤特性から創に浮腫や感染をもたらす可能性が高くなり，肉芽形成が円滑に進まないことがある。このように，軟膏基剤は浸出液の量や湿潤環境の程度に深く関係するファクターとなる。

褥瘡治癒に関与する局所環境因子

創傷治癒理論によると，①適正な湿潤環境の保持，②壊死組織の存在，③感染の有無，④細胞増殖因子の量，⑤酸素濃度の高低，⑥弱酸性のpH，⑦適切な保温――という7項目の局所環境因子が治癒に関係している(図1)。中でも，①が「細胞増殖に適した湿潤状態を保つ」，②が「清浄化によって壊死組織が存在しない創にする」，③が「感染兆候がなく，また不顕性感染(著明な感染兆候のない白っぽい膜で覆われたような状態)がない状態にする」，④が「細胞増殖を活性化するために必要なFGFなどの増殖因子を多く供給する」といった理由から，重要と思われる。

一般的に使われている湿潤環境という用語では，この①を用いている。湿潤環境は，湿潤状態が過剰であっても不足気味であっても肉芽形成に悪影響を与え

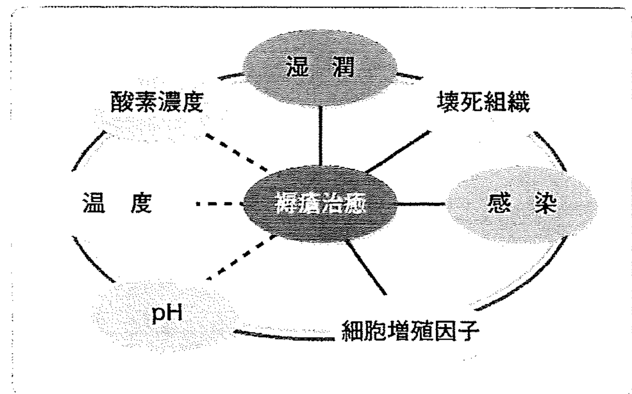

図1 創傷治癒に関与する局所環境因子

る。適正な湿潤状態とは，創面水分量が約60％の状態をいい，水分計（モイスチャーチェッカー：スカラ社，など）で簡便に測定することができる。このように褥瘡の水分量が多過ぎるときは，60％を超える余分な水分を吸収し，また60％を下回るときは水分を補い，水分量を引き上げる必要がある。この水分の吸収と供給に基剤が大きな役割を担っている。

基剤の効果

基剤は軟膏を製剤する際に必ず用いられ，この基剤がなくては，軟膏としての形態を維持できない。しかし，主薬の安定性や放出性を重要視するあまり，基剤の効果が創に与える影響についてはあまり研究されていない。

創傷に対しては，古来から木の皮や葉，樹液や動物の油などが創を覆うドレッシング材として利用されていた。創部を覆うことが痛みを軽減し，治癒に関係すると考えられていたからである。湿潤や乾燥の保持など，時代によって利用する目的は変化してきた。現在，ドレッシング材として市販されている製品もそのことを利用しており，基剤も同じである。そう考えると創の治癒に影響することは，ごく当然のことである。

一般に，褥瘡の治癒過程は壊死組織除去や肉芽形成，上皮化という段階を通過するが，それぞれの段階で基剤の効果が要求される。

壊死組織除去の過程では，浸出液の多いときは水溶性基剤によって創部の浸出液を吸収し，浮腫を抑制することで感染の発症を抑える。浸出液の少ないときは水分を補うために，水分含有量の多い乳剤性基剤によって壊死組織を浮き上がりやすくする（図2）。

肉芽形成過程では，前述の湿潤環境が特に重要となるが，湿潤の程度は適正でなければならない。浸出液が多いときは水溶性基剤を利用して吸収し，浮腫を軽減することで肉芽形成を促進する。また，浸出液が少ないときは，創へ水分を供給するために，水分含有量の多い乳剤性基剤で湿潤環境を形成する。

肉芽が十分に形成した上皮化の過程では，浸出液の多いときは上皮化に必要な湿潤状態を作るために水溶性基剤を用いることで上皮化が促される。浸出液が少ないときは，水分含有量の多い乳剤性基剤で不足した水分を補給することにより，上皮化に適した湿潤状態が作られ，上皮化が促進される。適正な湿潤状態の場合は，保湿効果のために油脂性基剤や油分の多い乳剤性基剤を用いる。

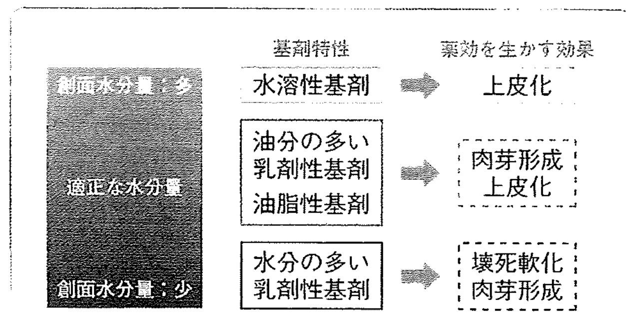

図2　基剤の特性による使い分け

基剤と湿潤環境

褥瘡の局所環境における湿潤状態は，肉芽形成や上皮化の段階で図3のような過程をたどると考えられる。図3は，治療開始当初に浸出液が多く，円滑に治癒過程が進行した場合を想定しているが，浸出液の少ない場合においても肉芽形成に必要な湿潤状態を保持することを表している。適正水分量は肉芽面において肉芽形成に適した湿潤状態と考え，過剰な湿潤（Wet）状態や湿潤状態が不十分（Dry）な場合において，肉芽形

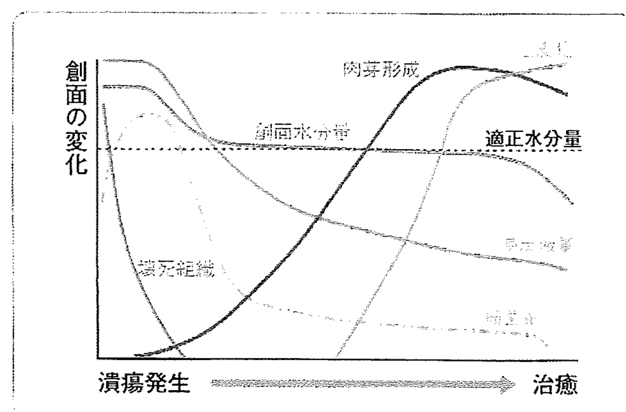

図3　創面の水分コントロール

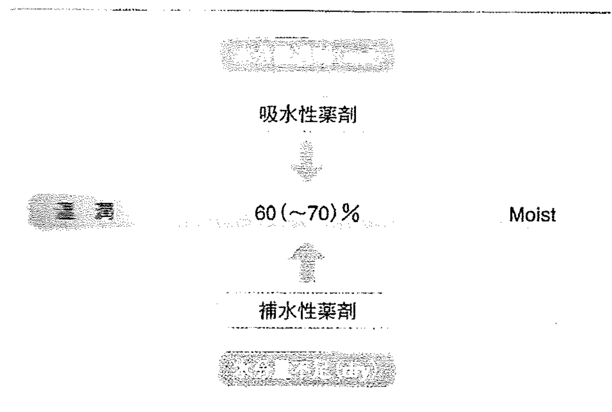

図4　薬剤による水分量のコントロール

[illegible]に適した湿潤状態に補正することが必要とされる（図4）。

例えば，浸出液が多い場合は水溶性基剤のマクロゴール軟膏，乾いた創には水分量の多い乳剤性基剤，湿潤保持には白色ワセリンのような油脂性基剤や，親水軟膏のような水分量の少ない乳剤性基剤を選択の基本とする。実際の臨床では，必ずしもこの図4の流れ通りにはならない場合が少なくないと考えられるが，基本的な概念として必要である。湿潤環境は，褥瘡治癒に関与する局所環境因子として配慮すべきとされており，基剤の選択もおのずと重要性を増している。軟膏では，基剤が湿潤環境に影響し，薬効成分は壊死組織の除去，肉芽形成や上皮形成の促進，あるいは感染制御などの効果を生かすことで有用性を発揮する。

褥瘡の治癒を妨げる要因

創が治癒するためには，先に述べた局所の適正な湿潤環境が必要であるが，高齢者では皮膚のたるみによって創の変形が絶えず発生する。創の変形は，たるんだ皮膚ではわずかな外力で起こり得る。その変形から創面に圧迫や摩擦が起こり，正常かつ円滑な細胞の増殖を妨げることになる。

骨の突出部位などのずれによる褥瘡が発生する場合では，創および創周囲の固定（fixation）（図5）が必要となる。具体的には，テープ類や固着性のドレッシング材を利用して創の変形を防ぐことが，治癒を促すために有効となる。また，創の変形により外用薬が創内にとどまらず，効果が発揮できない状態になることから，結果的に薬剤が効かないと誤解されることもある。薬剤が外力により創内にとどまりにくいときは，創を外力から保護するため，レストンパッドなどクッション性のある材料を用いることが有効な場合がある。外力などにより創面が悪影響を受けたり，薬剤が創内に停留できない状況を回避することが，薬物療法を有効に進めるうえで重要である。

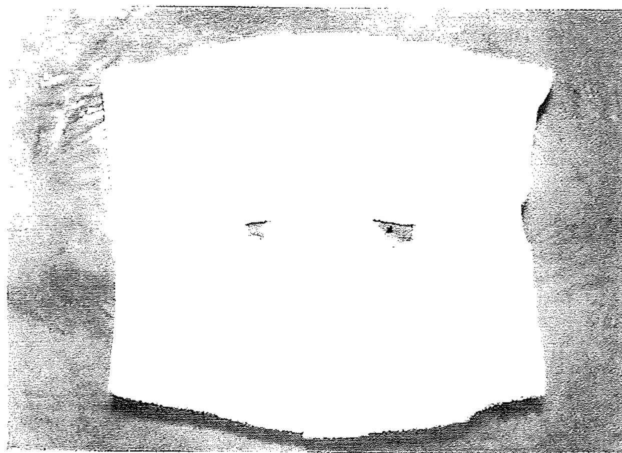

図5　fixation

褥瘡治療に用いる外用薬

表2は，DESIGN分類（26頁，「褥瘡ケアの実際―医師の立場から」参照）に基づき，薬効成分の作用から分類した外用薬一覧である。深さによっても使用する外用薬は異なり，浅い褥瘡と深い褥瘡とでは病態や治療目的が異なる。浅い褥瘡では皮膚の再生で治癒し，創の保護と湿潤環境の保持が重要となる。また，深い褥瘡では瘢痕形成で治癒し，壊死組織の付着や感染の有無，浸出液の量，ポケットの有無などの病態から薬効成分を選択し，それが軟膏であれば基剤を考慮する。軟膏以外の外用薬であれば，湿潤環境を保持するためにドレッシング材を併用するなどの配慮が求められる。

DESIGN分類は，褥瘡の病態を把握するツールとしては，これまでのツールに比べ評価項目は増えたが，いまだ不十分である。今後，さらに的確に病態を把握す

表2 DESIGN分類と主な外用薬

E→e	浸出液	カデックス軟膏，デブリサン，ユーパスタ
S→s	大きさ	亜鉛華軟膏，アクトシン軟膏，アズノール軟膏，ソフレットゲル，ソルコセリル軟膏，フィブラストスプレー，プロスタンディン軟膏，リフラップ軟膏
I→i	感染	イソジンゲル，カデックス軟膏，ゲーベンクリーム，フランセチン・T・パウダー，ヨードホルムガーゼ
G→g	肉芽組織	アクトシン軟膏，オルセノン軟膏，ソルコセリル軟膏，ソフレットゲル，フィブラストスプレー，プロスタンディン軟膏，リフラップ軟膏
N→n	壊死組織	エレース，カデックス軟膏，ゲーベンクリーム，デブリサン，フランセチン・T・パウダー，ブロメライン軟膏
P→−	ポケット	オルセノン軟膏，フィブラストスプレー，ユーパスタ

〔3）より〕

るために，詳細な評価項目が開発中となっている。また，それに適した外用療法の開発にも取り組まれている。

急性期褥瘡における外用薬の選択

褥瘡発生から約1～3週間は「急性期」と呼ばれ，褥瘡の局所病態が不安定であることが多いため，それ以降の病態が安定した慢性期褥瘡とは区別する。急性期では，湿潤環境を保ち，創面を保護することが基本とされ，白色ワセリンなどの油脂性基剤を選択することが多い。ワセリン基剤を使用しガーゼで覆う場合は，浸出液の乾燥によってガーゼが創面に固着することがあるため，ワセリン基剤を厚めにするか，ポリウレタンフィルムを併用するなどの配慮をする。また，感染を合併した場合には，非特異性抗菌活性を有する銀を含有し，かつ高い浸透性を持つ，水分含有率の多い乳剤性基剤を用いたゲーベンクリームを選択する。この場合，特異的抗菌活性を有する抗生物質含有軟膏は，耐性菌を生じる危険性から使用を避ける。

慢性期褥瘡における外用薬の選択

慢性期褥瘡については，発赤，水疱，びらんから真皮までの浅い潰瘍を「浅い褥瘡」とし，真皮を超えて深部組織にまで達する「深い褥瘡」とは分けて考える。これは治癒形態に大きな違いがあるからである。病態別外用薬の選択基準を褥瘡局所治療ガイドラインより，水分の供給に影響を与える外用薬から順に紹介する。

1．浅い褥瘡の場合

(1) 発赤には

創面保護を目的として，透明性の高いドレッシング材での被覆を第1選択とするが，外用薬は白色ワセリンを基剤に用いた軟膏を塗布し，創面の水分量を高め保湿する。

(2) 水疱には

水疱は破らずそのままで保護するが，破れたときには，びらん・浅い潰瘍の対応を行う。緊満している場合は穿刺する。

(3) びらん，浅い潰瘍には

浸出液など創面水分量を考慮したうえで，創面が観察可能なドレッシング材の被覆か，ワセリン基剤による創面保護，または上皮形成促進作用を有する以下の薬剤を用いる。

- 水分含有率の低い乳剤性基剤：油中水型〔リフラップ軟膏，リフラップシート，レフトーゼ貼付剤（塩化リゾチーム）〕
- 油脂性基剤：白色ワセリン〔アズノール軟膏，ハスレン軟膏（アズレン），亜鉛華軟膏，亜鉛華単軟膏，ウイルソン軟膏，サトウザルベ，サトウザルベ10，酸化亜鉛（酸化亜鉛），プラスチベース〔プロスタンディン軟膏（プロスタグランジン E_1）〕
- 水溶性基剤：マクロゴール〔アクトシン軟膏（ブクラデシン）〕

2．深い褥瘡の場合

(1) 壊死組織を除去するには（N→n）

浸出液など創面水分量を考慮したうえで壊死組織除去作用を有する以下の薬剤を用いる。

- 粉末製剤〔エレース（フィブリノリジン・デオキシリボヌクレアーゼ配合剤）〕
- 水分含有率の高い乳剤性基剤：水中油型〔ゲーベンクリーム（スルファジアジン銀）〕
- 粉末製剤〔フランセチン・T・パウダー（硫酸フラジオマイシン・トリプシン）〕
- 水溶性基剤：マクロゴール〔ブロメライン軟膏（ブロメライン）〕
- 水溶性基剤：マクロゴール＋ポリマービーズ〔デブリサン（デキストラノマー），カデックス軟膏（カデキソマーヨウ素）〕

(2) 肉芽形成を促進するには（G→g）

浸出液など創面水分量を考慮したうえで肉芽形成促進作用を有する以下の薬剤を用いる。

- 水分含有率の高い乳剤性基剤：水中油型〔オルセノン軟膏（トレチノイントコフェリル）〕
- 水分含有率の高いゲル基剤：ハイドロゲル〔ソフレットゲル（アルミニウムクロロヒドロキシアラントイネート），ソルコセリルゼリー（幼牛血液抽出物）〕
- 水分含有率の低い乳剤性基剤：油中水型〔リフラップ軟膏，リフラップシート，レフトーゼ貼付剤（塩化リゾチーム），ソルコセリル軟膏（幼牛血液抽出物）〕
- 液状スプレー剤〔フィブラストスプレー（トラフェルミン）〕
- 油脂性基剤：プラスチベース〔プロスタンディン軟膏（プロスタグランジンE_1）〕
- 粉末製剤〔イサロパン（アルミニウムクロロヒドロキシアラントイネート）〕
- 水溶性基剤：マクロゴール〔アクトシン軟膏（ブクラデシン），アラントロックス軟膏，アルキサ軟膏（アルミニウムクロロヒドロキシアラントイネート）〕

(3) 創を縮小するには（S→s）

浸出液など創面水分量を考慮したうえで創の縮小作用を有する以下の薬剤を用いる。

- 液状スプレー剤〔フィブラストスプレー（トラフェルミン）〕
- 水分含有率の高いゲル基剤：ハイドロゲル〔ソフレットゲル（アルミニウムクロロヒドロキシアラントイネート），ソルコセリルゼリー（幼牛血液抽出物）〕
- 水分含有率の低い乳剤性基剤：油中水型〔リフラップ軟膏，リフラップシート，レフトーゼ貼付剤（塩化リゾチーム），ソルコセリル軟膏（幼牛血液抽出物）〕
- 油脂性基剤：白色ワセリン〔アズノール軟膏，ハスレン軟膏（アズレン），亜鉛華軟膏，亜鉛華単軟膏，ウイルソン軟膏，サトウザルベ，サトウザルベ10，酸化亜鉛（酸化亜鉛）〕
  プラスチベース〔プロスタンディン軟膏（プロスタグランジンE_1）〕
- 粉末製剤〔イサロパン（アルミニウムクロロヒドロキシアラントイネート）〕
- 水溶性基剤：マクロゴール〔アクトシン軟膏（ブクラデシン），アラントロックス軟膏，アルキサ軟膏（アルミニウムクロロヒドロキシアラントイネート）〕

(4) 感染を抑制するために（I→i）

浸出液など創面水分量を考慮したうえで感染抑制作用を有する以下の薬剤を用いる。

- 水分含有率の高い乳剤性基剤：水中油型〔ゲーベンクリーム（スルファジアジン銀）〕
- 水溶性基剤：マクロゴール〔イソジンゲル，ネオヨジンゲル，ネグミンゲル（ポビドンヨード）〕
- 粉末製剤〔フランセチン・T・パウダー（硫酸フラジオマイシン・トリプシン）〕
- 水溶性基剤：マクロゴール＋白糖〔ユーパスタ，ソアナースパスタ，ドルミジンパスタ，イソジンシュガーパスタ，スクロードパスタ，ネグミンシュガー軟膏，ポビドリンパスタ（ポビドンヨードシュガー）〕
- マクロゴール＋ポリマービーズ〔カデックス軟膏（カデキソマーヨウ素）〕
- 粉末製剤〔カデックス，デクラート（カデキソマーヨウ素）〕

(5) 浸出液を吸収するために（E→e）

浸出液量など創面水分量を考慮したうえで浸出液吸収作用を有する以下の薬剤を用いる。

- 水溶性基剤：マクロゴール＋白糖〔ユーパスタ，ソアナースパスタ，ドルミジンパスタ，イソジンシュガーパスタ，スクロードパスタ，ネグミンシュガー軟膏，ポビドリンパスタ（ポビドンヨードシュガー）〕
- マクロゴール＋ポリマービーズ〔デブリサン（デキストラノマー），カデックス軟膏（カデキソマーヨウ素）〕

(6) ポケット形成をなくすために（P→−）

ポケットを消失させるために創内の壊死組織の清浄化，浸出液の抑制，肉芽形成などの条件が整っていることが必要であり，以下の薬剤を用いる。

- 水分含有率の高い乳剤性基剤：水中油型〔オルセノン軟膏（トレチノイントコフェリル）〕
- 液状スプレー剤〔フィブラストスプレー（トラフェルミン）〕
- 水溶性基剤：マクロゴール＋白糖〔ユーパスタ，ソアナースパスタ，ドルミジンパスタ，イソジンシュガーパスタ，スク

ロードパスタ，ネグミンシュガー軟膏，ポビドリンパスタ（ポビドンヨードシュガー）〕

おわりに

高齢社会が進み，在宅での介護・医療へと環境が大きく変わりつつあるが，受け入れる体制が整っていないのが実情である。そのために適切な介護・医療を受けられなかったり，提供できない状況が多くみられる。システムの構築もさることながら，チームとしての連携や活動が行われる状況を1日も早く作り上げる必要があり，薬局も医療機関として，医薬品を取り扱うだけでなく，積極的な貢献が求められる。褥瘡治療への関与はその布石になり得るだろう。

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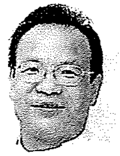
PROFILE

古田 勝経



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2004年　国立長寿医療センター薬剤部副薬剤部長
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ORIGINAL ARTICLE

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.

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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

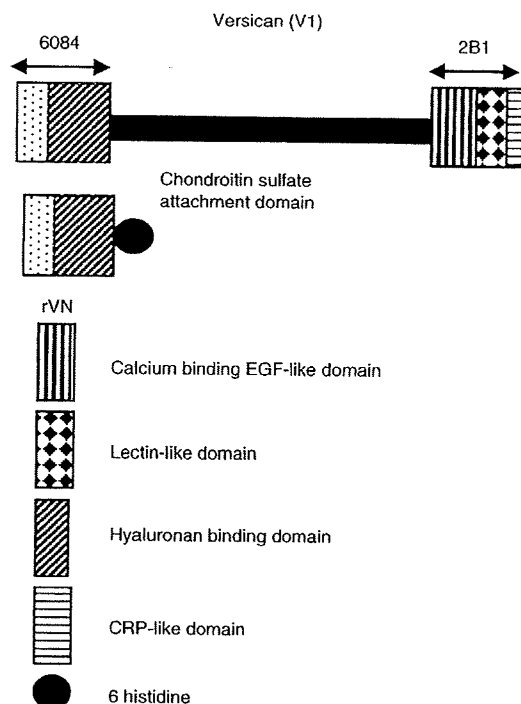

Figure 1. Schematic representation of human versican (V1) and the recombinant protein used in this study. A recombinant versican fragment covering the HABR was designed and designated rVN. The nomenclature of each domain and the antibody recognition sites are indicated.

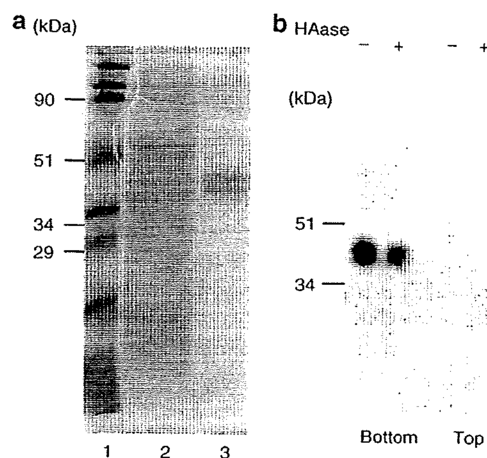

Figure 2. Expression and characterization of the HABR of human versican. (a) Conditioned media from 293 cells expressing rVN (lane 2) and the purified rVN polypeptide (lane 3) were resolved by 10% SDS–PAGE under non-reducing conditions. The molecular weights of standard marker proteins are indicated (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.

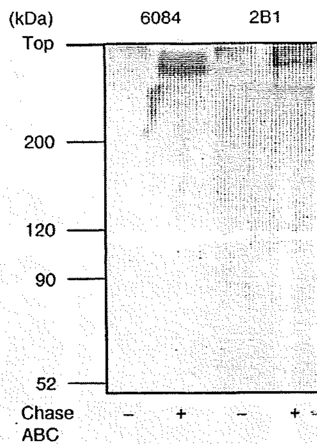

Figure 3. Specificity of polyclonal antibody 6084. Crude conditioned medium from normal skin fibroblasts was incubated with or without chondroitinase ABC (Chase). The samples were resolved by 7.5% SDS–PAGE, blotted onto membranes, and incubated with 2B1 (a mAb against the C-terminus of versican) or 6084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

~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

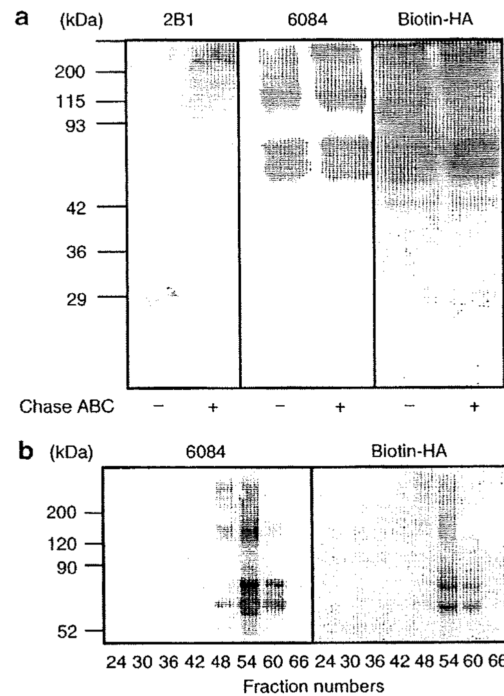

Figure 4. The HABR of versican is a major HA-binding component in skin. (**a**) Normal skin was extracted with 6 M guanidine hydrochloride and resolved by SDS-PAGE. (**b**) To compare between the immunoreactivity of 6084 and the HA-binding affinity, the extract was fractionated using a Sepharose CL-2B 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**.

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

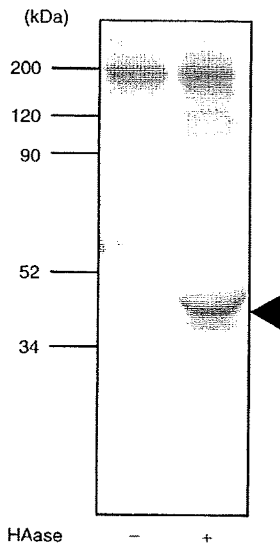

Figure 5. Hyaluronidase treatment releases small amounts of versican fragments. Normal skin pieces were incubated with *Streptomyces* hyaluronidase (+) or buffer alone (–) as described in Materials and Methods. The supernatants were resolved by SDS–PAGE and blotted with polyclonal antibody 6084. 6084-positive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

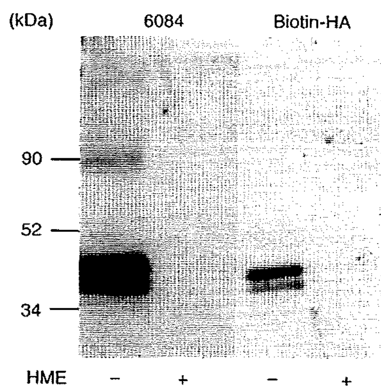

Figure 6. MMP-12 digests rVN and abolishes its reactivity to polyclonal antibody 6084. Purified rVN was incubated with human MMP-12 (+) or buffer alone (–). Aliquots of the samples were resolved by 10% SDS–PAGE, blotted onto membranes, and incubated with 6084 or biotin-conjugated HA. The reactivities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

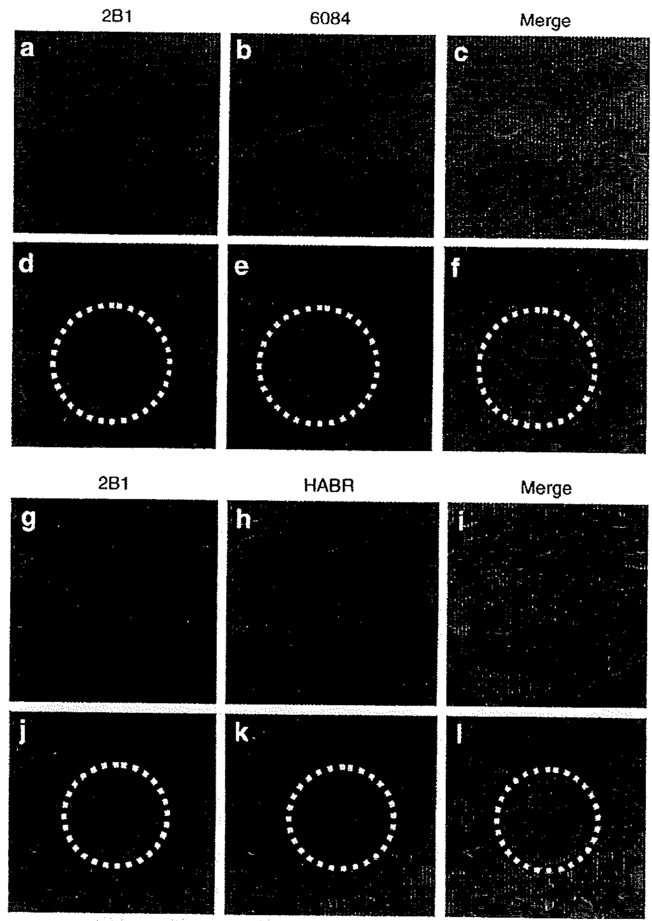

Figure 7. Versican in solar elastosis lesions is unable to bind HA. (a–f; g–l) Double-immunofluorescence staining of two skin regions with 2B1 (red) and 6084 (green) recognizing the C and N termini of versican, respectively, was performed on (a–c; g–i) normal skin and (outlined: d–f; j–l) solar elastosis 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.

Table 1. Kinematic viscosity of versican-bound microfibrils

	Non-treated microfibrils[1] (n=40)	MMP-12-treated microfibrils[1] (n=40)
Kinematic viscosity[2]	$1.263 \pm 0.026\ mm^2/s$	$1.177 \pm 0.021\ mm^2/s$

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

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

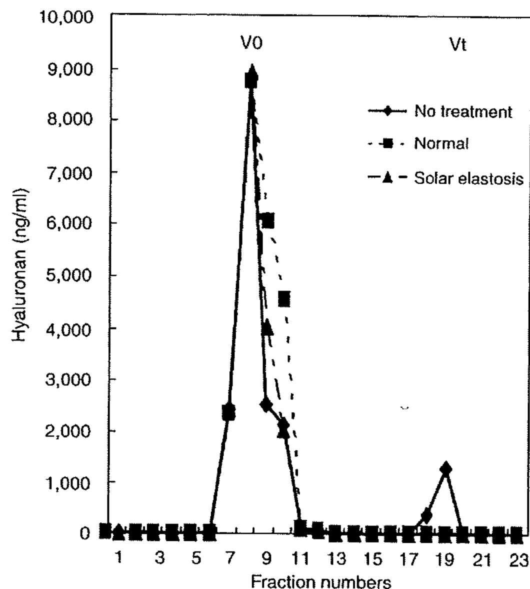

Figure 8. Hyaluronidase activity is not detected in solar elastosis. To exclude the possibility that activated hyaluronidase activity in solar elastosis reduced the accumulation of hyaluronan, skin pieces from normal and solar elastosis skin were minced and incubated with hyaluronan. The treated samples 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 *NheI* 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 *XhoI* restriction site at the 3′-end. The *NheI–XhoI* 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 ($\sim$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 ($\sim$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 ($\sim$9 μg) and rF23 ($\sim$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.

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Versican and Fibrillin-1 Form a Major Hyaluronan-Binding Complex in the Ciliary Body

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Purpose. In this study, biochemistry, molecular biology, immunohistochemistry, and electron microscopy techniques were used to examine whether versican, which is known to bind fibrillin-1, interacts with fibrillin-1 in the ciliary body and vitreous, and whether the versican in this complex binds to hyaluronan.

Methods. The new polyclonal antibodies against the amino and carboxyl termini of versican were raised and characterized. The mRNA expression levels of versican and fibrillin-1 were analyzed by RT-PCR and real-time PCR, and their protein levels were evaluated by Western blot analysis and immunohistochemistry. Isolation of versican bound to fibrillin-1-containing microfibrils from ciliary bodies was performed by extraction studies. Slot-blot analyses and rotary shadowing electron microscopy were applied to identify versican associated with fibrillin-1-containing microfibrils after gel filtration chromatography and density gradient centrifugation.

Results. The newly prepared polyclonal antibodies recognized amino and carboxyl termini of chicken versican. Versican, principally V0 and V1, was found to be securely bound to fibrillin-1-containing microfibrils, forming a major hyaluronan-binding structure in the ciliary nonpigmented epithelium. In addition, Western blot analysis revealed two cleaved complexes, the carboxyl-terminal end of versican bound to fibrillin microfibrils and the amino terminal end of versican bound to hyaluronan in the vitreous body.

Conclusions. Fibrillin-1, versican, and hyaluronan form a unique complex in the ciliary nonpigmented epithelium, and two cleavage products of this complex were shown to exist in the vitreous body. This newly clarified fibrillin-versican-hyaluronan (FiVerHy) complex and its cleavage products may be indispensable for the physiological properties important to the ciliary body and vitreous. (*Invest Ophthalmol Vis Sci.* 2008;49:2870–2877) DOI:10.1167/iovs.07-1488

One of the most important functions of the ciliary body is accommodation.[1–3] The ciliary muscle consists of longitudinal, circular, and radial fibers and contributes to accommodation. The distributions of some extracellular matrix components in the ciliary muscle have already been delineated, via immunohistochemical studies of collagen-I, -III, -IV, and -VI; elastin; fibrillin; fibronectin; and laminin.[4–9] Ciliary zonules arise from the vicinity of the apex of the nonpigmented epithelium of the ciliary processes of the ciliary body and insert into the lens capsule. The zonules anchor the lens to the wall of the eye and transmit accommodation forces from the ciliary muscle to the lens. To perform these physiological functions, the structural properties of these tissues must include both elasticity and strength.

The glycoprotein fibrillin is a major component of recognizable structural elements, designated microfibrils, in various connective tissues, and was reported to be the principal component of the ciliary zonules.[10] Microfibrils play important roles in the strength and elasticity of ocular connective tissues.[11] In the eye, microfibrils are also present in the vitreous body,[12] although their origin is unknown. In humans and chickens, there are three fibrillins (fibrillin-1, -2 and -3), each encoded by a distinct gene.[13] Fibrillin-1 and -2 perform compensatory functions in elastic fiber formation during development. Postnatal tissues require fibrillin-1, since fibrillin-2 is largely restricted to fetal development and to early postnatal life. The microfibrils of the ciliary zonules are almost exclusively composed of fibrillin.[11] Fibrillin-1 is involved in certain systemic connective tissue diseases with ocular manifestations, such as Marfan syndrome.[14] One of the major ocular complications in Marfan syndrome is dislocation of the lens induced by defective fibrillin-1-containing microfibrils in the ciliary zonules.

Versican, also known as PG-M, is a large hyaluronan-binding chondroitin sulfate proteoglycan that belongs to the lectican family.[15] Several different isoforms of versican (V0, V1, V2, and V3) containing different sets of chondroitin sulfate-attachment domains are generated by alternative splicing.[16] Versican, especially the V0 isoform, which has the most chondroitin sulfate attachment sites, plays important roles in retinal differentiation, particularly in the regulation of ganglion cells during retinal development.[17] Versican is widely expressed in many tissues, including the ciliary muscle and trabecular meshwork.[18,19] Although a previous study revealed that versican interacts with fibrillin-1 in some tissues,[20] it is unknown whether versican interacts with fibrillin-1 in the ciliary body and zonules. Chondroitin sulfate proteoglycans and hyaluronan were colocalized in the ciliary zonules and ciliary nonpig-

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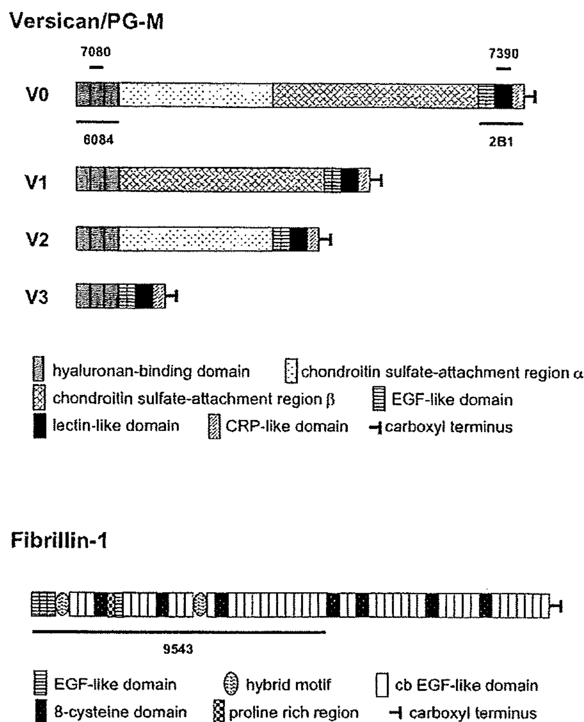

Figure 1. Schematic representation of the alternatively spliced isoforms of human versican (V0, V1, V2, and V3) and fibrillin-1 and the locations of the antigens recognized by antibodies. The epitopes for polyclonal antibody 7080 are located in the amino terminal region of versican containing the hyaluronan-binding domain. The epitopes for polyclonal antibody 7390 are located in the carboxyl-terminal region of versican containing the lectinlike domain. The epitope locations for antibodies 6084, 2B1, and 9543 are also shown. CRP, complement regulatory protein; cb, calcium binding.

mented epithelium of the rat eye,[21] but whether one of these proteoglycans is versican is unknown. Furthermore, previous studies demonstrated the presence of versican[22,23] and microfibrils[12] in the vitreous body, but the origin of these molecules and hyaluronan in the vitreous body has not yet been determined.

In the current study, we demonstrated a strong association of versican with fibrillin microfibrils in the ciliary body and furthermore showed that versican-bound fibrillin microfibrils represent a major hyaluronan-binding structure in this tissue. We also found two cleaved complexes, the carboxyl terminal end of versican bound to fibrillin microfibrils and the amino terminal end of versican bound to hyaluronan in the vitreous body. The recognition of the proposed fibrillin-versican-hyaluronan (FiVerHy) complex is significant for understanding the physiological functions and structural properties of these tissues and may be an important step for elucidating the etiology of some ocular diseases as described in the discussion.

Materials and Methods

To investigate the amino terminus and carboxyl terminus of versican separately, polyclonal antibodies 7080 and 7390, recognizing the amino and carboxyl termini of versican, respectively, were produced by Operon Biotechnology (Tokyo, Japan). The antibodies were raised by immunizing rabbits with the synthetic peptides [245]CYVDHLDGDVFHLTVPS[259] and [3242]GSTLQYENWRPNGPDS[3257] located within the hyaluronan-binding region and the lectin-like domain of human versican (NCBI accession no. NP 004376), respectively (Fig. 1). The polyclonal antibody 6084 against the amino terminus of versican is characterized elsewhere.[24] The polyclonal antibody 9543 against fibrillin-1[25] and the monoclonal antibody 2B1 against the carboxyl terminus of versican[26] are also characterized elsewhere. Peroxidase-conjugated goat IgG fractions against mouse immunoglobulins (IgG, IgA, and IgM) were purchased from Organon Teknika Corp. (Durham, NC). Anti-GST antibody was purchased from GE Healthcare (Little Chalfont, UK). Chondroitinase ABC (protease-free) was purchased from Seikagaku Corp. (Tokyo, Japan).

Peptide Fragments of Chicken Versican

PCR amplifications were performed with a chicken retina cDNA library[27] as a template and oligonucleotide primer pairs. We used the sense primer 5′-GTGATGTATGGAGTTGAGGACACAC-3′ (535-559) and the antisense primer 5′-AGTAGGCATCAAACTTGCTATCTGGG-3′ (1175-1150) to amplify a cDNA encoding the amino terminal of chicken versican. We used the sense primer 5′-CAGGATCCATGCAAAAGTAATCCCTGC-3′ (9907-9933) and the antisense primer 5′-GCGCCTTGAGTCCTGCCACGT-3′ (10830-10810) to amplify a cDNA encoding the carboxyl-terminal of chicken versican. The numbers indicate the nucleotide positions (GenBank accession no. D13542; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). Each primer was designed to contain *Bam*HI and *Xho*I sites. Each purified cDNA was ligated into the pGEX6p-1 vector (GE Healthcare). The ligated constructs were then transformed into *Escherichia coli* BL21 cells (Novagen, Madison, WI) for expression of glutathione *S*-transferase (GST) fusion proteins. Expression was induced by the addition of isopropyl-β-D-thiogalactoside. After lysis of the BL21 cells in PBS (pH 7.5), 1 mM PMSF, 10 mM DTT, 100 mM $MgCl_2$, 1.0 mg/mL lysozyme, and 20 U/mL DNase I, the released GST fusion proteins were purified (GSTrap FF; GE Healthcare). Positive colonies were checked by sequencing.

Western Blot Analysis

To test the binding affinities of the above-mentioned antibodies and biotinylated hyaluronan (b-HA) for versican, conditioned medium from human fibroblasts was concentrated by using *O*-diethylaminoethyl (DEAE)-Sephacel. The preparation of b-HA was described previously.[27] The bound fractions were washed with 0.3 M NaCl, 50 mM Tris-HCl (pH 7.5) and eluted with 4 M guanidine HCl, 50 mM Tris-HCl (pH 7.5). We used the DEAE partially purified sample for Western blot analyses. The eluted fractions were further separated by density gradient centrifugation.[28] The bottom fraction was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 3% to 8% polyacrylamide gels in nonreducing conditions. The separated proteins were electrotransferred to nitrocellulose membranes, blocked with 10% nonfat milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-Tween) for 1 hour and incubated with the antibodies 7080 (diluted 1:5000) and 7390 (1:2000) in PBS-Tween. The antibodies 6084 (1:5000), 2B1 (1:2000), and b-HA (1:500) were used for control staining experiments. After washing with PBS-Tween, the membranes were incubated with horseradish peroxidase (HRP)-conjugated protein A (Zymed, San Francisco, CA) for 7080 and 7390, anti-mouse IgG (Organon Teknika Corp.) for 2B1 and HRP-conjugated streptavidin (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) for b-HA. Finally, the membranes were developed (Western Lightning; PerkinElmer Life Sciences, Inc., Boston, MA) according to the manufacturer's instructions.

RNA Isolation and PCR Amplification

Eyes obtained from newly hatched chickens (White Leghorn) were washed with PBS in sterile conditions. All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The ciliary bodies were carefully dissected from the eyes in cooled PBS under a microscope.[16] Total RNA was obtained from these tissues and used for RT-PCR. For versican, we used specific primers and a cDNA template from newly hatched chicken retinas as

a positive control, since alternatively spliced isoforms of versican had been detected by RT-PCR, performed as described previously.[16] The primers for the RT-PCR amplifications of fibrillin-1 (Table 1) were chosen from the published sequence of chicken fibrillin-1 (NCBI accession no. U88872), and PCR amplification was performed in the same thermal cycler conditions.

The levels of the mRNAs for six versican isoforms in the ciliary body were measured by real-time PCR with the appropriate primers (Table 2). Primers were designed to yield products from each isoform as similar in size as possible. Product sizes were: V0(+), 173 bp; V1(+), 173 bp;V1(−), 134 bp;V2(+), 178 bp;V3(+), 136 bp; and V3(−), 139 bp. The sequence of each primer was selected on computer (Primer3Plus software; Wageningen Bioinformatics Webportal, Wageningen, The Netherlands). Specificity of the primer at the species level was verified by BLAST search of GenBank.

Immunohistochemical Analysis

Newly hatched chicken eyes were fixed in 10% formalin neutral-buffered solution (pH 7.4), for 4 hours at room temperature and embedded in paraffin. Antibodies (6084, 7080, 7390, and 9543) were used at 1:100 dilution in PBS containing 1% normal goat serum. Antibody binding was detected by fluorophore-labeled goat anti-rabbit IgG antibodies (Alexa Fluor 488; Invitrogen, Carlsbad, CA). For the detection of hyaluronan, biotinylated hyaluronic acid–binding protein (b-HABP; Seikagaku Corp.) was incubated instead of the primary antibody. Hyaluronidase (Seikagaku Corp.) was used for negative control of stainings by b-HABP. Nonimmune serum was used instead of the primary antibody as a negative control. For the detection of chondroitin sulfate, biotin-conjugated anti-proteoglycan ΔDi-0S, ΔDi-4S, and ΔDi-6S (Seikagaku Corp.) were used in the incubation instead of the primary antibody. Antibody binding was detected by streptavidin-FITC (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Immunolabeled tissue sections were observed by fluorescence microscope (Axioplan2, Axiophot2, and AxioCam; Carl Zeiss Meditec, Oberkochen, Germany) and analyzed with KS400 (Carl Zeiss Meditec).

Isolation of Versican Bound to Microfibrils from Chicken Ciliary Bodies

To extract versican-bound microfibrils, ciliary bodies from six newly hatched chickens were dissected. Our ciliary body identification procedures were as follows. Initially, we precisely removed the whole cornea and posterior eyeball to leave a "ciliary body ring" under microscopically controlled surgery. The "ciliary body ring" includes an outer scleral part and an inner ciliary body part. Then, we carefully separated the ciliary body from scleral tissues. In this step, however, there may have been some contamination from scleral tissues. As aggrecan is an element of chicken scleral cartilage, we checked for this molecule as a marker of contamination. Homogenized samples in 6 M guanidine HCl, 20 mM Na_2PO_4 (pH 7.8), and 500 mM NaCl for 72 hours at 4°C were centrifuged at 12,000 rpm for 15 minutes in a microcentrifuge (5415R; Eppendorf, Hamburg, Germany). A 200-μL aliquot of the supernatant was treated with chondroitinase ABC and analyzed by SDS-PAGE in a 3% to 8% gel in nonreducing conditions, to allow high-molecular-mass molecules to enter the gel. A separate 2-mL aliquot of the supernatant from the homogenized ciliary bodies was dialyzed against 4 M guanidine HCl and 0.1 M Tris-HCl (pH 8.0) and then fractionated on a 120-mL Sepharose CL-2B molecular sieve column (GE Healthcare Bio-Sciences Corp.) equilibrated in 4 M guanidine HCl and 0.1 M Tris-HCl (pH 8.0), at a flow rate of 0.3 mL/min. Fractions were collected every 3.6 mL (12 minutes/tube). Protein concentrations were determined with a BCA kit (Pierce Biotechnology Inc., Rockford, IL) with bovine serum albumin as the standard.

Slot-blot analyses were performed with a nitrocellulose filter spotted with 100-μL aliquots from odd fractions. The blots were blocked with 10% nonfat milk in PBS-Tween for 1 hour and then incubated with 6084 and 7080 (each diluted 1:5000), 7390 (1:500), 9543 (1:2000), b-HA (1:500), or biotinylated hyaluronan binding protein (b-HABP) (1:500) purchased from Seikagaku Corp. in PBS-Tween. After they were washed with PBS-Tween, the blots were incubated with HRP-conjugated protein A for the detection of antibodies and streptavidin for the detection of b-HA and b-HABP. The development procedure was performed as just described.

Table 1. Primers Used for PCR Amplifications

Primer	Position*	Sequence Chicken Fibrillin-1
FBN-f (S)	2089–2108	CCTAACATCTGTGTCTATGG
FBN-r (A)	2538–2519	AACTGTAATAGGGTTTGGTC

* Nucleotide positions. NCBI accession no. U88872.

Table 2. Primers Used for Real-time RT-PCR Amplifications of Chicken Versican

Primer	Position*	Sequence
V0(+) (S)	4291–4313	CAACCACAAGAGGTGTCTCCTAC
V0(+) (A)	4463–4441	GATTCTGCATCAGTATGGGTCTC
V1(+) (S)	1552–1575	GAAGTGGAGCACACTTACTCTGAA
V1(+) (A)	4505–4482	GAATCCTGCACAGAGTCTGAAGTA
V1(−) (S)	1135–1157	ACAGGCTTTCCTTACCCAGATAG
V1(−) (A)	4463–4441	GATTCTGCATCAGTATGGGTCTC
V2(+) (S)	4291–4313	CAACCACAAGAGGTGTCTCCTAC
V2(+) (A)	9994–9975	AACCTGGCAAACATGTACAG
V3(+) (S)	1552–1575	GAAGTGGAGCACACTTACTCTGAA
V3(+) (A)	9994–9975	AACCTGGCAAACATGTACAG
V3(−) (S)	1135–1157	ACAGGCTTTCCTTACCCAGATAG
V3(−) (A)	9994–9975	AACCTGGCAAACATGTACAG

* Nucleotide positions (GenBank accession no. D13542).

Density Gradient Centrifugation

Aliquots of the void volume fractions 13, 14, and 15 from gel filtration were brought to a density of 1.37 g/mL by the addition of CsCl.[28] A direct gradient was established by centrifugation at 40,000 rpm at 10°C for 45 hours (P90AT Hitachi, Tokyo, Japan). The gradients were partitioned into 12 fractions. Slot blots spotted with 20-μL aliquots of each fraction were created as has been described. To analyze the top fractions, we pooled fractions 1, 2, 3, and 4 and brought them to a density of 1.30 g/mL. A similar centrifugation procedure was performed, and the gradients were partitioned into 10 fractions. Slot blots spotted with 100-μL aliquots of each fraction were created. Samples on the membranes were stained with b-HABP before and after alkaline treatment with 0.2 M NaOH for 2 hours at room temperature.

Rotary Shadowing Electron Microscopy

Samples around the density of 1.29 g/mL, after density gradient centrifugation, were dialyzed against H_2O and rotary shadowed, as described previously.[29]

Vitreous Bodies

Vitreous bodies from six newly hatched chickens were extracted and subjected to further investigation, as described for the ciliary bodies.

Binding Assays

ELISAs were performed on samples after CsCl density gradient centrifugation. Samples were incubated in microtiter wells overnight at 4°C. The wells were then washed three times with PBS-Tween and samples were incubated for 1 hour at room temperature in 9543, 6084, 7390, or b-HA, which were threefold serially diluted, beginning with an initial dilution of 1:50. After the samples were washed three times with PBS-Tween, the samples were incubated for 1 hour at room temperature in HRP-conjugated protein A to detect the antibodies and streptavidin to detect b-HA. After they were washed again, color reaction was

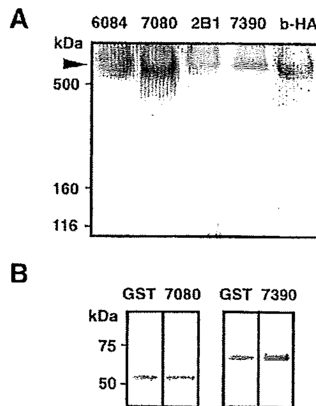

FIGURE 2. Characterization of the newly prepared polyclonal antibodies against versican. (A) Western blot analyses with the anti-versican antibodies 6084, 7080, 2B1, and 7390 on partially purified samples of conditioned medium from human fibroblasts. Versican was detected by all the antibodies examined (*arrowhead*). Versican was also stained by biotin-conjugated hyaluronan (b-HA). (B) Western blot analysis with 7080 and 7390 antibodies on GST fusion proteins matching the amino and carboxyl termini of chicken versican. Both fusion proteins were specifically stained with 7080 and 7390 antibodies.

achieved in the samples with 100 μL of soluble blue peroxidase substrate (Roche Diagnostics, Indianapolis, IN). Color absorbance was determined at 450 nm with a microplate reader (Model 680; Bio-Rad, Hercules, CA).

Reduction Procedures

Western blot analyses were also performed on extracts from chicken vitreous bodies under reducing conditions. Samples were incubated with or without 10 mM dithiothreitol (DTT) for 30 minutes at 37°C. Then, samples were filtered (Microcon YM-100 filter units; Millipore, Billerica, MA) at 12,000 rpm for 10 minutes in a microcentrifuge (5415R: Eppendorf) and subjected to SDS-PAGE in 10% polyacrylamide gels. The separated proteins were electrotransferred to nitrocellulose membranes, blocked with 10% nonfat milk in PBS-Tween for 1 hour, and incubated with 6084 (diluted 1:5000) or 7080 (1:5000).

All experiments were repeated three times, and the results are presented as the mean ± SE.

RESULTS

Characterization of Antibodies against Versican

The new polyclonal antibodies 7080 and 7390 raised against synthetic peptides of human versican were characterized by Western blot analysis of partially purified samples of human versican after chondroitinase ABC treatment. Human versican was specifically detected by 7080 and 7390, which recognize the amino and carboxyl terminus of versican, respectively (Fig. 2A, arrowhead). These antibodies, which potentially react with all forms of versican, detected only the V0 isoform, the alternatively spliced form with the most abundant sites for chondroitin sulfate attachment, according to molecular weight, suggesting that the human fibroblasts used for this experiment dominantly yield the V0 isoform under the present conditions. The previously characterized antibodies 6084 and 2B1, as well as b-HA, were used in positive control experiments for versican detection.

To verify that 7080 and 7390 react with chicken versican, we checked the reactivity of both antibodies with GST fusion proteins matching the amino and carboxyl termini of chicken versican expressed by *E. coli* BL21 cells, respectively, by Western blot analysis. Proteins that reacted with anti-GST antibody were also specifically stained with 7080 and 7390 (Fig. 2B), indicating that both reacted with chicken versican.

Identification of Versican and Fibrillin-1 mRNAs in Chicken Ciliary Bodies

RT-PCR amplifications were performed to detect expression of versican and fibrillin-1 in ciliary bodies from newly hatched chickens. Amplified cDNAs for all four versican isoforms (V0, V1, V2, and V3; data not shown), as well as fibrillin-1 (Fig. 3A), were detected. Although it is hard to rigorously compare relative expression levels among different mRNAs, we measured the levels of the mRNAs for six versican isoforms in the ciliary body by real-time PCR. This analysis showed that V0 and V1 are the major isoforms in the ciliary body (Fig. 3B).

Colocalization of Versican, Fibrillin-1, and Hyaluronan in Ciliary Body

Marked staining in ciliary zonules and diffuse staining in nonpigmented epithelium were observed by using 9543 polyclonal antibodies (Fig. 4, 9543). The 6084 polyclonal antibodies strongly stained the nonpigmented epithelium on ciliary processes (Fig. 4, 6084). Identical stainings as 6084 were observed by 7080 polyclonal antibodies (Fig. 4, 7080). The 7390 polyclonal antibodies similarly stained the nonpigmented epithelium especially and also stained ciliary zonules (Fig. 4, 7390). Similar staining was intensely found in the nonpigmented epithelium by biotin conjugated anti-proteoglycan ΔDi-6S and ΔDi-4S antibodies, but faintly by ΔDi-0S antibody (Fig. 4, ΔDi-6S, ΔDi-4S, and ΔDi-0S). Similar staining was also shown by b-HABP (Fig. 4, b-HABP), and the stained area in the tissues was

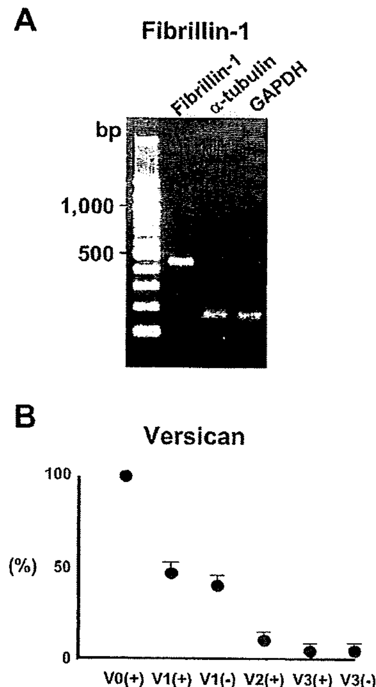

FIGURE 3. PCR analysis for fibrillin-1 and versican in chicken ciliary bodies. (A) RT-PCR analysis for fibrillin-1 expression. The band demonstrates the existence of fibrillin-1. As positive controls, RT-PCR amplifications of α-tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed. (B) Relative expression levels of versican isoforms. The levels of mRNAs for six isoforms of versican in the ciliary body were measured by real-time PCR. The analysis showed that V0 and V1 are the major isoforms in the ciliary body.

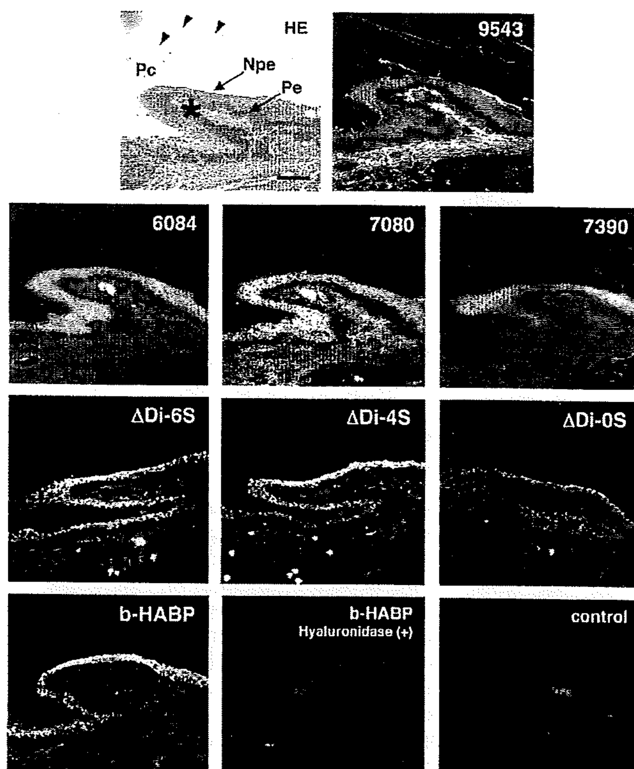

Figure 4. Immunohistochemical study of versican, fibrillin-1, and hyaluronan in chicken ciliary body. Marked staining in ciliary zonules and diffuse staining in nonpigmented epithelium were observed with 9543 polyclonal antibodies against fibrillin-1 (9543). The 6084 and 7080 polyclonal antibodies against amino terminus of versican strongly stained the nonpigmented epithelium (6084 and 7080, respectively). The 7390 polyclonal antibodies against carboxyl terminus of versican stained the nonpigmented epithelium and also stained ciliary zonules (7390). The nonpigmented epithelium that was stained by 6084, 7080, and 7390 polyclonal antibodies was reactive with biotin conjugated HABP (b-HABP). The area was not reactive with biotin-conjugated HABP after hyaluronidase treatment (b-HABP, hyaluronidase (+)). Similar staining was found in the nonpigmented epithelium by biotin conjugated anti-proteoglycan ΔDi-6S and ΔDi-4S antibodies, but the staining was faintly by ΔDi-0S antibody. For negative control of staining, only fluorophore-conjugated goat anti-rabbit IgG antibodies were used, without primary antibody (control). (✱) Ciliary process; Pc, posterior chamber; Npe, ciliary nonpigmented epithelium; Pe, ciliary pigmented epithelium; zonule fiber (*arrowheads*). Bar, 10 μm.

not reactive with b-HABP after hyaluronidase treatment (Fig 4; b-HABP, Hyaluronidase (+)).

Isolation of Versican-Bound Fibrillin-Containing Microfibrils from Chicken Ciliary Bodies

To isolate versican-bound microfibrils from ciliary bodies, chicken ciliary bodies were extracted with 6 M guanidine HCl, a nondegradative procedure for isolating microfibrils.[25] Western blot analysis revealed that fibrillin-1 monomers and the versican core protein, previously reported to be 350 kDa[10] and 550 kDa,[15] respectively, were not present in the extracts, even after chondroitinase ABC digestion, because they were not detected in the materials entering the running gels. Instead, both fibrillin-1 and versican remained in the stacking gel (data not shown), indicating that most of the fibrillin-1 and versican present in the extract was in the form of macroaggregates.

When extracts were sieved on Sepharose CL-2B, the protein concentration profile of the eluted fractions revealed two peaks (Fig. 5A). By slot-blot analysis, the fractions around the void volume (Fig. 5A, bar) reacted with antibodies 6084, 7080, and 7390 against versican, antibody 9543 against fibrillin-1, b-HA, and b-HABP. These results suggest that versican and fibrillin-1 form a complex and that this complex retains the ability to bind hyaluronan (Fig. 5B). Intense reactivity to b-HA was found in the low-molecular-weight fractions from 29 to 33, but these fractions showed only faint reactivity with b-HABP.

Next, the versican- and fibrillin-1-positive fractions around the void volume were separated by CsCl density gradient centrifugation. First, we analyzed a sample with an initial density of 1.37 g/mL (Fig. 6A). Slot-blot analysis revealed that the top fraction reacted with antibodies 6084, 7080, 7390, and 9543. This fraction also reacted with b-HA and b-HABP. To further separate the top fraction, a pooled sample of fractions 1, 2, 3, and 4 was again separated by CsCl density gradient centrifugation with an initial density of 1.30 g/mL. Subsequent slot-blot analyses revealed that the fractions around 1.29 g/mL reacted with antibodies 6084, 7080, 7390, 9543, and b-HA (Fig. 6B). Although there was faint staining of b-HABP in fractions 5 to 8 before alkaline treatment, strong reactivity was observed in fractions 3 to 8 after alkaline treatment.

Visualization and Characterization of Versican-Bound Microfibrils

The fractions around 1.29 g/mL after density gradient centrifugation were visualized by rotary shadowing electron microscopy. Vitreous body samples appeared as a multiple beads and strings structure (Fig. 7, vitreous body), characteristic of microfibrils.[20] By contrast, the morphology of the ciliary body microfibrils was different from that of the vitreous body microfibrils (Fig. 7, ciliary body). The ciliary body microfibrils showed additional projections (Fig. 7, ciliary body, arrows) from the interbead strands compared with the vitreous body microfibrils.

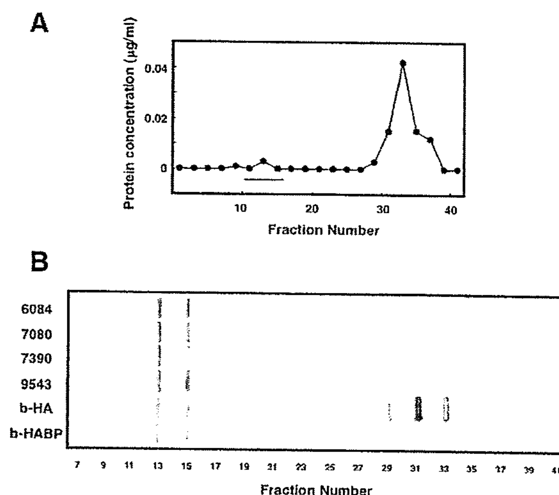

Figure 5. Isolation of FiVerHy complexes from chicken ciliary bodies by gel filtration chromatography. (A) Protein concentration profile of the eluted fractions after gel filtration chromatography. The position around the void volume is indicated (*bar*). (B) Slot-blot analyses for versican and fibrillin-1 with each antibody, and hyaluronan with b-HABP. Fractions in the peak closest to the void volume showed positive reactions with all the antibodies against versican and fibrillin-1, and b-HABP, suggesting that versican, fibrillin-1, and hyaluronan form macroaggregates in the ciliary body. Intense reactivity toward b-HA was observed in the low-molecular-weight fractions from 29 to 33, which contained versican but not fibrillin; however, these fractions showed only faint reactivity with b-HABP.