

The Ca^{2+} sensitivity curve suggests that the targets of the SCRAPPER-proteasome axis should include molecules that regulate Ca^{2+} -dependent neurotransmitter release at the presynapse and that these targets are not molecules such as Ca^{2+} channels, which directly regulate the Ca^{2+} influx, but are likely due to the modulation of the Ca^{2+} sensitivity of presynaptic machinery.

SCRAPPER Regulates Synaptic Transmission via RIM1

Are the effects of loss or gain of function of SCRAPPER on synaptic function mediated specifically through RIM1? RNAi-mediated knockdown of RIM1 in neurons from SCR-KO mice was able to reverse the change in mEPSC, which supports that SCRAPPER does indeed function via RIM1. Moreover, overexpression of RIM1 in WT neurons was sufficient to phenocopy the increased mEPSC frequency in SCR-KO mice. Thus, alteration in steady-state level of RIM1 is sufficient to modulate mEPSC and upstream signaling pathways that function through SCRAPPER can in turn regulate this process. At present we cannot exclude the possibility that monoubiquitination of RIM1 by SCRAPPER may also regulate RIM1 before RIM1 is polyubiquitinated and degraded by the proteasome. Mutation of SCRAPPER abolished the increase in mEPSC observed following treatment of WT neurons with proteasome inhibitors, indicating that SCRAPPER is the major mediator of ongoing proteasomal regulation of mEPSC frequency. The rapidity of MG132 action suggests that the effects are independent of transcriptional regulation. These results also indicate that there is robust proteasome activity for the regulation of Ca^{2+} -dependent synaptic vesicle release.

SCRAPPER Contributes to Regulation of Synaptic Plasticity

Synaptic plasticity is thought to be an important basis for learning and memory (Brown et al., 1990). PPF is one form of STP, the fundamental basis of synaptic plasticity. Ataxin-1 knockout mice show learning deficits and decreased hippocampal PPF, despite having normal LTP induction (Matilla et al., 1998). This indicates that PPF is related to learning. We showed that SCRAPPER functions as a regulator of PPF (i.e., synaptic plasticity), at presynapses.

SCR-KO mice displayed decreased PPF ratio. In addition, MG132-dependent upregulation of fEPSP was suppressed in SCR-KO animals. Our findings demonstrate that UPS-dependent regulation of PPF and mEPSC is mainly controlled by SCRAPPER. Based on analysis of RIM1 knockout mice in which the PPF value was increased at the hippocampal CA1 synapse (Schoch et al., 2002), together with the increased steady-state level of RIM1 in SCR-KO mice, we propose that increased RIM1 is a major contributor to the decreased PPF observed in SCR-KO mice. Thus, SCRAPPER appears to induce degradation of RIM1 complex via the proteasome, which regulates the PPF ratio. We note that overexpression of

Munc13 also increases mEPSC (Betz et al., 1998) and that synaptotagmin I increased the probability of vesicle fusion (Kreft et al., 2003). Hence, it is formally possible that upregulation of Munc13 and synaptotagmin could also contribute to the changes of PPF and mEPSC in SCR-KO mice.

Neural activity-dependent alteration of *Scrapper* expression directly implicates SCRAPPER as being involved in neural activity-dependent protein degradation, which in turn alters synaptic transmission efficiency. Regulation of RIM1 protein complex level is likely to cause both short- and long-term changes in the PPF ratio. That SCRAPPER can facilitate a long-term change in synaptic efficacy suggests that SCRAPPER could influence regulation of "the plasticity of plasticity" at the presynapse—i.e., the metaplasticity (Abraham and Tate, 1997). We have focused our investigation on presynaptic functions. However, as SCRAPPER is also localized to dendrites, there may be additional effects of SCRAPPER disruption on neuronal function and postsynaptic function. Finally, we speculate that the activation of certain neural networks does not occur in SCR-KO, because SCRAPPER may also serve as E3 for an unknown substrate in inhibitory neurons, which in turn may give rise to the inactivation of a certain population of excitatory neurons. In summary, we have identified a physiological role of SCRAPPER-dependent RIM1 ubiquitination for proteasomal degradation in presynaptic function. Many kinds of neuronal disorder/disease are caused by excessive neurotransmitter release. An attractive possibility is that SCRAPPER can be a potential target of new drug designs for the treatment of neuronal diseases.

EXPERIMENTAL PROCEDURES

Identification of SCRAPPER and Cloning of *Scrapper* Gene

Eleven hundred thirty genes with both neuron-restrictive silencing element and cAMP-response element were found using the Celera Discovery System (a search program that is no longer available). The F box domain was identified by the Pfam program. Cloning of the *Scrapper* gene was performed by RT-PCR, using cDNA from newborn mouse brain and *Scrapper*-specific primers. The NCBI accession number of *Scrapper* is EF649694.

Animals

Care and experiments with animals were in accordance with institutional guidelines and those of the National Institute of Health and the Animal Care and Use Committee (Mitsubishi Kagaku Institute of Life Science). C57BL/6 mice and Wistar SD rats were used.

Antibodies

Rabbit anti-SCRAPPER antibody was raised against amino residues 321–380 of mouse SCRAPPER expressed in bacteria. Other antibodies used are described in the Supplemental Data.

Miscellaneous Procedures

In situ hybridization for *Scrapper* mRNA was performed as described (Ikegami et al., 2006). Details are described in the Supplemental Data. Plasmid construction, cell culture, and Southern and western blotting were performed by conventional methods (Hatanaka et al., 2006c). Subcellular fractionation of mouse brain was performed as

described (Yao et al., 1999). Fractions were analyzed by western blotting. Stability of RIM1 was measured by the treatment of 20 μ g/ml cycloheximide (Hatanaka et al., 2006a) to cerebral cortical primary culture from WT or SCR-KO mice. RIM1 was knockeddown by the miR-RNAi system (Invitrogen).

Neuron Culture and Immunostaining

Hippocampal neurons were prepared (Kato et al., 2001) with minor modifications described in the Supplemental Data. Cultured cells were transfected with 1 μ g DNA with Lipofectamine2000 (Invitrogen). Primary cultured neurons from SCR-KO mice were prepared with P1-P3 mice. Immunofluorescence and immunohistochemical studies were performed as described (Yao et al., 2002) and imaged and quantified using confocal microscopy operated under manual control (Zeiss LSM5 PASCAL, Olympus FV-1000). For mixed culture, neurons were incubated separately with each DNA mixture, subsequently washed to remove any adherent DNA before plating then cocultured. The cells transfected independently with a vector expressing either nontagged RFP (DsRed2, Clontech), or different GFP-SCR constructs were plated together with other group neurons and used at 12–15 days in vitro for electrophysiological recordings. The mEPSC frequency recorded from neurons expressing either nontagged RFP or nontagged GFP displayed no significant difference between cells. The changes observed when recording from Red cells in the presence of green neurons transfected with different GFP-SCR constructs were interpreted as an effect generated in the presynaptic (green) cell.

Immunoprecipitation and In Vitro Pull-Down Assay

Immunoprecipitation of the mouse brain was performed as described (Yao et al., 1999). HEK293T cells were lysed 48 hr after transfection with a solution containing 50 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1% (v/v) Triton X-100, and a cocktail of protease inhibitors (Complete EDTA-free, Roche). Cell lysates were incubated with 5 μ g of antibody in 10 μ l of protein G-sepharose beads (Amersham) for 4 hr at 4°C. Immunoprecipitates were washed four times with ice-cold lysis buffer and were blotted with appropriate antibodies. In vitro binding using GST fusion proteins and cell extract was performed as described (Yao et al., 1999).

In Vitro Immunoprecipitation and Ubiquitination Assays

Recombinant Uba1 (E1), GST-UbcH5 (E2), His α 6-ubiquitin, APP-BP1/Uba3, GST-UbcH12, and NEDD8 were purchased from Medical and Biological laboratories Company, Limited. The NEDD8 system containing APPBP1/Uba3, UbcH12, and NEDD8 was added simultaneously to the mixture. SCRAPPER was immunoprecipitated with anti-SCRAPPER conjugated to Protein G sepharose, and denatured after being washed three times. The ubiquitination assay was carried out as described (Kawakami et al., 2001).

SCRAPPER Knockout and Transgenic Mice

We used homologous recombination in ES cells to mutate *Scrapper*. Exon 3 (which encodes the region including the F box domain) and a part of exon 4 (a recombination cassette that generates a frameshift) were replaced by a Neo selection cassette. Analysis of SCR-KO mice was performed on littermates derived from mating heterozygous mutant mice on a hybrid 129Sv/C57BL6 background and was confirmed with several independent litters derived from independent generations of heterozygous breeding. We established three independent mouse lines overexpressing GFP-fused SCRAPPER (TG-22, TG-26, and TG-31). The GFP-SCRAPPER transgene contains a CaMKII promoter, an EGFP tag, a *Scrapper*-coding region, and a polyadenylation signal. Details are described in the Supplemental Data. Two-dimensional analyses of the brain homogenate of *Scrapper* knockout mice were performed as described (Omori et al., 2002).

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed as described (Ikegami et al., 2006) and in the Supplemental Data. Quantitative analysis of TEM micrographs was performed as described (Altrock et al., 2003; Schoch et al., 2002).

Recording of mEPSC

mEPSC was recorded as described (Inoue et al., 2006), see the Supplemental Data. Cells for whole-cell recording configuration were selected on the status of RFP or GFP expression. The average frequency (Hz), amplitude (pA), rise time (ms), and decay time (ms) from each neuron were then averaged to give a value for the entire population. Statistical significance was determined using the two-tailed, paired Student's *t* test. **p* < 0.05 was considered to be statistically significant.

Electrophysiology of Hippocampal Slices

Hippocampal 300- μ m-thickness slices were prepared from ether-anesthetized 3- to 4-week-old SCR-KO mice as described in the Supplemental Data. PPF was measured by using two-paired 50, 100, 200, 300, and 400-ms interpulse interval stimuli. The PPF ratio (2nd/1st fEPSP slope) was evaluated in each interpulse interval (IPI).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and ten figures and can be found with this article online at <http://www.cell.com/cgi/content/full/130/5/943/DC1/>.

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Reduced cell anchorage may cause sarcolemma-specific collagen VI deficiency in Ullrich disease

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ABSTRACT

Background: *COL6* gene mutations are associated with Ullrich congenital muscular dystrophy (UCMD), which is clinically characterized by muscle weakness from early infancy, hyperlaxity of distal joints, and multiple proximal joint contractures. We previously reported that the majority of patients with UCMD have sarcolemma-specific collagen VI deficiency (SSCD). More recently, we found heterozygous *COL6A1* glycine substitutions in patients with UCMD with SSCD.

Objective: To elucidate how *COL6A1* glycine mutation leads to SSCD.

Methods: We evaluated the synthesis, formation, and binding of collagen VI to the extracellular matrix in fibroblasts with p.G284R mutation in *COL6A1*.

Results: Collagen VI was normally secreted into the cultured medium in fibroblasts harboring p.G284R mutation. When the medium with normal collagen VI was added to collagen VI-deficient fibroblast culture, collagen VI bound surrounding the cells, while collagen VI with p.G284R mutation did not. Cell adhesion of fibroblasts with p.G284R mutation was markedly reduced similarly to that of collagen VI-deficient cells. Interestingly, this reduction in adhesion of the cells with p.G284R mutation was recovered by the addition of the medium with normal collagen VI, which would suggest a therapeutic strategy for a replacement therapy.

Conclusion: Heterozygous glycine substitution in *COL6A1* may cause decreased binding of collagen VI microfibrils to the extracellular matrix resulting in sarcolemma-specific collagen VI deficiency. *Neurology*® 2007;69:1043-1049

Ullrich congenital muscular dystrophy (UCMD) is an inherited muscular disorder clinically characterized by muscle weakness, distal joint hyperlaxities, and proximal joint contractures.¹ Patients with UCMD show deficiency of collagen VI. We have previously demonstrated two modes of collagen VI deficiency: complete deficiency and sarcolemma-specific collagen VI deficiency (SSCD). In SSCD, collagen VI is present in the interstitium but is barely detectable in the sarcolemma.² The complete deficiency of collagen VI is associated with recessive mutations in collagen VI genes,²⁻⁶ but the primary cause of SSCD has not yet been determined.²

Collagen VI is an extracellular matrix (ECM) consisting of three chains: $\alpha 1$, 2, and 3, which are encoded by *COL6A1*, *COL6A2*, and *COL6A3* genes.⁷ Association of the three subunits to form monomers is by staggered assembly into dimers,⁸ which subsequently align to form tetramers. After being secreted, these tetramers associate end-to-end to form the characteristic beaded microfibrils.⁹⁻¹¹

Recently, heterozygous missense mutations that substitute the glycine in the Gly-X-Y amino acid repeat in the triple helical domain including p.G284R in *COL6A1* have been

Supplemental data at
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identified in patients with UCMD.¹²⁻¹⁴ Previous reports alluded to the effects of single amino acid substitutions on collagen VI structure and function in causing UCMD^{12,14,15} as well as in Bethlem myopathy,¹⁵⁻¹⁷ a milder autosomal dominant muscle disease allelic to UCMD.¹⁸⁻²² However, the molecular pathomechanism has not been clearly determined.

In this article, using fibroblasts with the p.G284R mutation, the synthesis, formation, and functions of collagen VI microfibrils were analyzed.

METHODS Clinical materials. All clinical materials used in this study were acquired with informed consent. Biceps brachii muscle was biopsied for diagnostic purpose. Based on our immunohistochemical and genetic screening of Japanese patients who were diagnosed with UCMD based on typical clinical features, i.e., muscle weakness, hyperextensibility of distal joints, and contractures of proximal joints, we found five patients with mutations in *COL6* gene: four with SSCD had a heterozygous c.850G>A (p.G284R) mutation in *COL6A1* gene, and one with complete deficiency harbored compound heterozygous mutations in *COL6A3*. In this study, we analyzed fibroblasts from foreskin biopsy which were available in two of the four patients with SSCD, and in the patient with complete deficiency of collagen VI.

Immunohistochemical and histologic staining. Immunohistochemical and histologic staining used in this study have been described previously.² Briefly, sections were incubated in mixtures of mouse monoclonal antibody against collagen VI (1:500 dilution) (ICN Pharmaceuticals) and rabbit polyclonal antibody against collagen IV (1:500 dilution) (Abcam Ltd.) for 1 hour. After phosphate buffered saline (PBS) washes, mixtures of anti mouse IgG Alexa 488, anti-rabbit IgG Alexa 568 conjugates (1:500 dilution) (Molecular probes) were applied for 30 minutes.

Cell culture. Fibroblasts from two patients with UCMD with p.G284R mutation, one with collagen VI complete deficiency, and two controls were cultured to reach confluence on 100 mm collagen I coated dishes in 10% fetal bovine serum/Dulbecco's modified Eagle's medium under humidified 5% CO₂ at 37 °C.

For enhancement of expression, cells were cultured in the presence of 0.25 mM L-ascorbic acid (Sigma) for 3 days. For cell detachment test, cultured fibroblasts were treated with 10 mM EDTA-PBS (pH. 8.0) at 37 °C for 1 hour. After washing with PBS, cells that remained on the dish were counted in a constant area (0.25 mm × 0.25 mm, total of 8 areas). Student *t* test was used for statistical analysis.

The cultured medium in each patient's cells was changed with the medium similar to which control or patient cells had been cultured for 3 days in the presence of 0.25 mM L-ascorbic acid. The cultured media for control or patient cells for exchanging were prepared by centrifugation at 2,000 rpm for 10 minutes. The amount of collagen VI in the clarified media was measured by Western blot using Quantity One software (PDI, Inc.). Cells were further cultured for 3

days in the exchanged medium containing relatively the same amount of collagen VI. They were subjected to cell detachment test and immunocytochemical staining.

For evaluating the substrate-retained collagen VI molecules, cells were mechanically removed by pipetting and the remaining proteins were extracted for Western blot analysis.

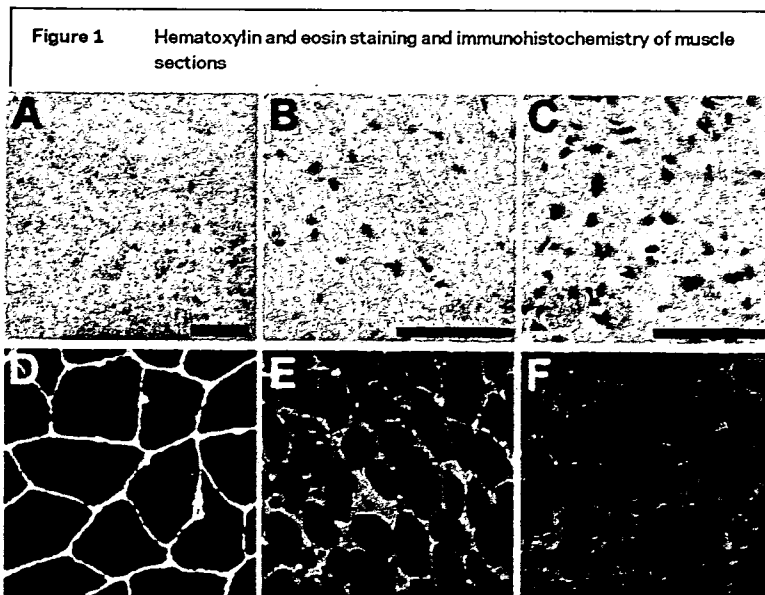
Immunocytochemical staining. The fibroblasts cultured on collagen I-coated cover slips were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.25% Triton X-100-PBS on ice for 10 minutes, followed by incubation with 1% bovine serum albumin in PBS for 1 hour. Cells were incubated for 1 hour in mixtures of mouse monoclonal antibody against collagen VI (1:500 dilution) (ICN Pharmaceuticals). After PBS washes, these were incubated with mixtures of antimouse IgG Alexa 488 and TOTO-3 to stain nuclei (1:600 dilution) (Molecular probes) for 30 minutes.

Western blot analysis. Proteins in the cultured medium and cell extract, as well as the proteins that remained on the dish after pipetting, were electrophoresed on 5–17.5% polyacrylamide gel under reduced condition and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation). The remaining proteins on dish with or without EDTA treatment were extracted with a solution containing 2 M thiourea, 7 M urea, 4% CHAPS, 10 mM Tris-HCl (pH 8.5). After blocking, the proteins were allowed to react with rabbit polyclonal antibody against the subunits of collagen VI (1:50 dilution) (Abcam) or rabbit polyclonal antibody against fibronectin (1:3,000 dilution) (Chemicon) and incubated with peroxidase-conjugated, anti-rabbit IgG (1:3,000 dilution) (TAGO Inc.). Visualization of proteins was done using ECL Western blotting detection reagents and analysis system (Amersham Biosciences).

Negative staining electron microscopy. Culture media from control and patient cells were purified by centrifugation and adsorbed onto thin bar grids covered with a thin layer of carbon for 5 minutes. The grids were washed with PBS, and stained with 2% phosphotungstic acid. The dried sample was observed in a HITACHI H-600 transmission electron microscope (Hitachi) operated at 120 kV accelerating voltage.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis. The remaining protein (50 µg) from control and UCMD cells after EDTA treatment were labeled with Cy3 and Cy5 minimal dyes (Amersham Biosciences), following manufacturer's instructions. Mixture of both labeled samples was subjected to isoelectric focusing for separation in the first dimension by IPG gels (covering the range pH 3 to 10) using the Ettan IPGphor isoelectric focusing system (Amersham Biosciences); SDS-PAGE was then performed on a 10% polyacrylamide gel in second dimension. The Cy3/Cy5 signals were separately detected using Typhoon 9400 (Amersham Biosciences). DeCyder software (Amersham Biosciences) was used for quantitation and comparison of Cy3 and Cy5 intensities of all spots. Protein mass fingerprint analysis based on matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on spots was performed for the identification of proteins (Hitachi Science Systems, Ltd.).

RESULTS Brief clinical summary of the patients with p.G284R mutation. The patients with p.G284R mutation in *COL6A1* showed typical



Muscle sections from normal control (A, D), patient with Ullrich congenital muscular dystrophy (UCMD) with p.G284R mutation (B, E), and patient with UCMD collagen VI complete deficiency (C, F) were stained with hematoxylin and eosin, and immunostained by anti-collagen VI antibody (D through F). Both collagen VI (green) and collagen IV (red) are present in sarcolemma in control muscle, as indicated in merged images (yellow, D). In contrast, collagen VI is only seen in the interstitium but not in the sarcolemma in the patients with UCMD with p.G284R mutation in *COL6A1* (E). In the case of complete deficiency of collagen VI, collagen VI is absent in the muscle section (F). Bars denote 50 μ m.

clinical phenotypes of UCMD, including muscle weakness, hyperextensibility of distal joints, and contractures of proximal joints. All were sporadic cases. No genotype-phenotype correlation between SSCD and complete deficiency of collagen VI was shown.

Localization of collagen VI in the skeletal muscle with p.G284R mutation. In the biopsied muscles from patients with UCMD with p.G284R mutation, double immunostaining of collagen VI and collagen IV, which is the major component of the basal lamina, revealed SSCD (figure 1, B and E). We also examined collagen VI microfibrils on electron microscopy; microfibrils were present in the interstitium, but they did not bind to the basement membrane (data not shown), similar to the previous report.² In the muscles from a patient with compound heterozygous mutations, collagen VI was completely deficient in the muscle (figure 1, C and F).

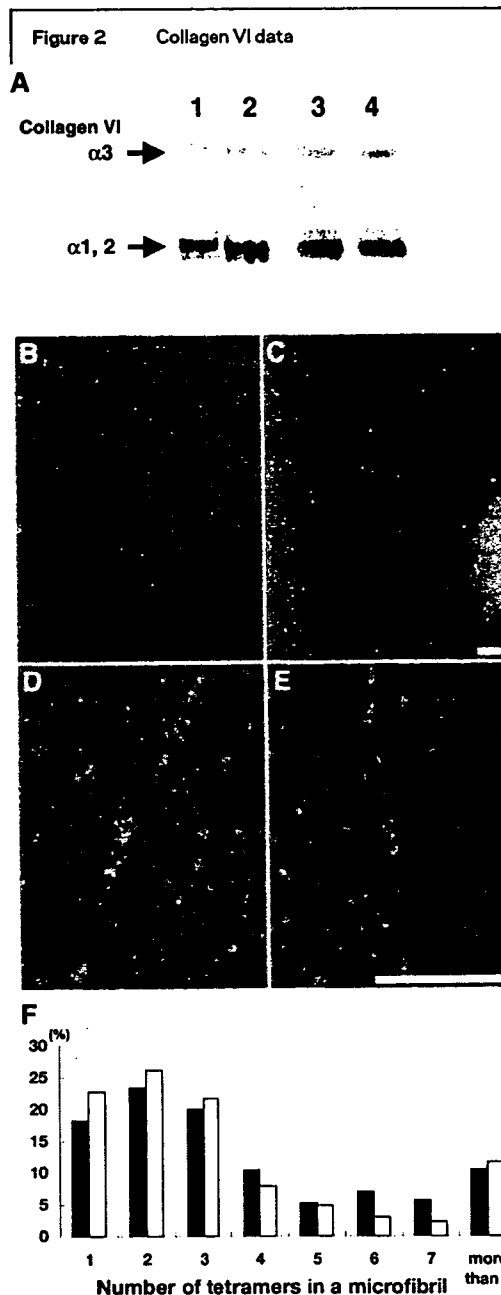
Analysis of collagen VI secreted in the cultured medium from patients with UCMD with p.G284R mutation. In fibroblasts from two patients with UCMD with the p.G284R mutation, collagen VI was present in the extracellular areas, as in control. On Western blot analysis, collagen VI subunits (α 1, 2, and 3) were detected in the whole extract from fibroblasts and in the cultured me-

dium, with similar size and amounts as in control cells (figure 2A). Ultrastructural analysis of collagen VI microfibrils with p.G284R mutation showed that tetramers were secreted from the cells; moreover, microfibrils were assembled in comparable length with control, including long microfibrils which consisted of more than 8 tetramers (figure 2, B through E). The shape and the length of each tetramer unit in microfibrils were also normal (figure 2, D and E).

Binding capacity of collagen VI with or without p.G284R mutation to ECM. In normal cells, collagen VI was localized in the ECM surrounding cells (figure 3A). On the other hand, collagen VI was absent in the ECM of cells from the patient with compound heterozygous mutations (figure 3E), consistent with the complete deficiency of collagen VI in muscle (figure 1F). When cultured medium of control cells containing normal collagen VI was added to these collagen VI-deficient cells, we proceeded to evaluate the binding of collagen VI to ECM on days 1, 2, and 3. Collagen VI was detected in the ECM of fibroblasts from patients with complete deficiency of collagen VI on days 2 and 3 (figure 3, B, C, and D). However, when cultured medium containing collagen VI with p.G284R mutation was added to collagen VI-deficient cells, collagen VI was only detected on day 3, in extremely reduced amount than that of normal cells after the incubation for 3 days (figure 3, F, G, and H).

Recovery of the adhesion ability of patients with UCMD's cells by treatment with the cultured medium containing normal collagen VI. In order to examine the effect of *COL6* mutation on the attachment of fibroblasts, we assessed cell adhesion of patients with UCMD's cells on a dish after treatment with EDTA. There was no difference in cell adhesion on dish in medium after washing with PBS among normal cells, p.G284R, and collagen VI-deficiency cells (data not shown). However, after EDTA treatment, in the cells with p.G284R mutation and collagen VI-deficient cells, the number of cells retained on the dish was reduced to approximately 30% of that of control ($p < 0.001$) (bars no. 1 in figure 4A).

Interestingly, by the addition of medium containing normal collagen VI to p.G284R mutated cells and collagen VI-deficient cells, the number of the retained cells on dish was restored to the level of control cells (p.G284R and collagen VI complete deficiency shown by bars no. 3, 5 in figure 4A). However, using the medium from p.G284R culture which contained the same amount of collagen VI did not restore the number



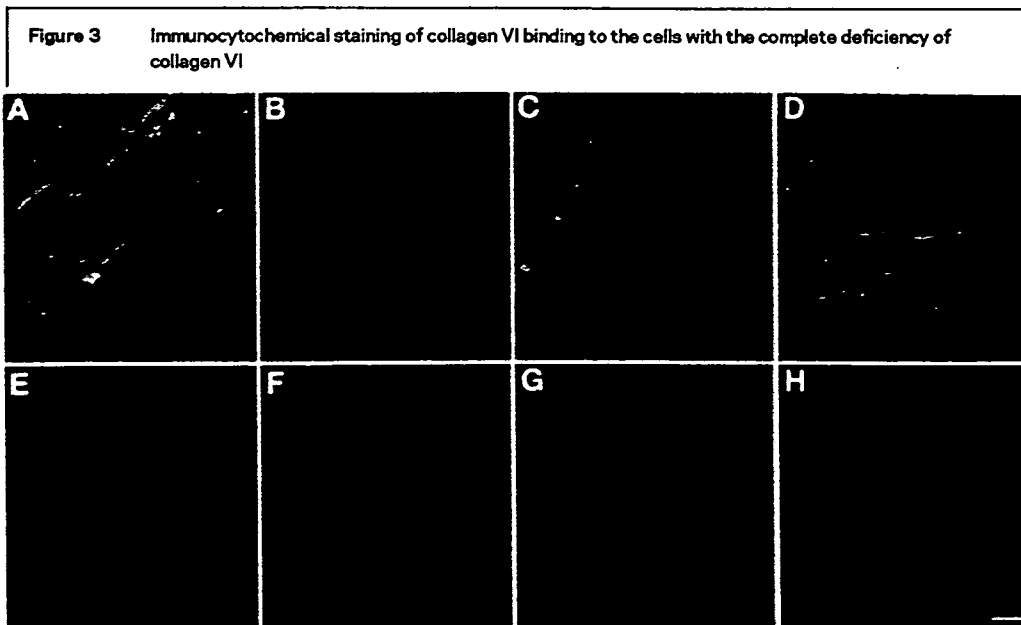
(A) Collagen VI in the fibroblasts or the cultured medium. Collagen VI is present in the extract of cells on dish (lane 1, control cells; lane 2, p.G284R mutation) and in the cultured medium of cells (lane 3, control cells; lane 4, p.G284R mutation). Collagen VI with p.G284R is produced and secreted to cultured medium in the same mass and amount compared to control cells. (B through E) Negative staining electron microscopy of secreted collagen VI. Collagen VI microfibrils in the cultured medium secreted from control cells (B, D) or cells with this p.G284R mutation (C, E) were visualized by negative staining electron microscopy. Long microfibrils are present in both control and patient (B, C). The magnified images show that the structure of tetramer is normal in length and shape. Bars denote 100 nm (D, E). (F) Quantitative analysis of association of collagen VI tetramers. The distribution of the numbers of tetramer in one microfibril is shown as a percentage of the total number of microfibrils. Black bars show the results of 231 microfibrils from control cells. White bars show 163 microfibrils from Ullrich congenital muscular dystrophy patient cells.

of collagen VI deficient cells retained on dish (bar no. 6 in figure 4A). Our analysis of substrate-retained collagen VI supported these findings. Only after treatment with the cultured medium containing normal collagen VI, we observed the recovery of substrate-retained collagen VI in the patient's cells with p.G284R mutation and collagen VI deficiency (figure 4B, lanes 3, 5). However, the addition of the cultured medium containing collagen VI with p.G284R mutation did not recover the reduction of the amount of collagen VI in the ECM of p.G284R mutated and collagen VI deficient cells (figure 4B, lanes 4, 6). In contrast, the amount of fibronectin bound to the substrate showed no difference between control and the mutant cells (figure 4B).

We further analyzed the remaining proteins bound to the substrate in order to examine influences of the alteration of collagen VI molecule to other proteins. In the 2D-PAGE analysis, only two groups of spots from 937 spots detected showed marked reduction (>fivefold) in the patient with p.G284R as compared to control. MALDI-TOF-mass spectrometry analysis revealed that these proteins were collagen VI $\alpha 1$ and $\alpha 2$. In contrast, the amount of the other substrate-bound proteins on dish was not greatly altered (see supplemental data at www.neurology.org).

DISCUSSION In this study, we used fibroblasts from patients with UCMD with heterozygous c.850G>A (p.G284R) mutation in *COL6A1* gene in the N-terminal region of the triple helical domain. Mutations in this region have been reported to be associated with UCMD and Bethlem myopathy.¹²⁻¹⁶ Muscles from all four patients with this mutation showed the characteristic features of SSCD, just as in previously reported patients.^{2,12,14,22} In our series, we were not able to establish genotype-phenotype correlation between SSCD and complete deficiency of collagen VI.

On electron microscopy, the shape and the length of collagen VI microfibrils secreted from cultured fibroblasts from the patients seemed to be normal, indicating that the mutation of p.G284R does not affect the production and formation of the microfibrils. Our data contradict previous reports showing that mutations in the triple helical domain of *COL6A1* are associated with abnormal tetramer formation and unnaturally long microfibrils.^{12,17} These results suggest that glycine substitution in the triple helical do-



Collagen VI (green) is clearly present in extracellular area of the control fibroblasts (A). In the cells of complete deficiency, collagen VI is absent (E). Culturing with control medium (B, 1 day; C, 2 days; D, 3 days) or in p.G284R medium (F, 1 day; G, 2 days; H, 3 days). In culture with control medium, collagen VI is present on dish on days 2 and 3; in p.G248R medium (F through H), collagen VI is detected on dish on day 3 in extremely reduced amount. Bar denotes 20 μ m. Nuclei of cells are stained with TOTO-3 (blue).

main may cause a variety of influences on the formation of collagen VI.

We evaluated the function of mutated collagen VI in p.G284R using patients with UCMD's fibroblasts. The immunocytochemical staining of the collagen VI deficient cells after incubation with cultured medium clearly show that collagen VI in the cultured medium of UCMD cells with p.G284R mutation had less binding capacity than that of control cells. These results suggest that the microfibrils with the mutated collagen VI α 1 chain alter the affinity of collagen VI to its binding partners. Previous studies showed the presence of aggregated filaments sometimes assuming dot/spot-like appearance in the fibroblasts with the same mutation p.G284R,¹⁴ which may support our contention that the alteration in the collagen VI microfibrils by this mutation can influence the interaction of collagen VI microfibrils in ECM.

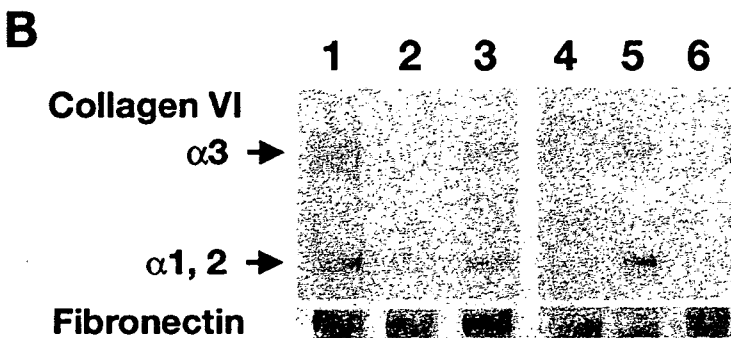
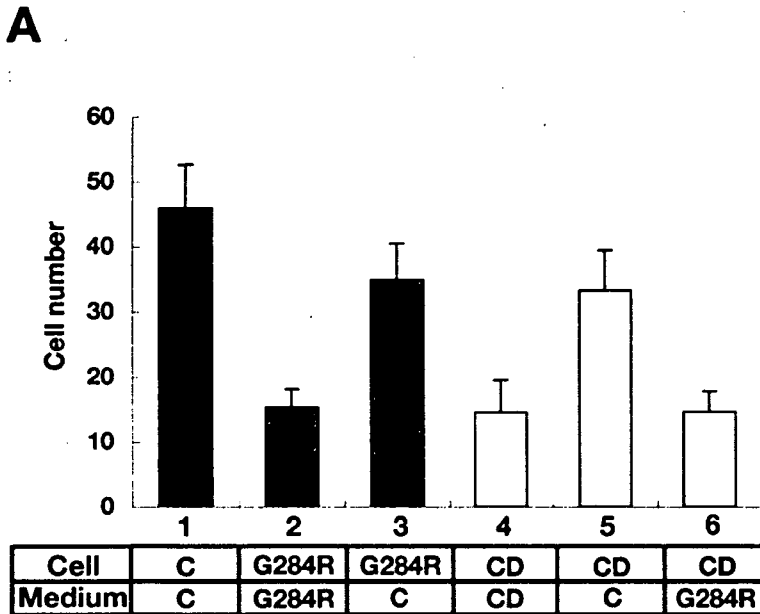
The results in the cell attachment test demonstrated that the adhesion ability of cells with the p.G284R and complete deficiency of collagen VI was less compared to that of control cells. The amount of the retained collagen VI on dish after the washing was reduced in the patients with UCMD (p.G284R and collagen VI complete deficiency) compared to control. These findings suggested that the reduction of the binding capacity by p.G284R mutation may cause an abnormality

in the adhesion of fibroblasts from patients with UCMD. Since collagen VI is thought to anchor cells to the ECM, loss of the binding capacity should result in reduced cell adhesion. Our findings indicate that the interaction of collagen VI is related to cell adhesion between fibroblasts and ECM.

The specific loss of collagen VI in sarcolemma of these patients, which is the pathologic finding in muscle sections, suggests a possibility that this feature may be caused by the reduction of binding capacity of collagen VI microfibrils to other molecules as a result of the heterozygous mutation in the *COL6A1* triple helical domain. Collagen VI microfibrils have been shown to bind to cell-surface receptors, integrins,²³ and NG2,^{24,25} and interact with several ECM components including fibrillar collagens,^{26,27} collagen IV,²⁸ fibronectin,^{29,30} biglycan, decorin,³¹⁻³³ and microfibril-associated glycoprotein-1 (MAGP-1).³⁴ Further studies on the binding partners of collagen VI in the ECM or on the cellular membrane may provide clues in understanding the function of collagen VI and the pathomechanism behind UCMD.

Our results on the mutant cells cultured in normal medium are of note. Interestingly, the adhesion ability of the cells with *COL6A1* p.G284R mutation and complete deficiency was restored by switching the medium to the one containing normal collagen VI microfibrils, indicating that

Figure 4 Recovery of binding ability of patients with Ullrich congenital muscular dystrophy (UCMD)'s cells by changing cultured medium from normal or p.G284R fibroblasts



(A) Number of remaining cells on the dish after detachment with EDTA solution. After changing into control medium (containing collagen VI), the number of cells became comparable to control (no. 1). On the other hand, UCMD cells, p.G284R (gray bar, no. 2), or collagen VI complete deficiency (open bar, no. 4), whose medium have not been changed, do not show recovery of cell attachment ($p < 0.001$, the number of counted area = 8). The cultured medium containing collagen VI with p.G284R likewise can not restore adhesion capacity of collagen VI complete deficiency cells (open bar, no. 6). Error bars indicate standard deviations. (B) The cells are cultured in three types of medium: original medium from cells (lanes 1, 2, 4); medium from control cells containing normal collagen VI (lanes 3, 5); or medium from cells with p.G284R mutation (lane 6). After the detachment of the cells by 10 mM EDTA, collagen VI is observed in control cells (lane 1). Cells with p.G284R mutation cultured in their own medium showed no detectable collagen VI (lane 2); however, after culture in the normal medium, collagen VI is observed in similar amounts to the control cells (lane 3). In collagen VI deficient cells, collagen VI is deficient in their own medium, but is detected when normal medium is used (lane 5). Interestingly, medium from p.G284R cells contains similar amounts of collagen VI protein to control cells (figure 2, lane 4), but collagen VI deficient cells cultured in this medium showed no detectable collagen VI (lane 6).

the function of mutant fibroblasts can be rescued by adding normal collagen VI. Moreover, recovering only by the addition of cultured medium containing normal collagen VI indicates that other functions and molecules of cells from pa-

tients with UCMD are normal. Results of recovery test using collagen VI-deficient fibroblasts suggested that collagen VI microfibrils with p.G284R mutation are less likely to interact with collagen VI binding partners, and thus, affecting the function of cells in attaching to other ECM proteins. These results demonstrated that the addition of normal collagen VI microfibrils possessing intact affinity to its binding partners rescues abnormalities in these cells from UCMD. This denotes that therapeutic use of normal collagen VI in patients with UCMD with SSCD may be plausible.

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Functionalized Nano-Magnetic Particles for an *In Vivo* Delivery System

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Nanotechnologies to allow the nondisruptive introduction of carriers *in vivo* have wide potential for therapeutic delivery system. We have prepared functional nano-magnetic particles ($d = 3$ nm) by silanization with (3-aminopropyl) triethoxysilane. For the purpose of functionalizing the surface of the nanoparticles with amino groups for subsequent cross-linking with pharmaceuticals and biomolecules. The extremely small particles were successfully introduced into living cells without any further modification to enhance endocytic internalization, such as the use of a cationic help. The cells containing the internalized particles continued to thrive, indicating that the particles have no inhibition effect for mitosis. In addition, the particles could be incorporated into the subcutaneous tissue of mouse's ear from ear skin and were able to be localized upon application of an external magnetic field. The functionalized nano-magnetic particles are expected to be useful as a new delivery tool.

Keywords: Nano-Magnetic Particle, Silanization, Living Cell, Endocytosis, Delivery System.

1. INTRODUCTION

Nanobioengineering^{1–4} is one of the most interesting areas of study to reveal basic questions and realize current challenges in biology and medicine by devising new experimental approaches that are enabled by nanotechnological platform. Especially, the efficient introduction of target molecules into the cell using nanomaterials^{5–8} is significant in medical field to realize the nondisruptive analysis and safety treatment for the disease.⁹

Nanometer-scale spheres are widely used for

- (1) gene therapy through the receptor on the plasma membrane^{10–12} and synthetic micelles,¹³
- (2) drug delivery^{14–16} as well as
- (3) bioengineering such as nanobiomachine¹⁷ and ligand purification^{18,19} because a particle can load plural impermeable chemical compounds and biomolecules and assist

in the transport of loaded molecules into the cell, although the destination of particles is not strictly controlled.

Nanoscale magnetic particles^{20,21} have attracted much attention because they were able to be transported to and concentrated at targeted locations by means of an external magnetic field. Recent reports indicate that magnetic nanoparticles with diameters < 100 nm are useful for medical applications, such as magnetic resonance imaging^{22–24} and delivery systems.^{25–28} Meanwhile, the conventional particles are coated by synthesis polymer to stabilize particle character,^{26,29} resulting in an increasing of particle diameter. Almost every magnetic particle showed several ten nanometer size.^{27–29} It induces difficulty of internalization of the particles to the cell. To improve the uptake of the particle as carrier by the cells, the method of introduction of cationic residues to the carriers which load target molecules is well used to facilitate endocytic introduction into living cells on most studies.^{27–29} However, there is a concern over the introduction of a carrier by the aid of cationic group regarding the cytotoxicity.

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Previously, one of the authors prepared nano-magnetic particles (NMPs) by mixing aqueous solutions of 3d transition metal chlorides ($MCl_2 \cdot nH_2O$) and a sodium metasilicate nonahydrate ($Na_2SiO_3 \cdot 9H_2O$) to obtain monodispersed NMPs in a single step. The particle size depended on the annealing temperature and time.³⁰⁻³⁵

We expect that extremely small particles can gain access to the surface of cells without steric hindrance between plasma membrane and themselves; therefore, particle can be introduced into the cells without the necessity for a cationic coating to help endocytic internalization. In this study, $\gamma\text{-Fe}_2\text{O}_3$ NMPs surrounded by amorphous SiO_2 are used (diameter (d) = 3 nm). We prepare functional NMPs that are added to functional group such as amino group on particle surface via a silanization procedure to covalently load desired molecules without the increasing of particle diameter. We also investigate the effect of the fluorescence reagent-modified nanoparticle on their cellular uptake without cationic help and by the accumulation of NMPs in mouse's ear under the influence of an external magnetic field.

2. MATERIALS AND METHODS

2.1. Preparation and Functionalization of NMPs

$\gamma\text{-Fe}_2\text{O}_3$ NMPs surrounded by amorphous SiO_2 (a- SiO_2) were prepared by mixing aqueous solutions of $FeCl_2 \cdot 4H_2O$ (WAKO Pure Chemicals Japan) and $Na_2SiO_3 \cdot 9H_2O$ (JUNSEI Chemical Japan). $FeCl_2 \cdot 4H_2O$ (10 mM) and $Na_2SiO_3 \cdot 9H_2O$ (10 mM) were each dissolved in 500 mL of distilled water, and the two solutions were mixed. After 10 minutes of stirring, the resulting precipitates were washed several times with distilled water and dried at 353 K in a water bath. The dried samples were crushed in a porcelain mortar and then annealed in air for 6 hours at 873 K in a furnace.

For functionalization of the NMP surfaces with amino groups (Fig. 1), 500 μL of (3-aminopropyl)triethoxysilane ($\gamma\text{-APTES}$; WAKO Pure Chemicals Japan) was added to the solid NMPs, and the reaction mixture was stirred at room temperature for 10 minutes and then heated to 403 K. After 20 hours at that temperature, the mixture was cooled and then washed three times with ultrapure water ($\geq 18.2 \Omega$, Milli-Q SP, Millipore Co.) and ethanol. The presence of the amino groups was confirmed by Fourier

transform infrared spectroscopy (FT-IR; Horiba FT-720). The morphology of the NMPs was investigated by transmission electron microscopy (TEM; JEM-1230, JEOL, Japan). The CuK_α X-ray powder diffraction (XRD) patterns ($\lambda = 0.154 \text{ nm}$) of the unfunctionalized NMPs and the amino-NMPs were measured at ambient temperature to confirm particle structure and diameter. Magnetization measurements were performed with a Quantum Design magnetic property measurement system, a superconducting quantum interference device (SQUID) magnetometer under an external field between -5 and 5 tesla (T) at 300 K.

To confirm the ability of the amino-NMPs to bind molecules to their surfaces and to visualize the introduction of NMPs into tissue, rhodamine (Rh)-labeled NMPs were prepared as follows. A 0.1 M dimethyl sulfoxide solution of 5- and 6-carboxytetramethyl rhodamine succinimidyl ester (Rh; SIGMA Co.; final concentration, 10 mM) was added to an aqueous solution of the amino-NMPs, and the total volume of the solution was brought to $\sim 500 \mu\text{L}$. The labeling reaction was allowed to proceed at 310 K for 10 minutes and was then quenched for 30 minutes at room temperature by the addition of formaldehyde (final concentration, 0.1 M). After reduction of the Schiff base by $NaBH_4$ (final concentration, 0.1 M), the resulting precipitates were washed several times with ultrapure water. The colloidal stability and surface electric charge of the Rh-NMPs were investigated by running the Rh-NMPs in 1% agarose gel (100 V, 10 minutes). These chemicals in the all experiments were used without further purification.

2.2. Cellular Uptake of Labeled NMPs

The resulting Rh-NMPs suspension [final number concentration, $1.4 \times 10^{12} \text{ mL}^{-1}$] and large-size particle as control (FluoreSphere beads standard which shows hydrophobic and non-electric charge ($d = 200 \text{ nm}$; invitrogen Co. California, U.S.)) [final number concentration, $4.8 \times 10^7 \text{ mL}^{-1}$] were independently injected into rat kangaroo kidney epithelium (PtK2) cells in cultured media and incubated for 24 hours. After the culture dishes were washed with phosphate-buffered saline buffer, PtK2 cells were fixed with 2.5% glutaric aldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 15 minutes at room temperature and then washed with PB. Osmium (0.1%) in 0.1 M PB was added to the cells at 277 K. The cells were rinsed with

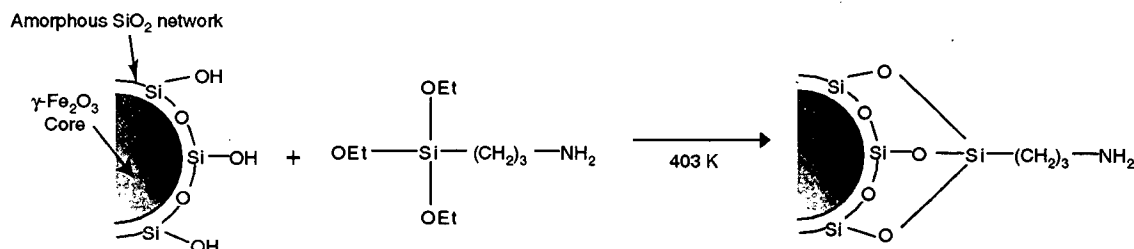


Fig. 1. Functionalization of the surface of NMPs with amino groups.

distilled water, dehydrated with ethanol, and embedded in epoxy resin (Epon 812). Ultrathin sections were cut with an ultramicrotome (Reichert-Nissei Ultracut N, Nissei Sangyo Co., Tokyo, Japan), collected on copper slot grids, stained with uranyl acetate/lead citrate, and then observed by TEM (Tecnai G2 Sphera, FEI, Hillsboro, OR, USA). To determine whether NMPs had cytotoxic effects on the cells, the cells containing the Rh-NMPs were cultured. Rh-NMPs were added to cultured-PtK2 cells in dishes. After 24 hours, the dishes were washed three times with culture medium to remove loosely bound particles from the cell surfaces, and the living cells were observed with a fluorescence microscope (Nikon TE-2000). The living cells were observed again after 5 days.

2.3. Localization of NMPs in Mouse Ear by Means of an External Magnetic Field

C57BL/6 mice were used in this study. All animal experiments were approved by the Ethical Committee for Experimental Animals of our institute and were performed according to international guidelines and the guidelines of the committee. The permanent magnet ($d = 7$ mm, surface flux density; 240 millitesla (mT)) was purchased from NIROKU Seisakusyo (Shiga, Japan).

The Rh-NMPs were mixed with Vaseline in a 1 : 1 (v/v) ratio to minimize desorption from the surface of the ear. The Rh-NMPs suspended in Vaseline were applied on the face aspect of one ear, and a magnet was fixed on the reverse side of the ear. A piece of aluminum foil that was folded multiple times was fixed similarly for use as a non-magnetic metal control.

3. RESULTS

3.1. Physical Characterization of NMPs

The presence of the amino groups on the amino-NMPs was confirmed by FT-IR. The typical O-H peak ($3100\text{--}3700\text{ cm}^{-1}$), the C-H peak ($2822\text{--}2982\text{ cm}^{-1}$), and C-N peak (1455 cm^{-1}) were detected in the spectrum of the amino-NMPs (Fig. 2(a)), whereas only the O-H ($3100\text{--}3900\text{ cm}^{-1}$) peak was observed in the spectrum of the unfunctionalized NMPs (Fig. 2(b)).

The number of amino groups on the particles was determined by FT-IR calibration to be equivalent to 320 nmol/mg. The number of amino groups per particle was derived in the following manner. The mass of $\gamma\text{-Fe}_2\text{O}_3$ (m_1) is given by Eq. (1):

$$m_1 = V\rho_{\gamma\text{-Fe}_2\text{O}_3} \quad (1)$$

where V is the volume of $\gamma\text{-Fe}_2\text{O}_3$ in the NMPs, and $\rho_{\gamma\text{-Fe}_2\text{O}_3}$ is the density of $\gamma\text{-Fe}_2\text{O}_3$. The number of moles of $\gamma\text{-Fe}_2\text{O}_3$ (x) can be calculated from the molecular weight of $\gamma\text{-Fe}_2\text{O}_3$ (Z_1):

$$x = m_1/Z_1 \quad (2)$$

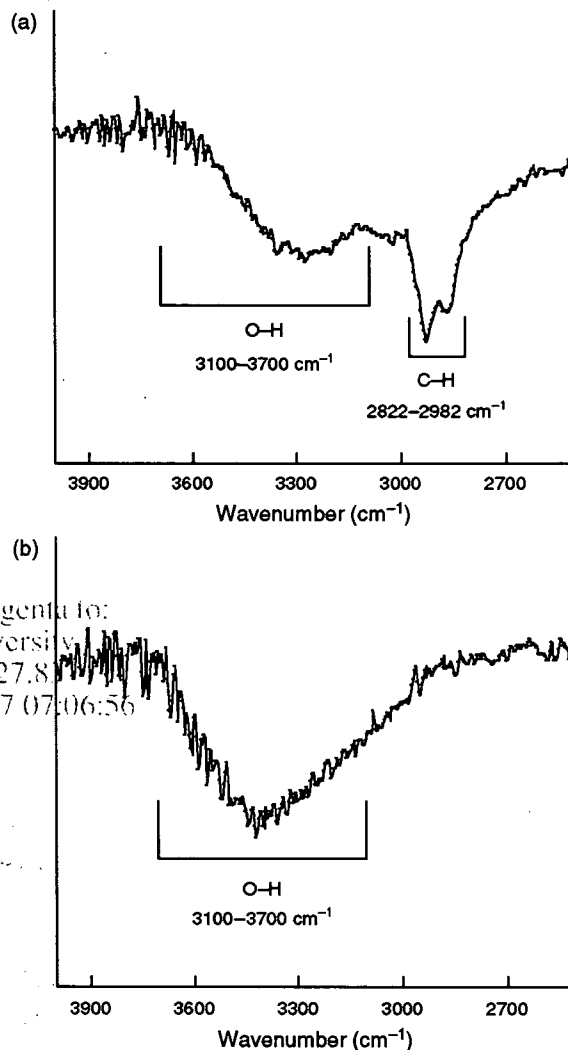


Fig. 2. FT-IR spectra of amino-NMPs (a) and unfunctionalized NMPs (b).

Assuming that the molar ratio of a-SiO₂ to $\gamma\text{-Fe}_2\text{O}_3$ is 1 : 1, the mass of a-SiO₂ (m_2) is given by Eq. (3):

$$m_2 = xZ_2 \quad (3)$$

where Z_2 is the molecular weight of a-SiO₂. Combining Eqs. (2) and (3) yields Eq. (4):

$$m_2 = m_1 Z_2/Z_1 \quad (4)$$

Because the mass of the NMPs is the sum of m_1 and m_2 , the mass of the NMPs (M) is given by Eq. (5):

$$M = m_1 + m_2 = V\rho_{\gamma\text{-Fe}_2\text{O}_3}(1 + Z_2/Z_1)/3 \quad (5)$$

If the NMPs are assumed to be spheres with radius r , V is given by Eq. (6) and M by Eq. (7):

$$V = 4\pi r^3/3 \quad (6)$$

$$M = 4\pi\rho_{\gamma\text{-Fe}_2\text{O}_3}r^3(1 + Z_2/Z_1)/3 \quad (7)$$

For $\rho_{\gamma\text{-Fe}_2\text{O}_3} = 4.9\text{ g/cm}^3$, $r = 1.5\text{ nm}$, $Z_1 = 159.7$, and $Z_2 = 60.1$, $M = 9.5 \times 10^{-17}\text{ mg}$. In addition, from the

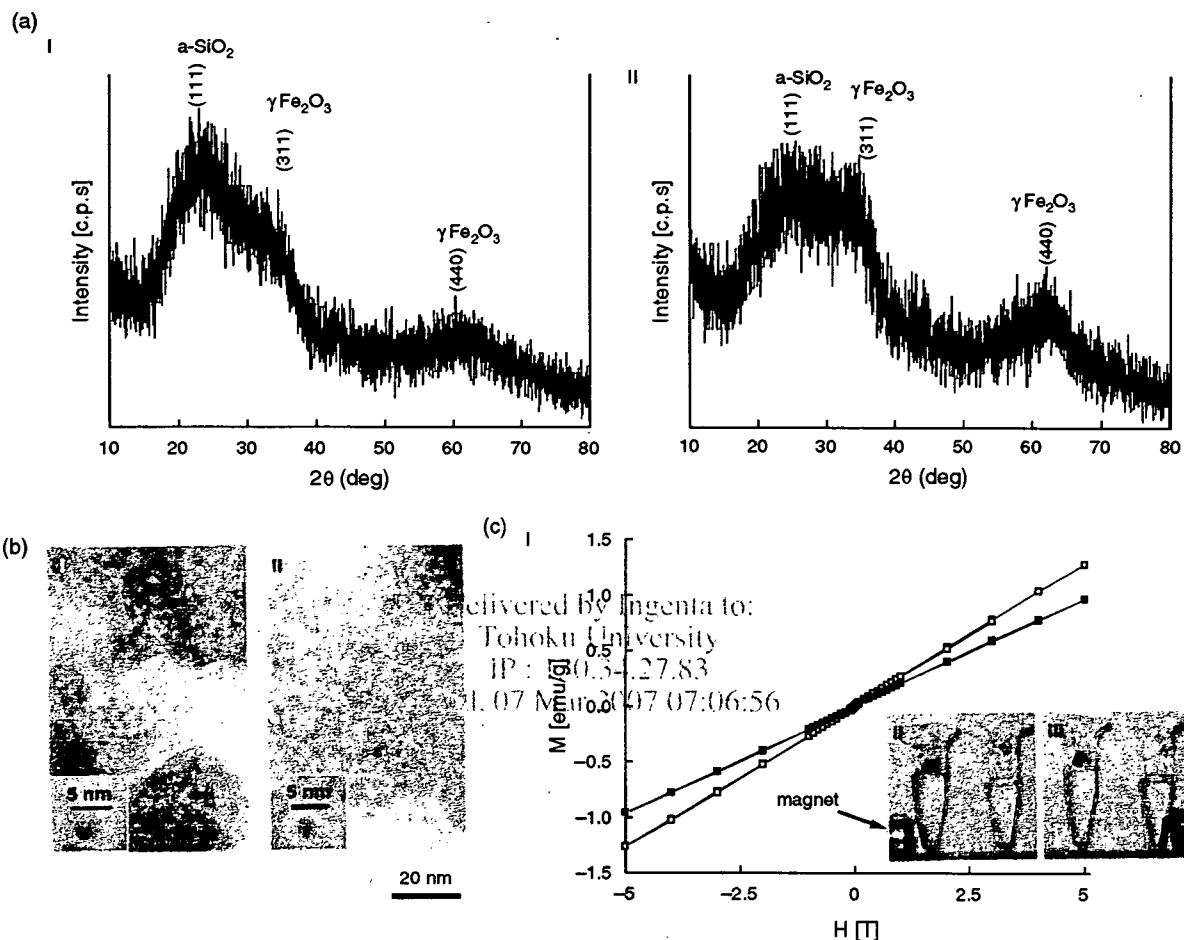


Fig. 3. The $\text{CuK}\alpha$ X-ray powder diffraction patterns of amino-NMPs (a-I) and unfunctionalized NMPs (a-II). TEM images of amino-NMPs (b-I) and unfunctionalized NMPs (b-II). M - H curves (c) for amino-NMPs (\square) and unfunctionalized NMPs (\blacksquare). Attraction of NMPs by an external magnetic field (340 mT) (b-II and III). Both photographs show amino-NMPs (left, blue sticker was posted on eppendorf tube) and unfunctionalized NMPs (right).

result of FT-IR calibration and the NMP mass (M), the number of moles of amino groups per particle was determined to be 3.0×10^{-23} (mol/particle). Using this value and the Avogadro constant (6.0×10^{23}), the number of amino groups per particle is calculated to be 18.

The XRD pattern of the amino-NMPs (Fig. 3(a-I)) indicated that the functionalized particles retained the spinel $\gamma\text{-Fe}_2\text{O}_3$ structure of the unfunctionalized NMPs (Fig. 3(a-II)). The broad peaks at $2\theta = 35$ and 60 degrees (Fig. 3(a-I), 3(a-II)) were confirmed to be the (311) reflection and the (440) reflection of $\gamma\text{-Fe}_2\text{O}_3$, respectively; and the peak below $2\theta = 30$ degrees was determined to have originated from amorphous SiO_2 .³⁶ The SiO_2 peak intensity was higher for the particles treated with $\gamma\text{-APTES}$ than for the unfunctionalized NMPs, and the increase was ascribed to an increase in the Si-O network due to silanization. The diameters of the amino-NMPs and the NMPs were independently estimated at 3 nm from the half-width of the XRD peaks for the amino-NMPs and NMPs.³¹ These results suggest that functionalization of the NMPs with amino groups did not affect their structure.

The TEM images showed no significant difference between the shape of the amino-NMPs (Fig. 3(b-I)) and that of the unfunctionalized NMPs (Fig. 3(b-II)). The number-average diameter of the amino-NMPs was determined to be about 3 nm, which is in good agreement with the XRD experimental value.

The SQUID results indicated that the magnetism of both the amino-NMPs and the unfunctionalized NMPs increased linearly with increasing magnetic field; that is, both types of particles showed paramagnetism (Fig. 3(c-I)). The NMP precipitates could be easily attracted from water solution by means of an external magnetic field regardless of whether or not they were functionalized (Fig. 3(c-II), 3(c-III)). These results indicate that the magnetism was basically unchanged by functionalization with the amino groups.

3.2. Uptake of NMPs by Living Cells

We investigated the intracellular space of the living PtK2 cells by TEM to confirm the introduction of the Rh-NMPs

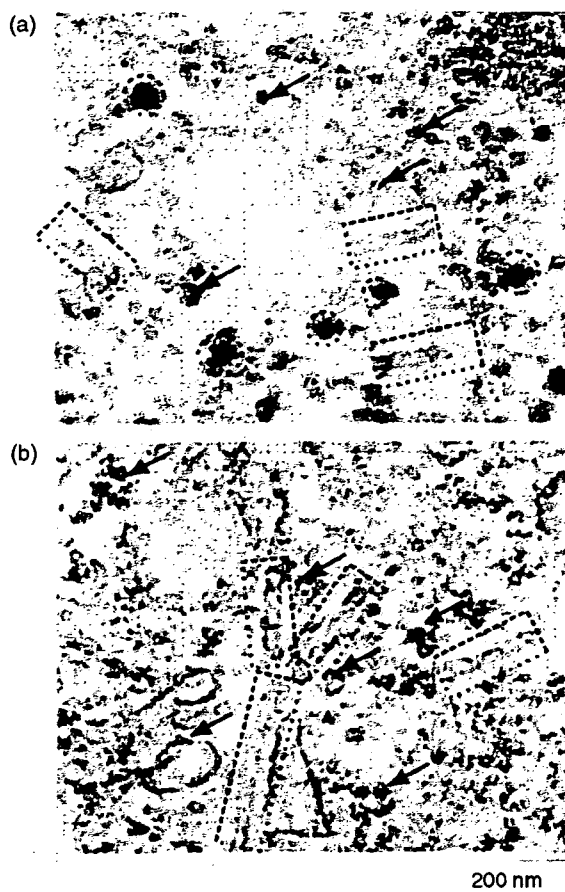


Fig. 4. TEM images of a PtK2 cell into which NMPs (a) and control particles ($d = 200$ nm; (b)) were introduced. The red dashed circles indicate aggregated NMPs, the yellow arrows indicate ribosomes, and the dotted rectangles indicate microtubules.

into the cells. In the TEM image of the cells into which NMPs had been introduced showed high-density spots (Fig. 4(a); red dashed circles). These can be identified as the aggregated NMPs. In the control TEM image (Fig. 4(b)), no large high-density spots that exhibit control particles ($d = 200$ nm) were observed, which indicates that no particularly large particles were introduced by endocytosis. These data suggested that the nano particles without cationic coating are introduced into cell and particle size may have significant influence for cellular uptake. Both images showed small dots (Fig. 4(a, b); yellow arrows). Judging from the shape and the massive number, they were not recognized as particles but possibly ribosomes. Microtubules (a type of cytoskeletal filament) with an outer diameter of 25 nm were observed (Fig. 4(a, b); representative examples are closed round by dotted rectangle), which indicates that NMPs had little effect on the cytoskeletal ultra structure.

To evaluate the cell toxicity of the NMPs, we cultured the PtK2 cells after uptake of the Rh-NMPs. After the cells were cultured for 24 hours, fluorescence was observed from inside the cells even after washing (Fig. 5(a)); in

contrast, no fluorescence was observed from the untreated cells as control (Fig. 5(b)). The PtK2 cells treated with the NMPs divided and continued to grow even after 5 days, at which time we observed low and diffuse fluorescence (Fig. 5(c)). Fluorescence intensity (F.I./ μm^2) is calculated. All fluorescence intensities are normalized with respect to that observed for F.I. of the untreated cells as control (Fig. 5(b)). The F.I. ratio of Figure 5(a) (the particle internalized cell after 1 day) to 5b (control) to 5c (cultured particle internalized cell for 5 days) is 8:1:2. Therefore, the cells containing the internalized particles continued to thrive, although fluorescent was low after cell culture, because the internalized particles were dispersed by cell division.

In addition, regarding the colloidal stability surface electric charge of the Rh-NMPs, as expected, no migration toward either the anode or the cathode occurred after 10 minutes at 100 mV, which indicates that the quench procedure of residual amino group on a particle succeeded (data not shown).

3.3. Specific Localization of NMPs by Means of an External Magnetic Field

Finally, we addressed the question of whether NMPs could permeate the epidermis into subcutaneous tissue by means of the magnetic force (Fig. 6(a)). The magnetization of the NMPs was assumed to be determined by the surface flux density (240 mT) and the $M-H$ curves (Fig. 3(c)), and therefore the magnetization of the particles was estimated to be 5.2×10^{-2} emu/g. We evaluated the localization of the Rh-NMPs in detail by examining a section of mouse ear stained with hematoxylin–eosin (Fig. 6(b)). Using confocal laser scanning microscopy (LSM5 PASCAL, Carl Zeiss Co.), we obtained differential interference contrast images and rhodamine fluorescence images of the mouse's external ear sections (Fig. 6(c-I), (II), and (III)). When a magnet was fixed on the reverse side of the ear, we evaluated the effect of the magnetic field by comparing the part of ear to which the magnet was directly attached (Fig. 6(c-I)) with the part that was not directly covered by the magnet (Fig. 6(c-II)). In the former case, strong fluorescence was observed, and the merged image clearly shows that the Rh-NMPs were localized at the hypodermis. In contrast, in the area outside of the external magnetic field, no fluorescence was observed (Fig. 6(c-II)), which indicates that no introduction or localization of NMPs occurred outside the area affected by the external magnetic field. In addition, in the control experiment using aluminum foil (a nonmagnetic metal) (Fig. 6(c-III)), no fluorescence was detected either. The relative fluorescence intensity in each section is shown in Figure 6(d). The ear sections to which the magnet was directly attached showed 5- and 7-fold higher intensity than the sections that were not directly covered by the magnet and those covered by the aluminum foil. These results were confirmed in duplicate experiments.

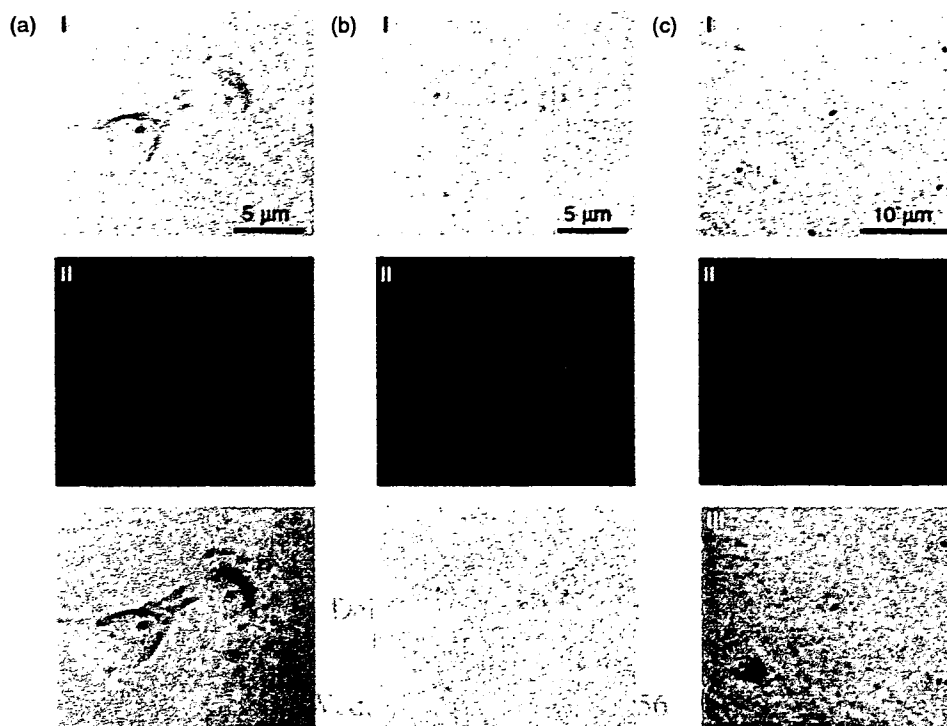


Fig. 5. PtK2 cells incubated with Rh-NMPs (a) and untreated PtK2 cells (b) cultured for 24 h; and PtK2 cells cultured for 5 days with Rh-NMPs (c). Bright-field micrographs of cells (I), fluorescence micrographs of Rh-NMPs (II), and merged images (III).

4. DISCUSSION

We studied the feasibility of *in vivo* introduction of functionalized NMPs. We demonstrated that functionalization of NMPs with amino groups via silanization permits covalent attachment of carbonyl compounds to the functionalized particles. We have simulated the number of amino group on a particle. Assuming that amino groups are symmetrically distributed on a particle, amino group should be existing on regular isosahedron. Hence, we suppose the number of amino groups per particle would be approximated to be 20. The XRD, TEM, and SQUID results indicated that functionalization of the particles with amino groups did not significantly affect the structure, size, or magnetism of the particles. γ -Fe₂O₃ particles with a size range of 5–6 nm exhibited ferromagnetic behavior at room temperature under an external field of 5 T with a maximum magnetization value of about 0.21 μ_B per Fe ion. Even 3-nm γ -Fe₂O₃ nanoparticles showed paramagnetic behavior with a large magnetization value (0.05 μ_B per ion). This remarkable characteristic makes these γ -Fe₂O₃ nanoparticles suitable as nanoscale ligands and carriers for next-generation delivery systems.

The second significant finding is *in vivo* introduction of functional NMPs in living cell and tissues without cationic coating. Other reports have managed to achieve about introduction of several ten nanometers particle to cell with cationic help.^{26–28} We suppose that several ten nanometers may hardly access to cell surface and fuse to endocytosed vesicle without cationic help due to steric hindrance and

the size. For uptake of our NMPs by cells, the small size of the nano particles allowed them to approach the cell surface more readily than particles with diameters of several ten nanometers or greater,³⁷ owing to less steric hindrance.

One possible explanation for resulting of aggregated NMPs in the cell (Fig. 3(a)) is that non-aggregated plural NMPs on cell surface may be included in an endocytosed vesicle, and aggregate together after endocytosis (pinocytosis) occurred. In addition, the cells containing the internalized particles continued to thrive, which implies that the cells did not severely suffer a damage that inhibits the cell division by incorporation of the particles. (However, the Trypan blue dye exclusion assay is needed to fully understand the potential cytotoxicity of NMPs.) We ascribe this result to the surface characteristics of the particles. The particles did not need to be modified with a cationic coating to be introduced into the cells. The residual amino groups of the functionalized particles could be converted to methylamino groups by means of a quench procedure. The functionalized NMPs were not influenced by an electric charge.

The experimental data in Figure 6 showed that the incorporation of the particles into the subcutaneous tissue of the mouse's ear was dependent on the presence of a magnetic field. Under the influence of a magnetic field (Fig. 6(c-I)), the NMPs were able to be concentrated in a quite localized area, which raises the possibility that the distribution of the NMPs in the body could be strictly controlled by the development of an appropriate guiding device. NMPs were capable of passing through areas of lower cell density;

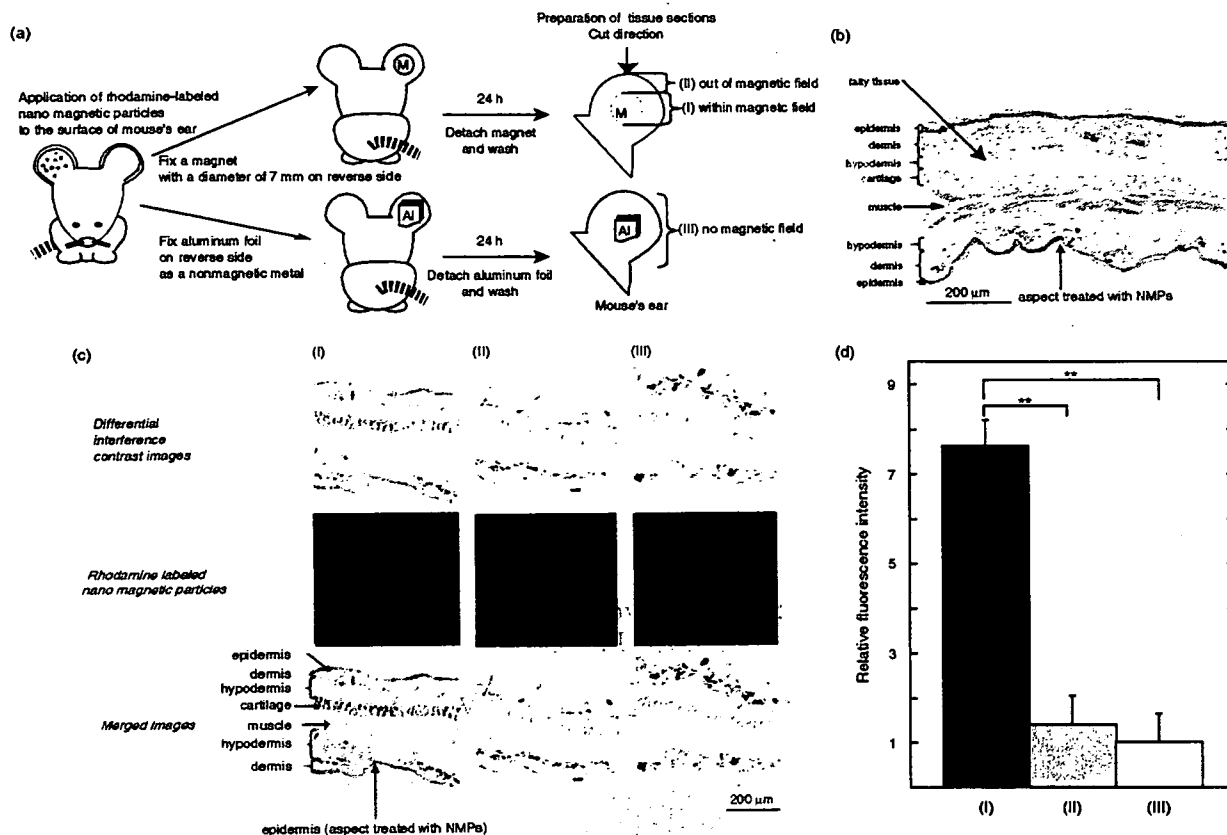


Fig. 6. Preparation of mouse external ear section for external magnetic field specific internalization of NMPs (a). Hematoxylin–eosin stained external ear section (b). Confocal laser scanning microscopy images of mouse external ear sections (c): differential interference contrast images (upper panel), rhodamine fluorescence images (middle panel), and merged images (lower panel). Localization of the NMPs ($d = 3$ nm) was investigated in the presence (c-I) and absence (c-II) of an external magnetic field and without the permanent magnet (aluminum foil control) (c-III). The relative fluorescence intensity was determined by dividing the sum of all pixel intensities. The values presented are mean \pm s.e.m. ($N = 13$). $**P < 0.01$ with Student's t test (d).

such as the epidermis and subcutaneous tissues, but were incapable of passing through areas of higher cell density, such as cartilage, even when an external magnetic field was applied. The different permeation abilities of the NMPs provide information about whether the particles can be delivered and retained in the body. The NMPs could not pass through the layers of epidermis, dermis, and the subcutaneous tissue in areas outside the influence of the magnetic field (Fig. 6(c-II)) or when no (Fig. 6(c-III)) magnetic field was present. Thus, we can localize NMPs to targeted region; hence they might apply to the *in vivo* imaging³⁸ and imaging mass spectrometry study³⁹ as imaging agent, although further analyses of issues such as the fate of NMPs that remain in the body for long periods, the possibility of their being absorbed by cartilage or bone, and their affinity for various organ tissues are necessary.

We believe that functionalized NMPs have great potential for future drug-delivery applications because the surface of the NMPs can be modified with various types of molecules. An ideal therapeutic method involves the targeted delivery of a drug in high concentrations to an affected site. If NMPs could be modified to recognize specific cell types, controlling the destination of a drug

using particles modified with a cell-selective moiety in addition to the drug might be feasible.⁴⁰ In a previous study, we took advantage of the recognition ability of a transporter on the cell surface membrane and designed amino acid-based prodrugs for a cell-specific delivery system.⁴¹ In a similar way, we might be able to utilize membrane proteins, such as receptors and transporters, as scaffolds for endocytic uptake of NMPs bearing both a drug and a moiety to act as a binding substrate for the membrane receptor or transporter.

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Truncated TrkB-T1 regulates the morphology of neocortical layer I astrocytes in adult rat brain slices

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Abstract

By altering their morphology, astrocytes, including those involved in the maintenance and plasticity of neurons and in clearance of transmitter, play important roles in synaptic transmission; however, the mechanism that regulates the morphological plasticity of astrocytes remains unclear. Recently, we reported that T1, a subtype of TrkB (a family of BDNF-specific receptors), altered astrocytic morphology through the control of Rho GTPases in primary astrocyte cultures. In this study, we extended this observation to investigate acute neocortical slices from adult rats. T1 siRNA-expression vectors were electroporated into astrocytes in neocortical layer I of living rats. In both normal slices and control vector-electroporated slices, BDNF induced the elongation of the astrocytic processes and increased the branching of processes in slices after 1 h incubation. In contrast, in T1 siRNA-electroporated slices, no such significant morphological changes were observed in the astrocytes. In addition, the number of synaptophysin⁺ sites in contact with GFAP⁺ processes increased in a BDNF–T1-dependent manner without the increase in the total synaptophysin⁺ sites. Therefore, the present study provides evidence of the regulation of layer I astrocytic morphology by the BDNF–T1 signal in adult rat neocortical slices.

Introduction

Neurotrophins and their specific tropomyosin-related kinase (Trk) receptors are known to be involved in the regulation of cell morphology during development (Bibel & Barde, 2000). Neurotrophins (NTs) belong to the nerve growth factor (NGF)-related gene family: NGF, brain-derived neurotrophic factor (BDNF), NT-3, and NT4/5. The Trk receptor family consists of three members: TrkA for NGF, TrkB for BDNF and NT-4/5, and TrkC for NT-3 (Barbacid, 1994). The *trkB* gene encodes at least three receptor subtypes (Klein *et al.*, 1990; Middlemas *et al.*, 1991). One such subtype is the full-length form (TK+) that includes tyrosine kinase in the cytosolic domain. The other two subtypes, T1 and T2, lack tyrosine kinases. T1 is expressed in both neurons and glial cells (Armanini *et al.*, 1995; Ohira *et al.*, 2005a, 2005b) whereas T2 is expressed primarily in neurons (Armanini *et al.*, 1995).

Recent studies have shed light on interactions between neurons and glial cells (Fellin & Carmignoto, 2004). In particular, it has been demonstrated that calcium entry into astrocytes modulates synaptic transmission (Bezzi *et al.*, 2004; Fiacco & McCarthy, 2004). In addition, astrocytic endfeet enwrap synapses (Ventura & Harris,

1999), i.e. those synapses referred to as tripartite synapses (Araque *et al.*, 2001). Interestingly, astrocytic processes surrounding active synapses are able to rapidly alter their morphology in acute slices from the brainstem (Hirrlinger *et al.*, 2004), hypothalamus (Langle *et al.*, 2003) and hippocampus (Benediktsson *et al.*, 2005) of infant- to pubertal-stage rodents. In contrast, alterations of fine neuronal structures such as dendrites and spines in the neocortex of adult mice hardly occur under normal conditions (Grutzendler *et al.*, 2002). Such results have suggested that the morphological alteration of astrocytes might be essential for the maintenance and plasticity of synaptic transmission, as well as for transmitter clearance. Therefore, it would be of great interest to clarify the mechanism(s) responsible for controlling astrocytic morphology.

Recently, we reported that T1 regulates astrocytic morphology via Rho GTPases in primary astrocyte cultures (Ohira *et al.*, 2005a). T1 also controls calcium entry into astrocytes (Rose *et al.*, 2003). In addition, BDNF release is highly regulated by neuronal activity (Hartmann *et al.*, 2001; Kohara *et al.*, 2001). Thus, these findings led us to postulate that BDNF release due to neuronal activity might induce morphological changes in astrocytes in the CNS.

For the initial evaluation of this hypothesis, we investigated the role played by endogenous T1 in the regulation of astrocytic morphology in acute slices of the adult rat neocortex. Using T1 small interfering RNA (siRNA)-expressing vectors that were electroporated into neocortical layer I of adult rats, we demonstrated that T1 regulated astrocytic

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