Type V Collagen Controls the Initiation of Collagen Fibril Assembly

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Vertebrate collagen fibrils are heterotypically composed of a quantitatively major and minor fibril collagen. In non-cartilaginous tissues, type I collagen accounts for the majority of the collagen mass, and collagen type V, the functions of which are poorly understood, is a minor component. Type V collagen has been implicated in the regulation of fibril diameter, and we reported recently preliminary evidence that type V collagen is required for collagen fibril nucleation (Wenstrup, R. J., Florer, J. B., Cole, W. G., Willing, M. C., and Birk, D. E. (2004) J. Cell. Biochem. 92, 113–124). The purpose of this study was to define the roles of type V collagen in the regulation of collagen fibrilogenesis and matrix assembly. Mouse embryos completely deficient in pro-α1(V) chains were created by homologous recombination. The col5a1−/− animals die in early embryogenesis, at approximately embryonic day 10. The type V collagen-deficient mice demonstrate a virtual lack of collagen fibril formation. In contrast, the col5a1+/− animals are viable. The reduced type V collagen content is associated with a 50% reduction in fibril number and dermal collagen content. In addition, relatively normal, cylindrical fibrils are assembled with a second population of large, structurally abnormal collagen fibrils. The structural properties of the abnormal matrix are decreased relative to the wild type control animals. These data indicate a central role for the evolutionary, ancient type V collagen in the regulation of fibrilogenesis. The complete dependence of fibril formation on type V collagen is indicative of the critical role of the latter in early fibril initiation. In addition, this fibril collagen is important in the determination of fibril structure and matrix organization.

Type V collagen is a member of the fibril subclass of collagens, which have in common a triple helical domain composed of an uninterrupted series of Gly-X-Y triplets. Type V collagen is a quantitatively minor component of predominantly type I collagen fibrils in most non-cartilaginous tissues. Several isoforms of type V collagen exist, which differ in the type and ratio of constituent chains, including heterotypic molecules containing type XI collagen chains. The most abundant and widely distributed isoform is α1(V)2α2(V), which forms heterotypic fibrils with type I collagen (1). The role of type V collagen in the organization and biological properties of collagenous extracellular matrix is poorly understood. Observations of an inverse correlation between type V collagen:type I collagen ratios and collagen fibril diameter in vitro fibril assembly experiments (2), cell cultures (3, 4), and in various tissues (5) have led to the hypothesis that type V collagen serves as a negative regulator of collagen fibril diameter (3–5). That function may be mediated by retention of the non-collagenous amino-terminal propeptide after type V collagen molecules are incorporated into fibrils (2, 6–9). This non-collagenous domain projects outward through the gap between adjacent type I collagen molecules, leaving major portions present on the fibril surface (1, 3, 7) where they may limit lateral growth of the fibril by steric hindrance and charge interactions (5, 11).

A novel role for type V collagen was suggested recently by the results of studies (4) of cultured dermal fibroblasts from patients with the heritable connective tissue disorder, Ehlers-Danlos syndrome. The fibroblasts had mutations that caused haploinsufficiency of COL5A1, which encodes pro-α1(V) chains. In those cultures, the total incorporation of collagen into collagen fibrils of the cell layer was reduced by half and was associated with a proportional decrease in fibril number. Because type V collagen comprises less than 5% of dermal collagen, the observed decrease in total collagen fibril formation was more than 1 order of magnitude greater than the expected reduction in collagen mass caused by the loss of contribution from one COL5A1 allele. The corresponding decrease in fibril number indicated that type V collagen may control the utilization of type I collagen during collagen fibril initiation in some tissues.

To further investigate the function of type V collagen in collagen fibril formation, in vivo studies were performed on mice in which col5a1 was inactivated by homologous recombination. The complete absence of a functioning col5a1 gene resulted in lethality at embryonic day 10 (E10), with evidence of cardiovascular failure at the time of fetal demise. Animals that are haploinsufficient for col5a1 manifest many of the clinical, biomechanical, morphologic, and biochemical features of the Ehlers-Danlos syndrome, classic type.

**EXPERIMENTAL PROCEDURES**

Generation of col5a1-deficient Mice—A col5a1 targeting vector was generated using PCR to amplify gene-specific 5′- and 3′-targeting arms from KG-1 ES cell DNA using Herculase (Stratagene, La Jolla, CA). PCR products were verified by sequence analysis. A 5′-targeting arm from a region that included part of exon 3 was designed utilizing the primers 5′-GATGAAATTC AAGCTTCAAGCTTCACGGTGGGCACAGAGACTGGA-3′ and 3′-GATGAAATTC AAGCTTCACGGTGGGCACAGAGACTGGA-5′. The abbreviations used are: E, embryonic day (e.g. E10); ES, embryonic stem (cell); CI, confidence interval.
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TA-3 and 5'—CAGGCAAGCTTGATCTCGATCCGCTTGGTATG-GAAAATTC-3'. These primers include HindIII restriction sites to facilitate cloning and a BamHI restriction site that introduces a unique restriction polymorphism. A 1.8-kb targeting arm was amplified by PCR digested with EcoRI and subcloned into the EcoRI site of pNTK-VKO (Stratagene). A 3'-targeting arm from a region that included part of exon 4 was designed utilizing the primers 5'-TAGGCGGAATTCCTCGGATGTAAGATTTGGAGTAC-3' and 5'-GAGTCGAATTCCTGGTATG-AAGCTTACCATGGCCTCAG-3'. These primers include EcoRI restriction sites (underlined). A 4.4-kb targeting arm was amplified by PCR, digested with EcoRI, and subcloned into the EcoRI site of pNTK-VKO. The col5a1 targeting vector was linearized with NotI, and 20 µg was electroporated into KG-1 ES cells using standard conditions (42). Positive ES cell clones were identified by Southern blot analysis using gene-specific 5' and 3' probes. ES cells targeted correctly were expanded and injected into C57BL/6 blastocysts. Germ line transmission was obtained by breeding. The genetic animals to either C57BL/6 or 129SVe. Wild type and recombinant alleles were detected by autoradiography using EnHanceTM (PerkinElmer Life Sciences) as the fluorescing agent.

Samples were washed with phosphate-buffered saline and then digested with pepsin as described previously (4). Collagen chains were separated by SDS-PAGE in 5% bisacrylamide gels under non-reducing conditions as described previously (4, 17). Radioactive proteins were separated by SDS-PAGE in 5% bisacrylamide gels under non-reducing conditions as described previously (4, 17). Radioactive proteins were separated by SDS-PAGE in 5% bisacrylamide gels under non-reducing conditions as described previously (4, 17). Radioactive proteins were separated by SDS-PAGE in 5% bisacrylamide gels under non-reducing conditions as described previously (4, 17). Radioactive proteins were separated by SDS-PAGE in 5% bisacrylamide gels under non-reducing conditions as described previously (4, 17).

Transmission Electron Microscopy—Mouse embryos at day 11, 12, or 14 were rinsed in phosphate-buffered saline, disaggregated physically into Dulbecco's modified Eagle's complete medium with 10% fetal bovine serum, 100 units of penicillin, and 100 µg/ml streptomycin, and cultured at 37°C, 95% humidity, and 5% CO2. For analysis of pro-collagens precipitated from the medium of cultured fibroblasts, samples were collected at day 12 and 14. For analysis of pro-collagens precipitated from the medium of cultured fibroblasts, samples were collected at day 12 and 14. For analysis of pro-collagens precipitated from the medium of cultured fibroblasts, samples were collected at day 12 and 14. For analysis of pro-collagens precipitated from the medium of cultured fibroblasts, samples were collected at day 12 and 14. For analysis of pro-collagens precipitated from the medium of cultured fibroblasts, samples were collected at day 12 and 14.

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Results

Targeted Disruption of colla1 and Survivability of Offspring—The region of colla1 to be targeted was identified in the Celera database of mouse genomic sequences. Two sequences flanking exons 3 and 4 were amplified from KG-1 ES cell DNA and cloned into a targeting vector containing a neo-mycin cassette (Fig. 1A). The construct was confirmed by sequence analysis. KG-1 ES cells were transfected with linearized target vector by electroporation. Southern blot analysis (Fig. 1B) of G418-resistant ES cell clones identified one of 300 clones containing the homologously recombined gene that replaced most of exons 3 and 4 with the neomycin cassette. Chimeric males that were generated using this clone were mated to C57BL/6 or 129SVe females to obtain germ line transmission. Genetic analysis (Fig. 1C) of offspring of heterozygous matings was consistent with preterm lethality of colla1−/− offspring, but embryos harvested between embryonic days 8.5 and 11 showed expected Mendelian ratios (Table I). colla1−/− embryos were found dead at day 11 and were resorbed completely by day 12 (data not shown).

Targeted Recombination of colla1 Prevents Gene Expression—Whole mount in situ hybridizations were performed in the presence and absence of ribonuclease protection assay probe. The probes were as follows: c5a1 antisense, 5'-CGTTAGAGGTGGT-ACATGAGGGGCC-3'; 5'-CATCATATAACCATCTATCTGGG-3', and 5'-CTCTATCGCTCTTGACAGGTT-3'. In Situ Hybridization—Whole mount in situ hybridizations were performed in the presence and absence of ribonuclease protection assay probe. The probes were as follows: c5a1 antisense, 5'-CGTTAGAGGTGGT-ACATGAGGGGCC-3'; 5'-CATCATATAACCATCTATCTGGG-3', and 5'-CTCTATCGCTCTTGACAGGTT-3'.

Measurement of Total Colagen Deposited in the Skin—Skin was harvested from 12-week-old animals. Two 4-mm punch biopsies were taken from the back and from the neck of every animal. The samples were washed with phosphate-buffered saline and then suspended in 6N HCl at 100 °C for 18 h for hydrolysis. Colorimetric analysis of the hydroxyproline content of each sample was performed after acid hydrolysis (12). The conversion ratio of 0.12:1.0 was used to convert micrograms of hydroxyproline to total collagen (12).

Photografting. For measurement of collagen fibril density, the dermis was divided into four equal regions. Analysis was performed for both the superficial and deep dermis. The superficial dermis was defined as the region subjacent to the epidermis, and the deep dermis was the lower fourth. Both regions were photographed in the central portion. Micrographs were taken at a magnification of ×31,680. Calibrated micrographs from each region were chosen randomly in a masked manner from the different regions. The micrographs were digitized, and all diameters were measured within a 2.0-µm mask. The mask was placed based on fibril orientation, that is, by cross-section and absence of cells. Diameters were measured along the minor axis of cross-sections using a RM Biometrics-Bioquant Image Analysis (Memphis, TN) system.

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The absence of type V collagen in the embryonal mesenchyme of col5a1−/− and wild type embryos was performed. Death of E10 Embryos Is Associated with a Lack of Mesenchymal Fibrils—The absence of type V collagen in the col5a1−/− mice was associated with a lack of collagen fibril formation in the mesenchyme. In wild type embryos, there were numerous small diameter fibrils located subectodermally (Fig. 5A) that were localized throughout the mesenchyme (Fig. 5C). In contrast, collagen fibrils were completely absent in the col5a1−/− yolk sac. Pooled blood is observed in the col5a1−/− yolk sac.
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Fig. 4. Whole mount in situ col5a1 expression in wild type embryos. A, C, D, and E, antisense probes are shown. B, a sense probe is shown. A, expression at E8.5 is detected in the branchial arches (black arrowheads), neural crest cells, and somites (red arrowhead). C and D, at E11, col5a1 is expressed throughout the ectoderm, with higher levels of expression within the somites (red arrowhead), condensing limb mesenchyme (red arrows), and umbilical vessels (*). E, at E11 the yolk sac shows expression in some yolk sac vessels (black arrow).

Fig. 5. Electron micrographs of dermal tissues. A and C, at embryonic day 10 in the wild type embryos, large numbers of small diameter fibrils are deposited homogeneously beneath the ectoderm (E) basement membrane (arrowhead) and in the predermal mesenchyme between mesenchyme (M) cells and collagen fibrils (small arrows). B and D, in the col5a1−/− embryos no fibrils are deposited in the predermal mesenchyme (D), and only a few abnormally large fibrils are found associated with the ectodermal basement membrane (C). Bar = 300 nm.

The Dermis of Mature col5a1−/− Mice Contains Abnormal Collagen Fibrils—The dermis of mature col5a1−/− mice has a single population of cylindrical collagen fibrils seen in cross-section (A) and longitudinally (B) (small arrows). The col5a1−/− have two intermixed fibril populations within the dermis (C and D). The first is a population of cylindrical fibrils that is comparable with those seen in the wild type dermis but larger in diameter (small arrows). The second population is composed of very large, heterogeneous fibrils with very irregular fibril contours (*) in cross-section (C). In the longitudinal section (D), the fibril surfaces are irregular, and the diameter is inconsistent along the fibril length. Bar = 300 nm.

Fig. 6. Electron micrographs of collagen fibril architecture in col5a1+/+ and col5a1−/− embryos. The collagen fibrils assembled by the −/− embryos are structurally abnormal. A, the wild type embryos assemble large numbers of small diameter collagen fibrils with circular profiles in the space subjacent to the ectodermal basement membrane. In contrast, the −/− embryos assemble far fewer fibrils. These fibrils have abnormally large diameters (B). The few fibrils assembled by the col5a1-deficient embryos have very irregular cross-sectional profiles (B, inset) compared with the small, cylindrical profile seen in the wild type embryos (A, inset). Bar = 100 nm.

Fig. 7. Transmission electron micrographs of the deep dermis from 12-week-old mice showing abnormal collagen fibrils in adult +/− animals. At postnatal week 12, the dermis of wild type mice has a single population of cylindrical collagen fibrils seen in cross-section (A) and longitudinally (B) (small arrows). The col5a1+/− have a single population of normal fibrils (Fig. 7, A and B). In contrast, the severe reduction in number and distribution, and resulting embryonic lethality. To further address the role of type V collagen in the regulation of fibrillogenesis, the effects of reduced copy number were studied in the postnatal dermis.

The Dermis of Mature col5a1−/− Mice Contains Abnormal Collagen Fibrils—The dermis of mature col5a1−/− mice is characterized by large numbers of structurally aberrant collagen fibrils (Fig. 7). The mature wild type dermis is composed of a relatively homogeneous population of cylindrical fibrils at 12 weeks (Fig. 7, A and B). In contrast, the
12-week-old col5a1+/− dermis contained cylindrical fibrils and a population of larger, abnormal fibrils with very irregular contours (Fig. 7, C and D). In addition to the structurally abnormal fibrils, the haploinsufficient animals were characterized by a decreased number of collagen fibrils present in the dermis. This decrease in fibril density observed in col5a1+/− versus wild type animals was found at all postnatal stages examined (postnatal day 10, 45, and 90) (data not shown). At postnatal week 12, col5a1+/− animals had −46% of the dermal collagen fibrils present in the wild type littermates (Fig. 8A). This difference was significant (p = 0.004) as found by comparing the mean number of fibril diameters in haploinsufficient (104, 95% CI, 68 to 140) and wild type (194, 95% CI, 158 to 230) animals. The reduced fibril number correlated with a reduction in skin collagen measured as the amount of hydroxyproline/mm² (p = 0.0002). Error bars represent means ± S.E.

**DISCUSSION**

Murine deficiency of the major type V collagen chain, proα1(V), causes death in early embryogenesis and is associated with the virtual absence of collagen fibrils. Morphological analyses of collagen fibrils in col5a1+/− and col5a1+/− mice and −/− mice embryos indicate that the collagen fibril number varies directly with col5a1 gene dose, that is, day 10 col5a1+/− embryos have virtually no collagen fibrils except for a small
number of abnormal structures in the primordial dermal-epidermal junction, whereas \( \text{col5a1}^{+/+} \) animals, which survive normally but phenotypically resemble the Ehlers-Danlos syndrome classic type (23), have approximately one-half the number of fibrils that wild type littermates have. Because type V collagen is a minor fibril component, whereas type I comprises over 90% of fibril collagen in most tissues, the data indicate that type V collagen has an essential regulatory role in collagen fibril initiation. This is the first observation that the deficiency of an extracellular matrix component prevents collagen fibril formation.

The failure of collagen fibrillogenesis is not associated with the reduced expression of type I collagen genes. Although the biochemical measurement of collagen from the dermis of \( \text{col5a1}^{+/-} \) animals correlated with the observed reduction in fibril number (Fig. 8), the \( \text{col1a1} : \text{col5a2} \) ratios from RNA harvested from tissues were similar to those in \( \text{col5a1}^{+/+} \) and \( \text{col5a1}^{+/-} \) animals (data not shown). Type I collagen was secreted efficiently from cultured embryonal fibroblasts from \( \text{col5a1}^{+/-} \) cells (Fig. 4) but was not deposited in the cell layer, in contradistinction to wild type cells. A similar pattern of reduced type I collagen utilization in the presence of normal type I collagen biosynthesis and relative type V collagen deficiency was observed in cultured dermal fibroblasts from patients with classic Ehlers-Danlos syndrome who had haploinsufficiency mutations in \( \text{COL5A1}^{+/-} \) (4).

Early collagen fibril formation events occur in channels at the cell surface of connective tissue fibroblasts (18). Recently, it has been suggested (24) that this process can begin intracellularly within Golgi-to-plasma membrane carriers during embryogenesis. Fibrils form via an intermediate stage that includes nucleation and unilateral elongation of short primary fibrils. These short fibrils later fuse, resulting in diameter enlargement and bidirectional growth (Fig. 10A, inset) (25–27). Thus, although the fibrillar irregularities observed in juvenile (postnatal day 10) and adult (postnatal week 12) \( \text{col5a1}^{+/-} \) mice may be accounted for in part by abnormal fusion events, abnormal fibril initiation is the only possible explanation for the morphological abnormalities observed in the limited number of fibrils present in \( \text{col5a1}^{-/-} \) embryos at E10, when collagen fibrils are first detected (Fig. 6B, inset). Thus, the data presented in this report support a role for type V collagen in nucleation or initial formation of fibrils. This function is consistent with previous observations by Birk et al. (1) that type V triple helical epitopes are buried within collagen fibrils where they may be among the earliest deposited molecules within a fibril. The retained amino-terminal propeptide, which is thought to project at right angles to the main axis of the fibril (7), may be the means by which the inverse relationship between fibril number and fibril diameter is controlled (see Ref. 28). Under conditions in which type V collagen is not limiting, new fibril formation may be favored over lateral expansion of existing fibrils at a given site (see Ref. 29). The limited number of large, irregular fibrils at the primordial dermal-epidermal junction of E10 \( -/- \) animals (Fig. 6) and the subpopulation of enlarged fibrils with very irregular borders observed in the dermis of \( \text{col5a1}^{+/-} \) animals (Fig. 7) may represent unregulated type I collagen self-assembly when available type V collagen is limiting (Fig. 10, B and C).

The role of type V collagen in fibril nucleation is also consistent with the hypothesis that \( \text{COL5A1}^{+/-} \) also is a more ancient gene than \( \text{COL1A1}^{+/-} \) (15, 30). Tillet et al. (31) have compared a narrow fibril collagen from the sea-pen \( \text{Veretillum cynomorium} \) with type V collagen and found that the fibrils from the sea-pen are thinner and have a more irregular structure than the type V fibrils from the mouse. This indicates that type V collagen is not the only factor that determines fibril diameter and structure.

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**Table II**

| Genotype   | Average median | Standard error | 95% Confidence limits | Lower  | Upper  |
|------------|----------------|----------------|-----------------------|--------|--------|
| \( \text{col5a1}^{+/-} \) | 90.64\(^{\ast} \) | 2.61           | 84.25–97.04           |        |        |
| \( \text{col5a1}^{+/-} \) | 107.96\(^{\ast} \) | 3.44           | 100.43–115.50         |        |        |

\(^{\ast} p = 0.003\)

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**Fig. 10. Schematic diagram of collagen fibril formation in \( \text{col5a1}^{+/-} \) (A), \( \text{col5a1}^{+/-} \) (B), and \( \text{col5a1}^{+/-} \) (C) animals.** A, under normal conditions, type V collagen fibrils are initiated at the surface of connective tissue cells to form fibril intermediates, which enlarge by lateral and end-to-end fusion events (inset). B, when type V collagen molecules (blue) are limiting because of haploinsufficiency, a reduced number of normal fibrils are nucleated through a regulated fibril assembly mechanism. Excess type I collagen molecules (black) may form morphologically abnormal aggregates through unregulated self-assembly so that both normal and abnormal populations of collagen fibrils are present (inset). C, in the state of complete type \( \text{col5a1} \) deficiency, only the self-assembly pathway may be present, leading to the presence of a very limited number of fibrils, all of which are morphologically abnormal (inset).
and noted structural similarities to vertebrate type V collagen, including the voluminous N-propeptide domain and the distribution of polar residues. Such collagens may represent ancestral forms of the minor vertebrate fibril collagens, and fibril initiation may be a residual function of type V collagen.

The death of col5a1−/− embryos at early organogenesis was associated with pooling of blood in the yolk sac, indicating the presence of cardiovascular abnormalities. It is difficult to determine whether this pooling is caused by cardiac defects or the loss of the gene within the vessels. Initial morphological studies show that the ventricles in col5a1-deficient embryos at E10 demonstrate a reduction in normal trabeculation and collagen content in contradistinction to wild type littermates. There was no apparent growth retardation at the time of death, and col5a1−/− embryos exhibited normal patterns of vessel formation, based on whole mount immunohistochemical staining with platelet endothelial cell adhesion molecule (CD31, BD Biosciences) (data not shown), although col5a1 was highly expressed in umbilical vessels and was present in the vessels in the yolk sac and the embryo (Fig. 4). It seems likely that in the absence of a collagenous component in the vessel wall, blood vessels do not have sufficient wall integrity to maintain a functioning yolk sac circulation, which is critical to survival of the embryo.

Collagen fibril formation is a complex process that requires participation of collagenous and non-collagenous elements. As expected, collagen fibrils fail to form in the absence of the major component (type I collagen) (32). However, complete murine deficiency of type III collagen, which, like type V, is a quantitatively minor component of collagen fibrils, does not prevent collagen fibrillogenesis (33). col5a1−/− animals perish from vascular rupture late in gestation, although some animals survive birth. Surprisingly, type I collagen-deficient mov-13 mice survive to postfertilization day 13 (34), compared with the col5a1−/− animals described in this report, which died ~3 days earlier. The differences in survival could be related to strain differences or possibly because of the residual type I collagen expression observed in some tissues in mov-13 mice (35). Another explanation for the earlier demise of col5a1−/− embryos compared with mov-13 mice may be that in the former embryonic demise is caused by the disruption of basement membrane formation rather than collagen fibril formation. Type V collagen has been localized to basement membranes (36), and embryonal disruption of basement membranes in col4a1 and col4a2 doubly deficient animals is also associated with lethal deme at approximately 10 days (37).

Defects in collagen fibril size, morphology, and content have also been observed in mice deficient in small leucine-rich repeat proteoglycans with collagen binding properties, including decorin, biglycan, fibromodulin, and lumican (38). Skin fragility phenotypes in decorin- and lumican-deficient mice have been associated with abnormal collagen fibril morphology, possibly as a consequence of abnormal lateral fusion/growth of collagen fibrils (39–41). Murine deficiency of tenascin X is also associated with abnormalities in fibril morphology and moderately reduced collagen content of skin (10). A comparison of the data presented in this report with the substantial body of knowledge of extracellular matrix deficiency states in mice indicates that no matrix component (save type I collagen itself) appears to exert the same level of control of fibril initiation as type V collagen. We propose that the control of collagen fibril formation is complex and that, in addition to transcriptional control of constituent molecules, post-translational control is exerted at the level of fibril nucleation; this process appears likely to be affected by the availability of type V collagen molecules.

Acknowledgments—We thank Biao Zuo, Kaitlin Petrella, Margarita Schmid, and Venette Davis for expert technical assistance.

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