Transfer of the $\alpha 5(\text{IV})$ Collagen Chain Gene to Smooth Muscle Restores *in Vivo* Expression of the $\alpha 6(\text{IV})$ Collagen Chain in a Canine Model of Alport Syndrome

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X-linked Alport syndrome is a progressive renal disease caused by mutations in the *COL4A5* gene, which encodes the $\alpha 5(\text{IV})$ collagen chain. As an initial step toward gene therapy for Alport syndrome, we report on the expression of recombinant $\alpha 5(\text{IV})$ collagen *in vitro* and *in vivo*. A full-length cDNA-encoding canine $\alpha 5(\text{IV})$ collagen was cloned and expressed *in vitro* by transfection of HEK293 cells that synthesize the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$, but not the $\alpha 3(\text{IV})$ to $\alpha 6(\text{IV})$ collagen chains. By Northern blotting, an $\alpha 5(\text{IV})$ mRNA transcript of 5.2 kb was expressed and the recombinant protein was detected by immunocytochemistry. The chain was secreted into the medium as a 190-kD monomer; no triple helical species were detected. Transfected cells synthesized an extracellular matrix containing the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains but the recombinant $\alpha 5(\text{IV})$ chain was not incorporated. These findings are consistent with the concept that the $\alpha 5(\text{IV})$ chain requires one or more of the $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, or $\alpha 6(\text{IV})$ chains for triple helical assembly. *In vivo* studies were performed in dogs with X-linked Alport syndrome. An adenoviral vector containing the $\alpha 5(\text{IV})$ transgene was injected into bladder smooth muscle that lacks both the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains in these animals. At 5 weeks after injection, there was expression of both the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains by smooth muscle cells at the injection site in a basement membrane distribution. Thus, this recombinant $\alpha 5(\text{IV})$ chain is capable of restoring expression

of a second $\alpha(\text{IV})$ chain that requires the presence of the $\alpha 5(\text{IV})$ chain for incorporation into collagen trimers. This vector will serve as a useful tool to further explore gene therapy for Alport syndrome. (*Am J Pathol* 2003, 162:873–885)

Alport syndrome is a hereditary disorder characterized by progressive nephropathy and ultrastructural abnormalities of the glomerular basement membrane (GBM) that is often associated with sensorineural deafness and ocular lesions.^{1–4} In affected males, progression to end-stage renal disease usually occurs by the third decade of life, whereas females express variable phenotypes. The pathogenesis of Alport syndrome has been linked to defects of type IV collagen, which is a major structural component of basement membranes. Type IV collagen exists as a family of triple helical isoforms assembled from three α -chains. In humans, six genetically distinct α -chains, designated $\alpha 1(\text{IV})$ to $\alpha 6(\text{IV})$, have been identified and each is encoded by a separate gene designated *COL4A1* to *COL4A6*, respectively.⁵ From their amino to carboxyl termini, mature collagen $\alpha(\text{IV})$ chains are characterized by a minor noncollagenous domain (~15 residues), a minor collagenous domain (~130 residues, 7S domain), a major collagenous domain (~1400 residues) containing 15 to 20 noncollagenous interruptions, and a carboxyl terminal noncollagenous domain (~230 residues, NC1 domain).⁶ Triple helices are formed intracellularly and secreted, whereupon they self-assemble to form dimers through their NC1 domains and tetramers through their 7S domains.

To date, more than 300 different mutations in the *COL4A5* gene have been identified in families with the more common X-linked form of Alport syndrome,^{4,7} whereas autosomal forms of the disease are associated

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with mutations in the *COL4A3* and *COL4A4* genes.^{8,9} These mutations lead to the assembly of a GBM that is abnormal with respect to morphology and composition. Whereas normal GBM contains the $\alpha 1(\text{IV})$ to $\alpha 5(\text{IV})$ chains, for most Alport patients, the GBM contains only the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains and lacks the $\alpha 3(\text{IV})$ to $\alpha 5(\text{IV})$ chains.^{10–13} Normal GBM contains two networks, the first consisting of the $\alpha 1(\text{IV})/\alpha 2(\text{IV})$ chains and the second of the $\alpha 3(\text{IV})/\alpha 4(\text{IV})/\alpha 5(\text{IV})$ chains.^{14,15} The existence of an $\alpha 3(\text{IV})/\alpha 4(\text{IV})/\alpha 5(\text{IV})$ network provides an explanation for the absence of each of these chains in Alport syndrome in the setting of *COL4A3*, *COL4A4*, or *COL4A5* mutations, in that all three chains are required for the assembly of this network.^{15,16}

There is no conservative or curative treatment currently available for those patients with Alport syndrome who have progressed to renal failure. However, as a result of advances in molecular genetic research, gene therapy is emerging as a possible treatment for some renal diseases,^{17,18} including Alport syndrome.^{19,20} An experimental model is essential for testing the feasibility of gene therapy for the treatment of Alport syndrome in humans. The model of X-linked Alport syndrome in Samoyed dogs mimics the human disease at the clinical, ultrastructural, and protein chemistry levels.^{21–23} The canine nephropathy arises because of a nonsense mutation in the *COL4A5* gene, resulting in ~90% reduction in the level of $\alpha 5(\text{IV})$ mRNA.²⁴ The GBM of affected male dogs lacks the $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ chains.^{25,26} As an initial step toward developing gene therapy for X-linked Alport syndrome, we report on the cloning of a full-length cDNA encoding canine $\alpha 5(\text{IV})$ collagen and its expression *in vitro* and *in vivo* using this canine model.

Experimental Procedures

cDNA Synthesis

Total RNA was extracted from normal canine testis using Trizol reagent (Invitrogen, Carlsbad, CA), then purified using the Straight A's mRNA isolation system (Novagen, Madison, MI). First- and second-strand cDNA was synthesized with the Marathon cDNA Amplification kit (Clontech, Palo Alto, CA). The full-length construct (Figure 1A) was generated as two overlapping fragments obtained by 5' and 3' rapid amplification of cDNA ends (RACE) and nested polymerase chain reaction (PCR). The primers were based on human $\alpha 5(\text{IV})$ sequence²⁷ (primers II and VI) and canine $\alpha 5(\text{IV})$ sequence²⁴ (primers I, III, IV, and V): I, 5' CTG GGT CTC CAG GCA AAC CCT GGT 3'; II, 5' TTC GTG CGG GTG CTG AAG GA 3'; III, 5' ATA CCT GGT AAG CCA GGG TCC CC 3'; IV, 5' GGT TTG CAG GGT CAG CCA GGA CCT 3'; V, 5' GGT GGT AAA GGA GAG CCT GGC CT 3'; VI, 5' TTA TGT CCT CTT CAT GCA CAC TT 3'.

5' and 3' RACE PCR was performed using the cDNA generated above as template, an adapter primer (5' CCA TCC TAA TAC GAC TCA CTA TAG GGC 3', Clontech) and primers I and IV, respectively. PCR was performed using rTth DNA polymerase-XL (Perkin Elmer, Norwalk,

Figure 1. Generation of a recombinant adenoviral vector encoding canine $\alpha 5(\text{IV})$ collagen (Ad $\alpha 5$). **A:** The cloned canine $\alpha 5(\text{IV})$ cDNA is characterized by 20 bp of 5' untranslated sequence followed by an open reading frame of 5076 bp. The translation initiation (ATG) and stop codon (TAA) are indicated, as is the site and sequence of an 18-bp insertion located at the boundary of exons 41 and 42 of the human cDNA sequence. The canine $\alpha 5(\text{IV})$ chain is 1691 amino acids in length with a predicted molecular weight of 162 kd. Illustrated are the signal peptide (SP) (residues 1 to 26, gray), the collagenous domain (residues 27 to 1462, white) that contains 22 noncollagenous interruptions (black), and the NC1 domain (residues 1463 to 1691, dark gray). The site and sequence of a 6-amino acid insertion is indicated as are the locations of the epitopes recognized by the monoclonal antibodies H52 and H53. **B:** A replication-deficient recombinant adenoviral vector containing an expression cassette consisting of the canine $\alpha 5(\text{IV})$ cDNA flanked by the major late promoter (MLP) of adenovirus serotype 2 and human growth hormone polyadenylation sequences (pA) was generated by homologous recombination in HEK293 cells. **C:** Recombinants with the desired genotype were identified using a triplex PCR-based strategy designed to amplify the 5' and 3' junctions of the expression cassette, as well as a portion of the adenoviral hexon gene (hex) that served as a positive control. AdCMVlacZ DNA serves as an internal negative control (neg).

CT) under the following conditions: 94°C for 1 minute (1×); 94°C for 30 seconds, 68°C for 4 minutes (35×); 72°C for 10 minutes (1×). Nested PCR was performed under the same conditions using aliquots of the amplified mixtures above as template and primer sets II and III and V and VI. The full-length cDNA was generated on subcloning of overlapping 5' and 3' halves at a common *Bgl*II restriction site in pBluescript II vector (Stratagene, La Jolla, CA), and its nucleotide sequence was determined by automated sequencing using primers providing at least twofold coverage of the entire cDNA. For expression studies, this construct was subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen).

Cell Culture and Transfection

All cell culture and transfection reagents were obtained from Invitrogen. HEK293 human embryonic kidney cells were obtained from the American Type Culture Collection

(Rockville, MD) and maintained in α -minimum essential medium (α -MEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B) by weekly subcultivation using 0.25% trypsin and 1 mmol/L ethylenediaminetetraacetic acid. Primary cultures of smooth muscle cells (SMCs) from normal and affected male dog bladder were established according to the protocol described by Baskin and colleagues.²⁸ SMCs (passage ≤ 5) were cultured on a substrate of type I collagen (1 μ g/cm²) (Sigma, St. Louis, MO) in M199 medium containing 10% fetal bovine serum, antibiotics, 100 μ mol/L α -MEM amino acids, and 1 $\times$ α -MEM vitamins. Cells were transiently transfected using lipofectamine and transgene expression was evaluated at 36 to 72 hours after transfection. For experiments examining the influence of ascorbate on collagen synthesis, cells were cultured and fed daily with Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and antibiotics with or without 2 mmol/L of L-ascorbate.

Northern Blot Analysis

Total RNA was extracted by lysing cells in Trizol reagent (Invitrogen). Samples (10 μ g) were separated by electrophoresis on 1% agarose-formaldehyde gels and transferred to Hybond membranes (Amersham, Cleveland, OH). The probes for $\alpha 1(IV)$ to $\alpha 6(IV)$ mRNAs were cDNAs for the respective canine NC1 domains generated as described previously²⁴ and labeled with ³²P-dCTP using the random primed DNA labeling system (Roche, Laval, Quebec, Canada). Detection was performed at -70°C using Kodak Biomax film with an intensifying screen.

Immunohistochemistry

The rat monoclonal antibodies H11, H22, H52, and H53 to the human $\alpha 1(IV)$, $\alpha 2(IV)$, and $\alpha 5(IV)$ collagen chains and B66 to the bovine $\alpha 6(IV)$ chain were used. Their specificity and reactive epitopes have been described previously.^{10,29} The mouse monoclonal antibodies mAb 1 and mAb 5 recognizing the human $\alpha 1(IV)$ and $\alpha 5(IV)$ NC1 domains, respectively, were obtained from Weislab AB (Lund, Sweden). For smooth muscle actin staining, a monoclonal mouse anti-human α -smooth muscle actin antibody was used (DAKO, Carpinteria, CA). Cells were seeded and transfected on glass coverslips, washed in phosphate-buffered saline (PBS), and fixed in acetone for 5 minutes at 4°C . Cryosections (5 μ m) were fixed in acetone for 10 minutes at 4°C and then pretreated in a 100 mmol/L acid-KCl solution (pH 1.5) for 10 minutes to expose epitopes. The procedure used for immunoperoxidase staining has been described previously.³⁰ For histochemical detection of β -galactosidase, cryosections were fixed in 2% formaldehyde/0.2% glutaraldehyde for 5 minutes, then washed in PBS and incubated in PBS containing 5 mmol/L $\text{K}_3\text{Fe}(\text{CN})_6$, 5 mmol/L $\text{K}_4\text{Fe}(\text{CN})_6$, 2 mmol/L MgCl_2 , and 2 mmol/L X-gal (5-bromo-4-chloro-3-indolyl β -D-galactopyranoside) at 37°C for color develop-

ment (1 to 16 hours). Counterstaining was performed with nuclear fast red.

For dual-immunofluorescent detection of the $\alpha 5(IV)$ and $\alpha 6(IV)$ chains, cryosections were fixed as described above, and then denatured in 6 mol/L urea, 0.1 mol/L glycine, pH 3.5, for 1.5 hours at 4°C . Sections were blocked in PBS containing 1.5% normal donkey and rabbit sera (Vector Laboratories, Burlingame, CA) and then incubated simultaneously with mAb 5 (1:50 dilution) and B66 (1:10 dilution) antibodies for 1 hour. Detection was performed using an fluorescein isothiocyanate-conjugated donkey anti-mouse antibody (1:100 dilution; Jackson ImmunoResearch, West Grove, PA) and a biotinylated rabbit anti-rat antibody (1:200 dilution, Vector Laboratories) for 1 hour, followed by tetramethyl-rhodamine isothiocyanate-conjugated streptavidin (1:50 dilution, Vector Laboratories) for 30 minutes.

Immunoprecipitation

The mouse monoclonal antibodies mAb 1, mAb 5, or anti-human α -smooth muscle actin (50 μ l) were incubated with 30 μ l of protein G agarose (Sigma) in 0.5 ml of PBS for 2 hours at 4°C . The beads were washed twice in PBS then resuspended in 10 μ l of 10% bovine serum albumin. Serum-free medium (1 ml) from transfected cells was buffered by the addition of HEPES to 25 mmol/L, then added to the beads and incubated with gentle mixing for 16 hours at 4°C . Immune complexes were collected by centrifugation and washed in 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40 and 0.5% sodium deoxycholate (4 $\times$ 5 minutes), then in the same buffer adjusted to 0.5 mol/L NaCl, 0.05% sodium deoxycholate (2 $\times$ 5 minutes), and finally in the latter buffer lacking NaCl (1 $\times$ 5 minutes). Samples were solubilized in Laemmli buffer and analyzed by Western blotting.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

For analysis of secreted proteins, cells were cultured in serum-free medium after transfection. Protein was precipitated with ethanol at -20°C then resuspended in 1% SDS. For collagenase digestion, samples were resuspended in 100 mmol/L Tris-HCl, pH 7.5, 10 mmol/L CaCl_2 containing 50 U/ml of purified bacterial collagenase (Worthington Biochemical, Freehold, NJ) and digested for 1 hour at 37°C , then precipitated as above. For analysis of the extracellular matrix, cells were washed in PBS, then incubated in PBS containing 0.5% Triton X-100 until detachment of the cell monolayer occurred. After extensive washes with PBS, the extracellular matrix remaining adherent to the culture dish was digested with collagenase then recovered as described above. Protein was quantified by DC protein assay (Bio-Rad, Hercules, CA). Native or reduced (100 mmol/L dithiothreitol) samples were subjected to SDS-PAGE using 4.5 to 12% linear gels as required. As a blotting control, type IV collagen

NC1 domain was prepared by collagenase digestion of GBM isolated from the kidneys of normal mixed breed dogs as described previously.³¹ After electrophoresis, gels were transferred to Immobilon-P membranes and the blots were blocked in 10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.1% Tween-20, 3% bovine serum albumin. Blots were incubated with primary antibodies (1:500 dilution) for 1.5 hours, followed by biotinylated rabbit anti-rat secondary antibody (1:1000 dilution, Vector Laboratories) for 1 hour, and then a peroxidase-conjugated avidin-biotin complex (Vector Laboratories) for 30 minutes. Detection was performed using LumiGLO chemiluminescent substrate (KPL Laboratories, Gaithersburg, MD).

Generation of a Recombinant Adenoviral Vector for the Canine $\alpha 5(\text{IV})$ cDNA

A recombinant adenovirus that expresses canine $\alpha 5(\text{IV})$ collagen (designated Ad $\alpha 5$) was constructed using the Adeno-Quest expression system (Quantum Biotechnology, Laval, Quebec, Canada). The vector contains an expression cassette at the E1 deletion site consisting of the full-length $\alpha 5(\text{IV})$ cDNA flanked by the major late promoter of adenovirus type 2 and human growth hormone polyadenylation sequences (Figure 1B). The $\alpha 5(\text{IV})$ cDNA was subcloned into the transfer plasmid pAdBM5PAG, then *Cla*I-linearized and co-transfected with Ad5CMVLacZ Δ E1/ Δ E3 DNA (9.4 to 100 map units) into HEK293 cells by calcium-phosphate precipitation. After transfection, cells were overlaid with α -MEM containing 10% fetal bovine serum, antibiotics, and 0.8% agar and cultured until plaques formed. Plaques were picked and eluted in α -MEM for 24 hours at 37°C. Viral eluates were amplified on HEK293 cells until a complete cytopathic effect was reached, at which point the medium was recovered and used for a second round of amplification. A multiplex PCR-based strategy was used to screen for recombinant viruses of the desired genotype. Primers were chosen that generated 960- and 496-bp amplicons spanning the 5' and 3' junctions of the $\alpha 5(\text{IV})$ cDNA with the transfer plasmid, respectively, as well as a 308-bp amplicon within the adenoviral hexon gene³² that served as a positive internal control (Figure 1C). Viral DNA was extracted from infected cells using the protocol described by Brown and colleagues.³³ PCR was performed under the following conditions: 95°C for 10 minutes (1 $\times$); 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes (35 $\times$); 72°C for 10 minutes (1 $\times$) using 200 ng of viral DNA as a template and the following primers: I, 5' TTC ACC TGG CCC GAT CTG G 3'; II, 5' TTC CTG GTG ACC GAG GGC CT 3'; III, 5' GCC CTC CCA TAT GTC CTT CCG AGT GAG AG 3'; IV, 5' GGC CAG AGC ATC CAG CCA TT 3'; V, 5' GCC GCA GTG GTC TTA CAT G 3'; VI, 5' CAG CAC GCC GCG GAT GTC 3'.

Positive clones were tested for expression of the $\alpha 5(\text{IV})$ transgene by infection of HEK293 cells followed by Western blot analysis of the culture medium. A selected clone was subjected to three rounds of plaque purification, then amplified on HEK293 cells and purified by double CsCl₂

equilibrium centrifugation. Viral titer was determined by plaque assay.

Expression of Recombinant Canine $\alpha 5(\text{IV})$ Collagen in Vivo

Ad $\alpha 5$ vector was injected into bladder smooth muscle of affected male dogs by direct visualization after general anesthesia and laparotomy. Before surgery and throughout the course of the study, dogs were placed on cyclosporin A (Neoral; Novartis Inc., Dorval, Quebec, Canada). Whole blood trough concentrations were maintained between 100 and 400 ng/ml as determined by weekly radioimmunoassay (Cyclo-Trac; Incstar, Stillwater, MN) using ethylenediaminetetraacetic acid-anti-coagulated whole blood. Injections (150 μ l) consisted of AdCMVLacZ (2×10^8 pfu, Quantum Biotechnology), Ad $\alpha 5$ (1×10^8 pfu), the same amount of Ad $\alpha 5$ spiked with AdCMVLacZ (2×10^7 pfu), or vehicle (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L MgCl₂, 5% sucrose). Each dog was administered a minimum of six injections that were marked with sutures. Dogs were euthanized and the injection sites were recovered and snap-frozen in OCT at various time points after injection ranging from 1 week (two dogs), 2 weeks (two dogs), 5 weeks (three dogs), to 7 weeks (one dog). Serial cryosections of bladder tissue were cut and transgene expression was evaluated by histochemical X-gal staining and immunostaining for type IV collagen.

Results

Nucleotide and Predicted Amino Acid Sequence of the Canine $\alpha 5(\text{IV})$ cDNA

Sequence analysis of the canine $\alpha 5(\text{IV})$ cDNA revealed 20 bp of 5'-untranslated sequence followed by a 5076-bp open reading frame coding for 1691 amino acids (Figure 1A). (This sequence has been deposited in GenBank with the accession number AY078501. Four corrections to the previously published 3' half of the canine $\alpha 5(\text{IV})$ collagen amino acid sequence²⁴ were identified; D₁N, W₂₃₄G, G₃₄₀^{*}PTGFQG (see text), and R₆₄₅A that were confirmed by sequencing a minimum of three different cDNA clones.) The canine cDNA shares $\geq 92\%$ identity at the nucleotide and amino acid levels to the human sequence. The deduced amino acid sequence predicts a protein of 162 kd with a 26-residue leucine-rich hydrophobic signal peptide as predicted using SignalP V1.1 software based on the method of Neilson and colleagues.³⁴ There is a 15-residue noncollagenous domain at the amino terminus that contains four of four conserved cysteine residues. The collagenous domain spans 1421 residues with 22 noncollagenous interruptions of 1 to 13 residues each. All interruptions are in identical locations as those in the human sequence, with 16 of these interruptions also sharing identical sequence. Of note, the canine $\alpha 5(\text{IV})$ cDNA was found to contain an 18-bp insertion at the site corresponding to the boundary of exons 41 and 42 of the human sequence (numbering according

Figure 2. Analysis of type IV collagen mRNA expression in transfected HEK293 cells. Northern blot analysis revealed an $\alpha 5(\text{IV})$ mRNA transcript of 5.2 kb that was expressed by $\alpha 5(\text{IV})$ -transfected cells ($\alpha 5$) but not mock-transfected controls (M), whereas signals of the expected size for the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ transcripts were detected at equivalent levels in both $\alpha 5(\text{IV})$ - and mock-transfected controls. Corresponding levels of 28S rRNA (bottom of lanes) served as loading controls.

to Zhou and colleagues³⁵). The identical insertion, which maintains the reading frame and preserves the Gly-X-Y repeat pattern within the collagenous domain, has been previously been reported in $\alpha 5(\text{IV})$ cDNA derived from normal human kidney³⁶ and has been shown to be the result of alternative splicing of two 9-bp exons located in intron 41 of the human *COL4A5* gene.³⁷ By reverse transcriptase-PCR and direct sequencing, the $\alpha 5(\text{IV})$ mRNA expressed in canine kidney, testis, and bladder smooth muscle were all found to contain this 18-bp insertion. The carboxyl terminal NC1 domain is 229 amino acids in length (the terminal seven residues are human sequence from the PCR primer). Eleven of 12 cysteine residues within the NC1 domain are conserved (the 12th cysteine is located in the terminal seven residues).

Expression of Recombinant Canine $\alpha 5(\text{IV})$ Collagen in Vitro in HEK293 Cells

The canine $\alpha 5(\text{IV})$ cDNA was expressed by transient transfection of HEK293 cells and the results were first assessed at the mRNA level by Northern blot analysis. Using an $\alpha 5(\text{IV})$ cDNA probe, a 5.2-kb transcript was detected in RNA from $\alpha 5(\text{IV})$ -transfected cells that was not present in mock-transfected controls (Figure 2). Signals for $\alpha 1(\text{IV})$ mRNA at 6.6 kb and for $\alpha 2(\text{IV})$ mRNA at 6.4 kb were detected at equivalent levels in both $\alpha 5(\text{IV})$ - and mock-transfected cells (Figure 2), whereas no signals for $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, or $\alpha 6(\text{IV})$ mRNA could be detected (results not shown). Expression of recombinant $\alpha 5(\text{IV})$ collagen at the protein level was first evaluated by immunostaining of transfected cells using the monoclonal antibodies H52 and H53 that recognize epitopes within the NC1 domain and the third noncollagenous interruption of human $\alpha 5(\text{IV})$ collagen, respectively. Positive staining was detected in a subset of $\alpha 5(\text{IV})$ -transfected cells, whereas mock-transfected cells were uniformly negative (Figure 3). Similar results were obtained using both antibodies,

Figure 3. Immunoperoxidase detection of recombinant canine $\alpha 5(\text{IV})$ collagen expressed by transfected HEK293 cells. Using antibody H53, a subpopulation of cells transfected with the $\alpha 5(\text{IV})$ cDNA ($\alpha 5$) displayed positive cytoplasmic staining for the $\alpha 5(\text{IV})$ chain, whereas mock-transfected controls (mock) were uniformly negative. Similar results were obtained using antibody H52. Hematoxylin counterstain; original magnifications, $\times 200$.

implying the full-length recombinant $\alpha 5(\text{IV})$ chain had been synthesized.

Western blot analysis was performed on protein precipitated from the serum-free culture medium of transfected cells. In reduced samples subjected to 6% SDS-PAGE analysis, blotting with antibody H53 revealed a band of 190 kd in samples from $\alpha 5(\text{IV})$ -transfected cells, but not mock-transfected controls (Figure 4A). Similar results were obtained with antibody H52 (results not shown). This species is in the size range expected for an $\alpha 5(\text{IV})$ chain monomer given its predicted molecular weight of 162 kd. The specificity of these results was confirmed through blotting of reduced samples with antibody H22, which detected a band of 200 kd representing an $\alpha 2(\text{IV})$ chain monomer present at equivalent levels in samples from both $\alpha 5(\text{IV})$ - and mock-transfected cells (Figure 4B). In nonreduced samples, blotting with antibody H53 revealed the 190-kd monomer as well as an additional high molecular weight $\alpha 5(\text{IV})$ -containing species of ~ 300 kd (Figure 4C). The high molecular weight $\alpha 5(\text{IV})$ -containing species was eliminated on reduction, which indicates that it contains disulfide-bonded $\alpha 5(\text{IV})$ chain monomers, however, it did not co-migrate with

Figure 4. Synthesis and secretion of type IV collagen α -chains by transfected HEK293 cells. Protein from the medium of $\alpha 5$ (IV)-transfected cells ($\alpha 5$) or mock-transfected controls (M) was analyzed by Western blotting using antibodies to the human $\alpha 1$ (IV), $\alpha 2$ (IV), and $\alpha 5$ (IV) chains under reducing or nonreducing conditions as indicated. **A:** In reduced samples subjected to 6% SDS-PAGE, blotting with antibody H53 detected the recombinant $\alpha 5$ (IV) chain as a 190-kd monomer in samples from $\alpha 5$ (IV)-transfected cells, but not mock-transfected controls. **B:** The specificity of these results was confirmed using antibody H22 that detected the $\alpha 2$ (IV) chain as a 200-kd monomer present in equivalent levels in samples from both $\alpha 5$ (IV)- and mock-transfected controls. **C:** In nonreduced samples, blotting with H53 detected the 190-kd $\alpha 5$ (IV) chain monomer and an additional $\alpha 5$ (IV)-containing species of ~300 kd in samples from $\alpha 5$ (IV)- but not mock-transfected cells. **D:** In nonreduced samples subjected to 4.5% SDS-PAGE, blotting with antibodies H11 revealed a weak band of ~300 kd and a higher molecular weight species that migrated just below the interface of the stacking and resolving gels. The latter $\alpha 1$ (IV) species co-migrated with $\alpha 2$ (IV) (not shown) in keeping with an $\alpha 1$ (IV)₂ $\alpha 2$ (IV) heterotrimer.

higher molecular weight bands blotting for the both the $\alpha 1$ (IV) chain (Figure 4D) and $\alpha 2$ (IV) chain (not shown) that represent $\alpha 1$ (IV)₂ $\alpha 2$ (IV) triple helices, implying that the ~300-kd $\alpha 5$ (IV)-containing species does not represent a trimer. The addition of ascorbate to the medium to promote collagen trimer formation did not result in $\alpha 5$ -containing trimers (results not shown).

To determine whether the recombinant $\alpha 5$ (IV) chain was associated with either the $\alpha 1$ (IV) or $\alpha 2$ (IV) chains in

Figure 5. The recombinant $\alpha 5$ (IV) chain does not undergo higher order assembly with the $\alpha 1$ (IV) or $\alpha 2$ (IV) chains. Medium from $\alpha 5$ (IV)-transfected HEK293 cells ($\alpha 5$) or mock-transfected controls (M) was immunoprecipitated with an antibody to the human $\alpha 1$ (IV) chain (mAb 1), the $\alpha 5$ (IV) chain (mAb 5) or an irrelevant control antibody (cont). Immunoprecipitates were subjected to 6% SDS-PAGE under reducing conditions and analyzed by Western blotting using antibodies to the human $\alpha 2$ (IV) and $\alpha 5$ (IV) chains as indicated. **A:** Blotting with antibody H22 revealed a 200-kd $\alpha 2$ (IV) chain monomer in samples from both $\alpha 5$ (IV)- and mock-transfected cells precipitated with mAb 1, but not in samples precipitated with the control antibody. **B:** No $\alpha 5$ (IV)-containing species were detected in replicate immunoprecipitates blotted using antibody H53. **C:** Blotting with antibody H53 revealed a 190-kd $\alpha 5$ (IV) chain monomer in samples from $\alpha 5$ (IV)-, but not mock-transfected, cells precipitated with mAb 5 and no bands were detected after precipitation with the control antibody. **D:** No $\alpha 2$ (IV)-containing species were detected in replicate immunoprecipitates blotted using antibody H22.

the ~300-kd species, the medium of transfected cells was immunoprecipitated with antibodies mAb 1 and mAb 5 to the human $\alpha 1$ (IV) and $\alpha 5$ (IV) chains, respectively. In samples from both $\alpha 5$ (IV)- and mock-transfected cells precipitated with mAb 1, a 200-kd band representing an $\alpha 2$ (IV) chain monomer was detected by blotting with antibody H22 that was not detected in replicate samples precipitated with an irrelevant control antibody (Figure 5A). Co-immunoprecipitation of the $\alpha 1$ (IV) and $\alpha 2$ (IV) chains is expected given they exist in an $\alpha 1$ (IV)₂ $\alpha 2$ (IV)

Figure 6. Collagenase digestion of recombinant $\alpha 5(\text{IV})$ collagen. Collagenase-digested protein from the medium (**A**) and extracellular matrix (**B**) of $\alpha 5(\text{IV})$ -transfected HEK293 cells ($\alpha 5$) and mock-transfected controls (**M**) was subjected to 12% SDS-PAGE under reducing conditions and analyzed by Western blotting using antibodies to the $\alpha 1(\text{IV})$, $\alpha 2(\text{IV})$, or $\alpha 5(\text{IV})$ chains as indicated. Normal canine GBM NC1 served as a positive control (**C**). Blotting with antibody H52 revealed a band of ~ 25 kd in samples from the medium of $\alpha 5(\text{IV})$ -transfected cells (but not mock-transfected controls) that co-migrates with $\alpha 5(\text{IV})$ NC1 domain monomer from canine GBM NC1. In replicate samples blotted with H22, a band of ~ 25 kd was detected at equivalent levels in samples from both $\alpha 5(\text{IV})$ - and mock-transfected cells that co-migrates with $\alpha 2(\text{IV})$ NC1 domain monomer from canine GBM NC1. Analysis of collagenase-digested protein from the extracellular matrix revealed that both $\alpha 5(\text{IV})$ -transfected cells and mock-transfected controls synthesize a matrix containing the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ collagen chains, which are detected as ~ 25 -kd and ~ 50 -kd NC1 domain monomers (MON) and dimers (DIM), respectively, but no $\alpha 5(\text{IV})$ NC1 species is detected.

heterotrimer. No $\alpha 5(\text{IV})$ -containing species were detected in replicate immunoprecipitates blotted using antibody H53 (Figure 5B). In samples from $\alpha 5(\text{IV})$ -transfected cells precipitated with mAb 5, a 190-kd band representing an $\alpha 5(\text{IV})$ chain monomer was detected by blotting with antibody H53 that was not detected in samples from mock-transfected cells and no bands were detected in samples precipitated with an irrelevant control antibody (Figure 5C). No $\alpha 2(\text{IV})$ -containing species were detected in replicate immunoprecipitates blotted using antibody H22 (Figure 5D). The failure of both the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains to immunoprecipitate with the $\alpha 5(\text{IV})$ chain indicates the ~ 300 -kd species does not represent a complex of the $\alpha 5(\text{IV})$ chain associated with either the $\alpha 1(\text{IV})$ or $\alpha 2(\text{IV})$ chains. This finding is consistent with the concept that these $\alpha(\text{IV})$ chains do not co-assemble into a trimeric molecule.¹⁶

Western blot analysis of collagenase-digested protein samples from the culture medium using antibody H52 revealed a strong band of ~ 25 kd in samples from $\alpha 5(\text{IV})$ -transfected cells, but not mock-transfected controls (Figure 6A), confirming the collagenous nature of the protein. This species co-migrated with an $\alpha 5(\text{IV})$ NC1 domain

monomer present in collagenase-digested canine GBM. No species of molecular weight greater than ~ 25 kd were detected. As a control, blotting of replicate samples using antibody H22 revealed an ~ 25 -kd $\alpha 2(\text{IV})$ NC1 domain monomer present in equivalent levels in samples from both $\alpha 5(\text{IV})$ - and mock-transfected cells (Figure 6A). The extracellular matrix of transfected cells was subjected to collagenase digestion. Western blotting using H11, H22, and H52 antibodies revealed NC1 dimer and monomer bands for both the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains but no $\alpha 5(\text{IV})$ -containing species (Figure 6B).

Expression of Recombinant Canine $\alpha 5(\text{IV})$ Collagen in Vitro in Smooth Muscle Cells

A replication-deficient adenoviral vector encoding canine $\alpha 5(\text{IV})$ collagen was generated by homologous recombination in HEK293 cells. A multiplex PCR-based strategy was used to screen for recombinant viruses of the desired genotype (Figure 1C). Of 41 clones that were screened, 9 yielded all three expected PCR products implying complete recombination. A subset of these also tested positive for the expression of recombinant $\alpha 5(\text{IV})$ collagen in HEK293 cells by Western blot analysis of the culture medium. A selected clone was plaque purified, amplified, then twice purified by CsCl_2 equilibrium centrifugation. Viral titer was determined by plaque assay on HEK293 cells and was routinely $\sim 1 \times 10^9$ pfu/ml. The recombinant $\alpha 5(\text{IV})$ chain encoded by this vector displayed properties identical to that synthesized in transfection studies using HEK293 cells (results not shown).

Expression studies using the Ad $\alpha 5$ vector were performed in primary cultures of SMCs from normal and affected dog bladder with the aim of demonstrating the biological activity of the recombinant $\alpha 5(\text{IV})$ chain. Normal canine bladder smooth muscle basement membrane contains the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains, whereas both proteins are absent in affected dogs.³⁸ Despite this, the expression $\alpha 6(\text{IV})$ mRNA in affected bladder remains at normal levels, implying that the absence of the $\alpha 6(\text{IV})$ chain reflects a defect at the level of protein assembly. We hypothesized that delivery of the $\alpha 5(\text{IV})$ transgene to affected SMCs might restore production of both the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains. Primary cultures of SMCs from normal and affected dog bladder were established and their identity was confirmed through immunostaining for α -smooth muscle actin. Both normal and affected SMCs expressed the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ collagen chains as detected by Northern blot analysis, immunostaining, and Western blotting of cell lysates (results not shown). However, the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains were no longer expressed at the mRNA level by normal SMCs or affected SMCs. Transduction of affected SMCs with Ad $\alpha 5$ vector resulted in high levels of expression of the recombinant $\alpha 5(\text{IV})$ collagen chain as detected by immunostaining, but no staining for the $\alpha 6(\text{IV})$ chain was detected at periods up to 5 days after infection (results not shown). Hence, in culture, both normal and affected primary bladder SMCs fail to recapitulate their *in vivo* phenotype with respect to ex-

pression of the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains. As a result, we chose to continue these studies at the *in vivo* level.

Expression of Recombinant Canine $\alpha 5(\text{IV})$ Collagen *in Vivo*

For *in vivo* expression of recombinant $\alpha 5(\text{IV})$ collagen, Ad $\alpha 5$ vector was injected into bladder smooth muscle of male dogs with X-linked Alport syndrome. Delivery of the Ad $\alpha 5$ vector to bladder smooth muscle is easily accomplished by laparotomy and direct injection. This is in contrast to the kidney, where efficient delivery of adenoviral vectors to the glomerulus requires specialized delivery methods such as extra-corporeal perfusion.^{20,39}

In vehicle-injected bladder of all dogs there was co-localization of the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains in the basement membranes underlying urothelial cells, in vessels, and surrounding smooth muscle cells (results not shown). In vehicle-injected bladder of normal dogs, strong staining for the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ collagen chains was detected whereas the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains were not detected in vehicle-injected bladder of affected dogs (results not shown). Injection of AdCMVLacZ (2×10^8 pfu) resulted in focal expression of the LacZ transgene by smooth muscle cells (Figure 7A). In affected dogs, injection of Ad $\alpha 5$ (1×10^8 pfu) resulted in focal expression of recombinant $\alpha 5(\text{IV})$ collagen by smooth muscle cells at the injection site (Figure 7B). However, transgene expression [LacZ and recombinant $\alpha 5(\text{IV})$ collagen] was limited to ~ 1 week after injection because of a robust immune response characterized by mononuclear cell infiltration and smooth muscle cell necrosis at the injection site (Figure 7C). Therefore, subsequent dogs were immunosuppressed with cyclosporin A to minimize the inflammatory response and prolong viral transgene expression. In these dogs, smooth muscle collected at 1, 2, 5, and 7 weeks after injection showed reduced inflammation and necrosis, except in the immediate vicinity of the sutures (results not shown). Furthermore, in immunosuppressed dogs injected with AdCMVLacZ (2×10^8 pfu), reporter gene expression was extended up to 5 weeks after injection (Figure 7D), although at reduced levels compared to the 1-week collection time point. At 5 weeks after injection, affected canine bladder injected with Ad $\alpha 5$ (1×10^8 pfu) showed focal expression of the recombinant $\alpha 5(\text{IV})$ chain by smooth muscle cells at the injection site that was distributed in a peripheral pattern around individual smooth muscle cells. This result implied export

of these proteins and would be consistent with localization to the basement membrane (Figure 7E). Similar results were obtained using both H52 and H53 antibodies in experiments involving replicate injections in three dogs. Expression of the $\alpha 5(\text{IV})$ transgene was detected at all periods up to 5 weeks after injection, but not at the 7-week time point. In sections adjacent to those that were positive for the $\alpha 5(\text{IV})$ transgene, there was co-expression of the $\alpha 6(\text{IV})$ collagen chain as detected by immunostaining using antibody B66 (Figure 7F). In tissues that were injected with AdCMVLacZ or vehicle alone, no expression of the $\alpha 6(\text{IV})$ chain was detected (results not shown). This implies that *de novo* synthesis of the $\alpha 6(\text{IV})$ chain was dependent on $\alpha 5(\text{IV})$ expression.

Dual immunofluorescent staining for the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains was performed to confirm these proteins co-localized within and surrounding individual smooth muscle cells at the injection site. Using normal dog kidney as a control, antibody mAb 5 stained the GBM and the basement membranes underlying Bowman's capsule and a subset of tubules in a discrete linear manner (Figure 7G). Within the same section, antibody B66 stained only the basement membrane of Bowman's capsule (Figure 7H). The $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains co-localized to Bowman's capsule but not to other $\alpha 5(\text{IV})$ -positive sites such as the GBM or selected tubular basement membranes (Figure 7I) confirming there was no cross-reactivity in the methods used for the detection of either antigen alone. Sections of Ad $\alpha 5$ -injected affected dog bladder stained using this procedure were positive for both the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains (Figure 7, J and K, respectively), and the signals co-localized in a pericellular distribution surrounding individual smooth muscle cells (Figure 7L).

Discussion

Currently the only treatment for end-stage renal disease in Alport syndrome is kidney transplantation or dialysis. The prospect of developing genetic therapies for the treatment of renal disease has been raised^{17,18} including Alport syndrome.¹⁹ Certain prerequisites must be met before embarking on treating a specific disease using gene therapy. These include: 1) an understanding of the pathophysiology of the disease, 2) knowing which gene(s) to correct, 3) knowing which cells to target, and 4) an available experimental model.

The molecular defects underlying X-linked Alport syndrome are mutations in the COL4A5 gene, which en-

Figure 7. Expression of adenoviral transgenes in dog bladder smooth muscle cells. **A:** In nonimmunosuppressed dogs, AdCMVLacZ (2×10^8 pfu) injection resulted in focal expression of the reporter gene by smooth muscle cells. **B:** In affected dogs, injection of Ad $\alpha 5$ (1×10^8 pfu) resulted in focal expression of the recombinant $\alpha 5(\text{IV})$ chain by smooth muscle cells at the injection site. **C:** Expression of both transgenes was limited to 1 week after injection because of an immune response characterized by mononuclear cell infiltration and smooth muscle cell necrosis. **D:** With immunosuppression, AdCMVLacZ (2×10^8 pfu) reporter gene expression was extended up to 5 weeks after injection. **E:** Affected dog bladder injected with Ad $\alpha 5$ (1×10^8 pfu) showed focal expression of the recombinant $\alpha 5(\text{IV})$ chain by smooth muscle cells at the injection site at 5 weeks after injection that was distributed in both the cytoplasm and also in a peripheral pattern consistent with localization to the basement membrane. **F:** In adjacent sections of those tissues that were positive for the $\alpha 5(\text{IV})$ transgene, there was co-expression of the $\alpha 6(\text{IV})$ collagen chain. Dual-labeled fluorescence microscopy was used to show co-localization of the $\alpha 5(\text{IV})$ chain (green) and $\alpha 6(\text{IV})$ chain (red). **G:** In control sections of normal dog kidney, mAb 5 stained the GBM and the basement membranes underlying Bowman's capsule and a subset of tubules. **H:** Within the same section, antibody B66 stained only the basement membrane of Bowman's capsule and smooth muscle of arterioles. **I:** As expected, the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains co-localized to Bowman's capsule and smooth muscle, resulting in a yellow fusion signal. Sections of Ad $\alpha 5$ -injected affected dog bladder stained using this procedure were positive for both the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains (**J** and **K**, respectively), with these proteins co-localizing and assuming a pericellular distribution surrounding individual smooth muscle cells (**L**). **A** and **D:** X-gal, nuclear fast red counterstain; **C:** hematoxylin and eosin; **B, E, and F:** immunoperoxidase; hematoxylin counterstain; **G–L:** immunofluorescence. Original magnifications: $\times 200$ (**A–D, G–I**); $\times 400$ (**E, F, J–L**).

codes the $\alpha 5(\text{IV})$ chain of type IV collagen. These mutations lead to a loss of the $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ chains from the GBM,^{10,40} which are contained within the same network.¹⁵ Because the life-threatening aspect of Alport syndrome is renal failure, therapy can be designed that targets the kidney, taking advantage of its isolated circulatory system for gene transfer. This approach has proven effective in studies involving the perfusion of porcine kidneys isolated from the systemic circulation using a separate oxygenated system and adenovirus as a vector.³⁹ Greater than 85% of glomeruli expressed the reporter gene, mainly by podocytes and endothelial cells. This approach overcomes the low efficiency of gene delivery to glomeruli using intra-arterial or ureteric injections of virus.¹⁸ Successful gene therapy of X-linked Alport syndrome requires transfer of an $\alpha 5(\text{IV})$ cDNA into the glomerular cells responsible for production of the GBM. The perfusion studies in normal pigs have demonstrated that transfer of an $\alpha 5(\text{IV})$ transgene with correctly localized expression to the GBM is possible.²⁰ However, because normal pigs were used, the biological activity of the $\alpha 5(\text{IV})$ transgene could not be assessed. The effectiveness of gene transfer will need to be worked out initially in an animal model. For human X-linked Alport syndrome there exists a well-characterized canine model that mimics the human disease at the clinical, ultrastructural, and protein chemistry levels.^{21–23} The genetic basis of the canine nephropathy is a nonsense mutation in the *COL4A5* gene that results in the generation of a premature stop codon²⁴ and leads to the loss of the $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ collagen chains from the GBM.^{25,26}

As a necessary first step toward developing a method of gene therapy for X-linked Alport syndrome using our canine model, we have cloned a full-length cDNA encoding canine $\alpha 5(\text{IV})$ collagen. This construct shares $\geq 92\%$ identity at the nucleotide and amino acid levels with the human sequence.²⁷ The sequence of the noncollagenous amino and carboxyl termini of the molecule (7S and NC1 domains, respectively) are highly conserved ($>97\%$ identical) as are the sites and sizes of interruptions in the Gly-X-Y repeat pattern within the collagenous domain. Such a degree of conservation presumably reflects the critical role these domains play in conferring functional properties to the molecule. The 7S and NC1 domains are known to be involved in intra- and intermolecular interactions between α -chains, while the noncollagenous interruptions may provide flexibility to the molecule, facilitating the formation of a crosslinked network in basement membranes.^{6,41,42}

By virtue of an 18-bp insertion at the site corresponding to the boundary of exons 41 and 42 of the human sequence, the $\alpha 5(\text{IV})$ cDNA derived from canine kidney, testis, and bladder encodes an $\alpha 5(\text{IV})$ chain that is longer than reported for the human $\alpha 5(\text{IV})$ cDNA derived from placental and umbilical vein endothelial cell cDNA libraries,⁴³ HT-1080 cells,²⁷ and lymphoblasts.³⁶ The insertion encodes two Gly-X-Y repeats, and thereby maintains the reading frame and conserves the Gly-X-Y repeat pattern within the collagenous domain. The identical insertion has been reported in $\alpha 5(\text{IV})$ cDNA derived from normal human kidney³⁶ and has recently been shown to be the

result of alternative splicing of the *COL4A5* gene.³⁷ We hypothesize the cDNA containing this 18-bp insertion is the form produced by cells that normally express the $\alpha 5(\text{IV})$ chain, whereas the shorter form may reflect illegitimate transcription of the *COL4A5* gene. It follows that an $\alpha 5(\text{IV})$ cDNA containing this insertion would be the preferred isoform to use for gene therapy of X-linked Alport syndrome.

The canine $\alpha 5(\text{IV})$ cDNA was capable of directing the expression of $\alpha 5(\text{IV})$ mRNA and protein in transiently transfected HEK293 cells. By Northern blot analysis, an $\alpha 5(\text{IV})$ mRNA transcript was detected in $\alpha 5(\text{IV})$ -transfected cells but not mock-transfected controls. Signals for the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ transcripts were detected at equivalent levels in both $\alpha 5(\text{IV})$ -transfected cells and mock-transfected controls, whereas the $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 6(\text{IV})$ transcripts are not endogenously expressed by HEK293 cells, nor was their expression induced after transfection with the $\alpha 5(\text{IV})$ cDNA. These results imply that expression of the $\alpha 5(\text{IV})$ transgene in HEK293 cells does not influence the expression of other type IV collagen α -chains.

The results of the Western blot analysis showed that transfected HEK293 cells synthesize and secrete the recombinant $\alpha 5(\text{IV})$ chain predominantly as a 190-kd monomer. The recombinant protein was full length and translated in the correct reading frame because it was recognized by two monoclonal antibodies to human $\alpha 5(\text{IV})$ collagen, one of which is directed against an epitope within the carboxyl-terminal NC1 domain. The observed molecular weight was slightly greater than that predicted based on its deduced amino acid sequence (162 kd). This discrepancy is most likely because of posttranslational processing events. Triple helical collagen IV molecules would have an apparent molecular weight of ≥ 500 kd, a value that is close to that observed experimentally.^{44,45} No $\alpha 5(\text{IV})$ -containing species of this size were observed, but rather, the predominant secreted form was a 190-kd monomer. There was, however, an $\alpha 5(\text{IV})$ -containing doublet of ~ 300 kd detected under nonreducing conditions that is closer to the expected size of a dimer formed from two α -chains, a species that is not known to occur *in vivo*. The exact biochemical nature of this species and its biological significance are undetermined.

Studies on the biosynthesis of fibrillar collagens have demonstrated that triple helical assembly of α -chains is required for secretion, and furthermore, that α -chains that are not incorporated into a triple helix are degraded intracellularly.⁴⁶ In the present study, both the $\alpha 2(\text{IV})$ and the recombinant $\alpha 5(\text{IV})$ collagen chains were found to be secreted in monomeric form to the culture medium suggesting that perhaps this pathway may not hold true for recombinant and/or nonfibrillar collagen chains. In this regard, our results are in agreement with those of a previous study that demonstrated the secretion of recombinant $\alpha 1(\text{IV})$ monomers by transfected CHO cells.⁴⁷ The $\alpha 1(\text{IV})$ chain may also be secreted as a monomer by HEK293 cells, but it cannot be detected with antibody H11 under reducing conditions, a finding in agreement with others.^{44,45}

Type IV collagen exists as a family of triple helical isoforms (protomers) arising from three α (IV) chains. The existence of six different α (IV) chains theoretically allows for a maximum of 56 distinct protomers differing in composition and stoichiometry. However, individual α (IV) chains do not randomly associate with each other to form triple helices, but rather are restricted to specific combinations that then form distinct supramolecular networks that vary from tissue to tissue. The α 1(IV) and α 2(IV) chains associate to form an α 1(IV)₂ α 2(IV) heterotrimer and comprise the classic network of type IV collagen that is ubiquitously expressed in basement membranes. In contrast, networks containing the α 3(IV) to α 6(IV) chains are restricted to specific sites that likely reflect their involvement in imparting specialized function. For example, in addition to the α 1(IV)/ α 2(IV) network, the GBM also contains a α 3(IV)/ α 4(IV)/ α 5(IV) network^{14,15} and smooth muscle basement membrane, an α 1(IV)/ α 2(IV)/ α 5(IV)/ α 6(IV) network.¹⁶ From these studies, it has been shown that the α 5(IV) chains form triple helical molecules with the α 3(IV), α 4(IV), or α 6(IV) chains. The results obtained through our *in vitro* expression studies of recombinant canine α 5(IV) collagen in HEK293 cells are consistent with these data. The recombinant α 5(IV) chain was secreted primarily as a 190-kD monomer implying that α 5(IV) homotrimers do not exist, nor does the α 5(IV) chain form heterotrimers with the endogenous α 1(IV) and α 2(IV) chains. Because HEK293 cells do not express the α 3(IV), α 4(IV), or α 6(IV) chains, it is reasonable that no triple helical molecules containing the α 5(IV) chain were detected. Similar conclusions have been reached in studies using HEK293 cells to express the full-length human α 3(IV) chain that also did not undergo spontaneous triple helical assembly,⁴⁸ and in CHO cells where co-expression of the α 1(IV) and α 2(IV) chains was necessary for triple helix formation.⁴⁷

Collagenase digestion of native basement membranes yields the NC1 domain of type IV collagen as a hexamer, derived from two triple helical molecules joined at their carboxyl termini.⁵ After denaturing gel electrophoresis, the hexamer dissociates into a series of bands with molecular weight of ~25 kD and ~50 kD, reflecting NC1 domain monomers (originating from a single protomer) and dimers (originating from two protomers linked by interchain disulfide bonds through their NC1 domains). Collagenase digestion of the secreted α 5(IV) chain followed by Western blot analysis yielded a single band at ~25 kD. This is the expected size of an NC1 domain monomer, confirming the collagenous nature of the translated protein. This implies, however, that the α 5(IV) chain did not dimerize with itself or with the α 1(IV) or α 2(IV) chains. This is not surprising because no α 5(IV)-containing triple helical collagen molecules were detected. Dimerization between collagen molecules is felt to occur only after triple helix formation and export of these molecules into the extracellular space.⁴⁶ At the same time, dimers containing the α 1(IV) and α 2(IV), but not the α 5(IV) NC1 domains could be detected in the collagenase-digested extracellular matrix from transfected cells indicating that they possess the biosynthetic pathways

necessary to assemble triple helical type IV collagen molecules and export these to the extracellular matrix.

The results of the *in vitro* expression studies demonstrate that the full-length recombinant α 5(IV) chain is synthesized; however, they do not address the biological activity of the recombinant protein. To resolve this issue, *in vivo* studies were conducted using a canine model for X-linked Alport syndrome that lacks the α 3(IV) to α 6(IV) chains as a result of a null mutation in the COL4A5 gene that leads to a premature stop codon.^{25,26,30,38} In these experiments, an adenoviral vector encoding α 5(IV) collagen was injected directly into bladder smooth muscle, an approach that circumvents the difficulties associated with achieving targeted gene delivery to less accessible α 5(IV)-containing sites such as the GBM. Normal bladder smooth muscle cells are ensheathed by a basement membrane composed of the α 1(IV), α 2(IV), α 5(IV), and α 6(IV) chains, whereas in affected male dogs, this basement membrane contains only the α 1(IV) and α 2(IV) chains. In affected male bladder, the levels of α 5(IV) mRNA are markedly reduced, presumably as a consequence of nonsense-mediated decay of the mutant mRNA. However, α 6(IV) mRNA continues to be expressed at normal levels in affected bladder which suggests that the absence of the α 6(IV) chain is due to a failure at the level of protein assembly.³⁸ The biochemical basis for this failure was revealed in a recent study which demonstrated that the α 5(IV) and α 6(IV) chains of the smooth muscle basement membrane exist as α 5(IV)₂ α 6(IV) heterotrimers that associate with α 1(IV)₂ α 2(IV) heterotrimers to form an α 1(IV)/ α 2(IV)/ α 5(IV)/ α 6(IV) network.¹⁶

We hypothesized that delivery of an adenoviral vector encoding α 5(IV) collagen to bladder smooth muscle cells of affected dogs may correct this underlying defect and restore expression of both the α 5(IV) and α 6(IV) chains. The results of our *in vivo* studies have confirmed this occurs. Ad α 5-infected smooth muscle cells synthesize the recombinant α 5(IV) chain, which assumes a peripheral distribution consistent with localization to the basement membrane. At 5 weeks after injection, expression of the α 6(IV) collagen chain was also detected, co-localizing with α 5(IV) collagen, as would be expected given that these chains are assembled in the same triple helical molecule and collagen network within the smooth muscle basement membrane.¹⁶ Expression of the α 6(IV) chain was detected 5 weeks after α 5(IV) collagen gene transfer, but not at 2 weeks or earlier, which suggests that the induction of α 6(IV) expression is time-dependent. The kinetics of this sequence of events, specifically onset and duration of expression, will need to be examined in greater detail using additional experiments. Other factors may also have affected detectability of the α 6(IV) chain: the B66 antibody used normally generates a weaker signal in dog tissues than the antibodies directed against the α 5(IV) chain and a trimer of α 5(IV) and α 6(IV) chains would be expected to contain twice as many α 5(IV) chains as α 6(IV) chains.¹⁶

In conclusion, we have shown that an adenoviral vector delivered to Alport smooth muscle cells *in vivo* can be used to express full-length recombinant α 5(IV) collagen that is exported, appears to localize to the basement

membrane, and is able to restore its missing partner chain, $\alpha 6(\text{IV})$ collagen. These findings constitute an important initial step toward a continuation of this work that focuses on delivery of the $\alpha 5(\text{IV})$ transgene to the glomeruli in affected dogs with the aim of reconstitution of the GBM. However, there are several hurdles that must first be cleared. Firstly, delivery of adenoviral vectors to the glomerulus is a technical challenge, but one that can be overcome using an organ perfusion system.^{20,39} Secondly, our results illustrate the requirement of immunosuppression for sustained expression of the $\alpha 5(\text{IV})$ transgene; without it, one or more of the restored collagen chains and/or viral antigens are recognized as novel proteins and the infected cells are eliminated by an immune response. This is a well-documented consequence of using first-generation (E1-deleted) adenoviruses for *in vivo* studies, because they are capable of inducing only transient expression (7 to 10 days) of foreign genes in immunocompetent hosts. If therapeutic $\alpha 5(\text{IV})$ collagen gene transfer in this model of Alport syndrome is to be successful, it will almost certainly require a vector that is not associated with these problems. Finally, Alport GBM lacks the $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ collagen chains; hence reconstitution of the GBM will require the expression of two other α -chains to be restored, rather than just one in the case of smooth muscle. Extensive research will need to be performed if gene therapy for Alport syndrome in humans is to become reality and ultimately this will depend on the results obtained from animal model work together with advances in gene therapy techniques.

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