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Prolongation of Transgene Expression by Coexpression of Cytokine Response Modifier A in Rodent Liver after Adenoviral Gene Transfer

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The short duration of expression of the transgenes is a major barrier to the clinical application of adenovirus-mediated gene therapy for hepatic enzyme deficiencies. Previous reports show that Fas-mediated apoptosis has a pivotal role in the rapid elimination of adenovirus-infected hepatocytes. After considering this result and our recent observation that murine hepatocytes can be protected from Fas-mediated apoptosis by expressing cytokine response modifier A (CrmA) *in vivo*, we hypothesized that CrmA coexpression could also prevent adenovirus-infected hepatocytes from rapid elimination and that this would make prolonged transgene expression achievable *in vivo*. To examine this, mice with congenital deficiency of lysosomal β-glucuronidase (GUSB) were infected with recombinant adenoviruses expressing both CrmA and GUSB, and the duration of transgene expression was evaluated. The serum GUSB activity in the mice injected with a recombinant adenovirus expressing GUSB only became undetectable 60 days after the injection, whereas higher than normal GUSB activity was observed for at least 120 days in mice injected with adenoviruses expressing both GUSB and CrmA. Furthermore, we showed that exogenous CrmA expression could prevent the adenovirus-infected hepatocytes from cell death induced by cytotoxic T lymphocytes *in vitro*. These observations indicate that transgene expression after administration of E1-deleted adenovirus is prolonged by coexpression of the anti-apoptotic protein CrmA.

Key Words: adenovirus, gene therapy, cytokine response modifier A, β-glucuronidase

INTRODUCTION

Recombinant adenovirus vectors are the most efficient vehicles for liver-directed gene therapy for hepatic enzyme deficiencies. The viruses can infect nondividing cells, such as hepatocytes, *in vivo* with very high efficiency. In addition, they are rapidly concentrated in the liver after systemic administration [1,2]. The therapeutic potential of these vectors has been shown in several animal models of liver-related genetic disorders, like phenylketonuria [3,4], tyrosinemia [5,6], ornithine transcarbamylase deficiency [7], hemophilia [8], Crigler–Najjar syndrome type I [8–10], and mucopolysaccharidosis type VII [1]. In all cases, excellent transgene expression was observed for a few days to weeks, but the therapeutic effect disappeared thereafter, because of the host antiviral immune response [11,12]. The short duration of the transgene expression mediated by adenovirus gene transfer is a major barrier to clinical application of adenovirus-mediated gene therapy for genetic disorders.

Various strategies to increase the duration of the transgene expression after adenovirus-based gene transfer have been evaluated in several laboratories. One approach involves deleting or mutagenizing all adenoviral genes. However, such "gutless" viruses also retain cellular and humoral immunogenicity because of the potent immunogenicity of the capsid protein of the virus [13]. Another strategy is directed at modifying the host to prevent an immune response to adenoviral proteins. Transient administration of immunosuppressive drugs such as cyclosporin A [8], FK506 [14], or CTLA-4Ig [1,15], or temporary elimination of tissue macrophages [16,17],

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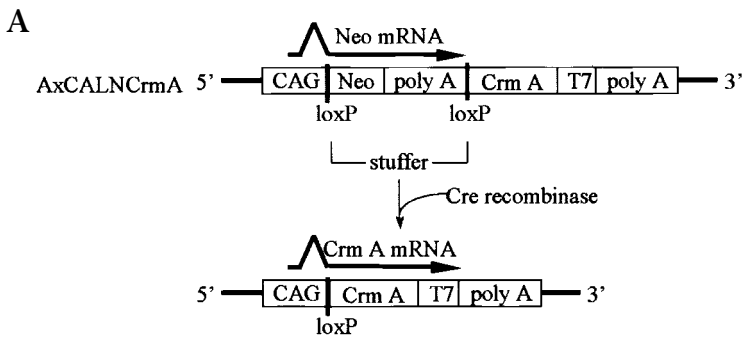
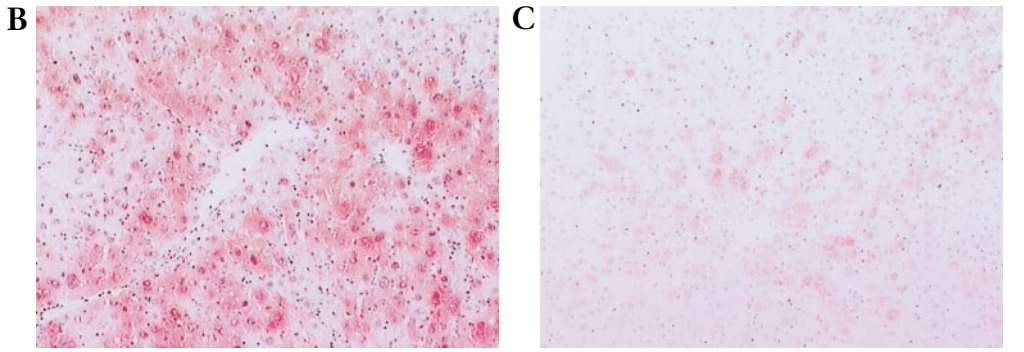


FIG. 1. Histochemical detection of Cre-dependent expression of exogenously transduced CrmA in mouse liver. (A) The activation process of CrmA gene expression in the CALNCrmA unit by Cre recombinase is shown. CAG, CAG promoter; Cre, Cre recombinase gene; poly(A), polyadenylation signal sequence; loxP, *loxP* sequence; Neo, neomycin-resistance gene; T7, T7 tag. Arrows show the orientation of the transcription. (B, C) We infected B6/MPSVII mice with 1×10^9 pfu of AxCALNCrmA and 1×10^9 (B) or 1×10^7 (C) pfu of AxCANCre. Seven days after the treatment, we stained the livers of the mice with anti-T7 antibody. The number of T7-positive hepatocytes increased with the increase of the dose of AxCANCre. Because we introduced the nucleotides encoding the T7 tag at the 3′ end of the CrmA gene, the T7-positive cells were expressing CrmA. Dose-dependent expression of CrmA is observed in the mouse liver.



increases the duration of transgene expression by inhibiting the T-cell activation that follows adenovirus infection. However, these treatments result in undesirable systemic and nonspecific immune suppression. To achieve adenovirus-specific host tolerization, several methods have been explored. Injection of recombinant adenoviruses during the neonatal period [8], intrathymic inoculation in adult rats [18], and oral tolerization to adenoviral proteins [19] permitted repeated administration of a recombinant adenovirus expressing human bilirubin-UDP-glucuronosyltransferase in Gunn rats (an animal model of Crigler–Najjar syndrome type I), resulting in prolonged amelioration of jaundice. However, there are concerns about the safety of lifelong tolerization to the antigens of adenoviruses, because of the possibility that naturally occurring infection by wild-type adenoviruses could cause systemic infection in the absence of a host immune response against them.

Previous reports demonstrated that the Fas-mediated apoptotic pathway is a major mechanism by which cytotoxic T lymphocytes (CTLs) eliminate hepatocytes infected with adenoviral vectors [20,21]. We hypothesized that protection of recombinant adenovirus-infected hepatocytes from apoptosis by simultaneous expression of an antiapoptotic protein should prolong the expression of transgenes introduced by adenoviral vectors. To test this, we generated a recombinant adenovirus expressing cytokine response modifier A (CrmA) [22]. Expression of the CrmA gene efficiently protected the hepatocytes from apoptosis and markedly increased the duration of expression of the adenovirally transferred lysosomal β-glucuronidase (GUSB) gene in mice with an inherited deficiency of this enzyme.

RESULTS

Prolongation of Transgene Expression in Mice with Mucopolysaccharidosis Type VII by Adenovirus-Mediated CrmA Coexpression

We previously demonstrated that exogenous CrmA expression could protect murine hepatocytes from Fas-mediated apoptosis *in vitro* and *in vivo* [23]. To determine whether CrmA coexpression can prolong the expression of an adenovirus-introduced transgene, we generated AxCALNCrmA, an adenovirus that expresses CrmA in the presence of Cre recombinase (Fig. 1A). After confirming that CrmA expression in mouse liver increased in accordance with the increase of the expression of Cre recombinase (Fig. 1B), B6/MPSVII mice (with inherited GUSB) were infected with AxCANCre and AxCAhGUS (an adenovirus expressing human GUSB; group A), and some of the mice were infected with AxCALNCrmA in addition (group B). We obtained serum samples from the mice periodically, and determined GUSB activities. In group A, the GUSB level decreased progressively to less than the normal GUSB activity within 60 days after the adenovirus injection. In contrast, the serum GUSB levels in group B mice decreased at a much slower rate, and more than 60-fold higher activity was still present at day 60 compared with the serum GUSB activity in normal mice (Fig. 2).

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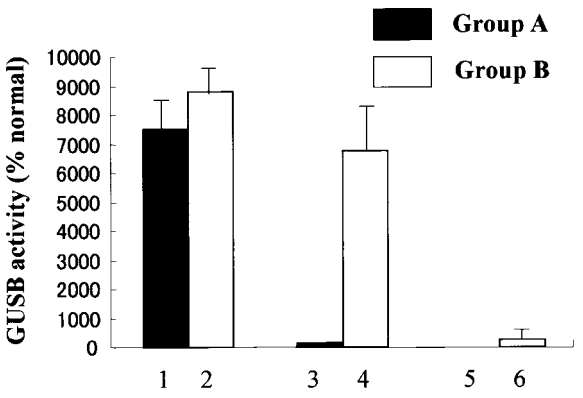

FIG. 2. GUSB activity in MPSVII mice transduced with AxCAhGUS, AxCALNCrmA, and AxCANCre. To evaluate the effect of CrmA coexpression on the delayed clearance of the transgene expression, B6/MPSVII mice (with congenital deficiency of GUSB) were infused with AxCAhGUS, AxCANCre, and AxCALNCrmA to obtain simultaneous expression of CrmA and human GUSB in the mouse liver. The titers of the injected adenoviruses in each group were shown in the text. Because lysosomal GUSB overexpressed in the liver was secreted into the systemic circulation, we evaluated transgene expression by monitoring the serum GUSB activity. The serum GUSB decreased rapidly in the mice of group A (closed bars); however, prolonged GUSB expression was observed in the mice of group B, which expressed both CrmA and GUSB in the liver (open bars). Lanes 1 and 2, 30 days after the treatment; lanes 3 and 4, 60 days after the treatment; lanes 5 and 6, 120 days after the treatment. GUSB activity is shown as the percentage of normal serum GUSB activity of B6 (+/+) mice. The data are expressed as mean ± SE.

Histochemical studies of liver and spleen showed similar differences in the GUSB activities between groups A and B (Fig. 3).

Detection of the Viral DNA in the Gene-Transduced Mouse Liver

We detected the viral genomes of AxCALNCrmA and AxCAhGUS in the mouse liver by PCR 120 days after the vector injection (Fig. 4). A clear 412-bp band was amplified from the liver DNA of the mice treated with AxCALNCrmA, indicating that CrmA expression persisted for > 120 days in the mouse liver (Fig. 4, lanes 1–3). On the other hand, the human GUSB cDNA was not detected in the livers of mice in which CrmA was not simultaneously expressed, even though human GUSB cDNA was initially transferred (Fig. 4, lanes 4, 5). These observations indicate that the exogenous CrmA coexpression allowed the survival of vector-transduced hepatocytes for > 120 days after the adenovirus administration.

Anti-adenovirus Neutralizing Antibodies in the Serum after Adenoviral Gene Transfer

We determined anti-adenovirus neutralizing antibodies at day 60 after adenovirus administration. All mice examined (group A, $n = 3$; group B, $n = 3$) had neutralizing antibody titers of 1:256 to 1:512 (group A, 1:256, 1:256, 1:512; group B, 1:256, 1:256, 1:256), indicating that CrmA expression did not influence the humoral immune response following the adenovirus infection.

Protection of Hepatocytes from CTL Cytotoxic Attack by Exogenous CrmA Expression

To test whether exogenous CrmA expression protects adenovirus-infected hepatocytes from the cell death induced by CTLs, we carried out CTL assays *in vitro*. We isolated T cells from the splenocytes of mice that had been injected with AxCALNCrmA 7 days before. We cocultured these cells with primary hepatocytes isolated from mice that had been infected with both AxCALNCrmA and AxCANCre (group I) or with AxCALNCrmA alone (group II). A remarkable increase of transaminase concentration in the medium was observed when we cocultured the effector T cells with target hepatocytes of group II at a ratio of 50:1. In contrast, there was a significantly smaller increase of transaminase concentration when we cocultured the T cells with group I hepatocytes (Fig. 5). These results demonstrate that exogenous CrmA expression efficiently protects adenovirus-infected hepatocytes from CTL cytotoxic attack.

DISCUSSION

We earlier demonstrated that CrmA expression in cultured murine hepatocytes was able to protect the cells against Fas-mediated apoptosis. On the basis of this observation, here we performed studies showing that coexpression of CrmA in the livers of intact mice results in prolonged expression of transgenes transferred by recombinant adenoviral vectors. Furthermore, we demonstrated *in vitro* that adenovirus-infected hepatocytes could escape from CTL cytotoxic stimulation by the expression of exogenous CrmA.

Several reports have demonstrated an important role for Fas-mediated apoptosis in the development of hepatitis [24–26]. Ogasawara *et al*. induced lethal acute liver failure in mice by the injection of agonistic anti-Fas antibody [26], indicating that hepatocytes are highly susceptible to Fas-mediated apoptosis. Kondo *et al*. [25] reported that adoptive transfer of hepatitis B antigen-specific CTLs into transgenic mice expressing hepatitis B surface antigen resulted in acute hepatitis, which was prevented by pretreatment with an antagonistic Fas antibody. These results strongly suggest an essential role of Fas-mediated apoptosis in the development of acute viral hepatitis B. These findings underscore the importance of direct cytotoxic attack on the virus-infected hepatocytes by CTLs through

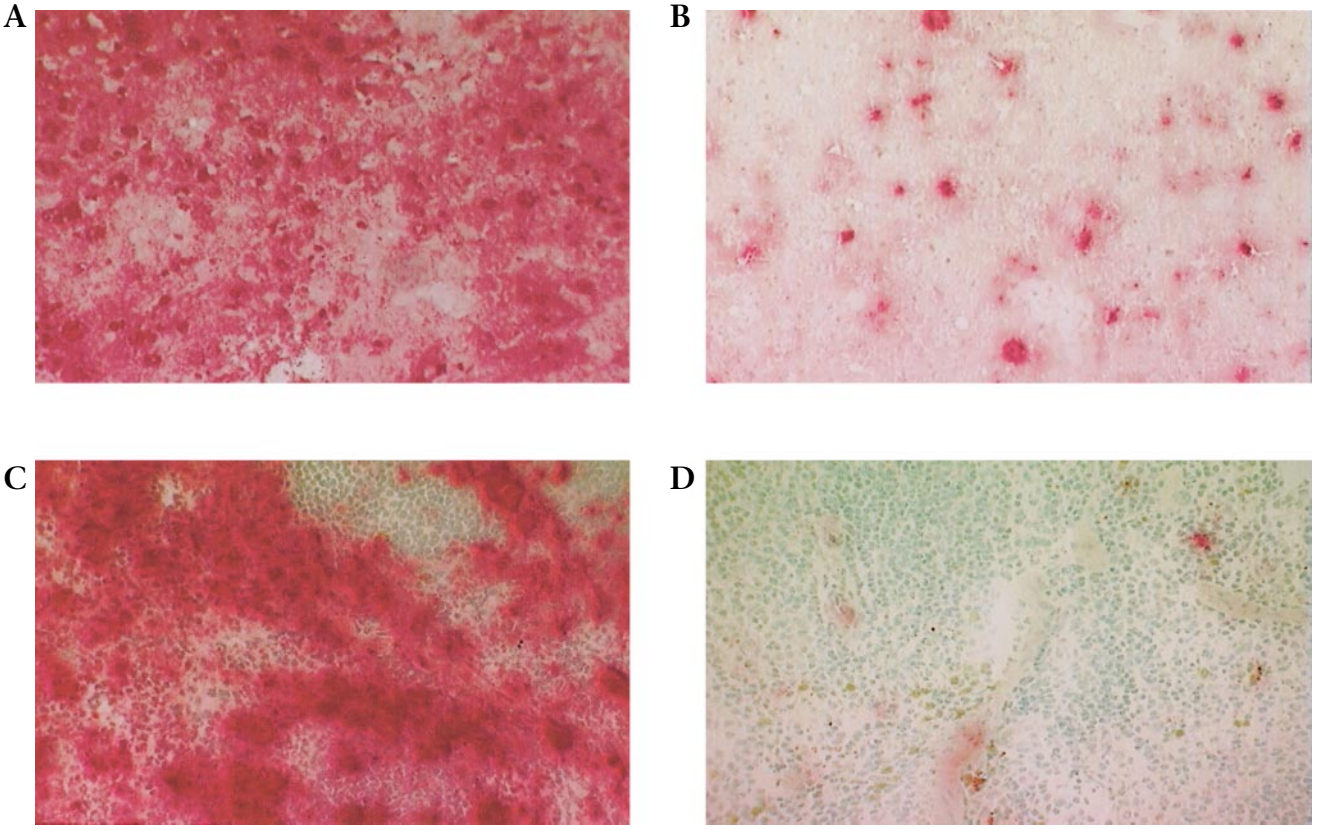

FIG. 3. Histochemical detection of GUSB-positive cells in liver and spleen We stained liver (A and B) and spleen (C and D) samples at 60 days after the virus administration with naphthol AS-BI β-D-glucuronide. The B6/MPSVII mice in group A showed few GUSB-positive cells in liver (B) and spleen (D). In contrast, many GUSB-positive cells were shown in the liver (A) and spleen (B) of the mice in group B. Original magnification, ×100.

Fas–Fas ligand interactions in the development of hepatitis B. Similar inflammatory changes were also observed in hepatitis induced by E1-deleted adenoviral vector infections. Yang *et al*. reported that the adenovirus-infected hepatocytes were destroyed directly by major histocompatibility complex (MHC) class I-restricted CTLs directed against the viral antigens [12]. In studies of mice with inherited deficiency of Fas or Fas ligand, we and others have shown previously that the Fas-mediated apoptotic pathway is pivotal in the induction of hepatocellular apoptosis by adenoviral vectors [20,21].

CrmA, a cowpox viral protein, binds to caspase 8 and thereby inhibits apoptosis triggered by ligation of Fas or tumor necrosis factor (TNF) receptors [27,28]. An antiapoptotic effect of the CrmA has been demonstrated not only in murine hepatocytes [22] but also in several immortalized cell lines or cancer cell lines established from lymphocytes [29], neuronal cells [30], or hepatocellular carcinoma cells [31]. For the transduction of the CrmA gene in rodent hepatocytes *in vivo*, we used an adenovirus containing a Cre/loxP switching system [32]. Using this system, we could circumvent overexpression of CrmA in 293 cells, in which the homologous recombination and propagation of adenoviral vectors were carried out. We had shown that simultaneous intravenous infusion of two viruses—an adenovirus containing a Cre-mediated switching expression cassette of rat Fas ligand and another adenovirus expressing Cre recombinase—allowed efficient Fas ligand gene expression in the rodent liver [33]. Here we have shown another example of successful gene expression using a Cre-mediated switching system.

The *in vivo* effect of CrmA expression in the mouse liver was investigated using mice with congenital GUSB deficiency (B6/MPSVII). There were several advantages to using these mice for this study. First, evaluation of the transgene expression by periodic monitoring of the serum GUSB concentration is possible, because overproduced GUSB is secreted into the systemic circulation [1]. Second, B6/MPSVII mice do not produce detectable levels of the antibody against human GUSB even after repeated injection of large doses of recombinant human GUSB protein

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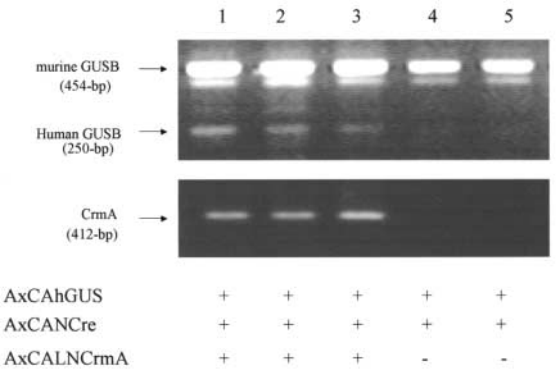

FIG. 4. Detection of the viral DNA in the livers of mice treated with AxCAhGUS, AxCANCre, and AxCALNCrmA. We carried out two PCR reactions to detect the viral DNA in the liver. In the first PCR, virally encoded human GUSB cDNA in AxCAhGUS produced a 240-bp band and the murine GUSB gene produced a 454-bp band, respectively. The other PCR reaction produced a 412-bp band derived from the CrmA gene. We isolated DNA from the livers of the mice of group B (lanes 1–3) and group A (lanes 4, 5). In all mice of group B, both a 240-bp band corresponding to human GUSB cDNA and a 412-bp band corresponding to the CrmA gene were detected, in addition to the 454-bp band amplified from murine GUSB gene (lanes 1–3). On the other hand, human GUSB cDNA was not amplified when we carried out PCR reactions using liver DNA from the mice of group A (lanes 4, 5). These results demonstrate that almost all vector-transduced hepatocytes were eliminated by day 140 in group A, but the vector-transduced hepatocytes were still surviving in group B.

[34]. This is in contrast to the genes for *Escherichia coli* β-galactosidase (LacZ), human α_1-antitrypsin, and green fluorescent protein, which are widely used as markers but exhibit significant immunogenicity in experimental animals, making them difficult to use for evaluation of the duration of transgene expression [35–37]. Third, easy and sensitive detection methods for GUSB activity in tissues or cells are available [38]. Using this model, we investigated the efficacy of CrmA coexpression *in vivo*. Coexpression of CrmA and GUSB in mice with GUSB deficiency resulted in prolonged expression of serum GUSB.

To confirm that CrmA expression is effective for protecting adenovirus-infected hepatocytes from cytotoxic stimulation by effector T cells *in vitro*, we carried out CTL assays. Hepatic cell lysis was induced in adenovirus-infected hepatocytes by coculturing them with T cells stimulated by adenovirus infection (Fig. 5). However, the hepatocytes were able to escape from lysis by expressing CrmA. These results demonstrate that exogenous CrmA expression protects hepatocytes from elimination by T-cell-mediated cytotoxic stimulation.

Although the protection of the vector-transduced hepatocytes from Fas-mediated apoptosis is a major factor, other biological CrmA function should also be taken into consideration. CrmA expression inhibits production of interleukin-1β, thereby reducing the inflammatory response following adenovirus infections in the recipient liver [21]. Furthermore, CrmA inhibits apoptosis triggered by the TNF receptor, which is another major effector of CTLs. TNFα is a major effector of CTLs; however, the contribution of TNFα to the induction of apoptosis in the transduced hepatocytes is considered to be weak. Several reports have shown that TNFα is involved in the activation of antigen-specific T cells rather than in effector cell killing [39].

Numerous attempts to prolong the duration of transgene expression after adenoviral gene transfer have been reported [3,14–17]; however, most of the strategies were based on the nonspecific suppression of T-cell activation, which causes systemic immune dysfunction. To achieve the prolongation of transgene expression, it is necessary to administer high doses of immunosuppressive drugs, and the side effect of these drugs cannot be neglected. In our studies, we demonstrated that the coexpression of CrmA did not interfere with the development of neutralizing antibodies against adenoviral vectors. This finding suggests that CrmA coexpression will make it possible to reduce the optimal dose of immunosuppressive drugs and thus to avoid the deleterious side effects of these drugs. Moreover, we demonstrated that coexpression of CrmA was effective for "moderate" prolongation of the transgene expression introduced by adenoviral vectors. This result indicates the possibility of "bridge use" of CrmA coexpression in adenovirus-mediated gene therapy for genetic disorders. In MPSVII or other lysosomal storage

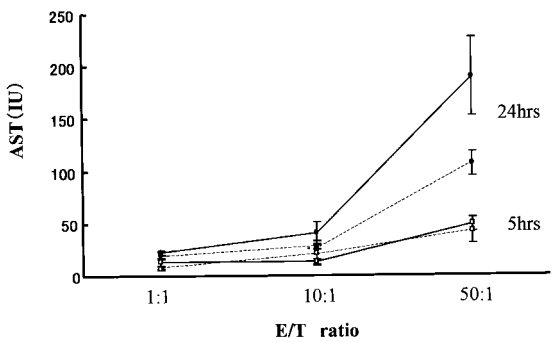

FIG. 5. Protection of adenovirus-infected hepatocytes from CTL cytotoxic attack by adenovirally transduced CrmA expression. We infected B6 (+/+) mice with both AxCALNCrmA and AxCANCre (group I), or with AxCALNCrmA alone (group II). Seven days after the adenovirus administration, we isolated hepatocytes and splenocytes. After restimulation of the splenocytes with adenovirus vector, we isolated T lymphocytes and used them as effector cells. We carried out the CTL assay by coculturing the two types of cells at different ratios (1:1, 10:1, 50:1). Hepatic cell lysis was evaluated by measuring the aspartate aminotransferase (AST) concentration in the medium. A significant difference of AST concentration was observed between group I and group II upon coculturing at an E/T ratio of 50:1 for 24 hours. Solid lines, group II mice ($n = 3$); dotted lines, group I mice ($n = 3$); filled circles, cocultured for 24 hours; open rectangles, cocultured for 5 hours. The data are expressed as mean ± SD.

disorders, bone marrow transplantation (BMT) is a well-accepted therapeutic strategy, and the long-term efficacy of BMT has already been demonstrated [33]. However, in some cases, it is difficult to find optimal donors, and patients have to wait for awhile. Adenovirus-mediated gene therapy accompanied by CrmA co-expression should be helpful in such situations.

Safety issues are a major concern when we consider the clinical protocols of adenovirus-mediated gene therapy, especially therapy targeted to the liver. Deleterious immunological and inflammatory reactions induced after adenoviral infection should be accelerated by rapid apoptosis of vector-infected hepatocytes. Because CrmA efficiently protects hepatocytes from virus-mediated apoptosis, CrmA coexpression should also reduce these reactions, and consequently make adenovirus-mediated gene therapy much safer.

Materials and Methods

Recombinant adenoviral vectors. The four kinds of E1/E3-deleted adenoviral vectors used in this study were generated based on the COS-TPC method described [40]. AxCANCre and AxCALacZ are adenoviruses expressing Cre recombinase and *E. coli* β-galactosidase under the control of the CAG promoter [41]. Both vectors were provided by Izumu Saito of the University of Tokyo, Japan. We cloned the 2.2-kb cDNA for human lysosomal β-glucuronidase [41], supplied by William Sly of St. Louis University (St. Louis, MO), into the unique *Swa*I site of cosmid pAxCAwt [42], and named the resulting construct pAxCAhGUS. We isolated the *Xho*I fragment containing the CrmA gene connected with a T7 tag (Novagen, Madison, WI) at the carboxy terminus from pH38T7CrmA [43], blunt-ended, and cloned into the *Swa*I site of cosmid pAxCALNLwt, and named the resultant construct pAxCALNCrmA. We generated adenoviruses termed AxCAhGUS and AxCALNCrmA through homologous recombination in 293 cells transfected with pAxCAhGUS and pAxCALNCrmA, respectively [1]. The titers of the virus stocks used in the study were determined as described [32].

Animals. We obtained syngeneic B6 (+/+) and B6/MPSVII (mps/mps) mice from a pedigree colony of B6.C-H-2^{bml}/ByBir-gus^{mps}/+ maintained at the National Children's Medical Research Center, Tokyo, Japan. All mice were maintained or treated in accordance with the guidelines of the animal committee of that facility.

Quantitative and histochemical analysis of GUSB. We divided B6/MPSVII mice into two groups. In group A ($n = 6$), the mice were infected with AxCAhGUS (1×10^9 pfu) and AxCANCre (1×10^8 pfu). In group B ($n = 6$), the mice were infected with AxCALNCrmA (1×10^9 pfu) as well as with AxCAhGUS (1×10^9 pfu) and AxCANCre (1×10^8 pfu). We measured GUSB activities in liver, spleen, and serum using a fluorometric assay as described [44]. Briefly, we homogenized the tissue samples of liver and spleen in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2% **[AU: VOL/VOL OR WT/VOL?]** Triton X-100, and 1 mM dithiothreitol, and centrifuged at 14,000 rpm for 1 minute to remove debris. We measured GUSB activities using 4-methylumbelliferyl-β-D-glucuronide (Sigma, St. Louis, MO) as a substrate. Histochemical analysis of GUSB activity was done on 10-μm-thick frozen sections using naphthol AS-BI β-D-glucuronide as a substrate.

Histochemical detection of CrmA in mouse liver. We detected CrmA expression in the mouse livers using the T7 tag located at the C terminus of the expressed CrmA. Portions of livers harvested 7 days after adenovirus administration were snap-frozen in liquid nitrogen and stored at –80°C until they were sectioned on a cryostat. The sections were air-dried and fixed in acetone at –20°C overnight, and then air-dried for 1 hour. We diluted mouse monoclonal T7-tag antibody conjugated with horseradish peroxidase (Novagen) at a ratio of 1:50 in PBS containing 2% BSA and 0.1% sodium azide. We developed color using the Alk Phos detection reagent kit (Novagen). Finally, we counterstained the sections with hematoxylin (Sigma).

Detection of viral DNA in the gene-transduced mouse liver. We extracted total DNA of liver samples using a QIAamp DNA Mini Kit (Qiagen Gmbh, Hilden, Germany). We detected viral DNA of AxCAhGUS using PCR to amplify a 254-bp partial cDNA for human GUSB. The sequences of the forward and reverse primers were 5′-CTGTGGCTGTCACCAAGAGC-3′ and 5′-GGACACTCATCGATGACCAC-3′, respectively. We detected viral DNA of AxCALNCrmA by PCR to amplify a 412-bp region of the CrmA gene using the primers 5′-GTTGATATCTTCACTGAGGGG-3′ and 5′-CCTTAAACTTGGGAATGTGCAC-3′ [22]. A 100-μl aliquot of the PCR reaction mixture contained 250 μM dNTPs, 10 pmol of the forward and reverse primers, 1 μg of the liver DNA, and 2.5 U of *Taq* DNA polymerase (TaKaRa, Shiga, Japan). Thirty cycles of the PCR reactions were carried out at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 60 seconds.

Anti-adenovirus neutralizing antibodies. We measured mouse anti-adenovirus neutralizing serum antibodies as described, with a minor modification [14]. Briefly, the serum samples were heat-inactivated at 55°C for 30 minute and diluted in the medium in 2-fold steps. Each diluted serum sample (0.1 ml) was mixed with 5×10^5 pfu of AxCALacZ (10 ml), incubated at 37°C for 90 minutes, and applied to nearly confluent 293 cells in a 96-well plate for 10 hours. The supernatant, containing serum and viruses, was then replaced by the normal medium for 18 hours, after which the cells were fixed and stained with X-gal. In the absence of neutralizing antibody, all of the cells were stained blue. We reported the titer of the neutralizing antibody for each serum sample as the highest dilution at which < 25% of the cells were stained blue.

Cytotoxic T lymphocyte assay. We infected B6 (+/+) mice with both AxCALNCrmA (5×10^8 pfu) and AxCANCre (5×10^8 pfu; group I), or with AxCALNCrmA alone (group II). We killed the mice in group II 7 days after the vector administration, and cultured splenocytes from these mice in GIT medium (Wako, Osaka, Japan). We then restimulated the cells with AxCALNCrmA (at a multiplicity of infection of 1–5) in the presence of μg/ml concanavalin A (Wako) for 4 days, and isolated T cells using Lympholyte-mice (Cedarlane, ON, Canada) and used them as effector cells. Adenovirus-infected primary hepatocytes isolated from mice in groups I and II were plated on collagen-coated 6-well plates in William's Medium E (1×10^5 cells/well), respectively. We harvested the stimulated effector cells, counted, and added to the primary hepatocyte cultures at a ratio of 1:1, 10:1, or 50:1, and incubated at 37°C for 5 or 24 hours. Hepatic cell lysis was evaluated by measuring the aspartate aminotransferase (ALT) concentration in the medium.

Acknowledgments

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