Distribution of PG-M/Versican Variants in Human Tissues and de Novo Expression of Isoform V3 upon Endothelial Cell Activation, Migration, and Neoangiogenesis in Vitro*

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We have carried out a comprehensive molecular mapping of PG-M/versican isoforms V0–V3 in adult human tissues and have specifically investigated how the expression of these isoforms is regulated in endothelial cells in vitro. A survey of 21 representative tissues highlighted a prevalence of V1 mRNA; demonstrated that the relative frequency of expression was V1 > V2 > V3 > V2, and showed that <15% of the tissues transcribed significant levels of all four isoforms. By employing novel and previously described anti-versican antibodies we verified a ubiquitous versican deposition in normal and tumor-associated vascular structures and disclosed differences in the glycanation profiles of versicans produced in different vascular beds. Resting endothelial cells isolated from different tissue sources transcribed several of the versican isoforms but consistently failed to translate these mRNAs into detectable proteoglycans. However, if stimulated with tumor necrosis factor-α or vascular endothelial growth factor, they altered their versican expression by de novo transcribing the V3 isoform and by exhibiting a moderate V1/V2 production. Induced versican synthesis and de novo V3 expression was also observed in endothelial cells elicited to migrate in a wound-healing model in vitro and in angiogenic endothelial cells forming tubule-like structures in Matrigel or fibrin clots. The results suggest that, independent of the degree of vascularization, human adult tissues show a limited expression of versican isoforms V0, V2, and V3 and that endothelial cells may contribute to the deposition of versican in vascular structures, but only following proper stimulation.

PG-M/versican is an evolutionarily conserved proteoglycan (PG) known to be precociously expressed during embryonic development (1–3) and present throughout adult life in the majority of the connective and nervous tissues (4–13). It was initially isolated and characterized biochemically and ultrastructurally from bovine aorta and chick embryo fibroblasts (14–18) and subsequently cloned and sequenced in cultured chick and human cells (19–21). Parallel studies in several animal species established that three isoforms, denoted V1, V2, and V3 could be generated by alternative splicing from a larger transcript denoted V0 (21–24). Tissue-restricted splice variants were also detected during some phases of embryonic development (25). The four primary versican isoforms vary in their relative content of the two glycosaminoglycan attachment domains, i.e. glycosaminoglycan-α and glycosaminoglycan-β, which are differentially omitted in the V2 and V1 isoforms and are entirely absent in the V3 one, contributing thereby to a significant side chain polymorphism in different versican variants.

In the adult, versican seems to be particularly enriched in vascular structures, where it links to components of the subendothelial basement membrane and to the elastic fibrils (26–29). In these tissue compartments, it is likely to be implicated in both the normal hemostasis of blood vessels as well as to be involved in some of the pathological conditions that may affect these tissue structures. In fact, versican is abundantly produced by vascular smooth muscle cells and its expression is enhanced during atherosclerosis and restenosis causing a higher sequestering of low density lipoprotein in these formations (27, 30–36). The PG has also been shown to be produced by one murine-transformed endothelial cell type, although it is presently unclear to what extent it affords a constitutive translational product of normal and diseased human endothelium. Similarly, no information is currently available about how endothelial versican expression may be modulated by factors influencing the activation state/differentiation of the cells, e.g. those operating during processes of inflammation, angiogenesis, and wound healing. Finally, due to the unavailability of immunological reagents that would distinguish the isoforms V0 and V3 from V1 and V2, there is currently scarce information about the relative tissue distribution of the four versican variants.

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The abbreviations used are: PG, proteoglycan; BSA, bovine serum albumin; DABCO, 1,4-diazabicyclo[2.2.2]octane; HIAEC, human iliac artery endothelial cells; HUVEC, human umbilical vein endothelial cells; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; RT, reverse transcriptase; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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To define the precise transcriptional map of versican isoforms in humans and to address their potential role in the pathophysiology of vascular tissues, we have determined the patterns of expression of these transcripts in the major organs/tissues of the adult and in primary vascular cells under resting and activated conditions.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—A panel of anti-versican mAbs was produced according to standard procedures by immunizing Balb/c female mice with isolated bovine aorta versican and screening the hybridomas against the immunogen and related antigens. Specificity of these mAbs has partially been described elsewhere (3, 37) and further details about their reactivities are detailed here. One of the mAbs, 12C5, was kindly provided by Richard Le Baron (Division of Life Sciences, University of Texas at San Antonio, San Antonio, TX). Monoclonal antibody (mAb) 5D5 (3, 5) was kindly provided by Firoz Rahemtulla (Laboratory of Dental Research, University of Alabama, Birmingham, AL). mAb 12C5 reacting with a conserved epitope contained within the N-terminal G1 versican domain was purchased from the Developmental Studies Hybridoma Bank (Iowa University, IA). mAb 2B1, known to bind specifically to the C-terminal G3 domain of human versican (39, 40), was purchased from Seikagaku Corporation (Japan). Anti-decorin mAb 6B6 was purchased from Seikagaku Corporation and mAb CS56 from Sigma. The anti-human perlecan antibody TE1 was kindly received from Renato Inzio (Department of Cell Biology and Pathology, The Thomas Jefferson University, Philadelphia, PA). Antiserum As73 against human collagen type VI was produced by immunization of rabbits with tetrameric pepsin-digested placental collagen type VI. The hybridoma clone H/FFN-7 producing a mAb against human fibronectin was purchased from the American Tissue Culture Collection (Rockville, MA).

**Solid-phase Binding Assays and TEM/Rotary Shadowing**—Bovine aorta versican was isolated as previously described (37, 41). Enzyme-linked immunosorbent assay with the various antibodies and enzyme digests was performed as described in previous studies (3). Ultrastructural analyses of antibody bindings to versicans were carried out by rotary shadowing and negative stainings performed as previously detailed (11, 41).

**Primary Vascular and Non-vascular Cells**—Primary human endothelial cells including HUVEC (umbilical cord), HIAEC (iliac artery), pulmonary artery, coronary artery, adult dermis microvasculature, neonatal dermis microvasculature, adult aorta, and primary human smooth muscle cells, including aorta and uterus were obtained from BioWhittaker Inc. (Walkersville, MD) at the first to third passage. HUVEC were also isolated according to standard procedures from umbilical cords collected in the laboratory. Endothelial cells were maintained in endothelial growth medium (BioWhittaker Inc.) supplemented with 1-glutamine (Sigma) and were consistently used prior to the fourth passage. Aorta and uterine smooth muscle cells were grown in smooth muscle growth medium (BioWhittaker Inc.). Hs913T (human fibrosarcoma) and Human intestinal smooth muscle cells were obtained from the American Tissue Culture Collection and were grown in Dulbecco’s modified Eagle’s medium with 10–20% fetal calf serum.

**Growth Factor and Cytokine Stimulations**—For stimulation with cytokines and growth factors, endothelial cells were pre-grown under low-nutrient conditions for 24 h in basal growth medium supplemented with 1% fetal calf serum. The following day, the cells were washed twice with PBS and incubated in serum-containing medium in the presence or absence of 50 ng/ml TNF-α (Chemicon International, Inc., Temecula, CA) or 100 ng/ml VEGF (Chemicon International, Inc.) for 4–48 h. Total RNA was extracted at 4, 8, 16, and 24 h and processed for RT-PCR (see below).

**RT-PCR**—Total cellular RNA was isolated from the different cell types using the RNA Fast kit (Molecular Systems, San Diego, CA) according to the manufacturer’s protocol. Total RNAs from the majority of human adult tissues were purchased from Clontech Laboratories. Cartilaginous RNA was kindly provided by Dr. Paul Di Cesare (The Hospital for Joint Diseases, New York, NY). RNA was also isolated with the above kit from healthy skeletal muscle kindly obtained from Dr. Carlo Minetti (G. Gaslini Pediatric Institute, Genova, Italy) and from mesenteric veins obtained from routine autopsies performed at the National Cancer Institute of Aviano. RT reactions were performed with 1 μg of total RNA using AMV-Reverse Transcriptase (Promega, Madison, WI). RNA was reverse-transcribed into first-strand cDNA using random hexamers primers. The primers used for the PCR amplification of versican isoforms were: PGM-A, 5′-GGCTTTGACCATCGGATTAC-3′; PGM-B, 5′-TCAACATCTCATGTCCTCCCTC-3′; PGM-C, 5′-TTCTTCCTCGGCGTATGGCTA-3′; PGM-D, 5′-CCAGCCCATAGTACATGCTCTC-3′. The sizes of the amplifications products were 405 bp for V0 splice form (primers PGM-B + PGM-C), 336 bp for V1 (primers PGM-A + PGM-C), 388 bp for V2 (primers PGM-B + PGM-C), and 498 bp for V3 (primers PGM-A + PGM-D). Tag DNA polymerase was obtained from Roche Molecular Biochemicals. RT-PCR amplification of a 400-bp fragment of glyceraldehyde-3-phosphate dehydrogenase cDNA served as positive internal control. Amplification products were resolved on 2% agarose gels stained with ethidium bromide.

**RNA Protection Assay**—Two sets of probes capable of discriminating between the four versican splice forms (3) were prepared by RT-PCR from total RNA isolated from Hs913T cells using primers PGM-B (sense) + PGM-C (antisense) and PGM-A (sense) + PGM-D (antisense). The probes were cloned into a pGEM-T vector (Promega, Madison, WI). [32P]UTP-labeled antisense riboprobes were synthesized from a SP6 promoter-driven RNA polymerase vector using primers PGM-A (antisense) and PGM-D (antisense) which were hybridized overnight with 10 μg of total RNA of each human tissue. The degree of intactness and calibration of the amount of input RNA were ascertained by using a human β-actin probe as reference. Post-hybridization procedures were performed using RPA II kit (Ambion) according to the instructions provided by the supplier. The RNP–protected fragments were run on a sequencing gel and autoradiographed, and the specific signals were quantified by computer-assisted densitometric scanning. Probe AD allowed the identification of the V3 isoform (429 bp), whereas probe BC hybridized with the V0 (405 bp), V2 (244 bp), and V1 (161 bp) ones. Assessment of the relative levels of expression of the different versican transcripts was accomplished by analyzing autoradiograms in the computer-assisted densitometric scanning using a Philips CCD video camera and the software GelScan. Densities of the autoradiographic bands obtained for each individual isoform were plotted accounting for the specific intensities in [32P] activities of the diversely sized fragments. Direct comparison of the relative levels of expression of the individual isoforms in each individual tissue/organ are reported as percent adopting the signal detected for the prevailing isoform of that given tissue/organ as 100%. The nature of the splicing sites of human versican allowed for a direct comparison of the expression levels of the V0–V2 isoforms in the same autoradiograms, whereas the protected signal for the V3 isoform was normalized to that obtained in the same autoradiogram for the cumulative expression of V0–V2 isoforms.

**Angiogenesis Assay**—For simulation of angiogenesis, endothelial cells were rinsed in PBS, trypsinized, and resuspended in endothelial growth medium at a density of 1–5 × 104 cells/ml. Human fibrinogen (kindly received from Luigi De Marco, The National Cancer Institute of Aviano) was diluted at 20 μg/ml in endothelial growth medium. An aliquot of 25 μl of this solution containing the resuspended cells was added to solidify by addition of 1 μg of human purified thrombin (kindly provided by Luigi De Marco) in Lab-Tek® plates (Nalge-Nunc International, Naperville, IL). The formed clots were equilibrated at 37 °C with 5% CO2 for 15 min and covered with 100 μl of medium and supplemented or not with 50 ng/ml TNF-α or 100 ng/ml VEGF. Cultures were then washed before being fixed in 4% paraformaldehyde in PBS, stained immunocytochemically, and photographed. An alternative assay involved Matrigel; a stock concentration of the specific batch (Becton Dickinson Labware, Bedford MA; 12.3 mg/ml) which was diluted at 1:1 with ice-cold serum-free Dulbecco’s modified Eagle’s medium, gently mixed, and dispensed into 6-well tissue culture plates (50–48-well plates, 0.15 ml/well). Plates were incubated at 37 °C for 30 min to form a uniform layer and cell suspensions of a similar density as indicated above were then seeded on top of the gels in the appropriate growth medium. Cultures were maintained under standard cell culture conditions for up to 72 h before being fixed and stained as described above. All cultures were routinely monitored by phase contrast microscopy at different time intervals. Total RNA was extracted from tube-forming endothelial cells using the RNA Fast Isolation kit and utilized for RT-PCR as described above.

**“Wound Healing-like” Assay**—For wound-healing simulation assays, HUVECs were seeded at 80% confluence in uncoated 6-well plates and...
grown to confluence. Confluent monolayers were starved for 4 h, thereby reduced levels of serum (1%), wounded by scraping away a swath of cells with a P1000 pipette tip and further cultured for up to 48 h. After scraping, cells were rinsed twice with PBS to remove wound-derived cell aggregates and debris and either immediately fixed in 4% paraformaldehyde or fixed after 4, 8, or 24 h of culture and processed for indirect immunostaining. Alternatively, unfixed cells at the above time intervals were processed for the isolation of total RNA to be used for RT-PCR analyses.

**Immunocytochemistry—**Healthy and tumor tissue specimens were obtained from surgical specimens of mammary carcinoma, hepatocarcinoma, colon carcinoma, melanoma, leiomyosarcoma, fibrosarcoma, malignant fibrohistiocytoma, and liposarcoma performed at the National Cancer Institute of Aviano (M.R.) and from routine autopsies carried out at the same institute. Tumor tissues were fixed in 4% paraformaldehyde in PBS, embedded in OTC compound (Miles Laboratories) and cryosectioned. Sections were air-dried, incubated with primary antibodies (undiluted supernatant or purified Iggs and ascites fluids at 1:100 dilution in PBS with 0.1 mg/ml BSA) at 4 °C overnight, washed and further incubated for 2 h at room temperature with fluorescein- or Texas Red-conjugated secondary antibodies diluted 1:100 in PBS/BSA. In some cases, to confirm the identity of the intracellular neoangiogenic vessels, sections from soft tissue sarcoma specimens where double-labeled with anti-versican antibodies and an anti-PECAM (platelet endothelial cell adhesion molecule) (Chemicon International, Temecula, CA) antibody, or anti-versican antibodies and biotinylated Ulex europaeus I lectin (revealed by fluorescently tagged streptavidin; Amersham Biosciences). In a number of other cases, incubation of tissue sections with anti-versican antibodies was preceded by digestions with collagenase or testicular hyaluronidase as previously described (3) to ascertain that immunolabelings were not compromised by epitope masking. For immunocytochemical staining, cells grown on plastic Lab-Tek® chamber slides, Matrigel or fibrin clots were washed twice in PBS with BSA 0.1% w/v (Sigma) and fixed in 4% paraformaldehyde for 10 min. Nonspecific binding sites were blocked by incubation with 2% v/v normal goat serum (Zymed Laboratories Inc.) in PBS/BSA 0.1% v/v for 30 min at room temperature. Cells were washed and incubated with fluorescein- or Texas Red-conjugated secondary antibodies diluted 1:100 in PBS/BSA. An alternative staining procedure adopted for immunolabeling with the “fixation-sensitive” mAb 5DS (3) consisted in direct incubation of live cells with the antibody diluted 1:50 in PBS/BSA at room temperature, followed by fixation with paraformaldehyde and incubation with secondary antibodies as described above. In both cases, stained cells were finally mounted with Mowiol 4.88 (Calbiochem-Novabiochem) supplemented with 2.5 mg/ml DABCO (Sigma) and as an anti-fading agent.

**Immunoblotting—**Bovine aorta versicans and nasal cartilage aggregates were solubilized in SDS-containing sample buffer in the presence of β-mercaptoethanol and resolved by SDS-PAGE on 3–8% gradient gels. Separated PGs were electrotransferred onto nitrocellulose membranes, which were subsequently saturated with α-casein and processed for immunoblotting using the panel of anti-versican antibodies as previously described (3). For immunohistochemical detection of versicans produced by activated endothelial cells, cell lysates were prepared from HUVEC grown for 48 h in the presence of 50 ng/ml TNF-α or 100 ng/ml VEGF. Lysis buffer was composed of 50 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 2% Triton X-100, and protease inhibitors. After lysis, the cell extract was subjected to centrifugation at 13,000 g for 30 min in the cold. Proteins were then fractionated by SDS/PAGE in a 4–20% acrylamide gel under reducing and non-reducing conditions and electroblotted onto nitrocellulose membranes overnight. Blots were blocked with dried milk in TBST (Tris-buffered saline with Tween 20) and incubated at 4 °C overnight with mAbs 12C5 or 2B1 (at 1:50 dilution in the blocking buffer). The membranes were washed, incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse secondary antibodies (at 1:1000 dilution in TBST) and further processed for chemiluminescent detection (ECL chemiluminescence kit; Amersham Biosciences) as described above.

**RESULTS**

**Versican Isoform mRNA Expression in Normal Adult Tissues—**As a first step, to define the expression pattern of versicans in human vascular structures, we determined the overall distribution pattern of the four versican isoforms in a panel of human adult tissues by conventional RT-PCR and RNase protection. The first approach asserted a ubiquitous transcription of the versican gene, the resulting transcripts of which were detected in 20 of the 21 tissues examined (Table I). The V1 isoform was found to be the most widespread, expressing in 19 of the 21 tissues, whereas the V2 one was the least frequently transcribed (Table I). More than 50% of the tissues/organs examined expressed detectable levels of all alternatively spliced transcripts, whereas 4 of the tissues/organs examined, namely liver, uterus, spleen, and cartilage expressed solely the V1 isoform (Table I). A more precise quantitative analysis of the expression levels of the different versican isoforms was then performed in the same tissues or organs by RNase protection. The latter assays confirmed the prevalence of the V1 isoform, showing that, on average, it accounted for more than 70% of all versican mRNA transcribed by each individual tissue or organ. These experiments also showed that the parental V0 isoform represented the one showing the lowest expression levels (Fig. 1). Furthermore, in several tissues, certain mRNAs that were detected by RT-PCR were found to be actually transcribed at minute levels, when accurately assessed. This finding thereby reduced the number of tissues or organs deemed to express significant quantities of all isoforms from 11 to a mere 3, including mammary gland, heart, and kidney (Fig. 1).

**Immunohistochemical Evidence for the Deposition of Versican Glycanation Variants in Normal Vascular Structures—**The above isoform mapping at the mRNA level corroborated previous findings suggesting that versicans V1 and V2 are the most widely distributed in human adult tissues. Because data on the tissue distribution of the core proteins of these isoforms have been reported elsewhere (8), we sought here to make use of a panel of mAbs produced against versicans to disclose possible differences in the distribution of putative glycanation variants of this PG in vascular tissues (Table II, Fig. 2). Enzyme-linked immunosorbent assay/dot-blotting (Table II), transmission electron microscopy/rotary shadowing data (data not shown), combined with differential enzyme digestions were initially

![Table I](http://www.jbc.org/)

| Tissue/organ          | Versican isoform |
|-----------------------|------------------|
| 1. Spleen             | V1, V2, V3       |
| 2. Stomach            |                  |
| 3. Mammary gland      | V1, V2, V3       |
| 4. Liver              | V1, V2, V3       |
| 5. Brain              |                  |
| 6. Heart              |                  |
| 7. Teeth              |                  |
| 8. Thymus             |                  |
| 9. Placenta           |                  |
| 10. Salivary gland    | V1, V2, V3       |
| 11. Kidney            | V1               |
| 12. Pancreas          | V1, V2, V3       |
| 13. Pons              | V1, V2, V3       |
| 14. Adrenal gland     | V1, V2, V3       |
| 15. Small intestine   | V1, V2, V3       |
| 16. Skeletal muscle   | V1, V2, V3       |
| 17. Lung              | V1               |
| 18. Cartilage         | V1               |
| 19. Skin              | V1               |
| 20. Uterus            | V1               |
| 21. Mesenteric veins  | V1               |
employed to assert that the mAbs indeed recognized glycana-
tion traits of the PGs. The experiments also confirmed that the
antibodies largely failed to react with several other ECM PGs
(Table II), as further demonstrated by additional immunoblot-
ting experiments involving intact and pre-digested bovine
aorta versicans (Fig. 3A). We also further characterized the previously described anti-
versican core protein antibody, mAb 5D5, by ultrastructural
means, such as to define its potential utility for examining
vascular versicans. Present and previously reported length
measurements of the "rotary shadowed" core proteins isolated
from bovine aorta, in conjunction with biochemical and immu-
nochemical data (3, 5, 37, 41), indicated that mAb 5D5 reacted
with an epitope common to V0, V1, and V2 isoforms and pre-
sumably reside within the initial segment of one of the globular
domains (Fig. 3B; no attempt was made to define which of these
was the one implicated).

Immunolabeling of tissue sections with this mAb and the
panel of anti-versican antibodies described hitherto detected a
substantial versican deposition in most tissues expressing sig-
ificant mRNA levels (Table II; Fig. 2). These observations
thereby confirmed previous immunochemical mappings of ver-
sicans V1 and V2 (8). The immunostaining patterns further
highlighted the abundance of versican in vascular structures of

![Diagram of versican isoforms and protected fragments](image)

**Fig. 1.** Quantitative analysis of versican isoform expression in healthy adult human tissues as determined by RNase protection. A, schematic diagram illustrating the primer combinations used for the quantification of the different versican transcripts (sizes of fragments are indicated within brackets). B–E, relative levels of V0–V3 mRNA expression in the tissues indicated by the corresponding number listed in the boxed area (upper left). The relative level of transcription of each isoform in a given organ/tissue was derived by normalization against the signal obtained for the organ/tissue that showed the highest expression of that specific isoform (see Experimental Procedures). Insets show representative protected bands in the indicated tissues.
The single letter code refers to immunolabeling of: larger arteries and veins (AV), smaller vessels (SV), and capillaries (C). No significant change in immunoreactivity when compared to untreated versican; ↑, 50–40% increase and 30–70% decrease in relative immunoreactivity, respectively. Enzyme abbreviations: Ch-ase, chondroitinase; Neu-ase, neuraminidase; N-Gly-ase, N-glycanase; Endo-ase, endoglycosidase H/F; Fuc-ase, fusidase.

* Immunized versicans were treated with the indicated enzymes and reacted with the antibodies in ELISA and dot-blot assays. Similar results were obtained with versicans isolated from bovine and human aorta and human ovarian follicular fluid, whereas no cross-reactivity was detected with a number of aggrecans from human, bovine and chick cartilage of the nasal and articular type, bovine and human tendon fibromodulin, human and murine perlecan, bovine cartilage decorin and biglycan.

† Equivalent results were obtained after digestion with chondroitinases ABC and ACII.

‡ Reacts with a recombinant fragment corresponding to the C-terminal lectin-binding G3 domain of human versican (kindly received from Anders Asberg, Department of Cell and Molecular Biology, University of Lund, Sweden); Key: —, no immunoreactivity; * +, immunoreactivity; +, no change in immunoreactivity when compared to untreated versican; —, —, no different versican isoforms were produced by these vascular structures.

versican variant revealed by these immunoprobes was not necessarily linked to diverse glycosaminoglycan composition, but rather were associated with divergent oligosaccharide profiles. For instance, mAbs 5C12 and 2C12 reacting with epitopes on fucose-containing oligosaccharide moieties of versicans failed to stain larger vessels of the dermis and skeletal muscle (Table II; Fig. 2). By contrast, mAb 2G5 reactive with an epitope present on N-linked type oligosaccharides that appeared unique for versicans, labeled larger vessels of these tissues, but failed to stain capillaries of any of the tissues/organs examined (Table II; Fig. 2).

We could also conclude that the differences in the observed staining patterns were not attributed to a diverse expression of different versican isoforms produced by these vascular structures because both mAb 2B1 and an antiserum reactive with the canonical N-terminal G1 domain consistently detected the PGs in all types of vascular structures, and did so in a rather uniform manner (Table II; data not shown). Although less likely, it cannot be precluded that some differences in immunolabeling may have been a result of a different accessibility of the epitopes in situ. Versican Isoform Transcription in Isolated Endothelial Cells—The in situ versican analysis indicated that not only could different isoforms be differentially distributed in human tissues/organs and their vascular compartments, but that a second level of polymorphism could be provided by different glycansation traits of the PGs deposited in these structures. However, these observations did not clarify whether versican, which is known to be a primary PG of smooth muscle cells, was also produced and secreted by endothelial cells. To approach this problem, we examined the versican transcription/translation in a number of primary human endothelial cells isolated from both arterial and venular blood vessels. These experiments showed that most endothelial cell types examined herein transcribed certain levels of two or all three of the V0–V2 isoforms, but consistently failed to express V3 (Fig. 3, C and D). However, immunohistochemistry with a number of antibodies directed against the globular or non-globular domains of versican failed to detect a significant secretion of any of these isoforms in cells maintained in resting conditions (Fig. 4, A and D). In contrast, a corresponding staining of isolated vascular and non-vascular smooth muscle cells with the same set of antibodies confirmed their deposition of elaborate versican-rich matrices (data not shown). Immunostaining of endothelial cells with antibodies to decorin, perlecan, collagen type VI, fibronect-
tin, and native chondroitin sulfates ascertained that the endothelial cells were capable of synthesizing other PG and non-PG endothelial growth medium molecules (not shown). Hence, the lack of versican synthesis in resting endothelial cells was not directly associated with a compromised ability of the cells to produce and release this type of macromolecules in the adopted culture conditions.

Modulation of Versican Expression by TNF-α/H9251 and VEGF—To determine whether expression of versican required activation of the cells, such as those elicited by cytokines and growth factors during inflammatory and wound-healing processes, and also implied an alteration in the isoform expression, resting endothelial cells were treated for different time periods with TNF-α or VEGF. In all endothelial cell types tested, and independently of the stimulating agent, transcription of the V3 isoform was induced within 4 h following stimulation (Fig. 4). A switch in isoform expression was also noted in human pulmonary artery endothelial cells after treatment with both TNF-α and VEGF, which coincidently elicited a down-regulation of the parental V0 isoform (Fig. 4). In endothelial cells, de novo expression of the V3 isoform was accompanied by the capability of the stimulated cells to secrete detectable amounts of versicans with different glycanation traits (Fig. 4, B, C, E, and F; data not shown). However, there was no possibility of discriminating between synthesis/secretion of the V1 and V2 isoforms from that of the parental V0 one, based solely upon the observed reactivity of the available antibodies. Notable was also the fact that, in contrast to the situation in smooth muscle cells, most of the versican synthesized by the endothelial cells was retained within the cytoplasm. This apparent intracellular retention did not seem to be influenced by the cell density and, hence, did not appear to depend on the degree of cell-cell contact.

We next examined whether stimulated endothelial cells were also capable of translating the up-regulated V3 mRNA. For this purpose, cell lysates were resolved by SDS-PAGE and immunoblotted in parallel with anti-G1 (mAb 12C5 or anti-Vc antiserum) and anti-G3 antibodies (mAb 2B1). In both cases a band at 65 kDa was detected in stimulated, but not in untreated, endothelial cells (Fig. 5).

Versican Expression in Endothelial Cells Migrating in Vitro—The transition from stationary to motile status in endothelial cells engaged in wound-healing processes is known to cause overall changes in gene expression. Therefore, to determine whether endothelial cells also altered their versican isoform expression and initiated synthesis of the PG when triggered to migrate, we adopted the classical in vitro model of “wound healing” simulation, i.e. the so-called “scratch assay.” HUVEC were allowed to grow to confluence under normal conditions and then a central area of the monolayer was scraped away. Within 24 h, endothelial cells had migrated to repopulate the empty culture surface area and under these conditions, the V3 isoform became detectable by RT-PCR already at 10 h following wounding (Fig. 6A). V3 expression further increased during the subsequent 14 h needed to complete the full coverage of the blotted surface area by the migrating cells (Fig. 6A). During this time interval, there was also a progressive V0/V2 versican synthesis, as detected by immunolabeling with mAb 5D5 (Fig. 6B). Conversely, there was no
obvious evidence for a significant secretion of the macromolecules, which seemed to remain confined to the cytoplasm.

Versican Production by Neoaangiogenic Endothelial Cells—

Immunohistochemical stainings of soft tissue sarcoma lesions demonstrated that versican expression was not only restricted to normal vessels, but occurred in tumor-associated capillaries and in the stromal component of the lesions (Fig. 7, A and B). This suggested that neoaangiogenic endothelial cells were fully capable of producing versican. This observation prompted us to investigate whether induction of capillary formation in primary endothelial cells, which is known to involve both cell movement and changes in cell-cell contact, was also effective in altering the transcription/translation of endothelial versican. For this purpose, HIAEC and HUVEC were grown in Matrigel or fibrin clots, in the presence or absence of VEGF and examined for their ability to transcribe and synthesize versican. In these conditions, cells organized into capillary-like structures within 72 h after plating (Figs. 7, C and D, and 8, A and B); maintained their constitutive V0–V2 isoform transcription, with the exception of HUVEC that consistently failed to express the V1; and additionally attained the ability to transcribe the V3 isoform (Fig. 7). Immunocytochemical stainings of these endothelial formations further demonstrated that neoangiogen...
genic endothelial cells acquired the capability to translate the versican mRNA into final PGs (Fig. 8, C–F). In fact, positive staining with mAb 5D5 was seen in HIAEC and their versican secretion was slightly enhanced in the presence of VEGF. Notably, most of the secreted versican appeared to remain associated with the tubule-like structures.

**DISCUSSION**

Previous studies have shown that versican is a ubiquitous endothelial growth medium component of human adult connective, supportive, and nervous tissues and that it is detectable in most of the principal organs of the body. Characteristically, cells with mesenchymal/fibroblastic traits are believed to express the V1 isoform as their predominant versican PG (6, 7, 12, 17, 42), whereas the V2 isoform has been proposed to be particularly enriched in adult human, bovine, and rat brains (5, 10, 13, 22, 43, 44). Both the V0 and V3 isoforms are known to be expressed in a limited number of tissues (23, 24, 35). Our comprehensive RT-PCR analysis of the versican isoform expression in the adult corroborates the data reported in these previous investigations, but refutes some other previous observations/conclusions. For instance, although we were able to assert the hypothesized ubiquity of the V1 isoform in the human body, we did not find evidence for V0 expression in some other tissues/organs such as liver. In this latter organ, V3 was also absent, suggesting that different isoform patterns may exist during fetal life and adulthood. This notion was particularly evident when considering that the V0 isoform appeared to be the prevalent one during early embryonic development (2, 3), but was seemingly the least represented in adult tissues. The quantitative analysis of isoform expression also showed that versican V0 was indeed found to be transcribed in the tissues previously reported to express this isoform, but its actual transcriptional levels in these tissues could be considered largely negligible. Similarly, versican V3 was found to be rather scarcely expressed in tissues to be considered of "reference" for this isoform, such as skin and brain. In addition, the greater levels of V1 versus V2 mRNA found here in normal human adult brain, and the higher amounts of V2 molecules reported to be extractable from bovine and rat brains (10, 44), indicate that these isoforms could be differentially translated in different species. A precise tissue distribution mapping of the V0 and V3 isoforms at the protein level awaits the availability of probes that would be capable of discriminating between these isoforms and distinguish them from V1 and V2.

Lower M<sub>r</sub> (50–70 kDa) versican polypeptides free of glycosaminoglycan chains have been identified immunochemically in brain, skin, and cartilage (5, 12, 13, 39, 45) using the anti-G1

FIG. 6. *Changes in versican isoform expression in endothelial cells induced to migrate in a wound-healing model in vitro.* A shows representative phase-contrast micrographs and the corresponding RT-PCR detection of versican isoform expression in HUVEC repopulating the central area of the culture dish at the indicated time intervals following scraping of a confluent monolayer. B shows representative immunofluorescent detection of versican V0/V2 expression in the same cells at the indicated time intervals, as revealed by staining with mAb 5D5.

FIG. 7. **Versican deposition in neoangiogenic vessels of human soft tissue sarcoma lesions and patterns of isoform expression in primary endothelial cells forming capillary-like structures in vitro.** A, micrograph showing the characteristic versican deposition detected by mAb 5D5 in neovessels and stroma of a metastatic fibrosarcoma lesion that had formed in the lung of the patient. B, similar staining of a leiomyosarcoma primary lesion with mAb 5C12. C and D, tubule-like structures formed by HIAEC (A) and HUVEC (B) after 72 h culture in Matrigel.
mAb 12C5, but not in bovine brain using an antiserum directed against the N terminus of versican (10), or when using the anti-G3 mAb 2B1 in these different tissues. Thus, it is generally believed that the molecule detected in these previous studies corresponds to a G1-containing proteolytic fragment of versican, which may be analogous to the glial hyaluronate-binding protein originally discovered in brain extracts (45). Hence, it would not represent the intact V3 isoform. ADAMTS-generated G1-containing versican fragments have recently been identified in human aorta (46), thereby demonstrating a naturally occurring proteolysis mechanism by which versican fragments encompassing the G1 domain could be generated.

Previous studies on transformed endothelial cells of the mouse (47) and on normal and diseased blood vessels in vivo (8, 32) have suggested that versican could represent a widespread constitutive ECM component of the adult endothelium. The five different endothelial cell types examined here were indeed found to exhibit a diverse versican isoform expression in their resting conditions, reinforcing the idea that endothelial cells of diverse vascular beds may exhibit different phenotypic characteristics. However, when explanted, the cells were not capable of synthesizing detectable amounts of versican in their non-stimulated state, irrespective of their proliferation status (i.e. when growing at low density or to confluency) and the presence of serum factors. These observations suggested to us two notions. First, mature endothelial cells are not specified to produce versican cell-autonomously. Second, the previously noted augmentation in versican expression by smooth muscle cells and lung fibroblasts upon stimulation with cytokines and growth factors, such as interleukin 1β (48), platelet-derived growth factor, TGF-β (42), and heparin (activating fibroblast growth factors; Ref. 49) may be essential for sustaining endothelial versican synthesis.

Isolated endothelial cells constitutively transcribed several of the versican isoforms with the exception of V3. Following activation by the pro-inflammatory cytokine TNF-α, or by the primary endothelial growth factor VEGF, this pattern was altered and a de novo expression of V3 mRNA could be detected. The translated isoform could also be identified by immunohistochemistry using the combination of anti-G1 and anti-G3 antibodies, which detected an identically sized SDS-PAGE band. Due to the dual identification of the polypeptide with the above antibodies, it is unlikely that the immunoblotted band represented two distinct proteolytic fragments derived from the core protein termini of a larger versican isoform. Stimulated endothelial cells were also found to initiate versican synthesis upon growth factor or cytokine induction, but these PGs did not appear to be fully secreted suggesting that the post-translational machinery was only partly activated. Versicans produced by stimulated cells were not characterized biochemically, but the different reactivity of anti-glycan moiety antibodies suggested that they were only to a certain extent post-translationally modified as those deposited in situ.

The significance of the up-regulated V3 expression in activated endothelium is presently unknown. However, recent data on V3-overproducing rat smooth muscle cells show a negative effect of this isoform in cell growth and migration but an apparent ability to enhance the cell-substratum avidity of the manipulated cells (50). Furthermore, if overexpression of this versican isoform is forced in vivo by retroviral transfer into a ballooned rat carotid artery, it promotes neointima formation and the assembly of well structured elastic fibers (51). Overall, these previous findings suggest that induced expression of versican V3 in endothelial cells could influence their interaction with the microenvironment. However, it is presently unclear how this would occur since there is no unambiguous biochemical data in the literature demonstrating that this versican isoform is actually secreted by the cells. In fact, even when overexpressed in versican-producing cells in vitro and in vivo, V3 appears to be retained within the cytoplasm (50, 51),4 despite comprising both globular domains embodying the necessary “secretion-promoting information”. Thus, the possibility remains that V3 may not be secreted because of not being fully processed in the Golgi apparatus and, if so, that its effect on cell behavior may be mediated by its modulation of intracellular processes.

In conclusion, we find that in primary human endothelial cells, synthesis and secretion of one or more of the versican isoforms seems to be associated with both migratory and differentiation-like processes of the cells and is subordinate to their cell cycle progression. In fact, whether cells were stimulated by TNF-α or VEGF, involved in neoangiogenic processes, or engaged in a wound healing situation, they consistently initiated versican production and transcribed additional isoforms. In particular, in all cases, there was a de novo expression of V3. Whether this was simply a causal effect of the mitosis-associated migration/differentiation program evoked within the cells or whether it is a prerequisite for the ensuing of this program remains to be determined. Tissue injury is, however, known to be associated with an up-regulation of versican (35, 44) and other PGs, such as decorin, biglycan, and syndecans, as well as with changes in the glycanation profiles of these PGs. Thus, it is highly probable that trauma also modulates the transcription pattern of versican by triggering that of novel isoforms such as V3.

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4 T. Shinomura, personal communication.
Distribution of PG-M/Versican Variants in Human Tissues and de Novo Expression of Isoform V3 upon Endothelial Cell Activation, Migration, and Neoangiogenesis in Vitro

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