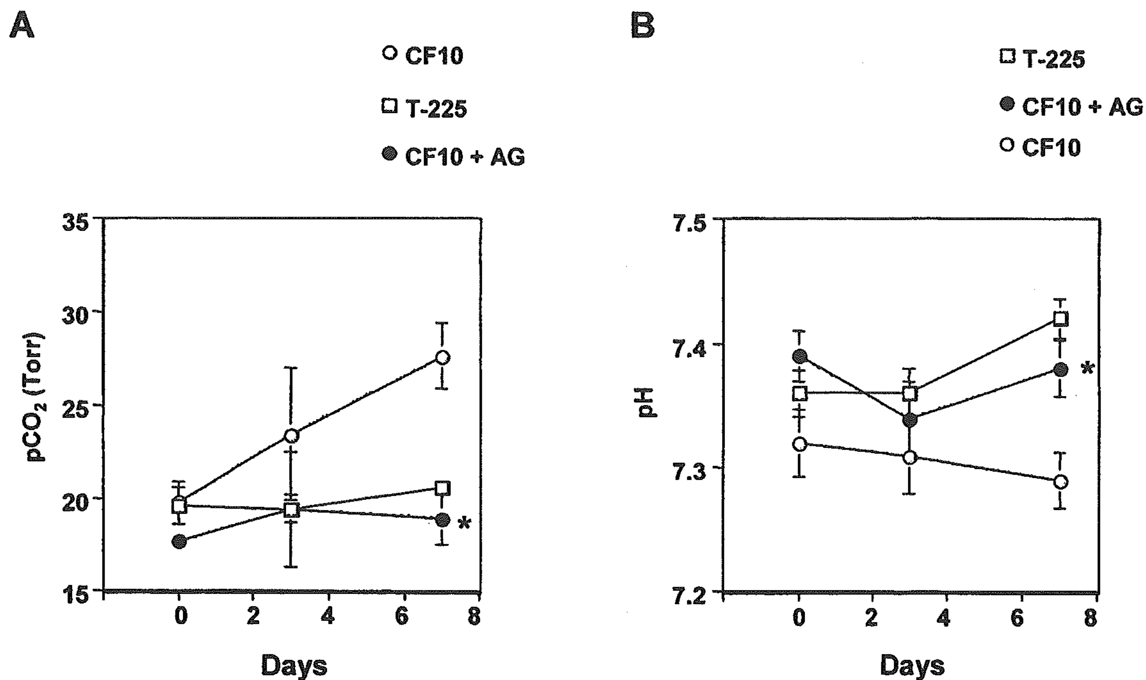

FIG. 1. Improved CO_2 and air exchange and maintenance of pH in conditioned medium after recombinant adenovirus infection. Subsequent to adenovirus infection in a normal flask with a surface area of 225 cm^2 (T-225) or a large culture vessel (a 10-tray Cell Factory [CF10] with a surface area of 6320 cm^2) in the presence or absence of active gassing (AG), CO_2 concentrations (**A**) and pH (**B**) in conditioned medium were determined ($n = 4$). Asterisk indicates $p < 0.05$ in comparison with a CF10 without AG.

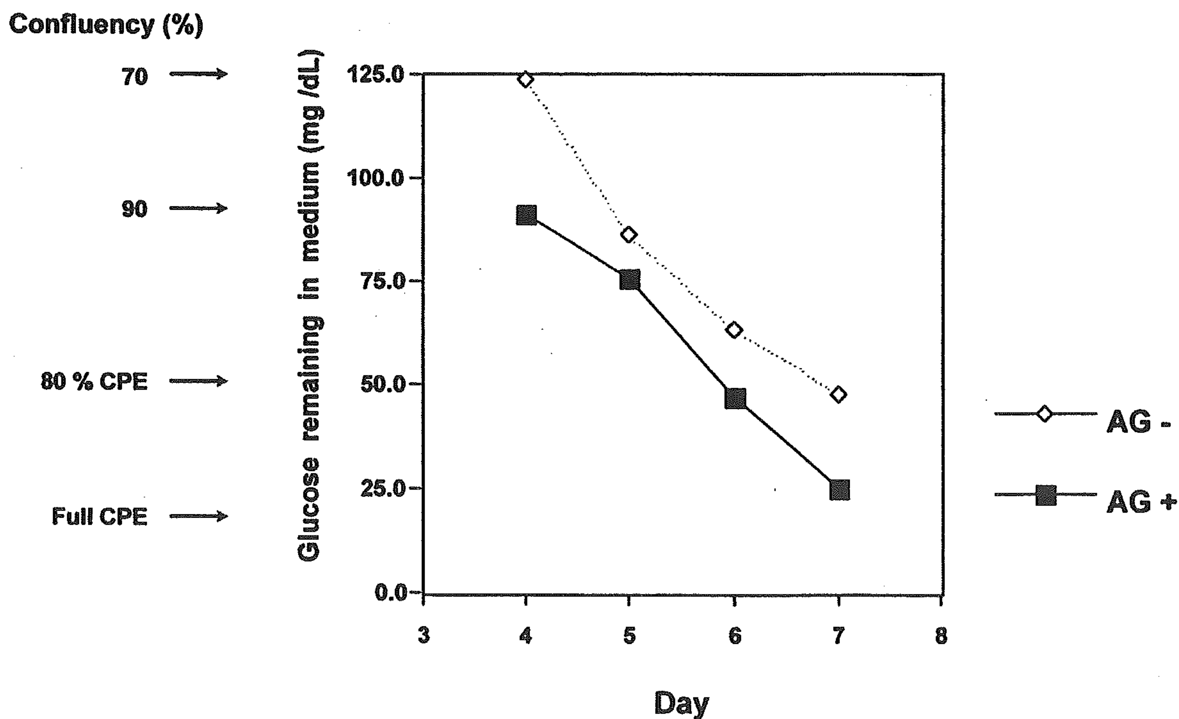

FIG. 2. Glucose reading to monitor cell growth. Glucose reading of culture medium was used as an index to monitor cell growth and cytopathic effect (CPE) in the CF10 to avoid the need for a specialized microscope. Cells were infected with recombinant adenovirus in the presence or absence of active gassing (AG) when 90% confluent.

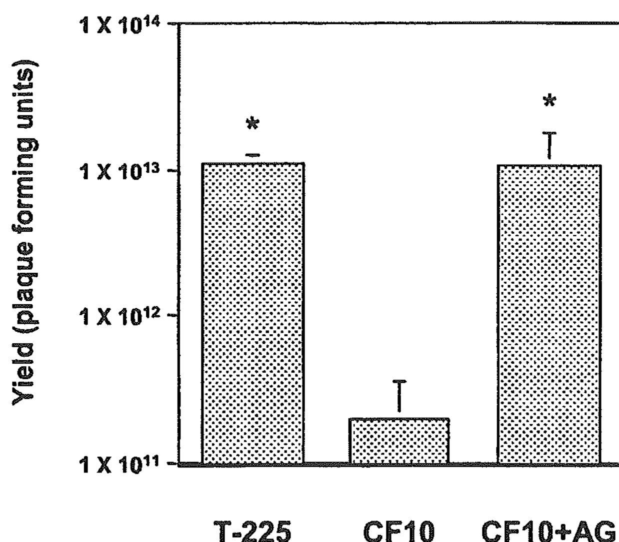

FIG. 3. Improved production of adenovirus vector. Adenovirus vector was propagated in 28 T-225 flasks ($n = 4$), a CF10 ($n = 3$), or a CF10 in the presence of active gassing (CF10 + AG, $n = 3$). Adenovirus vector expressing an EGFP reporter gene was generated in two independent experiments. The average number of plaque-forming units (PFU) was assessed by $TCID_{50}$. $^{*}p < 0.05$ in comparison with a CF10 without AG.

tor yield per cell was increased significantly, by 3.5 times (Table 3). Although vector yield was dependent on the transgene and construct, production of vector particles at up to 2.0×10^{13} genome copies per CF-10 was achieved.

Transduction of muscles with AAV vectors produced in a large culture vessel with active gassing

Five-week-old male Sprague-Dawley rats were injected with AAV1-enhanced green fluorescence protein (EGFP) (1×10^{11} genome copies per rat) via the anterior tibial muscle. Fifteen weeks after injection, the rats were sacrificed to confirm expression by fluorescence microscopy. The injected sites showed efficient expression of EGFP (Fig. 4).

TABLE 2. ENHANCED GAS EXCHANGE AND MAINTENANCE OF pH IN CONDITIONED MEDIUM AFTER PLASMID TRANSFECTION[a]

	pCO_2 *(Torr)*	*pH*
CF10	25.6 ± 1.1	7.23 ± 0.03
CF10 + AG	14.2 ± 0.1	7.40 ± 0.01

[a]Three days after plasmid transfection by using CF10 in the presence or absence of active gassing (AG), CO_2 concentrations and pH in the conditioned medium were estimated. Means ± standard deviations are shown ($n = 4$).

TABLE 3. IMPROVED YIELDS OF RECOMBINANT AAV TYPE 1 BY ACTIVE GAS EXCHANGE[a]

	Yield per vessel	*Yield per cell*
CF10	$(2.2 \pm 0.5) \times 10^{12}$	$(3.1 \pm 0.6) \times 10^{3}$
CF10 ± AG	$(1.0 \pm 0.7) \times 10^{13}$	$(1.1 \pm 0.7) \times 10^{4}$

[a]Titers of AAV1-EGFP were determined as genome copies by dot-blot analysis of DNase-treated stocks. AG, active gassing. Means ± standard deviations are shown ($n = 4$).

DISCUSSION

Successful vector production in a large culture vessel was achieved by improvement of CO_2 and air exchange along with maintenance of pH in the medium. Adenovirus production was enhanced by more than 50 times with the active gassing sys-

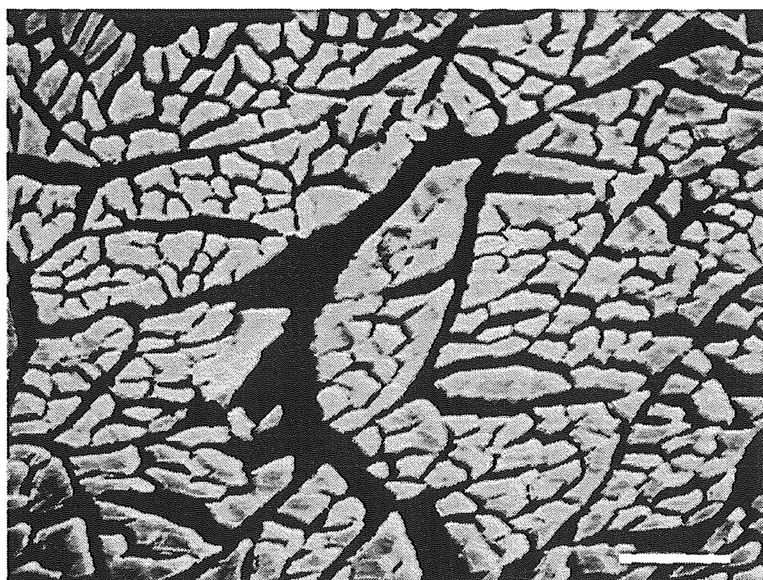

FIG. 4. Transduction of muscles with AAV vectors *in vivo*. Five-week-old male Sprague-Dawley rats were injected with AAV1-EGFP (1×10^{11} genome copies per body) via the anterior tibial muscle. Fifteen weeks after injection, the rats were killed to confirm expression by fluorescence microscopy. Scale bar: 100 μm.

tem. CF-10 with active gassing was also compatible with the three-plasmid transfection protocol for recombinant AAV production. When we used active gassing, the productivity of the AAV vectors was significantly increased.

In a direct comparison with vectors generated in ordinary culture flasks, viruses from the CF10 with active gassing were equivalent regarding function and bioactivity. The use of a CF10 with active gassing thus resulted in the production of vectors equivalent to those obtained in conventional culture dishes, but with a dramatically reduced workload. An average yield of approximately 1.0×10^{13} PFU requires as many as 28 T-225 flasks, according to our previous protocol. Alternatively, only one CF10 with active gassing was enough to achieve the same amount of virus. Furthermore, the PFU-to-particle ratio was also increased with the use of active gassing, suggesting improved bioactivity of the viruses. We used this system to amplify various adenovirus vectors. Although vector yield was dependent on the transgene and construct, a proportional increase in yield relative to surface area was achieved (data not shown).

The system was also compatible with plasmid transfection for recombinant AAV production. Active gassing combined with a large culture vessel significantly increased the productivity of the AAV vectors. The effect of enhanced gas exchange on the productivity of AAV vectors was less than the effect on the productivity of adenovirus vectors. Because lactate production accompanied by adenovirus replication is much greater than that with AAV, the protection of cells against pH drop by maintaining the CO_2 tension might be a plausible explanation for the preferential effect on adenovirus production. Transient transfection in a large culture vessel also provides a simple and flexible method of producing lentivirus-based vectors (Karolewski *et al.*, 2003). Therefore, our protocol would also be applicable to the efficient production of lentivirus- or retrovirus-based vectors.

Because this system fits into existing incubators and current vessels can readily be converted to the active gassing system, the viral production protocol using a CF-10 coupled with active gassing has practical utility for growing recombinant virus stocks in a limited laboratory space. This system has proven successful in our repeated manipulations and appears particularly promising. We have used the CF10 with the active gassing system in more than 400 vector preparations in the course of our more recent gene therapy experiments. The system allows us to perform a considerable number of *in vivo* experiments and to validate our studies.

ACKNOWLEDGMENTS

The authors thank Dr. Villy Nielsen, Ph.D. (Nunc, Roskilde, Denmark) and Mr. Hiroyuki Sano (Nalge Nunc International, Japan) for helpful discussions. We thank Avigen (Alameda, CA) for providing pAAV-LacZ and pAdeno. We thank Dr. Thomas Hope (University of Illinois at Chicago) for providing pBluescript II SK(+)WPRE-B11 and Dr. Jun-ichi Miyazaki (Osaka University Graduate School of Medicine, Japan) for pCAGGS. We also thank Ms. Miyoko Mitsu and Mr. Masataka Takahashi (Ieda Chemicals, Japan) for their encouragement and technical support. This study was supported in part by grants from the Ministry of Health, Labor, and Welfare of Japan; Grants-in-Aid for Scientific Research; a grant for the 21st Century Center of Excellence Program; and a matching fund subsidy from the High-Tech Research Center Project for Private Universities, through the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Address reprint requests to:
Dr. Takashi Okada
Division of Genetic Therapeutics
Center for Molecular Medicine
Jichi Medical School
3311-1 Yakushiji
Minami-Kawachi, Tochigi 329-0498, Japan

E-mail: tokada@jichi.ac.jp

Received for publication May 17, 2005; accepted after revision August 8, 2005.

Published online: September 21, 2005.

HUMAN GENE THERAPY 16:1212–1218 (October 2005)
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Technical Report

Large-Scale Production of Recombinant Viruses by Use of a Large Culture Vessel with Active Gassing

TAKASHI OKADA,[1] TATSUYA NOMOTO,[1] TORU YOSHIOKA,[1] MUTSUKO NONAKA-SARUKAWA,[1] TAKAYUKI ITO,[1] TSUYOSHI OGURA,[1] MAYUMI IWATA-OKADA,[2] RYOSUKE UCHIBORI,[1] KUNIKO SHIMAZAKI,[3] HIROAKI MIZUKAMI,[1] AKIHIRO KUME,[1] and KEIYA OZAWA[1,2]

ABSTRACT

Adenovirus and adeno-associated virus (AAV) vectors are increasingly used for gene transduction experiments. However, to produce a sufficient amount of these vectors for *in vivo* experiments requires large-capacity tissue culture facilities, which may not be practical in limited laboratory space. We describe here a large-scale method to produce adenovirus and AAV vectors with an active gassing system that uses large culture vessels to process labor- and cost-effective infection or transfection in a closed system. Development of this system was based on the infection or transfection of 293 cells on a large scale, using a large culture vessel with a surface area of 6320 cm^2. A minipump was connected to the gas inlet of the large vessel, which was placed inside the incubator, so that the incubator atmosphere was circulated through the vessel. When active gassing was employed, the productivity of the adenovirus and AAV vectors significantly increased. This vector production system was achieved by improved CO_2 and air exchange and maintenance of pH in the culture medium. Viral production with active gassing is particularly promising, as it can be used with existing incubators and the large culture vessel can readily be converted for use with the active gassing system.

OVERVIEW SUMMARY

Large-scale production of recombinant viruses, using a large culture vessel with active gassing, is superior to protocols using standard tissue culture plates or flasks because of the higher capacity for cell growth. Although a previous protocol for recombinant virus production in a large culture vessel had the problem of insufficient transduction efficiency resulting from inadequate gas exchange, a method to use active gassing successfully improved productivity of recombinant viruses. Development of a vector production system on a large scale, using commercially available large culture vessels, allows us to process labor- and cost-effective manipulation in a closed system.

INTRODUCTION

ADENOVIRUS AND ADENO-ASSOCIATED VIRUS (AAV) VECTORS are highly efficient for transduction in many gene therapy studies (Okada *et al.*, 2002b, 2004; Ito *et al.*, 2003; Nomoto *et al.*, 2003; Yamaguchi *et al.*, 2003; Mochizuki *et al.*, 2004; Yoshioka *et al.*, 2004; Liu *et al.*, 2005). However, current production methods rely on the manipulation of many individual flasks and are not generally considered appropriate for scaling-up of production because it would be a time-consuming and labor-intensive process. Therefore, alternative tissue culture vessels with higher capacity for cell growth, such as a 10-tray Cell Factory (CF10; Nalge Nunc International, Rochester, NY) with a surface area of 6320 cm^2, could be suitable for scaling-up of

[1]Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi 329-0498, Japan.
[2]Division of Hematology, Department of Medicine, Jichi Medical School, Tochigi 329-0498, Japan.
[3]Department of Physiology, Jichi Medical School, Tochigi 329-0498, Japan.

vector production (Okada *et al.*, 2002a). This device is easy to handle and can be used for efficient cell culture on a large scale in a closed system requiring only an air filter (Berger *et al.*, 2002; Tuyaerts *et al.*, 2002). Nevertheless, a previous protocol for recombinant virus production in the CF10 had the problem of insufficient scaling-up of vector production (Liu *et al.*, 2003). In that protocol, inadequate gas exchange between the culture vessel and the incubator might have been the cause of the inefficient yield.

We consequently adapted an active gassing system to generate large numbers of recombinant viruses in the CF10. The purpose of this active gassing is to control and maintain CO_2 tension and pH in the growth medium by passing a gas mixture through the CF10. For many types of cells, pH is an important parameter for controlling cell growth. This can be achieved by gassing with CO_2 in atmospheric air in the incubator. Enhanced gas exchange in a large culture vessel should improve both viral infectivity and plasmid transfection efficiency. In combination with the previously described method of using the CF10 (Okada *et al.*, 2002a), we have now created a simple and highly efficient system of producing vector stock on a large scale. Presented here is a labor- and cost-effective method for large-scale production of adenovirus and AAV vectors with an active gassing system that uses a large culture vessel to achieve transfection or infection in a closed system.

MATERIALS AND METHODS

Cell culture with active gassing

Propagation of vectors was based on the infection or transfection of human embryonic kidney-derived 293B cells (Yamaguchi *et al.*, 2003) by using either a flask with a surface area of 225 cm^2 (Falcon, T-225; BD Biosciences Discovery Labware, Bedford, MA) or the CF10, as described previously (Okada *et al.*, 2002a). Cells were cultured in Dulbecco's modified Eagle's medium and nutrient mixture F12 (DMEM–F12: Invitrogen, Grand Island, NY) with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 incubator. First, cells were plated at 2.3×10^6 cells per T-225 or at 6.5×10^7 cells per CF10 to achieve a monolayer at 20 to 40% confluency when cells initially attach to the surface of the flask. The volume of medium used per flask was 40 ml per T-225 or 1120 ml per CF10. Subsequently, cells were grown to a confluency of 70–90% over the next 48 to 72 hr for adenovirus infection or plasmid transfection. An aquarium pump (NISSO, Tokyo, Japan) was used to circulate air through the CF10 with 5% CO_2 and humidity control by an incubator. The CF10 was mounted with a bacterial air filter (bacterial air vents; Pall Gelman Sciences, Ann Arbor, MI) to connect the aquarium pump. The pump was connected to the gas inlet of the CF10 and the CF10 was placed inside the incubator, so that the incubator atmosphere was circulated through the CF10. The flow through the CF10 was maintained at 500 ml/min. Culture medium was sampled periodically, and the CO_2 concentrations and pH were estimated with a blood gas analyzer (Nova PHOX; Diamond Diagnostics, Holliston, MA). Glucose levels of the culture medium were also estimated with a glucose meter (Glutest Sensor, Glutest Ace GT-1640; Sanwa Kagaku Kenkyusho, Nagoya, Japan).

Construction and propagation of adenoviral vectors

A recombinant adenoviral vector, Ad-EGFP, was constructed using an adenoviral DNA–protein complex without a transgene insert (AVC2.null) (Okada *et al.*, 1998); it carried the cytomegalovirus (CMV) promoter, cloning sites, a simian virus 40 (SV40) intron, and the SV40 polyadenylation signal. To generate Ad-EGFP encoding enhanced green fluorescent protein (EGFP), a *Spe*I–*Cla*I fragment containing the *EGFP* cDNA excised from pEGFP-1 (BD Biosciences Clontech, Palo Alto, CA) was inserted into the *Xba*I and *Nsp*V sites in the DNA–protein complex, AVC2.null, using the direct *in vitro* ligation technique (Okada *et al.*, 1998). The ligated DNA–protein complex was introduced into 293 cells by the calcium phosphate transfection method. Viral plaques on 293 cells were isolated, amplified, and titrated by standard techniques. To amplify the vector in 293 cells, half the medium in the tissue culture flasks was exchanged with fresh DMEM–F12 containing 10% FBS 1 hr before infection. Cells were infected with the virus at 10 multiplicities of infection (MOI) per cell. Cells were incubated to reach full cytopathic effect, and crude viral lysate was purified by two rounds of CsCl two-tier centrifugation. The average number of plaque-forming units (PFU) was assessed on the basis of the 50% tissue culture infective dose. The number of vector particles was estimated by dot-blot hybridization of DNase I-treated stocks with plasmid standards.

Construction and propagation of AAV vectors

AAV1-EGFP, a recombinant AAV type 1 expressing the *EGFP* gene under the control of the CAG promoter (modified chicken β-actin promoter) with the CMV-IE enhancer, was generated by the following procedure. A *Bam*HI–*Xba*I fragment containing EGFP cDNA excised from pEGFP-1 and a *Hin*dIII fragment containing the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence excised from pBluescript II SK(+)WPRE-B11 (a gift from T. Hope, University of Illinois at Chicago, Chicago, IL) was cloned into an *Xho*I site of pCAGGS (a gift from J.-i. Miyazaki, Osaka University Graduate School of Medicine, Japan) to create pCAG-EGFP-WPRE, using an *Xho*I linker. The EGFP expression cassette in pCAG-EGFP-WPRE was ligated to *Not*I-excised pAAV-LacZ to form the proviral vector plasmid pAAV2-CAG-EGFP-WPRE. AAV viral stocks were prepared according to a previously described protocol (Okada *et al.*, 2002a) with minor modifications. Half the medium in tissue culture flasks was exchanged with fresh DMEM–F12 containing 10% FBS 1 hr before plasmid transfection. Subsequently, cells were cotransfected with 23 μg (per T-225) or 650 μg (per CF10) of each of the following plasmids: a proviral vector plasmid, AAV-1 chimeric helper plasmid p1RepCap (Mochizuki *et al.*, 2004), and adenoviral helper plasmid pAdeno, by a calcium phosphate coprecipitation method. Each of the vector and helper plasmids was added to 4 ml (per T-225) or 112 ml (per CF10) of 300 m*M* $CaCl_2$. This solution was gently added to an equal volume of 2× HEPES-buffered saline (HBS: 290 m*M* NaCl, 50 m*M* HEPES buffer, 1.5 m*M* Na_2HPO_4, pH 7.0) and immediately mixed by gentle inversion three times to form a uniform solution. This solution was immediately mixed with fresh DMEM–F12 containing 10% FBS outside the flasks to produce a homogeneous plasmid solution mixture. Subsequently, medium in the

culture flasks was entirely replaced with this plasmid solution mixture. At the end of incubation for 6 hr, the plasmid solution mixture in the culture flasks was replaced with prewarmed fresh DMEM–F12 containing 2% FBS. Cell suspensions were collected 72 hr after transfection and centrifuged at 300 × *g* for 10 min. Each cell pellet was resuspended in 2 ml (per T-225) or 56 ml (per CF10) of Tris-buffered saline (TBS: 100 m*M* Tris-HCl [pH 8.0], 150 m*M* NaCl). Recombinant AAV was harvested by three cycles of freeze–thawing of each resuspended pellet. Crude viral lysate was then purified twice by passage through a CsCl two-tier centrifugation gradient, as described previously (Okada *et al.*, 2002b). The viral stock was titrated by dot-blot hybridization of DNase I-treated stocks with plasmid standards. To confirm transgene expression with the propagated vector *in vivo*, 5-week-old male Sprague-Dawley rats were injected via the anterior tibial muscle with AAV1-EGFP (1×10^{11} genome copies per rat). Fifteen weeks after injection, the rats were sacrificed and expression was confirmed by fluorescence microscopy.

Statistical analysis

Statistical significance was determined on the basis of an unpaired, two-tailed *p* value and Student *t* test, and a *p* value less than 0.05 was considered significant.

RESULTS

Improved gas exchange and maintenance of pH in medium after recombinant adenovirus infection

Propagation of vectors was based on infection or transfection of 293 cells on a large scale. A minipump was connected to the gas inlet of the CF10 and placed inside the incubator, so that the atmosphere in the incubator, containing 5% CO_2, was circulated through the CF10. The gas flow for circulation through the CF10 was maintained at 500 ml/min. An appropriate gas flow rate was important to give a uniform distribution of the gas in the individual trays of the CF10. A flow less than 200 ml/min gave uneven distribution of the gas, and significantly influenced cell growth. Gas flow that was too high also disturbed the uniformity of cell density. Appropriate cell density and uniform distribution of cells are critical to achieve successful gene transduction. Application of active gassing significantly increased cell growth in the CF10 (Table 1). CO_2 concentrations in the media stayed at their initial levels when using either a T-225 or CF10 with active gassing (Fig. 1A). In contrast, the CO_2 concentration inside the CF10 increased subsequent to adenovirus infection in the absence of active gassing. The pH of culture medium in the CF10 with active gassing was close to that in the T-225 and significantly higher than that in the CF10 without active gassing (Fig. 1B).

Monitoring of cell numbers and time point for harvest

The glucose level was monitored as an index for tracing cell growth and cytopathic effect in the CF10 to avoid the necessity for a specialized microscope to monitor cells in the large culture vessel. The glucose level decreased with increasing cell confluency and progression of cytopathic effect (CPE) (Fig. 2). When 80% CPE was reached, the glucose level was reduced to about 50 mg/ml. When glucose levels were less than 25%, the cells showed full CPE and this was regarded as the appropriate time for harvest.

Improved adenovirus vector production in a large culture vessel with active gassing

We estimated the adenovirus vector yield propagated by using 28 T-225 flasks with a surface area of 225 cm^2, a CF10 with a surface area of 6320 cm^2, or a CF10 in the presence of active gassing. When active gassing was used with the CF10, the productivity of the adenovirus vectors was dramatically increased, by 53.4 times compared with that in the CF10 without active gassing (Fig. 3). The vector yield per producer cell in the CF10 was also significantly improved in the presence of active gassing (Table 1). The PFU-to-particle ratios for vectors produced in the T-225, CF10, and CF10 with active gassing were 1:7, 1:15, and 1:10, respectively.

Efficient AAV vector production in a large culture vessel with active gassing

Enhanced gas exchange in a large culture vessel should also improve vector production through plasmid transfection. AAV vectors were produced in a large vessel by a three-plasmid transfection adenovirus-free protocol (Okada *et al.*, 2002b). Three days after plasmid transfection, the CO_2 concentrations in medium from the CF10 in the presence of active gassing were significantly less than those without active gassing (Table 2). The pH of the culture medium in the CF10 with active gassing was also improved. The CF-10 with active gassing was compatible with the three-plasmid transfection protocol for recombinant AAV production. When we used active gassing, the vec-

TABLE 1. INCREASED CELL GROWTH AND VECTOR YIELD WITH ACTIVE CO_2 AND AIR EXCHANGE[a]

Flask	*Number of cells harvested*	*Vector yield per cell (PFU/cell)*
225-cm^2 flask	$(1.4 \pm 0.2) \times 10^9$ (per 28 flasks)	7.9×10^3
CF10	$(4.9 \pm 1.6) \times 10^8$	4.1×10^2
CF10 + AG	$(1.3 \pm 0.3) \times 10^9$	8.2×10^3

[a]At the time of cell harvest after adenovirus infection, cell growth and vector yield per cell in a CF10 with a surface area of 6320 cm^2 in the presence or absence of active gassing (AG) were compared with that in 28 flasks with a surface area of 225 cm^2 each.

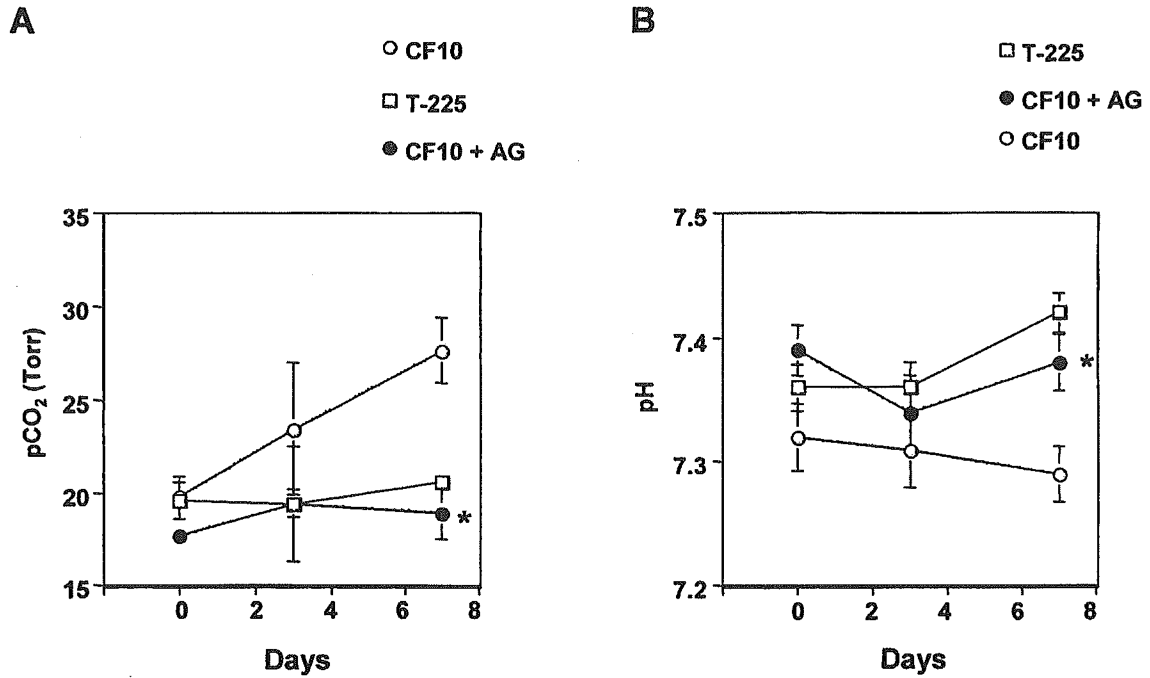

FIG. 1. Improved CO_2 and air exchange and maintenance of pH in conditioned medium after recombinant adenovirus infection. Subsequent to adenovirus infection in a normal flask with a surface area of 225 cm^2 (T-225) or a large culture vessel (a 10-tray Cell Factory [CF10] with a surface area of 6320 cm^2) in the presence or absence of active gassing (AG), CO_2 concentrations (**A**) and pH (**B**) in conditioned medium were determined ($n = 4$). Asterisk indicates $p < 0.05$ in comparison with a CF10 without AG.

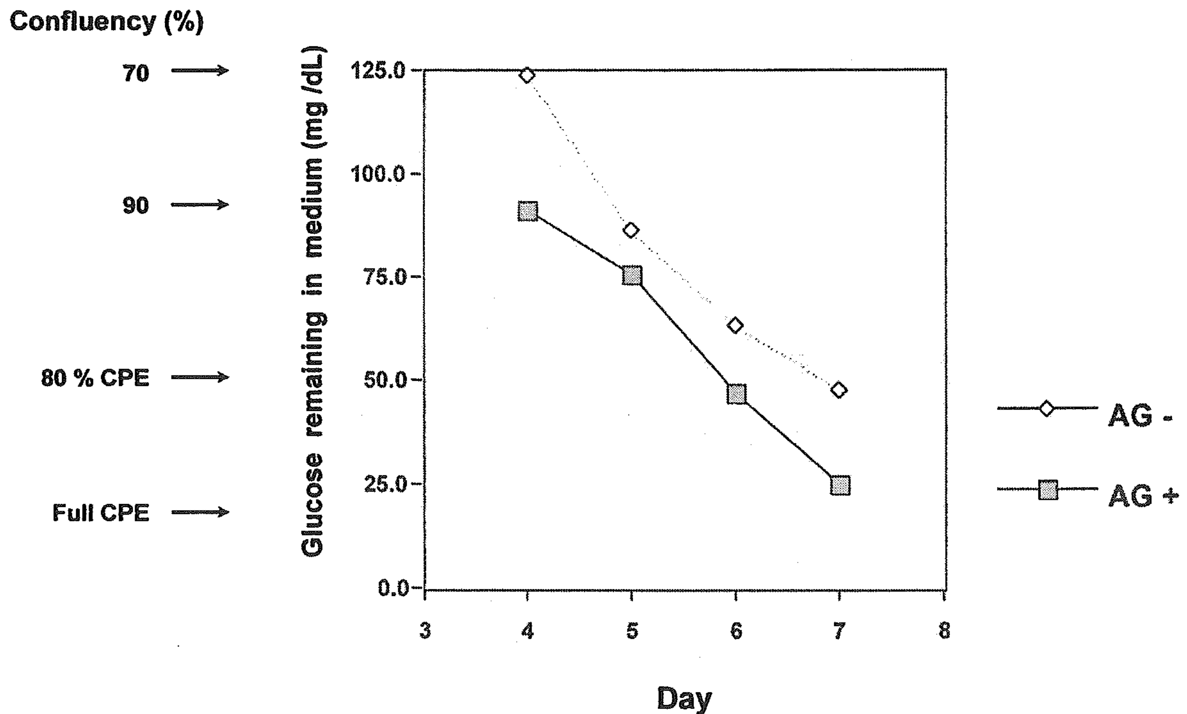

FIG. 2. Glucose reading to monitor cell growth. Glucose reading of culture medium was used as an index to monitor cell growth and cytopathic effect (CPE) in the CF10 to avoid the need for a specialized microscope. Cells were infected with recombinant adenovirus in the presence or absence of active gassing (AG) when 90% confluent.

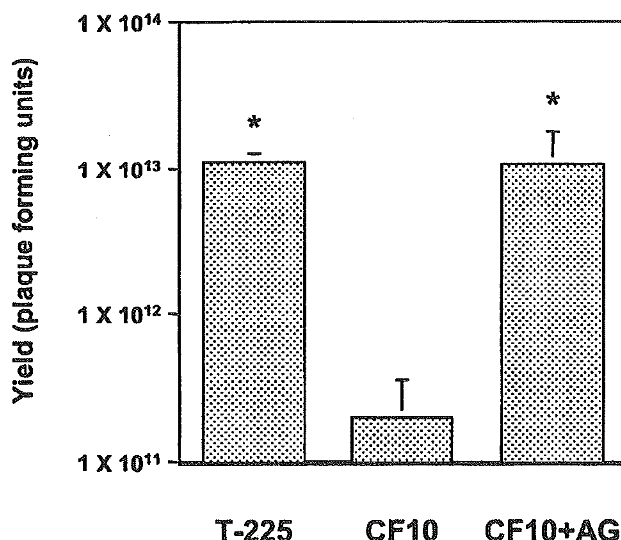

FIG. 3. Improved production of adenovirus vector. Adenovirus vector was propagated in 28 T-225 flasks ($n = 4$), a CF10 ($n = 3$), or a CF10 in the presence of active gassing (CF10 + AG, $n = 3$). Adenovirus vector expressing an EGFP reporter gene was generated in two independent experiments. The average number of plaque-forming units (PFU) was assessed by $TCID_{50}$. $^*p < 0.05$ in comparison with a CF10 without AG.

tor yield per cell was increased significantly, by 3.5 times (Table 3). Although vector yield was dependent on the transgene and construct, production of vector particles at up to 2.0×10^{13} genome copies per CF-10 was achieved.

Transduction of muscles with AAV vectors produced in a large culture vessel with active gassing

Five-week-old male Sprague-Dawley rats were injected with AAV1-enhanced green fluorescence protein (EGFP) (1×10^{11} genome copies per rat) via the anterior tibial muscle. Fifteen weeks after injection, the rats were sacrificed to confirm expression by fluorescence microscopy. The injected sites showed efficient expression of EGFP (Fig. 4).

TABLE 2. ENHANCED GAS EXCHANGE AND MAINTENANCE OF pH IN CONDITIONED MEDIUM AFTER PLASMID TRANSFECTION[a]

	pCO_2 *(Torr)*	*pH*
CF10	25.6 ± 1.1	7.23 ± 0.03
CF10 + AG	14.2 ± 0.1	7.40 ± 0.01

[a]Three days after plasmid transfection by using CF10 in the presence or absence of active gassing (AG), CO_2 concentrations and pH in the conditioned medium were estimated. Means ± standard deviations are shown ($n = 4$).

TABLE 3. IMPROVED YIELDS OF RECOMBINANT AAV TYPE 1 BY ACTIVE GAS EXCHANGE[a]

	Yield per vessel	*Yield per cell*
CF10	$(2.2 \pm 0.5) \times 10^{12}$	$(3.1 \pm 0.6) \times 10^{3}$
CF10 ± AG	$(1.0 \pm 0.7) \times 10^{13}$	$(1.1 \pm 0.7) \times 10^{4}$

[a]Titers of AAV1-EGFP were determined as genome copies by dot-blot analysis of DNase-treated stocks. AG, active gassing. Means ± standard deviations are shown ($n = 4$).

DISCUSSION

Successful vector production in a large culture vessel was achieved by improvement of CO_2 and air exchange along with maintenance of pH in the medium. Adenovirus production was enhanced by more than 50 times with the active gassing sys-

FIG. 4. Transduction of muscles with AAV vectors *in vivo*. Five-week-old male Sprague-Dawley rats were injected with AAV1-EGFP (1×10^{11} genome copies per body) via the anterior tibial muscle. Fifteen weeks after injection, the rats were killed to confirm expression by fluorescence microscopy. Scale bar: 100 μm.

tem. CF-10 with active gassing was also compatible with the three-plasmid transfection protocol for recombinant AAV production. When we used active gassing, the productivity of the AAV vectors was significantly increased.

In a direct comparison with vectors generated in ordinary culture flasks, viruses from the CF10 with active gassing were equivalent regarding function and bioactivity. The use of a CF10 with active gassing thus resulted in the production of vectors equivalent to those obtained in conventional culture dishes, but with a dramatically reduced workload. An average yield of approximately 1.0×10^{13} PFU requires as many as 28 T-225 flasks, according to our previous protocol. Alternatively, only one CF10 with active gassing was enough to achieve the same amount of virus. Furthermore, the PFU-to-particle ratio was also increased with the use of active gassing, suggesting improved bioactivity of the viruses. We used this system to amplify various adenovirus vectors. Although vector yield was dependent on the transgene and construct, a proportional increase in yield relative to surface area was achieved (data not shown).

The system was also compatible with plasmid transfection for recombinant AAV production. Active gassing combined with a large culture vessel significantly increased the productivity of the AAV vectors. The effect of enhanced gas exchange on the productivity of AAV vectors was less than the effect on the productivity of adenovirus vectors. Because lactate production accompanied by adenovirus replication is much greater than that with AAV, the protection of cells against pH drop by maintaining the CO_2 tension might be a plausible explanation for the preferential effect on adenovirus production. Transient transfection in a large culture vessel also provides a simple and flexible method of producing lentivirus-based vectors (Karolewski *et al.*, 2003). Therefore, our protocol would also be applicable to the efficient production of lentivirus- or retrovirus-based vectors.

Because this system fits into existing incubators and current vessels can readily be converted to the active gassing system, the viral production protocol using a CF-10 coupled with active gassing has practical utility for growing recombinant virus stocks in a limited laboratory space. This system has proven successful in our repeated manipulations and appears particularly promising. We have used the CF10 with the active gassing system in more than 400 vector preparations in the course of our more recent gene therapy experiments. The system allows us to perform a considerable number of *in vivo* experiments and to validate our studies.

ACKNOWLEDGMENTS

The authors thank Dr. Villy Nielsen, Ph.D. (Nunc, Roskilde, Denmark) and Mr. Hiroyuki Sano (Nalge Nunc International, Japan) for helpful discussions. We thank Avigen (Alameda, CA) for providing pAAV-LacZ and pAdeno. We thank Dr. Thomas Hope (University of Illinois at Chicago) for providing pBluescript II SK(+)WPRE-B11 and Dr. Jun-ichi Miyazaki (Osaka University Graduate School of Medicine, Japan) for pCAGGS. We also thank Ms. Miyoko Mitsu and Mr. Masataka Takahashi (Ieda Chemicals, Japan) for their encouragement and technical support. This study was supported in part by grants from the Ministry of Health, Labor, and Welfare of Japan; Grants-in-Aid for Scientific Research; a grant for the 21st Century Center of Excellence Program; and a matching fund subsidy from the High-Tech Research Center Project for Private Universities, through the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Address reprint requests to:
Dr. Takashi Okada
Division of Genetic Therapeutics
Center for Molecular Medicine
Jichi Medical School
3311-1 Yakushiji
Minami-Kawachi, Tochigi 329-0498, Japan

E-mail: tokada@jichi.ac.jp

Received for publication May 17, 2005; accepted after revision August 8, 2005.

Published online: September 21, 2005.

Preclinical Experiment of Auxiliary Partial Orthotopic Liver Transplantation as a Curative Treatment for Hemophilia

Saiho Ko,[1] Ichiro Tanaka,[2] Hiromichi Kanehiro,[1] Hideki Kanokogi,[1] Jun-ichi Ori,[2] Midori Shima,[2] Akira Yoshioka,[2] Alan Giles,[3] and Yoshiyuki Nakajima[1]

The cause of hemophilia is deficiency of coagulation factor VIII production in the liver, which can be cured by liver transplantation. Because the hepatic function of hemophilia patients is quite normal except for production of factor VIII, auxiliary partial orthotopic liver transplantation (APOLT) is beneficial in that patient survival is secured by preserving native liver even in the event of graft loss. However, it is not known whether the graft of APOLT would be enough to cure hemophilia. We evaluated the efficacy and feasibility of APOLT for hemophilia in a canine hemophilia A model that we established. Partial left liver graft was taken from the normal donor (blood factor VIII activity > 60%). The graft was transplanted to the hemophilia beagle dog (blood factor VIII activity < 5%) after resection of the left lobe preserving native right lobe. Changes in time of blood factor VIII activity and liver function parameters were observed after APOLT. APOLT and perioperative hemostatic management were successfully performed. The blood factor VIII activity increased to 30% after APOLT, and was sustained at least 6 weeks throughout the observation period without symptoms of bleeding. The result demonstrated sustained production of factor VIII in the hemophilia recipient after APOLT. Transplantation of approximately one third of whole liver resulted in cure of hemophilia. In conclusion, it is suggested that APOLT would be feasible as a curative treatment of hemophilia A to improve quality of life of the patients. (*Liver Transpl 2005;11:579-584.*)

The cause of hemophilia is deficiency of coagulation factor VIII production in the liver, which can be cured by liver transplantation from non-hemophilic donors. Starzl's group reported the first successful liver transplantation in a patient with hemophilia.[1] Subsequently, cure of coagulopathy after orthotopic whole liver transplantation has been reported in approximately 30 patients with hemophilia. In these recipients, liver transplantation was indicated for associated liver cirrhosis but not for cure of hemophilia.[2-7] Liver transplantation is sometimes performed for other lethal metabolic liver diseases; however, the indication of liver transplantation for cure of hemophilia itself is controversial, because coagulopathy of hemophilia can be controlled by replacement with external factor VIII and the risk of whole liver transplantation is too high in this situation. Nevertheless, repetitive replacement therapy induces inhibitor against factor VIII in the patients, which makes it difficult to control bleeding even with a large amount of factor VIII concentrates.[8,9] Together with hazard of viral infection via the factor VIII concentrates,[10] induction of inhibitor deteriorates quality of life in patients with hemophilia.

Recently, auxiliary partial orthotopic liver transplantation (APOLT) has been indicated for patients with acute liver failure, considering the possibility of recovery of the patient's own liver.[11-13] APOLT has also been performed for limited number of patients with metabolic liver diseases.[14-17] Because the liver function of patients with metabolic diseases remains normal except for production of the deficient metabolic factor, the patient survival is secured by preserved native liver even in case of graft loss. We thought that APOLT would be a choice for hemophilia patients to avoid long-term complications relating to replacement therapy with factor VIII concentrates. Before clinical application, it has to be evaluated whether the production of factor VIII from the graft of APOLT would be enough to cure hemophilia long term. However, there has been no report of an appropriate experimental model to evaluate the efficacy of APOLT for metabolic liver disease. In the present study we established a canine model of APOLT and evaluated the feasibility of APOLT using an inbred dog with hemophilia A as a preclinical study of curative treatment for hemophilia.

Abbreviation: APOLT, auxiliary partial orthotopic liver transplantation.

From the Departments of [1]Surgery and [2]Pediatrics, Nara Medical University, Kashihara, Nara, Japan; and [3]Department of Pathology, Queen's University, Kingston, ON, Canada.

Address reprint requests to Dr. Saiho Ko, First Department of Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan. Telephone: 81 744-29-8863; FAX: 81 744-24-6866; E-mail: saihoko@naramed-u.ac.jp

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Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/lt.20390

Materials and Methods

Animals

Nine young male beagle dogs were provided from the colony of an animal facility in the experimental institute of Nara Medical University. Eight of these normal beagle dogs were used for the establishment of the APOLT model. The remaining one normal beagle dog was used as the donor of APOLT for the hemophilia beagle dog. To obtain a hemophilia beagle dog to be the recipient of APOLT, two female dogs of carrier status of hemophilia were provided from Queen's University (Kingston, Canada).[20] These dogs were mated with normal beagle dogs and bred in the animal experiment institute of Nara Medical University. The offspring were screened for hemophilia by measuring blood factor VIII levels after birth. Then we obtained one male dog with blood factor VIII level of less than 5% that was used for the present study as a recipient of APOLT with long-term observation (body weight: 12.5 kg). Animal breeding and experimental procedures were performed according to the ethical guidlines approved by the institutional committee of Nara Medical University.

Transplantation Procedure

Partial liver graft of the left lobe was procured from the donor under the general anesthesia. The anesthesia and care of animals were performed as previously described.[21] The graft was perfused with 4°C cold lactate ringer solution ex vivo and stored in the same solution. The left portal vein and the left hepatic vein were prepared for vascular anastomosis. For arterial anastomosis, the common hepatic artery was prepared with the continuity of left hepatic artery. The left lobe of the recipient was resected, and the left lobe graft of the donor was transplanted to the recipient orthotopically, preserving the recipient's own right lobe. The left hepatic vein and left portal vein were anastomosed end to end by continuous suture using 5-0 and 6-0 prolene, respectively. After reperfusion, the hepatic artery of the graft was anastomosed to the recipient's common hepatic artery end to side using 8-0 prolene by microsurgical technique. Bile juice of the graft was externally drained through the abdominal wall via the silicon catheter to monitor the amount of bile production from the graft (Fig. 1). Central venous catheter was inserted via the jugular vein during operation for fluid supplement and blood sampling. These external catheters were covered with special experimental cloth on the dogs. Canine factor VIII concentrate was administered to the recipient to maintain hemostasis during operation, and discontinued at 20 hours after transplantation. The 0.16 mg/kg/d of tacrolimus was administrated intramuscularly daily from the day of transplantation. Glucose and electrolyte solutions were supplemented intravenously during the first postoperative week. Oral feeding was started from day 3. Water was given ad libitum. To establish this transplantation model, we performed APOLT four times using normal beagle dogs as both donors and recipients. The normal beagle recipients were explored by laparotomy on day 7. The grafted liver and native liver were evaluated by inspection, laser doppler tissue blood flowmetry (TBF-LN1, Unique Medical Co. Ltd., Tokyo, Japan), and histologic examination. Thereafter, we applied this transplantation method to the canine hemophilia recipient.

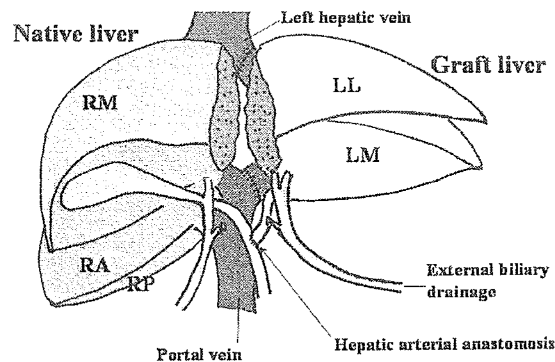

Figure 1. Procedure of APOLT. Left lateral (LL) and left medial (LM) segments of the donor liver were transplanted by vascular anastomosis of left hepatic vein and left portal vein after resection of the native left liver of the recipient. Native right liver (RM, RA, and RP) was preserved. Graft hepatic artery was anastomosed to the recipient in an end-to-side fashion.

Monitoring of Graft Function

The graft function was monitored from blood levels of factor VIII and serum total bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels. The blood samples were drawn from the central venous catheter or radial vein daily in the first week, every two days in the second and third weeks, and twice a week thereafter. The amount of bile production from the graft was monitored every day. Blood factor VIII levels of the recipient were determined at least twice a week. The measurement of blood factor VIII activity was performed as previously described.[20]

Results

APOLT from normal donors to normal recipients was successfully performed, and the postoperative recovery was uneventful in all recipients. These recipients received exploratory laparotomy on day 7. Gross appearance of the liver showed functioning partial liver graft and intact own liver, which were confirmed on histology. Laser doppler tissue blood flowmetry on day 7 revealed comparable hepatic blood circulation in the graft and own liver (Fig. 2).

The canine hemophilia recipient received APOLT from a normal beagle dog with the blood factor VIII activity of 60%. Postoperative course of the recipient is shown in Fig. 3. Blood factor VIII activity of the hemophilia recipient was less than 5% before APOLT. Perioperative hemostasis was maintained by the intrave-

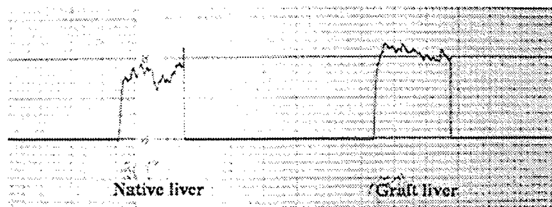

Figure 2. Laser doppler tissue blood flowmetry. Both native and grafted livers on day 7 showed comparable tissue blood flow. The results were reproductive in all four recipients.

nous infusion of canine factor VIII. While replacement therapy using canine factor VIII concentrate was stopped on day 1, the blood factor VIII activity remained higher than 30% during the first postoperative week (the half-life of externally supplemented factor VIII is known to be 8-12 hours). The recovery of the recipient was uneventful. Approximately 20 mL/d of bile juice from the left lobe graft was collected daily from day 2. The bile production and blood factor VIII levels began to decrease from day 9 without marked elevation of serum total bilirubin and aspartate aminotransferase levels. Blood factor VIII activity showed a significant drop to 3% on day 15. Deterioration of these parameters of graft function was observed several times, and these episodes were improved after the steroid pulse therapy. Especially, bile production of graft liver recovered immediately after the first dose of steroid pulse therapy. The factor VIII activity increased up to 40% on day 42. In spite of stable graft function, the recipient became ill around day 40. Antibiotic therapy

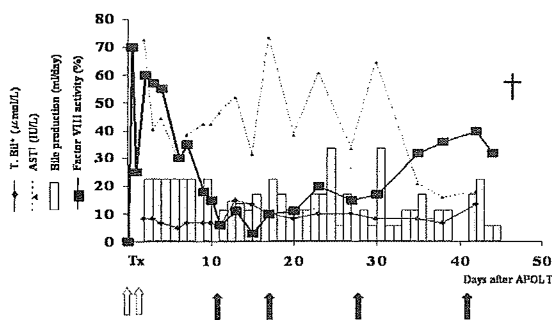

Figure 3. Posttransplantation course of the hemophilia recipient after APOLT. Graft function was monitored with coagulation factor VIII levels and daily amount of bile production from the partial liver graft. Open arrows indicate exogenous replacement of coagulation factor VIII concentrates. Closed arrows indicate intravenous injection of methyl-predonisolone at a dose of 10 mg/kg on two consecutive days. *Serum total bilirubin level. †Serum aspartate alanine aminotransferase level.

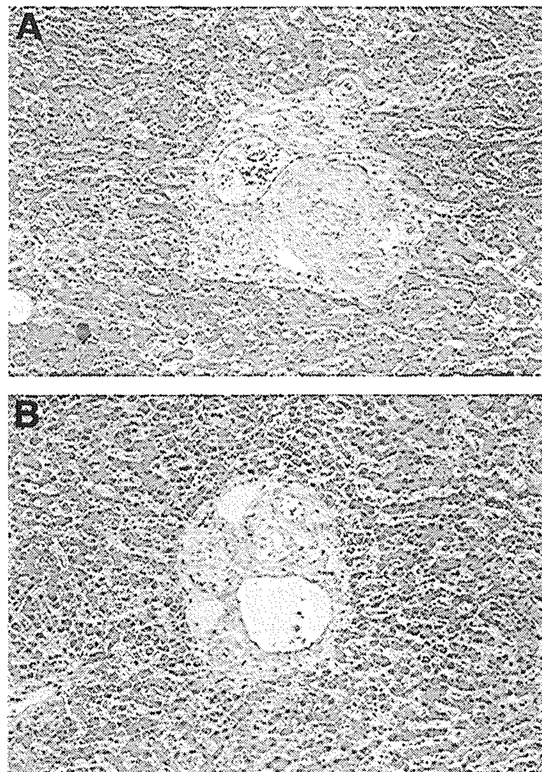

Figure 4. Histology of the graft and native liver on autopsy. The graft liver showed normal parenchymal structure and mild fibrous extension of the portal area including fibrotic change of bile ducts. (A) Hepatocytes of the sinusoid were not atrophic. (B) The native liver showed well-preserved parenchymal structure and hepatocytes. Original magnification, ×200.

and fluid resuscitation did not improve the condition of the recipient. The recipient was euthanized on day 46. Systemic exploration suggested that the cause of illness was severe pneumonia. The weights of the excised graft and the recipient's own liver were 110 g and 200 g, respectively. Both graft and native livers showed normal appearance without atrophy. No vascular complication was seen, and all anastomoses were patent. Histology of the graft liver showed well-maintained parenchymal structure and mild fibrous extension of the portal area. Native liver showed no abnormal findings (Fig. 4).

Discussion

The present study shows significant elevation of blood factor VIII activity and clinical cure of hemophilia in the canine hemophilia recipient after APOLT. The factor VIII activity reached 40%, which might represent maximum graft function considering that one third of

the whole liver from the donor with blood factor VIII activity of 60% was transplanted. Factor VIII was produced from the grafted partial liver for longer than 6 weeks, and hemophilia was clinically cured during observation. The result suggests the clinical feasibility and efficacy of APOLT as a cure of hemophilia. To our knowledge, this is the first report of experimental study of APOLT for metabolic liver disease.

Our APOLT model using a hemophilic dog was ideal to evaluate technical and functional aspects of this procedure as a curative treatment for metabolic liver diseases.

There has been no report of APOLT as a curative treatment for hemophilia, whereas two cases of living-related liver transplantation for cirrhosis in patients with hemophilia A and B have been reported recently.[22,23] Complete resection of the recipient's own liver before liver replacement is not necessary, because there is no structural damage or toxic effect of the own liver in patients with hemophilia. In this situation, APOLT has potential advantage that survival of the recipient can be secured by preserved native liver even in case of graft loss due to rejection or other complications. Another advantage of this procedure is that the requirement of graft volume is smaller than standard whole liver graft procedure, because of the support of preserved partial native liver.[25] Therefore, small partial liver graft from living donors can be safely used in this situation. Because hemophilia is not a lethal disease, it might be difficult to allocate cadaveric organs to patients with hemophilia. Then we believe that the applicability of living donor liver transplantation is important in clinical setting.

There is a technical problem of hemodynamical status of the partial liver graft side by side with the noncirrhotic native liver in APOLT recipients.[24-26] Graft dysfunction can be caused by impaired portal blood flow of the graft due to portal steal phenomenon by the preserved native liver, which can be detected as a sudden decrease of graft portal flow by doppler ultrasonography. While there was no significant correlation between the graft volume and occurrence of portal steal phenomenon, selective ligation of the native liver portal vein was needed to preserve graft function in some of the recipients after APOLT.[24,25] Yabe et al.[25] recommended preemptive transection of the native liver portal vein with satisfactory results. While this preventive procedure may result in atrophy of the native liver and loses the potential benefit of APOLT, native liver could exist even after selective ligation of the portal branch of the native liver long term.[24,25] In the present study, sudden decrease of bile production from the graft was seen several times in the canine hemophilia recipient. These events might be caused by portal steal phenomenon, because severe rejection was unlikely from the data of serum total bilirubin and AST levels. During deterioration of bile production and factor VIII activity the recipient was doing quite well, probably because of the preserved function of native liver. We speculated that unbalanced diversion of portal blood flow was triggered by mild edema of the graft liver, probably due to out flow block or other causes that can be reversed with steroid pulse therapy. However, the exact mechanism of portal flow diversion after APOLT has to be studied further.

There is no evidence that the incidence and severity of acute rejection in APOLT is higher than those in standard whole liver transplantation.[25,27] However, in APOLT even mild rejection can trigger the portal steal phenomenon, which might result in severe deterioration of graft function.[24,25] In the present study, intensive monitoring of acute rejection and portal blood flow of the graft and native liver were not possible due to the technical limitation of experimental facilities (liver biopsy could not be performed because of the ethical guidelines of animal experiments, and doppler ultrasonography could not be used for animals). When applied clinically, it should be emphasized that early diagnosis of rejection by frequent blood test or liver biopsy and intensive monitoring of graft blood flow by doppler untrasonography are critically important in APOLT.

Gene therapy is an alternative approach for clinical cure of hemophilia. Preliminary data of clinical trials suggest modest increases in plasma factor IX levels in some of hemophilia B patients after gene therapy.[28,29] Factor VIII is significantly more difficult to express in gene therapy systems than factor IX for a variety of reasons.[30] A clinical trial of factor VIII gene transfer for patients with hemophilia A demonstrated the levels of factor VIII activity less than 1% of normal level in many of enrolled patients, whereas bleeding frequency decreased.[31] Further improvement of gene transfer techniques is needed to obtain a stable long-lasting cure for hemophilia in this strategy.[32]

In the present experimental study, transplantation of approximately one third of the whole normal liver increased plasma factor VIII activity around 40% in a hemophilia A dog. Considering the clinical situations, left lateral segment graft would be enough to cure hemophilia even in adult patients. Even a much smaller graft than that in the present study may result in clinical cure of hemophilia, and it must be further studied. Considering the relative indication of liver transplanta-

tion for hemophilia, living donor liver transplantation would be a realistic strategy to obtain the graft. Procurement of left lateral segment graft is the safest method for living liver donors among several types of living liver donation.[33] At present, APOLT would be a choice for patients with hemophilia to improve their quality of life.

Acknowledgment

The authors thank the Chemo-Sero-Therapeutic Research Institute for the kind provision of canine cryoprecipitate.

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ORIGINAL ARTICLE

Haemostatic management of intraoral bleeding in patients with von Willebrand disease

Y Morimoto[1,2], A Yoshioka[3], M Sugimoto[3], Y Imai[2], T Kirita[2]

[1]*Department of Dental Anesthesiology, Graduate School of Dentistry, Osaka University, Suita, Osaka;* [2]*Department of Oral and Maxillofacial Surgery, Nara Medical University, Kashihara;* [3]*Department of Paediatrics, Nara Medical University, Japan*

OBJECTIVES: To develop plans for the haemostatic management of intraoral bleeding in patients with von Willebrand disease (VWD).
SUBJECTS AND METHODS: Thirty-seven episodes of haemostatic management of intraoral bleeding in 19 VWD patients were analysed retrospectively based on the medical records.
RESULTS AND CONCLUSIONS: When performing tooth extractions in patients with type 1 or 2A VWD [responsive to 1-deamino-8-D-arginine-vasopressin (DDAVP)], 0.35–0.4 μg kg^{-1} of DDAVP should be administered intravenously at three times.In patients with type 2A VWD (unresponsive to DDAVP) or patients with type 2B or 2N VWD, 50–90 U [as ristocetin cofactor (VWF:RCof)] kg^{-1} of a factor VIII concentrate containing von Willebrand factor (FVIII/VWF concentrate) should be administered twice in routine extractions, and four to six times in surgical extractions.Gingival bleeding related to primary teeth can be mostly managed by pressure haemostasis alone. However, when treating gingival bleeding caused by marginal periodontitis, it is often necessary to administer 0.4 μg kg^{-1} of DDAVP or 40–70 U (as VWF:RCof) kg^{-1} of a FVIII/VWF concentrate. As local haemostasis is difficult to achieve in bleeding from the tongue or labial or mandibular haematoma, it is necessary to administer 0.4 μg kg^{-1} of DDAVP or 60–80 U (as VWF:RCof) kg^{-1} of a FVIII/VWF concentrate.In addition, oral administration of 20 mg kg^{-1} day^{-1} of tranexamic acid should be combined with the regimens described above.
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Keywords: von Willebrand disease; intraoral bleeding; local haemostasis; 1-deamino-8-D-arginine-vasopressin; factor VIII concentrate

Correspondence: Yoshinari Morimoto, 1–8, Yamadaoka, Suita, Osaka 565-0871, Japan. Tel: +81 6 6879 2972, Fax: +81 6 6879 2975, E-mail: ysn-mori@dent.osaka-u.ac.jp
Received 8 March 2004; revised 13 August 2004; accepted 1 November 2004

Introduction

von Willebrand disease (VWD) is an autosomal disorder characterized by prolonged bleeding. In VWD, quantitative or qualitative abnormalities of von Willebrand factor (VWF), impaired platelet adhesion to vascular walls and platelet aggregation, thus result in a bleeding tendency (Sadler, 1994a). The mechanisms of action of FVIII/VWF are by complexing with FIII. Currently, haemostatic management protocols are being established for each type of VWD as classified by the VWF Subcommittee of the International Society on Thrombosis and Haemostasis (ISTH)/Scientific and Standardization Committee (SSC) (Sadler, 1994b; Eikenboom, 2001; Mazurier *et al*, 2001; Meyer *et al*, 2001; Rodeghiero and Castaman, 2001; Batlle *et al*, 2002; Laffan *et al*, 2004) (Table 1).

1-Deamino-8-D-arginine-vasopressin (DDAVP) administration increases the plasma levels of FVIII:C, VWF antigen (VWF:Ag) and ristocetin cofactor (VWF:RCof) for over an hour (Haemophilia of Georgia, 2000; Kasper, 2000; Mannucci, 2000, 2001; Pasi *et al*, 2004). The half-life of plasma FVIII/VWF is around 8–12 h (Haemophilia of Georgia, 2000; Kasper, 2000; Mannucci, 2000, 2001; Batlle *et al*, 2002; Pasi *et al*, 2004). Patients with type 1 VWD and some patients with type 2A VWD can be managed with DDAVP alone.

However, DDAVP is ineffective or contraindicated in some patients with type 2A VWD and patients with types 2B, 2N, 2M and 3 VWD. In these patients, 10–50 U (as FVIII:C) kg^{-1} of a FVIII/VWF complex concentrate is administered (Federici *et al*, 2000; Haemophilia of Georgia, 2000; Kasper, 2000; Mannucci, 2000, 2001; Piot *et al*, 2002) (Table 1).

However, few studies on the haemostatic management of surgical treatment in VWD have been undertaken and no consensus exists regarding therapeutic doses of replacement factors (Federici *et al*, 2000; Kasper, 2000; Cohen *et al*, 2001; Mannucci, 2001; Piot *et al*, 2002).

The amount of VWF in actual FVIII/VWF concentrates varies, with 1.6–2.9 units of VWF:RCof for every unit of FVIII:C, depending on the type of concentrate (Takahashi *et al*, 1987; Yoshioka *et al*, 1987; Fukui *et al*, 1988).

Table 1 Clinical and laboratory features in von Willebrand disease variants

Feature	*type 1*	*type 2A*	*type 2B*	*type 2N*	*type 2M*	*type 3*
Inheritance	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal recessive[a]	Autosomal dominant	Autosomal recessive
FVIII:C	Decreased	Normal/decreased	Normal/decreased	Decreased	Normal/decreased	Absent
Plasma VWF antigen	Decreased	Normal/decreased	Normal/decreased	Normal	Normal/decreased	Absent
VWF:ristocetin cofactor	Decreased	Decreased	Normal/decreased	Normal	Decreased	Absent
Plasma VWF multimers	Normal	Absent HMW multimers	Reduced/absent HMW multimers	Normal	Normal/presence of ultra HMW multimer	Absent
Platelet VWF multimers	Normal/decreased	Normal/absent HMW multimers	Normal	Normal	Normal	Decreased/absent
VWF function	Normal	Decreased affinity for GPIb	Increased affinity for GPIb	Decreased affinity for FVIII	Decreased affinity for GPIb	Absent
Ristocetin-induced platelet aggregation	Normal/decreased	Decreased	Enhanced sensitivity at low ristocetin concentrations	Normal	Decreased	Absent
Response to DDAVP	Increase in VWF and FVIII	Variable but increase in FVIII	Contraindicated for risk of thrombocytopenia	Variable but increase in FVIII (the half-life of FVIII is significantly reduced)	Variable	None
Population (%)	70	20				10

HMW, high-molecular weight; GPIb, glycoprotein Ib; FVIII, factor VIII; VWF, von Willebrand factor; DDAVP, 1-deamino-8-D-arginine-vasopressin.
[a]Compound heterozygosity.
Information in this table is from following references: Eikenboom (2001), Mazurier *et al* (2001), Meyer *et al* (2001), Rodeghiero *et al* (2001), Laffan *et al* (2004) and Pasi *et al* (2004).

We retrospectively analysed the haemostatic management of intraoral bleeding in VWD patients treated in our department and investigated drug dosage, frequency of administration, increases in plasma VWF:RCof and local haemostasis in relation to each disease type and treatment. Based on these results, we present specific protocols for the haemostatic management of intraoral bleeding in patients with VWD.

Subjects and methods

Subjects were 19 patients who were diagnosed with VWD at the Nara Medical University Hospital and underwent 37 episodes of haemostatic management for intraoral bleeding at the Department of Oral and Maxillofacial Surgery. The medical records of these patients were examined to ascertain gender, age at treatment, dental intervention or cause of intraoral bleeding, systemic haemostatic treatments (dosage, route, and frequency of DDAVP and FVIII/VWF concentrate administration), local haemostatic treatments and postoperative bleeding.

Two FVIII/VWF concentrates were used: Haemate P™ (FVIII:C:VWF:RCof, 1:2.9) (Yoshioka *et al*, 1987; Fukui *et al*, 1988) and Confact F™ (FVIII:C:VWF: RCof, 1:1.6) (Takahashi *et al*, 1987). The dosage of VWF:RCof per kilogram body weight (U kg^{-1}) was calculated based on the amount of VWF:RCof included in each preparation. In addition, the level of plasma VWF:RCof has been shown to increase by 1.4% when 1 U kg^{-1} of VWF:RCof is administered (Fukui *et al*, 1988), allowing the calculation of anticipated levels of VWF:RCof following administration of FVIII/VWF concentrate.

Results

Records of 19 patients (11 men and eight women; age range: 6–49 years) with a total of 37 treatment episodes were studied. Subtypes of VWD were diagnosed with the following frequencies: type 1 ($n = 7$), type 2A ($n = 9$), type 2B ($n = 2$) and type 2N ($n = 1$). None of the patients had type 2M or type 3 VWD. In type 1 or 2A VWD responsive to DDAVP, the drug was administered intravenously. For all treatments, 20 mg kg^{-1} day^{-1} of tranexamic acid was administered orally, for electives starting from 3 h prior to surgery and at admission when treating intraoral bleeding. Treatment was continued for 1–2 weeks.

For surgical extractions, pressure haemostasis was also performed using oxidized cellulose and the surgical wound was sutured but for routine extractions or gingival bleeding, a surgical acrylic splint was used for 7 days later. Different management regiments undertaken by us for type 1, 2A, 2B and 2N VWD is given in Tables 2–4.

Discussion

Based on the results of the present study, haemostatic management of intraoral bleeding in patients with VWD is summarized in Table 5.

Table 2 Haemostatic management of patients with type 1 von Willebrand disease

Treatment or haemostasis for	Occasion	Tooth number (causes of haemorrhage): Primary tooth	Tooth number (causes of haemorrhage): Permanent tooth (Causes of hemorrhage)	Agent	Dose $(kg)^{-1}$ and frequency: Day 0	Dose $(kg)^{-1}$ and frequency: Day 1	Dose $(kg)^{-1}$ and frequency: Day of splint removal	Local haemostasis
XLA	5[a]		6	DDAVP	0.37–0.4 μg	0.37–0.4 μg	0.37–0.4 μg	Splint
SR	3		5	DDAVP	0.35–0.4 μg	0.35–0.4 μg	0.35–0.4 μg	Suture, splint
	1		1	DDAVP	0.4 μg			Suture, splint
	1[b]		2	FVIII/VWF	90 U (126%)	70 U (98%)	70 U (98%)	Suture, splint
Scaling	1[c]			DDAVP	0.4 μg	0.4 μg		
GB	1[b]		1 (Pericoronitis)	FVIII/VWF	45 U (63%)			Splint
GB	1 (Postscaling)			FVIII/VWF	53 U (74%)			Compression
GB	2	5 (Mobility)						Compression
Haematoma of lower lip	1			DDAVP	0.4 μg			

DDAVP, 1-deamino-8-d-arginine vasopressin; FVIII/VWF, factor VIII/von Willebrand factor concentration; U, ristocetin cofactor (VWF:RCof) units; %, anticipated incremental percentage of VWF:RCof; XLA, extracted under local anaesthesia; SR, surgical extraction; GB, gingival bleeding.
[a]Two cases were performed at the same time of surgical extraction.
[b]Same patient.
[c]Scaling was performed at the same time of surgical extraction.

Table 3 Haemostatic management of patients with type 2A von Willebrand disease

Treatment or haemostasis for	Occasion	Tooth number: Primary tooth	Tooth number: Permanent tooth	Agent	Dose $(kg)^{-1}$ and frequency: Day 0	Dose $(kg)^{-1}$ and frequency: Day 1	Dose $(kg)^{-1}$ and frequency: Day 2	Dose $(kg)^{-1}$ and frequency: Day of splint removal	Local haemostasis
XLA	4	5	1	DDAVP	0.35–0.4 μg	0.35–0.4 μg		0.35–0.4 μg	Splint
	3	2	1	FVIII/VWF	54–88 U (76–123%)			54–88 U	Splint
	1	1[b]		FVIII/VWF	72 U (100%)	36 U (51%)	36 U	36 U	Splint
SR	2		4	FVIII/VWF	87–88 U (122–123%) × 2	87–88 U	87–88 U	87–88 U	Suture, splint
Scaling	1[a]			FVIII/VWF	88 U (123%) × 2				Splint
GB	4	1 (Mobility)[b]	3 (P 2: eruption 1[b])	FVIII/VWF	50–84 U (70–118%)				
GB	2	1 (Mobility)	2 (P)						Compression
Haematoma of mandible	1			FVIII/VWF	84 U (118%)				

DDAVP, 1-deamino-8-d-arginine vasopressin; FVIII/VWF, factor VIII/von Willebrand factor concentrate; U, ristocetin cofactor (VWF:RCof) units; %, anticipated incremental percentage of VWF:RCof; XLA, extracted under local anaesthesia; SR, surgical extraction; GB, gingival bleeding; P, marginal periodontitis.
[a]Scaling was performed at the same time of surgical extraction.
[b]Same patient.