liposome. In all experiments, five animals were used for each condition in all three of the experiments.

Characteristic immunogenicity of the HIV-HBc in mice and guinea pigs

To study the immune responses in mice, BALB/c mice were injected with an HIV-HBc chimeric protein and an HBc protein. Effector cells from the spleens of the mice immunized with HIV-HBc were generated by incubation with the V3 peptide in vitro. The stimulated effector cells significantly lysed target cells coated with the identical peptide but they did not lyse cells that were not coated (Fig. 3A). The induction of cytolytic response was specific to the HIV-antigen when it was expressed inside the chimeric protein particle; cytolytic activity was not detected when mice were immunized with an HBc protein alone and assayed for V3-specific CTL under the same condition (open triangle in Fig. 3A). Furthermore, the reactivity was restricted to the class I major histocompatibility complex, H-2^d, because cytolytic activity was not seen against allogeneic cells of BW5147 (H-2^k) and S49 (H-2^s) coated with the peptide (Fig. 3B) and the recognition of peptide 18IIIB and peptide 18MN are restricted by class I D^d molecule [28,42].

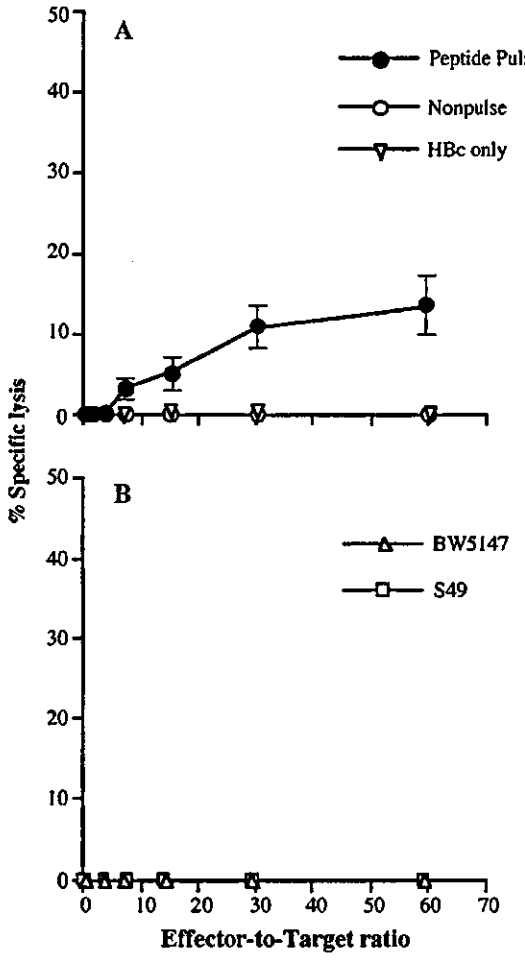

Fig. 3. Immunization of HIV-HBc chimeric antigen induces HIV-specific CTL in mice. (A) Cytolytic activity was measured against M12.4.5 target cells pulsed or not pulsed with the V3-tip peptide. The restimulated spleen cells from HIV-HBc-immunized mice were incubated with ^{51}Cr-labeled target cells that were either pretreated with the synthetic peptide (●), or untreated (○). As a control, the effector cell was prepared from HBc-immunized animals, and was incubated with ^{51}Cr-labeled target cells pretreated with the same synthetic peptide (▽). (B) Cytolysis was restricted to the major histocompatibility complex class I. Cytolysis was measured against BW5147 (△) and S49 (□).

The stimulatory effects of the HIV V3 peptide on the proliferative responses of spleen cells from the HIV-HBc immunized mice were tested 7 days after re-immunization with the same antigen. The V3 peptide stimulation did not enhance proliferations of the immune spleen cells at concentrations from 1 to 100 μg/ml, whereas HBc elicited more than 10 SI in all HIV-HBc- or HBc-immunized animals (data not shown).

Similarly, antibody titers of the sera from the above two groups specific for HIV-V3-tip antigen were all less than 10, which a value of 10 reflected an undetectable level of the antibody titer by the assay. However, HBc specific antibody titers were detected at 1:870–1150 in both groups of animals tested (data not shown).

Taken together, although these results demonstrate that immunization with a confined antigen (such as an HIV-V3 peptide expressed inside HIV-HBc particle) is able to induce HIV-specific CTL activity, neither antigen-specific $CD4^+$ T-cell nor humoral responses were observed. These results suggest that the HIV-HBc chimeric particle may induce HIV antigen-specific memory cells, but not induce effector cells effectively.

Booster injection of HVJ protein including HIV-liposome (HIV-HVJ-liposome) makes it possible to elicit $CD4^+$ T-cell response, enhanced CTL, and neutralization antibody production specific for the HIV-antigen

To study whether we could elicit marked HIV-specific immune responses to animals primed with the HIV-HBc chimeric protein, HVJ-protein was incorporated into HIV-liposome, which were used in antigen-primed animals as a booster injection. We initially characterized the effect of the incorporation of the HVJ protein into the liposome in a consecutive immunization strategy involving priming with HIV-HBc and boosting with anionic HIV_{HXB2}-HVJ-liposome. When HIV_{HXB2} V3-peptide was used, peptide-specific proliferative responses were detected with the addition of 5 μg/ml of the peptide to the culture of spleen cells from the immunized animals with the consecutive prime/boost regimen (Fig. 4). However, a lack of incorporation of the HVJ protein in HIV_{HXB2}-liposome in the booster antigen in the immunization strategy resulted in a marked decrease in the intensity of the proliferative response. In control animals that only had received a booster injection of HIV-HVJ-liposome, proliferative responses were not detected. Con A was used at a concentration of 2 μg/ml in spleen cell

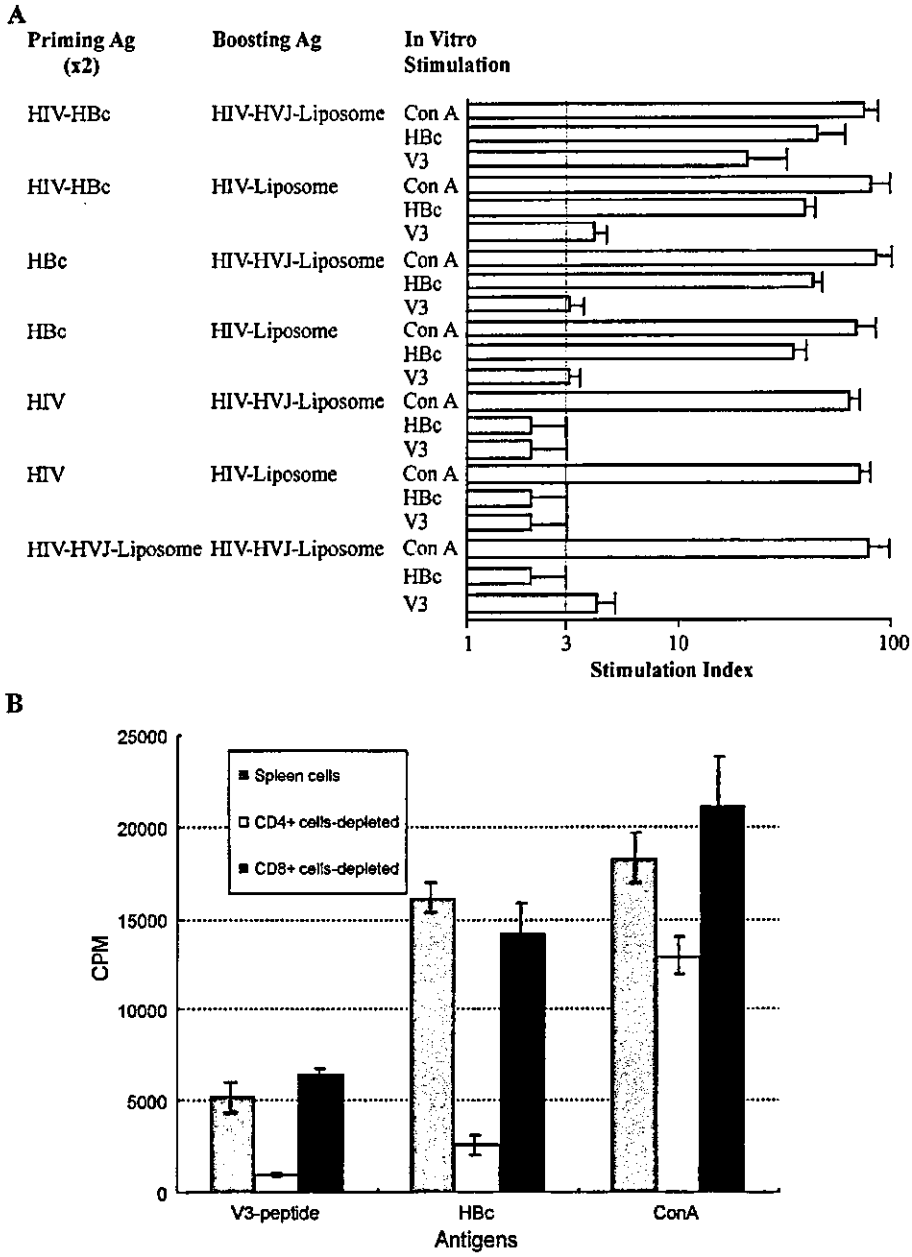

Fig. 4. $CD4^+$ T-cell proliferative responses specific for HIV-1 Env-V3 antigens. (A) The proliferative responses can be induced in animals immunized with the HIV-HVJ-liposome, which incorporated the HVJ protein into the HIV-liposome in a consecutive immunization regimen, involving priming with HIV-HBc and boosting with the HIV-HVJ-liposome. An anionic HIV-HVJ-liposome booster injection into HIV-HBc-primed guinea pigs resulted in a rise in lymphoproliferative responses to HIV-1 Env V3 antigens. The SIs of PBMC obtained from five guinea pigs in each group with various immunization regimens are shown in comparison to those obtained by stimulation with HIV-1_{HXB2} V3 circular peptide, HIV-1_{MN} V3 circular peptide, HBc protein, or medium alone. SIs are expressed as mean ± SEMs. Because results of SIs obtained by using HIV-1_{HXB2} V3 circular peptide and HIV-1_{MN} V3 circular peptide were roughly similar, the former results were shown. (B) Aliquots of spleen cells from mice vaccinated with the prime-boost regimen were depleted of $CD4^+$ or $CD8^+$ population before measuring the V3 peptide-specific proliferative responses.

cultures of normal animals and SI was always defined as more than 50. These results provide evidence that the incorporation of the HVJ protein into liposomes affects induction of a strong HIV-specific proliferative response in animals that had been immunized with the prime/boost regimen. Among the six groups, splenocytes from the mice in Group 1 (immunized with a prime-boost regimen) showed the highest levels of T-cell proliferative responses against the V3-loop peptides. The mean SI of each of the seven groups was 23.6 ± 12, 3.8 ± 2.4, 3.3 ± 1.3, 3.1 ± 1.5, 2.1 ± 1.3, 2.3 ± 1.4, and 4.4 ± 2.8, respectively (Fig. 4A). Depletion of the $CD4^+$ T-cell fraction dramatically reduced the proliferative responses from Group 1 to <10% (Fig. 4B). In contrast, proliferative activity was not affected by the depletion of the $CD8^+$ fraction from the cell suspensions.

We evaluated the effect of the anionic-type HIV-HVJ-liposome on induction of HIV-V3-specific CTL. The HIV-HVJ-liposome was administered to mice 3 weeks after immunization with HIV-HBc. CTL activity was clearly induced against syngeneic target cells pulsed with the HIV-1_{HXB2} V3 peptide (Figs. 5A and C) or HIV_{MN} V3 peptide (Figs. 5B and D) at an effector-to-target ratio from <1:6.25 in the mice inoculated with the booster injection of the HVJ-liposome that encapsulated the circular V3 peptide. However, in the booster injection of liposome that did not incorporate HVJ, the induction of HIV-specific CTL activities in the HIV-HBc primed animals was detected at an E/T ratio of 1:50–100. Furthermore, the CTL activity in the animals immunized with HVJ-HIV-liposome only was 18 ± 8.5% at 1:60 which is significantly less than that of prime-boost regimen consisting of HBc-HIV and HVJ-HIV-liposome (closed square in Figs. 5A and B). This result demonstrates that the incorporation of HVJ into liposome enhances CTL activity approximately 10-fold that of animals immunized with HVJ-unincorporated liposome (Figs. 5A and B). The enhanced induction of CTL activity by the HIV-HVJ-liposome is HIV-antigen specific and the reactivity was restricted to the class I-major histocompatibility complex, as is also shown in Fig. 3 (Figs. 5C and D).

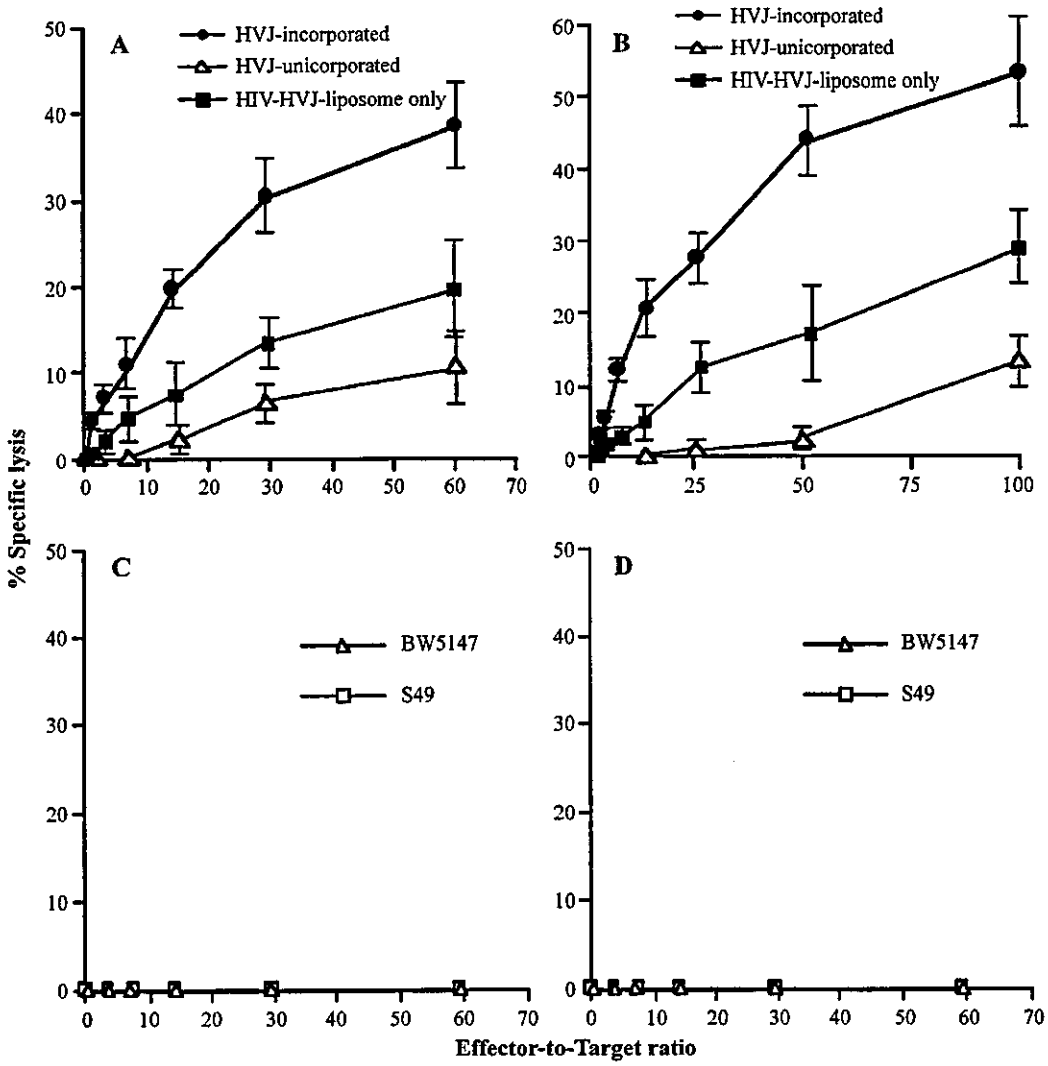

Fig. 5. Effect of the incorporation of the HVJ protein in terms of induction of HIV-1-specific CTL responses in the consecutively vaccinated mice primed with HIV-HBc followed by administration of HVJ-HIV-liposome. (A) Enhancement of HIV-1-specific CTL responses in the consecutively vaccinated mice with HIV-1_{HXB2}-HBc immunization followed by HIV-1_{HXB2}-HBc-liposome. Cytolytic activity was measured against M12.4.5 target cells pulsed or not pulsed with the V3-TIP peptide. The restimulated spleen cells from HIV-HBc-immunized mice followed by HIV-HVJ-liposome or followed by HIV-liposome were incubated with ^{51}Cr-labeled target cells. (●) cytolytic activity of V3-peptide-stimulated spleen cells from animals immunized with HIV-HBc priming followed by boosting with HVJ-incorporated HIV-liposome; (△) cytolytic activity of V3-peptide-stimulated spleen cells from animals immunized with HBc-HIV priming followed by boosting with HVJ-unincorporated HIV-liposome; and (■) cytolytic activity of V3-peptide-stimulated spleen cells from animals immunized with HVJ-incorporated HIV-liposome alone. (B) Similar enhancement of CTL activities was detected by a booster injection of HIV-1_{MN}-HVJ-liposome in animals with a prior immunization with HIV-1_{MN}-HBc injection. The results are expressed as the mean of three different experiments using five mice in each group. (C and D) Cytolysis was restricted to the major histocompatibility complex class I. Cytolysis was measured against BW5147 (△) and S49 (□).

HIV-V3-specific antibody responses were also induced in the HIV-HBc-primed animals boosting of the HIV-HVJ-liposome in the consecutive immunization regimen as well as HIV-HVJ-liposome immunization only. The guinea pigs of the two immunized groups similarly exhibited V3-binding antibody activity at serum dilutions more than 12,800 by HIV_{HXB2} V3 (Fig. 6A) or HIV_{MN} V3 (Fig. 6B) ELISA at 10 weeks after immunization. The serum antibody was purified from the HIV-HBc-immunized guinea pigs followed by the booster injection with HIV-HVJ-liposome or with HIV-liposome without HVJ, or from the animals immunized with HVJ-HIV-liposome only. PBMC-based virus neutralization assay with PBMC-passaged HIV_{LAI}, HIV_{MN} and HIV_{Th22} was used for the analysis (Fig. 6C). Measurements of inhibitory dose of 50% reduction of virus neutralization (ID_{50}) showed that the antibodies type-specifically neutralized the laboratory strain of clade B HIV_{LAI} and HIV_{MN2} with ID_{50} of serum antibodies from the HIV_{HXB2}- or HIV_{MN}-HVJ-liposome-boostered guinea pigs as well as from animals immunized with HIV_{MN}-HVJ-liposome only were approximately 6.5–15.5 μg/ml (Fig. 6C) but not HIV CRF01 AE, HIV_{Th22} (data not shown). However, virus neutralizations were not detected in sera from the HIV-HBc-immunized guinea pigs

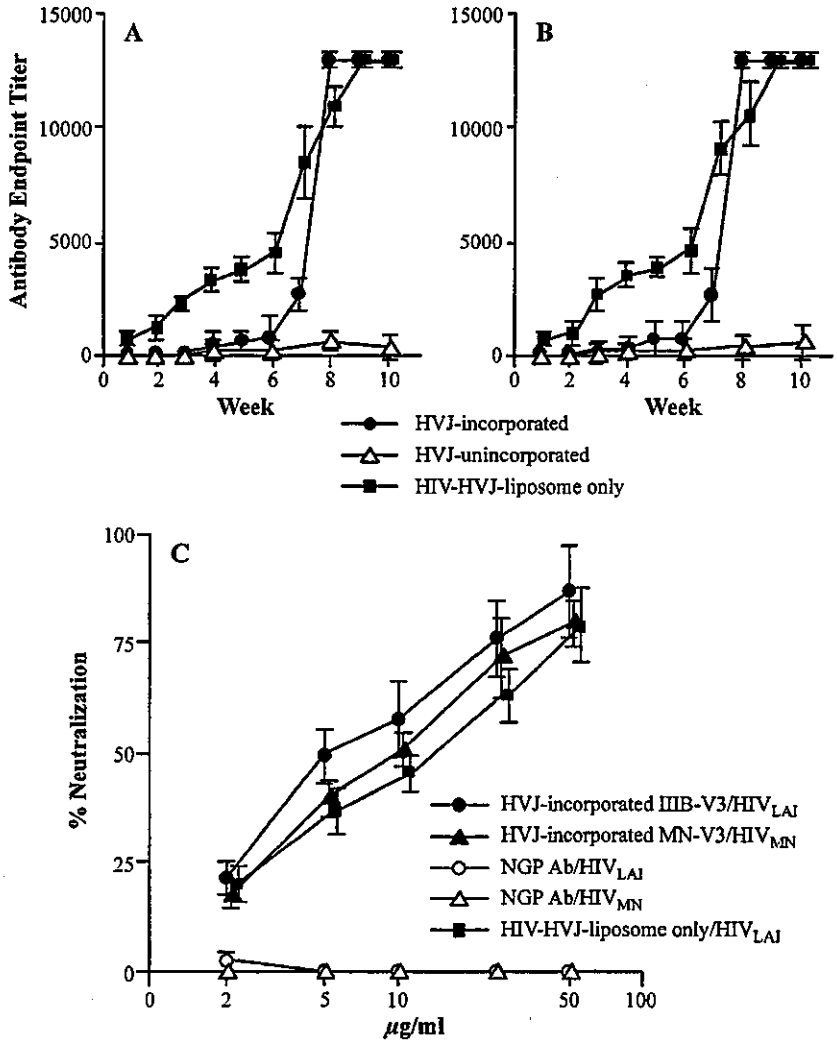

Fig. 6. HIV-1 antibody induction in a consecutive immunization regimen involving HIV-HBc immunization followed by the HIV-HVJ-liposome. HIV-1 antibody titers against HIV_{HXB2} V3 peptide (A) or HIV_{MN} V3 peptide (B) in the immunized animals were measured by ELISA with an endpoint dilution of immune sera. (●), sera from animals immunized with HBc-HIV priming followed by boosting with HVJ-incorporated HIV-liposome; (△) sera from animals immunized with HBc-HIV priming followed by boosting with HVJ-unincorporated HIV-liposome; and (■) sera from animals immunized with HVJ-incorporated HIV-liposome alone. (C) Detection of neutralization activity in the immune sera from the consecutively vaccinated animals. Neutralization activities are expressed as percentage inhibition, compared with control activity, and the mean of four different assays. (●), neutralization activity of serum IgG from animals immunized with HBc-HIV_{IIIB} priming followed by boosting with HVJ-incorporated HIV_{IIIB}-liposome; (▲) neutralization activity of serum IgG from animals immunized with HBc-HIV_{MN} priming followed by boosting with HVJ-incorporated HIV_{MN}-liposome; (○) neutralization activity of normal serum IgG against HIV_{IIIB}; (△) neutralization activity of normal serum IgG against HIV_{MN}; and (■) neutralization activity of serum IgG from animals immunized with HVJ-incorporated HIV-liposome alone.

following booster injections with HVJ-unicorporated HIV-liposome and in the preimmune animals or in naïve animals.

IL-12, IL-18, and IFN-γ productions are induced by stimulation with HVJ protein

We confirmed the HVJ protein-induced enhancement of IL-12, IL-18, and IFN-γ productions of spleen cells from animals immunized with HBc-HIV priming followed by HVJ-HIV-liposome boosting (Fig. 7). The enhanced production was specific to stimulation with HVJ protein for 24 h, but not with nonspecific culture with BSA. The result suggests that IL-18 may synergistically act with IL-12 to enhance IFN-γ production.

Discussion

This report describes a study, when an HVJ protein was incorporated into an HIV-liposome and used as a booster immunization in HIV-HBc-primed animals, the immunized animals demonstrated the induction of a strong HIV-specific $CD4^+$ T-cell response. The animals immunized with a consecutive immunization strategy were characterized. The analysis revealed enhanced cellular and humoral immunities. The findings thus suggest that the incorporation of the HVJ protein into the HIV-liposome significantly affects immunity in animals primed with HIV antigen encapsulated inside an HBc particle. Furthermore, the present results suggest that the HBc particle-based vaccine seems to be a suitable immunogen for an HIV-1 vaccine; this protocol effectively uses a booster immunization of an HIV antigen incorporated in an anionic HVJ-liposome.

In the present study, the HIV-liposome did not induce an HIV-specific proliferative response in HIV-HBc primed animals. However, the HVJ protein induced an immune response when it was incorporated into an HIV-liposome and used as a booster antigen. Moreover, a comparison of the T-cell proliferative responses inducing activity of both anionic-type and cationic-type HIV-HVJ-liposome demonstrated that the anionic liposome was more effective at inducing such activity than the cationic HIV-HVJ-liposome, when both are administered subcutaneously (data not shown). The effectiveness of the HVJ protein incorporation into the HIV-liposome at inducing the T-cell proliferative cell response does not seem to depend solely on the electrical charge, however. Instead, the effect depended on the route of antigen administration. This assumption was made because when the HIV-HVJ-liposome was administered nasally to mice, the HIV-HVJ-liposome was seen to induce antigen-specific CTLs and neutralizing antibody responses [47]. The different effects of HVJ-anionic and -cationic liposomes allow some inferences to be made about the antigen uptake rate into immune-competent cells. We previously developed a highly efficient method of gene transfer involving the entrapment of RNA or DNA using the HVJ protein to enhance the uptake of genes into target cells [48]. In that method, cationic lipids were used for the preparation of the liposome; the transgene expression level thereby significantly improved in cultured cells using this cationic-liposome gene delivery system [39,49]. Cellular uptake to targeted RNA complexed with an HVJ-cationic liposome was measured to be approximately 5 times higher than that of an HVJ-anionic liposome in cultured cell line cells [49]. In this study, we developed a highly efficient method for antigen immunization by delivering the antigen into cells using HVJ-anionic liposomes in experimental

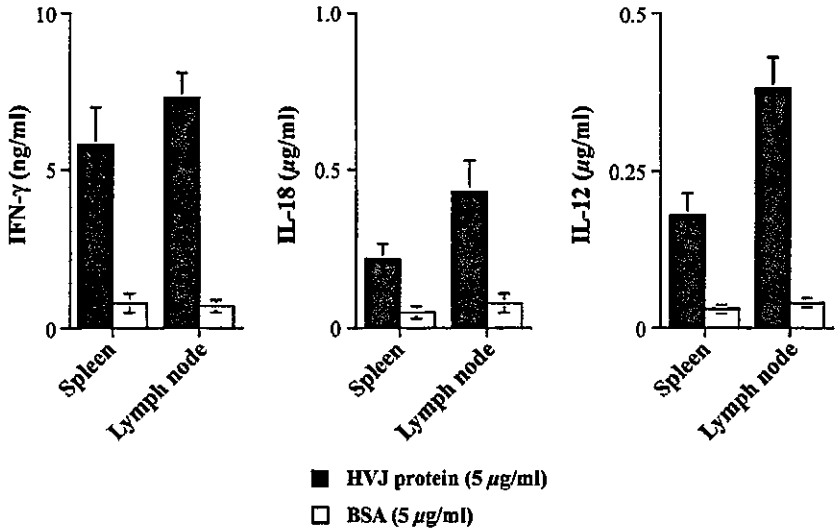

Fig. 7. IFN-γ, IL-18, and IL-12 secretion in HVJ extract-stimulated spleen cells from animals immunized with HBc-HIV priming followed by boosting with HVJ-HIV-liposome. Freshly isolated splenocytes were stimulated with 5.0 μg/ml of HVJ extract or same concentration of BSA for 24 h. Supernatants from separately cultured cells of five different animals were harvested and each cytokine amounts in supernatants were measured by ELISAs. The mean ± SD of three separate experiments is shown.

small animals. According to the present method, the anionic-liposome fused with HVJ was significantly effective at inducing an antigen-specific T-cell proliferative response. In fact, this method proved more effective than the use of antigen-primed animals that had received a booster injection of an HVJ cationic-liposome that was fused with an antigen (data not shown). Collectively, our results indicate that when the induction of antigen-specific T-cell immunity was targeted systemically, the use of an HVJ anionic liposome is suitable, because the antigen may not be localized at the injection site but rather be delivered throughout the body. Furthermore, antigen entrapment was enhanced by the effect of HVJ. However, when the immune induction is targeted locally, the cationic type of HVJ-liposome seems to be more effective as an antigen-delivery system, because the cationic HVJ-liposome effectively fused with the antigen and delivered the target antigen into cells located in a relatively limited region. Our observation also shows that HVJ protein is effective for the induction of an immune response. These findings suggest that enhancement of antigen uptake might be responsible for the induction of effective immune responses.

Concerning the adjuvant effect of the liposomes with HVJ protein, the HVJ protein appears to have the ability to enhance the secretion of immune enhancing cytokines, such as IL-12, IL-18, and IFN-γ, because those cytokines are released form splenocytes by in vitro culture with the HVJ-protein. Our results suggest that IL-18 released from stimulated macrophages may synergistically act with secreted IL-12 to stimulate enhanced production of IFN-γ. Stimulation of spleen cells or T-cell clones with HVJ protein-induced IFNs, TNF-α, and -β in vitro has been reported [50,51]. Thus, stimulation of lymphoid cells by HVJ protein might play a role in the enhanced induction of immunity. Pirhonen et al. [52] reported that the Sendai virus was able to enhance IL-18 level in macrophages, and that the data suggested that indirect immune activation by the effect of IL-18 produced by the stimulation of HVJ protein may also play a role in helper cell induction. This was thought to be because IL-18 can stimulate Th0 cells and promote the differentiation of cells to induce IFN-γ or IL-4 production in the presence or absence of IL-12 [53–55]. This co-stimulation of the immune system by a viral protein or component is commonly seen in the presence of other viruses or bacteria, for example, influenza virus and mycobacterium. The influenza fusion protein was similarly incorporated in fusigenic liposomes and used as a liposome-type adjuvant, namely, Virosome [56]. The advantage of the use of the HVJ protein is that it is known to be a highly fusigenic protein and has even previously been used for cell fusion to produce hybridoma. This method is expected to enhance the uptake of antigens into cells. The results show that repeated inoculations may be acceptable for in vivo use. In conclusion, it seems likely that HVJ protein-incorporated liposomes fused with antigen may enhance antigen uptake to the immunocompetent cells via the HVJ protein. Thus, the HVJ protein may also stimulate helper cells to differentiate and produce cytokines, thus enhancing immune responses.

As described above, the use of the HVJ protein for the preparation of HIV-liposomes allowed us to overcome the difficulty of immune induction induced by immunization of an antigen within the particles. Specifically, we were able to substantially induce a $CD4^+$ T-cell proliferative response. Furthermore, we observed a significant association among the induction of HIV-specific humoral response, and the enhancement of an HIV-specific CTL response due to immunization of HIV-HBc primed animals with the HVJ protein-incorporated HIV-liposome. These marked inductions of immunity are obtained by incorporating the HVJ protein into the anionic HIV-liposome, which were used as a booster antigen in a consecutive immunization regimen. The reason why the HVJ protein was able to induce HIV-specific immunity so effectively in the animal model may be that the consecutive immunization strategy, which employed the HVJ protein, can induce a marked $CD4^+$ T-cell response specific for HIV in the animal. We did not test a group immunized with the HVJ protein mixed with but not incorporated into the HIV-liposome. We speculate, however, that the HVJ protein was incorporated into the HIV-liposomes, and that after the incorporated HVJ peptide and HIV circular V3 peptide were mixed and trapped together on/in the liposome, the mixture was effective. If this is the case, the HVJ protein might be beneficial for forming the protein mixture that will work for the T-cell epitope. This speculation about the mechanism of helper response induction is also supported by the observation that when the HIV-V3 peptide was covalently constructed with an overlapping T-cell epitope peptide, it induced CTL in a non-emulsion adjuvant [57]. When the unlinked but mixed peptides were trapped together, the mixture also worked to some extent in a water-in-oil emulsion adjuvant [58,59]. The significance of the helper T-cell response has been demonstrated in other chronic viral infections [60]. The induction of a $CD4^+$ T-cell response in controlling HIV generation has recently shown that virus-specific T helper lymphocytes are critical for the maintenance of effective immunity in chronic viral infections [11]. The HIV-specific T-cell response is likely to be important in immunotherapeutic interventions [61] and vaccine development [62]. One of the explanations of the role of the $CD4^+$ T cells in CTL induction is that the $CD4^+$ T-cell help was mediated by binding with CD4 ligand on dendritic cells in the expansion of HIV-specific $CD8^+$ memory T-cell responses [63]. In contrast, in the absence of $CD4^+$ T-cell help, adequate CTL activity was not maintained and revealed the persistence of activated virus-specific CTL without effector function [64].

In conclusion, induction of a strong T-cell proliferative response has been obtained in a small animal model by using a consecutive immunization strategy that involved priming with HIV-HBc and boosting with the HIV-HVJ-liposome. Furthermore, it might be demonstrated that an efficacy test for viral challenges may be available.

Acknowledgments

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Transposon-independent increase of transcription by the Sleeping Beauty transposase

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Abstract

When a plasmid encoding the Sleeping Beauty (SB) transposase (pCMV-SB) was cointroduced with luciferase expression plasmid DNA into mouse skeletal muscle at a molar ratio of 4:1, luciferase gene expression was 5-times higher than the expression without pCMV-SB on day 28. This enhancement was not dependent on the presence of transposon (Tn) sequence in luciferase expression plasmid. Southern blot analysis failed to detect luciferase gene insertion into the host genome. Then, expression, a luciferase expression plasmid without Tn was cointroduced into HeLa cells with or without pCMV-SB. With pCMV-SB, the mRNA amount and the luciferase activity was 1.5 times and 2 times higher, respectively, than without pCMV-SB, even though the cells with pCMV-SB had a smaller copy number of luciferase plasmid than the cells without pCMV-SB. These results suggest that SB transposase enhances the transcription of an exogenous gene regardless of the presence of the Tn sequence.

Keywords: Transposon; Transposase; Gene expression; Transcription

Gene therapy requires the sustained expression of the therapeutic gene. Numerous vector systems have been developed for long-term gene expression [1–5].

Much attention has been paid to vertebrate transposons that were identified in teleost fish [6]. In the presence of transposase, the transposable elements can integrate into the host genome [7]. Sleeping Beauty (SB) transposase, which was reconstituted from inactive elements in the teleost fish genome, induces effective transposition in vertebrates [8]. The SB transposon is a Tc/mariner transposon that consists of two inverted terminal repeat (ITR) sequences of 225 bp that flank exogenous DNA [8]. SB transposase binds to two 30-bp sequences in each of the ITRs to bring the ends of the transposon together and to cleave the transposon from a donor plasmid for integration into another DNA sequence at AT-dinucleotide target sites [9]. Therefore, SB transposons integrate randomly into the host genome. Further investigations have been conducted to improve SB transposon-mediated gene insertion efficiency [10–12].

The SB transposon system has been widely used in gene therapy experiments. The SB transposon system has mediated long-term expression of factor IX gene for hemophilia [13], the fumarylacetoacetate hydrolase gene for tyrosinemia [14], and the laminin 5 gene in epidermal progenitor cells [15].

Thus, the SB transposon system is a promising tool for human gene therapy and gene insertion into the chromosomal genome by the SB transposon system has been widely noticed [16]. However, no previous reports have discussed the role of transposase on gene expression, especially its effect on transcription. Gene integration into the host genome was frequently detected in the nuclei of stable transformants [8]. But, does gene integration mediated by SB transposase occur so frequently in cells without any selection? Long-term gene expression by SB transposase was observed in the liver [13,14,17] and lung [18] by non-viral gene transfer methods. Linker-mediated PCR technology demonstrated transposition of pT/L into mouse chromosomal DNA in the lung [18]. However, there is no direct

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evidence to confirm gene integration into the chromosomal genome by Southern blot analysis.

We hypothesized that a mechanism other than gene insertion contributed to long-term gene expression by SB transposase. In this study, we found that SB transposase enhanced transcription of an exogenous gene on a plasmid independent of the presence of transposon sequences.

Materials and methods

Plasmid construction. pCMV-luciferase (pcLuci: 7.4 kb) was constructed by cloning the luciferase gene from the pGL3-Promoter Vector (Promega, Madison, WI, USA) into pcDNA3 (5.4 kb) (Invitrogen, San Diego, CA, USA) at the *Hin*dIII and *Bam*HI sites. pTn-SVNeo (4928 bp) and pCMV-SB (Sleeping Beauty: 4732 bp) were kind gifts from Dr. Perry Hackett (University of Minnesota, USA). pTn-Luci (6386 bp) was constructed by cloning the *Nru*I–*Nsi*I fragment that includes the luciferase gene from pcLuci into pTn-SVNeo at the *Nae*I site, which is compatible with the *Nru*I restriction site, and the *Nsi*I site. Plasmids were purified with the Qiagen plasmid isolation kit (Hilden, Germany).

In vitro transfection. HeLa cells were collected, washed with phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1 mM KH_2PO_4), and resuspended at a final concentration of 6×10^6 cells/800 μl PBS. The cell suspension (800 μl) was mixed with plasmid DNA in an electroporation cuvette (0.4-cm gap from Bio-Rad) and incubated for 10 min on ice. Electroporation was performed with Gene Pulser II (Bio-Rad Laboratories, CA) at 0.25 kV with 975 μF. The following ratios of pTn-Luci and pCMV-SB were used: 3.7 μg of pCMV-SB, 5.0 μg of pTn-Luci, and 33.7 μg of pcDNA3.1 (Invitrogen) for a 1:1 ratio; 14.9 μg of pCMV-SB, 5.0 μg of pTn-Luci, and 21.1 μg of pcDNA3.1 for a 4:1 ratio; and 29.7 μg of pCMV-SB, 5.0 μg of pTn-Luci, and 4.2 μg of pcDNA3.1 for an 8:1 ratio. In the experiment for Fig. 5, plasmid DNA was added as follows: 17.8 μg of pcDNA3.1 and 6.0 μg of pcLuci for the pcLuci plus pcDNA3.1 group; and 15.6 μg of pCMV-SB and 6.0 μg of pcLuci for the pcLuci plus pCMV-SB group. In both cases, pcDNA3.1 plasmid was added to equalize the copy number of plasmids among the groups.

In vivo transfection. For in vivo transfection, the following 4 plasmid combinations were used: 23.3 μg of pCMV-SB and 7.9 μg of pTn-Luci; 26.6 μg of pcDNA3.1 and 7.9 μg of pTn-Luci; 26.6 μg of pcDNA3.1 and 9.0 μg of pcLuci; and 23.3 μg of pCMV-SB and 9.0 μg of pcLuci. There was an equal copy number of total plasmids in the 4 groups. The plasmid mixtures were incorporated into HVJ-liposomes prepared as described previously [19]. Seven-week-old male Balb/c mice were anesthetized, and 50 μl of HVJ-liposomes containing the plasmid mixture were injected into the tibialis anterior muscles. Mice were killed by deep anesthesia with pentobarbital on days 3, 7, 14, 21, and 28 after injection. All mice were handled in a humane manner in accordance with the guidelines of the Animal Committee of Osaka University.

Luciferase assay. For in vitro analysis, HeLa cells were collected on day 3 after in vitro transfection and suspended in 500-μl cell culture lysis reagent. For in vivo analysis, mice were killed under deep anesthesia on days 3, 7, 14, 21, and 28. Tibialis anterior muscles were immediately resected and minced in 5 volumes of cell culture lysis reagent (Promega). The lysates were then clarified by centrifugation at 15,000 rpm for 10 min at 4 °C, and 20 μl of each supernatant was subjected to a luciferase assay using the Promega luciferase assay system and a Lumat LB 9501 luminophotometer as described previously [19].

Southern blot analysis. Total genomic DNA from tibialis anterior muscles was extracted on days 3 and 28 after injection as described previously [5] and suspended in 30 μl TE (10 mM Tris–Cl, 1 mM EDTA, pH 8). Two micrograms of genomic DNA was digested with *Eco*RV or with both *Eco*RV and *Hin*dIII and subjected to Southern blot analysis. ^{32}P-labeled luciferase gene was used as a probe.

PCR analysis. Genomic DNA was prepared from tibialis anterior muscles of mice on day 28 and amplified by PCR with oligonucleotide primers (5′-TCATGAACTCCTCTGGATCTACTG-3′ and 5′-AGAA TGTAGCCATCCATCCTTGTC-3′) to detect the luciferase gene using *Taq* polymerase. The amplification was performed with 1 cycle of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 57 °C, and 1 min at 72 °C. The final incubation was 5 min at 72 °C. The expected product size for the luciferase amplicon is a 466-bp fragment.

To detect the SB DNA fragment, genomic DNA was amplified by PCR with oligonucleotide primers (5′-AGATGGCATCATGAGG AAGG-3′ and 5′-TTCCTCCTGACAGAGCTGGT-3′) using *Taq* polymerase. The fragment was amplified with 1 cycle of 5 min at 94 °C, followed by 30 cycles of 30 seconds at 94 °C, 30 s at 56 °C, and 1 min at 72 °C. The final incubation was 5 min at 72 °C. The expected product size for the transposase (SB10) amplicon is a 294-bp fragment.

Northern blot analysis. On day 3 after in vitro transfection, HeLa cells were collected for Northern blot analysis. Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan). Ten micrograms of RNA was separated in a formalin gel and subjected to Northern blot analysis using a ^{32}P-labeled luciferase gene as a probe. G3PDH probe was used as an internal control.

Results

First, we determined the optimum ratio of pTn-Luci and transposase plasmid for luciferase gene expression in HeLa cells. As shown in Fig. 1, low gene expression was obtained when both plasmids were transferred to cells at an equimolar ratio. An excess amount of transposase plasmid gave rise to high luciferase activity on days 1, 3, and 7. Consequently, we decided to use a 1:4 ratio of pTn-Luci to transposase plasmid.

Next, we examined the effect of the Tn/transposase system on long-term gene expression in vivo. Either pTn-Luci or pcLuci was transferred into mouse tibialis anterior muscles with or without transposase plasmid using

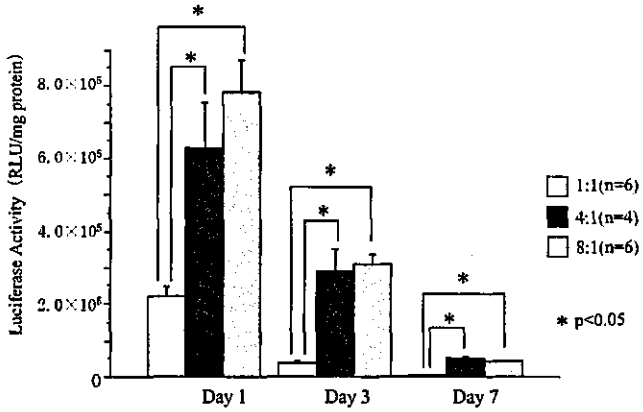

Fig. 1. Luciferase expression in vitro. The molar ratios of pCMV-SB to pTnLuci are 1:1, 4:1, and 8:1. Plasmids were transferred to HeLa cells by electroporation using Gene Pulser II (Bio-Rad Laboratories, CA) at 0.25 kV with 975 μF. On days 1, 3, and 7, cells were harvested to measure luciferase activity.

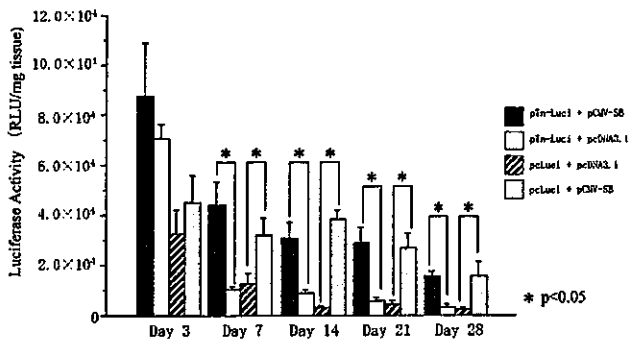

Fig. 2. Luciferase expression in vivo. On days 3, 7, 14, 21, and 28 after cointroduction of the luciferase gene (with or without Tn) with pCMV-SB into the tibialis anterior muscle, tibialis anterior muscle was harvested to measure luciferase activity. The number of animals used in this experiment was 20 on day 3, 14 on day 14, and 8 on days 7, 21, and 28.

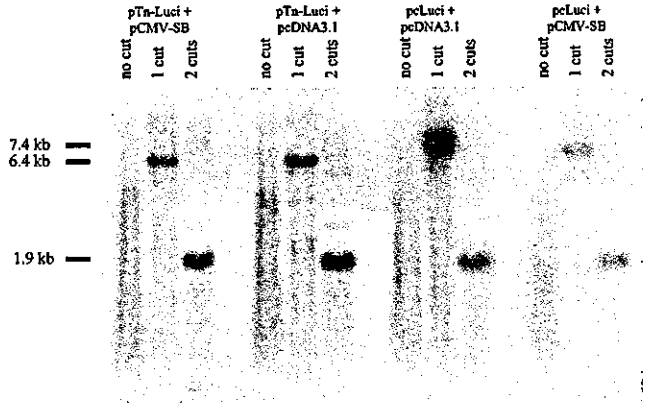

Fig. 3. Southern blot detection of the luciferase gene in muscle tissue. Total genomic DNA from the tibialis anterior muscle was extracted on day 3 after injection. Two micrograms of genomic DNA was undigested, digested with *Eco*RV (1 cut) or digested with both *Eco*RV and *Hin*dIII (2 cuts) and subjected to Southern blot analysis with ^{32}P-labeled luciferase gene as a probe. The bars indicate pc-Luci (7.4 kB), pTn-Luci (6.4 kB), and the luciferase gene (1.9 kB).

HVJ-liposomes (Fig. 2). By cotransferring pTn-Luci with transposase plasmid, the highest luciferase gene expression in muscle was obtained on day 3. Although the expression was decreased after day 3, luciferase was still expressed 28 days after the transfer. However, without the transposase expression vector, the luciferase activity peaked on day 3 and then rapidly decreased to a very low level. Surprisingly, pcLuci without Tn caused long-term gene expression when cotransferred with transposase plasmid. In fact, the luciferase gene expression pattern caused by the cotransfection of pcLuci and transposase plasmid was very similar to that caused by the Tn/transposase cotransfection.

We used Southern blots and PCR to examine the existence of the luciferase gene in muscle. Southern blot analysis showed that the luciferase plasmid was present in all samples at the same size as each plasmid, indicating that the plasmid did not integrate into the host genome (Fig. 3). However, Southern blots could not detect a signal in any group 28 days after transfer (data not shown). We increased the amount of DNA to 10 μg/lane, but no signal was detected (data not shown). However, the luciferase gene was amplified in all samples by PCR (Fig. 4A). Next, we examined the presence of transposase DNA in muscle. PCR analysis showed that transposase DNA was still present on day 28 in mice that received HVJ-liposomes containing the transposase plasmid with pTn-Luci or pcLuci (Fig. 4B). These results suggest that transposase activates transcription without interacting with Tn.

To test the possibility that transposase activates transcription, pcLuci was transferred to HeLa cells with or without the transposase expression vector. Southern blots detected epichromosomal luciferase plasmid in both groups 3 days after the transfer. The amount of luciferase plasmid was 1.7-times less when

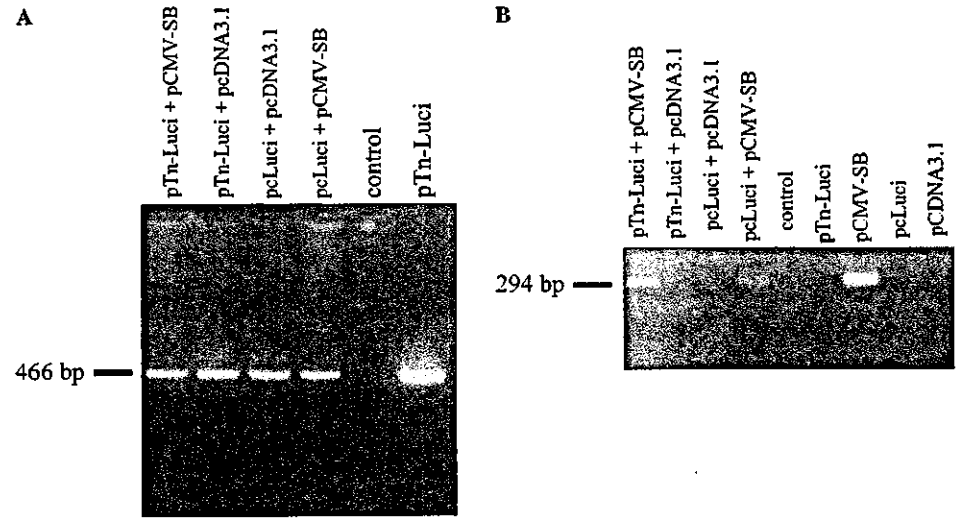

Fig. 4. Detection of the luciferase gene fragment (A) and the SB gene fragment (B). Genomic DNA was prepared from tibialis anterior muscles of mice 28 days after injection and amplified by PCR to detect the luciferase gene (466 bp) or the SB gene (294 bp). As a control, PCR was performed using a template of pTn-Luci, pCMV-SB, pcLuci or pcDNA3.1.1.

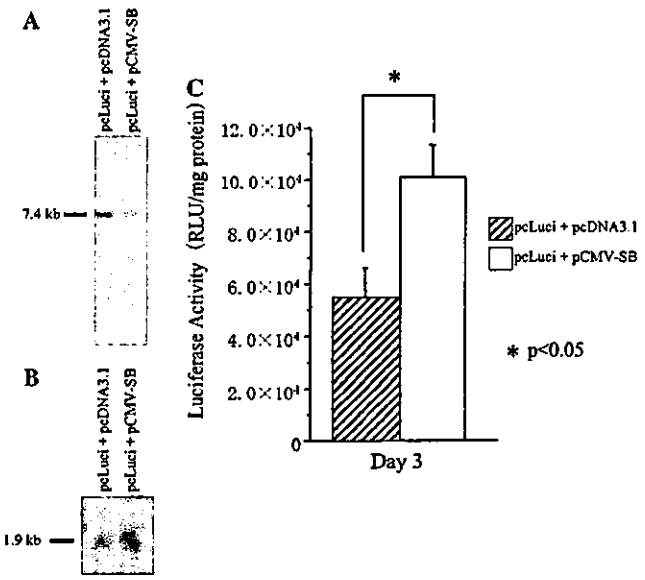

Fig. 5. Detection of luciferase gene (A), mRNA for luciferase (B), and luciferase activity (C) in HeLa cells. (A) On day 3, genomic DNA was prepared from HeLa cells transformed with pcLuci with and without SB plasmid. DNA was subjected to Southern blot analysis using ^{32}P-labeled luciferase gene as a probe. (B) On day 3, total RNA was prepared from HeLa cells transformed with pcLuci with and without SB plasmid. RNA was subjected to Northern blot analysis using ^{32}P-labeled luciferase gene as a probe. (C) On day 3, cell lysates were prepared from HeLa cells to measure luciferase activity.

cotransfected with the transposase plasmid than without the transposase plasmid (Fig. 5A). However, Northern blot analysis on day 3 revealed that 1.5-times more luciferase gene transcripts were detected in transfection with transposase vector than in transfection without transposase (Fig. 5B). The luciferase activity in cells cotransfected with transposase was approximately 2-times higher than that in cells without transposase (Fig. 5C).

Discussion

In this study, we found that transposase activates the transcription of cotransferred plasmid DNA even in the absence of transposon. The Tn/transposase system has been widely used for long-term gene expression since 1997 when fish transposase was reconstructed as Sleeping Beauty [8]. In all of these previous experiments, the transposase gene was cotransferred with a plasmid containing Tn, or a plasmid containing both Tn and transposase was constructed. When the optimum ratio of Tn and transposase for gene integration was studied, excess amounts of transposase inhibited transposition in culture [20–22]. Although the mechanism of transposition inhibition by excess transposase was not clearly elucidated, excess amounts of transposase have not been used in long-term gene expression studies. We transferred pTn-Luci with various amounts of the transposase plasmid. As shown in Fig. 1, excess amounts of transposase increased luciferase gene expression in cultured cells on days 1, 3, and 7. When pTn-Luci was transferred with an 8-fold excess of the transposase plasmid, cytotoxicity was observed. These results indicate that transposase might activate transcription on both the Tn plasmid and the host genome. Surprisingly, in the muscle gene transfer experiment, transposase activated luciferase gene expression in plasmid without Tn as well as in the Tn plasmid. On day 3, the luciferase gene existed epichromosomally without being integrated into the host genome. Since the luciferase gene was not detected by Southern blot on day 28, we could not determine if the luciferase gene was integrated into the host genome. However, no previous reports have showed Tn-transposase-mediated gene integration into the host genome by Southern blot analysis. Moreover, the amount of transposase plasmid exceeded the amount of Tn plasmid, and this condition is not recommended to induce transposition. Gene integration mediated by the Tn/transposase system has been shown in stable transformants of cultured cells after selection [8]. Without Tn, transposase did not enhance gene integration in stable transformants. Taken together, the integration of plasmid DNA without Tn into the host genome rarely occurred in muscle. Instead, transposase might enhance transcription of the transgene independent of Tn. This idea is supported by the data in Fig. 5, in which transposase increased transcription of Tn-deficient luciferase gene in HeLa cells without any selective pressure. Southern blots and PCR data did not indicate that SB affected long-term retention of epichromosomal plasmid DNA.

This is the first report of enhanced transcription by transposase. In *Arabidopsis*, FAR1 protein, which is related to the transposase of the type II MuDR family transposon, can activate transcription [23]. Epstein–Barr virus induces long-term gene expression by stable epichromosomal retention of the viral genome without integration into the host genome. When Epstein–Barr virus replicon plasmid containing oriP and EBNA-1 was used for transgene expression, long-term gene expression was also achieved [5]. When the mechanism of long-term gene expression was investigated, it was revealed that EBNA-1 enhanced transcription of the transgene by binding to oriP [24,25]. Even when gene integration does occur, gene expression cannot be maintained unless the chromatin structure of the integration site becomes active in transcription. The Tn/transposase system has been evaluated for long-term gene expression. We suspect that, in addition to playing a role in transposition, transposase plays a role in activating transcription of the transgene. To further analyse transposase function, the molecular complex containing transposase should be elucidated by coimmunoprecipitation.

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Intrathecal injection of HVJ-E containing HGF gene to cerebrospinal fluid can prevent and ameliorate hearing impairment in rats[1]

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SPECIFIC AIM

No satisfactory therapy for sensorineural hearing impairment is yet available because the auditory sensory epithelium (hair cell (HC)) and its associated neuron (spiral ganglion cell (SGC)) are hardly regenerated in mammalians. We developed a novel gene therapy strategy to prevent and ameliorate hearing impairment by administration of the hemagglutinating virus of Japan envelope (HVJ-E) vector containing hepatocyte growth factor (HGF) gene into the cerebrospinal fluid (CSF).

PRINCIPAL FINDINGS

1. Gene transfer into the inner ear region by intrathecal injection of HVJ-E vector

We developed an effective gene delivery system to the inner ear with minimum invasiveness. We used an HVJ-E vector system, a novel nonviral vector with fusion activity derived from hemagglutinating virus of Japan (HVJ) (Sendai virus). To examine the efficiency, distribution, and safety of the HVJ-E vector, we injected HVJ-E containing marker genes *lacZ* gene and luciferase gene intrathecally into the CSF of rats via the cisterna magna. No significant damage was observed in either the brain or ear tissues. β-gal expression was observed in the SGC and stria vascularis, as well as in the cerebral cortex, cerebellum, and medulla. Luciferase activity was detected in the cerebral cortex, medulla, and cochlea from rats injected with the HVJ-E containing luciferase gene but not in other organs such as the lung, spleen, or liver. These results showed that the HVJ-E vector reached the inner ear region after the intrathecal administration and transduced the gene into the tissues without significant damage.

2. In vivo transfection of the *HGF* gene into the subarachnoid space

We used HGF as a therapeutic molecule for hearing impairment. We administered HVJ-E containing the human *HGF* gene (*hHGF*) into the CSF and measured the protein level of HGF by ELISA. Human HGF protein was detected in the CSF of the rats transfected with *hHGF* even after 12 days of transfection (mean value 0.31 ng/mL on day 5). An increase of rat HGF was also observed in the CSF from the rats administered with *hHGF* (mean value 2.74 ng/mL on day 5). We immunocytochemically checked exogenous HGF expression in the SGCs obtained from rats inoculated with *hHGF* (pVAX1-*hHGF*) and compared the findings with the result from the control group using the control vector (pVAX1) alone. Human HGF was clearly observed in the cytoplasm of SGCs and the percentage of human HGF positive cells was >70%. We next examined the expression of c-Met, a tyrosine kinase receptor of HGF, on SGCs. In rats administered *hHGF*, the expression level of c-Met was greatly enhanced in SGC cytoplasm.

3. Protective and therapeutic effect of HGF on the inner ear damaged by kanamycin insult

We examined whether HGF can rescue the loss of the HC and SGC induced by kanamycin (KM) treatment. KM was used to mimic the clinical situation of hearing impairment, in which the HC is damaged and lost, leading to the degeneration of SGC due to the lack of neurotrophic substances and electric stimuli. Severe loss of the outer HC and partial loss of the inner HC were observed in the rats inoculated with KM and HVJ-E/pVAX1 (KM+vector group) (Fig. 1*I*). However, inner and outer HCs in the rats administered with KM and HVJ-E/pVAX1-*hHGF* (KM+HGF group), as well as those in the control rat, were well preserved (Fig. 1*H, J*). The number of surviving SGCs was assessed. A significant reduction of SGCs was observed in the KM +

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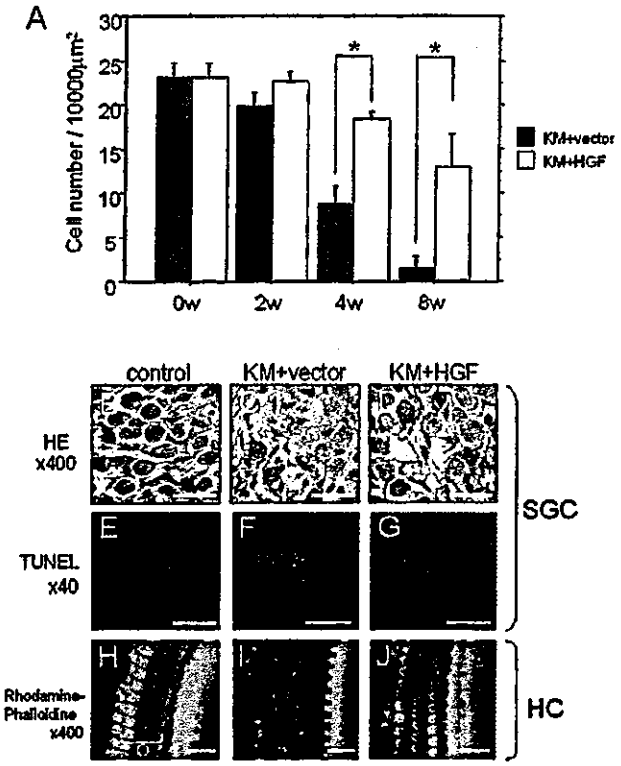

Figure 1. The protective effect of the HGF transgene on SGC and HC treated with kanamycin. Numbers of hematoxylin-positive cells of SGC of the rats treated with KM + vector or KM + HGF are counted at various time points (*A*) (n=6 for each group). Midmodiolar 10 μm cryosections from rats without treatment (control) (*B*), treated with kanamycin and HVJ-E containing control vector (*C*), or HVJ-E containing the human HGF gene (*D*) were stained with hematoxylin on wk 4. TUNEL staining of the contiguous sections of SGCs from the same rats as described above is shown in panels *E–G*. Fluorescent image of HC of the rats in the control, KM + vector, and KM + HGF groups is shown in panels *H–J*. O: outer hair cell, I: inner hair cell. Scale bar: *B–D, H–J)* 50 μm; *E–G)* 500 μm. *$P < 0.01$.

vector group 4 and 8 wk after KM administration (Fig. 1*A*). On the other hand, in the KM + HGF group, the cochlea showed significantly more surviving SGCs on wk 4 and 8 than the KM + vector group. On wk 8, the surviving cell count in the KM + HGF group was ~sixfold higher than those in the KM + vector group (13.3±3.2 cells/10000 μm^2 vs. 2.2±1.8 cells/10000 μm^2, P<0.05) (Fig. 1*A*). These results show that *HGF* gene transfer has a protective effect on HC and SGC survival. Light microscopic examination demonstrated there were many cells showing vacuolated cytoplasm and nuclei containing clumped chromatin in the KM + vector group (Fig. 1*C*). In the KM + HGF group, however, there were considerably fewer cells with such an appearance and most cells had an appearance similar to the control (Fig. 1*B, D*). TUNEL staining of SGC showed lower numbers of positive cells in the KM + HGF group than the KM + vector group and control rats (Fig. 1*E–G*). These results indicate that the death of HC and SGC in response to KM treatment could be inhibited by the intrathecal HVJ-E inoculation of the *HGF* gene. Hearing functions before and after KM treatment was also assessed by auditory brainstem response. Hearing impairment was prevented when the *HGF* gene was administered shortly before KM treatment (**Fig. 2***B*); even after induction of impairment by KM, hearing function could be recovered (Fig. 2*C*).

CONCLUSIONS AND SIGNIFICANCE

We demonstrated that intrathecal injection of HVJ-E containing *hHGF* into the CSF prevented the loss of HC and SGC by inhibition of apoptosis and showed a high therapeutic potential for both the prevention and treatment of hearing impairment. The success of this

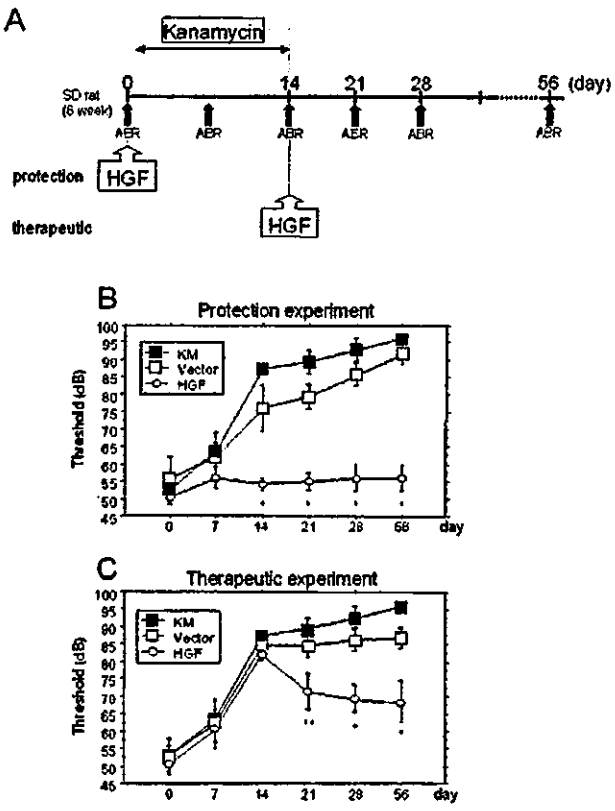

Figure 2. Hearing function of rats treated with KM, KM + vector, or KM + HGF was evaluated by auditory threshold using ABR. *A)* Time course of the experiment. In the protection experiment (*B*), rats treated with the HVJ-E containing control vector (vector) or HVJ-E containing the human *HGF* gene (HGF) immediately prior to the kanamycin insult underwent evaluation of the auditory threshold on days 0, 7, 14, 21, 28, and 56. In the therapeutic experiment (*C*), rats were treated with the HVJ-E containing control vector (vector) or HVJ-E containing the human *HGF* gene (HGF) 14 days after kanamycin insult and the auditory threshold was measured at each time point. KM means the auditory threshold of rats treated only with kanamycin. Six rats were used in each group. Mean and SD of each value are indicated.

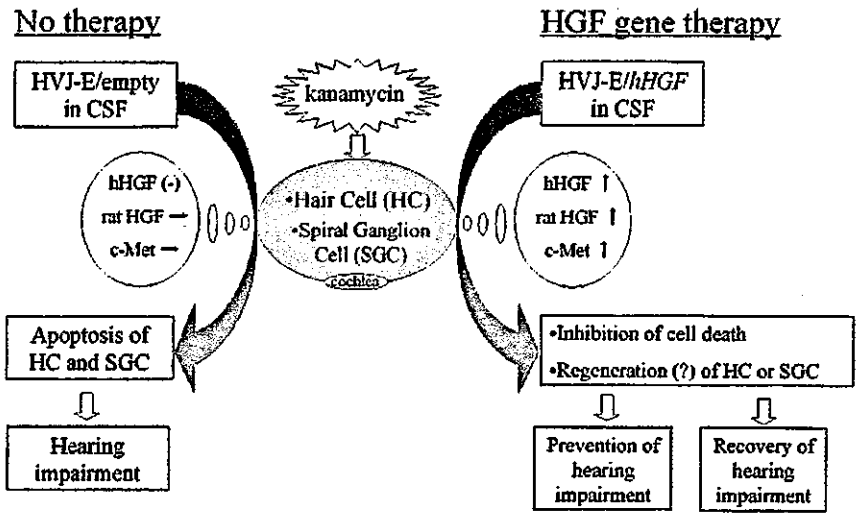

Figure 3. Schematic diagram.

gene therapy is due to two novel issues. One is the novel nonviral vector system; another, the therapeutic molecule with multiple functions.

We used the HVJ-E vector system as a delivery method to the inner ear. HVJ-E vector is constructed by treating HVJ with mild detergent and centrifugation in the presence of plasmid DNA. Our previous studies demonstrated the successful delivery of DNA using this method in vitro and in vivo. In this study, we injected the HVJ-E vector into the CSF to avoid invasion of the inner ear by direct injection to the cochlea. It is thought that HVJ-E most likely spread via the cochlear aqueduct, which connects the CSF to the perilymphatic space of the cochlea. Although safety issues regarding the dissemination of the vector beyond the targeted cochlea need to be addressed, this approach is advantageous, especially for bilateral cochlear gene therapy.

Several neurotrophic factors have been used as therapeutic molecules for the auditory systems. HGF, however, has not been used for this purpose so far. HGF was first identified as a potent mitogen for mature hepatocytes and proved to have multiple functions such as angiogenetic, anti-apoptotic, and neurotrophic activities. These functions of HGF can be enhanced by a positive feedback mechanism, mediated by an essential transcription factor, ETS. In this study, the biological effects of HGF appeared to be up-regulated multifold by such a feedback mechanism, although the level of human HGF in CSF was much lower than rat HGF after stimulation by human HGF. Therefore, *HGF* gene therapy for the auditory system is thought to have several advantages over the earlier gene therapy using neurotrophic factors. Further study of vascular function in the cochlea after HGF gene transfer will provide novel information regarding cochlear function. Moreover, another possibility exists: this study implies that HGF could cause the regeneration of HC or SGC.

Thus, *HGF* gene therapy is a potent candidate for treatment of auditory impairment. This research provides new insight and an approach for clinical treatment for hearing impairment by the combination of the *HGF* gene and the HVJ-E vector system. FJ

Novel Therapeutic Strategy to Treat Brain Ischemia

Overexpression of Hepatocyte Growth Factor Gene Reduced Ischemic Injury Without Cerebral Edema in Rat Model

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Background—Although cerebral occlusive disease leads to cerebral ischemic events, an effective treatment has not yet been established. An ideal therapeutic approach to treat ischemia might have both aspects of enhancement of collateral formation and prevention of neuronal death. Hepatocyte growth factor (HGF) is a potent angiogenic factor that also acts as a neurotrophic factor. Thus, in this study, we examined the therapeutic effects of HGF on brain injury in a rat permanent middle cerebral artery occlusion model.

Methods and Results—Gene transfer into the brain was performed by injection of human HGF gene with hemagglutinating virus of Japan–envelope vector into the cerebrospinal fluid via the cisterna magna. Overexpression of the HGF gene resulted in a significant decrease in the infarcted brain area as assessed by triphenyltetrazolium chloride staining, whereas rats transfected with control vector exhibited a wide area of brain death after 24 hours of ischemia. Consistently, the decrease in neurological deficit was significantly attenuated in rats transfected with the HGF gene at 24 hours after the ischemic event. Stimulation of angiogenesis was also detected in rats transfected with the HGF gene compared with controls. Of importance, no cerebral edema or destruction of the blood-brain barrier was observed in rats transfected with the HGF gene.

Conclusions—Overall, the present study demonstrated that overexpression of the HGF gene attenuated brain ischemic injury in a rat model, without cerebral edema, through angiogenic and neuroprotective actions. In particular, the reduction of brain injury by HGF may provide a new therapeutic option to treat cerebrovascular disease. **(*Circulation*. 2004;109:424-431.)**

Key Words: gene therapy ■ nervous system ■ stroke ■ cerebral ischemia ■ angiogenesis

Cerebral occlusive disease caused by atherosclerosis of the cerebral arteries or Moyamoya disease often causes global ischemia of the brain. Although such a condition leads to not only cerebral ischemic events but also neuropathological changes, including dementia,[1,2] an effective treatment to improve brain ischemic injury has not yet been established. Ischemic stroke induces active angiogenesis, particularly in the ischemic penumbra, which correlates with longer survival in humans.[3] However, the natural course of angiogenesis is not sufficient to compensate for the hypoperfusion state. Therefore, novel therapeutics are needed to treat these patients. Because angiogenic growth factors stimulated the development of collateral arteries in animal models of peripheral and myocardial ischemia, a concept called therapeutic angiogenesis,[4,5] the therapeutic implications of angiogenic growth factors to treat cardiovascular disease were recently described. The efficacy of therapeutic angiogenesis using vascular endothelial growth factor (VEGF) gene transfer has been reported in human patients with critical limb ischemia or myocardial infarction.[6,7] Thus, the strategy for therapeutic angiogenesis using angiogenic growth factors should be considered for the treatment of patients with ischemia. From this viewpoint, therapeutic angiogenesis must be an effective therapy for cerebral ischemia, resulting in the prevention of future stroke. Indeed, several angiogenic growth factors, such as fibroblast growth factor, hepatocyte growth factor (HGF), and VEGF were applied to prevent the extension of focal ischemic injury in animal models.[8–10]

However, the simple effects of stimulation of angiogenesis might not be enough to treat brain ischemia, because neurons are highly sensitive to hypoxia–ischemia. Given this susceptibility and their postmitotic nature, the development of

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effective protective therapeutic strategies is also essential. In particular, pyramidal neurons in the CA1 subfield of the hippocampus are known to be the most vulnerable to cerebral ischemia.[11] After transient occlusion of the bilateral common carotid arteries in the gerbil, delayed neuronal death begins in CA1 pyramidal neurons a few days after recirculation, during which time no energy crisis or morphological change is observed. Therefore, prevention of delayed neuronal death might be of therapeutic value. Thus, several neurotrophic growth factors, such as brain-derived neurotrophic factor,[12,13] were reported to prevent the extension of focal ischemic injury in animal models. To consider both aspects of brain ischemic injury, the ideal growth factors should have both functions of angiogenesis and neurotrophic actions. Because HGF is a well-known potent pleiotropic cytokine that exhibits mitogenic, motogenic, and morphogenic activities in a variety of cells,[14] HGF has a neuroprotective effect in vitro and in vivo.[15-17] Here, we demonstrated that gene transfer of HGF into the subarachnoid space could cause beneficial effects on neurological symptoms through the prevention of brain injury and stimulation of angiogenesis without any apparent toxicity in a rat model.

Methods

Preparation of HVJ-Envelope Vector

A hemagglutinating virus of Japan (HVJ)-envelope vector was prepared as described previously.[18,19] Briefly, the virus suspension (15 000 hemagglutinating units) was inactivated by UV irradiation (99 mJ/cm^2) and mixed with plasmid DNA (400 μg) and 0.3% Triton-X. After centrifugation, it was washed with 1 mL balanced salt solution (10 mmol/L Tris-Cl, pH 7.5, 137 mmol/L NaCl, 5.4 mmol/L KCl) to remove the detergent and unincorporated DNA. After centrifugation, the envelope vector was suspended in 100 μL PBS. The vector was stored at 4°C until use. To produce an HGF expression vector, human HGF cDNA (2.2 kb) was inserted into a simple eukaryotic expression plasmid that uses the cytomegalovirus promoter/enhancer.[20] The control vector was expression vector plasmid with the same structure, including the promoter, but not containing HGF cDNA.

In Vivo Gene Transfer Into Subarachnoid Space in Normal Rats

Injection of the HVJ-envelope vector into the cisterna magna was performed for gene transfer to the brain of Wistar male rats (270 to 300 g; Charles River Japan, Atsugi, Japan).[19] The head of each animal was fixed in the prone position, and the atlanto-occipital membrane was exposed through an occipitocerebral midline incision. A stainless cannula (27 gauge; Becton Dickinson) was introduced into the cisterna magna (subarachnoid space). HVJ-envelope vector (100 μL) containing human HGF gene was infused at a speed of 50 μL/min after removal of 100 μL of cerebrospinal fluid (CSF). Then, the animals were placed head down for 30 minutes. No behavioral change, such as convulsion or abnormal movement, was observed. All procedures were conducted in accordance with Osaka University guidelines.

To investigate the effects of HGF gene transfer on cerebral ischemia, a rat permanent middle cerebral artery (MCA) occlusion model was used in the present study. To generate the MCA occlusion model, the right MCA was occluded by placement of poly-L-lysine–coated 4-0 nylon around the origin of the MCA.[21] The right common carotid artery, right external carotid artery, and right internal carotid artery were isolated via a midline incision. Then, 4-0 nylon was inserted from the right external carotid artery and advanced 20 mm. The right external carotid artery was ligated with 6-0 nylon. To examine transfection of the HGF gene in the CSF, 100 μL CSF was collected 5 and 12 days after gene transfer. The concentration of HGF was determined by enzyme immunoassay using anti-human or anti-rat HGF antibody (Institute of Immunology, Tokyo, Japan).[17] The antibody against human HGF reacts with only human HGF, and not with rat HGF.

Histological Examination

For immunohistochemical staining for c-met, rats were killed 5 days after gene transfer by transcardial perfusion fixation with normal saline followed by 4% paraformaldehyde. The brain was removed, postfixed, and cut on a vibratome at 40 μm. After blocking, free-floating sections were incubated in 3% normal goat serum and anti–c-met antibody (SP 260, 1:250; Santa Cruz), followed by anti-rabbit fluorescent antibody (1:1000, Alexa Flour 488, Molecular Probes). For in situ end-labeling of fragmented DNA, brain at 1 day after MCA occlusion was fixed with 10% formalin and processed for paraffin embedding. Terminal dUTP nick end-labeling (TUNEL) of apoptotic cells was measured with an ApopTag Plus Peroxidase In Situ Apoptosis Detection kit (Integren Inc). Counterstaining was performed by immersing slides in methyl green in 0.1 mol/L sodium acetate solution (pH 4.0) for 5 minutes at room temperature.

Evaluation of Effect of HGF Gene Transfer on Infarcted Area

The right MCA was occluded at 5 days after gene transfer into the subarachnoid space. Rats were killed 24 hours after occlusion, and the brain was removed within 3 minutes of death. Coronal sections were made at +3.7, +1.0, −0.8, −3.3, and −5.3 mm from the bregma, and brain slices were immersed in 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Nakalai tesque) in normal saline at 37°C. This procedure can be used as a reliable marker of ischemic damage.[22] To assess the ischemic area, we calculated the hemispheric lesion area (HLA) in coronal sections. The corrected HLA was calculated as HLA (%)=[LT−(RT−RI)]/LT×100, where LT is the area of the left hemisphere, RT is the area of the right hemisphere, and RI is the infarcted area.

Behavior Examination

For behavior assessment, we used a simple protocol[23] to evaluate neuromuscular function that uses the following categories (maximum score is 4). Forelimb flexion: Rats were held by the tail on a flat surface. Paralysis of the forelimbs was evaluated by the degree of left forelimb flexion. Torso twisting: Rats were held by the tail on a flat surface. The degree of body rotation was checked. Lateral push: Rats were pushed either left or right. Rats with right MCA occlusion showed weak or no resistance against a left push. Hindlimb placement: One hindlimb was removed from the surface. Rats with right MCA occlusion showed delayed or no placement of the hindlimb when it was removed from the surface.

Evaluation of Cerebral Edema After Permanent MCA Occlusion

The brain was removed within 3 minutes of death after 24 hours of MCA occlusion. The brain was divided into the intact hemisphere and the infarcted hemisphere. The wet weight was measured quickly, and the brain was dried in an oven at 110°C for 24 hours.[24] Then the dry weight was measured. The water content of these samples was calculated as water content (%)=(wet wt−dry wt)×100/wet wt.

Evaluation of Blood-Brain Barrier Permeability With Evans Blue Dye

To evaluate the effect of HGF on blood-brain barrier (BBB) permeability, Evans blue dye was used as a marker of albumin extravasation.[25] Evans blue dye (2% in saline, 3 mL/kg) was injected via the femoral vein under halothane anesthesia at 6 hours after MCA occlusion. Three hours after Evans blue dye injection, the rats were anesthetized with sodium pentobarbital and perfused with physiological saline.[24] Coronal sections were made at +1.0 and −0.8 mm from the bregma. To check the existence of infarction, a coronal

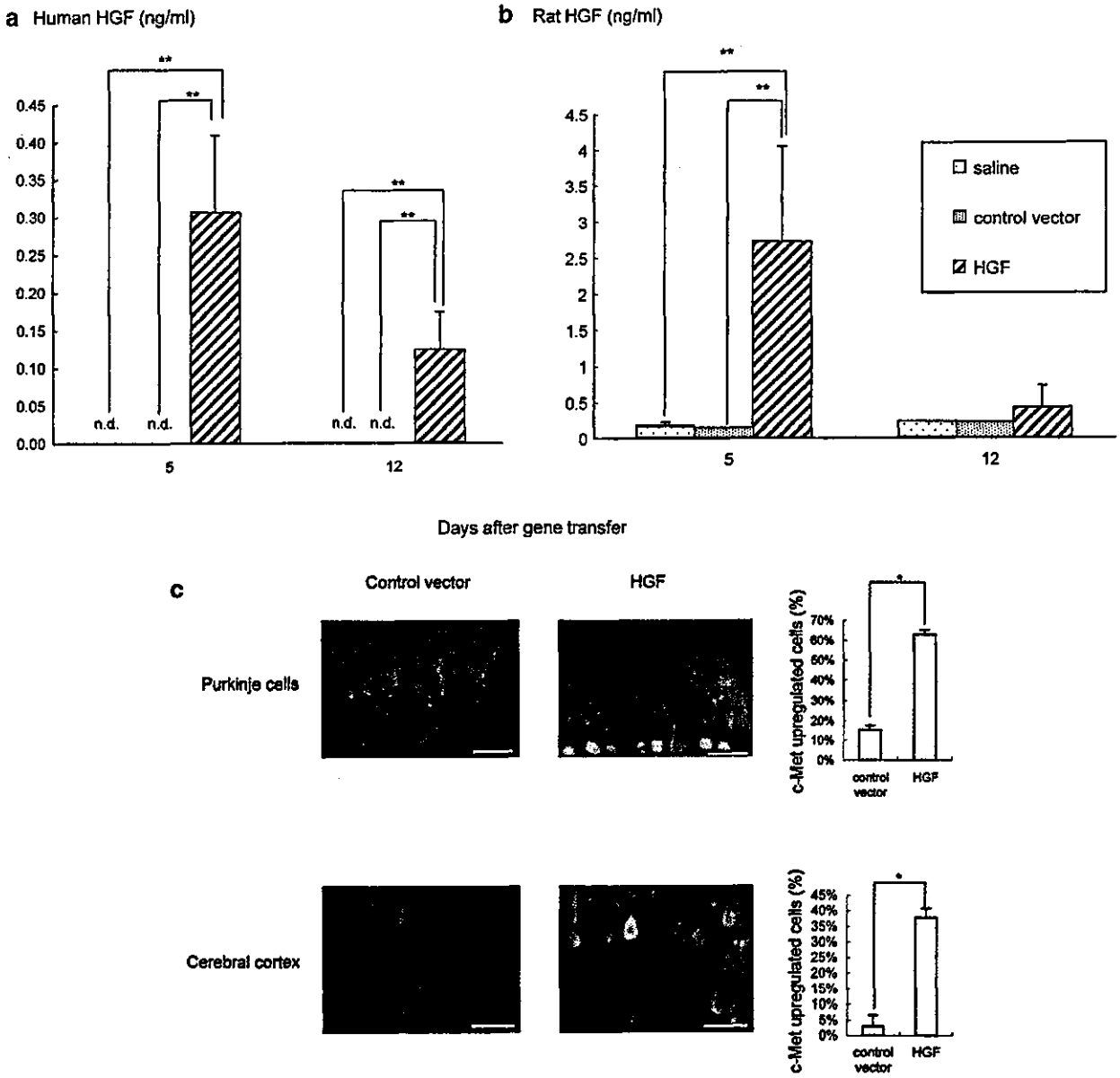

Figure 1. Concentrations of human (a) and rat HGF (b) in CSF at 5 and 12 days after gene transfer. Saline indicates rats injected with saline; Control vector, rats transfected with control vector; HGF, rats transfected with human HGF vector; n.d., not detected; $^{**}P<0.01$. c, Immunohistochemical staining for c-met at 5 days after gene transfer in cerebral cortex and injected site (cerebellum). Top, bar=50 μm; bottom, bar=25 μm.

section at +1.0 mm was stained with TTC as described above. Leakage of Evans blue dye was calculated as leakage (%)=[LT−(RT−RB)]/LT×100, where LT is the left hemisphere, RT the right hemisphere, and RB the area stained blue.

Evaluation of Capillary Density

By use of a recently developed microangiographic technique,[26] capillary density and blood-brain leakage were evaluated in the cerebral cortex after MCA occlusion. This technique allows evaluation of BBB function as well as vascular pattern. Briefly, fluorescent albumin solution was prepared by reconstituting 500 mg bovine desiccate albumin-fluorescein isothiocyanate (Sigma-Aldrich) in 50 mL PBS. The solution was injected via the jugular vein at a rate of 1 mL/min (10 mL/kg) 24 hours after MCA occlusion. The same amount of blood was withdrawn before the injection to avoid systematic blood pressure elevation. Brain was fixed in 10% formalin solution, cut in the coronal plane at 100 μm, and mounted with a Prolong Antifade Kit (Molecular Probes Inc). Because regional variation in brain capillary density has been reported,[26] we set the region of interest at the surface of the cerebral cortex (width, 0.625 mm; depth, 0.8 mm). The region of interest was set as the region supplied by the anterior cerebral artery, because the area was adjacent to the area supplied by the MCA. Five consecutive sections in each rat were observed with a confocal laser microscope (Bio-Rad). The acquired images were imported into Adobe Photoshop (version 7.0, Adobe System), and the color of the image was inverted. Then, the area or length of vessels was analyzed with an Angiogenesis Image Analyzer (version 1.0, Kurabo).

Statistical Analysis

All values are expressed as mean±SEM. ANOVA with subsequent Duncan's test was used to determine the significance of differences in multiple comparisons. Differences with a probability value of $P<0.05$ were considered significant.