Versican Is Expressed in the Proliferating Zone in the Epidermis and in Association with the Elastic Network of the Dermis

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Abstract. The expression of the large chondroitin sulfate proteoglycan versican was studied in human adult skin. For this purpose, bacterial fusion proteins containing unique portions of the versican core protein were prepared. Polyclonal antibodies against the fusion proteins specifically reacted with versican from a proteoglycan fraction of MG63 osteosarcoma cells. In immunohistochemical experiments, the affinity-purified antibodies localized versican in the *stratum basale* of the epidermis, as well as in the papillary and reticular layers of the dermis. An apparent codistribution of versican with the various fiber forms of the elastic network of the dermis suggested an association of versican with microfibrils. Both dermal fibroblasts and keratinocytes expressed versican in culture during active cell proliferation. In line with the observation that versican is absent in the suprabasal layers of the epidermis where keratinocytes terminally differentiate, culture conditions promoting keratinocyte differentiation induced a down-regulation of versican synthesis. In Northern blots versican mRNA could be detected in extracts from proliferating keratinocytes and dermal fibroblasts. Comparison of RNA preparations from semi-confluent and confluent fibroblast cultures demonstrated decreasing amounts of versican mRNA at higher cell densities. This inverse correlation of versican expression and cell density was confirmed by indirect immunofluorescence staining of cultured fibroblasts and keratinocytes. The localization of versican in the basal zone of the epidermis as well as the density dependence of versican in cell cultures suggest a general function of versican in cell proliferation processes that may not solely be confined to the skin.

LARGE aggregating proteoglycans form an integral part of the extracellular matrix of a wide range of tissues such as the aorta, brain, cartilage, placenta, sclera, skin, and tendon (for review see references 16, 22, 32). Their core proteins are mainly substituted with chondroitin/dermatan sulfate side chains and they are able to interact with hyaluronic acid. Although a large amount of biochemical data is available for these proteoglycans, until recently little was known about their structural relationships. To date, the entire primary structures of three members of this family of aggregating proteoglycans have been determined based on their cDNA sequences. This includes: the large cartilage proteoglycan aggrecan from rat (10), human (11), and chicken (6) sources, the human fibroblast-derived versican (44) and its chicken homologue PG-M (36), and rat neurocan, a chondroitin sulfate proteoglycan expressed in brain (31). The core protein structures of aggrecan, versican, and neurocan are similar, being composed of link protein-like elements at the amino terminal end, followed by a glycosaminoglycan attachment region of variable length and finally EGF-like elements, a lectin-like domain and a complement regulatory protein-like sequence at the COOH-terminus. In aggrecan, EGF- and complement regulatory protein-like elements can be missing due to alternative splicing events (1, 11). Whereas the amino and carboxy-terminal domains of aggrecan, versican and neurocan show a high degree of similarity with 40 to 60% identical amino acid residues; the central portions of the core proteins are unique.

Versican was originally cloned from cDNA libraries derived from human fetal lung fibroblasts IMR-90 (23) and from human placenta (44). A series of cells express versican in vitro as judged by Northern blotting. This includes, in addition to IMR-90 fibroblasts, MG63 osteosarcoma cells (23), skin and gingiva fibroblasts (20), and arterial smooth muscle cells (35). Versican has been isolated from the culture medium of MG63 cells and of Chinese hamster ovary cells transfected with the full-length cDNA. Experiments using recombinant versican demonstrated a high affinity binding to hyaluronic acid through its amino-terminal domain (25).

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The chicken equivalent of versican, PG-M, binds to hyaluronic acid, fibronectin, and type I collagen in vitro (43). Fibroblasts in culture secrete two alternative splice forms of PG-M, which differ in the length of the central glycosaminoglycan attachment region (36). PG-M is transiently expressed during the precartilage condensation process in the developing limb bud of the chicken, disappearing with the onset of cartilage matrix deposition (21). Other immunoreactive tissues in chicken embryos include aorta, lung, cornea, brain, skeletal muscle, and dermis (42).

Data on the tissue expression of human versican are sparse. Virtual identity of versican sequences with partial amino acid sequences of the 60-kD glial hyaluronate binding protein, GHAP, suggested that versican (or at least a portion of it) may be present in nervous tissues (28, 44). The recent isolation of a brain chondroitin sulfate proteoglycan with NH_2-terminal sequences identical to versican supports this notion (29). Versican-like proteins seem to occur in a number of other tissues (reviewed in reference 22). However, a clear identification of these proteoglycans has been hampered by the lack of well characterized antibodies. Extensive sequence similarities between the large aggregating proteoglycans as well as the high degree of core protein substitution with glycosaminoglycans and N- and O-linked oligosaccharides make the generation of monospecific antibodies difficult.

We therefore employed a fusion protein strategy to prepare polyclonal antibodies exclusively recognizing unique portions of the versican core protein and used these antibodies to study the regulation of versican expression in human skin and on keratinocytes and dermal fibroblasts in culture.

Materials and Methods

Preparation of Bacterial Fusion Proteins and Fusion Protein Columns

The bacterial expression system, including vectors and bacterial strains, were kindly provided by Dr. Dieter Stüber (Hoffmann-La Roche, Basel, Switzerland). Fusion proteins were prepared essentially according to Stüber et al. (37). PCR was used to isolate two specific versican cDNA portions: (*a*) fragment A included bases 1,335 to 1,967 and (*b*) fragment D bases 5,241 to 6,569 (44). The upper strand oligonucleotide primers, synthesized on a 391 DNA Synthesizer (Applied Biosystems, Inc., Foster City, CA) included at the 5′ end two variable nucleotides, an SfiI restriction site, the codons for the Factor Xa cleavage sequence, followed by 20 nucleotides of versican sequences. The lower strand primers contained two variable nucleotides, a NotI restriction site, an inverse complementary stop codon sequence, followed by 20 nucleotides of inverse complementary versican sequences. Using versican cDNA cloned into pBluescript (Stratagene, La Jolla, CA) as template, 20 PCR cycles were run on a thermocycler using a PCR amplification kit (Perkin-Elmer, Norwalk, CT). The cycling conditions consisted of a denaturing step at 94°C for 1 min, primer annealing at 55°C for 1 min and an extension for 4 min at 72°C. The final extension was prolonged to 10 min. All reactions were performed in a volume of 100 μl and included 1 ng of the starting plasmid template. Resulting fragments were digested with SfiI and NotI (Boehringer Mannheim, Mannheim, Germany), run on agarose gels, electroeluted and subcloned in the SfiI/NotI restriction sites of the expression vector. For this purpose, we first modified the expression vector pDS56/RBSII,6xHis (= pDS9) by ligating a synthetic cassette containing an SfiI and a NotI site between the BamHI and PstI sites (pDS9-Cassette) (Fig. 1). The resulting constructs were sequenced over the ligation sites to confirm correct in frame cloning of the versican fragments. Constructs were transformed into M15[pREP4] cells. Transformed cells were grown in LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. Expression of the fusion proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside (Boehringer Mannheim) to a final concentration of 2 mM. 5 h after induction, cells were spun down at 6,000 *g*

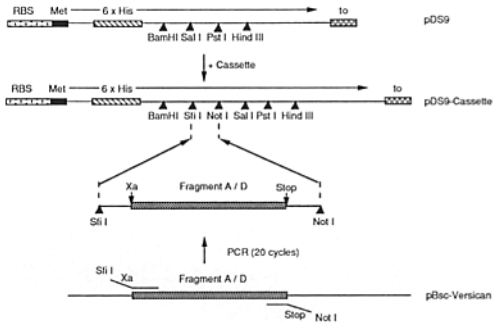

Figure 1. Strategy used for the preparation of bacterial expression constructs. *RBS*, ribosomal binding site; *to*, universal translation terminator; *Xa*, coagulation Factor Xa cleavage site.

for 10 min and the pellet was extracted with 6 M guanidine-HCl/100 mM sodium phosphate, pH 8.0, for 1 h at room temperature. The solution was cleared of bacterial debris by centrifugation at 10,000 *g* for 10 min and the supernatant was directly applied to a 5-ml Ni^{2+}-NTA agarose (Diagen, Düsseldorf, Germany) affinity column. After washing with 8 M urea/100 mM sodium phosphate/10 mM Tris, pH 8.0, the fusion proteins were eluted stepwise by lowering the pH to 6.3 and 5.9. Fractions were analyzed by SDS-PAGE and compared with total bacterial extracts prepared by boiling bacterial samples in SDS sample buffer.

For the preparation of bacterial control extracts, M15[pREP4] cells were transformed with the expression vector without insert. Propagation, induction, and extraction with 6 M guanidine or SDS sample buffer were done as described above. The guanidine extract was dialyzed against 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3, precipitated with 3 vol ethanol and lyophilized.

Purified fusion proteins A and D as well as the dialyzed guanidine extract of a control culture were coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

Preparation and Purification of Polyclonal Antibodies

Fusion proteins A and D, respectively, were emulsified in incomplete Freund's adjuvant and used to immunize New Zealand white rabbits according to standard protocols (15).

Antisera were affinity purified on a Sepharose 4B column (CNBr-activated; Pharmacia) coupled with bacterial control extract followed by absorption to the corresponding fusion protein column. Eluted antibodies were precipitated by addition of $(NH_4)_2SO_4$ to 50% saturation and resuspended in TBS/1% BSA/0.02% sodium azide. Preimmune control serum was treated analogously.

Cell Cultures

Keratinocytes were released from normal human skin by trypsinization and cultured in serum-free keratinocyte growth medium (KGM) containing 0.09 mM calcium (2), 50 μg/ml bovine pituitary extract (BPE), and 5 ng/ml recombinant human EGF (GIBCO BRL, Gaithersburg, MD). In experiments with higher calcium concentrations, $CaCl_2$ was added into the medium of semiconfluent keratinocyte cultures to yield a final concentration of 1.5 mM for the last 48 h prior to immunostaining. Normal human fibroblasts were grown out of skin explants in DME with 20% FCS, and cultured in the same medium with 10% FCS in later passages. For indirect immunofluorescence staining experiments, cells from the first or second passage were grown on glass cover slips. Keratinocytes were seeded at densities varying from 2,000 to 20,000 cells/cm^2 and incubated for 4 d, and dermal fibroblasts at densities between 4,000 to 40,000 cells/cm^2 and grown for 2 d prior to staining.

MG63 osteosarcoma cells (CRL 1427; American Type Culture Collection, Rockville, MD) were grown in IMDM supplemented with 5% FCS, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml amphotericin (all from GIBCO BRL).

Isolation of Proteoglycans from Cell Cultures

Keratinocytes and dermal fibroblasts were seeded at a cell density of 3,000 and 4,000 cells/cm^2, respectively, and grown for 48 h in the corresponding media mentioned above. Subsequently, BPE/EGF and serum were removed

and the cells were incubated for another 48 h. Versican-enriched fractions were prepared by anion exchange chromatography from conditioned medium and cell layers of both human keratinocytes and skin fibroblasts as well as from MG63 osteosarcoma culture medium. In detail, 10 ml Q-Sepharose (Pharmacia) per liter and PMSF and sodium azide to final concentrations of 1 mM and 0.02%, respectively, were added to spent medium and incubated on a shaker at 4°C overnight. After short centrifugation at 500 *g*, the supernatant was removed and the resin was packed into a column. After successive washing with 6 M urea/10 mM EDTA/1 mM PMSF/Tris, pH 8, and with the same buffer containing 0.3 M NaCl, a versican-enriched fraction was eluted with 1 M NaCl.

Cells were extracted with 4 M guanidine/50 mM Tris, pH 8, containing protease inhibitors (10 mM EDTA, 1 mM PMSF, 2 μM leupeptin, and 2 μM pepstatin) for 4 h at 4°C. Subsequently, the guanidine concentration was adjusted to 0.3 M by diluting with 50 mM Tris, pH 8.0, plus inhibitors. 2 ml Q-Sepharose was added per 100 ml diluted extract. The subsequent steps were performed as described for the medium preparation.

Fractions containing versican were selected based on immunoreactivity in a dot blot, diluted with an equal volume of H_2O and precipitated with 3 vol of ethanol. Chondroitinase ABC (ICN, Costa Mesa, CA) digestions were carried out overnight at 37°C, using 2 U/ml of enzyme and 10 μg/ml ovomucoid (Sigma, Buchs, Switzerland) in 40 mM Tris-acetate, pH 8.0. Proteoglycan preparations from cell extracts were digested with 10 U/μl benzonase (Merck, Darmstadt, Germany) for 1 h at 37°C before the addition of chondroitinase ABC.

Electrophoresis and Immunoblotting

SDS sample buffer extracts of bacteria and fusion proteins or proteoglycan fractions were run under reducing conditions on 12.5 or 4–15% Phastgels (Pharmacia), respectively, using SDS-buffer strips and stained with Coomassie blue or processed for diffusion blotting at 70°C for 20 min (according to the manufacturer's recommendations). The efficiency of blotting was tested by including prestained molecular weight standards (Bio-Rad Laboratories, Richmond, CA) in each electrophoresis run. Blots were blocked with 3% low fat milk in TBS. First antibodies were used 1:1,000. The following steps were performed using the ProtoBlot AP Western blot detection system (Promega Biotec, Madison, WI).

Tissue and Cell Staining

Immunohistochemical stainings were done on methanol-fixed, 5-μm cryosections of normal human skin using the immunoperoxidase Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). Incubation with the first antibodies was performed at 4°C overnight. The subsequent steps were carried out according to the instructions of the manufacturer. Processed sections were counterstained for 1 min in Mayer's hematoxylin solution.

In some sections, elastic fibers were stained with resorcin-fuchsin in combination with a fast red nuclear stain.

For indirect immunofluorescence experiments, cells were fixed and permeabilized in methanol for 15 min at −20°C. First antibody incubation was done at room temperature overnight. FITC-labeled anti-rabbit antibodies (Dakopatts, Glostrup, Denmark) were used as second antibodies.

RNA Isolation and Northern Hybridization

Total RNA was isolated from cultured keratinocytes and skin fibroblasts as described by Chomczinski and Sacchi (7). 5 and 20 μg total RNA were run on 0.8% agarose-formaldehyde gels, transferred to GeneScreen Plus nylon filters (New England Nuclear, Boston, MA) and UV cross-linked. Amounts of loaded RNA and integrity of the samples were verified by staining with 0.04% methylene blue in 0.5 M sodium acetate, pH 5.2 (17). Versican-specific RNAs were detected with a digoxygenin-labeled anti-sense riboprobe covering bases 2,342 to 4,462 of the versican cDNA (44). The riboprobe was prepared from a pBluescript KS construct with a DIG RNA labeling kit using T7 RNA polymerase (Boehringer Mannheim). Blocking, hybridization, washing, and color detection were carried out according to the manufacturer's recommendations.

Results

Expression of Versican Core Protein Fragments in Bacteria

Two unique portions of the human versican core protein were chosen for the preparation of fusion proteins in a bacterial expression system (Fig. 2). Usually, 1 liter of bacterial culture yielded between 3 and 10 mg of versican fragments A or D, which could be isolated from cell extracts by affinity purification on a metal chelating column charged with nickel (Fig. 3). This resin specifically binds with high affinity to a histidine-rich sequence in the leader portion of the fusion protein permitting a one-step removal of contaminating bacterial proteins. The resulting preparations contained more than 95% pure fusion proteins.

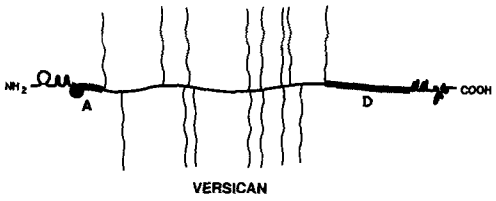

Figure 2. Localization of versican core protein sequences A and D (*shaded areas*) used for antibody preparation. Versican model modified from reference 44.

Polyclonal Antibodies Directed Against Versican Fusion Proteins Recognize Intact Versican

Antisera obtained from rabbits immunized with recombinant proteins A or D were strongly reactive with the corresponding fusion proteins in immunoblots (Fig. 3). Neither bacterial proteins of cells transformed with pDS9 cassette alone nor the amino-terminal fusion sequence were recognized by the antibodies as antisera directed against one fusion protein did not cross-react with bacterial extracts containing the other fusion protein (data not shown). The crude antisera were purified on an affinity-column coupled with extracted protein from bacterial control cultures followed by absorption on the corresponding fusion protein columns. Both polyclonal antibodies, anti-A and anti-D, reacted with intact versican, partially purified from MG63 culture medium. On 4–15% SDS-polyacrylamide gels, versican re-

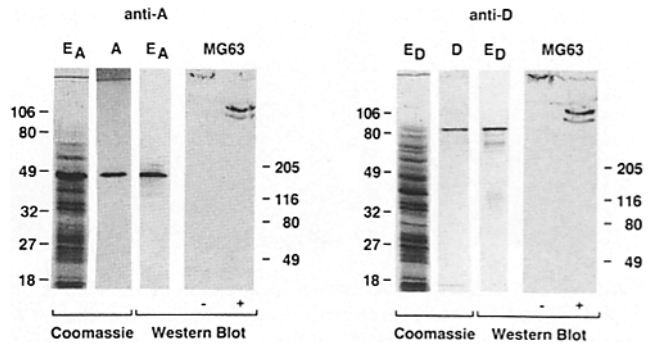

Figure 3. Characterization of polyclonal antibodies specific for versican. SDS-extracts of bacteria expressing A or D fragments of versican (E_A and E_D) and purified fusion proteins (*A* and *D*) were electrophoresed on 12.5% SDS-polyacrylamide gels and either stained with Coomassie blue or subjected to Western blotting as indicated. The immunoreactivity of the affinity-purified antibodies A and D was tested on versican in a proteoglycan fraction from MG63 osteosarcoma cells. The samples were separated on 4–15% SDS-polyacrylamide gels either before (−) or after (+) digestion with chondroitinase ABC and processed for immunoblotting. The molecular masses of standard proteins are indicated in kD.

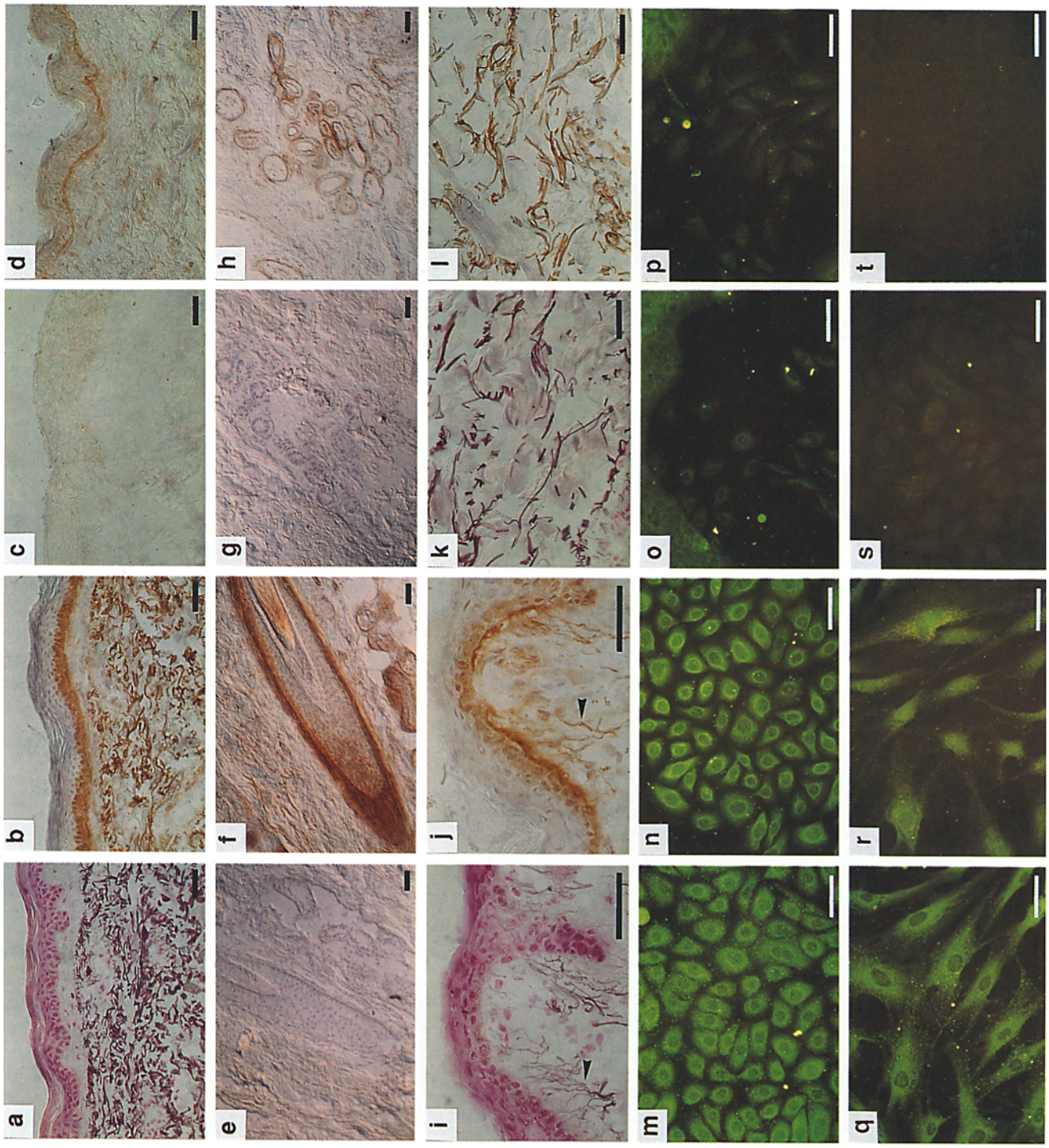

Figure 4. The immunohistochemical localization of versican in cryosections of adult human skin using anti-A (*d*) and anti-D antibodies (*b*, *f*, *h*, *j*, and *l*) is compared with resorcin-fuchsin staining of the elastic network (*a*, *i*, and *k*). Versican is localized in the basal cell layer of the epidermis (*b* and *j*), in the hair follicle (*f*), and sweat glands (*h*), and in association with the elastic fibers in the dermis (*b*, *j*, and *l*). The arrowheads mark co-localization of versican staining with oxytalan and elaunin fibers in the papillary layer (*i* and *j*). The control reactions included: blocking of the anti-D immunoreaction by addition of 50 μg/ml fusion protein D (*c*) or replacing the first antibody with preimmune serum (*e* and *g*). Micrographs *m* to *t* show indirect immunofluorescence stainings of keratinocytes grown in conditions promoting cell proliferation (*m* and *n*) or terminal differentiation (*o* and *p*) and of dermal fibroblasts (*q* and *r*) using anti-A (*m*, *o*, and *q*) and anti-D antibodies (*n*, *p*, and *r*) or preimmune serum (*s* and *t*). Bars, 50 μm.

mained at the point of loading. After digestion with chondroitinase ABC, two bands with molecular masses larger than 300 kD were recognized by both polyclonal antibodies (Fig. 3).

Versican Is a Component of the Dermal–Epidermal Interface

Immunostaining of normal human skin with both versican-specific antibodies A and D exhibited a similar, distinct staining pattern, although the antibodies against the A fragment regularly produced a weaker signal. Staining of the basal keratinocytes in the interfollicular epidermis, hair follicles, and sweat glands was prominent (Fig. 4, *b*, *d*, *f*, and *h*). In the epidermis, one to two basal keratinocyte layers displayed a diffuse pattern of the immunoreaction product, with the basolateral aspect of the cells staining most strongly. It was not evident whether the basement membrane itself stained positive. Trials to split the basement membrane zone through the lamina densa using neutral buffers containing 1 M NaCl (12) were successful in inducing dermal–epidermal separation, but simultaneously the high salt buffer eluted versican from the skin specimen abolishing the immunoreaction. Other splitting methods, such as incubation of the skin at 60°C, destroyed both the structure of dermal–epidermal junction and the versican epitopes, thus precluding a definitive evaluation.

In the dermis, papillary and reticular fibrillar networks stained positive with anti-versican antibodies (Fig. 4, *b*, *j*, and *l*). The patterns of the dermal networks closely resembled those obtained with resorcin-fuchsin (Fig. 4, *a*, *i*, and *k*), a stain used in traditional histology to discern the elastic fibers in the reticular dermis and the so called elaunin and oxytalan fibers in the papillary layer (9). The immunoreactive fibrils in the reticular dermis were of large diameter running mostly parallel to the skin surface (Fig. 4 *l*). Another kind of immunoreactive fibrillar structure, thinner in diameter, originated from these thick fibers. Vertically arranged to the surface of the skin, they entered the upper dermis, fanning out into thin branches that inserted into the basement membrane (Fig. 4 *j*).

The specificity of the immunoreaction was demonstrated by control stainings with preimmune sera (Fig. 4, *e* and *g*) and competitive inhibition of the binding of antibodies to tissue epitopes with the corresponding fusion proteins in solution. Addition of 50 μg/ml of fusion protein D to the anti-D antibodies completely abolished the positive immunoreaction in the tissue (Fig. 4 *c*), whereas addition of similar amounts of fusion protein A had no effect on the binding of anti-D antibodies (not shown).

Keratinocytes and Fibroblasts Synthesize Versican In Vitro: Evidence for an Association of the Expression with Cell Density

Normal human keratinocytes were first cultured under conditions that support proliferation but not differentiation (2), using a low calcium concentration of 0.09 mM. Under these conditions the cells strongly expressed versican as assessed by immunofluorescence staining with antibodies A and D (Fig. 4, *m* and *n*). An intracellular, diffuse staining in the keratinocytes was observed. However, when the calcium concentration in the culture medium was increased to 1.5 mM, a condition that induces terminal differentiation of the keratinocytes (2), versican expression was turned off (Fig. 4, *o* and *p*). Aggregated, stratifying, and differentiating cells stained weakly, but individual keratinocytes growing next to the stratifying islands were negative.

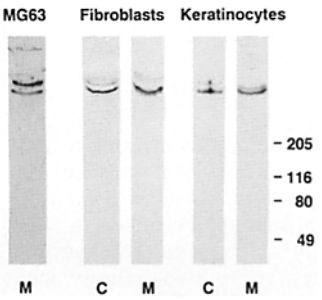

Figure 5. Detection of versican in cell layer (*C*) and medium fraction (*M*) of dermal fibroblasts and keratinocytes, comparison with versican from MG63 osteosarcoma cells. Proteoglycan preparations were electrophoresed on 4–15% SDS-PAGE and subjected to immunoblotting with antibodies against the D fragment. The concentration of versican in the samples has been adjusted to yield similar signal intensities (dilution factors: 3× and 80× for the fibroblast cell layer and medium fraction, respectively; keratinocyte fractions are both undiluted). The molecular masses of standard proteins are indicated in kD.

Similarly to keratinocytes in culture, human skin fibroblasts synthesized versican in vitro, exhibiting a diffuse intracellular or pericellular staining but without a detectable deposition in the extracellular space (Fig. 4, *q* and *r*). Since the lack of deposition might be due to diffusion of the proteoglycan into the culture medium, immunoblotting experiments on cell layer extracts and medium fractions of cultured human keratinocytes and dermal fibroblasts were performed.

Human skin fibroblasts and keratinocytes in low calcium medium both secreted versican into the culture medium as demonstrated on immunoblots (Fig. 5). For semi-quantitative analysis, the dilutions of the versican-enriched fractions were adjusted, in order to give bands of similar intensities on immunoblots. The comparison of the cell layer and medium fractions of cultured dermal fibroblasts revealed that only a very small amount of versican was retained in the cell layer fraction. In contrast, roughly equivalent quantities of versican could be isolated from cell layer and medium of keratinocytes grown under culture conditions promoting cell proliferation. In general, fibroblasts expressed considerably more versican than keratinocytes. Unlike the conditioned medium of MG-63 cells, where two bands were recognized in immunoblots by the versican-specific antibodies after chondroitinase ABC digestion, the cell layer and medium fractions of keratinocytes and skin fibroblasts contained mainly the lower molecular mass component (Fig. 5). No difference between experiments performed with anti-D and anti-A antibodies were observed (not shown). All proteoglycan fractions used in the immunoblot experiments were derived from semi- to subconfluent cultures, as confluent cultures of keratinocytes, in particular, yielded barely detectable amounts of versican.

In Northern blots, versican-specific mRNAs could be found in total RNA preparations of both keratinocyte and fibroblast cultures (Fig. 6). The probe hybridizing to a central, unique portion of versican recognized two duplex bands in the size range of 10 and 12 kb. Only by increasing the amount of total RNA loaded to 20 μg, versican-specific mRNA could be detected in Northern blots of extracts from

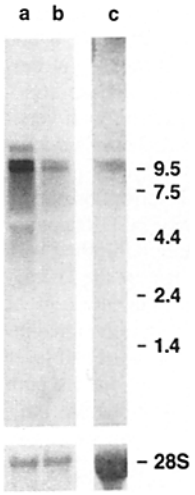

Figure 6. Detection of versican mRNA in extracts from dermal fibroblasts and keratinocytes on Northern blot. Total RNA preparation from semi-confluent (*a*) and confluent (*b*) fibroblast cultures and from keratinocytes, grown at low density under conditions permitting cell proliferation (*c*), were separated on 0.8% agarose gels and processed for Northern blotting. 5 μg (*a* and *b*) and 20 μg (*c*) of total RNA were loaded. Internal standard: 28S ribosomal RNA stained with methylene blue. The sizes of RNA standard molecules are indicated in kilobases.

low density keratinocyte cultures (6,300 cells/cm²). No hybridization was observed in 20 μg of total RNA from confluent keratinocytes grown in either low or high calcium medium (not shown). In comparison to keratinocytes, a much higher versican expression was observed in RNA extracts of dermal fibroblasts. Semi-confluent fibroblast cultures gave stronger versican-specific signals than cells at confluency (Fig. 6).

In line with the results from the Northern blots, the intensity of cell stainings with anti-versican antibodies in indirect immunofluorescence experiments correlated inversely with the culture density (Fig. 7). Keratinocytes plated at low densities revealed a bright immunofluorescence (Fig. 7 *a*). With the exception of dividing cells, a decreasing immunoreactivity with versican antibodies was observed in the progression to subconfluent (Fig. 7 *b*) and finally confluent keratinocyte cultures (Fig. 7 *c*), the latter staining being only slightly above background levels. A similar density dependence, however less pronounced, could also be demonstrated in indirect immunofluorescence experiments with dermal fibroblasts in culture (Fig. 7, *d–f*). Again, mitotic cells displayed a constantly bright staining signal.

Discussion

Versican belongs to the family of large aggregating proteoglycans. Other members of the family, which have been characterized in terms of their primary structure, are aggrecan (10) and neurocan (31). Shared features of these three proteoglycans are a hyaluronic acid binding domain at the NH_2-terminal end and EGF-like, lectin-like, and complement-regulatory protein-like sequences in the COOH-terminal portion of the core protein. The intermediate stretches of the core proteins are unique in each of these proteoglycans. Due to the extensive sequence similarities in the amino- and carboxy-terminal domains of versican, aggrecan, and neurocan, and due to the high degree of carbohydrate substitution, we expected that antibodies generated by immunization with native versican would recognize epitopes shared with other proteoglycans and glycoproteins. To avoid such cross-reactivity, specific fragments of the versican core protein were prepared in a bacterial expression system. This way antigenic sites could be restricted to sequences unique to versican. In addition, two core protein portions with a low probability for attachment of glycosaminoglycan side chains were chosen, in order to minimize potential blocking of antibody access through sterical hindrance.

Both affinity-purified antibodies against the A or D fragment reacted with intact versican in immunoblots of a proteoglycan-enriched fraction isolated from MG63 culture medium. After chondroitinase ABC digestion the antibodies revealed two high molecular mass core proteins. The two bands most likely reflect versican isoforms generated by alternative splicing events, as two duplex bands in the range of 10 and 12 kb can be detected in Northern blots. The duplex patterns observed for both putative splice forms may originate from alternative polyadenylation sites, as the original versican cDNA sequence contained a polyadenylation signal 350-bp upstream of the 3′ end, but no poly-A tail (23). Further evidence for the existence of alternative splice forms of versican is provided by recent data of Shinomura and co-workers (36), who have sequenced the entire cDNA of two splice variants of PG-M, the chicken analogue of versican. Based on sequence similarities, the published versican sequence corresponds to the shorter splice product of PG-M (36). cDNA cloning of the long version of human versican is currently under way in our laboratory.

Both affinity-purified antibodies specific for versican could be used for immunohistochemical staining experiments on cryostat sections. Although polyclonal antibodies against the D fragment gave consistently a more intense signal than antibodies against fragment A, no differences in the actual staining patterns were observed. In human skin the antibodies revealed an apparent co-localization of versican with the elastic network of the dermis. Versican seemed to be associated with all three components of the elastic fiber system: oxytalan, elaunin, and elastic fibers (9). The thin oxytalan fibers are arranged perpendicularly to the dermal–epidermal junction. They branch out from elaunin fibers and intersect the basal lamina. Mainly thick elastic fibers are observed in the reticular layer of the dermis. Both elastic and to a lesser extent also elaunin fibers consist of an amorphous core of insoluble polymeric elastin covered with bundles of 10–12-nm thick microfibrils. Lacking the amorphous elastin core, oxytalan fibers are composed solely of microfibrils (24). As anti-versican antibodies also stain tissue structures strongly resembling oxytalan fibers, an association with microfibrils appears very likely. Several proteins have been described to be associated with microfibrils (reviewed in reference 27). Among them, fibrillin, a 350-kD protein (33) and MAGP, a 31-kD protein (13) seem to be the principal structural components. Therefore, fibrillin and MAGP have to be considered prime candidates in the search for potential ligands of versican in the elastic network.

At present, two different large chondroitin/dermatan sulfate proteoglycans have been isolated from skin tissue or dermal fibroblasts in culture: (*a*) a heterodimeric disulfide-bridged proteoglycan with no hyaluronic acid binding activity from human embryonic skin fibroblasts (4); and (*b*) a large aggregating proteoglycan derived either from human (5, 8, 19, 34), rat (14) or chicken sources (26). Versican seems to be closely related or identical to the second proteoglycan, as both bind to hyaluronic acid and yield similar core protein bands on SDS–polyacrylamide gels after chondroitinase ABC digestion (14, 34). Interestingly, embryonic skin

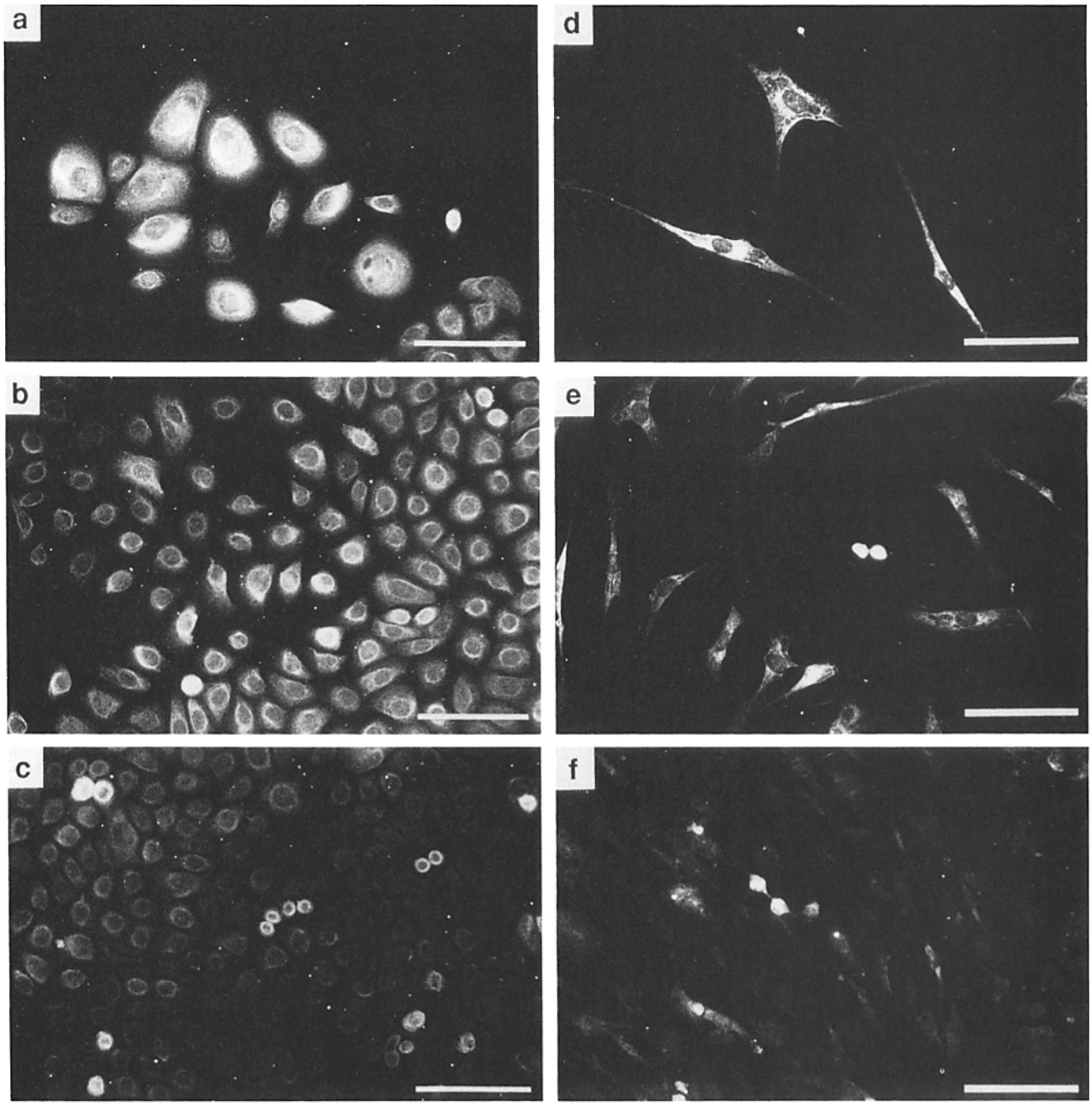

Figure 7. Immunostainings of keratinocytes (*a–c*) and fibroblasts (*d–f*) with anti-D antibodies reveal a decrease in fluorescence signals with increasing densities of the cultures. Keratinocytes were seeded at initial densities of 2,000 (*a*), 10,000 (*b*) and 20,000 (*c*) cells/cm^2, fibroblasts at densities of 4,000 (*d*), 12,000 (*e*), and 40,000 (*f*) cells/cm^2. The cells were cultured for 4 d (*a–c*) or 2 d (*d–f*) prior to staining. Bar, 100 μm.

fibroblasts express comparable amounts of the two core protein forms (34) resembling the pattern obtained from MG63 osteosarcoma culture medium, whereas the skin fibroblasts from adult donors used in our experiments mainly synthesize the lower molecular mass form. This variation may be evidence for a differential expression of the two versican isoforms in normal adult compared to embryonic or tumor tissues.

Apart from fibroblasts, keratinocytes are also able to express versican in vivo and in vitro, as demonstrated by immunohistological techniques, Western and Northern blots. Although the cell staining of proliferating keratinocytes in culture with antibodies against versican was stronger in comparison to fibroblasts, keratinocytes express relatively smaller amounts of versican. The difference may be explained by the observation that, unlike keratinocytes, fibroblasts mainly secrete versican into the culture medium.

In the epidermis, versican is restricted to the zone of keratinocyte proliferation suggesting an involvement in growth and differentiation of epidermal cells. Accordingly, only proliferating keratinocytes in low calcium cultures express ver-

sican, whereas culture conditions promoting terminal differentiation seem to down-regulate the production of versican. The actual synthesis rate of versican may be controlled by interaction with neighboring keratinocytes as a clear decrease in versican-specific immunoreactivity with increasing cell density was observed. In line with this observation, versican mRNAs could only be detected in preparations from low density keratinocyte cultures. However, a clear assessment of differences in levels of transcription can not be made based on the Northern blot experiments, as versican mRNAs seem to be present only at very low abundance even in low density keratinocyte cultures.

Similar to keratinocytes, the down-regulation of versican expression is also observed in dermal fibroblast cultures. The substantial decline in versican mRNA levels from semi-confluent to confluent cultures can clearly be demonstrated on Northern blots. Previous studies, analyzing glycosaminoglycans synthesis in keratinocytes (30) and fibroblasts (18) noted a significant reduction in chondroitin sulfates from semi-confluent to confluent cultures. This alteration may at least partly be attributed to the down-regulation of versican core protein expression.

The search for putative functions of versican should also include its high affinity ligand (25), hyaluronan. Hyaluronan has been localized in the dermis as well as in the basal and spinal layer of the epidermis (39). Analogous to versican, hyaluronan synthesis is inversely correlated with the cell density of fibroblast cultures (18). In vivo, cells usually proliferate in hyaluronan-rich matrices (reviewed in 40 and 41). Hyaluronan may be involved in the detachment and rounding during mitosis of fibroblasts (3) and eventually also keratinocytes (38). Similarly, we have observed a strong immunofluorescence signal with versican antibodies on rounded fibroblasts and keratinocytes in mitosis. In the epidermis, secretion of versican and hyaluronan by proliferating keratinocytes could provide a highly hydrated matrix facilitating upward movement of the dividing cells into the suprabasal layers. The down-regulation of the versican expression by differentiating keratinocytes may subsequently allow tighter cell surface heparan sulfate proteoglycan- and integrin-mediated cell–cell contacts in the spinal layer of the epidermis.

Extracellular matrices rich in versican and hyaluronan may more generally function as modulators of cell proliferation and migration. Thus, the association of cell proliferation with versican expression by human epidermal and dermal cells could provide a valuable model system, to study the function of versican in other epithelial and mesenchymal tissues.

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