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Can Alport syndrome be treated by gene therapy?

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Alport syndrome, also termed hereditary nephritis, was initially described in 1927 by A.C. Alport [1] as an inherited kidney disease characterized by hematuria and sensorineural deafness. Later, ocular lesions were also associated with the syndrome and, with the introduction of the electron microscope, irregularities and disruptions in the glomerular basement membrane (GBM) were shown to be typical for this disorder as well [2, 3]. The disease is highly progressive and usually leads to renal failure during adolescence or before middle age.

The disease is primarily inherited as an X-chromosome linked dominant trait with an estimated gene frequency of 1:5,000 to 1:10,000, but autosomal forms also exist [2, 3]. The defective gene in X-linked Alport syndrome was located in 1988 and 1989 to the long arm of the X chromosome [4–6]. In 1990, a gene encoding a novel basement membrane (type IV) collagen $\alpha 5$ chain was discovered and localized to the Alport gene region on chromosome X, and this was soon followed by identification of mutations in this gene in Alport patients [7, 8]. More recently, mutations have also been reported in the genes for the $\alpha 3$ and $\alpha 4$ type IV collagen chains in the rarer autosomal forms of Alport syndrome [9–11].

Presently, over 200 different mutations are identified in type IV collagen genes in Alport patients, most of them in the COL4A5 gene that encodes the α_5 chain of type IV collagen [12–16]. These mutations can be considered responsible for abnormalities in the structural framework of the GBM, with the result of kidney manifestations. Identification of the genetic defects have opened up the question if the disease can be treated by gene therapy. In this article we discuss the potential for the development of gene therapy of Alport syndrome in the light of current knowledge of the pathomechanisms of the disease and our recent progress on gene transfer into kidney and the molecular biology of type IV collagen.

Clinical features of Alport syndrome

Patients with Alport syndrome usually have recurrent microscopic or gross hematuria in childhood, earlier in males than in females. Affected males, and in rare cases also females, usually develop end-stage renal disease. The hearing loss, if present, is sensorineural and primarily affects high tones. Electron microscopy usually reveals thinning and thickening of the GBM with longitudinal splits into thin layers with a basket-weave pattern. These changes are most evident in male patients, except in boys of very young age. Lenticonus, a peculiar change of lens shape, is also frequently observed in Alport patients [2, 3]. The disease is inherited and family history is present in 85% of cases. The remaining 15% may represent new mutations [2]. A large proportion of patients with renal failure and mutations in type IV collagen do not have hearing loss or eye lesions [12–16]. Thus, the classical clinical definition for the Alport syndrome, that is, hereditary nephritis with hearing loss, does not apply to all patients with type IV collagen defects. It is, therefore, important that clinicians consider Alport syndrome as a possible diagnosis for patients with hematuria and/or renal failure, even though they do not have hearing deficiency, ocular lesions or family history. Gregory et al [3] have recently described the criteria for diagnosis of Alport syndrome (Table 1). According to their proposal, at least four of the ten criteria must be satisfied to establish diagnosis in an Alport syndrome family.

Molecular properties of the glomerular basement membrane

The filtration barrier of the kidney consists of the fenestrated capillary endothelium, the GBM, and the epithelial podocytes that are separated by the slit and connected by a thin diaphragm. Similar to other basement membranes in the body, the GBM consists of type IV collagen, laminin, proteoglycan and nidogen/entactin [17]. Type IV collagen, the main structural component of the GBM, forms a special type of quite flexible but tightly crosslinked protein meshwork. The laminin molecules also form a network that is linked to the type IV collagen network through nidogen (entactin). The proteoglycan components are believed to contribute to the filtration of macromolecules as an anionic filtration barrier.

In Alport syndrome, the structure of the type IV collagen network is substantially weakened due to mutations in isoforms of this protein that are crucial for the GBM function. Knowledge about the biosynthesis, structure and genetics of this protein is of fundamental importance for consideration of experiments aimed at gene therapy.

Type IV collagen: Genes, biosynthesis and tissue distribution

Type IV collagen is a basement membrane specific protein that exists in several isoforms [17]. Similarly to other types of collagen, the type IV collagen molecule is composed of three α chains that contain numerous consecutive Gly-X-Y repeats, where proline and hydroxyproline are frequently located in positions X and Y. The presence of glycine as every third amino acid is essential, as

Table 1. Criteria for the clinical diagnosis of Alport syndrome according to Gregory et al [3]

1. Family history of nephritis of unexpected hematuria in a first degree relative of the index case or in a male relative linked through any numbers of females.
2. Persistent hematuria without evidence of another possibly inherited nephropathy such as thin GBM disease, polycystic kidney disease, or IgA nephropathy.
3. Bilateral sennsorineural hearing loss in the 2,000 to 8,000 Hz range. The hearing loss develops gradually, is not present in early infancy, and commonly presents before the age of 30 years.
4. A mutation in COL4An (where $N = 3$, 4, or 5).
5. Immunohistochemical evidence of complete or partial lack of the Alport epitope in glomerular, or epidermal basement membranes, or both.
6. Widespread GBM ultrastructural abnormalities, in particular thickening, thinning, and splitting.
7. Ocular lesions including anterior lenticonus, posterior subcapsular cataract, posterior polymorphous dystrophy, and retinal flecks.
8. Gradual progression to ESRD in the index case of at least two family members.
9. Macrothrombocytopenia or granulocytic inclusions.
10. Diffuse leiomyomatosis of esophagus or female genitalia, or both.

Four of the ten criteria should be fulfilled.

it is the only amino acid small enough to fit into the center of the triple helix in collagenous proteins. The long collagenous structure in type IV collagen is interrupted by several non-collagenous domains along the molecule. The interruptions are considered to give flexibility to the molecule and facilitate the formation of a network in basement membranes. In addition to the long collagenous domain, type IV collagen α-chains contain a noncollagenous domain, NC1, at the carboxyl end.

In the mammalian genome to date six different genes have been found for type IV collagen. Each one of the COL4A1 - COL4A6 genes encodes a different α-chain, α1-α6. The genes are located pairwise in an unusual head-to-head fashion on chromosomes 2, 13 and X [17, 18]. The six genes are likely to have evolved through duplication and inversion of an ancestral gene with subsequent two further rounds of duplication resulting in the three head-to-head located gene pairs on different chromosomes. The type IV collagen genes are large, over 100 kb, and complex, containing 46 to 52 exons [19–23].

Transcription of the α chain genes occurs as normally for proteins, but during translation in the cisternae of the rough endoplasmic reticulum the nascent α chains undergo several enzymatic post-translational modifications (Fig. 1). These include hydroxylation at the 4 and 3 positions of prolyl residues by prolyl-4- and prolyl-3-hydroxylases, respectively, hydroxylation of lysyl residues by lysylhydroxylase, and glycosylation of most hydroxylysyl residues by two glycosyl transferases to form glucosyl-galactosyl-hydroxylysyl residues (a review on collagen biosynthesis is in [24]). Hydroxyproline is essential in collagen, where it is required the maintenance of a stable triple helix through the formation of hydrogen bonds between the α chains. In type IV collagen about 60% of the prolyl residues are hydroxylated to 4-hydroxyproline and about 5% to 3-hydroxyproline, and about 90% of the lysyl residues are hydroxylated with about 90% of them containing glucosyl-galactosyl disaccharides. The hydroxylysyl residues are believed to stabilize cross-links between molecules, but the function of carbohydrates is unknown. In the cisternae three chains assemble into a triple helix, after which the post-translational modifications cease as the enzymes do not react with α chains in a triple-helical protein. The triple-helical monomers are then secreted out from the cell. Collagen α chains that do not form a triple helix are degraded intracellularly.

Once secreted out from the cells, the triple-helical molecules assemble by end-to-end aggregation and lateral alignment into a tightly crosslinked network structure (Fig. 2). Two molecules bind to each other through disulfide bonds at their carboxyl termini by the association of the NC1 domains and four molecules assemble, also via disulfide bridges, at the amino termini. Of the six genetically distinct type IV collagen chains, the α1 and α2 chains are ubiquitous in all basement membranes. In contrast, the α3, α4, α5 and α6 chains are minor component chains with a more restricted tissue distribution and thus probably specialized functions. For example, molecules containing α3, α4 and α5 chains are particularly abundant in the adult GBM [25], where they are believed to form α3:α4:α5 heterotrimers. As yet, there is no direct evidence that the α3, α4 and α5 chains form the trimeric molecule in the GBM, but several studies have revealed that those chains are co-expressed in the same tissues [26–29]. At the protein assembly level however, there are still several possibilities for how the α3(IV), α4(IV), and α5(IV) chains are organized with respect to: (a) each other forming triple-helical molecules that vary in kind and composition of chains (relative abundance), and (b) which triple-helical molecules, containing α1(IV) to α6(IV) chains, interact through NC1-NC1 association forming a network. Possibly, the α5(IV) chain forms triple-helical molecules separate from the α3(IV) and α4(IV) chains, or it forms triple-helical molecules together with the α3(IV) and α4(IV) chains. At the suprastructure level, the chains appear to be distributed about at least two networks: an α1(IV)/α2(IV) network and an α3(IV)-α5(IV), and, possibly, α6(IV) network. Clearly, the α5(IV) is critical for the α3(IV) and α4(IV) incorporation and for the GBM function, as revealed by the renal pathology caused by mutation in the *COL4A5* gene. Knowledge of the composition of the α-chains and their organization in GBM is fundamental to the understanding of the molecular basis for hereditary nephritis. In any case, basement membranes composed of molecules containing the α3, α4, and α5 chains are believed to be stronger due to the presence of high content of cysteine residues and, thus, disulfide crosslinks [28, 29]. The strength of the structural network is considered to be particularly important in basement membranes, such those of renal glomeruli (GBM) and the lens capsule, which do not have supportive collagen fibers. Therefore, mutations in the genes for any of the three α chains of the main GBM type IV collagen isoform are believed to result in the weak network and hematuria, the main characteristic of Alport syndrome.

Molecular pathology of Alport syndrome

Alport syndrome is a type IV collagen disease. Mutations have been described in the *COL4A5* gene in over 200 cases [12–16], and mutations have also been identified in the *COL4A3* and *COL4A4* genes in autosomal recessive forms of the disease [9, 11]. Interestingly, there are several observations indicating that the α3(IV) and α4(IV) chains are abnormal in the X-linked form of Alport syndrome even though both are encoded by autosomal genes [26, 30, 31]. It is unclear how a mutation in the α5(IV) gene accounts for the abnormalities in these other α(IV) chains, but it is possible that one or more mechanisms that link the incorporation of α3(IV), α4(IV), and α5(IV) chains could operate at the protein

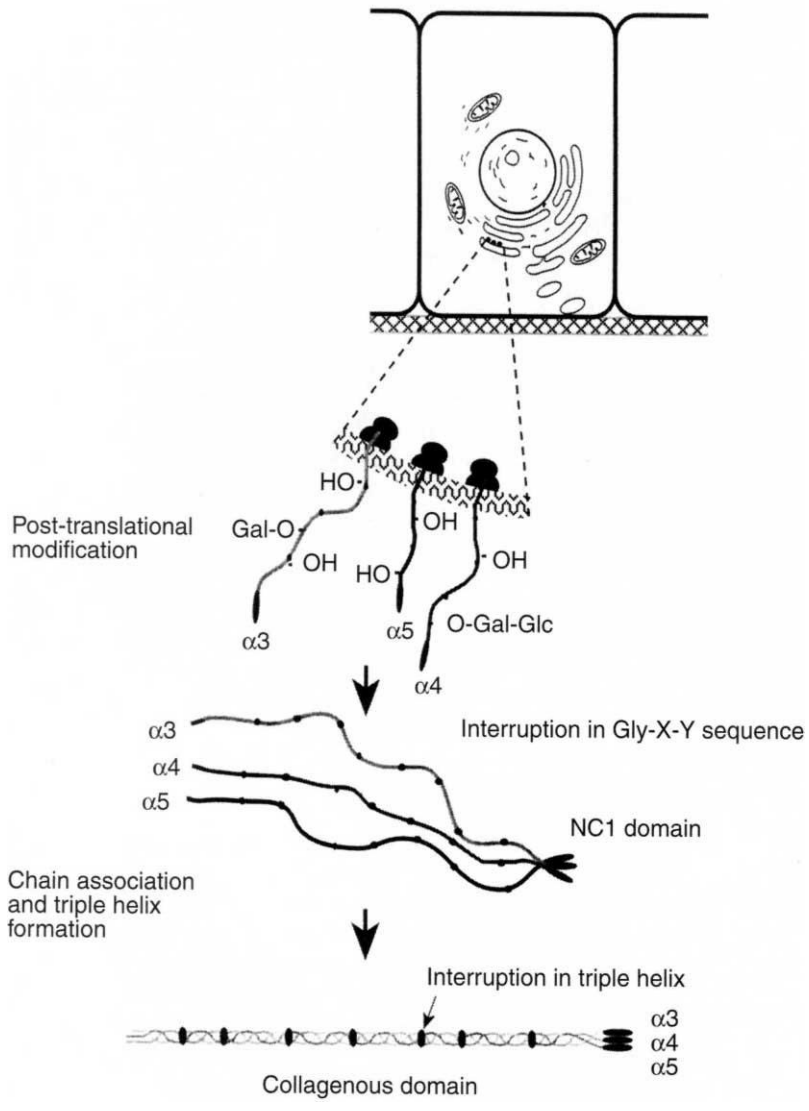


Fig. 1. *Biosynthesis of type IV collagen in a cell resting on a basement membrane follows the pattern of that for collagenous proteins, in general [24].* Following transcription and processing of the primary transcript the mRNA is transported to the cytosol to ribosomes where translation occurs. Post-translational hydroxylations and glycosylations take place on the nascent α chains in the cisternae of the rough endoplasmic reticulum. This is followed by chain association, disulfide bonding and formation of the triple helix of three appropriate α chains. Single chains not incorporated into triple helices are degraded, while the triple-helical molecules are transported through the Golgi apparatus to the extracellular space where supramolecular assembly occurs.

assembly and/or at the translational/transcriptional level [32]. At the protein level, events at both triple helix formation and supramolecular assembly need to be considered. For example, an abnormal α5(IV) chain could lead to faulty heterotrimer assembly, if the α3(IV), α4(IV), and α5(IV) chains are present in heterotrimers, resulting in the absence of all these chains in GBM of patients with X-linked Alport syndrome. Alternatively, the α3(IV) and α4(IV) chains may be in trimers distinct from those containing the α5(IV) chain, the latter being necessary for the incorporation of α3(IV) and α4(IV) chains containing trimers into the supramolecular assembly of collagen type IV molecules. Consequently, abnormality of α5(IV)-containing trimers in X-linked Alport syndrome could lead to absence of the α3(IV)- and α4(IV)-containing trimers from the GBM. At the translational/transcriptional level, expression of the α3(IV), α4(IV), or α5(IV) chains might be coordinated, so the transcription or translation of the α3(IV) chains might be impaired secondary to a mutation in the α5(IV) gene in the X-linked disease. The protein assembly and the translational/transcriptional mechanisms need not be mutually exclusive.

Almost all the mutations identified to date differ between families. No clear correlation can be found between the nature of a mutation and the respective phenotype. A small single amino acid substitution can cause as severe symptoms as a large deletion of almost the entire collagen gene. This, together with the complexity of the collagen genes, makes DNA-based diagnosis of Alport syndrome particularly difficult. About 15% of the mutations characterized to date are large gene rearrangements, such as deletions, insertions, inversions or duplications [12–16]. The rest are small mutations, mainly single base changes, in addition to small deletions, insertions or duplications. The mutations can result in a complete absence of the protein (α-chain) in question, a truncated protein or in a malfunctional protein. A loss of the carboxyterminal noncollagenous domain would prohibit the formation of heterotrimers. Several mutations in Alport syndrome involve replacement of a glycine residue in the collagenous domain by another amino acid. Glycine mutations are frequently also the cause of other collagen disorders such as *osteogenesis imperfecta* [33]. Based on the knowledge obtained from studies on *osteogenesis imperfecta,* it has been suggested that the abnormally

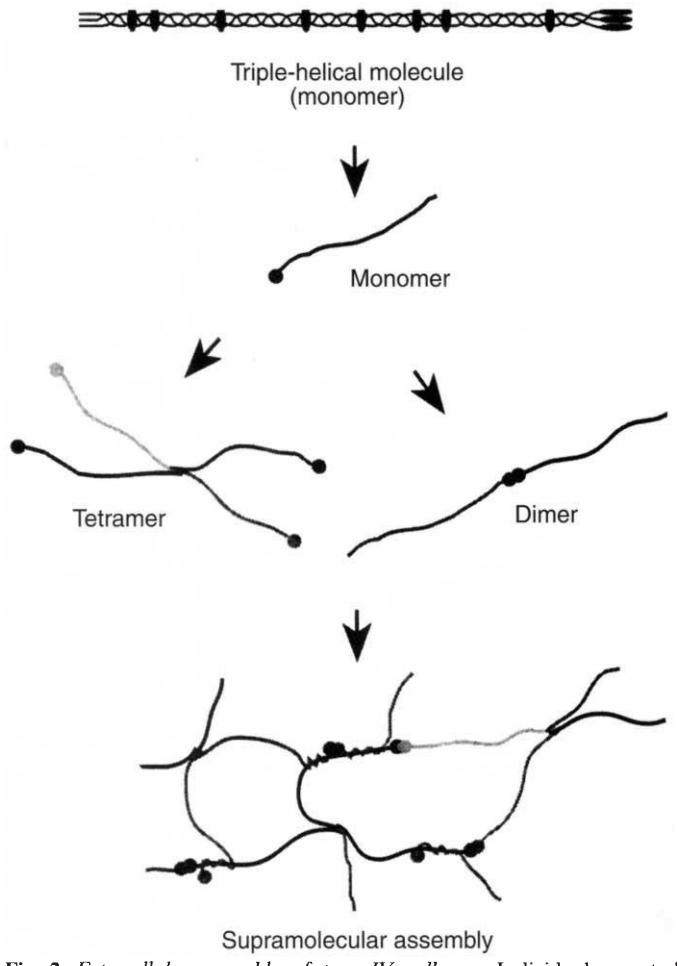


Fig. 2. *Extracellular assembly of type IV collagen.* Individual secreted triple-helical molecules assemble by end-to-end associations into a network-like structure into which other basement membrane proteins are bound in a largely unknown fashion. In reality the type IV collagen network structure also contains more complex laterally aligned molecules. (Modified from [17]; used with permission).

folded triple helices are susceptible to degradative enzymes leading to partial or total absence of all the $\alpha 3$, $\alpha 4$ and $\alpha 5$ chains. As a result, the structural framework of the GBM that requires type IV molecules containing these polypeptide chains, becomes structurally weak and disrupted.

Can Alport syndrome be treated by gene therapy?

To date, there is no satisfactory and curative conservative treatment available for Alport syndrome [34]. Patients developing end-stage renal disease are treated by hemodialysis, and also by kidney transplantation whenever possible. However, about 5% of transplanted Alport males develop anti-GBM nephritis and lose the allografted kidneys.

As a result of the advances in molecular genetic research, gene therapy may be developing into a real possibility for the treatment of a variety of hereditary diseases in the future. Although gene therapy has not yet come of age as a real alternative for treatment of human diseases, extensive research efforts are being made in that direction. Alport syndrome, which primarily affects the renal glomeruli, is an attractive disease target for gene therapy for the following reasons. First of all, as it almost solely affects the kidney glomeruli, extrarenal complications not being life-threatening or occurring in all patients, the therapy can, at least initially, be targeted to the kidney alone. Secondly, the kidney, with its well separated circulatory system lends itself extremely well to organ targeted gene transfer. Thirdly, the turnover of type IV collagen is relatively slow, the half-life probably being over one year. However, extensive research still needs to be carried out before we can expect to be able to do successful gene therapy of Alport syndrome in humans, and there are numerous questions that need to be answered before we know if gene therapy will become possible for this disease. In the following we address some of the key questions that researchers encounter today.

Is it possible to achieve effective gene transfer targeted to the endothelial and epithelial cells of renal glomeruli?

The GBM is surrounded by endothelial and epithelial cells, which both are believed to contribute to the biosynthesis of the GBM type IV collagen. Due to lack of sensitivity, methods such as *in situ* hybridization have not confirmed whether one or both cell types contribute by the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains to the GBM. However, from the gene transfer point of view, these cells can be considered as the prime target.

Our recent work indicates that the targeting of genes to these cells may not become a significant problem, even though previous work with gene transfer into kidneys has been disappointing. Intra-arterial injections of the viruses have yielded very low if any gene delivery to glomerular cells [35, 36], but using liposomes, together with sendai virus, transfer efficiency has been reported to reach 15% of the glomeruli [37]. Recently using a kidney perfusion method (Fig. 3), we could obtain up to 85% transfer efficacy into pig glomeruli with an adenovirus vector containing the β-galactosidase reporter gene (Fig. 4) [38]. The key to this high transfer efficacy is probably the prolonged contact time between cells and viruses. The perfusion method is relatively easy to apply to the kidney since it can be applied *in situ*, and may also prove useful for the transfer of genes to other organs with well separated blood supply.

Is it possible to obtain life-lasting and controlled expression of the transferred gene?

This is a serious problem as it is the main hurdle of gene therapy in general, and it poses one of the main challenges that research in this field faces today. The adenovirus used in our experiments provides, in its current form, expression for only six to eight weeks, as it remains extrachromosomal and is not integrated into the genome. Even though the half life of type IV collagen is estimated to be over a year, this means that the treatment would need to be periodically repeated during life with

Fig. 4. *Immunolocalization of β-galactosidase in a glomerulus.* The protein can be observed mainly in podocytes while staining of endothelial and mesangial cells is weaker and less frequent (immunoperoxidase, ×700).

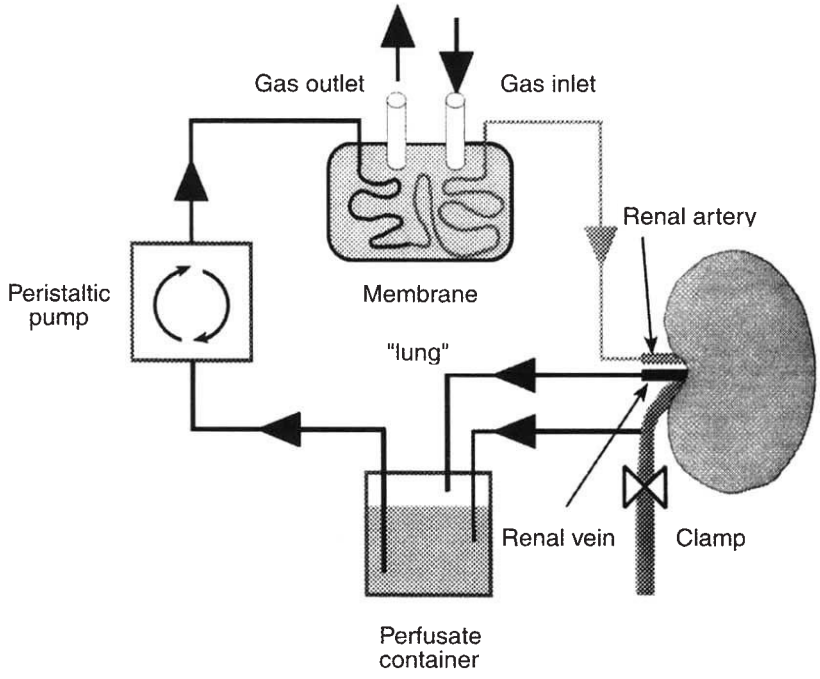
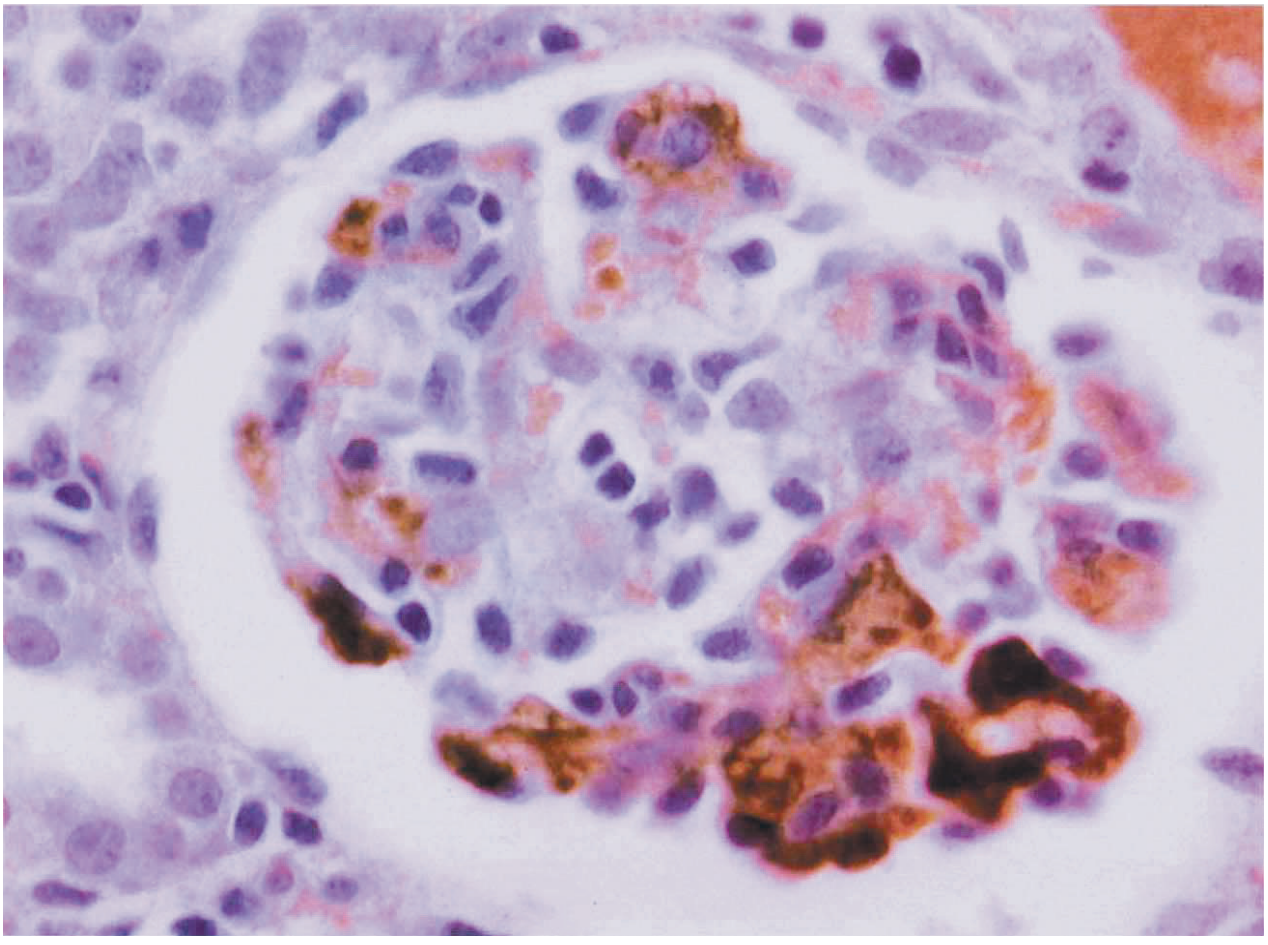


Fig. 3. *Scheme of the kidney perfusion system used for gene transfer into pig kidneys [38].* The closed-circuit system consists of a reservoir for the perfusate, a peristaltic pump, an oxygenator and a thermostatic heater. The renal vein and artery are cannulated and connected to the tubing perfusion system. The effluent from the ureter is recovered into the reservoir. The 350 ml perfusate contains the recombinant virus solution, porcine blood cells (17% hematocrit), 25,000 U heparin, 20,000 U penicillin and 20,000 μg streptomycin in Krebs-Ringer solution.



current methods, which would entail mean repeated surgical operations. Retroviruses would theoretically be better, but they cannot integrate large DNA inserts; also, they are only integrated into replicating DNA, which is not the case for the glomerular epithelial and endothelial cells that, for practical purposes, are non-dividing cells. Therefore, successful future gene therapy of Alport syndrome will depend on future developments in research on gene transfer vectors.

Will a transferred type IV collagen cDNA or gene coupled to an appropriate promoter be expressed in a controlled manner and incorporated into normal GBM type IV collagen molecules?

We have already been able to express the full-length α5(IV) collagen chain in cultured cells using plasmids, and are in the process of doing similar studies with adenoviruses containing the α5 cDNA. There is evidence that the incorporation of the α3, α4 and α5 chains into GBM is a coordinated event, with factors acting at the transcriptional, translational and/or protein assembly levels [32, 39]. The fact that the production of these three chains is coordinated is probably a favorable thing. This means that the limiting factor of intracellular synthesis of trimers (homo- or hetero-) is likely to become the availability of the two other α-chains. Thus, if one chain is produced in excess, it will be degraded intracellularly (see biosynthesis above). It has been shown that α1(IV) mRNA can exist 3- to 15-fold longer than α2(IV) mRNA, even though the ratio of the chains in the protein was 2:1, respectively [40]. Possibly any promoter can be used to produce the α5(IV) chain in excess. However, specific targeting or the right level of expression may be achieved by using the promoter of the α5(IV) chain gene. If the production of the defective chain can be corrected to some extent, secretion of normal molecules is likely to occur, even in the presence of some abnormal malfunctional molecules. Female patients with X-linked Alport's are obligate mosaics for a mutant *COL4A5* gene and provide a natural experiment for partial production of a normal α5 chain, which gene therapy would also attempt to create. Female patients often have mild disease and avoid end-stage renal disease, providing and encouraging observation that total replacement of mutant with normal α5-chains would not be necessary in any gene therapy treatment designed. However, only experimental work will prove or disprove of these speculations.

Can corrected type IV collagen trimers be incorporated into the GBM and restore its structure and function?

This is still an open question which will have to be addressed experimentally in animal models. Our work in the Samoyed dog model of X-linked hereditary nephritis (see below) has shown that carrier female dogs, in which glomeruli are chimeric for the *COL4A5* mutation, have a mixture of abnormal and normal GBM in early stages of the disease. Later in life, however, the GBM begins to take on a more normal appearance throughout all glomeruli [41]. One explanation for this observation is that normal molecules can spread within the GBM to restore the structure. Thus, incorporation of type IV collagen trimers into the GBM may occur with restoration of its structure and function, thereby eliminating a major obstacle for gene therapy of Alport's disease.

Are there appropriate animal models for gene therapy studies on Alport syndrome?

An animal model for a human disease is essential for testing the feasibility of gene therapy of diseases in humans. From the Alport syndrome point of view, the situation is quite favorable as models for both the X-linked and autosomal forms have been described in dogs. For example, a model for the X-linked disease has been described in Samoyed dogs [42, 43]. Hemizygous male dogs develop hematuria at the age of three months and end-stage renal disease usually at nine months of age. The disease is caused by a nonsense mutation in the X chromosomal *COL4A5* gene and is both clinically and morphologically similar to that in humans [43]. Such dogs may prove excellent models for gene therapy experiments.

Conclusion

As a treatment, somatic gene therapy is a new type of treatment aiming directly at the causative defect, not only at the symptoms. Gene therapy has not really come of age yet, but extensive efforts are being made in numerous laboratories to explore the realistic possibilities for this new treatment. In spite of many hurdles, there is still cautious optimism among researchers about the possibilities for developing lasting gene therapy protocols for several inherited disorder.

Identification of the genes affected in Alport syndrome has raised questions about the possibilities for gene therapy, and in this article we have discussed the potentials for the development of such therapy for Alport syndrome. Based on the knowledge we have about the type IV collagen chains affected in the disease, we consider it possible both theoretically and practically to correct, at least transiently, the abnormal GBM type IV collagen network by transfer of a normal α(IV) gene into cells that synthesize the protein *in vivo*. We have been able to transfer reporter genes with high efficiency into glomerular cells *in vivo* by kidney perfusion, and will soon be able to explore if the defect can actually be corrected in dogs with Alport syndrome. If those experiments yield promising results, gene therapy of human Alport patients may become a possibility some day. However, future gene therapy of this disease, as for any disease for that matter, will depend largely on the development of better gene transfer vectors, and on more knowledge about the pathophysiology of the disease and regulation of the genes to be treated.

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