and inhibition of tumor invasion by the membrane-anchored glycoprotein **RECK.** *Proc Natl Acad Sci U S A* 1998, **95(22)**:13221-13226.
20. Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA: **Matrix metalloproteinases: a review.** *Crit Rev Oral Biol Med* 1993, **4(2)**:197-250.
21. Gueders MM, Foidart JM, Noel A, Cataldo DD: **Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs in the respiratory tract: potential implications in asthma and other lung diseases.** *Eur J Pharmacol* 2006, **533(1-3)**:133-144.
22. DeGraba TJ: **Immunogenetic susceptibility of atherosclerotic stroke: implications on current and future treatment of vascular inflammation.** *Stroke* 2004, **35(11 Suppl 1)**:2712-2719.
23. Zhao BQ, Wang S, Kim HY, Storrie H, Rosen BR, Mooney DJ, Wang X, Lo EH: **Role of matrix metalloproteinases in delayed cortical responses after stroke.** *Nat Med* 2006, **12(4)**:441-445.
24. Vihinen P, Kahari VM: **Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets.** *Int J Cancer* 2002, **99(2)**:157-166.
25. Spinale FG, Coker ML, Heung LJ, Bond BR, Gunasinghe HR, Etoh T, Goldberg AT, Zellner JL, Crumbley AJ: **A matrix metalloproteinase induction/activation system exists in the human left ventricular myocardium and is upregulated in heart failure.** *Circulation* 2000, **102(16)**:1944-1949.
26. Burrage PS, Mix KS, Brinckerhoff CE: **Matrix metalloproteinases: role in arthritis.** *Front Biosci* 2006, **11**:529-543.
27. Overall CM, Kleifeld O: **Towards third generation matrix metalloproteinase inhibitors for cancer therapy.** *Br J Cancer* 2006, **94(7)**:941-946.
28. Aimes RT, Quigley JP: **Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments.** *J Biol Chem* 1995, **270(11)**:5872-5876.
29. Birkedal-Hansen H: **Proteolytic remodeling of extracellular matrix.** *Curr Opin Cell Biol* 1995, **7(5)**:728-735.
30. Schoser BG, Blottner D, Stuerenburg HJ: **Matrix metalloproteinases in inflammatory myopathies: enhanced immunoreactivity near atrophic myofibers.** *Acta Neurol Scand* 2002, **105(4)**:309-313.
31. Choi YC, Dalakas MC: **Expression of matrix metalloproteinases in the muscle of patients with inflammatory myopathies.** *Neurology* 2000, **54(1)**:65-71.
32. Kieseier BC, Schneider C, Clements JM, Gearing AJ, Gold R, Toyka KV, Hartung HP: **Expression of specific matrix metalloproteinases in inflammatory myopathies.** *Brain* 2001, **124(Pt 2)**:341-351.
33. von Moers A, Zwirner A, Reinhold A, Bruckmann O, van Landeghem F, Stoltenburg-Didinger G, Schuppan D, Herbst H, Schuelke M: **Increased mRNA expression of tissue inhibitors of metalloproteinase-1 and -2 in Duchenne muscular dystrophy.** *Acta Neuropathol (Berl)* 2005, **109(3)**:285-293.
34. Kherif S, Lafuma C, Dehaupas M, Lachkar S, Fournier JG, Verdiere-Sahuque M, Fardeau M, Alameddine HS: **Expression of matrix metalloproteinases 2 and 9 in regenerating skeletal muscle: a study in experimentally injured and mdx muscles.** *Dev Biol* 1999, **205(1)**:158-170.
35. Nakamura A, Yoshida K, Ueda H, Takeda S, Ikeda S: **Up-regulation of mitogen activated protein kinases in mdx skeletal muscle following chronic treadmill exercise.** *Biochim Biophys Acta* 2005, **1740(3)**:326-331.
36. Yamada H, Saito F, Fukuta-Ohi H, Zhong D, Hase A, Arai K, Okuyama A, Maekawa R, Shimizu T, Matsumura K: **Processing of beta-dystroglycan by matrix metalloproteinase disrupts the link between the extracellular matrix and cell membrane via the dystroglycan complex.** *Hum Mol Genet* 2001, **10(15)**:1563-1569.
37. Matsumura K, Zhong D, Saito F, Arai K, Adachi K, Kawai H, Higuchi I, Nishino I, Shimizu T: **Proteolysis of beta-dystroglycan in muscular diseases.** *Neuromuscul Disord* 2005, **15(5)**:336-341.
38. Zhong D, Saito F, Saito Y, Nakamura A, Shimizu T, Matsumura K: **Characterization of the protease activity that cleaves the extracellular domain of beta-dystroglycan.** *Biochem Biophys Res Commun* 2006, **345(2)**:867-871.
39. Shimatsu Y, Yoshimura M, Yuasa K, Urasawa N, Tomohiro M, Nakura M, Tanigawa M, Nakamura A, Takeda S: **Major clinical and histopathological characteristics of canine X-linked muscular dystrophy in Japan, CXMDJ.** *Acta Myol* 2005, **24(2)**:145-154.
40. Sharp NJ, Kornegay JN, Van Camp SD, Herbstreith MH, Secore SL, Kettle S, Hung WY, Constantinou CD, Dykstra MJ, Roses AD, *et al.*: **An error in dystrophin mRNA processing in golden retriever muscular dystrophy, an animal homologue of Duchenne muscular dystrophy.** *Genomics* 1992, **13(1)**:115-121.
41. Shimatsu Y, Katagiri K, Furuta T, Nakura M, Tanioka Y, Yuasa K, Tomohiro M, Kornegay JN, Nonaka I, Takeda S: **Canine X-linked muscular dystrophy in Japan (CXMDJ).** *Exp Anim* 2003, **52(2)**:93-97.
42. Fujiwara A, Shibata E, Terashima H, Shishido A, Nishiki J, Yoshida K, Miyauchi K, Madachi A, Matsuura N: **Evaluation of Matrix Metalloproteinase-2 (MMP-2) Activity with Film in situ Zymography for Improved Cytological Diagnosis of Breast Tumors.** *Breast Cancer* 2006, **13(3)**:272-278.
43. Shiraga M, Yano S, Yamamoto A, Ogawa H, Goto H, Miki T, Miki K, Zhang H, Sone S: **Organ heterogeneity of host-derived matrix metalloproteinase expression and its involvement in multiple-organ metastasis by lung cancer cell lines.** *Cancer Res* 2002, **62(20)**:5967-5973.
44. Kaneyoshi T, Nakatsukasa H, Higashi T, Fujiwara K, Naito I, Nouso K, Kariyama K, Kobayashi Y, Uemura M, Nakamura SI, Iwasaki Y, Tsuji T: **Actual invasive potential of human hepatocellular carcinoma revealed by in situ gelatin zymography.** *Clin Cancer Res* 2001, **7(12)**:4027-4032.
45. Nakada M, Nakamura H, Ikeda E, Fujimoto N, Yamashita J, Sato H, Seiki M, Okada Y: **Expression and tissue localization of membrane-type 1, 2, and 3 matrix metalloproteinases in human astrocytic tumors.** *Am J Pathol* 1999, **154(2)**:417-428.
46. Ohashi K, Nemoto T, Nakamura K, Nemori R: **Increased expression of matrix metalloproteinase 7 and 9 and membrane type 1-matrix metalloproteinase in esophageal squamous cell carcinomas.** *Cancer* 2000, **88(10)**:2201-2209.
47. Kaji M, Moriyama S, Sasaki H, Saitoh Y, Kiriyama M, Fukai I, Yamakawa Y, Mitsui A, Toyama T, Nemori R, Fujii Y: **Gelatinolytic activity of matrix metalloproteinase in lung cancer studied using film in situ zymography stamp method.** *Lung Cancer* 2003, **39(2)**:125-130.
48. Nakano Y, Niida S, Dote K, Takenaka S, Hirao H, Miura F, Ishida M, Shingu T, Sueda T, Yoshizumi M, Chayama K: **Matrix metalloproteinase-9 contributes to human atrial remodeling during atrial fibrillation.** *J Am Coll Cardiol* 2004, **43(5)**:818-825.
49. Frederiks WM, Mook OR: **Metabolic mapping of proteinase activity with emphasis on in situ zymography of gelatinases: review and protocols.** *J Histochem Cytochem* 2004, **52(6)**:711-722.
50. Corbi AL, Kishimoto TK, Miller LJ, Springer TA: **The human leukocyte adhesion glycoprotein Mac-1 (complement receptor type 3, CD11b) alpha subunit. Cloning, primary structure, and relation to the integrins, von Willebrand factor and factor B.** *J Biol Chem* 1988, **263(25)**:12403-12411.
51. Davis CE, Harris JB, Nicholson LV: **Myosin isoform transitions and physiological properties of regenerated and re-innervated soleus muscles of the rat.** *Neuromuscul Disord* 1991, **1(6)**:411-421.
52. Birkedal-Hansen H, Taylor RE: **Detergent-activation of latent collagenase and resolution of its component molecules.** *Biochem Biophys Res Commun* 1982, **107(4)**:1173-1178.
53. Guerin CW, Holland PC: **Synthesis and secretion of matrix-degrading metalloproteases by human skeletal muscle satellite cells.** *Dev Dyn* 1995, **202(1)**:91-99.
54. Daimon E, Wada Y: **Role of neutrophils in matrix metalloproteinase activity in the preimplantation mouse uterus.** *Biol Reprod* 2005, **73(1)**:163-171.
55. Gilbert SJ, Wotton PR, Tarlton JF, Duance VC, Bailey AJ: **Increased expression of promatrix metalloproteinase-9 and neutrophil elastase in canine dilated cardiomyopathy.** *Cardiovasc Res* 1997, **34(2)**:377-383.
56. Ohtake Y, Tojo H, Seiki M: **Multifunctional roles of MT1-MMP in myofiber formation and morphostatic maintenance of skeletal muscle.** *J Cell Sci* 2006, **119(Pt 18)**:3822-3832.
57. Echizenya M, Kondo S, Takahashi R, Oh J, Kawashima S, Kitayama H, Takahashi C, Noda M: **The membrane-anchored MMP-regulator RECK is a target of myogenic regulatory factors.** *Oncogene* 2005, **24(38)**:5850-5857.

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REVIEW

Gene therapy for Duchenne muscular dystrophy

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Gene therapy has great potential to treat Duchenne muscular dystrophy. Among many proposed strategies to deliver a therapeutic gene to muscle, recombinant adeno-associated virus-mediated gene transfer is the most promising. The recent isolation of new adeno-associated virus serotypes from human and nonhuman primates provides the opportunity to develop vectors that can achieve the long-term expression of a therapeutic gene in muscles of the entire body without detrimental effects. To translate the results from small animal models to clinical trials in humans, further work using larger animal models, such as dystrophic dogs or nonhuman primates, is required. This review also discusses recent progress in other gene transfer-related therapeutic approaches, including targeted exon skipping and gene correction.

Duchenne muscular dystrophy (DMD), which affects one in 3300 males, is a devastating, progressive, muscle-wasting disease caused by mutations in the dystrophin gene [1,2]. Skeletal muscles in DMD are characterized by myofiber degeneration and progressive fibrous and fatty changes. There is, currently, no way to prevent muscle fiber necrosis and patients suffer severely from respiratory and cardiac complications in the second decade of life. The *DMD* gene is among the largest genes known, spanning 2.4 Mb at Xp21 and encoding a 427-kDa subsarcolemmal cytoskeletal protein, dystrophin, and several shorter isoforms (Dp260, Dp140, Dp116 and Dp71). The full-length dystrophin protein is composed of four domains: an N-terminal actin-binding domain, a central rod domain consisting of 24 spectrin-like repeats, a cysteine-rich domain and a C-terminal domain. Dystrophin binds actin at the N-terminal domain, β-dystroglycan at the cysteine-rich domain and dystrobrevin and syntrophins at the C-terminal domain, forming the dystrophin–glycoprotein complex (DGC) at the sarcolemma (Figure 1) [3]. A lack of dystrophin at the sarcolemma causes secondary loss of the DGC and other functional molecules, such as neuronal nitric oxide synthase (nNOS) [4] and aquaporin-4 [5]. Importantly, mutations in the genes encoding other members of the DGC cause several different types of muscular dystrophy. The mechanism of the degeneration and death of dystrophin-deficient myofibers is not yet fully understood, but it is believed that myofibers lacking dystrophin and the DGC at the cytoplasmic membrane are mechanically weak and highly susceptible to contraction-induced injury. As a result, the affected muscle experiences continuous cycles of myofiber death and regeneration, resulting in the gradual loss of myofibers and contractile force. In addition to mechanical weakness, abnormalities in calcium handling and changes in mitogen-activated protein (MAP) kinase and GTPase signaling in dystrophin-deficient muscle have been reported and proposed as underlying processes of muscular dystrophy [6,7].

At present, only corticosteroids are reported to effectively attenuate the progress of the disease [8], and current treatment options focus on respiratory and cardiac assistance and improvement of quality of life. Many research groups are still attempting to develop an effective therapy for DMD. In this review, we describe recent progress in gene and related therapies for DMD.

Recombinant adeno-associated virus vector: a promising tool for delivery of dystrophin gene to skeletal muscles

Among several gene transfer vectors and methods developed to date, the adeno-associated virus (AAV) vector is the most suitable to introduce the exogenous gene into postmitotic, nondividing myofibers. An AAV is a tiny, nonpathological, replication-defective virus, with a 4.7-kb single-stranded DNA genome, belonging to the parvovirus family. AAV vectors induce fewer immunological and inflammatory responses *in vivo* than adenovirus vectors [9]. Although the virus genome persists predominantly in episomal form, expression of the transferred gene lasts months to years in adult skeletal muscle. To date, more than 100 AAVs with distinct virus genome sequences have been isolated from humans, nonhuman primates and other species [10]. They display varying

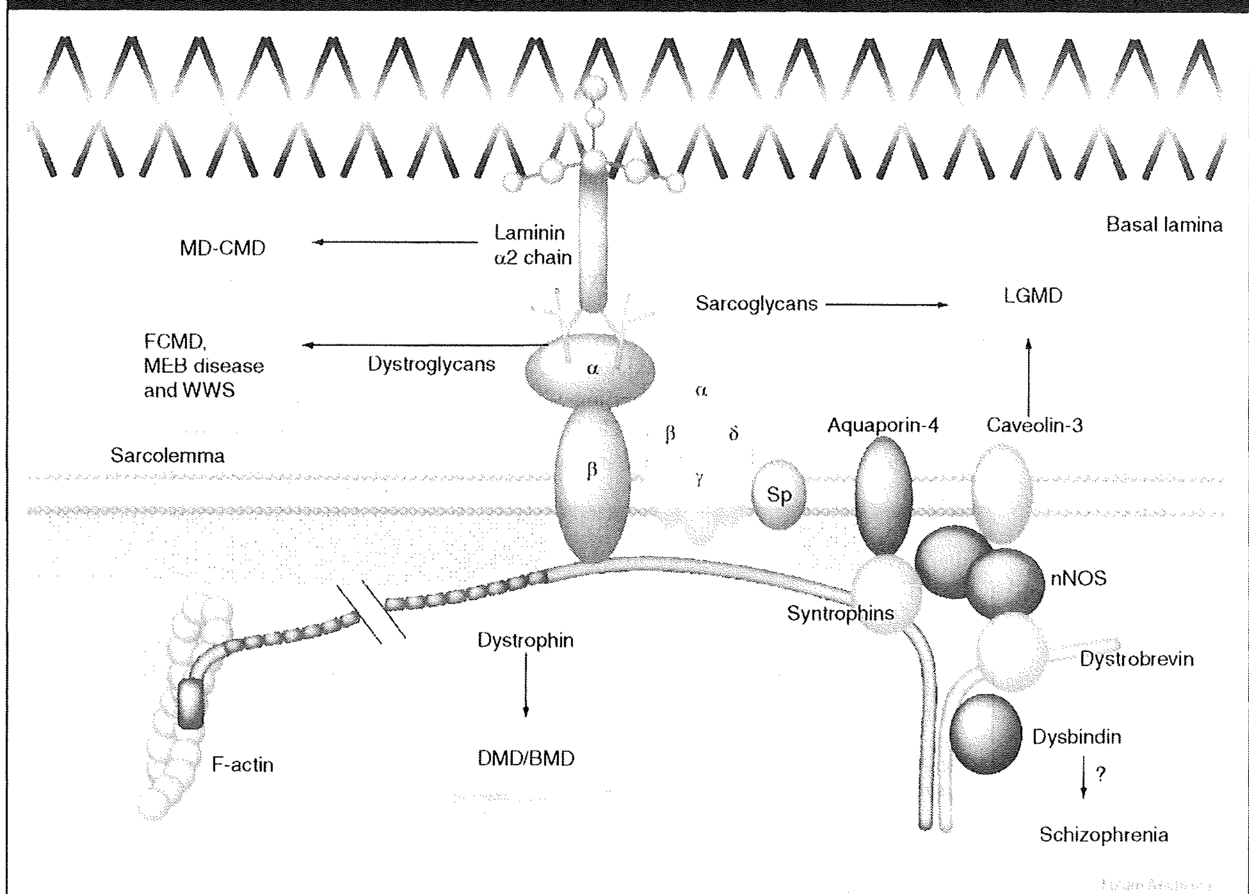
Keywords: adeno-associated virus vector, Duchenne muscular dystrophy, dystrophin, exon skipping, gene therapy

degrees of similarity in their capsid proteins and show diverse tissue tropisms. More than nine AAV vectors have already been developed and evaluated in animal models as a tool for gene transfer *in vivo* (Table 1) [10]. Although the molecular mechanisms of tissue and cell tropisms of AAV vectors are not fully explained, they are likely to use different cellular receptors for entry into and binding to the host cells. The expression of the therapeutic genes is not permanent, mainly because recombinant (r)AAV does not replicate in the host and is barely incorporated into the genome of satellite cells. They are diluted out with the turnover of myofibers and, therefore, repeated administrations are required. New AAV serotypes would provide good options for follow-up treatments because they have the potential to evade pre-existing neutralizing antibodies against the previously used AAV serotype. However, to avoid the risks of *in vivo* vector delivery, it is important to better understand the vectors and the natural infection with the corresponding virus.

Generation of microdystrophin suitable for use in rAAV vectors

The rAAV vector is a promising tool for gene transfer to DMD muscle, but the limitation of the insertion size to 4.9 kb excludes incorporation of

Figure 1. Dystrophin forms the large dystrophin–glycoprotein complex at the sarcolemma, linking the basal lamina to the cytoskeletal actin.



Mutations in the dystrophin gene end in the secondary loss of dystrophin–glycoprotein complex and other functional molecules, such as nNOS and aquaporin-4. Mutation in the laminin α2 chain gene causes congenital MD. Abnormal glycosylation of α-dystroglycan is commonly observed in FCMD, MEB disease and WWS. Abnormal glycosylation of α-dystroglycan also causes abnormalities in the eye and the CNS. Mutations in any of four sarcoglycan genes (α, β, γ and δ) result in LGMD. These observations emphasize the importance of dystrophin and associated molecules for muscle integrity. The dysbindin (*DTNBP1*) gene is one of the several putative susceptibility genes for schizophrenia.

BMD: Becker MD; DMD: Duchenne MD; FCMD: Fukuyama-type congenital MD; LGMD: Limb girdle MD; MD: Muscular dystrophy; MD-CMD: Merosin-deficient congenital MD; MEB: Muscle–eye–brain; nNOS: Neuronal nitric oxide synthase; WWS: Walker–Warburg syndrome.

Table 1. Characterization of nine serotypes of AAV vectors.

Serotype	Amino acid homology to AAV2 (%)	Isolated from	Tissue tropism				Delivery system
			Skeletal muscle	*Heart*	*Liver*	*CNS*	
1	84	NHP	+++	++	+	+	Local
2	100	Human	+	+	+	+	Local
3	88	Human		+	±	+	Local
4	64	NHP		+	±	+	Local
5	61	Human	+	+	++	++	Local
6	84	Human	++	++	+	+	Local, systemic
7	83	NHP	+++	+++	++	++	Local, systemic
8	84	NHP	+++	+++	+++	++	Local, systemic
9	83	Human	+++	+++	+++	++	Local, systemic

AAV: Adeno-associated virus; NHP: Nonhuman primate.
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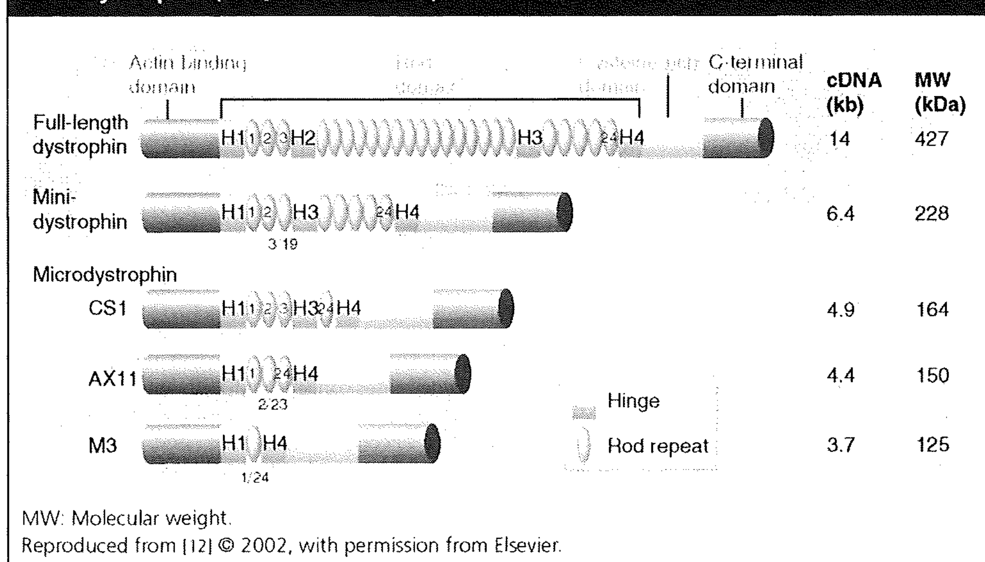
a full-length dystrophin gene (14-kb mRNA, 11-kb open reading frame). To overcome this drawback, several groups have designed small dystrophins in which the long, central rod domains are largely deleted, and tested their functions in dystrophin-deficient *mdx* mice [11]. The functions of three types of microdystrophins (CS1, AX11 and M3; Figure 2) have been tested on microdystrophin-transgenic, dystrophin-deficient *mdx* mice, and it was observed that over-expressed CS1 with four repeats and three hinges almost completely ameliorated dystrophic phenotypes [12]. Therefore, a rAAV2 vector was constructed expressing CS1, driven by a skeletal muscle-specific muscle creatine kinase promoter [13], and was injected it into the anterior tibialis muscles of immunocompetent adult *mdx* mice. A total of 24 weeks after injection, 50% of myofibers, on average, expressed microdystrophin and the treated muscles demonstrated improved contractile force [14].

Systemic delivery of rAAV-serotype 6, 8 & 9 vectors

Systemic delivery systems for the treatment of DMD require improvement to enable transfer of the therapeutic genes to the complete musculature of the body, especially to the heart and diaphragm. Gregorevic and colleagues reported that intravenous injection of rAAV6 vectors efficiently delivered a microdystrophin gene to the

Figure 2. Structure of full-length dystrophin and constructs of mini- and microdystrophin (CS1, AX11 and M3).



MW: Molecular weight.
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muscles of an adult mouse and the ratio of microdystrophin-positive fibers was increased when co-injected with vascular endothelial growth factor [15]. The widespread expression of microdystrophin was sufficient to correct susceptibility to contraction-induced injury and to lower serum creatine kinase levels [15]. Wang and colleagues, and Nakai and colleagues, demonstrated that AAV8 was more efficient than AAV6 or AAV1 at attaining systemic gene transfer, especially to the cardiac muscles of mice or hamsters, without pharmacological intervention [16,17]. More recently, Inagaki and colleagues reported that AAV9 vectors demonstrated robust systemic transduction in mice [18]. Remarkably, rAAV9 is superior to rAAV8 for gene delivery to cardiac muscle by systemic vector administration [18]. The molecular basis of the high transduction efficiency via the bloodstream is not fully understood, but these results are encouraging for researchers who are developing gene therapies for DMD patients. On the other hand, however, AAV8 or 9 vectors also increase transduction of nonmuscle tissues, such as liver (Table 1), which may be deleterious.

AAV vectors for human muscle

Animal models are indispensable for the evaluation of the efficacy and safety of AAV-mediated gene therapy of DMD, but a recent report on clinical gene transfer studies for hemophilia B demonstrated that the data obtained in preclinical studies in animals are not always predictive of vector efficacy in humans [19]. Certain human populations are exposed to AAVs in daily life: 50–96% are seropositive for AAV2 and at least a third have a neutralizing antibody to AAV2 [10]. Therefore, prior exposure to AAV2 explains the unsatisfactory results of clinical trials using rAAV2-factor IX gene transfer on hemophilia B patients [19]. The new serotypes of AAVs are reported to be prevalent in human and nonhuman primates. Prescreening of patients for neutralizing antibodies against the vector serotype and transient immune suppression would be required to avoid the elimination of rAAV particles by neutralizing antibodies.

Minidystrophin coded by two AAV vectors (dual vector system)

Microdystrophin proteins, with 3–4 spectrin-like repeats in the rod domain, do not completely compensate for the lack of full-length dystrophin. Among the constituents of DGC and its binding proteins, the expression of nNOS cannot be recovered through the introduction of microdystrophin. In an attempt to introduce a therapeutic gene larger than 4.7 kb into target cells, the trans-splicing approach, in which the gene is split between two rAAV vectors, each containing part of an intron with either a splice-acceptor or a splice-donor sequence, has been developed. After formation of head-to-tail concatamers, trans-splicing of the two RNA transcripts from the two different expression cassettes removes the intervening sequence, producing a functional mRNA larger than could be delivered in a single vector. This approach was employed to deliver a minidystrophin to *mdx* muscle [20], however, the coordinated nature of transcription and splicing makes this strategy highly inefficient *in vivo*.

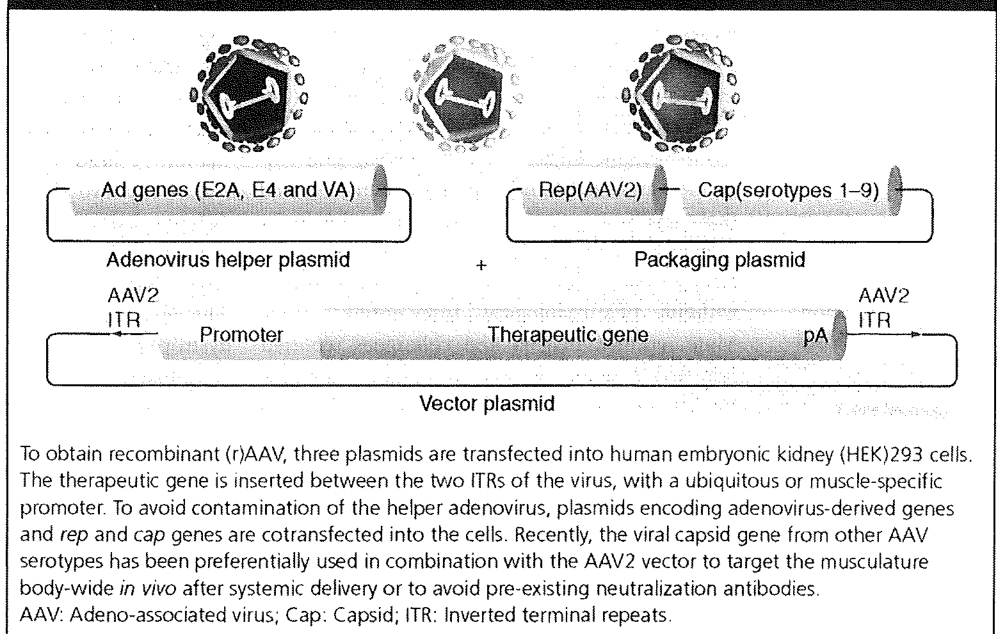
Production of AAV vectors on a large scale

rAAV vector plasmids are generated by deleting the viral genome except for the inverted terminal repeats. To obtain recombinant AAV particles, double [21] or triple [22] transfection of the plasmids into human embryonic kidney (HEK)293 cells is performed to provide rep and capsid proteins and adenoviral helper functions (Figure 3). The obtained AAV vectors are further purified by CsCl gradient sedimentation or ion-exchange chromatography. In the case of rAAV2, it is estimated that at least 1×10^{13} vg/kg is required to treat humans with hemophilia, whereas the titer of the vectors prepared by this standard method is approximately $2–5 \times 10^{13}$ genome copies from 1×10^{9} HEK293 cells [13]. In clinical trials, an inexpensive, safe, large-scale system must be developed for the production of AAV. For example, Urabe and colleagues described a highly powerful production of rAAV using non-mammalian cell culture [23]. Okada and colleagues described a large-scale AAV vector production with active gassing [24].

Safety issues

Currently, most research on AAV-mediated gene transfer focuses on the systemic delivery of therapeutic genes via the blood circulation. Some have demonstrated the effectiveness of high-pressure arterial [25] or venous [26] infusion. These procedures seem to be powerful in transducing the therapeutic genes into targeted muscle groups, but the safety should be tested carefully in larger animal models. In particular, the mutagenic and carcinogenic potentials of recombinant genomes should be investigated, in addition to their potential for germline transfer after systemic delivery.

Figure 3. Production of pseudo-typed adeno-associated virus vectors in HEK293 cells.



To obtain recombinant (r)AAV, three plasmids are transfected into human embryonic kidney (HEK)293 cells. The therapeutic gene is inserted between the two ITRs of the virus, with a ubiquitous or muscle-specific promoter. To avoid contamination of the helper adenovirus, plasmids encoding adenovirus-derived genes and *rep* and *cap* genes are cotransfected into the cells. Recently, the viral capsid gene from other AAV serotypes has been preferentially used in combination with the AAV2 vector to target the musculature body-wide *in vivo* after systemic delivery or to avoid pre-existing neutralization antibodies.
AAV: Adeno-associated virus; Cap: Capsid; ITR: Inverted terminal repeats.

Gutted adenoviral vectors expressing full-length dystrophin

Adenoviral vectors infect both dividing myoblasts and terminally differentiated muscle fibers, and possess a large insert capacity. However, early generations of adenoviral vectors, however, elicited substantial immune reactions in immunocompetent *mdx* mice and, hence, a rapid loss of transgene expression [27,28]. To circumvent this problem, a 'gutted' adenoviral vector, from which most viral DNA sequences are deleted, has been developed. Gutted adenoviral vectors are capable of carrying the large dystrophin gene together with regulatory sequences, and show reduced immunotoxicity compared with conventional adenoviral vectors [29–31]. Preparation of the gutted adenovirus vector requires a conventional adenovirus to supply replication and packaging functions *in trans*, and therefore has a high risk of helper virus contamination that may elicit immunological reactions upon delivery to tissues. In addition, recombinant adenoviral vectors remain comparatively toxic, especially in the liver, when administered systemically and have yet to achieve comparable transduction efficiency compared with AAV vectors.

Other vectors

A lentiviral vector is an alternative option for *in vivo* gene transfer into skeletal muscle. Kobinger and colleagues demonstrated that a lentiviral vector encoding minidystrophin targeted both satellite cells and myofibers of *mdx* mice and provided functional correction *in vivo* [32].

Direct injection of naked plasmid into dystrophic animals

Direct injection of a naked plasmid containing a full-length dystrophin cDNA into the muscles of DMD patients has been proposed as a promising treatment to restore the expression of dystrophin. The efficiency was low in animal models [33,34] and in a Phase I gene therapy clinical trial [35], but the dystrophin expression is relatively stable and evoked no signs of humoral or cellular immune responses. Experiments using mouse models demonstrated that the efficiency of gene transfer can be enhanced by electroporation coupled with the intramuscular application of hyaluronidase [36,37]. However, combination of electroporation and hyaluronidase administration would act to damage the muscle. The application to DMD patients is questionable. Hydrodynamic delivery of naked plasmid DNA expressing full-length dystrophin into the *mdx* mice has been reported to be effective [38]. Dystrophin expression was seen in 1–5% of the myofibers of the targeted muscle group of the hind limb for an extended period. To protect dystrophin-deficient muscles from muscle degeneration, repeated administration of plasmids would be required.

Ex vivo gene transfer into myogenic cells

Cell-mediated therapy can be used to deliver the normal dystrophin gene to dystrophic muscle. In particular, *ex vivo* transfer of a functional dystrophin gene into patients' satellite cells (myogenic progenitor cells usually located between myofibers and muscle basal lamina in a dormant state) and their progeny (myoblasts) is an attractive option for cell-based therapies for DMD since several methods to freshly purify satellite cells from muscle have been established [39,40]. A lentivirus vector would be the first choice for *ex vivo* mini- or micro-dystrophin gene transfer into autologous myogenic cells because it can infect freshly isolated satellite cells without lowering their proliferation and differentiation potential [Ikemoto *et al.*, Unpublished Data]. Stem cells other than satellite cells, such as muscle side population (SP) cells [41–43], mesoangioblasts [44], and AC133-positive human stem cells [43], have been reported to participate in muscle regeneration. Muscle SP cells are isolated by their ability to efflux Hoechst dye. Bachrach and colleagues demonstrated that SP cells from *mdx* (5cv) mice transduced with microdystrophin *ex vivo* were transplanted successfully via the tail vein and delivered human microdystrophin to the skeletal muscle of nonirradiated *mdx* (5cv) mice [45]. Recently, Dezawa and colleagues reported a novel method to induce muscle progenitor cells from human bone marrow stromal cells with a high efficiency [46].

Correction of endogenous genes

Gene conversion using chimeraplasts attempts to correct point mutations of the *DMD* gene in the cell. The first generation of chimeraplasts comprises hybrid RNA/DNA molecules that are homologous to a targeted gene, yet include one mismatched base. These hybrid nucleotides trigger gene conversion from a mutant to a functional allele via intranuclear DNA mismatch repair mechanisms. Injection of chimeric oligonucleotides into *mdx* mice resulted in the expression of full-length dystrophin in muscle fibers at the site of injection [47]. Gene correction mediated by chimeraplasts has also been demonstrated in the dystrophic golden retriever dog [48]. A second generation gene editing tool is a linear DNA oligonucleotide, 25-mer or longer containing a single central mismatch. This tool repaired single point mutations in the dystrophin gene with efficiencies comparable to that seen with chimeric RNA/DNA oligonucleotides, but yielded more consistent results [49]. Approximately 20% of DMD patients have single point mutations and, therefore, are potential targets of this therapeutic approach. However, gene repair techniques may not work for all mutations. Further data on the efficacy of the correction *in vivo* are required, using a range of point mutations of the dystophin gene.

Targeted exon skipping

Antisense oligonucleotides

DMD and *mdx* muscles have a few revertant fibers that express functional dystrophin [50,51]. This phenomenon is explained by aberrant splicing, which omits one or more exons and, as a result, restores a disrupted reading frame and dystrophin expression. Based on this observation, forced exon skipping is being developed as a future treatment to restore dystrophin expression from the mutated DMD gene in humans. The main tools for targeted exon skipping are antisense oligonucleotides (AOs). 2′-*O*-methyl-modified RNA on a phosphorothioate backbone, endowed oligonucleotides with greater resistance to nuclease degradation and, therefore, additional increases in stability were achieved [52–54]. Direct intramuscular injection of 2′-*O*-methyl phosphorothioate AOs resulted in a significant increase in the number of dystrophin-positive fibers (20%) in *mdx* mouse muscle [55]. Phosphoro-amide morpholino oligonucleotides have also proven to be effective in producing functional dystrophin in dystrophin-deficient muscle [56]. Weekly intravenous injections of morpholino AOs induced the expression of functional levels of dystrophin body-wide in skeletal muscles of the dystrophic *mdx* mouse and improved muscle function [57]. Based on the successful results in animal models, a clinical trial using AOs has already started in Leiden and is about to commence in the UK. Theoretically, AO-based exon skipping is applicable to 80% of dystrophin gene mutations. Furthermore, it is estimated that targeting just 12 exons restores the open reading frame of 75% of all deletions responsible for DMD.

AAV-mediated exon skipping

AOs display a limited half-life *in vivo*, and administration of AOs to patients must be repeated weekly or monthly. To obtain a longer-term effect, rAAV1 vectors expressing a modified U7 small nuclear RNA gene were used to direct exon skipping in *mdx* mice [58]. Following a single, high-pressure injection of the rAAV1/U7 vector

into the femoral artery of mdx mice, normal levels of dystrophin expression were restored and sustained for over 6 months. Although the initial study was limited to delivery to a single limb, this technique could be coupled with systemic delivery of AAV vectors of new serotypes.

Insulin-like growth factor-1 & myostatin blockade rescue dystrophin-deficient muscle

Myostatin (also known as growth and differentiation factor [GDF]8) is a transforming growth factor (TGF)-β family member that negatively regulates skeletal muscle growth, as evidenced by the increased musculature of the mice with a null mutation in this gene [59]. Mutation of the myostatin gene has also been found in human [60]. The myostatin-null child was reported to be muscular without any health problems at 4.5 years of age [60]. Myostatin blockade in *mdx* mice results in increases in both muscle mass and muscle strength and reductions in muscle fiber degeneration and serum creatine kinase levels [61]. Based on this observation, the recombinant human antibody against myostatin (MYO-029) is now being tested on adult muscular dystrophy patients.

Increased insulin-like growth factor (IGF)-1 within *mdx* myofibers reduces the breakdown of dystrophic muscle during the acute onset of muscle degeneration [62]. This mechanism of action can partly account for the long-term reduced severity of the dystrophic pathology in *mdx* mice overexpressing mIGF-1 and provides opportunities for therapeutic strategies [63].

Conclusion

Almost 20 years have passed since the discovery of dystrophin. Unfortunately, we have yet to find an effective therapy that can mitigate the dystrophic process. Numerous approaches are currently being explored, but many suffer from a variety of drawbacks. Among the gene therapy approaches to DMD under investigation, rAAV-mediated gene transfer is the most promising but still faces several obstacles. Other therapeutic approaches, including cell therapy and pharmacological intervention, would be used in complement with AAV-microdystrophin gene transfer.

Future perspective

An important step towards the clinical use of gene therapy is the evaluation of the efficacy and safety of gene transfer methods and protocols using animals larger than mice. We have established a beagle-based canine X-linked muscular dystrophy (CXMD) colony at the National Institute of Neuroscience in Japan ($CXMD_J$) and reported their severe phenotypes [64]. Beagle-based $CXMD_J$ is smaller and easier to handle than golden retriever CXMD, and is, therefore, a useful model for DMD. Preclinical studies using nonhuman primates would also be informative before clinical trials. Importantly, there are so many variables, even in a single treatment, such as myostatin blockade with antibodies, that more trials will be needed.

At present, gene therapy trials and related strategies face various hurdles and difficulties. Effective treatment of DMD may be achieved through a combination of different therapeutic approaches; for example, a combination of AAV vector-mediated gene transfer plus corticosteroid administration or myostatin blockage.

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Executive summary

Introduction

- Duchenne muscular dystrophy (DMD) is caused by mutations in the *DMD* gene, which encodes a 427-kDa subsarcolemmal cytoskeletal protein, dystrophin.
- At present, there is no treatment to arrest the progression of DMD and patients generally suffer from respiratory and/or cardiac complications in the second decade of life.
- Among several therapeutic strategies for this disease, recombinant adeno-associated virus (rAAV)-mediated gene transfer is the most promising.

Executive summary

Viral vector-mediated gene therapy

- AAV vectors drive long-term expression of the therapeutic gene in skeletal muscle *in vivo*, but the insertion size is limited to 4.9 kb.
- Functional, rod domain-deleted dystrophin (microdystrophin) can be incorporated into AAV vectors.
- New serotypes of AAV vectors have been isolated and developed as gene-transfer vectors, some of which transport the therapeutic genes to all the muscles of the body after systemic delivery.

Ex vivo *gene transfer into myogenic stem cells*

- Cell-mediated therapy can be used to deliver a normal dystrophin gene to dystrophic muscle in the hope that the delivered cells will participate in muscle-fiber regeneration in dystrophic muscle, express dystrophin and improve muscle function.
- Muscle satellite cells, side population cells, mesangioblasts, AC133-positive cells and bone marrow stromal cells are expected to be potential cell sources for cell-mediated therapy.

Gene correction & exon skipping using antisense oligonucleotides

- Chimeraplasts, which are chimeric RNA/DNA oligonucleotides homologous to a targeted gene (except for the inclusion of one mismatched base) can be used to direct the correction of a mutation by inducing preferential gene conversion from a mutant to a functional allele.
- Exon skipping using antisense oligonucleotides (AOs) targets transcribed RNA molecules to omit a nonsense mutation and restore a disrupted reading frame.
- Weekly intravenous injections of morpholino phosphorodiamidate (morpholino) AOs induce the expression of functional levels of dystrophin in skeletal muscles body-wide in the dystrophic *mdx* mouse.

Myostatin & insulin-like growth factor-1

- Blockage of myostatin and delivery of insulin-like growth factor-1 are effective to improve dystrophic phenotypes and the contractile force of dystrophin-deficient muscle.

Future perspective

- Preclinical studies using dystrophic dogs and nonhuman primates would be informative before human clinical trials.
- To overcome this devastating disease, multiple, diverse therapeutic strategies should be combined.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM: Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell* 50, 509–517 (1987).
2. Ray PN, Belfall B, Duff C *et al.*: Cloning of the breakpoint of an X;21 translocation associated with Duchenne muscular dystrophy. *Nature* 318, 672–675 (1985).
3. Engel AG, Ozawa E: Dystrophinopathies. In: *Myology (3rd Edition)*. Engel AG, Franzini-Armstrong C (Eds). McGraw-Hill, NY, USA, 961–1025 (2004).
   - • **Excellent text on muscular dystrophies.**
4. Brenman JE, Chao DS, Xia H, Aldape K, Bredt DS: Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* 82, 743–752 (1995).
5. Yokota T, Miyagoe Y, Hosaka Y *et al.*: Aquaporin-4 is absent at the sarcolemma and at perivascular astrocyte endofeet in α1-syntrophin knockout mice. *Proc. Japan Acad.* 76(Ser. B), 22–27 (2000).
6. Rando T: The dystrophin–glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. *Muscle Nerve* 24, 1575–1594 (2001).
7. Batchelor CL, Winder SJ: Sparks, signals and shock absorbers: how dystrophin loss causes muscular dystrophy. *Trends Cell. Biol.* 16, 198–205 (2006).
8. Biggar WD, Harris VA, Eliasoph L, Alman B: Long-term benefits of deflazacort treatment for boys with Duchenne muscular dystrophy in their second decade. *Neuromuscul. Disord.* 16, 249–255 (2006).
   - • **Long-term study that demonstrates that deflazacort has a very significant impact on the health and quality of life of boys with Duchenne muscular dystrophies (DMD), and is associated with few side effects.**
9. Blankinship MJ, Gregorevic P, Chamberlain JS: Gene therapy strategies for Duchenne muscular dystrophy utilizing recombinant adeno-associated virus vectors. *Mol. Ther.* 13, 241–249 (2006).
   - •• **Comprehensive review of recent progress in adeno-associated virus (AAV)-mediated gene therapy for DMD.**
10. Gao G, Vandenberghe LH, Wilson JM: New recombinant serotypes of AAV vectors. *Curr. Gene Ther.* 5, 285–297 (2005).
    - •• **Interesting review of the isolation strategy, evolution of new AAV serotypes, and their impact on gene therapy.**
11. Athanasopoulos T, Graham IR, Foster H, Dickson G: Recombinant adeno-associated viral (rAAV) vectors as therapeutic tools for Duchenne muscular dystrophy (DMD). *Gene Ther.* 11, 109–121 (2004).
    - •• **Comprehensive review of recent progress in AAV-mediated gene therapy for DMD.**
12. Sakamoto M, Yuasa K, Yoshimura M *et al.*: Micro-dystrophin cDNA ameliorates dystrophic phenotypes when introduced into *mdx* mice as a transgene. *Biochem. Biophys. Res. Commun.* 293, 1265–1272 (2002).
13. Yuasa K, Sakamoto M, Miyagoe-Suzuki Y *et al.*: Adeno-associated virus vector-mediated gene transfer into dystrophin-deficient skeletal muscles evokes enhanced immune response against the transgene product. *Gene Ther.* 9, 1576–1588 (2002).
14. Yoshimura M, Sakamoto M, Ikemoto M *et al.*: AAV vector-mediated microdystrophin expression in a relatively small percentage of *mdx* myofibers improved the mdx phenotype. *Mol. Ther.* 10, 821–828 (2004).
15. Gregorevic P, Blankinship MJ, Allen JM *et al.*: Systemic delivery of genes to striated muscles using adeno-associated viral vectors. *Nat. Med.* 10, 828–834 (2004).

16. Wang Z, Zhu T, Qiao C *et al.*: Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nat. Biotechnol.* 23, 321–328 (2005).
- **Authors demonstrate that AAV8 vector is capable of introducing the therapeutic gene into the musculature of the whole body.**
17. Nakai H, Fuess S, Storm TA, Muramatsu S, Nara Y, Kay MA: Unrestricted hepatocyte transduction with adeno-associated virus serotype 8 vectors in mice. *J. Virol.* 79, 214–224 (2005).
18. Inagaki K, Fuess S, Storm TA *et al.*: Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. *Mol. Ther.* 14, 45–53 (2006).
19. Manno CS, Pierce GF, Arruda VR *et al.*: Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat. Med.* 12, 342–347 (2006).
20. Lai Y, Yue Y, Liu M *et al.*: Efficient *in vivo* gene expression by trans-splicing adeno-associated viral vectors. *Nat. Biotechnol.* 23, 1435–1439 (2005).
21. Grimm D, Kern A, Rittner K, Kleinschmidt JA: Novel tools for production and purification of recombinant adeno-associated virus vectors. *Hum. Gene Ther.* 18, 2745–2760 (1998).
22. Xiao X, Li J, Samulski RJ: Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J. Virol.* 72, 2224–2232 (1998).
- **One of the pioneering papers in adenovirus-free AAV preparation methods.**
23. Urabe M, Ding C, Kotin RM: Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum. Gene Ther.* 13, 1935–1943 (2002).
24. Okada T, Nomoto T, Yoshioka T *et al.*: Large-scale production of recombinant viruses by use of a large culture vessel with active gassing. *Hum. Gene Ther.* 16, 1212–1218 (2005).
25. Gonin P, Arandel L, Van Wittenberghe L, Marais T, Perez N, Danos O: Femoral intra-arterial injection: a tool to deliver and assess recombinant AAV constructs in rodents whole hind limb. *J. Gene Med.* 7, 782–791 (2005).
26. Su LT, Gopal K, Wang Z *et al.*: Uniform scale-independent gene transfer to striated muscle after transvenular extravasation of vector. *Circulation* 112, 1780–1788 (2005).
27. Ishii A, Hagiwara Y, Saito Y *et al.*: Effective adenovirus-mediated gene expression in adult murine skeletal muscle. *Muscle Nerve* 22, 592–599 (1999).
28. Yamamoto K, Yuasa K, Miyagoe Y *et al.*: Immune response to adenovirus-delivered antigens upregulates utrophin and results in mitigation of muscle pathology in *mdx* mice. *Hum. Gene Ther.* 11, 669–680 (2000).
29. Dello Russo C, Scott JM, Hartigan-O'Connor D *et al.*: Functional correction of adult mdx mouse muscle using gutted adenoviral vectors expressing full-length dystrophin. *Proc. Natl Acad. Sci. USA* 99, 12979–12984 (2002).
30. Matecki S, Dudley RW, Divangahi M *et al.*: Therapeutic gene transfer to dystrophic diaphragm by an adenoviral vector deleted of all viral genes. *Am. J. Physiol. Lung Cell Mol. Physiol.* 287, L569–L576 (2004).
31. Dudley RW, Lu Y, Gilbert R *et al.*: Sustained improvement of muscle function one year after full-length dystrophin gene transfer into *mdx* mice by a gutted helper-dependent adenoviral vector. *Hum. Gene Ther.* 15, 145–156 (2004).
32. Kobinger GP, Louboutin JP, Barton ER, Sweeney HL, Wilson JM: Correction of the dystrophic phenotype by *in vivo* targeting of muscle progenitor cells. *Hum. Gene Ther.* 14, 1441–1449 (2003).
33. Braun S, Thioudellet C, Rodriguez P *et al.*: Immune rejection of human dystrophin following intramuscular injections of naked DNA in *mdx* mice. *Gene Ther.* 7, 1447–1457 (2000).
34. Liu F, Nishikawa M, Clemens PR, Huang L: Transfer of full-length *Dmd* to the diaphragm muscle of *Dmd* (*mdx*/*mdx*) mice through systemic administration of plasmid DNA. *Mol. Ther.* 4, 45–51 (2001).
35. Romero NB, Braun S, Benveniste O *et al.*: Phase I study of dystrophin plasmid-based gene therapy in Duchenne/Becker muscular dystrophy. *Hum. Gene Ther.* 15, 1065–1076 (2004).
36. Schertzer JD, Plant DR, Lynch GS: Optimizing plasmid-based gene transfer for investigating skeletal muscle structure and function. *Mol. Ther.* 13, 795–803 (2006).
37. Murakami T, Nishi T, Kimura E *et al.*: Full-length dystrophin cDNA transfer into skeletal muscle of adult *mdx* mice by electroporation. *Muscle Nerve* 272, 37–41 (2003).
38. Zhang G, Ludtke JJ, Thioudellet C *et al.*: Intraarterial delivery of naked plasmid DNA expressing full-length mouse dystrophin in the *mdx* mouse model of Duchenne muscular dystrophy. *Hum. Gene Ther.* 15, 770–782 (2004).
39. Montarras D, Morgan J, Collins C *et al.*: Direct isolation of satellite cells for skeletal muscle regeneration. *Science* 309(5743), 2064–2067 (2005).
40. Fukada S, Higuchi S, Segawa M *et al.*: Purification and cell-surface marker characterization of quiescent satellite cells from murine skeletal muscle by a novel monoclonal antibody. *Exp. Cell Res.* 296(2), 245–255 (2004).
41. Asakura A, Seale P, Girgis-Gabardo A, Rudnicki MA: Myogenic specification of side population cells in skeletal muscle. *J. Cell Biol.* 159, 123–134 (2002).
42. Uezumi A, Ojima K, Fukada S *et al.*: Functional heterogeneity of side population cells in skeletal muscle. *Biochem. Biophys. Res. Commun.* 341, 864–873 (2006).
43. Bachrach E, Li S, Perez AL *et al.*: Systemic delivery of human microdystrophin to regenerating mouse dystrophic muscle by muscle progenitor cells. *Proc. Natl Acad. Sci. USA* 101(10), 3581–3586 (2004).
44. Sampaolesi M, Torrente Y, Innocenzi A *et al.*: Cell therapy of α-sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblasts. *Science* 301, 487–492 (2003).
45. Torrente Y, Belicchi M, Sampaolesi M *et al.*: Human circulating AC133+ stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. *J. Clin. Invest.* 114, 182–195 (2004).
46. Dezawa M, Ishikawa H, Itokazu Y *et al.*: Bone marrow stromal cells generate muscle cells and repair muscle degeneration. *Science* 309, 314–317 (2005).
47. Rando TA, Disatnik MH, Zhou LZ: Rescue of dystrophin expression in *mdx* mouse muscle by RNA/DNA oligonucleotides. *Proc. Natl Acad. Sci. USA* 97, 5363–5368 (2000).
48. Bartlett RJ, Stockinger S, Denis MM *et al.*: *In vivo* targeted repair of a point mutation in the canine dystrophin gene by a chimeric RNA/DNA oligonucleotide. *Nat. Biotechnol.* 18, 615–622 (2000).
49. Bertoni C, Morris GE, Rando TA: Strand bias in oligonucleotide-mediated dystrophin gene editing. *Hum. Mol. Genet.* 14, 221–233 (2005).

50. Hoffman EP, Morgan JE, Watkins SC, Partridge TA: Somatic reversion/suppression of the mouse *mdx* phenotype *in vivo*. *J. Neurol. Sci.* 99, 9–25 (1990).
51. Fanin M, Danieli GA, Cadaldini M *et al.*: Dystrophin-positive fibers in Duchenne dystrophy: origin and correlation to clinical course. *Muscle Nerve* 18, 1115–1120 (1995).
52. Wilton SD, Lloyd F, Carville K *et al.*: Specific removal of the nonsense mutation from the *mdx* dystrophin mRNA using antisense oligonucleotides. *Neuromuscul. Disord.* 9, 330–338 (1999).
53. Mann CJ, Honeyman K, Cheng AJ *et al.*: Antisense-induced exon skipping and synthesis of dystrophin in the *mdx* mouse. *Proc. Natl Acad. Sci. USA* 98, 42–47 (2001).
54. Mann CJ, Honeyman K, McClorey G, Fletcher S, Wilton SD: Improved antisense oligonucleotide induced exon skipping in the *mdx* mouse model of muscular dystrophy. *J. Gene Med.* 4, 644–654 (2002).
55. Lu QL, Mann CJ, Lou F *et al.*: Functional amounts of *dystrophin* produced by skipping the mutated exon in the *mdx* dystrophic mouse. *Nat. Med.* 9, 1009–1014 (2003).
56. Fletcher S, Honeyman K, Fall AM *et al.*: Dystrophin expression in the *mdx* mouse after localised and systemic administration of a morpholino antisense oligonucleotide. *J. Gene Med.* 8, 207–216 (2006).
57. Alter J, Lou F, Rabinowitz A *et al.*: Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. *Nat. Med.* 12, 175–177 (2006).
•• **Demonstrates that weekly intravenous injections of morpholino antisense oligonucleotides induced expression of functional levels of dystrophin in skeletal muscles body-wide in the dystrophic *mdx* mouse, with resulting improvement in muscle function.**
58. Goyenvalle A, Vulin A, Fougerousse F *et al.*: Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. *Science* 306, 1796–1799 (2004).
•• **Describes persistent exon skipping of the mutated *DMD* gene in *mdx* muscle due to a single administration of an AAV vector expressing antisense sequences linked to a modified U7 small nuclear RNA.**
59. McPherron AC, Lawler AM, Lee SJ: Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. *Nature* 387, 83–90 (1997).
60. Schuelke M, Wagner KR, Stolz LE *et al.*: Myostatin mutation associated with gross muscle hypertrophy in a child. *N. Engl. J. Med.* 350, 2682–2688 (2004).
61. Bogdanovich S, Krag TO, Barton ER *et al.*: Functional improvement of dystrophic muscle by myostatin blockade. *Nature* 420, 418–421 (2002).
•• **Demonstrated that antimyostatin antibody treatment effectively improved the phenotypes of dystrophic *mdx* mice.**
62. Shavlakadze T, White J, Hoh JF, Rosenthal N, Grounds MD: Targeted expression of insulin-like growth factor-I reduces early myofiber necrosis in dystrophic *mdx* mice. *Mol. Ther.* 10, 829–843 (2004).
63. Barton ER, Morris L, Musaro A, Rosenthal N, Sweeney HL: Muscle-specific expression of insulin-like growth factor I counters muscle decline in *mdx* mice. *J. Cell Biol.* 157, 137–148 (2002).
64. Shimatsu Y, Yoshimura M, Yuasa K *et al.*: Major clinical and histopathological characteristics of canine X-linked muscular dystrophy in Japan, $CXMD_J$. *Acta Myol.* 24, 145–154 (2005).
• **Describes the phenotypes of a canine model of muscular dystrophy in the Beagle.**
65. Inagaki K, Nakai H: Recent progress in AAV vector (Japanese). *Cell Mol. Ther.* 6, 3–10 (2006).

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Granular Swollen Epithelial Cells: A Histologic and Diagnostic Marker for Mitochondrial Nephropathy

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Abstract: Focal segmental glomerulosclerosis (FSGS) is a progressive kidney disease, and mitochondrial disease known to be a primary malady for secondary FSGS. Mitochondrial nephropathy with FSGS is diagnosed by genetic analysis or electron microscopy when it is suspected. As adequate morphologic features to diagnose mitochondrial nephropathy by light microscopy are lacking, this study used 10 cases with genetically proven mitochondrial disease and analyzed the kidney samples obtained by biopsy (n = 7) or autopsy (n = 3). We found granular swollen epithelial cells (GSECs) among the distal tubuli and collecting ducts in all patients, whereas such features were absent in IgA nephropathy, primary FSGS, and interstitial nephritis. Ultrastructural analysis of GSECs displayed accumulation of abnormal-shaped mitochondria in GSECs. To test whether GSECs were really associated with mitochondrial mutations, laser-captured single GSECs in 1 case with a position where 3,271 mutation were measured using a single-cell PCR analysis. This revealed that the mutant load of GSECs was significantly higher than normal-appearing epithelial cells within the same sample ($63.4 \pm 17.8\%$ vs. $32.5 \pm 4.6\%$; $P < 0.0001$). This is direct evidence that GSEC is a characteristic cellular feature, indicating cells with mutant mitochondrial DNA accumulation. In addition, the incidence of GSECs did not correlate with serum creatinine levels, proteinuria, percent glomerulosclerosis, tubulointerstitial changes, or arteriolar hyalinosis, suggesting that GSECs per se may not cause tissue damage. In conclusion, GSEC is a distinct morphologic feature suggesting mitochondrial nephropathy and is a useful tool to identify secondary FSGS on the basis of mitochondrial abnormalities.

Key Words: mitochondrial nephropathy, focal segmental glomerulosclerosis, mitochondrial disease

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Focal segmental glomerulosclerosis (FSGS), a progressive kidney disease often leading to chronic renal failure, consists of heterogeneous disorders, including idiopathic or secondary forms. The majority of FSGS exists as a secondary form associated with various systemic diseases, including remnant kidney, vesicoureteral reflux, obesity, and virus-associated, genetic, drug-induced, and cyanotic heart disease.[1,3,13,15] Determination of secondary FSGS is important because the therapeutic strategy is different depending on the primary disease. Among several diseases accompanying secondary FSGS, mitochondrial disease is unique for its clinical features and the mechanism of FSGS occurrence.[6,10,12,14]

Mitochondrial diseases are multisystemic disorders that primarily involve skeletal muscle, the central nervous system, and cardiac muscle.[4,7] The diseases also complicate kidney disease of various features, including FSGS, Fanconi syndrome, and Bartter syndrome.[2,6,8,10,12,14,20,21] The diagnosis of mitochondrial diseases is usually made with blood samples, but is often difficult because the proportion of mutant mitochondrial DNA (mtDNA) varies in different cell types. Furthermore, mitochondrial abnormalities basically occur in a cell-specific manner, and thus, abnormalities in a blood sample do not necessarily fully explain that a mitochondrial abnormality is the cause of organ damage. In this context, direct examination of mitochondrial abnormalities in damaged tissue in situ is more convincing for a diagnosis. Site-specific mitochondrial abnormalities have been established by myopathies accompanied by mitochondrial disease. If clinical symptoms of muscle diseases sufficiently suggest mitochondrial abnormalities in the muscle, modified Gomori trichrome staining of a muscle biopsy sample may reveal ragged-red fibers (RRFs) and abnormal mitochondria accumulation. This morphologic identification can be confirmed biochemically by staining for succinate dehydrogenase and cytochrome c oxidase (COX), enzymes specific for mitochondria. In contrast, no specific clinical symptom pointing to mitochondrial nephropathy is known. Particularly the case absence for typical symptoms of "mitochondrial myopathy,

encephalopathy, lactic acidosis, and stroke-like episodes" (MELAS) often reveal only deafness or diabetes mellitus (DM) as the clinical symptom of mitochondrial disease.[12] Furthermore, for a diagnosis of isolated kidney disease with mitochondrial abnormalities, for example, FSGS, genetic mitochondrial analysis is started by a suspicion from kidney histology. Presently, morphologic evidence for mitochondrial abnormalities is assessed by electron microscopy with marked increase of abnormal-shaped mitochondria in podocytes or tubular epithelial cells.[5,12,14] However, sampling error for electron microscopic material may greatly affect the finding of sporadic abnormal cells, and thus, identifying abnormal cells by light microscopy may be helpful.

We have noted, but not proven, that the abnormal morphology in the tubular cells of mitochondrial nephropathies is distinguished by the presence of numerous mitochondria under a light microscope (Figs. 1, 2). We defined these abnormal epithelial cells, seen especially in distal tubuli and collecting ducts, as granular swollen epithelial cells (GSECs). In this study, we analyzed the presence of GSECs in various mitochondrial nephropathies that had already been diagnosed by genetic examinations of blood or skeletal muscle, and investigated the association between GSECs and clinical or histologic findings. In addition, we measured the mutant load of GSECs to determine whether GSECs are an abnormal cell with mutant mtDNA accumulation, similar to RRFs in skeletal muscle, using a single-cell PCR analysis.

Our data suggest that GSECs, primarily in the distal tubuli or collecting ducts, have distinct morphologic features suggesting mitochondrial nephropathy and are a useful tool in identifying secondary FSGS with mitochondrial abnormalities.

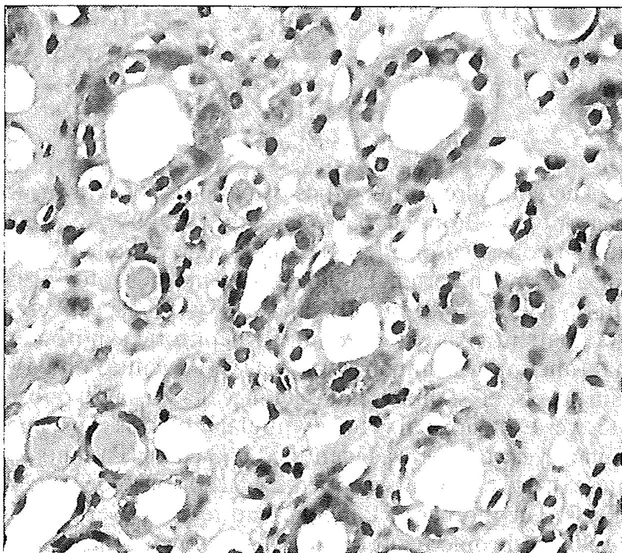

FIGURE 1. Light microscopic view of the granular swollen epithelial cell (GSEC). Arrows show GSECs in the collecting ducts (Masson trichrome stain × 200).

MATERIALS AND METHODS

Pathologic Analyses

We analyzed 7 biopsied kidneys from patients who had mtDNA position A3243G mutations and autopsied kidneys from 3 cases with T3271C mutations, including Kearns-Sayre syndrome, Pearson syndrome, and MELAS. Almost all biopsied patients were collected to our hospital (Kashiwa Hospital, The Jikei University School of Medicine, Chiba, Japan) for the diagnostic consultation from 7 institutions. Seven hundred ten kidney biopsies were analyzed between January 2001 to November 2009 and 15 patients were diagnosed as primary FSGS in our hospital. Among them, 1 patient (Patient 2) was FSGS related to genetically proven mitochondrial disease according to the finding of GSEC. Disease controls were randomly sampled 10 cases of IgA nephropathy (10 cases), primary FSGS (10 cases), and acute tubulointerstitial nephritis (10 cases). Formalin-fixed paraffin sections were stained with hematoxylin and eosin (HE), Masson trichrome, periodic acid-Schiff, and periodic-acid methenamine silver (PAM). A GSEC was defined as a granular swollen single epithelial cell in collecting ducts or distal tubuli. Masson trichrome stain is the best way to detect GSEC and PAM stain is suitable to distinguish GSEC from protein droplets in cases with proteinuria because the absorptive protein droplets are positive for PAM but not GSEC. We classified the extent of GSECs on the basis of the maximum number of GSECs in cross sections of collecting ducts or distal tubuli in a square high-power (× 400) field for 20 fields: mild = 1 to 2 cells, moderate = 3 to 5 cells, and severe = > 5 cells. Other histologic findings, including interstitial fibrosis or tubular atrophy and arteriolar hyalinosis, were classified using the Banff classification for transplanted kidneys.[22] Electron microscopic analysis was carried out in 5 biopsied cases (Patients 1 to 5).

Measurements of mtDNA Mutant Loads in GSECs by Single-cell PCR Analysis

Paraffin-embedded kidney tissue from an autopsied case with a T3271C mutation was cut into 4-μm sections and stained with HE. After dehydration, we dissected each of 20 GSECs and normal-appearing epithelial cells from collecting ducts using a LM200 laser microdissection system (Arcturus Engineering Inc., Mountain View, CA). A PicoPure DNA Extraction Kit (Arcturus) was used to take out DNA from individually dissected cells. PCR amplification (30 s at 94°C, 30 s at 50°C, 30 s at 72°C, for 30 cycles) was carried out with a forward primer (5′-GGCAGAGCCCGGTAATC-3′: 3238 to 3254) and a reverse primer (5′-TAAGAAGAGGAATTGAACCTC TGACCTTAA-3′: 3272 to 3301) using an ABI 9700 thermal cycler (Applied Biosystems, Foster City, CA). PCR products were digested with AflII (Takara, Tokyo, Japan) for 24 hours, and restriction fragments (30 and 34 bp) were detected on 4% agarose gel (NuSieve 3:1

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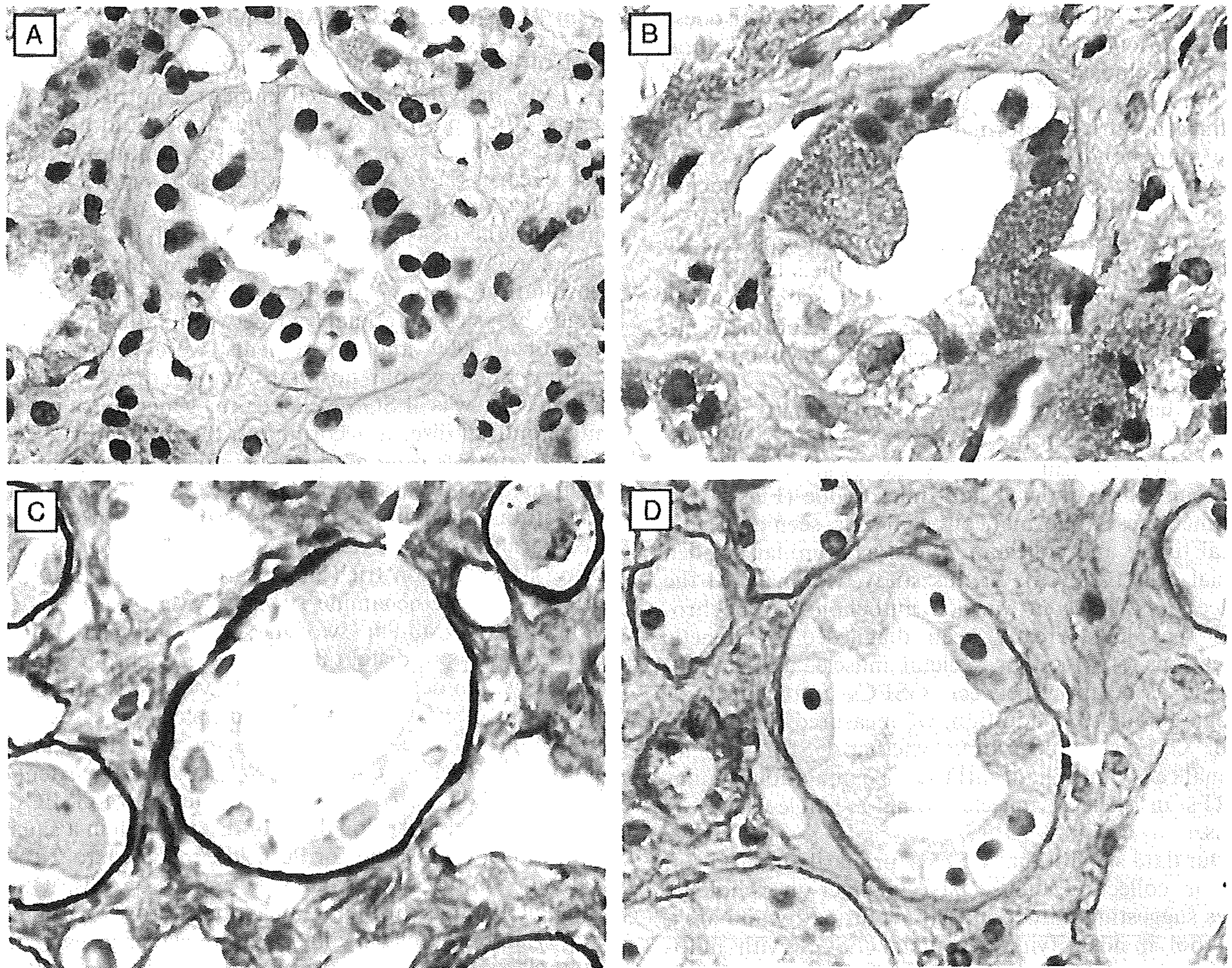

FIGURE 2. Higher magnification views of granular swollen epithelial cell (GSEC) by light microscopy. Arrowheads show GSECs in the collecting duct. (A) hematoxylin and eosin (HE) stain, (B) Masson trichrome stain, (C) periodic-acid methenamine (PAM) stain, (D) periodic acid-Schiff stain (× 600).

Agarose; Lonza Rockland Inc., Rockland, ME). This PCR method was described earlier.[9,19] To measure the mutant load, the intensity of a fragment was determined using LabWorks (Bio Medical Equipment Service Co., Louisville, KY). A standard curve was constructed using a series of plasmid DNAs of known mutant proportions.

Data Analysis

Data are presented as means ± SD. Mutant load groups were compared using the Mann-Whitney *U* test. The comparison of clinical and histopathologic findings among the groups was evaluated by Kruskal-Wallis test and Sheffe F-test. Correlations were analyzed using Spearman rank test. Data were analyzed using GraphPad Prism ver. 5.01. *P* values < 0.05 were deemed to indicate statistical significance.

RESULTS

Clinical Characteristics

The clinical characteristics of patients are shown in Tables 1 to 3. Biopsied patients with mtDNA A3243G mutation consisted of 5 female and 2 male patients: their mean age, mean serum Cr level, and mean proteinuria were 19.7 years, 0.81 mg/dL, and 1.87 g/d, respectively. Only 1 patient (Patient 2) exhibited nephrotic syndrome. Renal function was impaired in 2 cases (Patients 3 and 5). Serum levels of sodium, potassium, calcium, and phosphate were all normal. Renal tubular acidosis was not seen in any case. DM was identified in 3 cases (Patients 2, 3, and 5). Five patients (Patients 1, 2, 4, 5, and 7) had deafness. The mother of Patient 1 had received peritoneal dialysis for chronic renal failure of unknown origin. The mothers of Patients 2, 3, and 7 had DM. The younger brother of Patient 1 and the mother of Patient 6 also had A3243G mutations.

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TABLE 1. Clinical Characteristics of Biopsied Patients With mtDNA A3243G Mutations

Patient no.	Age (y)/Gender	s-Cr (mg/dL)	Ccr (mL/min)	Proteinuria (g/d)	Complications, Symptoms	Outcome of the Kidney
1	14/F	0.7	125	2.0	Deafness	Dialysis
2	25/F	0.5	115	4.0	DM, deafness	Renal insufficiency
3	22/F	1.6	30.8	0.9	DM	Transplantation
4	25/F	0.6	73.3	2.3	Deafness	Dialysis
5	35/M	1.5	45.9	0.5	DM, deafness	Renal insufficiency
6	12/F	0.6	97.6	1.6	None	Unknown
7	5/M	0.2	166	1.8	Deafness	Unknown

TABLE 2. Clinical Characteristics of Autopsied Patients

Patient no.	Age (y)/Gender	Original Disease	Complications of the Kidney	Other Complications, Symptoms
8	20/F	KSS (mtDNA deletion)	Fanconi syndrome chronic renal failure	Retinitis pigmentosa, DM, deafness, atrioventricular block, muscle weakness
9	10/F	Pearson syndrome (mtDNA deletion)	Chronic renal failure	DM, muscle weakness, pancreatic insufficiency
10	36/F	MELAS (T3271C mutation)	Acute renal failure	Stroke-like episodes, DM, muscle weakness

KSS indicates Kearns-Sayre syndrome.

TABLE 3. Clinical Backgrounds of the Control Groups and Biopsied Patients With mtDNA A3243G Mutation

	IgA Nephropathy (n = 10)	Primary FSGS (n = 10)	TIN (n = 10)	Biopsied Patients With A3243G Mutation (n = 7)
Male/Female	5/5	7/3	3/7	2/5
Age (y)	37.7 ± 17.2	42.5 ± 12.5	45.3 ± 28.5	19.7 ± 10.0
s-Cr (mg/dL)	0.76 ± 0.24	1.07 ± 0.57	2.81 ± 2.19*	0.81 ± 0.53
Proteinuria (g/d)	0.75 ± 0.36	6.10 ± 3.04†	0.50 ± 0.45	1.87 ± 1.23

*$P < 0.05$, versus IgA nephropathy, primary FSGS and biopsied patients with A 3243G mutation.
†$P < 0.01$, versus IgA nephropathy, TIN and biopsied patients with A 3243G mutation.
FSGS indicates focal segmental glomerulosclerosis; TIN, tubulointerstitial nephritis.

TABLE 4. Histopathologic Findings

Patient no.	GSEC	Global Sclerosis (%)	FSGS Lesion (%)	IF/TA	Arteriolar Hyalinosis
1	Mild	0/6	1/6 (16.7)	Mild	Mild
2	Moderate	3/10 (30)	1/10 (10)	Moderate	Moderate
3	Moderate	3/28 (10.7)	11/28 (39.2)	Moderate	Mild
4	Moderate	6/48 (12.5)	4/48 (8.3)	Mild	Mild
5	Moderate	1/12 (8.3)	1/12 (8.3)	Mild	Mild
6	Moderate	3/10 (30)	1/10 (10)	Mild	None
7	Mild	3/12 (25)	1/12 (8.3)	Mild	Moderate
8	Mild	196/283 (69.2)	12/283 (4.2)	Severe	Severe
9	Severe	156/234 (66.6)	3/234 (1.3)	Severe	Mild
10	Severe	8/362 (2.2)	0/362	None	None

FSGS indicates focal segmental glomerulosclerosis; IF/TA, interstitial fibrosis and tubular atrophy.

All autopsied cases were female and they all had DM. The patient with Kearns-Sayre syndrome had Fanconi syndrome and chronic renal failure, and the patient with Pearson syndrome also had chronic renal failure. The patient having MELAS with a T3271C mutation had episodes of acute renal failure.

Histopathologic Findings

Histologic findings are summarized in Tables 4 and 5. Various extents of tubulointerstitial fibrosis and tubular atrophy were observed in 9 patients. GSECs were recognized in all patients, but no case of IgA nephropathy, primary FSGS, or acute tubulointerstitial nephritis

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TABLE 5. Histopathologic Findings of the Control Groups and Biopsied Patients With mtDNA A3243G Mutation

	IgA Nephropathy (n = 10)	Primary FSGS (n = 10)	TIN (n = 10)	Biopsied Patients With A3243G Mutation (n = 7)
GS (%)	10.0 ± 8.17	1.61 ± 2.13	4.17 ± 6.13	16.6 ± 11.7*
FSGS lesion (%)	1.61 ± 2.13	13.2 ± 9.60**	0.71 ± 2.24	14.4 ± 11.3†
IF/TA	0.30 ± 0.48	0.30 ± 0.48	0.70 ± 0.82	1.29 ± 0.48‡
ah	0.40 ± 0.69	0.60 ± 0.69	0.60 ± 0.48	1.14 ± 0.69
GSEC	0	0	0	1.71 ± 0.48§

IF/TA, ah and GSEC are shown as the mean score of the grades (1: mild, 2: moderate, 3: severe).
*$P < 0.05$, versus Primary FSGS and TIN.
†$P < 0.05$, versus IgA nephropathy and TIN.
‡$P < 0.05$, versus IgA nephropathy and Primary FSGS.
§$P < 0.01$, versus IgA nephropathy, Primary FSGS and TIN.
ah indicates arteriolar hyalinosis; FSGS, focal segmental glomerulosclerosis; GS, global sclerosis; GSEC, glanular swollen epithelial cell; IF/TA, interstitial fibrosis and tubular atrophy; TIN, tubulointerstitial nephritis.

was observed. Electron micrographs from 5 patients (Patients 1 to 5) also showed epithelial cells with increased numbers of mitochondria in the distal tubuli (Figs. 3A, B), some of which had coil-like arranged cristae (Fig. 3B, inset). Although the accumulation of abnormal mitochondria was sometimes seen in proximal tubuli by electron microscopy, it is difficult to identify by light microscopy, probably because abundant cytoplasma and osmiophilic features in normal proximal tubular epithelium are hard to distinguish and even abnormal mitochondria is increased. Electron microscopic views showed mosaic patterns consisting of mitochondria-abundant epithelial cells and normal-appearing epithelial cells in the same cross sections of distal tubuli (Fig. 3A).

Regarding glomerular lesions, FSGS lesions were observed in 9 patients. Electron microscopy revealed podocytes containing marked proliferating and irregular mitochondria in 4 patients who had FSGS lesions.

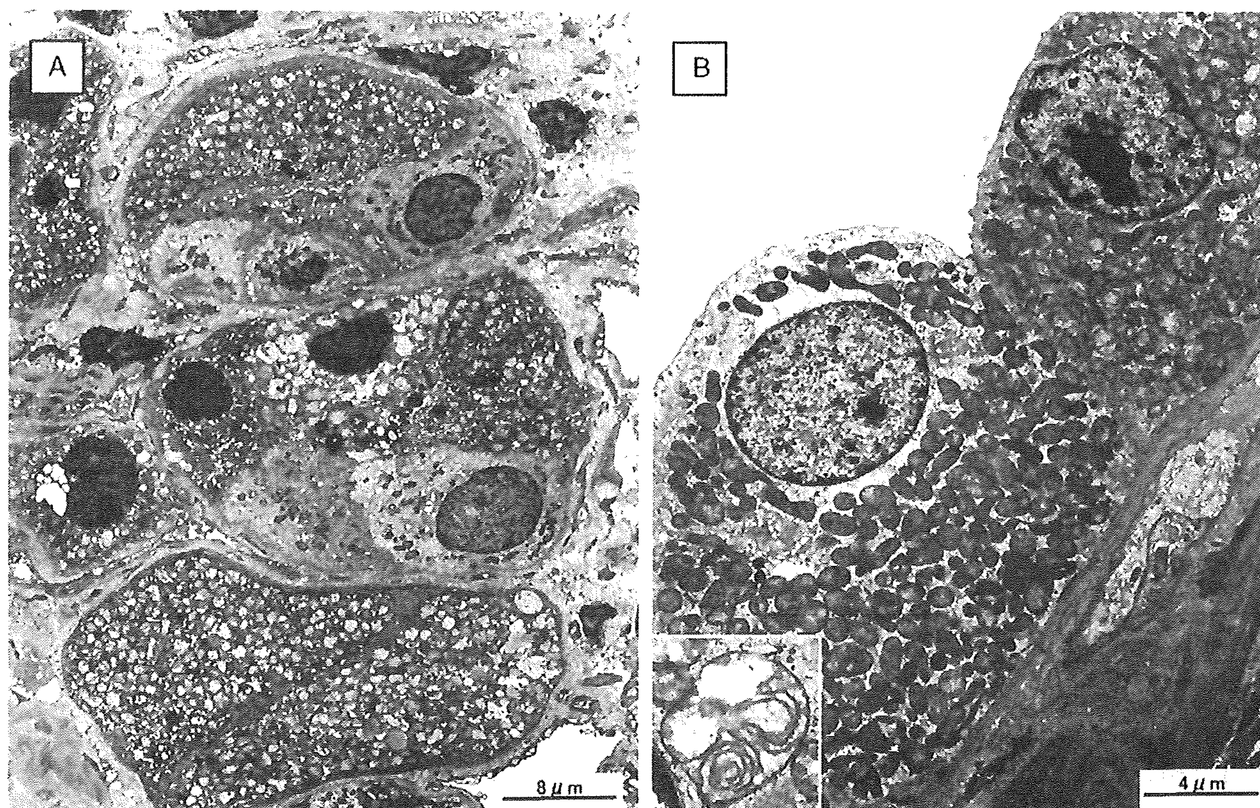

FIGURE 3. Electron microscopic views of the distal tubuli. A, A mosaic pattern consisting of mitochondria-abundant epithelial cells and normal-appearing epithelial cells is recognized in the distal tubules (×1500). B, High magnification of the distal tubular epithelial cell reveals a markedly increased number of mitochondria (×3000) and some of which were coil-like arranged cristae (inset, ×40,000).

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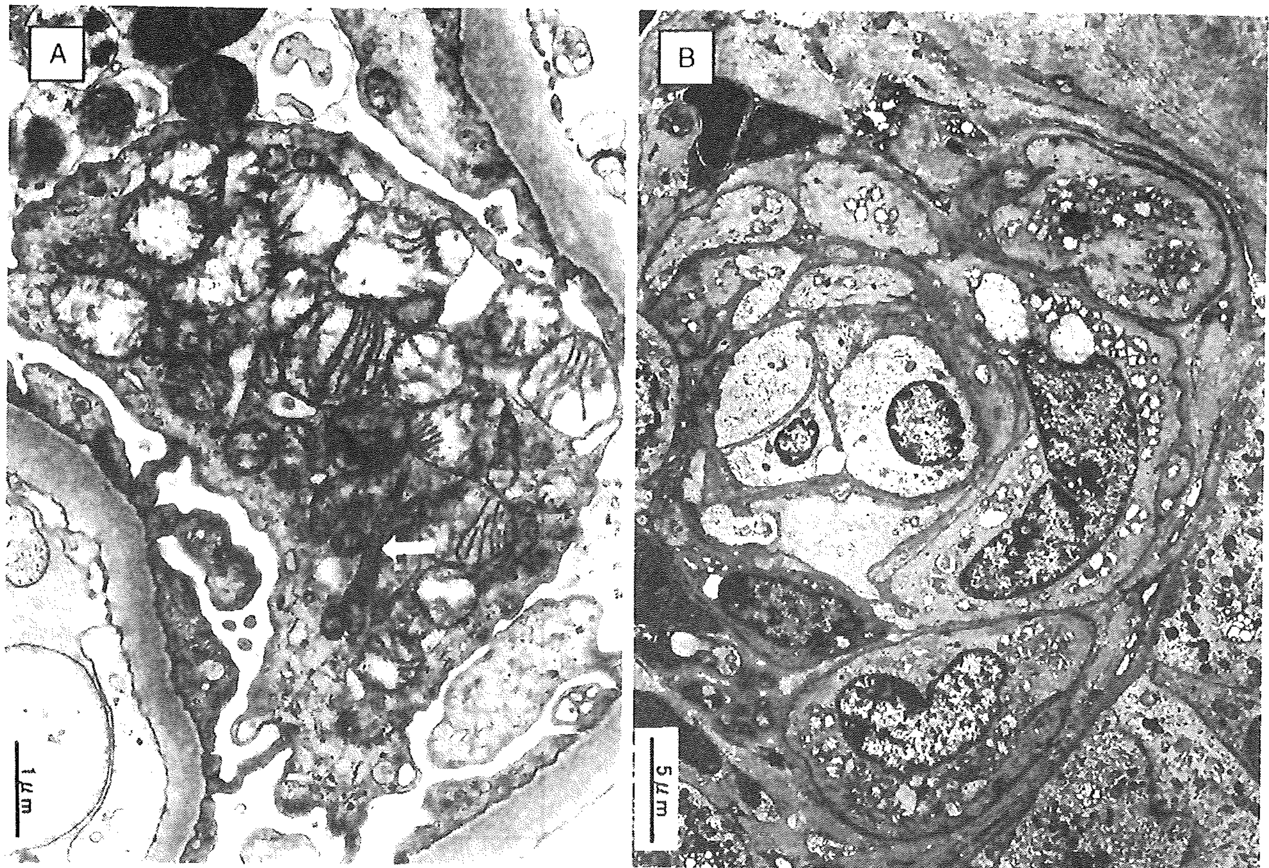

FIGURE 4. Electron microscopic views of a podocyte and an arteriole in Patient 3. A, Abnormal mitochondria increased in a podocyte. The size and shape of mitochondria are irregular, including swollen and ballooning mitochondria. One of these has a unicorn-shaped giant mitochondria (arrow; ×5000). B, Increased number of mitochondria in arteriolar smooth muscles (×2000).

The size and shape of the mitochondria were irregular, including swollen and ballooning mitochondria (Fig. 4A). Of these, 1 had a "unicorn-shaped" giant mitochondria (Fig. 4A, arrow).

Concerning vascular lesions, arteriolar hyalinosis was identified in 8 patients. Patient 3 showed increased numbers of mitochondria in arteriolar smooth muscle cells on the basis of electron microscopy (Fig. 4B).

The Grade of GSECs and Clinical or Pathologic Parameters

No significant correlation was observed between the grade of GSECs and renal function, including serum Cr levels and proteinuria. Histologically, the grade also did not correlate with the score of interstitial fibrosis and tubular atrophy, arteriolar hyalinosis, or the proportion of global or focal segmental sclerosis (Fig. 5).

Mitochondrial DNA Mutant Loads in GSECs

A DNA sample from each microdissected GSEC was amplified by PCR and digested with AflII. Two cleaved fragments (34 and 30 bp) were detected (Fig. 6A). The mutant load of GSECs was significantly higher than in normal-appearing epithelial cells (Fig. 6B; 63.4 ± 17.8% vs. 32.5 ± 4.6%, $P < 0.0001$).

DISCUSSION

Cells with mitochondrial abnormalities have been identified at the ultrastructural level, revealing the accumulation of morphologically abnormal mitochondria within the cytoplasm. The abnormal cells reported in mitochondrial nephropathy are often present in tubular cells and podocytes[5,8,12,14,21] as seen during electron microscopy. As the distribution of abnormal cells in situ is sporadic rather than ubiquitous, identifying them by light microscopy within this tissue may be useful for the diagnosis of mitochondrial nephropathy.

We found mitochondrial-rich cells among tubular epithelial cells by Masson trichrome staining and noted that the accumulation of mitochondria in these cells may indicate mitochondrial abnormalities.

As shown in Figures 1 and 2 single expanded epithelial cells among tubular cells were fully stained by Masson trichrome staining, suggesting mitochondrial

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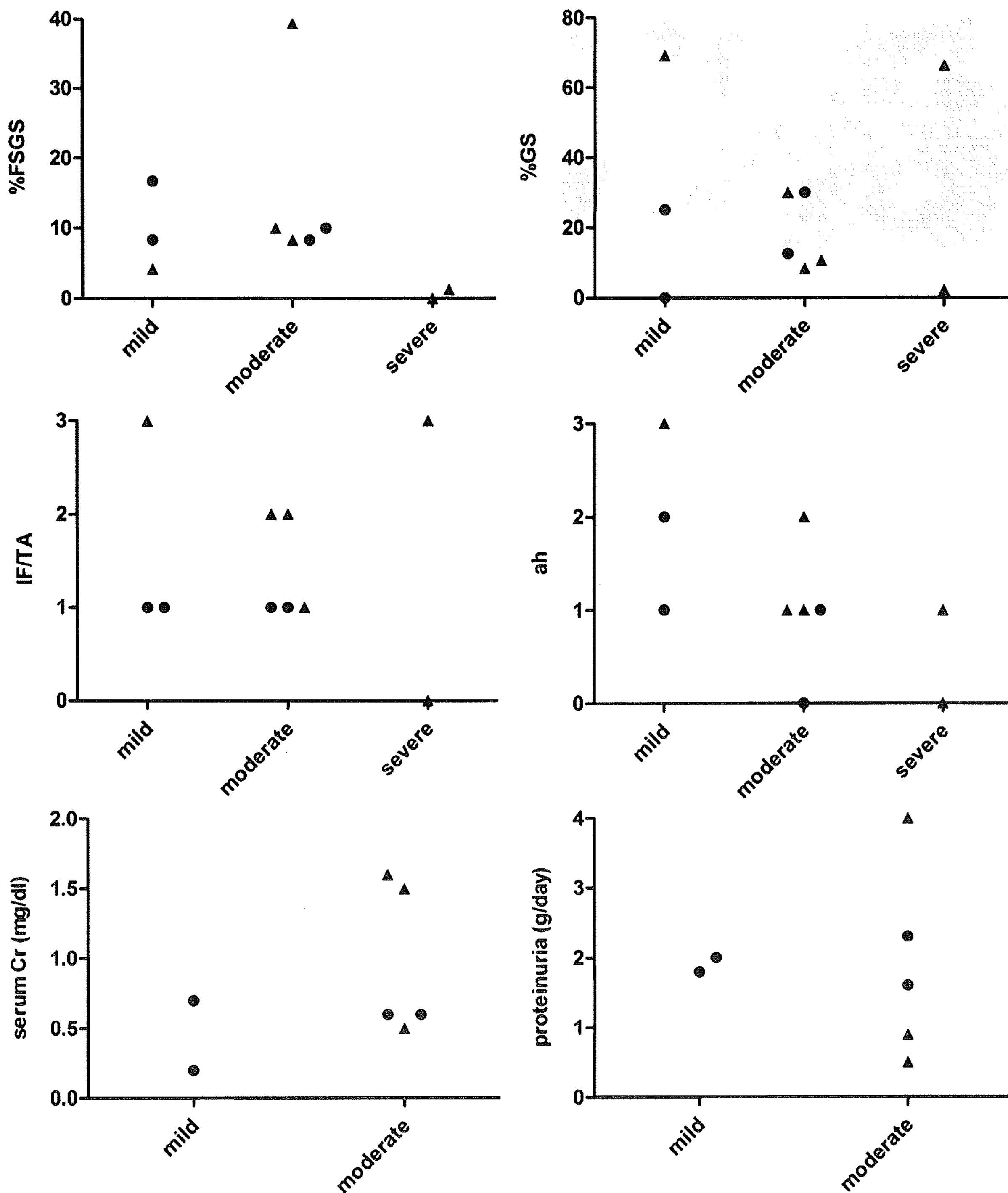

FIGURE 5. The association between the granular swollen epithelial cell (GSEC) grade (x-axis) and pathologic or clinical parameters (y-axis). ah indicates arteriolar hyalinosis; % focal segmental glomerulosclerosis (FSGS), proportion of focal segmental glomerulosclerosis; %GS, proportion of global sclerosis; IF/TA, interstitial fibrosis, and tubular atrophy. The grades of IF/TA and ah were classified as: 1, mild; 2, moderate; 3, severe. Clinical data of serum Cr and proteinuria were obtained from biopsied patients (n = 7). ▲: Diabetes mellitus (DM) patients. Serum Cr and proteinuria in patients of autopsy cases were omitted (n = 3).

proliferation. These cells indeed showed accumulation of morphologically abnormal mitochondria (Fig. 3).

In this study, we examined 10 cases with genetically proven mitochondrial disease and found that GSECs were present in all kidney samples obtained by biopsy or autopsy.

GSECs were recognized not only in patients with the A3243G mutation, but also in patients with some

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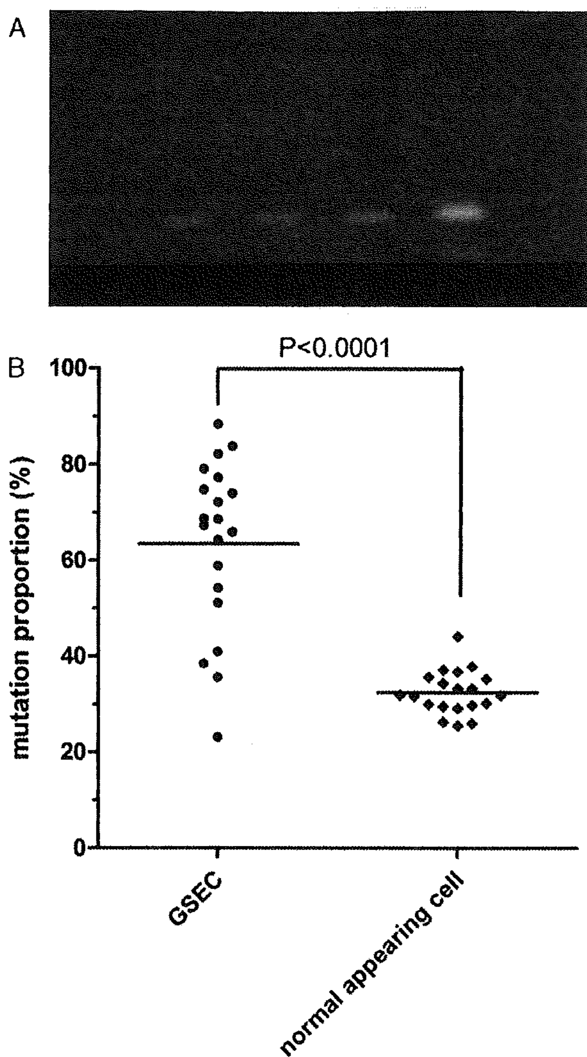

FIGURE 6. Identification of the T3271C mutation and comparisons of the mutant loads in granular swollen epithelial cell (GSECs) and normal-appearing epithelial cells. A, Each lane showed the results of PCR amplification from a single GSEC. DNA samples from each microdissected GSEC were amplified by PCR and digested with AflII. Two cleaved fragments (34 and 30 bp) were detected. B, The mutant load in GSECs was significantly higher than in normal-appearing epithelial cells (63.4 ± 17.8% vs. 32.5 ± 4.6%; *P* <0.0001).

mitochondrial diseases. Thus, GESCs may not reflect specific genetic mitochondrial abnormalities. We found no GSECs in specimens from cases of IgA nephropathy, primary FSGS, or acute tubulointerstitial nephritis, suggesting that such epithelial features may be related to mitochondrial nephropathy.

To define whether morphologically determined GSECs really had mitochondrial mutations, we measured the mutant load of GSECs and normal-appearing epithelial cells from the collecting duct by single-cell PCR analysis using each GSEC that was laser-microdissected from paraffin-embedded kidney tissue. For this procedure, we modified the single-fiber PCR analysis earlier used for RRFs in cases of MELAS.[17] As biopsy samples contain few GSECs, we applied this method to autopsy samples from a patient who had MELAS with a T3271C mtDNA mutation. We found a higher mutant load in GSECs than in normal-appearing cells, indicating that the GSECs represent mutant mitochondria accumulation, similar to that of RRFs in skeletal muscle. This is consistent with earlier studies reporting that the proportion of mutant mtDNA was higher in RRFs than in non-RRFs in muscle.[16,17] Simultaneously, we identified a few GSECs with low mutant loads. As shown in Figure 6, about 20% of GSECs showed low mutant loads. The reason for this phenomenon is unclear, but it may be argued that RRFs often maintain COX activity in patients with MELAS. Moreover, these COX-positive RRFs show that mitochondrial proliferation is not always an unsuccessful response to mitochondrial dysfunction. Extensive normal mitochondrial proliferation can provide functional compensation.[17]

Although GSECs indicate mtDNA mutations in kidney-resident cells, whether GSECs cause tissue damage resulting from FSGS or Fanconi syndrome is not known. Notably, the grade of GSECs did not correlate with percent FSGS. This is not inconsistent because mitochondrial abnormalities occur in a cell-specific manner and the frequency of GSECs in tubular cells does not necessarily correlate with FSGS caused by podocyte dysfunction, as shown by abnormal mitochondria in podocytes.[12] Yamagata et al showed that a common mtDNA deletion was observed primarily in glomerular epithelial cells using in situ PCR analysis, and they suggested the possibility that this mtDNA deletion may be the cause of glomerular epithelial damage.[23] Indeed, 4 of the 5 cases in this study in which electron microscopy was carried out showed that podocytes also contained proliferating abnormal mitochondria. In this context, we did not notice apparent GSECs in podocytes in cases with FSGS. Although the reason is unknown, podocytes may show a variety of morphologies with large and loose cytoplasm, and thus features of GSECs seen in the tubular cells are hard to detect in the case of podocytes. Other histologic parameters (IF/TA, percent GS, arteriolar hyalinosis) and clinical parameters (serum Cr levels, proteinuria) did not correlate with the grade of GSEC. This may have occurred because the proportion of mutant mtDNA varies in different cell types owing to the heteroplasmic character of mitochondrial disease and because the tissue injury depends on the damaged cells. Nakada et al showed that in mice with mutant mtDNA carrying a 4696-bp deletion (ΔmtDNA4696), the appearance of COX-negative mitochondria was limited to tissues with more than 85% ΔmtDNA4696, and that it was correlated with the onset of disease phenotypes.[18] For our patients, functional abnormalities might not have occurred because the mutant load was not sufficiently high in the GSECs and the number of GSECs was limited.

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