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Type VIII Collagen in Murine Development

Association with Capillary Formation *in Vitro*[a]

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INTRODUCTION

Initial studies on type VIII collagen were performed with cultured cells. From vascular and corneal endothelial cells, astrocytoma cells, and a limited number of other transformed and normal cellular populations, collagenase-sensitive polypeptides with molecular mass of 180 kDa (EC [endothelial collagen] 1), 125 kDa (EC 2), and/or 100 kDa (EC 3) were isolated that were structurally distinct from other collagen types (for a review, see Reference 1). These chains were related structurally, gave rise to pepsin-resistant fragments of 50 kDa (based on collagenous peptide standards) or 65 kDa (based on globular protein standards), were secreted independently of prolyl and lysyl hydroxylation, lacked large globular propeptides and interchain disulfide bonds, and were sensitive to a wide spectrum of proteases. These observations led to an extended chain or "interrupted helix" model for the structure of type VIII collagen, in which each of the three (possibly nonidentical) chains (180 kDa) that comprised the native molecule contained pepsin-resistant (G-X-Y) domains (50 kDa) that were connected by noncollagenous sequences. An alternative model describes type VIII collagen as three identical chains of 61 kDa that form a triple helix with noncollagenous domains at each end.[2]

Resolution or replacement of these models required biochemical characterization of protein isolated from tissues. A major contribution was the isolation of pepsin-resistant fragments of type VIII collagen from bovine corneal Descemet's membrane, a tissue that is particularly enriched in this protein.[3] Purification and sequence analysis of two fragments (M_r 50 kD) from pepsin-extracted Descemet's membrane showed unique N-terminal regions containing several interruptions within (G-X-Y) triplet repeats.[4] Monoclonal antibodies specific for the 50-kD fragments showed that type VIII collagen had a limited distribution in the fetal calf: it was prominent in peri-

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chondrium/periosteum and, to a lesser extent, in cartilage matrices. Moreover, several derivatives of the neural crest stained positively for type VIII collagen, including Descemet's membrane, ocular sclera, meninges surrounding the brain and optic nerve, cranial mesenchyme, and glial cells.[5]

In the present study, we describe temporal and tissue-specific expression of type VIII collagen in the developing mouse. Based on immunohistological localization, selected tissues were extracted and probed for type VIII collagen by immunoblot analysis. The results provide evidence that tissue-specific posttranslational processing occurs, that more than one type of α chain is present, and that type VIII collagen might function in endothelial cell differentiation and organization.

MATERIALS AND METHODS

Cell Culture and Metabolic Labeling

Human astrocytoma cells (251-MG) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum.[6] Metabolic labeling and pulse-chase studies were performed in the presence of 64 μg/ml of β-aminopropionitrile (β-APN) and 50 μg/ml sodium ascorbate as previously described.[6]

Bovine aortic endothelial (BAE) cells were grown in DMEM containing 10% fetal calf serum. Certain strains of these cells, isolated by dilution cloning from parent endothelial cultures, manifested a sprouting phenotype as described by Cotta-Pereira *et al.*[7] This growth pattern is characterized by a transition from polygonal to elongated cells, lack of contact inhibition, and the eventual establishment of a network of branching, hollow, tube-like structures. In this study, monolayers and spontaneously forming endothelial tubes were examined immunohistologically for the presence of type VIII collagen (see below).

Extraction of Type VIII Collagen from Tissues

Type VIII collagen was isolated from pepsin-treated bovine corneal Descemet's membranes by differential salt precipitation followed by molecular sieve chromatography under denaturing conditions as described by Kapoor *et al.*[4] These polypeptides migrated on SDS-PAGE with apparent M_r of 50,000 (based on collagenous peptide standards) or 65,000 (based on globular protein standards). Further purification of the pepsin-resistant type VIII collagen fragments was achieved by high performance liquid chromatography (HPLC), which resolved 2 unique components (50K-A and 50K-B).[4] Peptides derived from CNBr digestion of 50K-A and 50K-B were also purified by HPLC prior to amino acid sequence analysis. Several peptides were subjected to automated Edman degradation as previously described,[4] and sequencing was performed by Dr. W. R. Gray (University of Utah, Salt Lake City, UT). Organs from embryonic mice were homogenized in 1 ml 0.01-M ethylenediamine tetraacetic acid (EDTA) (pH 7.5), 5 mM benzamidine HCl, 5 mM N-ethylmaleimide, and 0.01 M ϵ-amino-*n*-caproic acid, and were then incubated for 1 h at room temperature after addition of 10 μl Triton X-100 to the homogenization buffer. NaCl was subsequently

added to a final concentration of 1 M, and samples were incubated in the presence of DNase II (Sigma Chemical Co., St. Louis, MO) (0.5 mg/ml) for 1 h at room temperature.[4] One-half of each sample was acidified in 0.5 M acetic acid prior to digestion with pepsin (Serva, Heidelberg, FRG) (0.25 mg/ml) for 12 h at 0 °C. All samples were lyophilized and analyzed by SDS-PAGE on 8% polyacrylamide gels.

Immunological Studies

Polyclonal antibodies against native type VIII collagen (50K-A and 50K-B) from bovine Descemet's membrane were raised in rabbits as previously described.[4] The antisera were fractionated by precipitation in 20% ammonium sulfate (weight to volume ratio), and IgG was further affinity adsorbed on a Sepharose-type V collagen column. By both immunoblotting and ELISA, the preparation showed reactivity specifically toward type VIII collagen and did not recognize collagen types I-V, bovine serum albumin, laminin, or fibronectin.[4]

Immunoblotting was performed essentially as described by Kapoor *et al.*,[4] with the following modifications: lyophilized samples were solubilized in SDS-PAGE buffer, and the proteins were resolved on 7% SDS-polyacrylamide gels. The separated proteins were electrotransferred to nitrocellulose membranes for 16 h at 4 °C. Nonspecific binding was blocked by incubating the nitrocellulose membranes with 0.1% nonfat dry milk, 0.05% Tween in phosphate-buffered saline (PBS) containing 0.1% sodium azide for 16 h at room temperature. The blots were next incubated with rabbit anti-bovine type VIII collagen antibody (diluted 1:1000 in PBS), washed with several changes of blocking solution, and subsequently incubated with 1×10^7 cpm of [^{125}I]-Protein A (immunological grade, New England Nuclear). The nitrocellulose membranes were washed with PBS (0.05% Tween), air-dried, and exposed to an X-ray film (Kodak) at −70 °C.

Localization of type VIII collagen in mouse tissues was performed by means of an immunoperoxidase technique. Embryos were isolated from mated mice on days 11 to 19 of gestation (24 h after the appearance of a vaginal plug was considered the end of day 1).

Several fixation protocols were tested in an attempt to retain immunoreactivity and improve tissue preservation. Fixation in Karnovsky's solution for 3 h at 4 °C provided the best results. Tissues were embedded in paraffin. Sections of 5 μm were dehydrated and rinsed with 70% methanol containing 3% H_2O_2 for 30 min to inactivate endogenous peroxidases. Nonspecific binding was reduced by treatment with 1% normal goat serum in PBS for 2 h at 4 °C. Subsequently, the sections were exposed to the primary antibody at a dilution of 1:100 in PBS, in a humidified chamber at 4 °C for 1-2 h. After extensive washes in PBS, the sections were exposed sequentially to biotinylated goat anti-rabbit IgG for 1 h, several rinses in PBS, and an avidin-biotin-peroxidase complex for 30 min at 4 °C (ABC Reagent, Vector Labs, Burlingame, CA).[8] The complex was developed by exposing the cells to a solution of 3-3' diaminobenzidine-4 HCl (1 mg/ml in 0.05 M Tris-HCl, pH 7.5, containing 0.02% H_2O_2) for 10 min. After a rinse in tap water, the sections were counterstained with a solution of 0.5% toluidine blue, dehydrated, clarified, and mounted in Permount. Photomicroscopy was performed with a Zeiss photomicroscope II and Kodak color 160 ASA film.

RESULTS AND DISCUSSION

Biosynthesis of Type VIII Collagen in Vitro

To ascertain the molecular size of newly synthesized intracellular type VIII collagen, pulse-chase experiments were performed on a human astrocytoma cell line (FIG. 1). These cells produce predominantly type VIII collagen and a low level of type IV collagen; other types have not been observed.[6] When cell layers (CL), pulsed for 5 min with [^{3}H]-proline, were lysed directly in hot SDS-PAGE sample buffer containing dithiothreitol (DTT) and run immediately on a 5% acrylamide gel, a band of molecular mass 125 kDa (arrow) was observed 25 min after removal of the radiolabel. This band was degraded by bacterial collagenase (data not shown) and exhibited a lower mobility on SDS-PAGE in the hydroxylated form (compare lanes with protein synthesized in the presence of ascorbate with lanes with protein synthesized in the absence of ascorbate). A protein of similar molecular mass was also observed in the culture medium (FIG. 1, M), within 15 min after 1-h pulse. This band was both collagenase- and ascorbate-sensitive, and accumulated in the culture medium for up to 3 d with no apparent processing.[6] In BAE cells, the 125-kDa component (EC 2) was also the major secreted form of type VIII collagen, with lesser amounts of EC 1 (180 kDa) and EC 3 (100 kDa).[1] At no time during their synthesis or secretion were the chains disulfide-bonded.

It therefore appeared that the initial biosynthetic product of type VIII collagen in human astrocytoma cells was the 125-kDa chain, which was later shown to react with a monoclonal antibody directed against the pepsin-resistant fragments of Descemet's membrane type VIII collagen.[5] The molecular sizes that we have reported for type VIII collagen secreted *in vitro* (180, 125, and 100 kDa) are different from the

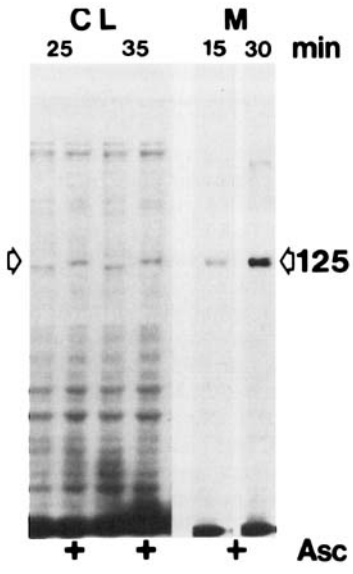


FIGURE 1. Biosynthesis of type VIII collagen *in vitro:* pulse-chase analysis. Human astrocytoma cells in serum-free DMEM containing β-APN were pulsed with 20 μCi/ml of [^{3}H]-glycine and 40 μCi/ml of [^{3}H]-proline; chases in nonradioactive medium containing Gly and Pro are shown from 5-35 min (m). Cell layers (CL, left panel) were labeled for 5 min, washed twice, lysed after 25- and 35-min chases directly into hot SDS-PAGE sample buffer, and resolved on a 5% polyacrylamide gel under reducing conditions. The first of each pair of lanes was labeled in the absence and the second in the presence (+) of ascorbate (Asc). *Hollow arrow* indicates an intracellular ascorbate-sensitive protein of M_r 125,000 that was apparent within 30 min after addition of radiolabeled amino acids. Proteins secreted into the culture medium (M, right panel) were recovered by precipitation in 10% trichloroacetic acid and resolved by SDS-PAGE as described for cell layers. Cells in this case were pulsed for 1 h in the presence of ascorbate and chased for 15 and 30 min. Within 75 min, a major biosynthetic product of M_r 125,000 was observed. (From Alitalo *et al.*[6] Reprinted by permission from *Journal of Biological Chemistry.*)

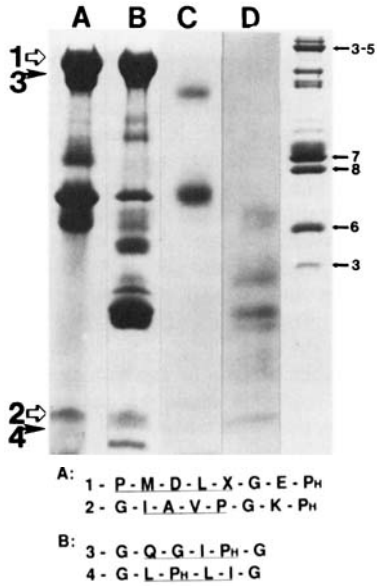


FIGURE 2. Amino acid sequences of interruptions within triple helix-forming domains of type VIII collagen CNBr-derived peptides. The 12.5% polyacrylamide gel shows a Coomassie blue stain (A, B) and a fluorescent autoradiogram (C, D) of type VIII collagen peptides produced by cleavage with CNBr. Type VIII collagen was isolated from pepsin-treated bovine Descemet's membrane (A, B) and from pepsin-treated radiolabeled culture medium proteins secreted by bovine corneal endothelial cells (C, D). Prior to cleavage with CNBr, two pepsin-resistant fragments (50 K-A; and 50 K-B), which displayed M_r of 50,000 by collagen standards and 65,000 by globular protein standards, were purified by HPLC; *lanes* A and C represent 50 K-A, while *lanes* B and D contain 50 K-B. The *lane* on the far right contains CNBr-derived peptides of type I collagen, which have been identified by numbers [e.g., 3 corresponds to α1(I)-CB3]. The proteins from cell culture correspond closely to those derived from tissue (i.e., C is similar to A, and D is similar to B). Much of the starting material in *lanes* A and B (peptide 1 and peptide 3, respectively) was uncleaved. Limited amino acid sequence analysis was performed on peptides 1 and 2 (*lane* A, *hollow arrows*) and peptides 3 and 4 (*lane* B, *solid arrows*), as shown in one-letter code. X indicates an unidentified residue, and P_H indicates hydroxyproline. Interruptions in the (Gly-X-Y) sequential triplets are *underlined.* (From Kapoor *et al.*[4] Reprinted by permission from *Biochemistry.*)

61-kDa chains recovered from rabbit corneal endothelial cell culture medium as described by Benya and Padilla.[2] These authors presented evidence for β- and γ-forms of parent α1(VIII) chains (61 kDa, based on collagen standards) that associated intermolecularly by noncovalent, acid-labile bonds. While pepsin treatment of EC 2 produced fragments of chain M_r 65,000 (based on globular protein standards), the pepsin-resistant fragment(s) of the 61-kDa collagen comigrated with a 43-kDa globular protein standard.[2] Although the reasons for the difference between type VIII collagen from rabbit corneal endothelial cells vs. bovine (aortic and corneal) endothelial and human astrocytoma cells are not clear, possible explanations include: (a) the existence of tissue- and/or species-specific isoforms; (b) differences in posttranslational processing, possibly relating to its biosynthesis *in vitro;* (c) the presence of more than one type VIII collagen chain; and (d) the use of alternate transcriptional mechanisms (e.g., differential splicing or start site utilization) by the same type VIII collagen gene. We have begun to examine some of these possibilities by characterizing type VIII collagen produced *in vivo,* as shown in the succeeding figures.

Sequence Analysis of Type VIII Collagen Purified from Descemet's Membrane

Type VIII collagen was extracted from pepsin-treated bovine Descemet's membranes, and two fragments were purified to homogeneity by HPLC.[4] As shown in FIGURE 2, these fragments were subjected to cyanogen bromide (CNBr), and the

cleavage products (shown in lanes A and B) were subsequently resolved by HPLC. Amino acid sequences from the uncleaved, pepsin-resistant fragment (A, peptide 1 and B, peptide 3) and from 2 CNBr peptides (A, peptide 2 and B, peptide 4), are shown below the gel pattern in FIGURE 2. Sequences were selected that showed interruptions within the (G-X-Y) triplet repeats. These "non-triple-helical" sequences were not similar to those present in the triple-helical segment of the $\alpha 1$, $\alpha 2$, or $\alpha 3$(VI) collagen chains—a type that contains several interruptions.[9] The discontinuities in type VIII collagen, however, resembled the kinds of imperfections in the triple helices of types IX and X collagen: GXGXY and GXYXYG.[10] The significance of this apparent similarity cannot be appreciated until the complete sequence of type VIII collagen is available. This information would also establish whether the two pepsin-resistant fragments (FIG. 2, lanes A and B) are contained within the same chain or represent different chains of type VIII collagen.

FIGURE 2 also shows the CNBr peptide patterns of type VIII collagen synthesized by bovine corneal endothelial cells *in vitro* (lanes C and D). Two fragments from pepsin-treated EC 2 were resolved by HPLC, cleaved with CNBr, and separated by SDS-PAGE. The pattern of fragments shown in lane C matches that in lane A, and peptides in lane D are similar to those in lane B. These data confirm that the type VIII collagen chain or chains produced *in vitro* correspond structurally to those extracted from tissue.

Synthesis of Type VIII Collagen during Murine Development

In an earlier study we showed that type VIII collagen was present in several fetal bovine tissues that were derived from the neural crest.[5] These tissues included calvarium, meninges of brain and spinal cord, ocular sclera, and Descemet's membrane. Type VIII collagen has also recently been observed in astrocytes and Müller cells of the day-3 postnatal rat retina (T. Reh and H. Sage, unpublished observations). In addition, fibrillar staining was apparent in perichondrium and periosteum, while certain cartilage matrices exhibited a more diffuse pattern corresponding to anti-type VIII collagen IgG-reactive epitopes. Given the rather limited distribution of this collagen and its appearance during fetal calf development, we surmised that it might perform a role in the ontogeny of certain organs during mammalian embryogenesis. Using polyclonal, anti-type VIII collagen IgG specific for EC 2 and the 65-kD pepsin-resistant fragments of type VIII collagen (FIG. 2, 4, 5), we stained sections of staged whole mouse embryos. Controls included the use of preimmune IgG from the same animal prior to injection of antigen, and the omission of primary antibody. In both cases, no staining was observed.

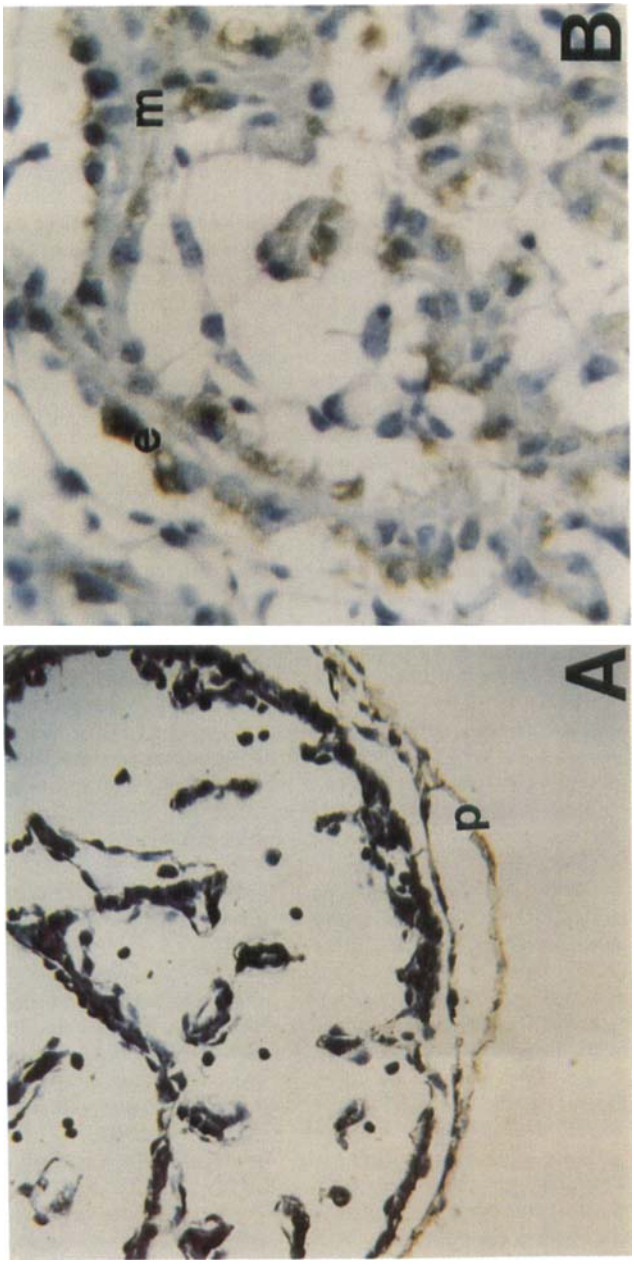
Positive, fibrillar stain specific for type VIII collagen was observed in 11-18 d cranial mesenchyme, in 8-d and 18-d placental capillaries, and in the developing heart from 11 to 17 d. An example of the appearance of type VIII collagen during murine cardiogenesis is shown in FIGURE 3. Both the pericardium (p) and premyocardium were stained in the 12-d heart (FIG. 3A). FIGURE 3B shows in greater detail the presence of type VIII collagen-positive endothelial cells (e) and cardiac myocytes (m), which form trabeculae of heart tissue at this relatively early developmental stage. In the 17-d embryonic heart, staining for type VIII collagen was confined to the subendothelium (including smooth muscle cells) (s) of the large vessels associated with this organ (FIG. 3C). By day 18 (just prior to birth), type VIII collagen could no longer be detected immunohistologically in the embryonic heart (FIG. 3D). The antibodies also recognized epitopes in hearts of chicken embryos (data not shown).

Western blotting was performed on whole mouse embryonic and neonatal organs with anti-type VIII collagen IgG. Similar results were obtained with both unextracted tissue (e.g., lysed directly in hot SDS-PAGE sample buffer) and tissue extracted as described in the legend to FIGURE 4. Several immunopositive bands appeared in embryonic and postnatal hearts, as indicated in FIGURE 4A by the hollow arrows with their corresponding globular molecular weights. During embryogenesis (12, 15, and 18 d), the principal species were 125 kDa, 65 kDa, and 55 kDa. After birth (2, 5, and 14 d), there was a general decrease in molecular size, with major bands appearing at 55 kDa, 40 kDa, and 15 kDa. With increasing age, there was an apparent accumulation of the 40-kDa and 15-kDa species, and a decrease in the 55-kDa species. Some bands of higher molecular mass were also present (e.g., 175 kDa and 125 kDa). Pepsin-treated hearts from the same developmental stages produced a different spectrum of anti-type VIII collagen IgG-reactive proteins, as shown in FIGURE 4B. At the embryonic ages tested, cardiac type VIII collagen was not pepsin-resistant (12, 15, and 18 d). In contrast, after birth, there were several immunoreactive species of 250 kDa, 150 kDa, 70 kDa (a doublet), and 60 kDa. Bands of similar molecular mass were also seen in the 14-d lung and thymus, the most prominent of which migrated with an apparent molecular mass of 70 kDa (FIG. 4B). These results, as well as data from the brain at similar developmental stages, have been summarized in TABLE 1.

The presence of several proteins that reacted with the polyclonal antibodies specific for type VIII collagen and the occurrence of certain molecular size classes within a given tissue are suggestive of (a) complex posttranslational processing and/or cross-linking; (b) tissue-specific isoforms; or (c) several chains comprising the type VIII collagen molecule. The 125-kDa chain present in the heart, brain, lung, and thymus could correspond to the EC 2 polypeptide that has been described in studies performed with cultured cells.[1] Similarly, the 175-kDa species could correspond to EC 1. The 65-kDa and 55-kDa forms could be processed from the 125-kDa polypeptide or could correspond to another chain of type VIII collagen. The three smaller polypeptides (55 kDa, 40 kDa, and 15 kDa), which were seen principally in postnatal hearts, appear to have resulted from processing of the 125/65-kDa precursor(s) (TABLE 1). The 61-kDa α1(VIII) chain from rabbit corneal endothelial cells described by Benya and Padilla,[2] which exhibited a molecular mass of approximately 80 kDa based on globular protein standards, was not observed. Explanations for this absence include both species- and tissue-specific gene products and/or processing at the posttranslational level.

It was interesting that type VIII collagen in the embryonic heart did not survive incubation with pepsin. However, the protein in embryonic brain tissue exhibited pepsin-resistant, triple-helical regions (TABLE 1). This result again could be related to differences in the rates of maturation of these two tissues, which would be reflected in the degree of cross-linking or other posttranslational changes. A 70-kD pepsin-resistant fragment of type VIII collagen was found consistently in postnatal mouse heart, lung, and thymus, but a 43-kDa pepsin-resistant α1(VIII) form, as described in the rabbit corneal endothelium,[2] was not observed. The significance of these apparently triple-helical regions of type VIII collagen will not be appreciated until the overall structure of the molecule has been elucidated.

Since type VIII collagen was initially described in vascular endothelial cells but was not subsequently detected in calf aortic endothelium,[5] it was of interest to ascertain the distribution of this protein in the early embryonic vasculature. As shown in FIGURE 5A, type VIII collagen was localized specifically to capillaries of the 18-d mouse placenta, whereas the endothelial cells lining the maternal sinuses (s) were negative. Similar results were obtained with 8-d placental tissue (data not shown). Since we had previously proposed that type VIII collagen might be synthesized by endothelial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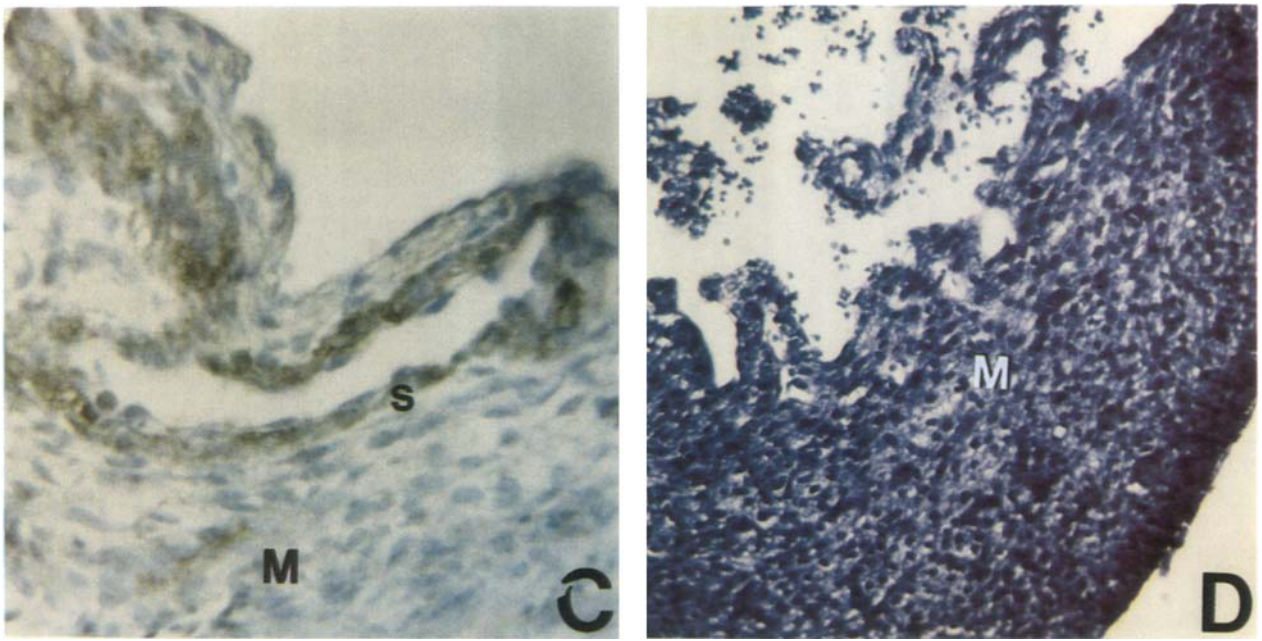

FIGURE 3. Appearance of type VIII collagen during murine cardiogenesis. Hearts from 12-d (**A**), 13-d (**B**), 17-d (**C**), and 18-d (**D**) mouse embryos were fixed in Karnovsky's solution and embedded in paraffin. Five-μm sections were exposed to anti-type VIII collagen IgG, and immune complexes were visualized by the peroxidase technique in conjunction with avidin, biotin, and diaminobenzidine. Sections were counterstained with toluidine blue. Immunopositive type VIII collagen, visualized as a brown reaction product, was present in the pericardium (p), as well as the endocardium (e) and premyocardium (m), of 12-d and 13-d embryonic hearts (A, B). At 17 d (C), staining was largely confined to the subendothelium (s) of large vessels associated with a more fully developed myocardium (M). At 18 d (D), type VIII collagen was no longer apparent by immunohistological techniques in the embryonic mouse heart. Magnification: 65× (A and D); 152× (B and C).

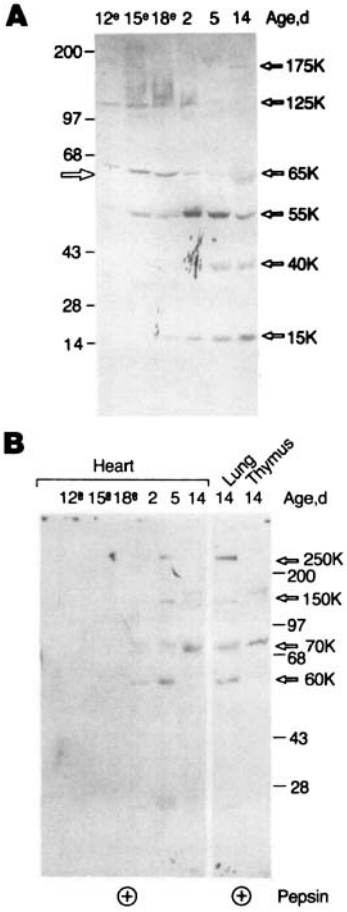

FIGURE 4. Identification of type VIII collagen in mouse tissues by immunoblotting. Tissues from 12-d, 15-d, and 18-d mouse embryos (e) and from 2-d, 5-d, and 14-d neonates were homogenized in the presence of protease inhibitors and 1% Triton X-100 and treated with DNase II. One-half of each sample was digested with pepsin (0.25 mg/ml) in 0.5 N acetic acid at 0 °C for 12 h. Samples were lyophilized, resolved by SDS-PAGE (7% gel) under reducing conditions, electrophoretically transferred to nitrocellulose membranes, and exposed sequentially to anti-type VIII collagen IgG and [^{125}I]-Protein A. Panel **A** shows a fluorescent autoradiogram of heart tissue prior to pepsin treatment. Major immunoreactive bands have been denoted by *hollow arrows* on the *right,* followed by an estimated molecular mass in kilodaltons based on globular protein standards (shown on the *left*). *Large hollow arrow* on the *left* indicates the position of migration of a pepsin-extracted type VIII collagen standard from bovine Descemet's membrane (M_r 65,000). Major immunoreactive bands of 125 kDa and 65 kDa were apparent in embryonic hearts. After birth, these species were significantly diminished, and bands of 55 kDa, 40 kDa, and 15 kDa were present. Panel **B** represents heart tissues from the same developmental stages as shown in A, as well as 14-d postnatal lung and thymus, after pepsin treatment ⊕. Mobilities of globular protein molecular weight standards are indicated; major immunoreactive bands are indicated by *hollow arrows* on the *right.* In embryonic (e) heart tissues, type VIII collagen bands of 125 kDa and 65 kDa (see A) were extensively degraded by pepsin. After birth, pepsin-resistant species were apparent (250 kDa, 150 kDa, 70 kDa [doublet], and 60 kDa). A major band of 70 kDa was also present in 14-d lung and thymus.

cells at phases of cellular growth that occur during embryogenesis or culture shock,[1,5] we examined the distribution of this protein in capillary tube-like structures formed from BAE cells *in vitro.* The endothelial cells contributing to these tubes stained positively for von Willebrand factor and demonstrated endocytosis of acetylated low density lipoprotein, two criteria that attested to the homogeneity of the strains. Cross-sections of the branching networks revealed lumina enclosed by 1-4 endothelial cells.[11] Staining of a capillary-like tube formed beneath a confluent monolayer of BAE cells with anti-type VIII collagen IgG is shown in FIGURE 5B. Type VIII collagen is normally not synthesized in significant amounts by confluent BAE cells.[1] However, type VIII collagen-positive cells were apparent in the walls of the growing tubes, while most areas of the quiescent monolayer were negative. Immunostaining of these cultures with antibodies against types IV and V collagen produced negative results; type III collagen, which is constitutively produced by BAE cells, stained the extracellular matrix fibrils surrounding these structures (data not shown). Type VIII collagen might therefore play a role in endothelial cell organization and differentiation, as shown in this system of angiogenesis *in vitro.*

Significance of the Distribution of Type VIII Collagen in the Mouse Embryo

Type VIII collagen was first detected immunohistologically in the embryonic mouse heart at day 11 of development. It was a prominent component of the pericardium, epimyocardium, and possibly the endocardium. In a manner similar to that described for the origin of vascular primordia, the heart is thought to be derived from the endocardial (endothelial) tube within the splanchnic mesoderm (this process is understood best in the chicken embryo). Markwald and coworkers have described an epithelial-mesenchymal transition during the formation of atrioventricular mesenchyme in the developing chicken heart.[12] Formation of prevalvular mesenchyme, which later gives rise to the valves and septa of the heart, occurs by a regionally specific interaction of the atrioventricular endothelium with the myocardium. Endothelial cells are thought to invade the myocardial basement membrane (also referred to as "cardiac jelly") in response to an inductive signal from the myocardial extracellular matrix.[13] An interesting observation was the initiation of expression of type I procollagen by activated atrioventricular endothelial cells coincident with the formation of the prevalvular mesenchyme.[14] The presence of type VIII collagen at endocardial-myocardial interfaces in the developing mouse heart might facilitate a differentiation event that eventually leads to the morphogenesis of cardiac septa and valves by myocardial fibroblasts.

The initiation of type I procollagen synthesis was also seen during the assembly of cultured endothelial cells into capillary-like tubes, a process characterized by relatively high levels of type VIII collagen production (FIG. 5B). Since type I collagen is also found in the ventricular wall, the presence of type VIII collagen in 11-17 d ventricular myocardium is not inconsistent with its possible role as an extracellular matrix component that facilitates certain types of cellular differentiation. At day 17

TABLE 1. Temporal Sequence of Appearance of Immunoreactive Type VIII Collagen in Murine Tissues[a]

		Molecular Size (kDa)[b]	
Age	Tissue	Intact	Pepsin-Treated
Embryo			
12-18 d	Heart	125, 65, 55	ND[c]
	Brain	125, 65	135, 80, 65, 60
Neonate			
2 d	Heart	125, 65, 55	70, 60
	Brain	125, 65	
5 d	Heart	55, 40, 15	250, 150, 70, 60
14 d	Heart	175, 55, 40, 15	70
	Lung	125, 65	70
	Thymus	125, 65	250, 70, 60

[a] Tissues were processed in the presence of protease inhibitors and were treated with pepsin as described in MATERIALS and METHODS. Proteins in the presence of dithiothreitol were resolved by SDS-PAGE, transferred electrophoretically onto nitrocellulose sheets, and probed sequentially with anti-type VIII collagen IgG and [^{125}I]Protein A.

[b] All molecular weights were based on comparative migration with globular protein standards.

[c] No immunoreactive proteins were detected.

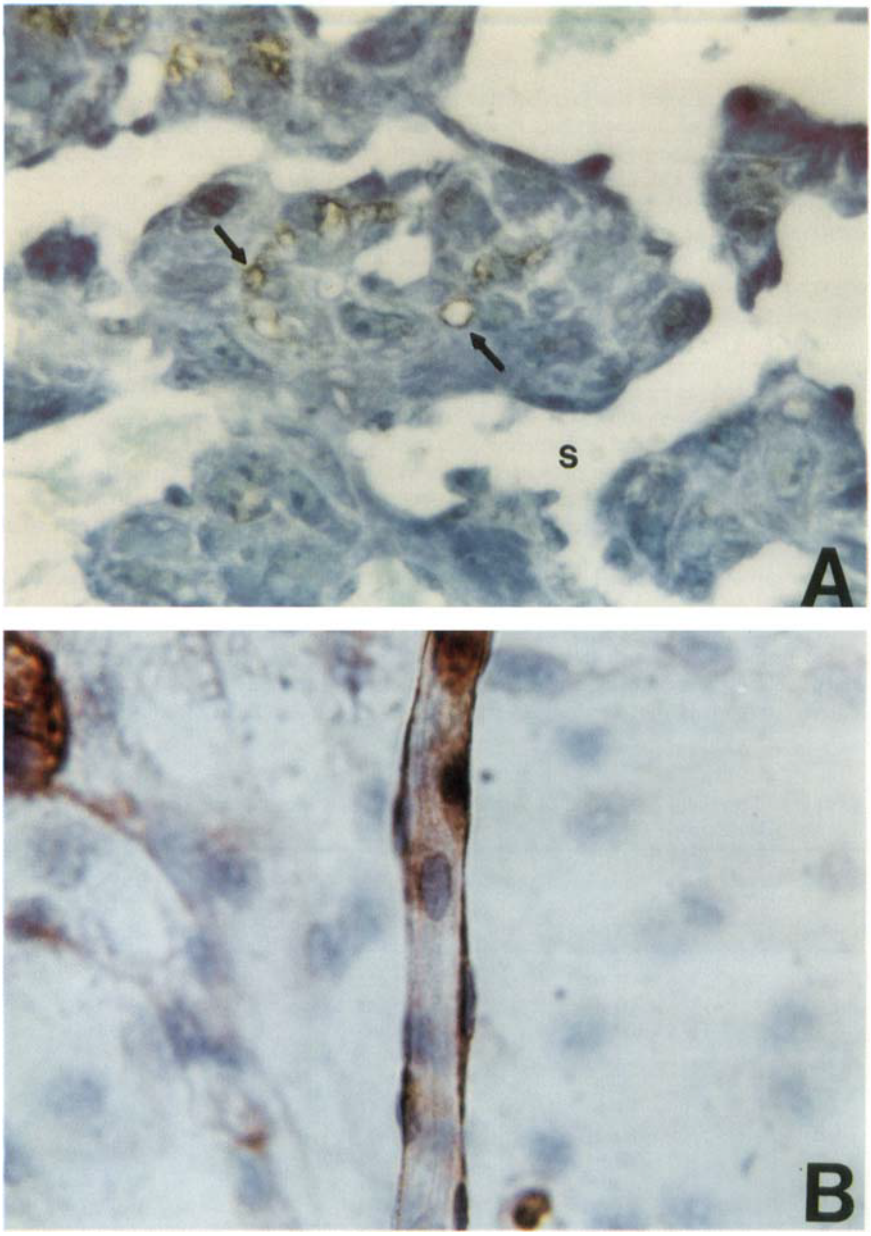

FIGURE 5. Distribution of type VIII collagen in placental capillaries and in cultured endothelial tubes. Placental tissue attached to 18-d mouse embryos (**A**) was fixed in Karnovsky's solution. Paraffin sections were exposed to anti-type VIII collagen IgG, and immune complexes were identified by staining with an avidin-biotin-peroxidase reagent and diaminobenzidine. Type VIII collagen was localized around numerous capillaries in the placenta (*arrows*) but was absent from endothelial cells lining the maternal sinuses (s). Confluent cultures of BAE cells, grown in

of murine embryogenesis, however, staining for type VIII collagen was largely confined to the subendothelium of large vessels associated with the heart, with sparse filaments still present in the well-developed myocardium (FIG. 3C). Although we could not detect this collagen in the 18-d heart, it was clearly present—most likely in a processed form—in late embryonic and neonatal animals, as shown by immunoblotting of extracted tissue (FIG. 4A). The immunoreactive epitopes on type VIII collagen may be masked at these later stages of cardiogenesis.

We have previously noted that there was often concurrent expression of types II and VIII collagen in fetal tissues (e.g., cornea, endochondral bone, and at epithelial-mesenchymal interfaces in neurocranium).[5] In this regard it is interesting that type II collagen has been found at the interface between the epimyocardium and endocardium of the developing chicken heart.[15] The function of type II collagen, like that of type VIII collagen, appears to be independent of chondrogenesis for some tissues.

Although several structural similarities have been noted between type VIII and type X collagen,[1,2] the distribution of these proteins in tissues is clearly different. In both avian and bovine tissues, monoclonal antibodies localized type X collagen to skeletal hypertrophic cartilage (e.g., fracture callus, growth plate).[16,17] This protein was found in no other tissues, with the exception of bone, which might contain diminished amounts of type X collagen.[17]

In conclusion, we suggest that type VIII collagen functions in certain aspects of cellular organization and differentiation. Moreover, its various molecular weight forms might reflect tissue- or species-specific processing, as well as the presence of several related chains. A situation analogous to that described for type VI collagen could pertain to the chain composition of the type VIII collagen molecule. Although the triple-helical domains of the $\alpha 1$, $\alpha 2$, and $\alpha 3$ chains of type VI collagen are similar in size (approximately 38 kDa), their intact molecular masses range from 140-250 kDa.[9] In this regard one could imagine an extended $\alpha 1$(VIII) chain (e.g., 125-180 kDa) forming a triple helix with two other α(VIII) chains (e.g., 65-80 kDa). The triple-helical domain would correspond to approximately 50kDa (based on collagenous protein standards). To reconcile the shortened helical and the extended chain models that have been proposed for type VIII collagen,[1,2] it might be pertinent to consider the structures of the homologous collagen types IX and XII. The $\alpha 1$ (XII) chain has an extended globular domain that is absent from the type IX collagen chains and is approximately seven times larger than the homologous triple-helical region.[18]

Although the α chains of types IX and XII collagen are encoded by distinct genes, they are clearly very closely related. By analogy, the various molecular weight species that have been ascribed to type VIII collagen may reflect several distinct but related gene products. Cloning of the type VIII collagen gene(s), in parallel with characterization of the protein structure and posttranslational processing, will prove invaluable in advancing our understanding of this unusual collagen.

DMEM containing 10% fetal calf serum, developed a sprouting phenotype and, within 1-2 months, formed extensive networks of capillary tube-like structures. One of these tubes, shown in panel **B**, has been stained with anti-type VIII collagen IgG and avidin-biotin-peroxidase reagent. Cells forming the walls of the tube stained positively for type VIII collagen, while quiescent cells in the monolayer to the right of the tube were generally negative. Counterstains were toluidine blue (A) and hematoxylin and eosin (B). Magnifications: 152× (A) and 255× (B).

SUMMARY

Bovine endothelial and human astrocytoma cells, and a limited number of other normal and malignant cells, synthesize three chains that have been identified as type VIII collagen (180 kDa, 125 kDa, and 100 kDa). Digestion with pepsin converts these forms to major fragments of 65 kD (based on globular protein standards).[1] In this study we have examined the structure and distribution of type VIII collagen in developing mice by immunohistological and immunoblotting techniques. Temporal and tissue-specific expression was observed in embryonic heart, cranial mesenchyme, and placental capillaries. Western blotting of embryonic and neonatal tissues showed major species of 125 and 65 kDa in the brain, placenta, heart, lung, and thymus. The predominant band in pepsin-treated tissues was 60-70 kDa, with additional forms of 250 and 150 kDa in neonatal heart and lung. Type VIII collagen was also synthesized by endothelial cells, forming capillary tubes *in vitro*. We suggest that type VIII collagen functions in cellular organization and differentiation, and that its various forms reflect not only tissue-specific processing but the presence of several related chains.

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