Dystrophin Acts as a Transplantation Rejection Antigen in Dystrophin-Deficient Mice: Implication for Gene Therapy

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Duchenne muscular dystrophy (DMD) is a common X-linked recessive disease caused by a defect in dystrophin. Normal myoblast transplantation and dystrophin gene transfer have been expected to correct the deficiency in the muscles, but their clinical application has been hampered by the limited preservation of dystrophin-positive myofibers. In this study, we investigated the mechanism for immunologic rejection of normal C57BL/10 (B10) myoblasts transplanted into dystrophin-deficient mdx mice, an animal model of Duchenne muscular dystrophy. We found that mdx mice develop CTL specific for dystrophin itself, which were CD8 dominant and restricted by H-2Kb. We identified several antigenic peptides derived from dystrophin that bind to H-2Kb and are recognized by the mdx anti-B10 CTL. Immunologic tolerance against dystrophin was successfully induced by i.v. injection of these peptides before B10 myoblast transplantation, which resulted in sustained preservation of dystrophin-expressing myofibers in mdx mice. These results demonstrate that dystrophin is antigenic in dystrophin-deficient mice and that immunologic regimen would be necessary to achieve the persistent expression of introduced dystrophin in the muscles of dystrophin-deficient individuals.

**Materials and Methods**

**Mice**

Four to six-week-old male C57BL/10 (B10) and mdx mice were purchased from Clea Japan (Tokyo, Japan).

**Preparation of myoblasts**

The muscle tissues from dystrophin-transgenic mdx mice were provided by Dr. Jeffrey S. Chamberlain (University of Michigan, Ann Arbor, MI) (11). Myoblasts were prepared by collagenase disaggregation as described previously (17, 18). Briefly, femoral muscles were finely minced with scissors;
transferred to a flask containing RPMI 1640 medium supplemented with 10% FCS, 50 mM l-glutamine, 50 mM 2-ME, 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 mg/ml Fungizone (culture medium) supplemented with 0.02% collagenase (Sigma, St. Louis, MO); and stirred for 60 min in a 37°C water bath. Cell suspensions were filtered through nylon mesh, suspended in the culture medium, and overnight cultured on collagen-coated flasks to remove adherent fibroblasts. Then, the myoblasts were collected by gentle pipetting. The viability of myoblasts was >85% as assessed by trypan blue exclusion. These cells were >95% myoblasts as estimated by immunostaining with anti-desmin Ab.

**Myoblast transplantation**

Myoblasts (1 x 10^6 cells/mouse) were injected into the soleus muscle in right leg of mdx mice. Mice were sacrificed 2, 4, or 6 wk after the implantation, and the muscles surrounding the implanted site were removed for histologic examination.

**Immunohistochemical staining**

Soleus muscle tissue samples were snap-frozen in OCT embedding medium (Miles, Elkhart, IN) and stored at −80°C until processing. Five-μm frozen sections were prepared and air dried for 30 min before use. Each section was fixed with cold acetone for 10 min. After blocking with 5% normal goat serum in PBS for 30 min, sections were incubated with rat anti-mouse CD4 mAb (RM4-5, PharMingen, San Diego, CA) or rat anti-mouse CD8 mAb (30-2-13, PharMingen) followed by FITC-conjugated goat anti-rat IgG Ab (Amersham, Arlington Heights, IL), or FITC-conjugated mouse anti-human dystrophin mAb (Chemicon International, Temecula, CA) that cross-reacts with mouse dystrophin (19). In some experiments, two-color staining was performed using Cy3-conjugated goat anti-rat IgG Ab (Amersham). Nonspecific staining was evaluated using isotype-matched control IgG (PharMingen).

**Induction of mdx anti-B10 CTL**

Lymphocytes (1 x 10^6/ml), isolated from lymph nodes and spleen of mdx mice that had been transplanted with B10 myoblasts 2 to 6 wk previously, were cultured with irradiated (2000 rad) B10 myoblasts (2 x 10^6/ml) in 24-well plates for 5 days.

**Cytotoxicity assay**

Myoblasts (1 x 10^6 cells) were labeled with 1 μCi/ml of Na251CrO4 (Amersham) in the culture medium at 37°C for 60 min. After washing, the 51Cr-labeled myoblast target cells (1 x 10^6 cells) were mixed with various numbers of effector cells (1 x 10^4 to 1 x 10^5) at an E:T cell ratio of 10:1—40:1 in 200 μl of the culture medium in V-bottom 96-well plates. After 6-h incubation at 37°C, 100-μl aliquots of the supernatants were collected using a harvesting frame (Skatron, Lier, Norway), and radioactivity was counted on a gamma scintillation counter (Wallac Oy, Turku, Finland). Cytotoxic activity was assessed by the percent lysis, calculated by the following formula: 

\[
\% \text{l} \text{ysis} = 100 \times \left(1 - \frac{R_{\text{Spontaneous}}}{R_{\text{Test}}} \right) 
\]

where R Spontaneous and R Test are the radioactivity counts of the spontaneous release and total release, respectively.

Total release was obtained by lysis with 1% Triton X-100. Spontaneous release was obtained in the absence of effector cells. In some experiments, cytotoxic assay was performed in the presence of 10 μg/ml each of anti-mouse CD4 (GK1.5), CD8 (30-2-13), H-2D^b (KH95), H-2K^b (AF6-88.5), or H-2K^b (SFI-1.1) mAbs (PharMingen).

**Preparation of peptides**

Octamer or nanomer peptides with the H-2K^b binding motif (15, 16) were identified in the mouse dystrophin amino acid sequence predicted from the entire coding region of cDNA (5) by computer searching. Ten representative (p-1 to p-10) and a control OVA peptide (SIINFEKL) that binds to K^b with high affinity (20) were synthesized using a peptide synthesizer (Applied Biosystems, Foster City, CA) and verified by HPLC.

**H-2K^b stabilization assay**

Binding of peptides to H-2K^b was assessed by stabilization of K^b on RMA-S cells as previously described (15). Briefly, serially titrated peptides in DMEM containing 0.25% BSA were added to RMA-S cells that had been cultured overnight at 26°C. After incubation at room temperature for 30 min, the temperature was shifted to 37°C, and the incubation was continued for 60 min. At the end of the incubation, remaining surface K^b molecules were quantitated by staining with FITC-conjugated B8.24.3 mAb (American Type Culture Collection, Rockville, MD) followed by FACS analysis. This mAb binds to K^b in a conformation-dependent manner, but independently of the peptide bound (15). Peptide concentrations that stabilize 50% of peptide-receptive K^b molecules on RMA-S cells were calculated from the linear fluorescence intensity values and the least square fit analysis as previously described (15).

**Peptide-dependent cytotoxicity**

51Cr-labeled mdx myoblasts were preincubated with 10 ng/ml of each peptide in the culture medium at room temperature for 15 min. Then, 1 x 10^4 cells were subjected to the 6-h 51Cr release assay as described above.

**Tolerance induction by peptide administration**

Recipient mdx mice were administered i.v. with 50 μg of each of the indicated peptides or PBS at 4 wk of age. At 8 wk of age, these mice were implanted with B10 myoblasts as described above. Six weeks after transplantation, all mice were sacrificed. Leg muscles were subjected to histologic examination as described above. Lymph node and splenic lymphocytes were subjected to CTL induction as described above.

**Statistical analysis**

Student’s t test was used to determine the statistical significance of differences between groups.

**Results**

**Immunologic rejection of B10 myoblasts in mdx mice**

It has been shown that i.m. transplanted B10 myoblasts fused to mdx myofibers and converted them from dystrophin negative to dystrophin positive (6). However, the expression of dystrophin was not long lasting. The mechanism for this disappearance of dystrophin-positive myofibers has not been clarified. We first examined the B10 myoblast transplantation into mdx mice histologically. As shown in Figure 1, dystrophin-positive myofibers were clearly detectable at the site of B10 myoblast injection into mdx mice 2 wk after the transplantation, but were greatly decreased 6 wk after transplantation. A massive infiltration of mononuclear cells surrounding the myofibers was noted at the latter time, which was mainly composed of CD8+ T cells. In contrast, no infiltration of mononuclear cells or CD8+ T cells was observed at the site of mdx myoblast injection into mdx mice (not shown). The observed histologic features of B10 myoblast transplantation into mdx mice 6 wk after transplantation were reminiscent of typical immunologic rejection of allografts (21). Since genetic backgrounds of B10 mice and mdx mice are identical except for the defective expression of dystrophin in mdx mice, we supposed that dystrophin might act immunogenically in dystrophin-deficient mdx mice.

**Dystrophin acts as a CTL target Ag in mdx mice**

To verify the immunologic rejection of B10 myoblasts in mdx mice, we examined the development of CTL that lyse B10 myoblasts in B10 myoblast-transplanted mdx mice. As shown in Figure 2, a marked CTL activity that lysed B10 myoblasts, but not mdx myoblasts, could be induced in mdx lymphocytes 6 wk after transplantation.

To verify whether the generated mdx anti-B10 CTL is directed to dystrophin, we used myoblasts from dystrophin-transgenic mdx mice as a target. As shown in Figure 3, mdx anti-B10 CTL lysed the dystrophin-transgenic mdx myoblasts, but not mdx myoblasts, more efficiently than B10 myoblasts. This may reflect higher expression of dystrophin in the transgenic mice due to multiple copies of the transgene (11). These results indicated that the dystrophin-deficient mdx mice develop dystrophin-specific CTL that lyse normal myoblasts expressing dystrophin.

**Identification of dystrophin-derived antigenic peptides**

Blocking experiments with mAbs to CD4, CD8, and MHC class I molecules indicated that mdx anti-B10 CTL activity is dependent on CD8+ T cells and is restricted by H-2K^b (Fig. 4). A similar blocking pattern was observed in the mdx anti-B10 CTL cytotoxicity against the dystrophin-transgenic mdx myoblasts (data not
shown). To further characterize the antigenic nature of dystrophin, we synthesized several octamer or nanomer peptides from the amino acid sequence of mouse dystrophin, which have potential H-2Kb binding motifs according to the definition of Udaka et al. and Rammensee et al. (15, 16) (Fig. 5). Actual binding of these peptides to H-2Kb was verified by stabilization assay of RMA-S cells (15). The peptides p-1 and p-10 exhibited high affinity binding comparable to the control OVA peptide; p-2, p-3, p-4, and p-8 exhibited moderate binding; and p-5, p-6, p-7, and p-9 exhibited low binding. When these peptides were preincubated with mdx myoblasts, p-1, p-2, and, to a lesser extent, p-8 conferred susceptibility to the mdx anti-B10 CTL cytotoxicity compared with the control OVA peptide (Fig. 6). These results indicated that at least a part of the mdx anti-B10 CTL is directed to the H-2Kb-binding dystrophin peptides.

**Induction of graft tolerance by preadministration of dystrophin peptides**

Immunologic tolerance has been successfully achieved by i.v. administration of antigenic peptides on some occasions (22). By
using the dystrophin antigenic peptides identified in Figure 6, we tried the induction of tolerance against dystrophin in mdx mice to facilitate the engraftment of normal myoblasts. The mdx mice at 4 wk of age were administered i.v. a mixture of p-1, p-2, and p-8 4 wk before the transplantation of B10 myoblasts. Six weeks after transplantation, mdx anti-B10 CTL induction and graft histology were examined. As shown in Figure 7, CTL development against B10 myoblasts was significantly suppressed by preadministration of p-1, p-2, and p-8. In contrast, preadministration of neither a mixture of p-6 and p-7 nor the control OVA peptide suppressed CTL development. Histologic examination of the transplanted site indicated a sustained preservation of dystrophin-positive myofibers in association with greatly reduced infiltration of CD8 T cells in the p-1, p-2, and p-8-treated mice compared with that in the p-6- and p-7-treated mice (Fig. 8), suggesting that B10 myoblast survival was facilitated by elimination of dystrophin-specific CD8 CTL. These results also imply that the dystrophin-derived peptides did actually act as the predominant target Ag in the normal myoblast rejection in dystrophin-deficient mdx mice.

Discussion

Transplantation of normal myoblasts has been expected to be one strategy correcting the muscle deficiency in DMD patients (7–9). However, clinical trials have to date been unsuccessful due to the limited preservation of dystrophin-expressing myofibers in the DMD patients (9), the mechanisms for which have not been clarified. In cases of allogeneic myoblast transplantation, host immune responses against major and/or minor histocompatibility Ags on the donor myoblasts may lead to allograft rejection, as in the cases of other organ transplantation. Consistent with this idea, a typical cellular rejection has been observed at the transplantation sites of allogeneic or xenogeneic myoblasts into dystrophin-deficient mdx mice (21). In this respect, the transplantation of B10 myoblasts into mdx mice provides a unique opportunity to address the feasibility of normal myoblast transplantation without interference by histoincompatibility, since the genetic backgrounds of B10 and

FIGURE 3. Cytotoxic activity of mdx anti-B10 CTL against dystrophin-transgenic mdx myoblasts. Splenocytes from mdx mice that had been implanted with B10 myoblasts 6 wk previously were in vitro stimulated with B10 myoblasts for 5 days. Cytotoxic activity was then tested against B10 (▲), mdx (●), or dystrophin-transgenic mdx (●) myoblasts in a 6-h 51Cr release assay at the indicated E:T cell ratios. Data represent the mean ± SD of triplicate samples. Similar results were obtained in two independent experiments.

FIGURE 4. H-2Kb restriction and CD8 dependence of mdx anti-B10 CTL. The mdx anti-B10 CTL were generated as described in Figure 3. Cytotoxic activity was then tested against B10 myoblasts in a 6-h 51Cr release assay at an E:T cell ratio of 40 in the presence or the absence of mAbs (10 μg/ml) to the indicated antigens. Data represent the mean ± SD of triplicate samples. * indicate p < 0.01 compared with the control. Similar results were obtained in three independent experiments.

FIGURE 5. Binding of dystrophin-derived peptides to H-2Kb. Octamer or nanomer peptides with H-2Kb anchoring residues were identified in the mouse dystrophin amino acid sequence, and 10 peptides were synthesized (p-1~ to p-10). An OVA-derived peptide (p-OVA) that binds to H-2Kb with high affinity was also synthesized as a control. Binding of these peptides to H-2Kb was assessed by the Kb stabilization assay as described in Materials and Methods. Kb binding is expressed as the log concentration of peptide required for 50% stabilization in M (SD50).

FIGURE 6. Identification of dystrophin-derived target peptides for mdx anti-B10 CTL. The mdx anti-B10 CTL were generated as described in Figure 3. Cytotoxic activity was then tested against mdx myoblasts that were preincubated with 10 ng/ml of the indicated peptides in a 6-h 51Cr release assay at an E:T cell ratio of 40. Peptides p-4 and p-5 were omitted due to their limited solubility. Data represent the mean ± SD of triplicate samples. * indicates p < 0.05, and ** indicates p < 0.01 compared with the control OVA peptide. Similar results were obtained in three independent experiments.
B10 myoblasts in a 6-h 51 Cr release assay at an E:T cell ratio of 40. Data
ated as described in Figure 3. Cytotoxic activity was then tested against
the primary cause of normal myoblast elimination in
blasts further verified that the CTL response against dystrophin is
sides in association with the reduction of CTL against B10 myo-
vival was facilitated by preadministration of the dystrophin pep-
m
mdx

FIGURE 8. Histologic examination of B10
myoblast rejection in dystrophin peptide-treated
mdx mice. mdx mice were i.v. administrated 50
μg of each of peptides p-1, p-2, and p-8; p-6 and p-7; p-OVA; or PBS 4 wk
before B10 myoblast transplantation. The mdx anti-B10 CTL were gener-
ated as described in Figure 3. Cytotoxic activity was then tested against
B10 myoblasts in a 6-h 31Cr release assay at an E:T cell ratio of 40. Data
represent the mean ± SD of triplicate samples. * indicates p < 0.01 com-
pared with the PBS control. Similar results were obtained in three inde-
pendent experiments.

mdx mice are identical except for the defect in dystrophin in the
latter. Unexpectedly, however, the preservation of B10 myoblasts in mdx mice was also limited, as shown in Figure 1. Histologic
examination suggested a contribution of immunologic rejection
mediated mainly by CD8⁺ T cells, although their infiltration was
weaker and slower compared with those in xeno- and allotomyoblast transplantsations (21). We then revealed that the mdx mice raise
CTL against B10 myoblasts, which recognize dystrophin-derived
peptides in the context of H-2Kb. The fact that B10 myoblast sur-
vival was facilitated by preadministration of the dystrophin pep-
tides in association with the reduction of CTL against B10 myo-
blasts further verified that the CTL response against dystrophin is
the primary cause of normal myoblast elimination in mdx mice. In
normal adults, the immune system has been rendered tolerant
against peptides from self proteins potentially including dystro-
phin. In mdx mice that innately lack dystrophin, however, such a
tolerance against dystrophin has not been established, and the dys-
trophin-derived peptides would be regarded as a foreign Ag. In the
present study we could successfully induce CTL tolerance that led
to prolonged graft survival by preadministration of the target pep-
tides. We administered the mixture of p-1, p-2, and p-8 peptides
because it was considered to be more effective to induce tolerance
against dystrophin based on the cytotoxicity developed against
each peptide, as shown in Figure 6. A similar peptide-induced CTL
tolerance has been induced by preadministration of a lymphocytic
choriomeningitis virus peptide (23, 24) or human adenovirus type
5 early region 1 (Ad5E1) peptides (25, 26). Particularly in the
latter case, engraftment of Ad5E1-expressing tumor cells was fa-
cilitated by peptide preadministration, as in our case of B10 myo-
blast transplantation. Therefore, preadministration of dystrophin-
derived antigenic peptides would be applicable for facilitating
normal myoblast engraftment in DMD patients. Alternatively, im-
munosuppressants such as FK506, anti-LFA-1, and anti-ICAM-1
mAbs or CTLA4Ig, which often induce allograft tolerance (27–
30), would be also useful.

Our present findings using the B10 myoblast transplantation into
mdx mice have great relevances to gene therapy, since the B10
myoblasts mimic autologous myoblasts in which dystrophin was
introduced. Transduction of the dystrophin gene into the muscle of
DMD patients has an advantage, excluding the histoincompatibil-
ity-associated allogeneic myoblast transplantation. It has been
shown that adenovirus-mediated transfer of a dystrophin minigene
into mdx mice efficiently corrected the dystrophic degeneration and
physical symptoms in injected muscles (31, 32). However, it has
also been shown that cellular immune responses to adenoviral ant-
genics limit transgene expression in immunocompetent mice, and
some immunosuppressive regimen, such as cyclosporin A and cy-
clophosphamide or CTLA4Ig treatment, was required for long
term expression of the transgene (33–37). Moreover, it has been
shown that cellular and humoral immune responses to xenogeneic
transgene products limit the stability of gene expression (37, 38).
Although the immunogenicity of human dystrophin in DMD pa-
tients has not been noticed, it has been known that neutralizing Abs
against human factor IX are occasionally raised in hemophilia B
patients that have genetic deficiency in factor IX (39). This further
implies that an individual with an innate defect of a certain protein
is not tolerant against the introduction of that protein and elicits
immune responses for eliminating the foreign protein, as we dem-
onstrated in the present study. It should also be noted that the
muscle has been a favorable site of DNA vaccination for eliciting
cellular and humoral immune responses (40–43). Therefore, some

FIGURE 7. Suppression of mdx anti-B10 CTL induction by preadmin-
istration of dystrophin peptides. The mdx mice were administrated i.v. 50
μg of each of peptides p-1, p-2, and p-8; p-6 and p-7; p-OVA; or PBS 4 wk
before B10 myoblast transplantation. The mdx anti-B10 CTL were gener-
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FIGURE 8. Histologic examination of B10
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mdx mice. mdx mice were i.v. administrated 50
μg of each of peptides p-1, p-2, and p-8 (right);
p-6 and p-7 (middle); or PBS (left) 4 wk before
B10 myoblast transplantation. Frozen sections of
soleus muscle from untreated mdx mice or trans-
planted mdx mice 6 wk after the transplantation
were stained with anti-dystrophin (green) and
anti-CD8 (red) mAbs. Original magnification,
×200. Representative samples from six mice in
each group are shown.
immunologic regimen would be generally required to achieve persistent expression of the transgene products in the muscle for gene therapy of genetic deficiencies such as DMD and hemophilia B.

Acknowledgments

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References