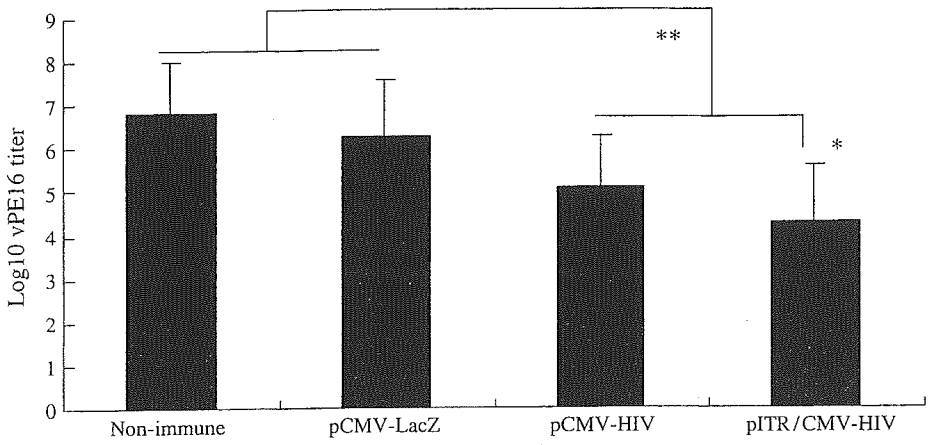

Figure 8. Resistance of immunized mice to infection by vaccinia virus (vPE16) expressing the HIV *env* gene. One week after the last immunization, immune mice were challenged intravenously with 5 x 10^7 pfu of vPE16 virus. Vaccinia virus titers were measured 6 days after virus challenge. Data represent the average of 10 mice/group

of the encoded antigen, and thereby boosts vaccine immunogenicity. Introducing these ITRs into plasmids encoding the *LacZ* or HIV *env* gene under the control of a CMV promoter significantly enhanced protein production (Figures 2 and 3). The effect of the ITR was mediated by the CMV promoter, since no effect was observed in plasmids containing ITR but not CMV. Mice immunized with ITR-enhanced plasmids generated significantly higher antigen-specific Ab and cell-mediated immune responses (Figures 5, 6 and 7).

Current results confirm and extend earlier observations establishing that plasmids encoding ITRs can efficiently transfect host cells, and induce protein expression [19,20]. Furthermore, ITR-introduced DNA vaccine induces higher antigen-specific immune responses *in vivo*. Our findings are also in general agreement with data from Flotte *et al.* indicating that the ITR sequence can enhance the functional activity of the AAV p5 promoter [21]. Indeed, in our hands, AAV-ITR alone did not significantly promote gene expression (Figures 2 and 3). Additional studies performed in our laboratory confirm that plasmid introducing the AAV ITR but lacking the CMV promoter is virulently inactive (data not shown).

The mechanism of AAV integration is not yet fully understood. Recombinant AAV randomly integrates its transgene into mammalian cells [22–25], but most of them apparently as an episomal form for long-term expression [26]. To investigate whether the higher expression in the ITR-introducing plasmid was caused by chromosomal integration or episomal persistence, we transfected the pCMV-LacZ and pITR/CMV-LacZ plasmid into HEK 293 cells and counted LacZ-positive cells for 49 days. We found that the amount of positive cells was rapidly reduced in each plasmid-transfected cells by 21 days and positive cells in pITR/CMV-LacZ-transfected cells were 15–20% higher than the pCMV-LacZ-transfected cells. However, by 49 days post-transfection, there were 25 and 4 positive cells per 10^4 cells using pITR/CMV-LacZ and pCMV-LacZ, respectively (Figure 4). These results suggest that ITRs of plasmid without AAV helper components may play a role in chromosomal integration or episomal persistence. Single-stranded AAV genome has to be synthesized to double-stranded DNA then integrate to the host genome. Therefore, double-stranded DNA plasmid with ITRs may be more efficient for chromosomal integration than the single-stranded AAV genome *in vivo* [27]. In this study, we are not sure whether the increased chromosomal integration or episomal persistence greatly affected immunogenicity of the HIV vaccines with or without ITRs, since the number of positive cells was not high (Figure 4).

To increase the DNA vaccine-induced HIV-specific immune responses, RIBI adjuvant, a detoxified form of lipid A derived from the lipopolysaccharide of *Salmonella Minnesota* R595, was used as a vaccine adjuvant [28]. Early studies [29,30] showed that the adjuvant activity of RIBI reflects its ability to activate macrophages and stimulate IFN-γ and interleukin 2 (IL-2) [31] production, known to be essential for the induction of Th1-derived cell-mediated immunity. Moreover, inclusion of RIBI improved the transfection efficiency of pITR/CMV-LacZ and pCMV-LacZ into HEK 293 cells (data not shown).

Taken together, current results indicate that DNA vaccines containing both AAV ITRs and the CMV promoter induce higher antigen-specific antibody and IFN-γ responses than vaccines with a CMV promoter alone, and that AAV ITRs act by enhancing the activity of the CMV promoter rather than a promoter itself.

Acknowledgements

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RESEARCH ARTICLE

Suicide gene therapy using AAV-HSVtk/ganciclovir in combination with irradiation results in regression of human head and neck cancer xenografts in nude mice

T Kanazawa[1,2,3], H Mizukami[1,3], T Okada[1,3], Y Hanazono[1,3], A Kume[1,3], H Nishino[2], K Takeuchi[4], K Kitamura[5], K Ichimura[2] and K Ozawa[1,3,6]

[1]*Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan;* [2]*Department of Otolaryngology, Jichi Medical School, Tochigi, Japan;* [3]*CREST, Japan Science and Technology Corporation (JST), Tochigi, Japan;* [4]*Department of Anatomy, Jichi Medical School, Tochigi, Japan;* [5]*Department of Otolaryngology, School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan ; and* [6]*Department of Hematology, Jichi Medical School, Tochigi, Japan*

The application of adeno-associated virus (AAV) vectors to cancers is limited by their low transduction efficiency. Previously, we reported that γ-ray enhanced the second-strand synthesis, leading to the improvement of the transgene expression, and cytocidal effect of the herpes simplex virus type-1 thymidine kinase (HSVtk) and ganciclovir (GCV) system. In this study, we extended this in vitro findings to in vivo. First, the laryngeal cancer cell line (HEp-2) and HeLa were treated with AAVtk/GCV, the number of surviving cells was reduced as the concentration of GCV increased. Furthermore, the 4 Gy irradiation enhanced the killing effects of AAVtk/GCV by four-fold on HeLa cells and 15-fold on HEp-2 cells. Following the in vitro experiments, we evaluated the transgene expression and the antitumor activity of the AAV vectors in combination with γ-ray in nude mice inoculated with HEp-2 subcutaneously. The LacZ expression was observed in the xenografted tumors and significantly increased by γ-ray. The AAVtk/GCV system suppressed the tumors growth, and γ-ray augmented the antitumor activity by five-fold. These findings suggest that the combination of AAVtk/GCV system with radiotherapy is significantly effective in the treatment of cancers and may lead to reduction of the potential toxicity of both AAVtk/GCV and γ-ray.

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Keywords: *adeno-associated virus vector; herpes simplex thymidine kinase; irradiation; animal experiments; head and neck neoplasms*

Introduction

Current therapy for the head and neck cancer utilizes an aggressive multimodal approach with surgery, radiotherapy, and chemotherapy, depending on the tumor stage and patient characteristics. Despite advances in the therapeutic approaches, no substantial improvement in efficacy and survival has occurred over the past several decades. Conventional palliative treatments, such as chemotherapy, are often toxic and sometimes ineffective. In addition, no effective salvage therapy is available for whom standard treatment fails. Therefore, new treatments are needed both to improve survival in the long term and to obtain worthwhile, less toxic palliation in the short term.

Recent progress in gene therapy technologies has made it possible to develop novel therapeutic strategies for intractable cancers. Head and neck cancer is particularly well suited for gene transduction strategies. First, its location in the upper aerodigestive tract allows easy access for vector delivery and assessment of response. Second, targeting of gene transduction can be achieved by direct injection of the vector into the tumor. Finally, metastases mostly occur late in head and neck cancer progression, making local disease responsible for most of the morbidity and mortality.[1,2] Therefore, any improvement in local disease control implies benefits for patients. At present, several virus vectors such as retroviral, adenoviral and adeno-associated virus (AAV) vectors[3,4] have been utilized for the experiments of cancer gene therapy. AAV is a non-pathogenic virus with a single-stranded DNA genome.[5,6] AAV vectors have emerged as a useful alternative to other vectors,[7] and AAV have been evaluated in preclinical and clinical models for cystic fibrosis,[8] Parkinson's disease[9] and Hemophilia B.[10] AAV vectors have a broad host range and can transduce head and neck cancer cells.[11] However, an obstacle to these applications is a low transgene expression efficiency, mainly due to a limited second-strand synthesis.[12,13] Recently, γ-ray irradiation has been reported to enhance the second-strand synthesis of the AAV vector genome and improve the transgene expression.[14–16] In our previous study, we demonstrated that γ-rays enhance AAV-mediated transgene expression in maxillary sinus cancer cells *in vitro*.[11] Thus, an AAV

Correspondence: K Ozawa, Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji Minamikawachi, Tochigi 329-0498, Japan
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vector encoding suicide gene would kill target cells more efficiently when combined with γ-ray irradiation therapy. Although the herpes simplex virus type-1 thymidine kinase (HSVtk)/ganciclovir (GCV) system has shown to be effective for controlling tumor growth in animal models,[17–20] this therapeutic approach alone sometimes fails to eradicate cancer cells, and tumors recur thereafter. Thus, alternative therapeutic modalities, such as combination therapy should be considered.[21,22]

In this study, we demonstrate effective suicide gene therapy using the AAV vector in combination with γ-ray irradiation, enhancing the antitumor activity both *in vitro* and *in vivo*.

Results

Comparison of the transduction efficiency of AAVLacZ between HeLa and HEp-2 cells

HeLa or HEp-2 cells were transduced with recombinant AAV with *Escherichia coli* LacZ expression cassette (AAVLacZ) at titers from 1×10^4 to 1×10^6 particles/cell (Figure 1a). The percentage of positive HEp-2 cells for 5-bromo-4-chloro-indonyl-β-D-galactopyranoside (X-gal) staining reached 72% when the cells were transduced with 1×10^6 particles/cell of AAVLacZ. The transduction efficiency of HEp-2 cells was almost as high as that of HeLa cells. Figure 1b shows the β-galactosidase expression level in HeLa and HEp-2 cells quantified by enzyme-linked immunosorbent assay (ELISA), when the cells were transduced with AAVLacZ at titers from 1×10^3 to 1×10^5 particles/cells. The amount of β-gal in both cells increased along with the increased concentration of AAVLacZ.

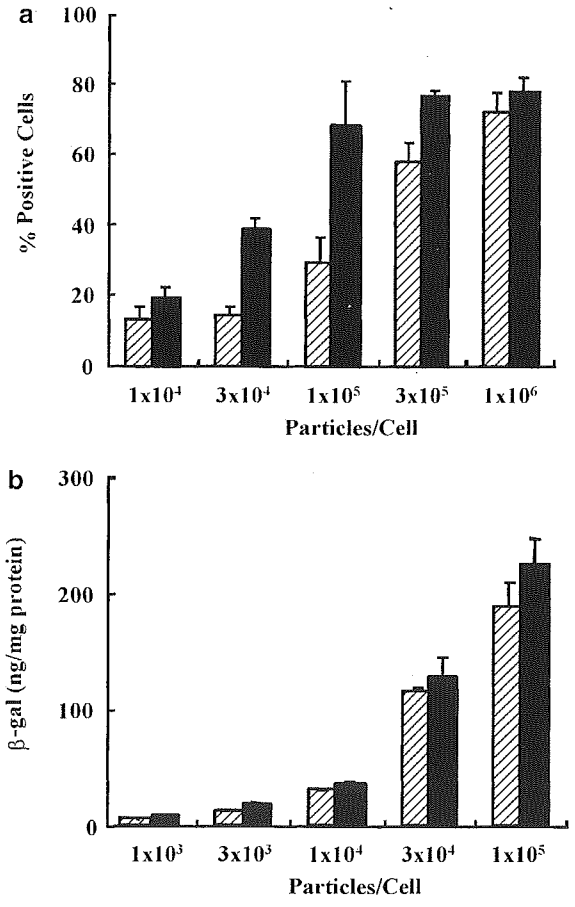

Figure 1 AAVLacZ expression in HeLa or HEp-2 cells. (a) Subconfluent HeLa (closed bar) or HEp-2 cells (hatched bar) were transduced with AAVLacZ at various doses ranging from 1×10^4 to 1×10^6 particles/cell. At 36 h post-transduction, the cells were fixed and stained with X-gal, and then positive cells were counted. (b) Subconfluent HeLa (closed bar) or HEp-2 cells (hatched bar) were transduced with 1×10^3 to 1×10^5 particles/cell of AAVLacZ. Thirty-six hours after transduction, the cells were lysed and β-gal was assayed by using the β-gal ELISA kit. Each bar represents the mean ± s.d.

Effect of γ-ray irradiation on AAV-mediated transgene expression

γ-Rays have been shown to increase the transduction efficiency with AAV vectors, mainly by accelerating the rate of leading-strand synthesis of the AAV vector genome. According to our previous study,[11] optimal transduction efficiency was obtained when the cells were transduced with 1×10^3 particles/cell of AAVLacZ immediately after irradiation. In Figure 2, γ-ray irradiation significantly increased LacZ expression in HeLa and HEp-2 cells in a dose-dependent manner. (one-way ANOVA: $P < 0.01$).

γ-Rays enhance the second-strand synthesis of the AAV genome in HeLa and HEp-2 cells

To examine whether the second-strand synthesis of the AAV vector genome occurs more efficiently in γ-ray-irradiated cells, HeLa and HEp-2 cells were subjected to

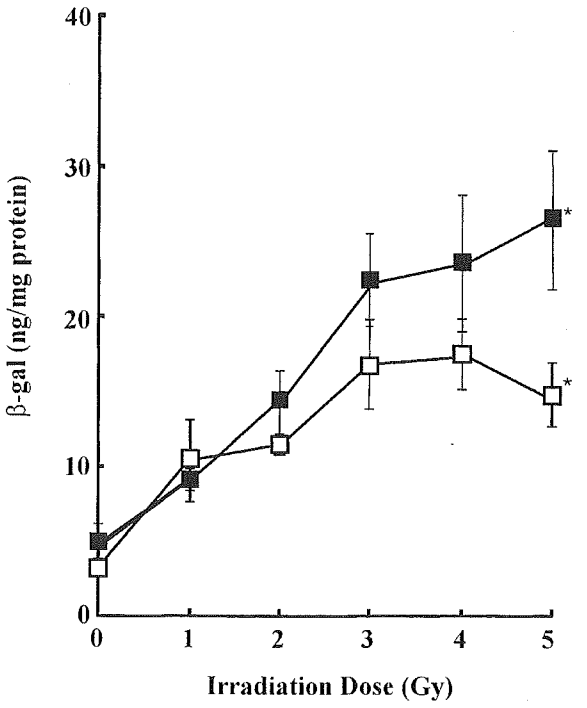

Figure 2 Effect of γ-ray on AAV vector-mediated transgene expression. HeLa (closed square) or HEp-2 cells (open square) were transduced with 1×10^3 particles/cell of AAVLacZ immediately after γ-ray irradiation at doses ranging from 0 to 5 Gy. Thirty-six hours after transduction, the expression levels of LacZ were assayed by using the β-gal ELISA kit. Data were statistically analyzed by one-way ANOVA ($P < 0.01$). Each data point represents the mean ± s.d.*

0, 2, or 4 Gy of γ-ray irradiation, and then transduced with 1×10^4 particles/cell of AAVLacZ. Forty-eight hours after transduction, total DNA was isolated, treated with mung bean nuclease, and then loaded on 1% agarose gels. After transfer to nylon membranes, signals corresponding to the AAVLacZ genome were detected (Figure 3). Mung bean nuclease was used to digest the single-stranded DNA and to clearly visualize the double-stranded replicative form (RF) of the AAV vector genome. The RF was almost equal to 4.7 kb fragment derived from pAAVLacZ in size. At the dose of 4 Gy, in both HeLa and HEp-2 cells, the intensity of signal corresponding to the RF increased significantly, suggesting that the augmented transgene expression was associated with the conversion of the AAV vector genome to the double-stranded RF.

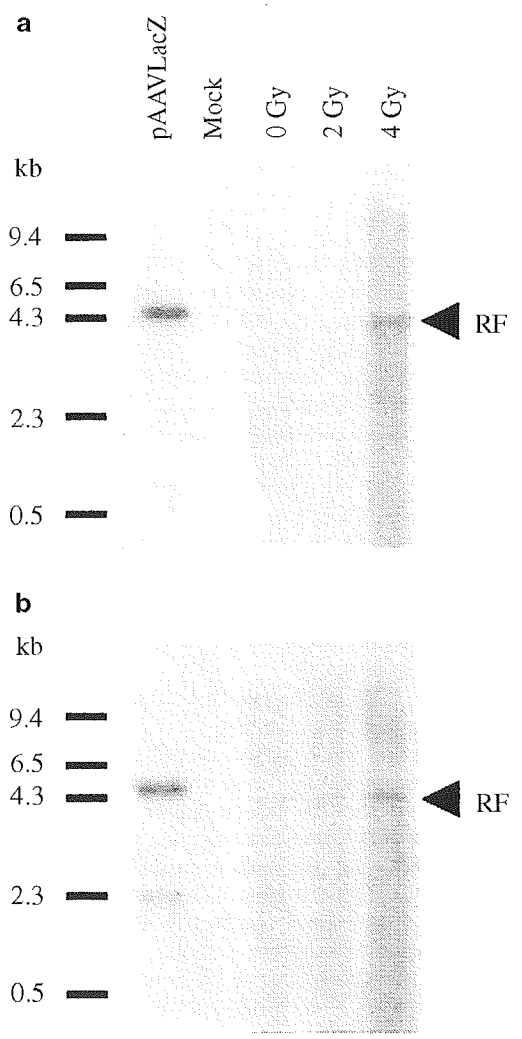

Figure 3. Second-strand synthesis of AAVLacZ genome after γ-ray irradiation in HeLa or HEp-2 cells. HeLa (a) or HEp-2 cells (b) were transduced with 1×10^4 particles/cell of AAVLacZ immediately after 0, 2, or 4 Gy of γ-ray irradiation. Two days later, total DNA was isolated in a low-salt condition. After mung bean nuclease treatment, the DNA samples were loaded on 1% agarose gels, transferred onto nylon membranes (Hybond N⁺, Amersham), and then hybridized with a radiolabeled CMV-specific probe. Signals were detected by using an imaging analyzer. Lane 1, a 4.7 kb fragment derived from pAAVLacZ; lane 2, mock transduced; lanes 3–5, AAVLacZ-transduced immediately after 0-, 2-, or 4-Gy irradiation respectively. RF: the double-stranded replicative form.

GCV treatment

Figure 4 shows the killing effect of various concentrations of GCV on HeLa and HEp-2 cells transduced with 1×10^5 particles/cell of recombinant AAV with HSVtk expression cassette (AAVtk) (closed bar). When the AAVtk-transduced cells were treated with 1 μg/ml of GCV, 75% of HeLa cells and 35% of HEp-2 cells were killed. As the concentration of GCV was increased, surviving cells were reduced and 98% of HeLa cells and 96% of HEp-2 cells were killed by exposure to 10 μg/ml of GCV, which was significantly higher than the killing rate in AAVLacZ-transduced cells (hatched bar) or mock-transduced cells (open bar) (two-way ANOVA: $P<0.01$).

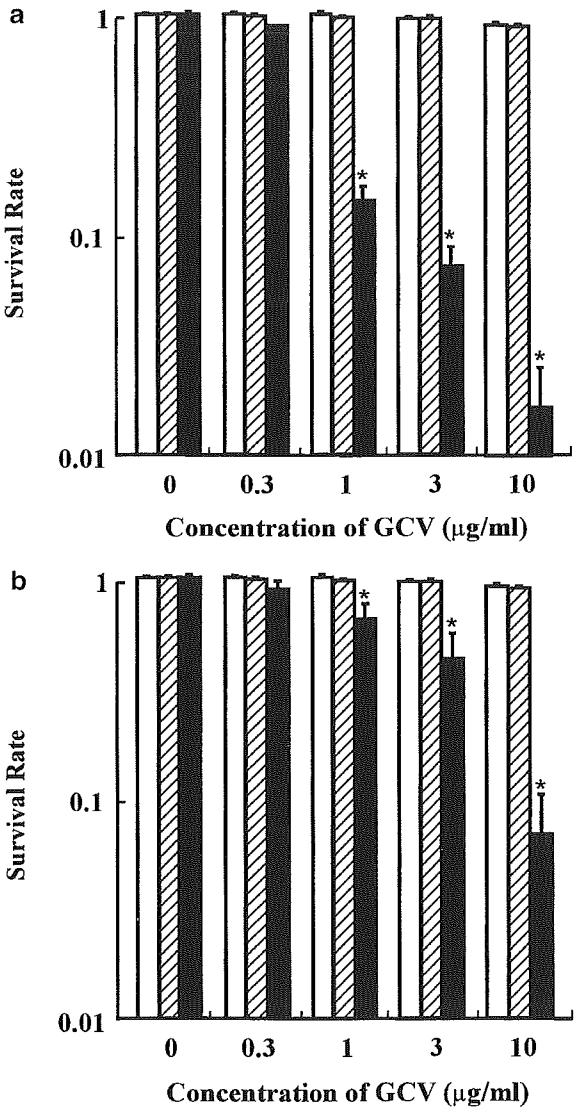

Figure 4 Survival of HeLa and HEp-2 cells upon AAVtk/GCV. HeLa (a) or HEp-2 cells (b) were mock transduced (open bar), transduced with 1×10^5 particles/cell of AAVLacZ (hatched bar), or AAVtk (closed bar). Twenty-four hours after transduction, the cells were exposed to different concentrations of GCV. After a 7-day incubation in the presence of GCV, surviving cells were counted. Data were analyzed by two-way ANOVA. Asterisks mean that the data obtained for AAVtk transduction were significantly different from those with or without transduction of AAVLacZ ($P<0.01$). Each bar represents the mean ± s.d. ($n=3$).

Enhanced cytocidal effect of the AAVtk/GCV system by γ-ray irradiation

To facilitate comparison of the therapeutic outcome, the killing effect of GCV (3 μg/ml) on HeLa or HEp-2 cells transduced with various doses of AAVtk with or without γ-ray irradiation was evaluated (Figure 5). When HeLa and HEp-2 cells were transduced with 3×10^4 particles/cell of AAVtk without irradiation, 48% of the HeLa cells and 40% of the HEp-2 cells were killed by the exposure to the GCV. As the dose of AAVtk was increased, the number of surviving cells were reduced, and 98% of the HeLa cells and 95% of the HEp-2 cells were killed when transduced with 3×10^5 particles/cell of AAVtk, which was significantly higher than the killing rate in the case of AAVLacZ-transduced cells as expected. To investigate whether γ-ray enhances the killing effect of AAVtk/GCV, HeLa and HEp-2 cells were irradiated with 2 or 4 Gy of γ-ray immediately before the transduction with various doses of AAVtk, and then cultured in 3 μg/ml of GCV. When the HeLa cells were transduced with 3×10^4 particles/cell of AAVtk, 70% of the 2 Gy irradiated cells and 85% of the 4 Gy irradiated cells were killed by the addition of GCV. When the HeLa cells were transduced at 3×10^5 particles/cell, γ-ray irradiation enhanced the killing effects of AAVtk/GCV system by four-fold. γ-Ray irradiation also enhanced the killing effects on HEp-2 cells by 15-fold. The enhancement by γ-ray irradiation was calculated from the ratio of 4 Gy irradiated survival rate to non-irradiated survival rate. These results show that γ-ray irradiation enhances the killing effects of AAVtk/GCV system significantly (two-way ANOVA: $P<0.01$).

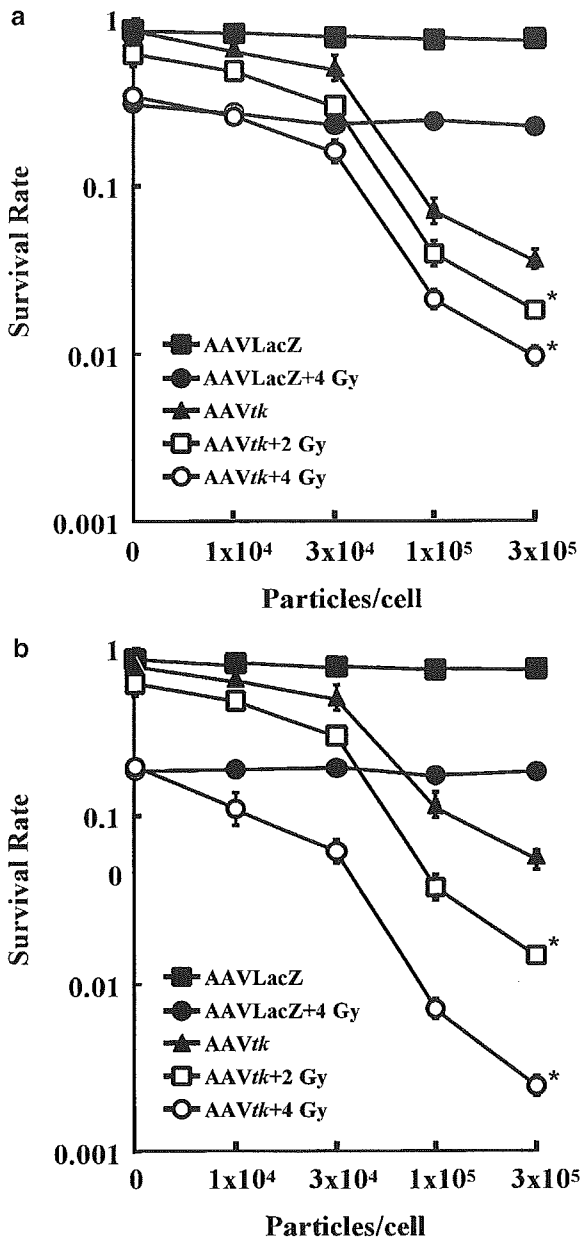

Figure 5 Enhancement of the cytocidal effect of AAVtk by γ-ray irradiation. HeLa (a) or HEp-2 cells (b) were transduced with various doses of AAVLacZ or AAVtk with or without γ-ray irradiation. Twenty-four hours after transduction, the cells were treated with 3 μg/ml of GCV. After a 7-day incubation in the presence of GCV, surviving cells were counted. Closed squares: AAVLacZ-transduced cells; closed circles: AAVLacZ-transduced cells with 4 Gy irradiation; closed triangles: AAVtk-transduced cells; open squares: AAVtk-transduced cells with 2 Gy irradiation; open circles: AAVtk-transduced cells with 4 Gy irradiation. Asterisks mean that the AAVtk-transduced and irradiated cells were significantly different from the AAVtk-transduced and non-irradiated cells ($P<0.01$). Each data point represents the mean ± s.d.

γ-Ray enhances the transgene expression in vivo

To assess the enhancement of transgene expression by γ-ray irradiation *in vivo*, the AAVLacZ-transduced tumors were stained with X-gal (Figure 6). Compared with non-irradiated control, the tumors irradiated at 4 Gy showed increased number of X-gal-positive cells. To quantify the amount of β-galactosidase, we homogenized the non-irradiated or 4 Gy irradiated tumors and measured with the β-gal ELISA kit. As shown in Figure 7, the amount of β-galactosidase in 4 Gy irradiated tumors was 2.5 times larger than that in non-irradiated tumors (one-way ANOVA: $P<0.01$).

Suppressive effects of combination therapy upon tumor growth in vivo

To examine the killing effect of this system *in vivo*, tumor nodules were established in the flanks of BALB/c nude mice by subcutaneous injection of the HEp-2 cells. Once tumors were established, those animals were irradiated at 4 Gy and then directly injected with 1×10^{12} particles of AAVLacZ or AAVtk in the tumors. Administration of GCV (50 mg/kg) or phosphate-buffered saline (PBS) intraperitoneally twice a day was started at 24 h after the vector injection and continued for 2 weeks. The tumors were measured every 3 days.

Five treatment groups of 4 or 5 animals each were established: Group 1 is the AAVLacZ-transduced animals with PBS administration (n=4). Group 2 is the AAVtk-transduced animals with GCV administration (n=5). Group 3 is the AAVLacZ-transduced animals with 4 Gy irradiation and GCV administration (n=5). Group 4 is the AAVtk-transduced animals with 4 Gy irradiation and PBS administration (n=4). Group 5 is the AAVtk-transduced animals with 4 Gy irradiation and GCV administration.

The representative growth curve of the tumors after treatment is shown in Figure 8. The AAVtk/GCV system suppressed the growth rate of xenografted tumors by

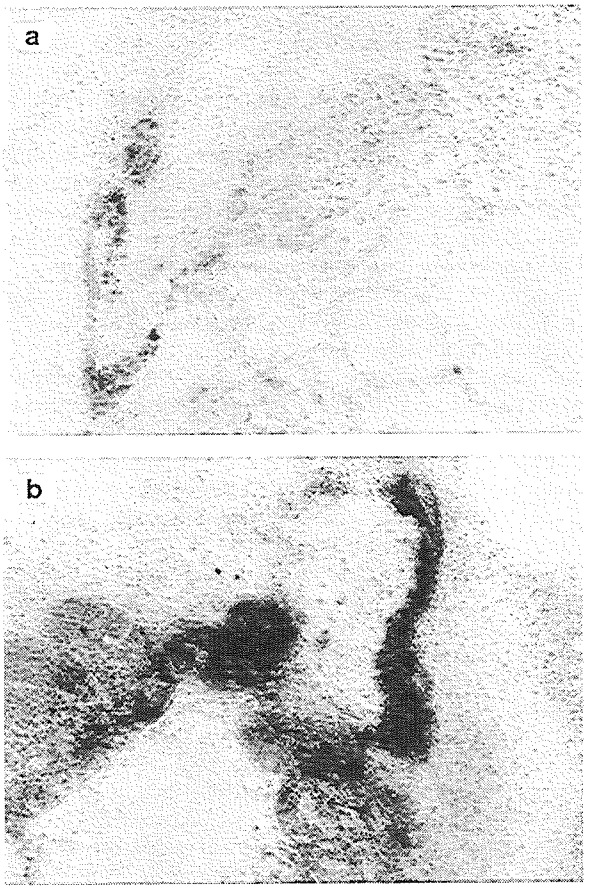

Figure 6 The effect of γ-ray irradiation on AAV-mediated transgene expression in subcutaneous tumors in BALB/c mice. The tumors were established by HEp-2 cells into the flanks of the mice xenografts. (a) Transduction with 5×10^{11} particles/tumor of AAVLacZ without γ-ray irradiation. (b) Transduction with AAVLacZ immediately after 4 Gy irradiation. Three days after AAVLacZ injection, the tumors were excised from mice and frozen in OTC embedding compound on a liquid-nitrogen bath. Cryostat sections were made and fixed with 0.05% glutaraldehyde in PBS. Histochemical staining for β-galactosidase activity was performed in the sections as described in Materials and methods.

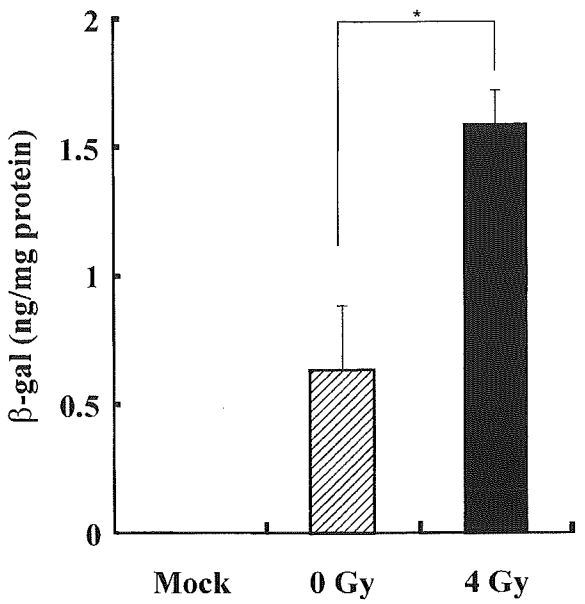

Figure 7 γ-Ray increased the expression level of β-galactosidase. The tumors were established by HEp-2 cells into the flanks of the mice xenografts. The tumors were injected with 5×10^{11} particles of AAVLacZ immediately after γ-ray irradiation at the dose of 0 or 4 Gy. Three days after AAVLacZ injection, the tumors were excised, homogenized, and assayed for the amount of β-galactosidase. Data were analyzed by one-way ANOVA. Asterisk means that the β-galactosidase expression level in 4 Gy irradiated tumors was significantly different from that in non-irradiated tumors ($P<0.01$). Each bar represents the mean ± s.d. ($n=3$).

half, and γ-ray irradiation augmented the antitumor activity five-fold as assessed by the relative tumor volume to the controls. The combined approach with AAVtk/GCV system and γ-ray irradiation suppressed the tumor growth for 30 days. These data were analyzed by two-way ANOVA ($P<0.01$).

Discussion

In this study, we demonstrated that γ-ray irradiation enhanced AAV vector-mediated transgene expression and cytocidal effect of AAVtk/GCV on target cells. These findings were also shown in animal experiments, ie γ-ray irradiation enhanced transgene expression and killing effect of the AAVtk/GCV upon the grafted tumors.

There is a possibility that the killing effect was contributed by HSVtk/GCV and γ-ray irradiation without AAV vectors. However, we made several experiments about the second-strand synthesis utilizing AAVLacZ and demonstrated that the enhanced killing effect can be explained by the higher conversion efficiency of AAV vector genome to double-stranded form. Although several studies have been reported on the higher conversion efficiency, the mechanism by which a genetic stress enhances the second-strand synthesis is not fully understood.[12–16] Qing *et al*[23] reported that dephosphorylation of the single-stranded D sequence-binding protein facilitated second-strand synthesis of the AAV vector genome. Sanlioglu *et al*[24] reported that the enhancement of AAV vector transduction by UV and adenovirus E4orf6 correlated with induction of two distinct molecular conversion pathways, and UV led to increased abundance of circular AAV vector genome. Some studies have shown that DNA repair synthesis is required for efficient transduction rather than replicative cellular DNA synthesis.[13,25] The machinery of cellular DNA repair synthesis may play an important role in converting the single-stranded AAV vector genome to a double-stranded form, which is activated by γ-ray irradiation.

Several studies reported that AAV vectors were useful for the treatment of cancers in model experiments. Kunke *et al*[3] showed that expressing the antisense of human papillomavirus early gene effectively killed the tumors derived from cervical cancer cells. Suicide gene therapy, in particular, HSVtk-expressing AAV vectors, was reported in the application to several kinds of cancers. The AAV vectors expressing HSVtk and interleukin 2 effectively killed glioma cells implanted into brains of nude mice.[26] The expression of HSVtk driven by a liver-specific promoter via AAV vectors in tumors experimentally produced by implantation of hepatocellular carcinoma cells successfully retarded the tumor progression.[27] We previously demonstrated the enhancement of the cytocidal effect of AAVtk/GCV system by

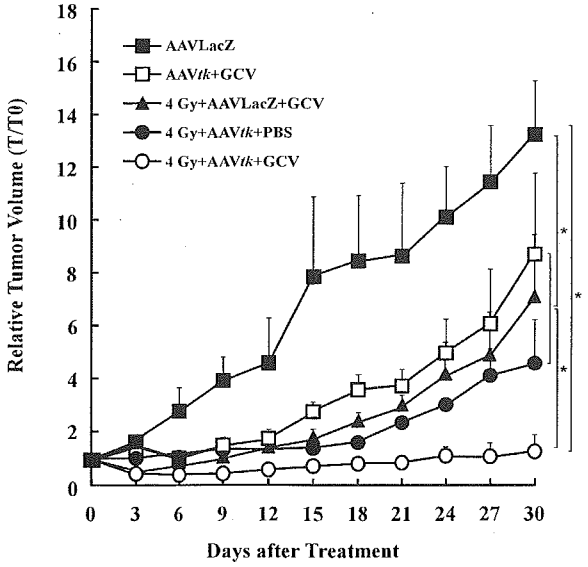

Figure 8 In vivo tumor growth inhibition with AAVtk/GCV treatment and γ-ray irradiation. The tumors derived from HEp-2 cells were established in the flanks of BALB/c nude mice by subcutaneous injection of the cell lines. Once established (a diameter of 4–5 mm), the tumors were irradiated at 4 Gy and then directly injected with 1×10^{12} particles of AAVLacZ or AAVtk. Administration of GCV (50 mg/kg) or PBS intraperitoneally twice a day was started 24 h after the virus injection and continued for 2 weeks. The tumors were measured every 3 days with calipers in two perpendicular diameters. Closed squares: AAVLacZ-injected animals (n=4); open squares: AAVtk-injected animals with GCV administration (n=5); closed triangles: AAVLacZ-injected animals with 4 Gy irradiation and GCV administration (n=5); closed circles: AAVtk-injected animals with 4 Gy irradiation and PBS administration (n=4); open circles: AAVtk-injected animals with 4 Gy irradiation and GCV administration. The relative tumor volume (T/T_0) was calculated as the ratio of tumor volumes at any time after the treatment (T) to that before the treatment (T_0). Data were analyzed by two-way ANOVA ($P<0.01$). Each data point represents the mean ± s.d.

γ-ray *in vitro*.[11] The AAVtk/GCV system effectively killed the cancer cells depending on the concentration of GCV. Moreover, when the cancer cells were irradiated prior to transduction with AAVtk, they were killed more efficiently in a dose-dependent manner.

To extend these *in vitro* findings to an animal model, in the present study, we investigated the enhancement of AAV-mediated transgene expression by γ-ray irradiation in nude mice. To confirm whether γ-ray enhances the transgene expression, the xenografted tumors were injected with AAVLacZ. γ-Ray irradiation increased the number of X-gal-positive cells and the amount of β-galactosidase as expected in the tumors *in vivo*. Furthermore, significantly higher growth inhibition of the xenografted tumors was observed in the 4 Gy irradiation plus AAVtk/GCV group compared with the AAVtk/GCV system alone, the 4 Gy irradiation plus AAVLacZ/GCV, or the AAVtk/PBS. The combination therapy using other vectors and irradiation has been reported,[21,22] but the augmentation of the second-strand synthesis by γ-ray irradiation is unique to AAV vectors.

Apoptosis induced by γ-ray irradiation enhances the bystander effects, which may allow nearby untransduced cells to take up the apoptotic vesicles containing phosphorylated toxic GCV metabolites.[21] Thus, the combination therapy of AAV-mediated suicide gene therapy with radiotherapy or other genotoxic stress such as chemotherapy seems to be valuable for the treatment of cancers.

Recently, the HSVtk mutants with improved GCV-mediated killing and bystander effect have been developed.[28–30] Since GCV has side effects such as pancytopenia[31] and acute renal failure,[32] the concentration of GCV should be kept as low as possible. In this study, the concentration of GCV was higher than the standard experiments, this concentration was chosen because the difference in the killing effects was most prominent among the groups, and the animals well tolerated throughout the study.

Our model would be another alternative to improve AAV-mediated suicide gene therapy of cancer. Although several studies were reported on combining radiotherapy and viral vector mediated gene therapy,[22,33] our therapeutic model made it with lower dose of irradiation. AAV-mediated suicide gene therapy and γ-ray irradiation may provide a more effective and safer alternative for the treatment of head and neck cancer.

Materials and methods

Cell lines

To compare the transgene expression between head and neck cancer cells and the cells used in the standard experiments, we used HeLa cells other than the head and neck cancer cells. HeLa cells and a human laryngeal carcinoma cell line, HEp-2 cells (a gift from the Cell Resource Center for Biomedical Research, Tohoku University), were cultured in DMEM/F12 (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Irvine Scientific, Santa Ana, CA, USA) at 37°C in 5% CO_2.

Plasmids

The plasmid pAAVLacZ contains the cytomegalovirus (CMV) promoter, human growth hormone first intron, *E. coli* LacZ gene, and SV40 early polyadenylation sequence between two inverted terminal repeats. A 1.8 kb DNA fragment encoding the HSVtk gene was obtained by double digestion with *Hinc* II and *Pvu* II of plasmid M2[34] (a gift from Dr Y Mishina, Yokohama City University, Japan) and subcloned into the pAAVLacZ in the place of the LacZ gene (pAAVtk). pW1909 is an AAV helper plasmid harboring rep/cap sequences, in which the p5 promoter was moved to downstream of the poly A signal to enhance AAV vector production.[35] An adenovirus helper plasmid plAd5 contains the adenovirus early genes: E2a, E4, and VA.[36]

AAV vector production

AAV vectors were produced based on the plasmid transfection.[36] Briefly, subconfluent 293 cells were co-transfected with AAV vector plasmid, pW1909, and adenovirus helper plasmid by a calcium phosphate precipitation method. Recombinant AAV was harvested by three cycles of freeze/thaw. The vector solution was then purified twice on a CsCl gradient as described previously.[36] The vector titer was determined by a quantitative dot blot hybridization of DNase-treated stocks.

Transduction of HeLa or HEp-2 cells with AAV vectors

One day before transduction, 1×10^5 cells were plated onto 3.5 cm dishes in triplicate. The cells were transduced with different amounts of AAVLacZ.

Assay for β-galactosidase activity

Thirty-six hours after transduction with AAVLacZ, the cells were fixed by 0.05% glutaraldehyde in PBS and stained with X-gal (Takara, Tokyo, Japan) in PBS containing 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, and 1 mM $MgCl_2$.[37] For each dish, 500 cells were counted by light microscopy and the percentage of blue stained cells was determined. An average of three wells was determined for each variable. In addition, the amount of β-galactosidase was quantified by using the β-gal ELISA kit (Boehringer-Mannheim, Hilden, Germany).

The enhancement of transgene expression by γ-ray irradiation

To examine whether γ-ray enhances the transgene expression in HeLa and HEp-2 cells, the cells were plated at a density of 1×10^5 cells/well in six-well culture plates 24 h prior to irradiation at doses of 0–5 Gy. Irradiation was done using GAMMACELL-40 (Atomic Energy of Canada, Ottawa, Canada) at a dose rate of 0.83 Gy/min. The cells were then transduced with 1×10^3 particles/cell of AAVLacZ immediately after irradiation. Thirty-six hours after transduction, we measured the amount of β-galactosidase with the β-gal ELISA.

Analysis of the second-strand synthesis of the vector genome

HeLa and HEp-2 cells grown in 10 cm dishes (1×10^6/dish) were transduced with 1×10^4 particles/cell of AAVLacZ immediately after irradiation. Two days later, total DNA was isolated under a low-salt condition (10 mM Tris–HCl [pH 7.5], 5mM EDTA, and 0.5% sodium dodecyl sulfate) to prevent annealing of the AAVLacZ genomes.[38] Forty micrograms of genomic DNA digested with 80 units of mung bean nuclease (Takara, Tokyo, Japan) was resolved on 1% agarose gels, transferred to nylon membranes (Hybond N^+; Amersham, Buckinghamshire, UK) and then hybridized with CMV promoter-specific probe radiolabeled with random primer labeling kit (Amersham, Buckinghamshire, UK) in 50% formamide, 6 × SSC, 0.5% SDS, 5 × Denhardt's solution, and 100 μg/ml of denatured salmon sperm DNA at 42°C overnight. The membranes were washed, and then analyzed by using an image analyzer (BAS-1500, Fuji, Tokyo, Japan).

GCV treatment

The cells were plated and transduced with AAVtk at the dose of 1×10^5 particles/cell. Twenty-four hours after transduction with AAV vectors, culture media were replaced by fresh media containing various concentrations of GCV ranging from 0 to 10 μg/ml. After a 7-day incubation in the presence of GCV, surviving cells were counted. The survival rate was calculated from the ratio of the number of surviving cells to the number of cells not treated with GCV. To evaluate the synergistic effect of the AAVtk/GCV system and γ-ray irradiation, the cells were irradiated at doses of 2 or 4 Gy just before AAV vector transduction.

Animal experiments

Female BALB/c nude mice (between 4 and 5 weeks of age) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and maintained in the specific pathogen-free animal facility at Jichi Medical School. The HEp-2 cells (2×10^6/mouse) with 25% Matrigel (Collaborative Research, Bedford, MA, USA) were injected subcutaneously into the flanks of the mice. The tumors were irradiated by the GAMMACELL-40.

Assay for β-galactosidase in tumors

To examine whether γ-rays enhance the transgene expression *in vivo*, 5×10^{11} particles of AAVLacZ were injected into the tumors in a diameter of 4–5 mm immediately after 4 Gy irradiation. Three days after AAVLacZ was injected, the tumors were excised from mice and frozen in OTC embedding compound on a liquid-nitrogen bath. Cryostat sections (20 μm thick) were made with a Cryotome CR-502 (Nakagawa, Tokyo, Japan) and were fixed with 0.05% glutaraldehyde in PBS. Histochemical staining for β-galactosidase activity was performed in the sections, as described above and counterstained with nuclear fast red. Furthermore, to quantify the amount of β-galactosidase in the non-irradiated or 4 Gy irradiated tumor, these samples were homogenized for 10 s in a tissue homogenizer. The homogenates were centrifuged twice and aliquots of the supernatants were prepared for the assay with β-gal ELISA kit (Boehringer-Mannheim). Four mice were used in each treatment group.

In vivo gene therapy

To examine the tumor growth inhibition, 1×10^{12} particles of AAVtk or AAVLacZ as control were injected into the tumors immediately after 4 Gy irradiation. Administration of GCV (50 mg/kg) or PBS intraperitoneally twice a day was started 24 h after the virus infection and continued for 2 weeks. The tumors were measured every 3 days with calipers in two perpendicular diameters. The tumor volume was calculated as $0.5 \times L \times W^2$, where L is the length (mm) and W is the width (mm).[39] The tumors reaching a diameter of 4–5 mm were used as the starting point for the study of tumor growth or regression.

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Clinical Evaluation of a Recombinant Factor VIII Preparation (Kogenate) in Previously Untreated Patients with Hemophilia A

A. Yoshioka,[a] K. Fukutake,[b] J. Takamatsu,[c] A. Shirahata,[d]
and the Kogenate Post-Marketing Surveillance Study Group

[a]Department of Pediatrics, Nara Medical University, Kashihara; [b]Department of Clinical Laboratory Medicine, Tokyo Medical College, Tokyo; [c]Department of Blood Transfusion, Internal Medicine I, Nagoya University School of Medicine, Nagoya; [d]Department of Pediatrics, University of Occupational and Environmental Health, Kitakyushu, Japan

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Abstract

The safety and efficacy of a recombinant factor VIII (rFVIII) preparation (Kogenate) for the treatment of bleeding episodes was studied in previously untreated patients (PUPs) with severe, moderate, and mild hemophilia A. Patient peripheral blood samples taken at baseline and at 3, 6, 9, 12, 18, and 24 months after the first infusion were evaluated for FVIII inhibitor antibodies by the Bethesda assay, for antibodies formed against trace proteins derived from the rFVIII production process, and for general changes in laboratory test results. Samples for general laboratory testing were also drawn every 6 months after the first 24 months. Hemostatic efficacy was assessed by physicians, and adverse events were recorded throughout the study period. Forty-three PUPs (30 with FVIII:C <1%; 10 with FVIII:C 1%-5%; and 3 with FVIII:C >5%) aged 3 months to 32 years were enrolled at 33 centers in Japan. Patients were studied for a mean of 51 months (range, 11-80 months), and the mean exposure time was 83 days (range, 2-571 days). The incidence of occurrence of FVIII inhibitors was 34.9% (high responders [≥10 Bethesda U/mL], 11.6%; low responders [0.5-<10 Bethesda U/mL], 23.3%). The median cumulative exposure time of inhibitor detection was 12 days, indicating inhibitor development at an early stage after the start of infusion of this preparation. Hemostasis was achieved with a single dose of Kogenate in 94.8% of the 951 bleeding episodes recorded in the study. Transient increases in antibodies against baby hamster kidney proteins and antimouse immunoglobulin G were observed in 14.0% and 18.6% of patients, respectively. Anti-rFVIII seroconversion was observed in 18.6% of patients and only in patients with inhibitor antibodies. Antibody responses to trace proteins were not correlated with drug-related adverse events with the exception of FVIII activity inhibition in PUPs with anti-rFVIII seroconversion. These data indicate that Kogenate is safe and effective for the treatment of bleeding in PUPs with hemophilia A. *Int J Hematol.* 2003;78:467-474.

Key words: Recombinant factor VIII; Kogenate; Clinical study; Previously untreated patients with hemophilia A; Inhibitor; Antibody to foreign protein

1. Introduction

A recombinant factor VIII (rFVIII) preparation (Kogenate; Bayer, Berkeley, CA, USA) was developed to reduce the risk of patient infection with pathogenic viruses, a long-standing problem for human plasma-derived FVIII (pdFVIII) products, and to decrease the need for human blood as a raw material.

Recombinant FVIII was produced in 1984 by introducing the complementary DNA for the human blood–coagulating FVIII into baby hamster kidney (BHK) cells [1,2]. Kogenate was manufactured by the production system set up by Miles Laboratories (presently Bayer Corporation) in the United States.

Early clinical trials [3,4] of this preparation conducted in countries including Japan (1988) were followed by early-stage trials (stage I and II) in Japan (1988-1989) covering 5

Correspondence and reprint requests: A. Yoshioka, Department of Pediatrics, Nara Medical University, Shijo-cho 840, Kashihara, Nara 634-8522, Japan; 0744-29-8881; fax: 0744-24-9222 (e-mail: ayped@naramed-u.ac.jp).

patients with severe hemophilia A at 2 institutions. In these trials, Kogenate exhibited an in vivo recovery, a half-life in the blood, and satisfactory hemostatic effects and safety that were comparable to those of the conventional pdFVIII preparation (Koate-HT; Bayer) [5]. In addition, a multicenter long-term infusion study (stage III) of Kogenate was conducted from June 1989 to February 1991 by 13 institutions covering 20 previously treated patients, including children. As a result, it was reported that (1) Kogenate exhibited an in vivo recovery and a half-life comparable to those of the pdFVIII preparation, (2) Kogenate exhibited a high hemostatic effect, and (3) no production of inhibitors and antibodies to rFVIII was observed [6].

In July 1993, this product was approved for the first time in Japan as an rFVIII preparation and was launched in September of the same year. Postmarketing surveillance (PMS) included a prospective study from September 1993 to March 1999 that investigated the safety and hemostatic efficacy of rFVIII in previously untreated patients (PUPs) with hemophilia A. In addition, the PMS required evaluation of the production of FVIII inhibitors and whether antibodies against rFVIII, BHK protein, and mouse immunoglobulin G (IgG) were generated. This PMS was required for a period of 2 years or more and included institutions participating in the Kogenate PMS Study Group.

2. Materials and Methods

2.1. Subjects

Eligible subjects were patients who had not received previous treatment with blood transfusion or a blood preparation and had given consent to participate in the PMS study.

Patients who had been given a blood preparation other than FVIII concentrates once or twice were also included. Institutions participating in the study are shown in Table 1.

Table 1.
Kogenate Postmarketing Surveillance Study Group

Hospital	Department
Asahikawa Kosei Hospital	Pediatrics
Asahikawa Red Cross Hospital	Pediatrics
Iwate Medical University School of Medicine	Pediatrics
Iwaki Kyoritsu General Hospital	Pediatrics
Shinshu University, Faculty of Medicine	Pediatrics
Nagano Red Cross Hospital	Pediatrics
Nagano Children's Hospital	Hematology/Oncology
Isesaki Municipal Hospital	Pediatrics
Saitama Medical School	Pediatrics
Saitama Children's Medical Center	Hematology/Oncology
Chiba University, Faculty of Medicine	Pediatrics
Tokyo Medical College	Clinical Laboratory Medicine
Ogikubo Hospital	Hematology
Tokyo Medical and Dental University	Pediatrics
Kanagawa Children's Medical Center	Hematology
St. Marianna University School of Medicine	Pediatrics
Nagoya University, School of Medicine	Blood Transfusion, Internal Medicine I
Nagoya City University Medical School	Pediatrics
Nara Medical University	Pediatrics
Senboku National Hospital	Pediatrics
Osaka City General Hospital	Pediatrics (Internal Medicine)
Saiseikai Ibaraki Hospital	Pediatrics
Kinan General Hospital	Pediatrics
Kashiwara City Hospital	Pediatrics
Bell Land General Hospital	Pediatrics
Hiroshima University Medical School	Pediatrics
Onoda General Hospital	Pediatrics
Ehime University, School of Medicine	Pediatrics
Yamaguchi University School of Medicine	Pediatrics
University of Occupational and Environmental Health, Japan	Pediatrics
Medical Bulletin of Fukuoka University	Pediatrics
Saga Medical School	Pediatrics
Kurume University School of Medicine	Pediatrics

2.2. Study Period

The study period was July 1993 through March 1999.

2.3. Assays and Timing of Observations

2.3.1. FVIII Inhibitor Test (Bethesda Assay)

Inhibitor measurements were made with the Bethesda assay [7] at each institution at baseline and at 3, 6, 9, 12, 18, and 24 months after infusion. When any inhibitor was detected, remeasurement was made at the Department of Pediatrics, Nara Medical University, for confirmation. A measured value of 0.5 Bethesda unit (BU)/mL or greater was considered positive.

2.3.2. Tests of Antibodies against rFVIII, BHK Protein, and Mouse IgG (Anti-C7F7 Protein)

Serum was collected simultaneously with inhibitor measurement, and levels of total IgG, IgG1, IgG2, IgG3, and IgG4 antibodies and IgM antibody in each serum sample were measured by means of enzyme-linked immunosorbent assay (ELISA) as previously reported by the Department of Pediatrics, Nara Medical University [8].

A positive result for anti-rFVIII antibody was defined as an absorbance greater than 0.1 in the IgG4 antibody test. However, if the IgG4 antibody test result was negative, and the result for total IgG antibodies or any one of the IgG1, IgG2, or IgG3 antibodies was positive (absorbance $\geq$0.1), the status was judged a false positive and was included in the rate of positive responses.

Anti-BHK protein and antimouse IgG antibody levels were considered positive in the following cases: (1) The absorbance for total IgG antibodies was 0.1 or greater; (2) the absorbance for total IgG antibody was less than 0.1, the absorbance of any one of the IgG1, IgG2, IgG3 or IgG4 antibodies was 0.1 or greater, and the blocking assay [8] response was 50% or higher.

2.3.3. Blocking Test

Nonspecific positive results were assessed by combining samples of the patient's blood serum with a volume of anti-

gen (rFVIII, BHK, and C7F7) exceeding 10 times the amount in the patient's blood serum and evaluating the sample by ELISA. When the absorbance of a sample was blocked 50% or more (optical density less than one half), the assay result was considered positive.

2.3.4. General Laboratory Tests

Measurements were made at each institution at baseline and at 3, 6, 9, 12, 18, and 24 months after infusion. After 24 months, measurements were made every 6 months. The following groups of parameters were measured: hematologic tests (erythrocyte count, hemoglobin volume, hematocrit value, leukocyte count, differential leukocyte count, and platelet count), blood biochemistry (aspartate aminotransferase, alanine aminotransferase (GPT), alkaline phosphatase, γ-glutamyl transpeptidase, total bilirubin, direct bilirubin, lactate dehydrogenase, blood urea nitrogen, creatinine, total protein, serum albumin, sodium, potassium, and chloride), urinalysis (protein, glucose, and sediment), and immunologic tests (human immunodeficiency virus antibody, hepatitis B surface antigen, and antibodies to hepatitis B surface antigen, hepatitis B core antigen, hepatitis C virus, cytomegalovirus, and Epstein-Barr virus).

2.4. Collection of Samples

Several conditions were set before the collection, preservation, and transportation of samples.

2.4.1. Baseline Testing

For baseline blood samples before the first infusion, the sample was collected just before infusion or as early as 4 weeks before infusion. However, if a patient required immediate treatment with rFVIII or if collecting the blood all at once was not possible given the volume required and the timing of the collection, only antibody testing for trace proteins was conducted. Other tests were performed immediately after the administration of rFVIII or within one week after the first infusion at the latest.

2.4.2. FVIII Inhibitor Testing

It was preferable to collect blood samples more than 3 days after the infusion of rFVIII. For example, 0.5 mL of plasma was frozen at –70°C at each institution prior to measurement. If an inhibitor was detected, an additional 2 bottles of plasma of 0.5 mL each (total volume, 1 mL) were frozen at –70°C at the institution and then sent to the Department of Pediatrics, Nara Medical University, for confirmation.

2.4.3. Trace Protein Antibody Testing for Antibodies against rFVIII, BHK Protein, and Mouse IgG (Anti-C7F7 Protein)

Two 0.25-mL bottles of serum samples (total volume, 0.5 mL) were frozen at –70°C at each institution and then sent to the Department of Pediatrics, Nara Medical University.

2.4.4. General Laboratory Tests

When the GPT value exceeded twice the normal value, measurements were made every 2 weeks until the value decreased to twice the normal value or lower.

2.5. Evaluation

2.5.1. Hemostatic Effect

The hemostatic effect of Kogenate on bleeding symptoms was rated for each bleeding episode by physicians and patients using the following categories:

1. Markedly effective, hemostasis attained within 1 to 2 days;
2. Effective, hemostasis attained within 3 to 4 days;
3. Slightly effective, hemostasis attained within 5 to 7 days;
4. Ineffective, hemostasis not attained within 7 days;
5. Aggravated, condition aggravated.

2.5.2. Safety

For patients with adverse reactions, including abnormal changes in laboratory test values, detailed records were taken about the symptoms, their severity, the dates of onset, any causal relationships to this preparation, the subsequent course of the symptoms, the actions taken, and the outcomes. In addition, a follow-up investigation was made until the patient recovered from the adverse reaction(s) or the laboratory test value returned to the normal level.

Safety evaluation included tests for the production of FVIII inhibitor antibodies and the production of antibodies against residual trace proteins (rFVIII, BHK protein, and mouse IgG).

2.5.3. Usefulness

Subjective evaluation of rFVIII therapy usefulness was made by the attending physician at the end of the investigation according to the following 5 categories: very useful, useful, slightly useful, indecisive, and not useful.

2.5.4. Statistics

Comparisons between groups were made with the χ^2 test. Probability values were derived by means of a 2-tailed significance test. P values <.05 were considered to indicate statistical significance.

3. Results

3.1. Subjects

Forty-seven patients were enrolled, and 43 patients were eligible for evaluation. Thirty-one patients were described as having "severe" hemophilia A (FVIII:C, <1%), 9 patients had "moderate" disease (FVIII:C, 1%-5%), and 3 patients had "mild" disease (FVIII:C, >5%). The ages of the evaluable patients ranged from 3 to 386 months (32.2 years) with a mean of 26 months. The observation periods ranged from

11 to 80 months (mean, 51 months). The exposure times ranged from 1 to 48 days with a median of 12 days.

3.2. Production of FVIII Inhibitor Antibodies

Of the 43 patients evaluated, inhibitor production was noted in 15 patients for an inhibitor incidence of 34.9% (Table 2). Five patients exhibited high titers of inhibitors (≥10 BU/mL) for an incidence of 11.6% (5/43). The peak titers of the inhibitors in these 5 patients ranged from 13.1 to 975 BU/mL, and all 5 patients had severe cases (FVIII:C, <1%). Kogenate treatment was discontinued in 1 of these patients (patient no. 2) and continued in the 4 other patients with high inhibitor titers.

The hemostatic effect of Kogenate in the 5 patients with high responder inhibitor levels was satisfactory before the formation of inhibitors. After the production of inhibitors, anti-inhibitor drugs (Feiba Immuno, Proplex, Autoplex; Baxter Healthcare Corporation, Deerfield, IL, USA) were given to 3 patients (nos. 1, 2, and 5) at the time of bleeding. Immune-tolerance therapy with the regular infusion of Kogenate FVIII was introduced for 2 patients (nos. 1 and 5), and 1 patient (no. 2) was treated with another pdFVIII preparation. The hemostatic effects for the 2 patients (nos. 1 and 5) who received Kogenate were satisfactory, and therefore Kogenate treatment was continued.

Ten patients exhibited low inhibitor titers (0.5-<10 BU/mL) for an incidence of 23.3% (10/43). Eight patients who developed low inhibitor titers had severe disease, and 2 had moderate disease (FVIII:C, 1%-5%). Five of these patients had inhibitor titers ranging from 0.5 to ≤1.0 BU/mL. In 7 of the 10 patients, the inhibitors were transient, and even after the detection of inhibitors the hemostatic effect of Kogenate was satisfactory in each patient. Thus, the infusion of rFVIII was continued. In the 3 remaining patients, however, the disappearance of the low inhibitor titers was not confirmed within the observation period.

The median cumulative exposure time for the detection of inhibitors was 12 days (range, 1-48 days) in high responders, 10.5 days (range, 1-38 days) in low responders, and 12 days (range, 1-48 days) in all 15 patients. The cumulative doses until the development of inhibitors in high responders and low responders were not significantly different ($P = .306$).

3.3. Production of Antibodies against rFVIII, BHK Protein, and Mouse IgG

Of the 43 patients evaluated (Table 2), positive results for anti-rFVIII antibodies were observed in 8 patients, and a false positive was observed in 1 patient who was later confirmed to be negative. The rate of positive conversion was 18.6% (8/43). None of the 8 patients who tested positive for anti-rFVIII antibodies had tested negative by the end of observation period in this PMS study. All 5 patients with high titers of inhibitor antibodies were positive for anti-rFVIII antibodies. Three of the 10 patients with low titers of inhibitor antibodies had anti-rFVIII antibodies. In the 8 patients who tested positive for anti-rFVIII antibodies, the production of inhibitor antibodies was observed concurrently. Anti-rFVIII antibody test results were negative in 6 of the 7 patients in whom the presence of inhibitors was of low titer and transient.

The exposure time from the day of the initial infusion to the onset of positive anti-rFVIII antibodies was longer than the exposure time required for the development of inhibitors (Table 3), but not significantly so ($P = .167$).

Of the 43 patients evaluated, positive results for anti-BHK protein antibodies were observed in 6 patients, a 14.0% (6/43) rate of positive conversion (Table 2). The positivity was transient in all of these patients.

Of the 43 patients evaluated, positive test results for antimouse IgG antibodies were observed in 8 patients for an incidence for becoming positive of 18.6% (8/43). The positivity was transient in all 8 patients (Table 2).

All patients who tested positive for anti-BHK protein antibodies also tested positive for antimouse IgG antibodies (Table 2). Of those patients who tested positive for anti-BHK protein antibodies or antimouse IgG antibodies, the development of inhibitors was observed in 2 patients.

Patients who tested positive for anti-BHK protein antibodies and patients who tested positive for antimouse IgG antibodies displayed no adverse reactions, such as anaphylactic symptoms, and no problems with hemostatic efficacy were noted.

3.4. Results of Treatment for Bleeding Episodes

The 43 patients experienced 951 observed bleeding episodes. When bleeding occurred at 2 or more sites at the same time, each additional bleeding site was counted as an individual bleeding episode. An analysis of bleeding sites indicated that subcutaneous bleeding was the most prevalent location with 454 observed episodes (47.7%), followed by 241 episodes (25.3%) of joint bleeding, 86 episodes (9.0%) of oral cavity bleeding, 84 episodes (8.8%) of muscle bleeding, 21 episodes (2.2%) of open hemorrhage, 21 episodes (2.2%) of bleeding from sites not mentioned, 18 episodes (1.9%) of epistaxis, 12 episodes (1.3%) of gingival bleeding, 8 episodes (0.8%) of intracranial bleeding, 4 episodes (0.4%) of hematuria, 1 episode (0.1%) of bleeding as a result of tooth extraction, and 1 episode (0.1%) of gastrointestinal tract bleeding. Analysis of the number of infusions required for the treatment of each episode indicated that hemostasis was achieved with a single dose of rFVIII in 937 bleeding episodes, or 98.5% of the total episodes. Thus, a therapeutic effect was obtained with a single dose in almost all bleeding episodes (Table 4).

The physicians' subjective evaluations of the hemostatic efficacy of rFVIII therapy for each bleeding episode were "markedly effective" in 702 episodes (73.8%), "effective" in 200 episodes (21.0%), "slightly effective" in 34 episodes (3.6%), "ineffective" in 11 episodes (1.2%), and "aggravated" in 4 episodes (0.4%). Thus, the combined effectiveness rate of markedly effective and effective responses was 94.8% (902/951 episodes) (Table 5).

The evaluations of hemostatic efficacy per administration of rFVIII (total, 1496 infusions) were markedly effective in 810 infusions (54.1%), effective in 399 infusions (26.7%), slightly effective in 145 infusions (9.7%), ineffective in 67 infusions (4.5%), and aggravated in 75 infusions

Table 2.
List of All 43 Previously Untreated Patients (PUPs)*

Patient No.	Age, mo	Severity†	Transience (FVIII Inhibitor)	Antibodies to rFVIII (ELISA)	Peak Titer of FVIII Inhibitor, BU/mL	Antibodies to BHK protein (ELISA)	Antibodies to Mouse IgG (ELISA)	Observation Period, mo	Immune-Tolerance Treatment	Bypass Therapy
High responder‡										
1	6	S		+	975	–	–	54	Yes	Yes
2	9	S		+	102	+	+	55	Yes	Yes
3	5	S		+	53	–	–	43		
4	13	S		+	50	–	–	23		
5	9	S		+	13.1	–	–	33	Yes	Yes
Low responder‡										
6	13	S		+	3.3	–	–	47		
7	15	S		+	2	–	–	59	Yes	
8	23	S	Yes	+	1	–	–	38		
9	6	S		±§	2.3	–	–	23		
10	19	S	Yes	–	7.2	–	+	42		
11	21	S	Yes	–	4.6	–	–	80		
12	23	Mo	Yes	–	0.7	–	–	80		
13	3	Mo	Yes	–	0.5	–	–	56		
14	83	S	Yes	–	0.5	–	–	38		
15	26	S	Yes	–	0.5	–	–	35		
				18.6% (8/43)	34.9% (15/43)					
16	9	S		–	<0.5	+	+	58		
17	6	S		–	<0.5	+	+	75		
18	9	S		–	<0.5	+	+	72		
19	8	S		–	<0.5	+	+	75		
20	36	Mo		–	<0.5	+	+	75		
21	6	Mo		–	<0.5	–	+	50		
22	8	S		–	<0.5	–	–	52		
23	13	S		–	<0.5	–	–	47		
24	19	S		–	<0.5	–	–	69		
25	60	Mo		–	<0.5	–	–	63		
26	39	Mo		–	<0.5	–	–	80		
27	9	S		–	<0.5	–	–	25		
28	6	S		–	<0.5	–	–	71		
29	12	Mi		–	<0.5	–	–	71		
30	21	Mi		–	<0.5	–	–	57		
31	8	S		–	<0.5	–	–	28		
32	386	Mo		–	<0.5	–	–	62		
33	36	Mo		–	<0.5	–	–	43		
34	11	S		–	<0.5	–	–	52		
35	10	Mo		–	<0.5	–	–	50		
36	4	S		–	<0.5	–	–	21		
37	4	S		–	<0.5	–	–	11		
38	11	S		–	<0.5	–	–	42		
39	11	S		–	<0.5	–	–	38		
40	9	S		–	<0.5	–	–	58		
41	12	S		–	<0.5	–	–	46		
42	8	S		–	<0.5	–	–	20		
43	75	Mi		–	<0.5	–	–	74		
						14.0% (6/43)	18.6% (8/43)			

*FVIII indicates factor VIII; ELISA, enzyme-linked immunosorbent assay; BU, Bethesda unit; BHK, baby hamster kidney; IgG, immunoglobulin G.
†S indicates severe (FVIII:C, <1%); Mo, Moderate (FVIII:C, 1%~5%); Mi, Mild (FVIII:C, >5%).
‡High responder, ≥10 BU/mL; Low responder, 0.5-<10 BU/mL.
§The IgG4 antibody result was negative, and the total IgG antibody result or any one of IgG1, IgG2, or IgG3 antibody results was positive (ELISA absorbance ≥0.1); result later confirmed to be negative.

(5.0%). The combined effectiveness rate of markedly effective and effective responses was 80.8% (1209/1496 infusions) (Table 6).

The evaluations of effectiveness by bleeding site showed that the effectiveness rates were as follows: subcutaneous bleeding, 90.7% (536/591); joint bleeding, 82.5% (353/428); muscle bleeding, 59.4% (111/187); oral cavity bleeding, 64.8% (92/142); intracranial bleeding, 44.9% (22/49); open hemorrhage, 100% (30/30); epistaxis, 90.0% (18/20); gingival bleeding, 100% (15/15); hematuria, 100% (5/5); bleeding

Table 3.
Status of 15 Previously Untreated Patients (PUPs) Who Developed Inhibitors*

Patient No.	Age, mo	Severity†	Time from First-Time Infusion to Anti-rFVIII Antibody Development, d	Time from First-Time Infusion to FVIII Inhibitor Development, d	Exposure Time from First-Time Infusion to Anti-FVIII Antibody Development, d	Exposure Time from First-Time Infusion to FVIII Inhibitor Development, d	Total Amount of Replacement to Anti-FVIII Antibody Development, U	Total Amount of Replacement to FVIII Inhibitor Development, U
High responder‡								
1	6	S	241	94	19	12	6000	4250
2	9	S	238	23	100	48	62,558	34,263
3	5	S	850	90	NE	2	29,915	500
4	13	S	237	237	46	46	78,750	78,750
5	9	S	56	19	2	1	32,520	4100
			324.4§	92.6§	32.5‖	12‖	36,148.6§	23,552.6§
Low responder‡								
6	13	S	271	89	11	1	8164	1164
7	15	S	138	138	7	7	3500	3500
8	23	S	88	88	38	38	10,000	10,000
9	6	S	255	157	27	14	9189	5689
10	19	S	NE	565	NE	7	NE	2398
11	21	S	NE	478	NE	17	NE	8500
12	23	Mo	NE	201	NE	1	NE	500
13	3	Mo	NE	623	NE	15	NE	5185
14	83	S	NE	566	NE	22	NE	26,000
15	26	S	NE	557	NE	5	NE	5000
				346.2§	19‖	10.5‖		6793.6§
					23¶	12¶		

*rFVIII indicates recombinant factor VIII; NE, not evaluated.
†S indicates severe (FVIII:C, <1%); Mo, Moderate (FVIII:C, 1%~5%); Mi, Mild (FVIII:C, >5%).
‡High responder, ≥10 BU/mL; Low responder, 0.5-<10 BU/mL.
§Average of 5 high responders (patients nos. 1-5) or 10 low responders (patient nos. 6-15).
‖Median of 5 high responders (patient nos. 1-5) or 10 low responders (patient nos. 6-15).
¶Median of the 15 inhibitor results (patient nos. 1-15).

from tooth extraction, 100% (2/2); and gastrointestinal tract bleeding, 100% (1/1) (Table 6).

3.5. Laboratory Test Values

Of the 43 patients, an increased GPT level (106 IU/L) was noted in 1 patient (no. 40). The symptom was mild and transient. This patient had not been infected with hepatitis B or C virus, and the patient had not received a vaccine inoculation. Therefore, the cause of the elevated GPT level was unknown.

3.6. Usefulness

Overall therapeutic usefulness was evaluated by taking the hemostatic effects, adverse reactions, and laboratory test values into consideration.

Table 4.
Number of Infusions per Bleeding Episode Classified by Bleeding Site

Bleeding Site	Bleeding Episodes, n	No. of Infusions			
		1	2	3	4 or More
Subcutaneous	454 (47.7%)	450	4	0	0
Joint	241 (25.3%)	238	3	0	0
Oral cavity	86 (9.0%)	86	0	0	0
Muscle	84 (8.8%)	82	2	0	0
Open hemorrhage	21 (2.2%)	21	0	0	0
Not mentioned	21 (2.2%)	21	0	0	0
Epistaxis	18 (1.9%)	18	0	0	0
Gingival	12 (1.3%)	12	0	0	0
Intracranial	8 (0.8%)	3	4	1	0
Hematuria	4 (0.4%)	4	0	0	0
Tooth extraction	1 (0.1%)	1	0	0	0
Gastrointestinal tract	1 (0.1%)	1	0	0	0
Total	951 (100.0%)	937 (98.5%)	13 (1.4%)	1 (0.1%)	0 (0.0%)

Table 5.
Hemostatic Efficacy of Kogenate for Each Bleeding Episode

Bleeding Site	Bleeding Episodes, n	Hemostatic Efficacy: Markedly Effective, n	Effective, n	Slightly Effective, n	Ineffective, n	Aggravated, n	Not Mentioned, n	Effectiveness Rate (Markedly Effective + Effective)
Subcutaneous	454	349	89	11	4	1	0	96.5% (438/454)
Joint	241	170	59	10	1	1	0	95.0% (229/241)
Oral cavity*	86	67	10	4	4	1	0	89.5% (77/86)
Muscle	84	52	25	5	1	1	0	91.7% (77/84)
Open hemorrhage	21	15	6	0	0	0	0	100.0% (21/21)
Not mentioned	21	18	2	0	1	0	0	95.2% (20/21)
Epistaxis	18	14	3	1	0	0	0	94.4% (17/18)
Gingival	12	7	5	0	0	0	0	100.0% (12/12)
Intracranial	8	5	0	3	0	0	0	62.5% (5/8)
Hematuria	4	3	1	0	0	0	0	100.0% (4/4)
Gastrointestinal tract	1	1	0	0	0	0	0	100.0% (1/1)
Tooth extraction	1	1	0	0	0	0	0	100.0% (1/1)
Total	951	702 (73.8%)	200 (21.0%)	34 (3.6%)	11 (1.2%)	4 (0.4%)	0 (0.0%)	94.8% (902/951)

*Except for gingival and tooth extraction.

Of the 43 patients eligible for the usefulness evaluation, the rating of "very useful" was given to 31 patients (72.1%), and "useful" was given to 10 patients (23.3%). Combining these 2 ratings gave an overall useful rate of 95.3% (41/43). There were no cases of "not useful."

4. Discussion

One of the most important problems yet to be overcome in the treatment of hemophilia is the production of alloantibodies (inhibitors) to coagulation factors. Inhibitor antibodies have been reported in 5% to 25% of hemophilia A patients [9-13]. However, the mechanism by which the antibody is produced in some patients but not in others has yet to be clarified [14].

In Japanese clinical trials of a purified FVIII/von Willebrand factor preparation [15], an rFVIII preparation (Kogenate [6], Reconate [16]), and a monoclonal antibody–refined FVIII preparation (Crosseight M [17]) covering previously treated patients, no development of inhibitors was observed in any of the participating patients.

However, because previously treated patients are those in whom inhibitors did not develop in previous replacement therapy, such patients are inappropriate for samples to determine the real incidence of inhibitors for each preparation. Therefore, the prospective evaluation of safety and efficacy in PUPs was warranted. Overseas trials of an rFVIII preparation have summarized that inhibitors developed in 28.1% to 30.5% of patients [18]. The incidence of antibodies in PUPs with severe disease after treatment with a plasma-derived preparation has been reported to be 10% to 52% [19].

In the present study, we prospectively investigated the safety of Kogenate, especially in terms of the production of inhibitors and various antibodies, and examined Kogenate's hemostatic effects during long-term administration as part of the PMS. The study was conducted for an average of 4.1 years (range, 11-80 months), including follow-up investigations. The study covered 951 bleeding episodes, 1496 infusions of this preparation, and the administration of 1,671,352 U of Kogenate.

The 34.9% (15/43) incidence of inhibitors with this preparation is slightly higher than the incidences of 19.8% to

Table 6.
Hemostatic Efficacy of Kogenate for Each Infusion

Bleeding Site	Infusions, n	Hemostatic Efficacy: Markedly Effective, n	Effective, n	Slightly Effective, n	Ineffective, n	Aggravated, n	Not Mentioned, n	Effectiveness Rate (Markedly Effective + Effective)
Subcutaneous	591	382	154	30	15	10	0	90.7% (536/591)
Joint	428	209	144	47	4	24	0	82.5% (353/428)
Muscle	187	59	52	24	28	24	0	59.4% (111/187)
Oral cavity*	142	72	20	15	18	17	0	64.8% (92/142)
Intracranial	49	22	0	27	0	0	0	44.9% (22/49)
Open hemorrhage	30	19	11	0	0	0	0	100% (30/30)
Not mentioned	26	18	6	0	2	0	0	92.3% (24/26)
Epistaxis	20	14	4	2	0	0	0	90.0% (18/20)
Gingival	15	8	7	0	0	0	0	100% (15/15)
Hematuria	5	4	1	0	0	0	0	100% (5/5)
Tooth extraction	2	2	0	0	0	0	0	100% (2/2)
Gastrointestinal tract	1	1	0	0	0	0	0	100% (1/1)
Total	1496	810 (54.1%)	399 (26.7%)	145 (9.7%)	67 (4.5%)	75 (5.0%)	0 (0.0%)	80.8% (1209/1496)

*Except for gingival and tooth extraction.

30.6% that have been reported so far with rFVIII preparations [19-21]. However, the incidence of high responders (≥10 BU/mL), who pose a great problem in the replacement therapy, was 11.6% (5/43), an incidence nearly equal to that previously reported [19]. The remaining 10 patients had low inhibitor titers. Of these 10 patients, the symptom was transient in 7 patients. Analysis of the 31 patients with severe disease revealed an incidence of inhibitors of 41.9% (13/31), and the incidence of high responders in this group was 16.1% (5/31). On the other hand, the incidences of high responders in PUPs with severe disease and given a monoclonal antibody–purified FVIII preparation have been reported to be 10% [22] to 24% [23]. The incidence in our present study was nearly equal to those of these earlier reports.

The median exposure time in 15 patients from the time of infusion to the development of inhibitors was 12 days. Scharrer et al [19] reported that the development of inhibitors was seen after 9 to 18 days of administration. In our study, inhibitors also developed at an early stage after the start of administration.

Antibody testing by the ELISA method revealed 8 patients (18.6%) positive for anti-rFVIII antibodies, 6 patients (14.0%) positive for anti–BHK protein antibodies, and 8 patients (18.6%) positive for antimouse IgG antibodies. Of the 15 patients exhibiting the development of inhibitors, 6 patients tested negative for anti-rFVIII antibodies, and 7 patients exhibited the transient presence of inhibitors (all with low titers). Because these figures agree, we consider the ELISA antibody test used in the present study to be useful for confirming the presence of inhibitors in patients. The inhibitor positivity was transient in all of the patients positive for anti–BHK protein antibodies and in all of the patients positive for mouse IgG antibodies. Because the development of inhibitors was observed in only 2 patients positive for anti–BHK protein antibodies or mouse IgG antibodies, the presence of these antibodies is considered to have no relationship to the development of inhibitors.

Patients positive for anti–BHK protein antibodies and positive for antimouse IgG antibodies displayed no anaphylactic or other symptoms, and no problems were observed in the everyday use of this preparation [24].

Sufficient hemostasis was attained with a single dose of this preparation in 98.5% of the bleeding episodes. The hemostatic effect evaluated in terms of bleeding episodes was 94.8% (902/951), and the effectiveness rate of the hemostatic effect of infusions was 80.8% (1209/1496 infusions). Thus, a high effectiveness rate was obtained in this study, as was obtained in the clinical trials conducted at the time of Kogenate development [6].

From these results, we conclude that Kogenate is an effective and safe FVIII preparation for the treatment of PUPs.

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Management of haemophilia B inhibitor patients with anaphylactic reactions to FIX concentrates

M. SHIBATA*, M. SHIMA*, H. MISU†, Y. OKIMOTO‡, J. C. GIDDINGS§ and A. YOSHIOKA*
**Department of Pediatrics, Nara Medical University, Kashihara, Nara, Japan; †Department of Hematology and Oncology, Hyogo Prefectural Kobe Children's Hospital, Kobe, Japan; ‡Department of Hematology and Oncology, Chiba Children's Hospital, Chiba, Japan; and §Department of Haematology, University of Wales College of Medicine, Cardiff, UK*

Summary. Allergic reactions to concentrates containing factor IX (FIX) are serious complications in the treatment of haemophilia B patients with inhibitor. We have established a therapeutic protocol for such cases using an initial skin test followed by stepwise infusions of FIX concentrates under hydrocortisone cover. We have successfully treated three patients whose treatment with FIX had been suspended.

Keywords: anaphylaxis, factor IX, haemophilia B, inhibitor, management

Introduction

Some haemophilia B patients with inhibitors experience allergic reactions to factor IX (FIX) concentrates [1]. In such cases, the therapy utilizing products containing significant amounts of FIX including prothrombin complex concentrate (PCC) and activated prothrombin complex concentrate (APCC) is usually contraindicated and haemostatic control becomes extremely difficult. Although treatment with recombinant activated factor VII (FVIIa) is generally recommended [2], it is not always therapeutically effective. Only a few reports describing successful management of such patients, utilizing products containing FIX [3,4] are available. In the present study, we have devised a straightforward therapeutic protocol for such cases and have successfully treated three patients.

Patients

All patients had severe haemophilia B, the clinical course of which is summarized in Table 1. They have been treated with FVIIa as anaphylactic reactions appeared, but not responded well to FVIIa and suffered progressive haemophilic arthropathy.

Correspondence: Dr Midori Shima, Department of Pediatrics, Nara Medical University, 840 Shijo-cho, Kashihara, Nara, 634-8522 Japan.
Tel.: +81-744-29-8881; fax: +81-744-24-9222;
e-mail: mshima@naramed-u.ac.jp

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Protocol

All studies were conducted after full informed consent. Immediate hypersensitivity was excluded by negative intracutaneous reactions before i.v. infusions of FIX-containing products. Skin tests were performed using 0.1 ml of FIX preparations diluted tenfold in sterile physiological saline. Reactions were assessed after 15 min. In the presence of negative skin tests, 50–100 U of FIX was administered i.v. under hydrocortisone cover (5–10 mg kg^{-1}). Subsequently, the dose of FIX was increased by 100–300 U a day in a stepwise manner until therapeutic levels were reached and then hydrocortisone was gradually reduced (Fig. 1). Cardiovascular and respiratory measurements were continuously monitored during infusion.

Results and discussion

Case 1 had no anaphylactic reaction after infusion of up to 1600 U (107 U kg^{-1}) of FIX concentrate. On day 10 after administration, the inhibitor titre increased to 6 BU mL^{-1}. Immediately after injection of FIX increased to 3000 U (200 U kg^{-1}), an anaphylactic skin eruption was evident. The FIX concentrate was replaced with 800 U of PCC (53 U kg^{-1}), and no allergic reaction occurred. Seven days later, inhibitor decreased and became undetectable. Further infusions were given daily in an attempt to consolidate the antibody depletion. The inhibitor titre fluctuated, however, between 2 and 4 BU mL^{-1}. Thirteen

Table 1. Clinical course of three haemophilia B patients with inhibitor.

Case	Birth date	Date of diagnosis of haemophilia B	Date of inhibitor detection	Date of first anaphylactic reaction	Maximum inhibitor titre (BU mL^{-1})	Date of resuming FIX concentrate
1	January 1992	February 1992	September 1992	September 1992 (cyanosis, apnea)	3.0	May 1996
2	November 1990	January 1992	February 1992	February 1992 (urticaria, decrease in blood pressure)	11.0	September 1996
3	June 1994	December 1994	February 1995	February 1995 (severe systemic urticaria)	1.8	April 1995

Table 2. Results from resuming of FIX concentrates.

Case	Anaphylactic reaction	Inhibitor titre	ITI therapy	Current treatment
1	+ (urticaria)	Increased to 6 BU mL^{-1} on day 10	Failed (result in nephrotic syndrome)	PCC or APCC
2	–	Increased to 1.8 BU mL^{-1} on day 8	Succeeded	FIX concentrate
3	–	undetectable	–	FIX concentrate

PCC, prothrombin complex concentrate; APCC, activated prothrombin complex concentrate.

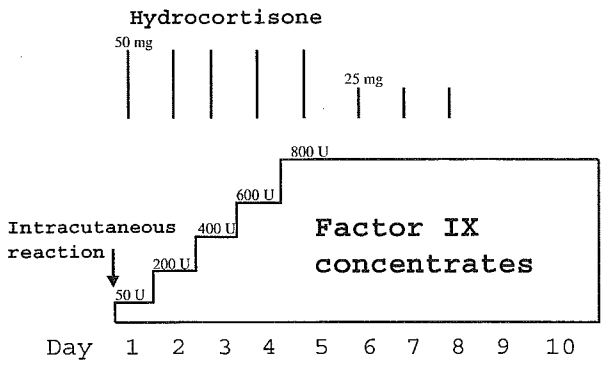

Fig. 1. Protocol for the treatment of haemophilia B with anaphylactic reactions. The dose of FIX concentrate and hydrocortisone indicated for case 3.

months later, the patient developed nephrotic syndrome and immune tolerance induction (ITI) therapy was suspended. Subsequently, the patient has been given PCC or APCC on demand. No allergic reactions have recurred and premedication with hydrocortisone has not been necessary. Case 2 had no evidence of anaphylactic reactions to 1500 U of FIX concentrate (75 U kg^{-1}). At day 8 the inhibitor titre was 1.8 BU mL^{-1}. Nevertheless, therapeutic levels of circulating FIX:C (50 U dL^{-1}) were detected after injection. Treatment was continued thrice a week for ITI therapy. After 2 months the inhibitor was eliminated and has remained undetectable. Clinically, the incidence of haemarthroses has been dramatically decreased. Case 3 also had no evidence of anaphylaxis up to 800 U (120 U kg^{-1}) of FIX concentrate, and we confirmed normal recovery of FIX:C in his plasma. Furthermore, FIX inhibitor was not detected following completion of the protocol (Table 2).

Precise mechanisms of developing anaphylaxis to FIX remain to be determined. Dioun *et al.* [4] detected anti-FIX IgE antibody in their patients with anaphylactic reactions. In our studies, IgE antibody was measured as described previously [4] but not detected in any of our subjects (data not shown).

Although the inhibitor and anaphylaxis in case 3 was transient, the treatment utilizing FIX is usually contraindicated for such patients in almost all facilities. In these circumstances our step-wise protocol of FIX replacement combined with immunosuppression is essential before administering conventional therapeutic amounts of concentrates. Recent reports proposed that ITI therapy in these patients with allergy to FIX was prone to cause nephrotic syndrome [5]. Our finding in case 1 was consistent with the hypothesis. Although we could not help suspending ITI therapy, even in this patient we have successfully secured haemostasis utilizing PCC or APCC, which contains FIX. In conclusion, individuals with anaphylaxis can be treated successfully with FIX-containing products using a carefully managed therapeutic protocol.

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