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Inhibition of Fas-Mediated Fulminant Hepatitis in CrmA Gene-Transfected Mice

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Hyperimmune response via Fas/Fas-ligand and perforin/granzyme pathways may be essential in pathogenesis of virus-induced fulminant hepatitis. CrmA inhibits activation of caspases and granzyme B, suggesting it may block these pathways. We investigated whether CrmA expression would inhibit Fas-associated lethal hepatitis in mice. We successfully generated AxCALNLCrmA, a recombinant adenovirus expressing CrmA gene with a Cre-mediated switching cassette. We increased CrmA expression level in the liver transfected with AxCALNLCrmA (10^9 pfu) by increasing administration dose (10^7–10^9 pfu) of AxCANCre, a recombinant, adenovirus-expressing Cre gene. Injection of anti-Fas antibody into the control mice rapidly led to animal death due to massive liver apoptosis, while the apoptosis was dramatically reduced in the CrmA-expressed mice. The animal survival increased with an increase of CrmA expression. The formation of active caspase-3 was markedly inhibited in the *crmA*-transfected hepatocytes *in vitro*. These results suggest that *crmA* is an effective gene that can inhibit immune-related liver apoptosis. © 2000 Academic Press

Key Words: **fulminant hepatitis; apoptosis; hepatocytes; CrmA; AxCALNLCrmA; AxCANCre; anti-Fas antibody; Cre-loxP system; caspase 3; caspase 8.**

Cells infected with viruses can be targeted for destruction by immune-competent cells (1). In hepatitis B virus (HBV) transgenic mice, both FasL- and perforin/granzyme-dependent signal pathways were activated by cytotoxic T cells to induce hepatocyte death *in vivo* (2). In clinical liver transplantation to patients infected with HBV, reinfection of the virus may lead rapidly to graft failure (3, 4). However, HBV recurrence after transplantation was much lower in patients with fulminant B hepatitis than in those with viral B cirrhosis, which is related to the presence of viral replication prior to transplantation (5). It is suggested that a hyperimmune response is essential in the pathogenesis of HBV-induced fulminant liver failure (1, 2).

Therefore, an ideal strategy for treating fulminant hepatitis would be to protect the hepatocytes against severe attack by the host's immune system by transfecting anti-apoptotic genes. Previous studies have shown that the cytokine response modifier A (CrmA) protein, a member of the serine proteinase inhibitors (serpin), encoded by the gene of the cowpox virus, interferes with the host's antiviral immune responses (6). CrmA inhibits the proteolytic activity of the IL-1β-converting enzyme (ICE; caspase-1), a cysteine proteinase, as well as granzyme B, a serine proteinase (7). In this study, we transfected *in vivo* a recombinant adenovirus vector expressing the *crmA* gene into mouse livers in order to investigate its protective effect on immune-mediated fulminant hepatitis.

Researchers have reported that activation of Fas and FasL systems in the liver may play an essential role in the development of fulminant hepatitis caused by virus infection or endotoxin (8–10). The administration of the agonistic anti-Fas antibody to mice rapidly induces severe damage to the liver by massive apoptosis, indicating that this animal model would be a good system for investigating human fulminant hepatitis (9, 11). Also, cell apoptosis induced by the binding of the FasL or anti-Fas antibody to Fas involves an activation of caspase 1 (12, 13). Therefore, we administered the anti-Fas antibody to mice with *crmA* gene-transfected livers

Abbreviations used: HBV, hepatitis B virus; CrmA, cytokine response modifier A; pfu, plaque forming units; MOI, multiplicity of infection; *neo*, neomycin-resistant gene; poly A, polyadenylation signal; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; ALT, alanine aminotransferase; AST, asparate aminotransferase.

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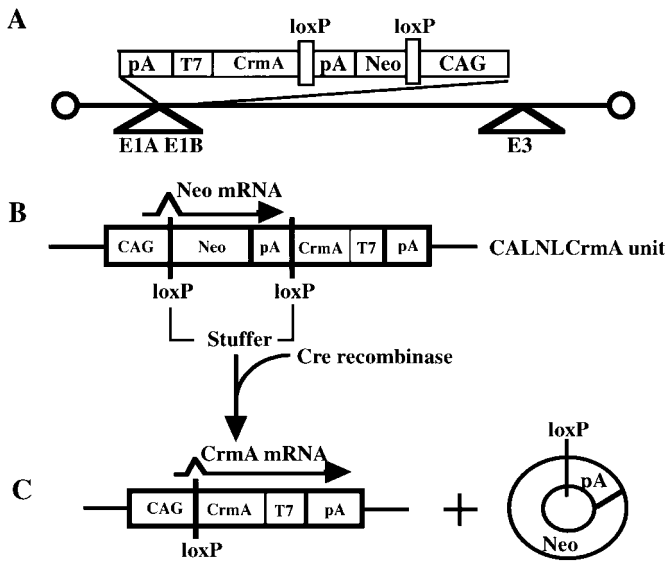

FIG. 1. Schematic illustration for the constructs of expression vector. (A) Structure of E1/E3-deleted recombinant adenovirus vector, AxCALNLCrmA. (B) Activation process of the CrmA gene in the CALNLCrmA unit by Cre recombinase. CAG, the CAG promoter; Neo, neomycin-resistant gene; pA, polyadenylation signal; T7, T7-tag; CrmA, cytokine response modifier A; loxP, loxP sequence. Arrows show the orientation of the mRNA transcription. A triangle under the adenovirus genome represents the deletion of an adenoviral sequence.

to study whether the gene expression could save the animal's life from fatal hepatitis.

MATERIALS AND METHODS

Generation of recombinant adenovirus-expressing crmA and cre genes. We constructed a recombinant adenovirus vector, AxCALNLCrmA, which carries a Cre-loxP on/off-switching unit (Fig. 1A), based on the COS-TPC method described in previous studies (14, 15). In brief, we constructed a cosmid-containing, Cre-mediated, switching expression cassette, pAxCALNLCrmA, by the following method. We inserted a XhoI fragment from pH38T7CrmA (16), which included C-terminal T7-tagged *crmA*, into the *Swa*I site of a cosmid vector pAxLNLw (17). The *crmA* was a cowpox virus white-pock variant (CPV-W2) (Accession No. m14217), and we prepared the T7-tag from pET21b (Novagen, Madison, WI). As a preliminary, we checked that the T7-tag did not inhibit the function of the CrmA.

We co-cultured 293 cells with the pAxCALNLCrmA and an adenovirus DNA-terminal protein complex digested at several sites with *EcoT22I*. Thus, we generated a recombinant AxCALNLCrmA through a homologous recombination in the 293 cells and purified them by double-cesium, step-gradient ultra-centrifugation. We checked the purified AxCALNLCrmA for the deletion of the E1A region, both by restricted digestion of the viral DNA and by PCR amplification, using primers specific for E1A (18). We have described previously the recombinant adenovirus expressing a modified Cre gene, AxCANCre (17, 19).

Administration of adenovirus vectors and anti-Fas antibody into mice. For this study, we purchased male Balb/c mice between the ages of 8 and 12 weeks from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). We transfected the mice with adenoviral vectors, according to each experimental protocol. Three days after transfection, we injected vein a purified hamster monoclonal antibody, agonistic anti-Fas antibody (Jo2, PharMingen, San Diego, CA), via the tail at a dose of 10 μg/mouse.

Hepatocyte isolation and culture. We isolated mouse hepatocytes using a modification of the method described by Soda *et al.* (20). We anesthetized the mice with ether, and exposed the portal vein with a ventral midline abdominal incision. Into this vein we inserted a fluid-filled 24-gauge catheter (3/4 inches). We slowly introduced into the catheter 20 ml of 37°C perfusion buffer, containing 137 mM NaCl, 4.69 mM KCl, 1.17 mM NaH_2PO_4, 0.65 mM $MgSO_4 \cdot 7H_2O$, 1.0 mM EGTA, 10 mM HEPES, pH 7.2. This was followed by 10 ml of an enzyme solution (0.5 mg/ml collagenase type I, 1 mg/ml, 66.7 mM NaCl, 6.70 mM KCl, 4.76 mM $CaCl_2 \cdot 2H_2O$, 100 mM HEPES, pH 7.5), which then passed through the liver. We then excised the liver immediately, diced it into small pieces, and placed these into 10 ml of the collagenase solution that also contained 0.001% DNase I. The digested tissue was then filtered through a sterile nylon mesh. We centrifuged the cell suspension at 600*g* for 5 min. We then resuspended the hepatocyte pellets in Williams medium (10% FCS, 10 mM HEPES, 1 μg/ml fungison, 10^{-6} mM insulin, 10^{-6} mM dexamethazon, 50 ng/ml EGF, 100 U/ml penicillin, and 100 μg/ml streptomycin, pH 7.1) and cultured them. Cell viability was determined by trypan blue dye-exclusion, and over 90% of the cells were viable.

We cultured the hepatocytes from the mice that had been cotransfected with AxCALNLCrmA [10^9 plaque-forming units (pfu)] and AxCANCre (10^9 pfu) and those from the control mice in six-well culture plates with or without anti-Fas antibody (1 μg/ml) in the presence of actinomycin D (0.5 μg/ml). Twenty-four hours after culture, we observed the cell morphology by a phase-contrast microscope (Olympus IX70, Tokyo). To detect apoptosis, we collected hepatocytes from each well, washed them with PBS and fixed them for 30 min in PBS containing 1% glutaraldehyde at room temperature. After centrifugation, we resuspended the cells in 25 μl of 200 μM Hoechst 33342 (Wako, Osaka). We then observed the nuclear morphology with a fluorescence microscope (Olympus BX50).

In situ assay for DNA fragmentation. We used terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method with an Apop Tag Plus Kit (Oncor, Gaithersburg, MD) to detect DNA fragmentation. Cryosections (6 μm) were fixed in 10% neutral buffered formalin in a coplin jar. These sections were quenched in 0.5 to 1% hydrogen peroxide in PBS for five minutes at room temperature and incubated at 37°C for one hour with deoxynucleotidyl transferase (TdT) and dioxigenin-conjugated dUTP (9) in 38 μl of reaction buffer. We terminated the reaction with a prewarmed working strength stop/wash buffer for 30 min at 37°C. To visualize the incorporated dUTP, we incubated the sections with a peroxide-conjugated anti-digoxigenin antibody for 30 min at room temperature, then washed them three times and incubated again with a 3,3′-diaminobenzidine (DAB) substrate working solution for three to six minutes at room temperature. This reaction was terminated by washing with distilled water, and then the sections were counterstained with hematoxylin and mounted for observation. The negative controls were prepared by substituting PBS for the TdT enzyme in the reaction mixture.

Immunohistology. For detecting CrmA expression, we harvested liver samples three days after vector administration, snap-froze them in liquid nitrogen, then stored them at −80°C until they were sectioned on a cryostat. The sections (6 μm) were air dried and fixed in acetone at −20°C overnight, followed by air drying for one hour. We treated them with a mouse monoclonal anti-T7-Tag antibody conjugated alkaline phosphatase (Novagen), diluted to 1:50 in PBS solution containing 2% bovine serum albumin and 0.1% sodium azide. Color was developed with an Alk Phos detection reagent kit (Novagen). Finally, we counterstained the sections with hematoxylin (Sigma, St. Louis, MO). To obtain quantitative data on the amount of positively staining present, we used an image analyzer (Fuji HC-2000-equipped Olympus microscope (BH-2) interfaced with MacSCOPE version 2.3.3, Fujifilm, Tokyo, Japan), and calculated the

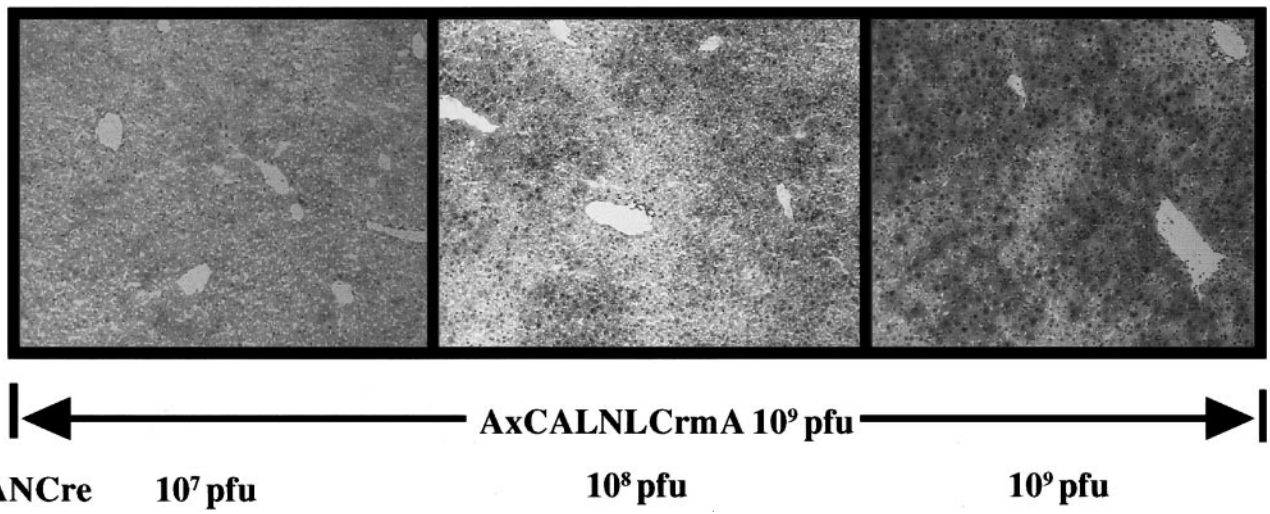

FIG. 2. Gene expression of a mouse liver transfected *in vivo* with CrmA. We intravenously injected recombinant adenovirus vectors, AxCALNLCrmA (10^9 pfu) combined with AxCANCre (10^7–10^9 pfu), into Balb/c mice and performed immune staining with anti-T7-tag antibody on their liver sections three days after gene transfection. Since the switching-on structure of AxCALNLCrmA expresses a fusion protein of CrmA and T7-tag, we can detect the CrmA-expressing cells as T7-tag-positive cells. The CrmA expression level increased with an increase of the AxCANCre dose (10^7–10^9 pfu). We observed approximately 80% of the T7-tag positive cells, calculated with an image analyzer, in the sections by administering 10^9 pfu of AxCALNLCrmA combined with 10^9 pfu of AxCANCre. Original magnification: ×100. The data are representative of three separate experiments.

percentage of positive area to whole area in a randomly chosen appropriate field.

For CD11b-staining, we prepared 6 μm of cryocut sections from the *crmA*-transfected livers 24 h after administration of the anti-Fas antibody. After one hour of incubation with rat monoclonal antibody for CD11b (Ly-40, Serotec, Oxford, UK), diluted to 1:100 in a PBS solution containing 2% bovine serum albumin and 0.1% sodium azide, the secondary antibody, goat anti-rat IgG conjugated alkaline phosphatase (SC2021, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) diluted at 1:100 in the above working solution, was added and incubated for another hour. We developed the color with the Alk Phos detection reagent kit and counterstained with hematoxylin.

Western blotting analysis for detecting CrmA and caspase-3. We prepared cell lysates and/or extracted protein of the tissues with a 100 μl 4× SDS sample buffer, and boiled 10 μl of the protein lysates for 5 min. Protein concentration was determined with a DC protein assay kit (Bio-RAD, Hercules, CA) using bovine serum albumin as a standard. Proteins were separated by 10% SDS–PAGE then transferred to nitrocellulose filters. We blocked the filters with a TBST buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20) containing 5% skimmed milk and incubated for one hour with a mouse monoclonal anti-T7-Tag antibody (Novagen) or a rabbit anti-caspase-3 antibody (PharMingen) at 1:500. We used horseradish peroxidase (HRP) conjugated goat anti-mouse (PharMingen) or anti-rabbit antibodies (Sigma) at 1:1000 as a second antibody. We washed the filters in the TBST buffer four times for 10 min between each step then detected the immune complexes by the ECL chemiluminescence method (Amersham, Buckinghampshire, UK).

Assay for caspase activity. We determined activities of caspases 1, 3 and 8 with a colorimetric protease assay kit (MBL, Nagoya, Japan) for ICE/caspase-1, CPP32/caspase-3, and FLICE/caspase-8. We suspended the cell pellets in 50 μl of chilled cell lysis buffer and incubated on ice for 10 min. After centrifugation at 10000*g* for 1 min, supernatants were stored at −70°C. Proteolytic reaction was carried out in reaction buffer, containing 50 μg of cytosolic protein extracts and 20 nM acetyl-Try-Val-Ala-Asp-pNA (Ac-YVAD-pNA), acetyl-Ile-Glu-Thr-Asp-pNA (Ac-IETD-pNA), acetyl-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA). We incubated the reaction mixtures at 37°C for 4 h, and measured the formation of p-nitroanilide at 405 nm using a Wallac1420 ALBOsx microtiter plate reader (Amersham).

Transaminase levels in the serum and culture medium. The concentrations of alanine aminotransferase (ALT) and asparate aminotransferase (AST) in the mouse serum and in the culture medium were determined using a Vision kit (Abbott, Abbott Park, IL), according to the manufacturer's protocol.

Statistical analysis. We compared the survival times of the experimental animals among the groups by Gehan's generalized Wilcoxon test. The significance of the differences in ALT and AST concentrations was assessed among the different groups with Student's unpaired *t* test. We considered a probability of $P < 0.05$ to be significant in all our studies.

Ethics. We conducted all our experimental protocols in accordance with the policies of the Animal Ethics Committee of the National Children's Medical Research Center.

RESULTS

CrmA Expression in Mouse Livers Transfected with the Gene Using the Cre-loxP System

As shown in Fig. 1A, the AxCALNLCrmA consisted of a CAG promoter, a stuffer sequence, *crmA*, a T7-tag gene, and a polyadenylation signal (polyA) sequence. The stuffer sequence was made up of a neomycin-resistant gene (*neo*) and another polyA sequence flanked by a pair of *loxP* sites to prevent the expression of the downstream *crmA* (*crmA*-off structure). After transfection into animal livers, the AxCALNLCrmA initially expressed the *neo* but not the *crmA* (Fig. 1B). When a sufficient amount of functional Cre recombinase was supplied to the switching unit by the transfection of AxCANCre, the stuffer DNA was excised and

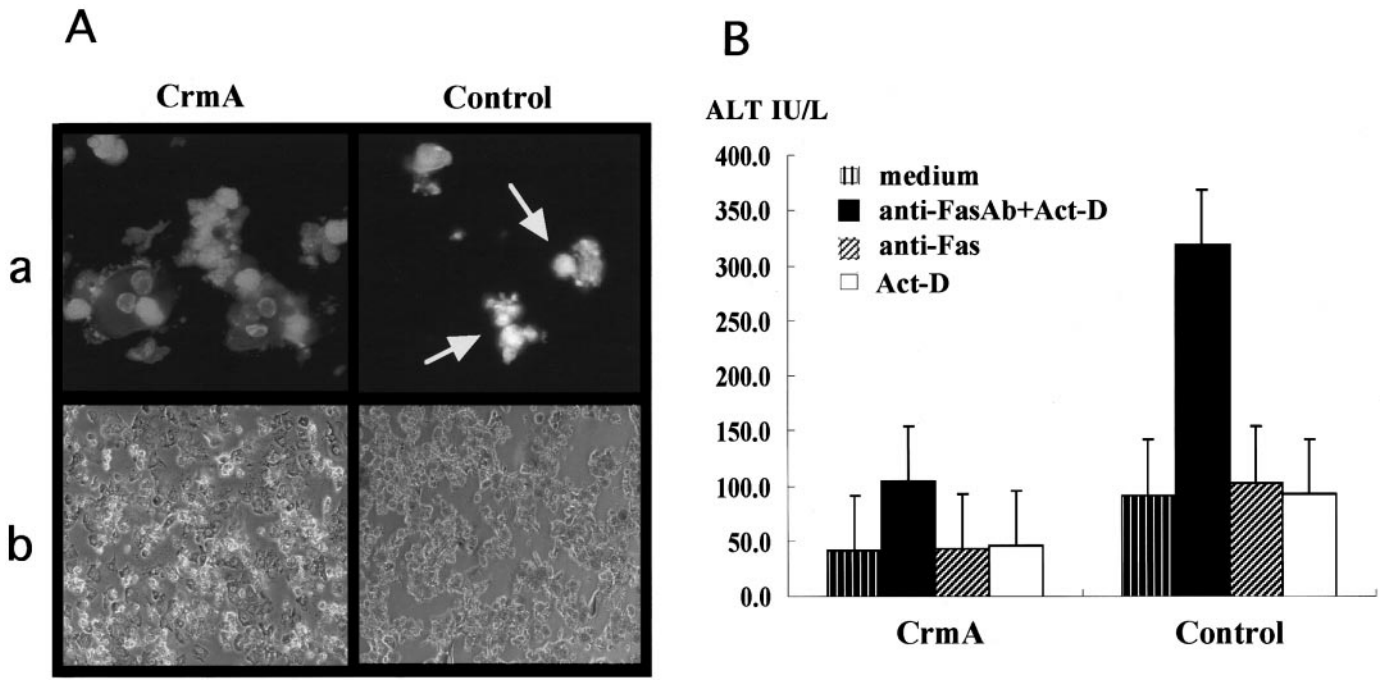

FIG. 3. *In vitro* inhibitory effect of CrmA expression on anti-Fas antibody-induced hepatocyte apoptosis. (A) We incubated hepatocytes that had been isolated from gene-transfected mice with agonistic anti-Fas antibody at 1 μg/ml in the presence of actinomycin D at 0.5 μg/ml in six-well culture plates for 24 h. After incubation, phase-contrast microscopy (b; original magnification $\times$100) revealed a large number of cells in the control group had detached from the bottom of the culture well due to cell death, but this was not seen in the CrmA-expressed hepatocytes. When the cells were stained with Hoechst 33342, we observed nuclear condensation and fragmentation in the control cells, but not in the CrmA gene-transfected cells, by fluorescence microscopy (a; original magnification, $\times$400). The data are representative of three separate experiments. (B) The cell death in the control hepatocytes was also confirmed by a high level of ALT concentration in the control cells treated with anti-Fas antibody and actinomycin D. We obtained a significant ($P < 0.01$) difference of ALT levels between the CrmA-transfected cells and the control cells. There was no significance between the CrmA-expressed cells and the control hepatocytes when these cells were treated with a medium, anti-Fas antibody alone, or with actinomycin D alone. The data show the mean $\pm$ SD from six mice in each group.

separately formed a circular DNA, resulting in the conjunction of the CAG promoter with the *crmA* gene via a single loxP site (14). The consequent structure, as shown in Fig. 1C, was enabled to express the *crmA* under the control of the CAG promoter (*crmA*-on structure). Using this system, we successfully generated *in vivo* the CrmA protein in the mouse livers; that is, the AxCALNLCrmA transfection expressed CrmA that was dependent on the administration dose of the AxCANCre. In addition, an advantage of using adenovirus vector was its high gene transduction efficacy in the liver and its multiple infections of the hepatocytes (21).

We injected Balb/c mice intravenously with AxCALNLCrmA in combination with AxCANCre. We detected the expression level of the CrmA protein in the gene-transfected hepatocytes by immune staining with an anti-T7-tag antibody, because the switching-on structure of AxCALNLCrmA expresses a fusion protein of the T7-tag and CrmA. As shown in Fig. 2, CrmA expression was dependent on the administration doses (10^7 to 10^9 pfu) of AxCANCre, under a constant dose (10^9 pfu) of AxCALNLCrmA (Fig. 2). Approximately 80% of the hepatocytes were positively stained by the administration of AxCALNLCrmA at 10^9 pfu and AxCANCre (10^9 pfu).

In Vitro Inhibition of Anti-Fas Antibody-Induced Apoptosis in crmA-Transfected Hepatocytes

We cultured isolated hepatocytes from the *crmA*-transfected mice by co-transfection of AxCALNLCrmA (10^9 pfu) and AxCANCre (10^9 pfu) with an agonistic anti-Fas antibody at a concentration of 1 μg/ml in the presence of actinomycin D (0.5 μg/ml). Phase-contrast microscopy 24 h after incubation (Fig. 3A-b) showed that a large number of cells in the control group were detached from the bottom of culture well due to cell death, but we did not see this condition in the CrmA-expressed hepatocytes.

We then stained the cells with Hoechst 33342 and observed them with fluorescence microscopy (Fig. 3A-a). When we treated the control hepatocytes that had no gene-transfection with the antibody and actinomycin D, we could clearly see the apoptotic hepatocytes with nuclear condensation and fragmentation. How-

ever, the hepatocytes isolated from the *crmA*-transfected mice demonstrated a marked reduction of apoptotic cells when compared to the control hepatocytes. We also confirmed cell death in the control group by a high level of transaminase in the cultured medium, while its level was markedly lower in the medium of CrmA-expressed hepatocytes (Fig. 3B).

In Vivo Inhibition of Anti-Fas Antibody-Induced Lethal Hepatitis by CrmA Expression

Three days after the *in vivo* gene transfection into the mice, we intravenously injected the anti-Fas antibody at 10 μg/mouse. We designed five groups based on the administration dose of the viral vectors. We injected AxCANCre at doses of 10^9, 10^8, and 10^7 pfu in Groups 3, 4 and 5, in combination with 10^9 pfu of AxCALNLCrmA. The controls were the group without any gene-transfection (Group 1), to which we had administered AxCALNLCrmA alone at 10^9 pfu (Group 2). We then examined the effect of the CrmA expression on the animal survival rate from Fas-mediated hepatic failure. When injected with the anti-Fas antibody, all mice, except for one, in the control groups died within 24 h, but the expression of the *crmA* gene dramatically improved the survival rate (Fig. 4A). The number of survivors after antibody treatment increased markedly in proportion to the expression levels of CrmA, and no animal died after administration of 10^9 pfu of AxCANCre combined with AxCALNLCrmA (10^9 pfu). In addition, the serum levels of transaminase decreased with increased levels of *crmA* expression (Fig. 4B).

The TUNEL and hematoxylin and eosin (HE) stainings (Fig. 5) revealed that fulminant hepatic failure with massive hepatocyte apoptosis was induced by treatment with the anti-Fas antibody in the group injected with AxCALNLCrmA alone [CrmA (−)]. In contrast, apoptosis was dramatically reduced in the group expressing CrmA [CrmA (+)].

As shown in Fig. 6, we saw a marked infiltration of CD11b-positive cells in the crmA-non-expressed livers 24 h after anti-Fas antibody treatment (b), while these cells were decreased remarkably in the livers expressed *crmA* gene (c).

Decreased Activity of Caspase-3 by CrmA Gene Expression

We transfected hepatocytes isolated from normal murine livers with AxCALNLCrmA, with or without AxCANCre, at a multiplicity of infection (MOI) from 0.01 to 1.0 and incubated them for 24 h. An anti-Fas antibody (1 μg/ml) and actinomycin D (0.5 μg/ml) were then added to the culture medium. Western blotting (Fig. 7A) demonstrated that the active form (17 kD) of caspases-3 appeared in the control hepatocytes with a transfection of AxCALNLCrmA alone, while this form

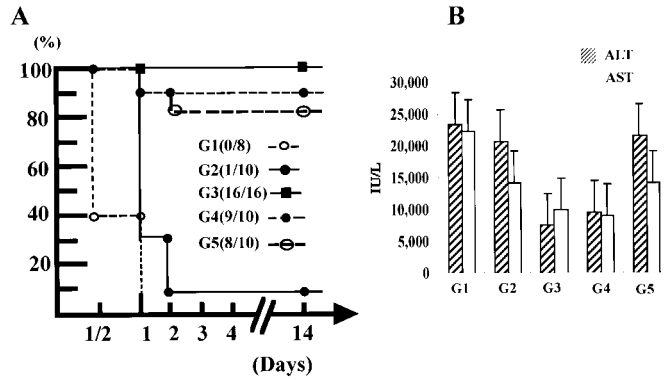

FIG. 4. Survival rate of CrmA gene-transfected mice and serum level of transaminase after administration of anti-Fas antibody. (A) We transfected the mice with 10^9 pfu of AxCALNLCrmA in combination with different doses (10^7, 10^8, and 10^9 pfu) of AxCANCre. Three days after gene transfection, we injected an agonistic anti-Fas antibody at a dose of 10 μg/mouse via the tail vein. The animal survival rate increased with an increase of the CrmA expression level. Groups 1 and 2 were the controls without gene transfection and with 10^9 pfu of AxCANCre alone. Groups 3, 4, and 5 were transfected with 10^9, 10^8, and 10^7 pfu of AxCANCre and a constant dose (10^9 pfu) of AxCALNLCrmA. Gehan's generalized Wilcoxon test: $P < 0.01$, Group 1 versus Group 3, 4 or 5; NS, Group 1 versus Group 2. (B) Twenty-four hours after introduction of the anti-Fas antibody, we detected ALT and AST levels in the sera from the gene-transfected mice. The levels decreased, depending on the dose (10^7–10^9 pfu) of AxCANCre, under a constant dose (10^9 pfu) of AxCALNLCrmA. Group 1 and Group 2 were transfected without any gene and with AxCALNLCrmA-alone at 10^9 pfu. Groups 3, 4, and 5 were administered 10^9, 10^8 and 10^7 pfu of AxCANCre under a constant dose (10^9 pfu) of AxCALNLCrmA. The data shows the mean ± SD from six mice in each group. Unpaired Student's *t* test: $P < 0.01$ in ALT level, Group 1 versus Groups 3 and 4; $P < 0.05$ in AST level, Group 1 versus Groups 3 and 4; NS in ALT and AST levels, Group 1 versus Groups 2 and 5.

was completely absent in the cells co-transfected with both AxCALNLCrmA and AxCANCre at an MOI of 1.0 in each. We also observed an MOI-dependent increase of CrmA expression.

Inhibitory Effect of CrmA on Activation of Caspases 3 and 8

The activity of caspase-3 and caspase-8 was increased in the hepatocytes by the treatment of anti-Fas antibody, but caspase-1 was not (Fig. 7B). When we expressed CrmA with co-transfection of AxCALNLCrmA and AxCANCre at a multiplicity of infection (MOI) from 0.01 to 1.0, we observe an MOI-dependent inhibition in the activation of caspases 3 and 8 (Fig. 7B). The result showed that the caspases 3 and 8 played an important role in the anti-Fas antibody-induced apoptosis and CrmA protected the apoptotic cell death via an inhibition of a caspase activation.

DISCUSSION

To explore the participation of CrmA expression using the AxCALNLCrmA with a Cre-mediated switch-

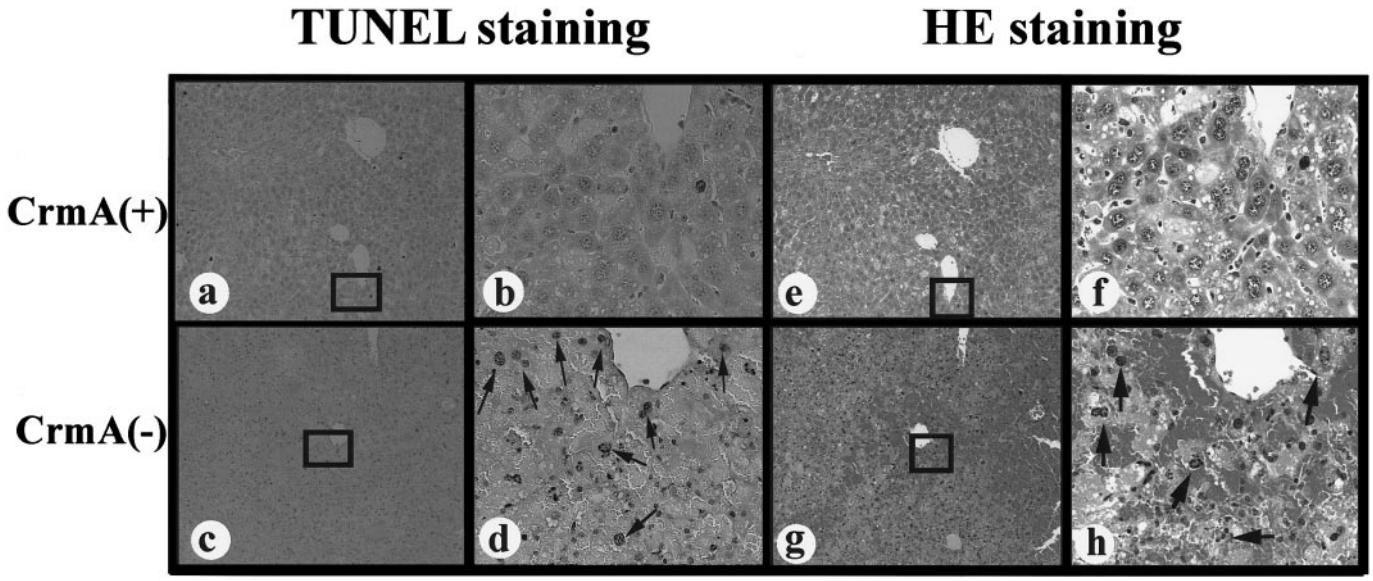

FIG. 5. Histological findings in the CrmA gene-transfected liver after administration of an anti-Fas antibody. Twenty-four hours after injection of the anti-Fas antibody, we observed a number of TUNEL-positive cells in the CrmA-negative liver (c and d), but no TUNEL-positive cells in the CrmA-expressed hepatocytes (a and b). HE staining revealed a number of hepatocyte deaths, accompanied by massive hemorrhage, in the CrmA-negative liver (g and h), while we did not see these results in the CrmA-positive liver (e and f). We administered 10^9 pfu of AxCALNLCrmA combined with 10^9 pfu of AxCANCre in the CrmA(+) group, but only AxCANCre (10^9 pfu) was injected in the CrmA(−) group. The arrows indicate apoptotic cells. (b, d, f, and h) Enlargements of the boxed areas in a, c, e, and g. Original magnification: a, c, e, and g, ×40; b, d, f, and h, ×400. The data are representative of three separate experiments.

ing system, we then attempted to induce fulminant hepatitis by administering the agonistic anti-Fas antibody intravenously into the mice transfected with or without the *crmA* gene. Injection of the antibody into the control mice resulted in rapid animal death by inducing massive hepatocyte apoptosis, while the survival rate increased in the AxCALNLCrmA (10^9 pfu)-transfected mice with the increase of the doses we administered (10^7 to 10^9 pfu) of AxCANCre. When we administered 10^9 pfu of AxCANCre, at which point the expression rate was approximately 80% of the whole hepatocytes, no animal death was induced with the anti-Fas antibody. In addition, a number of CD11b-positive cells infiltrated the control liver after treatment with the anti-Fas antibody, while these cells were markedly decreased in the antibody-treated livers that had been expressed the *crmA* gene. Since CD11b is a maker of granulocytes, we believe the infiltrated cells play a role in eliminating apoptotic hepatocytes. We also demonstrated that the hepatocytes isolated from the CrmA-expressed mice that were treated *in vitro* with the anti-Fas antibody, in the presence of actino-

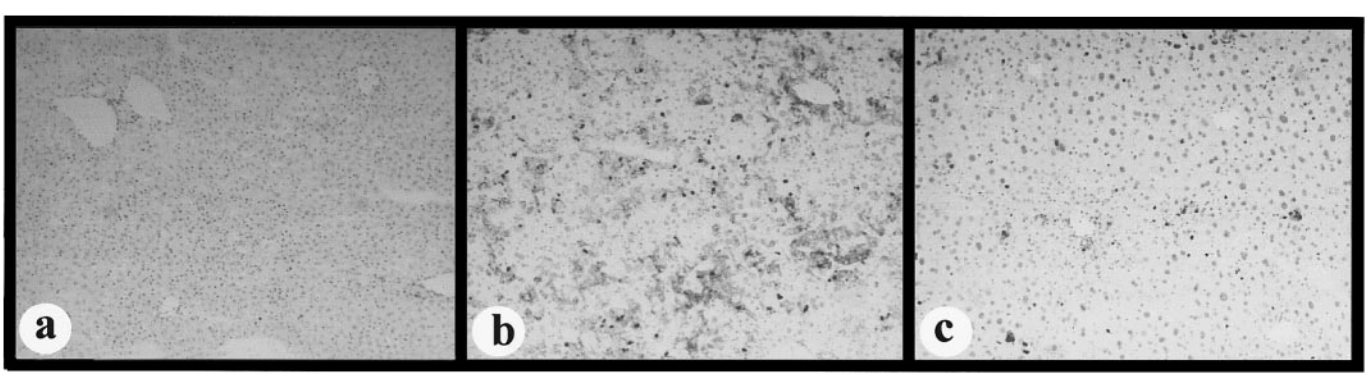

FIG. 6. Infiltration of CD11b-positive cells in the liver after administration of the anti-Fas antibody. Twenty-four hours after administration of the anti-Fas antibody, we observed the infiltration of a large number of CD11b cells in the control liver transfected with 10^9 pfu of AxCANCre alone (b). However, the cell infiltration was markedly reduced in the group treated with 10^9 pfu of AxCALNLCrmA and 10^9 pfu of AxCANCre (c). We saw no cell infiltration in the anti-Fas antibody-administered group pretreated with 10^9 pfu of AxCALNLCrmA and 10^9 pfu of AxCANCre (a). Original magnification: ×100. The data are representative of three separate experiments.

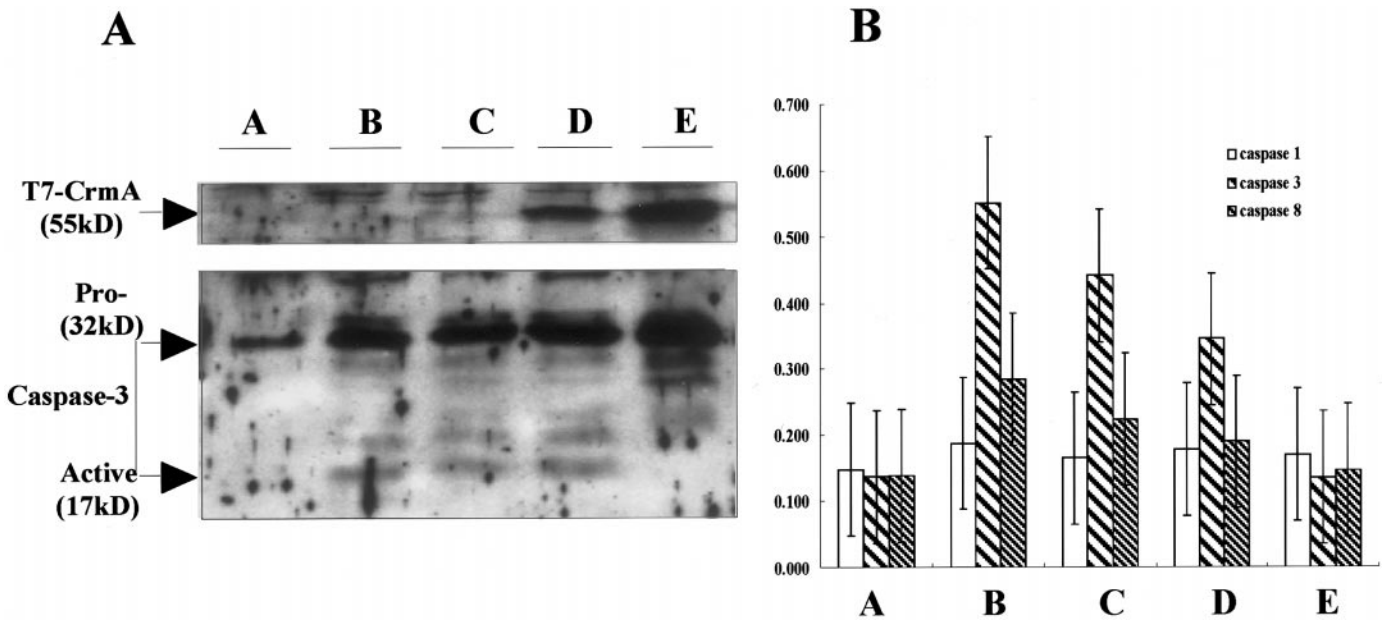

FIG. 7. Effect of CrmA on caspases 3 and 8 in the hepatocytes treated with anti-Fas antibody. Hepatocytes were cotransfected *in vitro* with AxCALNLCrmA and AxCANCre at an MOI of 0.01 to 1.0. (A) We detected the expression of CrmA and the active form of caspase-3 by Western blotting. We obtained an MOI-dependent increase of the CrmA protein and a decrease of the active-form (17 kD) of caspase-3. (B) The colorimetric protease assay demonstrated an MOI-dependent decrease in the activities of caspase 3, and caspase 8 by the treatment of anti-Fas antibody, whereas we did not see any remarkable change in caspase-1 activity. A, the control hepatocytes with neither gene transfection nor anti-Fas antibody treatment; B, the hepatocytes transfected with AxCALNLCrmA alone at an MOI of 1.0; C, those with AxCALNLCrmA and AxCANCre at an MOI of 0.01 in each; D, with AxCALNLCrmA and AxCANCre at an MOI of 0.1; E, with AxCALNLCrmA and AxCANCre at an MOI of 1.0. The data are representative of three separate experiments.

mycin D, had much less apoptotic cell death than the hepatocytes that were not gene-transfected. Actinomycin D is needed for *in vitro* induction of Fas-mediated apoptosis in some cells to inhibit *de novo* protein synthesis by inhibiting mRNA transcription (22). Taken together, our results indicated that *crmA* is an effective gene that strongly inhibits massive hepatocyte apoptosis.

On the other hand, Bcl-2, an anti-apoptotic protein located in mitochondria, has been shown to inhibit Fas-mediated apoptosis (11). Recent study proved that this effect differed in cell types, some of which were not inhibited (23). Activation of caspase cascade plays a critical role in the Fas-mediated apoptosis. Caspase-8, one of the initiator caspases, is well known to activate directly or indirectly effector caspases including caspases 3 and 7. In the indirect pathway, the caspase-8 acts on mitochondrial membrane to release cytochrome c, which subsequently activates caspase-9 and then effector caspases (24). The members of Bcl-2 family, such as Bcl-2 and Bcl-XL, block the release of mitochondrial cytochrome c, although they do not block the activation of caspase 8. Thus, the Bcl-2 may not completely inhibit Fas-associated apoptosis.

The *crmA* gene encodes a 38 kD protein whose amino acid sequence is similar to those of members of the serpin superfamily (25). Initially, the CrmA protein was demonstrated to decrease the inflammatory response to viral infection by inhibiting caspase 1 (26, 27). The protein also inhibits caspase-8, and so inhibits downstream caspases (28). In this study, *crmA* transfection in the isolated hepatocytes inhibited the formation of an active structure (17 kD) of caspase-3 from its pro-form (32 kD). Activated caspase-3 is one of the key proteins that directly induce the activation of the DNA fragmentation factor (29). The colorimetric protease assay also demonstrated that the treatment of anti-Fas antibody activated caspase-3 and caspase-8 in the control hepatocytes, while the CrmA-expression markedly inhibited their activation. The inhibition of caspase-3 activation may not be induced by the direct effect of CrmA, but by the inhibition of upstream caspases, including caspases 1, 4, and 8 (30). Therefore, the anti-apoptotic activity of CrmA was initially achieved by the inhibition of caspase-8. This suggests that Fas-mediated apoptosis was completely inhibited by expressing *crmA* gene, that is markedly different from the anti-apoptotic action of Bcl-2. In addition, previous studies showed that an activation of caspase-1 was involved in Fas-mediated apoptosis (13, 31), although we did not observe its activation in the anti-Fas antibody-treated hepatocytes. This result is in agreement with the experiences by other investigators (32, 33).

From the clinical point of view, fulminant hepatitis is an immoderate disease that causes acute and severe impairment of liver function. HBV infection commonly

induces the disease (34). The host's immune responses target the HBV-infected hepatocytes for apoptotic cell death (1, 35). Therefore, apoptosis is critical as a host defense system for eliminating virus-infected cells, but over-reaction of the immune system results in rapid patient death due to massive hepatocyte apoptosis.

A large number of patients worldwide are chronically infected with HBV (36). Some of them are candidates for liver transplantation. However, recurrence of HBV infection is a major problem in these patients after transplantation and frequently induces aggressive disease leading to liver failure (34, 37). In saving the patient's life during the aggressive phase of the disease, there is a possibility we can control HBV infection by using anti-virus drugs and/or antibodies specific for HBV (34, 38). In this study, we demonstrated a potent protective effect of CrmA expression on the liver damage induced by the anti-Fas antibody. CrmA also inhibits the granzyme B produced by activated cytotoxic T cells. Therefore, expression of CrmA renders the cells resistant to being killed by the T cells (6, 39). These findings suggest that *crmA* gene-transfection may be a potent therapeutic treatment for clinical patients suffering from immune-mediated fulminant hepatitis.

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