

Negamycin-therapy in Skeletal and Cardiac Muscles of *mdx* Mice

Masayuki Arakawa⁽¹⁾, Masataka Shiozuka⁽¹⁾, Yuki Nakayama⁽²⁾, Takahiko Hara⁽²⁾, Masa Hamada⁽³⁾, Daishiro Ikeda⁽³⁾, Yoshikazu Takahashi⁽³⁾, Ryuichi Sawa⁽⁴⁾, Yoshiaki Nonomura⁽⁴⁾, Kianoush Sheykholeslami⁽¹⁾, Kenji Kondo⁽¹⁾, Kimitaka Kaga⁽⁴⁾, Shin'ichi Takeda⁽⁵⁾, and Ryoichi Matsuda⁽¹⁾

(1)Department of Life Sciences, The University of Tokyo, Tokyo, Japan

(2)Department of Tumor Biochemistry, Tokyo Metropolitan Institute of Medical Sciences, Tokyo, Japan. (3)The Institute of Microbial Chemistry, Tokyo, Japan

(4)Department of Otolaryngology, The University of Tokyo, Tokyo, Japan

(5)Department of Molecular Therapy, National Institute of Neuroscience, Tokyo Japan

Abstract

The ability of aminoglycoside antibiotics to promote read-through of nonsense mutations has attracted interest in these drugs as potential therapeutic agents in genetic diseases. However, the toxicity of aminoglycoside antibiotics may result in severe side effects during long-term treatment. In this study, we report that negamycin, a dipeptide antibiotic, also restores dystrophin expression in skeletal and cardiac muscles of *mdx* mice, a most widely accepted animal model for Duchenne muscular dystrophy (DMD) with a nonsense mutation in the dystrophin gene, and in cultured *mdx* myotubes. Dystrophin expression was detected up to 10% of normal levels, confirmed by immunoblotting. The toxicity of negamycin found to be less toxic than gentamicin. Furthermore, negamycin bound to a partial sequence of the eukaryotic rRNA-decoding A-site. We suggest that negamycin is a new therapeutic candidate for DMD and other genetic diseases caused by nonsense mutations.

Keywords : *mdx* mouse, dystrophin, Negamycin, nonsense mutation, muscular dystrophy

Basic Appl Myol 13(6): 313-320, 2003

Introduction

Aminoglycoside antibiotics are known to decrease translational fidelity and cause read-through of termination signals in prokaryotic and eukaryotic cells [7, 19, 37]. Considerable attention has therefore been paid on aminoglycoside antibiotics as potential therapeutic agents for genetic diseases. Recently, gentamicin has been used in clinical trials for the treatment of cystic fibrosis, Hurler's disease, infant neuronal ceroid and lipofuscinosis, diseases that are caused by nonsense mutations [4, 9, 22, 33].

The most common myopathy in children is Duchenne muscular dystrophy (DMD), which is a lethal X-linked recessive disorder affecting 1 in 3,500 live-born males characterized by mutations in the dystrophin gene. DMD patients and the *mdx* mice, are attempted treated pharmacologically with corticosteroids and corticosteroid-sparing agents, however, the use of corticosteroids is accompanied by significant side

effects and is of limited and short-lived benefits [14, 16, 23]. On the other hand, 65% of patients with DMD the gene exhibit gross rearrangement (predominantly deletion or duplication), while the remaining 35% have a dystrophin gene with either nonsense or other point mutations.

Pre-clinical trials to develop drugs need reliable animal models of the disease. The *mdx* mouse, an animal model for DMD, carries a nonsense mutation (CAA to TAA) at nucleotide position 3,185 in exon 23 of the dystrophin gene [6, 34]. This causes an absence of dystrophin as part of the cytoskeleton of muscle and, thus, the dystrophin-associated glycoprotein complex in the sarcolemma. Identifying clinically useful methods to suppress the nonsense mutation within the dystrophin gene would be of benefit to a significant number of patients with DMD.

In 1999, Barton-Davis *et al.* reported that gentamicin restores dystrophin function to skeletal muscles of *mdx* mice [3]. Besides, gentamicin is currently in clinical

trials for DMD patients. However, aminoglycoside antibiotics are associated with numerous side effects such as auditory and renal malfunctions, and excessive use can lead to the emergence of drug-resistant bacteria.

The serious side effects of gentamicin led us to search for other possible drug candidates to suppress nonsense mutations. Uehara *et al.* reported that negamycin (δ -hydroxy- β -lysine linked with methylhydrazinoacetic acid), a dipeptide antibiotic discovered in 1970, also induces the read-through of stop codons in a prokaryotic translation system [17, 35]. We therefore injected negamycin subcutaneously into *mdx* mice over period of 2 to 4 weeks. Immunofluorescence and immunoblotting analyses revealed that dystrophin protein expression was restored in skeletal and cardiac muscles of negamycin-treated *mdx* mice. The restoration of dystrophin was also found in immortalized *mdx* skeletal muscle cells (*mdxSV40-T* cells) prepared by introducing a temperature-sensitive SV40-T gene into *mdx* skeletal muscle cells using a retroviral vector.

It has been reported that aminoglycoside antibiotics bind to the A-site of rRNA and change its conformation [13, 24, 38]. In 1999, Griffey *et al.* showed the interaction between aminoglycoside antibiotics and the rRNA-decoding A-site of prokaryotes and eukaryotes using electrospray ionization mass spectroscopy (ESI-MS) [15]. To understand the mechanism of the read-through activity induced by negamycin during protein synthesis, we demonstrate here the interaction between a partial sequence of rRNA and negamycin, as estimated by time-of-flight mass spectroscopy (TOF-MS).

Dystrophin deficiency in skeletal muscle of *mdx* mouse can be partially remedied by negamycin. These results predict that negamycin is a new therapeutic candidate for DMD and other genetic diseases caused by nonsense mutations.

This paper is a revision and expansion of two earlier studies, 'Negamycin can restore dystrophin in *mdx* skeletal muscle'[1] and 'Negamycin restores dystrophin expression in skeletal and cardiac muscles of *mdx* mice'[2].

Materials & Methods

Antibiotic treatment in mice

The amount of negamycin administered was adjusted to be equivalent to the number of molecules of gentamicin administered as described by Barton-Davis *et al.* [3]. Male *mdx* mice (7–8 weeks old, $n = 45$, or 4-week-old, $n = 5$) were injected subcutaneously with 0.2 ml of negamycin (1.2×10^{-5} mol/kg body weight (BW)) in PBS daily for 2 or 4 weeks. Untreated male *mdx* mice and normal male C57BL10 mice (7–8 weeks old, $n = 6$) were also injected subcutaneously with only

0.2 ml of PBS daily. Animal experiments were carried out according to the manual of the University of Tokyo.

Vital staining with Evans Blue dye

Evance Blue dye was used as a marker of myofiber damage and histology was used to assess the muscle degeneration and regeneration, as described by Matsuda *et al.* [27]. All animals were given an intraperitoneal injection of Evans Blue dye (2% EB in PBS, 0.1 ml/10g, BW) 12 h prior to sacrifice.

Dystrophin staining of mouse tissues

Immunofluorescent staining was carried out on 7–10 μ m transverse cryosections of the TA muscle and ventricular muscle from experimental mice. The cryosections were blocked for 15 min with 10%HS-PBS at room temperature and then washed in PBS. These specimens stained with a rabbit polyclonal anti-dystrophin (C-terminal region) antibody and detected with FITC conjugated anti-rabbit IgG antibody (Amarsham).

Negamycin treatment in mdx mouse skeletal muscle cells

Cell culture of *mdx* mouse skeletal muscle cells (*mdxSV40-T*) was carried out according to the procedure of Arakawa *et al.* [2]. Cells (2.0×10^5 cells/35-mm dish) were grown in growth medium (20% FBS in DMEM) at 32°C for 7 days in gelatin-coated culture dishes. Growth medium was replaced with differentiation medium (10% HS in MEM) and cultured at 38°C for next 5 days. After myotube formation, negamycin (4.0×10^{-7} mol/ml, 100 μ g/ml, was added daily for 7 days to the cultures.

Dystrophin staining of cultured skeletal muscle cells

The cultured cells were fixed in methanol for 10 min at -20°C and then washed with PBS. Fixed cells were blocked for 30 min with 10% HS-PBS at room temperature, washed extensively with PBS and then stained with the monoclonal antibody MF20 against sarcomeric muscle myosin heavy chain or rat monoclonal anti-dystrophin antibody, and detected using a Alexa Fluor secondary antibodies.

Immunoblot analysis

Partial enrichment of the dystrophin/dystrophin-associated glycoprotein complex from muscle tissues of left hindlimb (600 mg wet weight) and heart (100 mg wet weight) isolated from *mdx* or B10 mice using the lectin wheat germ agglutinin-Sepharose CL-6B (Sigma). Immunoprecipitation from cultured cells was carried out using protein A-Sepharose CL-4B (Sigma) with rabbit polyclonal anti-dystrophin antibody as described previously [2].

Negamycin-therapy in *mdx* mice

The protein concentrations of SDS-treated samples were estimated by the microbeuret method [21] and was adjusted to 20 or 100 $\mu\text{g}/20\mu\text{l}$, with SDS sample buffer. The samples were subjected to electrophoresis on 4%–8% gradient SDS-PAGE. Proteins were transferred to a PVDF membrane (Millipore) and treated with a blocking buffer. The membrane was treated with monoclonal anti-dystrophin C-terminal antibody (DYS2: Novocastra) followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 antibody (ZYMED) and then treated with an Enzyme Chemiluminescence (ECL) solution (Amersham Pharmacia Biotech). The pattern was visualized by exposing the membrane to X-ray film (Hyperfilm ECL, Amersham Pharmacia Biotech). For further details of these protocols, see Arakawa *et al.* [2].

Interaction between negamycin and rRNA (A-site)

HPLC-purified mammalian partial rRNA sequences (A-site:

5'-GGCGUCGCUACUUCGGUAAAAGUCGCC-3':
27 mer) were synthesized by the TAKARA Biotechnology Co. A reaction mixture containing 5.0×10^{-8} mol of negamycin and 5.0×10^{-9} mol of the rRNA sequence was prepared in distilled water to a total volume of 1ml. The formation of a negamycin:rRNA complex was examined by the Orthogonal Acceleration TOF-MS (AccuTOF, JEOL). Measurements were performed at conditions produced by a 50 $\mu\text{l}/\text{min}$ syringe pump at temperatures under 100°C . To prepare a modified A-site, bold nucleotides were changed in the partial, 1406-1494 loop region of the A-site (modified A-site: 5'-GGCGACUCUUCUUCUUCGGAAGAGUCGCC-3', 26 mer).

Changes in body weights of *mdx* mice treated with negamycin

Subcutaneous injection of male *mdx* mice (7-week-old, $n = 4$ per each dose) with negamycin was performed at various doses: 1.2×10^{-5} mol/kg (1x), 1.2×10^{-4} mol/kg (10x), 6.0×10^{-4} mol/kg (50x), and 1.2×10^{-3} mol/kg (100x) each day for 2 weeks. Gentamicin sulfate (1.2×10^{-3} mol/kg) was also injected into different male *mdx* mice (7-week-old, $n = 2$). As a control, PBS was injected into *mdx* mice every day. Body weights were measured every day.

Ototoxicity

Male *mdx* mice (7 weeks old, $n=2$) were treated with 1.2×10^{-4} mol/kg (10x) negamycin, gentamicin or PBS(control) for 7 days. Hearing ability was determined by the auditory brainstem response, according to the method of Shapiro *et al.* [32].

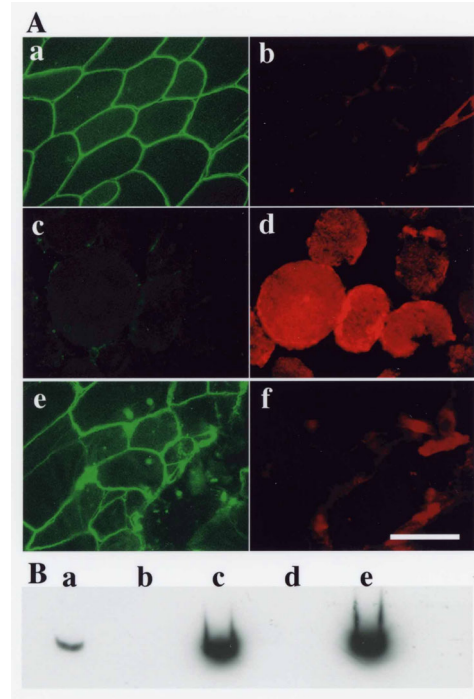


Figure 1. Dystrophin expression in skeletal muscles of negamycin-treated and untreated *mdx* mice. **A**, Immunofluorescent staining of dystrophin (**a**, **c**, and **e**) and EB staining (**b**, **d**, and **f**) of degenerating fibers. **a** and **b**, normal B10 mouse; **c** and **d**, untreated *mdx* mouse; **e** and **f**, negamycin-treated *mdx* mouse. Bar = 100 μm . **B**, Immunoblot analysis of full-length dystrophin proteins from the left hindlimb muscles. **a**, negamycin-treated *mdx* mouse; **b**, sample buffer; **c**, negamycin-treated *mdx* mouse (ten times the amount of protein used in **a** was loaded); **d**, untreated *mdx* mouse; **e**, B10 control mouse.

Results & Discussion

Dystrophin accumulates in negamycin-treated dystrophic muscles

Immunostainings of dystrophin were carried out on TA muscles harvested from *mdx* mice that had received daily injections of negamycin (1.2×10^{-5} mol/kg BW) for 2 weeks (Fig. 1A). The number of dystrophin-positive fibers was greater in *mdx* TA muscle compared with controls. An average of 6% of 350–550 total TA muscle fibers in six negamycin-treated *mdx* mice were dystrophin-positive. Furthermore, the number of EB-positive fibers in the *mdx* mice decreased from 17% in untreated mice to 5% with negamycin treatment. Dystrophin accumulation was confirmed by immunoblots of partially enriched, WGA-purified dystrophin/dystrophin-associated glycoprotein complex (Fig. 1B). We estimate that the dystrophin level in the skeletal muscle of negamycin-treated *mdx* mice is roughly 10% that in

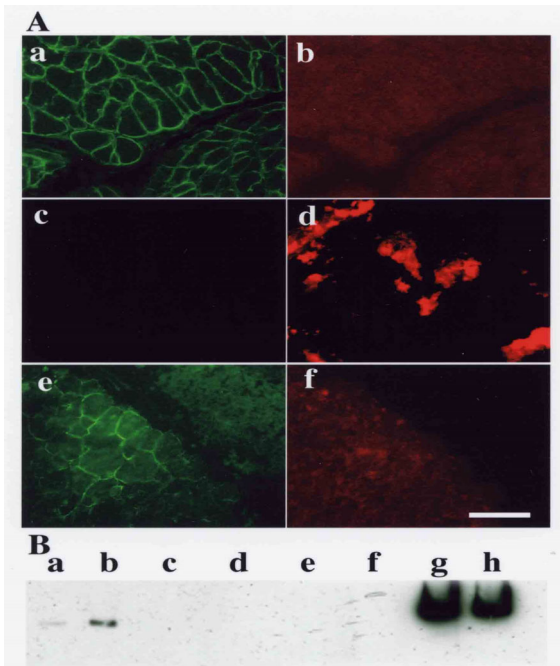


Figure 2. Dystrophin expression in cardiac muscles of negamycin-treated and untreated *mdx* mice. **A**, Immunofluorescent staining of dystrophin (**a**, **c**, and **e**) and EB staining (**b**, **d**, and **f**) of degenerating fibers. **a** and **b**, B10 control mouse; **c** and **d**, untreated *mdx* mouse; **e** and **f**, negamycin-treated *mdx* mouse. Bar = 40 μ m. **B**, Immunoblot analysis of full-length dystrophin protein from cardiac muscles. **a** and **b**, two individual, negamycin-treated *mdx* mice; **c** and **f**, empty well; **d** and **e**, two individual untreated *mdx* mice; **g** and **h**, two individual normal B10 mice.

normal skeletal muscles. Dystrophin-positive fibers were not observed in cardiac muscle of *mdx* mice treated with negamycin for 2 weeks (data not shown). The restoration of dystrophin was observed in the cardiac muscles of *mdx* mice treated with negamycin after prolonged (4 weeks) negamycin treatment (Fig. 2A). There were no EB-positive fibers in the dystrophin-positive area. The full-length dystrophin protein was also detected by immunoblot analysis in the cardiac muscles of negamycin-treated *mdx* mice (Fig. 2B). The dystrophin level in the cardiac muscle was also estimated to be less than 10%. Using WGA-conjugated affinity beads, the dystrophin/dystrophin-associated protein complex was successfully enriched from muscles of negamycin-treated *mdx* mouse. This study indicated that the accumulation of dystrophin-associated glycoprotein might also be recovered by the restoration of dystrophin in muscles of negamycin-treated animals. Moreover, with reference to Fig. 1, serum creatine kinase activity was decreased 35% in *mdx* mice treated with negamycin for 2 weeks (data not shown). Accordingly, it is reasonable to conclude that the

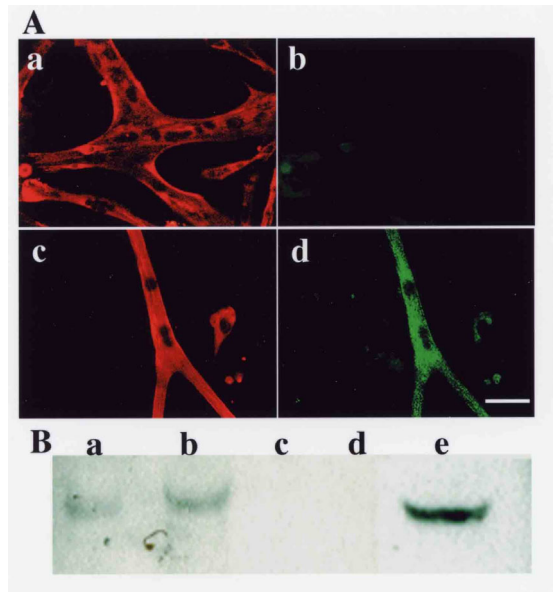


Figure 3. Dystrophin expression in cultured *mdx* skeletal muscle cells. **A**, Untreated (**a** and **b**) and negamycin-treated (100 μ g/ml) (**c** and **d**) cultured *mdx* myotubes. Immunofluorescent staining of myosin heavy chain (**a** and **c**) and dystrophin (**b** and **d**). Bar = 40 μ m. **B**, Immunoblot of dystrophin in cultured SV40-T or C2C12 cells. **a** and **b**, two separate cultures of myotubes treated with negamycin for 1 week; **c**, untreated *mdx* myotubes; **d**, empty well; **e**, C2C12 myotubes.

physiologic function of the muscle cell membrane is present not only in the TA muscle but also in other muscles in the whole body by negamycin treatment. However, muscle contraction profiles in negamycin-treated and untreated *mdx* mice remained to be compared in detail.

Dystrophin accumulates in negamycin-treated mdx skeletal muscle cells in culture

In order to study the restoration of dystrophin in cultured *mdx* skeletal muscle cells, we first established *mdxSV40-T* cell line. Muscle cell differentiation of *mdxSV40-T* cells was confirmed morphologically as well as immunohistochemically by the expression of the sarcomeric myosin heavy chain (Fig. 3A). After 1 week of negamycin treatment (4.0×10^{-7} mol/ml, 100 μ g/ml), dystrophin was detected immunohistochemically in an average of 6% of a total of 340 sarcomeric myosin heavy chain-positive myotubes from 4 different experiments, and dystrophin accumulation was confirmed by immunoblot analysis (Fig. 3B). Dystrophin was not detected in untreated *mdxSV40-T* cells.

Interaction between negamycin and the eukaryotic rRNA A-site

Analysis of its binding affinity should allow conclusions to be drawn on how negamycin affect rRNA. The mass measurement accuracy and resolution of MS easily provides this information in a rapid and sensitive manner. In the case of rRNA-aminoglycoside antibiotic and RNA-argininamide complex analysis, changes in the chemical shift of the rRNA complex were caused by binding to other small molecules [8, 15, 24, 30]. In the present study, to examine the functional relationship between negamycin and its read-through activity, we investigated the interactions between negamycin and the eukaryotic rRNA-decoding A-site using TOF-MS. The ESI-MS was obtained from a mixture of 5 nmol partial eukaryotic rRNA A-site and 50 nmol negamycin in distilled water. A partial eukaryotic rRNA A-site was detected at a mass number (MN) of 8625.51 (M+H) by TOF-MS (Fig. 4A). The negamycin/eukaryotic rRNA A-site complex (MN: 9368.31), corresponding to a 3:1 ratio of negamycin to eukaryotic rRNA was detected (Fig. 4B). Under the same condition, binding of gentamicin to the eukaryotic rRNA A-site was detected (data not shown). To examine the affinity site more closely, binding of negamycin and a modified rRNA A-site, which was modified 5-nucleotids in the loop region as to block to form match pairs in the eukaryotic rRNA A-site sequence, was studied under these same conditions. The modified rRNA was often conjugated with Na⁺ or K⁺ ions, which appeared as complexes on mass spectra (Fig. 4C). However, no interaction between negamycin and the modified A-site was detected (Fig. 4D). The eukaryotic rRNA A-site showed stoichiometric and stereospecific affinities that were significantly higher than those of the modified rRNA A-site. Our results indicate that an interaction occurs involving three molecules of negamycin and one molecule of rRNA. Negamycin/eukaryotic rRNA complex might also induce a local conformational change in the eukaryotic rRNA and cause the read-through of the nonsense mutation.

Negamycin is less toxic than gentamicin in *mdx* mice

It is well known that aminoglycoside antibiotics are associated with severe side effects. Although these antibiotics are used routinely for the treatment of bacterial infections, they often cause nephrotoxicity and ototoxicity. We examined negamycin toxicity by measuring changes in body weight and hearing acuity in drug-treated mice. Male *mdx* mice (7-week-old) were injected subcutaneously with varying doses of negamycin for 2 weeks. The body weights of mice treated with lower doses of negamycin (1x and 10x the minimal effective dose) and untreated control increased gradually (Fig. 5); however, the body weights of mice treated with higher doses of negamycin (50x and 100x)

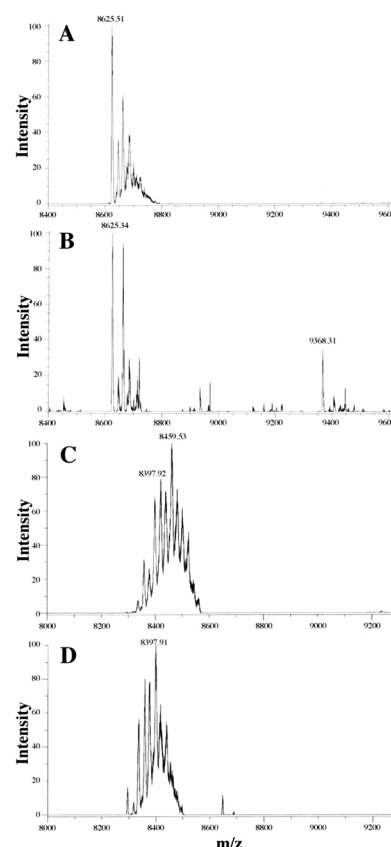


Figure 4. Interaction between negamycin and *a* eukaryotic partial rRNA A-site. Deconvolution of a mass spectrum of the eukaryotic rRNA A-site (MN: 8625.51, **A**), the negamycin/rRNA A-site complex (MN: 9368.31, **B**), modified RNA (8459.72: RNA+3Na⁺+2K⁺, 8397.73: RNA+Na⁺+2K⁺, **C**), and negamycin/modified RNA (8397.91: RNA+Na⁺+2K⁺, **D**)

decreased. In particular, it is noteworthy that the highest

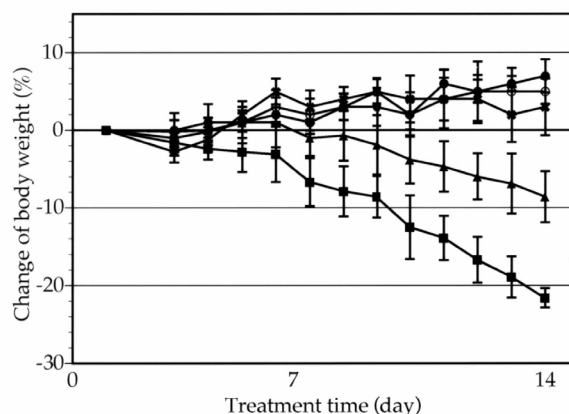


Figure 5. Changes in body weights of *mdx* mice during negamycin treatment. *Mdx* mice were treated with negamycin and weighed every day for 2 weeks. *Mdx* mice treated with PBS (open circles), or with negamycin at 1.2×10^{-5} mol/kg (closed circles), 1.2×10^{-4} mol/kg (crosses), 6.0×10^{-4} mol/kg (closed triangles), or 1.2×10^{-3} mol/kg (closed squares).

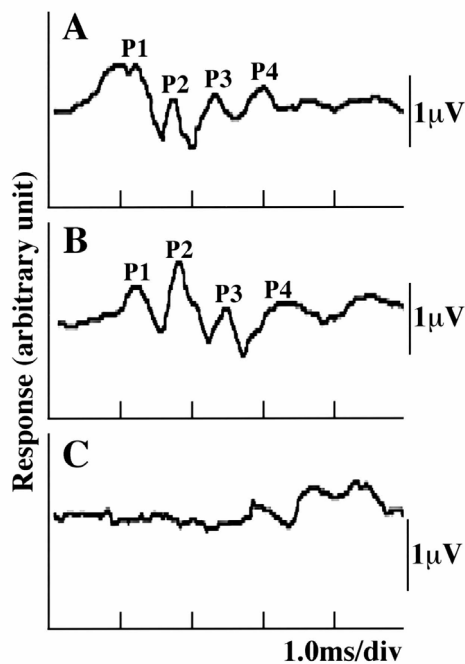


Figure 6. Changes in auditory brainstem response. Audiometric profiles according to the auditory brain stem response of antibiotic-treated mice are shown. **A**, control mouse, four positive peaks, P1, P2, P3, and P4, were recorded; **B**, negamycin-treated mouse, four positive peaks, similar to those in the control mouse, were recorded; **C**, gentamicin-treated mouse, the four positive peaks were absent.

dose of negamycin was not lethal. In contrast, a high dose of gentamicin (1.2×10^{-3} mol/kg/day BW, 100x) killed all mice tested within 4 h (data not shown). Compared with gentamicin, negamycin treatment exhibited lower toxicity. While the antibacterial activity of negamycin resembles that of gentamicin, it is possible that negamycin may have different side effects.

Furthermore, to investigate the ototoxicity, we conducted otologic studies on untreated and antibiotic-treated mice. Auditory brainstem responses to sounds ranging from 95 to 25 decibels (dB) were recorded. In both untreated and negamycin-treated *mdx* mice, the normal P1, P2, P3, and P4 peaks of an auditory brainstem response were observed. On the other hand, gentamicin-treated mice lacked all waves of auditory brainstem response under 80 dB (Fig. 6). This study indicates that hearing loss under 80 dB was obvious only in the gentamicin-treated mice, and not in negamycin-treated mice. There is considerable interest in understanding the mechanisms underlying negamycin toxicity so that negamycin can be used to treat a variety of medical conditions. Long-term side effects of negamycin treatment remained to be clarified.

Intriguing results were reported on pharmacological treatment of dystrophin-deficiency in *mdx* mice or

patients of DMD. Barton-Davis *et al.* reported that gentamicin could restore functional dystrophin in *mdx* mice [3]. Although, Dunant *et al.* reported that gentamicin treatment failed to lead to a significant increase in full-length dystrophin protein expression in *mdx* mice [12]. In human trials, Politano *et al.* reported that one of four gentamicin-treated DMD patients exhibited increased dystrophin re-expression by immunohistochemistry and Western blot analysis [29]. In contrast, Wagner *et al.* and Serrano *et al.* reported that the dystrophin protein was not detectable by immunohistochemistry or immunoblot analysis in clinical trials of DMD/BMD patients treated with gentamicin [31, 36]. These discrepancies of gentamicin clinical trials can probably be explained by the likely variations in the purity of commercially available gentamicin sulfate and in the ratios of its three distinct isomers.

Therapeutic significance of negamycin treatment

The mild Becker-type muscular dystrophy (BMD) is also caused by mutations in various portions of the dystrophin gene [10]. Hoffman *et al.* reported that in several cases of BMD, the phenotype was improved by dystrophin expressed at a level greater than or equal to 20% of normal [18]. Although we have no direct evidence as to whether 10% of the normal dystrophin level observed in this study would be sufficient to improve muscle performance *in vivo*, it is likely to be far better than a complete lack of dystrophin.

Danko *et al.* reported that dystrophin-positive fibers, called revertant fibers, were observed in 1% or fewer of the skeletal muscle fibers of 8-week-old *mdx* mice [11]. These dystrophin-positive *mdx* muscle fibers presumably arose from alternative splicing and/or a second mutation that skipped the primary mutation in exon 23. The number of revertant fibers increases as *mdx* mice age [25]. In the present study, the percentage of dystrophin-positive fibers in the skeletal muscle of *mdx* mice treated with negamycin was an average of 6%, which is significantly higher than the percentage of revertant fibers. In age-matched *mdx* skeletal and cardiac muscles, no truncated or shortened forms of dystrophin were detected in negamycin-treated *mdx* mice.

Many human genetic diseases have a relatively high proportion of premature stop codon. Nonsense mutations are found in approximately 5%-15% of patients with DMD [23]. To overcome nonsense mutations, one can expect that the naturally occurring suppressor tRNA may play important roles. Especially in the case of selenoprotein synthesis, the selenocysteyl tRNA carries the anticodon for UGA, a stop codon [5, 39]. Unfortunately, however, the selenocysteyl tRNA function in for selenoprotein synthesis only in a presence of selenocysteine insertion sequence (SECIS) in the 3' untranslated region (UTR) of the mRNA, and SECIS is found only in the mRNAs encoding for

selenoproteins. Except for selenocysteyl-tRNA, the level of other suppressor tRNAs is not high enough to overcome common nonsense mutations. Therefore, it is reasonable to search new drugs which induce read-through of premature stop codons.

The read-through of termination codons during normal protein synthesis may also be considered a source of side effects of negamycin. However, according to the cDNA databases that include the dystrophin gene, two or more stop codons usually exist near the termination codon in the UTR (data not shown). Extensive read-through of termination signals during protein synthesis may not occur. Both the type of stop codon and the nucleotide sequence surrounding the nonsense mutation have been reported to be critical for the read-through activity of gentamicin and other aminoglycoside antibiotics [20, 26, 28]. Therefore, the type of nonsense mutation and surrounding nucleotide sequence affected by the read-through activity induced by negamycin should be investigated.

We emphasize, however, that negamycin has not yet been approved for use in humans.

Acknowledgements

The writing of this paper was made possible through grants from Nervous and Mental Disorders (RM; 11B-1), Research in Brain Science from the Ministry of Health, Labor and Welfare, Japan (RM), the Fugaku Foudation (RM), Japan Foudation for Aging & Health (MS), Waseda University (MS), and Japan Science Society (MS; Sasakawa Scientific Research Grant).

Address correspondence to :

Ryoichi Matsuda, Dept. of Life Sciences, The Univ. of Tokyo, 3-8-1 Komaba, Tokyo 153-8902, Japan. Phone: +81-3-5454-6637 / Fax: +81-3-5454-4306. E-mail: ryoichi@matsuda.c.u-tokyo.ac.jp

References

- [1] Arakawa M, Nakayama Y, Hara T, Shiozuka M, Kondo S, Kitamura T, Takeda S, Matsuda R: Negamycin can restore dystrophin in *mdx* skeletal muscle. *Acta Myol* 2001; 20: 154-158.
- [2] Arakawa M, Shiozuka M, Nakayama Y, Hara T, Hamada M, Ikeda D, Takahashi Y, Sawa R, Nonomura Y, Sheykholeslami K, Kondo K, Kaga K, Takeda S, Matsuda R: Negamycin restores dystrophin expression in skeletal and cardiac muscles of *mdx* mice. *J Biochem* 2003; (in press)
- [3] Barton-Davis ER, Cordiner L, Shoturma DI, Leiland SE, Sweeney HL: Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of *mdx* mice. *J Clin Invest* 1999; 104: 375-381.
- [4] Bedwell DM, Kaenjak A, Benos DJ, Bebok Z, Bubien JK, Hong J, Tousson A, Clancy JP, Sorscher EJ: Suppression of a CFTR premature

- stop mutation in a bronchial epithelial cell line. *Nat Med* 1997; 3: 1280-1284.
- [5] Berry MJ, Banu L, Harney JW, Larsen PR: Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons *EMBO J* 1993; 12: 3315-3322.
- [6] Bulfield G, Siller WG, Wight PA, Moore KJ: X chromosome-linked muscular dystrophy (*mdx*) in the mouse. Comparison with Duchenne muscular dystrophy. *Proc Natl Acad Sci USA* 1984; 81: 1189-1192.
- [7] Burke JF, Mogg AE: Suppression of a nonsense mutation in mammalian cells *in vivo* by the aminoglycoside antibiotics G-418 and paromomycin. *Nucleic Acids Res* 1985; 13: 6265-6272.
- [8] Chow CS, Bogdan FM: A Structural Basis for RNAMinus signLigand Interactions. *Chem Rev* 1997; 97: 1489-1513.
- [9] Clancy JP, Bebok Z, Ruiz F, King C, Jones J, Walker L, Greer H, Hong J, Wing L, Macaluso M, Lyrene R, Sorscher EJ, Bedwell DM: Evidence that systemic gentamicin suppresses premature stop mutations in patients with cystic fibrosis. *Am J Respir Crit Care Med* 2001; 163: 1683-1692.
- [10] Dalkilic I, Kunkel LM: Muscular dystrophies: genes to pathogenesis. *Curr Opin Genet Dev* 2003; 13:231-238.
- [11] Danko I, Chapman V, Wolff JA: The frequency of revertants in *mdx* mouse genetic models for Duchenne muscular dystrophy. *Pediatrc Res* 1992; 32: 128-131.
- [12] Dunant P, Walter MC, Karpati G, Lochmuller H: Gentamicin fails to increase dystrophin expression in dystrophin-deficient muscle. *Muscle Nerve* 2003; 27: 624-627.
- [13] Fourmy D, Recht MI, Blanchard SC, Puglisi JD: Structure of the A site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic. *Science* 1996; 274: 1367-1371.
- [14] Granchelli JA, Pollina C, Hudecki MS: Pre-clinical screening of drugs using the *mdx* mouse. *Neuromuscul Disord* 2000; 10: 235-239.
- [15] Griffey RH, Hofstadler SA, Sannes-Lowery KA, Ecker DJ, Crooke ST: Determinants of aminoglycoside-binding specificity for rRNA by using mass spectrometry. *Proc Natl Acad Sci USA* 1999; 96: 10129-10133.
- [16] Griggs RC, Moxley RT 3rd, Mendell JR, Fenichel GM, Brooke MH, Pestronk A, Miller JP, Cwik VA, Pandya S, Robinson J: Duchenne dystrophy: randomized, controlled trial of prednisone (18 months) and azathioprine (12 months). *Neurology* 1993; 43: 520-527.

Negamycin-therapy in *mdx* mice

- [17] Hamada M, Takeuchi T, Kondo S, Ikeda Y, Naganawa H, Maeda K, Okami Y, Umezawa H: A new antibiotic, negamycin. *J Antibiot* 1970; 23: 170-171.
- [18] Hoffman EP, Kunkel LM, Angelini C, Clarke A, Johnson M, Harris JB: Improved diagnosis of Becker muscular dystrophy by dystrophin testing. *Neurology* 1989; 39: 1011-1017.
- [19] Howard MT, Frizzell RA, Bedwell DM: Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations. *Nat Med* 1996; 2: 467-469.
- [20] Howard MT, Shirts BH, Petros LM, Flanigan KM, Gesteland RF, Atkins JF: Sequence specificity of aminoglycoside-induced stop codon readthrough: potential implications for treatment of Duchenne muscular dystrophy. *Ann Neurol* 2000; 48: 164-169.
- [21] Itzhaki RF, Gill DM: A micro-biuret method for estimating protein. *Anal Biochem* 1964; 9: 401-410.
- [22] Keeling KM, Brooks DA, Hopwood JJ, Li P, Thompson JN, Bedwell DM: Gentamicin-mediated suppression of Hurler syndrome stop mutations restores a low level of alpha-L-iduronidase activity and reduces lysosomal glycosaminoglycan accumulation. *Hum Mol Genet* 2001; 10: 291-296.
- [23] Khurana T, Davies K: Pharmacological strategies for muscular dystrophy. *Nat Rev Drug Discov* 2003; 2: 379-390.
- [24] Loo JA, DeJohn DE, Du P, Stevenson TI, Ogorzalek Loo RR: Application of mass spectrometry for target identification and characterization. *Med Res Rev* 1999; 19: 307-319.
- [25] Lu QL, Morris GE, Wilton SD, Ly T, Artem'yeva OV, Strong P, Partridge TA: Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. *J Cell Biol* 2000; 148: 985-995.
- [26] Manuvakhova M, Keeling K, Bedwell DM: Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system. *RNA* 2000; 6: 1044-1055.
- [27] Matsuda R, Nshikawa A, Tanaka H: Visualization of dystrophic muscle fibers in *mdx* mouse by vital staining with Evans blue: evidence of apoptosis in dystrophin-deficient muscle. *J Biochem* 1995; 118: 959-964.
- [28] Namy O, Hatin I, Stahl G, Liu H, Barnay S, Bidou L, Rousset JP: Gene overexpression as a tool for identifying new trans-acting factors involved in translation termination in *Saccharomyces cerevisiae*. *Genetics* 2002; 161: 585-594.
- [29] Politano L, Nigro G, Nigro V, Piluso G, Papparella S, Paciello O, Comi LI: Gentamicin administration in Duchenne patients with premature stop codon. Preliminary results. *Acta Myol* 2003; 22: 15-21.
- [30] Puglisi JD, Tan R, Calnan BJ, Frankel AD, Williamson JR: Conformation of the TAR RNA-arginine complex by NMR spectroscopy. *Science* 1992; 257: 76-80.
- [31] Serrano C, Wall C, Moore SA, King W, Barresi R, Campbell KP, Noticewala P, Brown RH, Nagarja HN, Mendell JR: Gentamicin treatment for muscular dystrophy patients with stop codon mutations. *Neurology* 2001; 56(Suppl): A79.
- [32] Shapiro SM, Moller AR, Shiu GK: Brain-stem auditory evoked potentials in rats with high-dose pentobarbital. *Electroencephalogr Clin Neurophysiol* 1984; 58: 266-276.
- [33] Sleat DE, Sohar I, Gin RM, Lobel P: Aminoglycoside-mediated suppression of nonsense mutations in late infantile neuronal ceroid lipofuscinosis. *Europ J Paediat Neuro* 2001; 5: 57-62.
- [34] Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, Barnard PJ: The molecular basis of muscular dystrophy in the *mdx* mouse: a point mutation. *Science* 1989; 244: 1578-1582.
- [35] Uehara Y, Hori M, Umezawa H: Negamycin inhibits termination of protein synthesis directed by phage f2 RNA *in vitro*. *Biochim Biophys Acta* 1974; 374: 82-95.
- [36] Wagner KR, Hamed S, Hadley DW, Gropman AL, Burstein AH, Escolar DM, Hoffmann EP, Fischbeck KH: Gentamicin treatment of Duchenne and Becker muscular dystrophy due to nonsense mutations. *Ann Neurol* 2001; 49: 706-711.
- [37] Wilhelm JM, Jessop JJ, Pettitt SE: Aminoglycoside antibiotics and eukaryotic protein synthesis: stimulation of errors in the translation of natural messengers in extracts of cultured human cells. *Biochemistry* 1978; 17: 1149-1153.
- [38] Yoshizawa S, Fourmy D, Eason RG, Puglisi JD: Sequence-specific recognition of the major groove of RNA by deoxystreptamine. *Biochemistry* 2002; 41: 6263-6270.
- [39] Zavacki AM, Mansell JB, Chung M, Klimovitsky B, Harney JW, Berry ML: Coupled tRNA(Sec)-dependent assembly of the selenocysteine decoding apparatus. *Mol Cell* 2003; 11: 773-781.