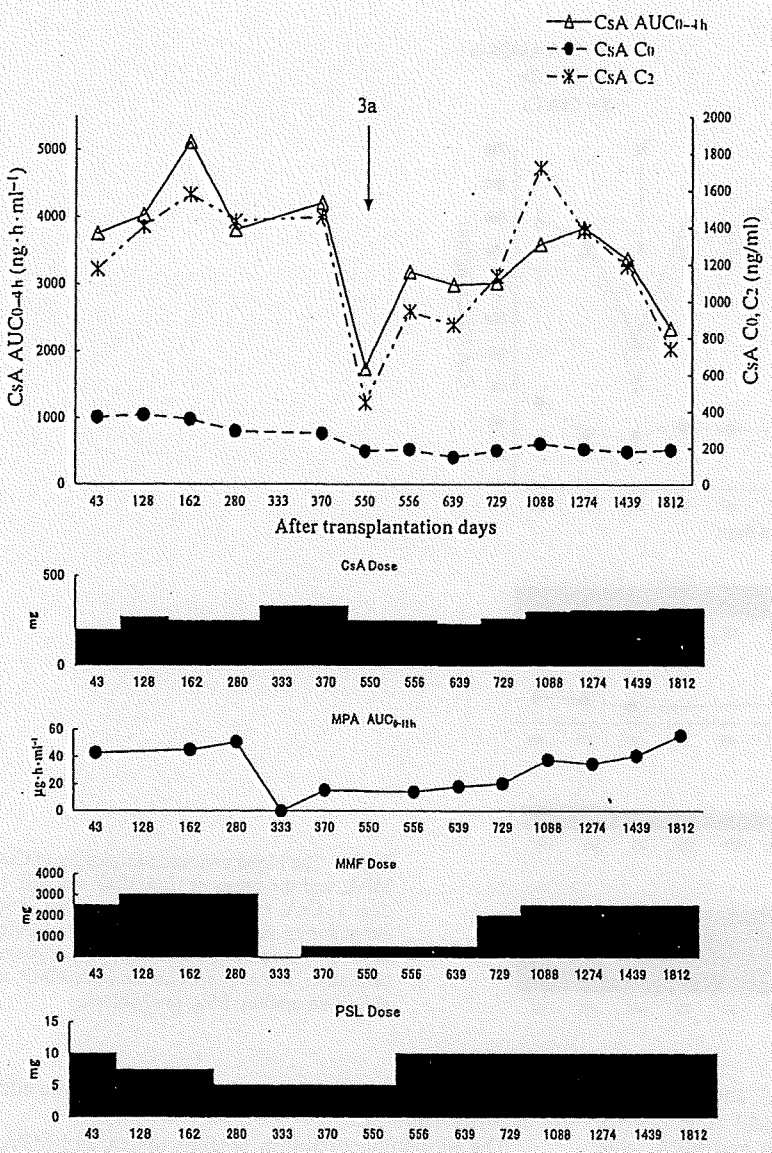

Fig2. The blood concentration profiles of CsA and MPA, and the doses of CsA, MMF and PSL in patient 2.

was at 420 mg/day, C0 at 308 ng/ml, C2 at 607 ng/ml, and the AUC0-4h at 2,408 ng·h·ml^{-1}; the dose of MMF was 1 g/day, AUC0-12h of MPA was 20.8 μg·h·ml^{-1}, and the dose of PSL was at 20 mg/day. The grade of acute rejection improved following a 3-day course of pulse therapy with MP at 1 g/day. The C0 of CsA was at the target level with few variations, but despite that, acute rejection of ISHLT grade 3a occurred twice, and the patient sustained a pressure fracture of a vertebra because of PSL. In view of these findings, CsA was changed to FK.

Patient 2 (Acute Rejection)

A woman in her 40s with DCM as the underlying disease underwent cardiac transplantation after being on a NCVC extracorporeal left ventricular assist system (LVAS) (Toyobo, Tokyo, Japan)[21] HLA (A, B, DR) compatibility was 2/6, and CMV antibody was (+) for the donor and (+) for the recipient. At the time of the transplant, Panel Reactive Antibody was (–), as the cross-match test. Specific anti-HLA antibodies against the donor were found in the recipient. In addition, owing to concern about the possibility of a renal function disorder because of long-term use of the LVAS, immunosuppressive therapy was begun with OKT-3, then switched to the 3-drug combination therapy.

The blood concentration profiles of CsA and MPA, and the doses of CsA, MMF and PSL are shown in Fig2. On day 333 post-transplant, the patient's leukocyte count had decreased to 3,400/μl, so MMF treatment (3 g/day) was stopped and the dose of CsA was increased from 280 to 330 mg/day. However, the serum creatinine level increased mildly to 1.3 mg/dl. On day 370 post-transplant, MMF treatment was reinstated at 0.5 g/day. CsA was maintained at 330 mg/day. The C0 of CsA was at 275 ng/ml, C2 at 1,452 ng/ml, and AUC0-4h at 4,204 ng·h·ml^{-1}. In addition, the AUC0-12h of MPA was 15.3 μg·h·ml^{-1}. subsequently, the serum creatinine level increased to 1.1 mg/dl and the CsA dose was decreased from 330 to 250 mg/day in order to obtain a target C0 of CsA of ≈200 ng/ml. On day 550 post-transplant, a myocardial biopsy was performed and acute rejection of ISHLT grade 3a was identified. At this point C0 was at 182 ng/ml, C2 at 445 ng/ml, AUC0-4h at 1,735 ng·h·ml^{-1}, the dose of MMF was 0.5 g/day, and that of PSL was 5 mg/day. Blood MPA level was not measured. After a 3-day course of pulse therapy with MP 1 g/day, the PSL dose was increased to 10 mg/day and acute rejection improved on day 556 post-transplant. The patient's leuko-

day 550 in patient 2. Although the MPA AUC_{0-12h} decreased to 15.3 $\mu g \cdot h \cdot ml^{-1}$ on day 370, the high CsA AUC_{0-4h} (4,204 $ng \cdot h \cdot ml^{-1}$) and C_2 (1,452 ng/ml) were maintained, which may have prevented acute rejection in patient 2. Patient 3 did not experience acute rejection during the MMF washout period from day 99 to day 262, which may be attributed to the high AUC_{0-4h} (4,019 $ng \cdot h \cdot ml^{-1}$) or C_2 (1,249 ng/ml). These findings suggest that the high CsA AUC_{0-4h} (>4,000 $ng \cdot h \cdot ml^{-1}$) or C_2 (>1,200 ng/ml) might prevent acute rejection, even if the MPA AUC_{0-12h} is 30 $\mu g \cdot h \cdot ml^{-1}$ or less.

We calculate the AUC of CsA in heart transplant patients who are admitted for myocardial biopsy and monitor the C_0 and C_2 levels (the reference levels) of outpatients to determine the dose of CsA. However, there was no clear link between the risk of acute rejection and CsA C_0 levels in these 3 patients. It has been reported that, in determining the appropriate dose, monitoring of the absorption profile is more important than conventional C_0 monitoring of CsA.[4–8] The AUC_{0-4h} is the important parameter of the absorption profile; however, a 1-point monitoring strategy needs to be developed for predicting the AUC_{0-4h} in clinical practice, in particular for outpatients?[22] It has been reported that C_2 is the most accurate surrogate marker for AUC_{0-4h},[4–6] and has been found to be a better marker for rejection and nephrotoxicity than C_0.[4,5] Our experience also suggests that the CsA C_2 values changed in relation to the AUC_{0-4h}. Cantarovich et al[6,9,23] report a clinical benefit of CsA C_2 monitoring (as opposed to C_0 monitoring) in long-term heart transplant patients. The C_2 target levels of their study were as follows: 0–3 months, 600–800 ng/ml; 4–6 months, 500–700 ng/ml; >6 months, 400–600 ng/ml. Other groups report that high C_2 values (1,015±422 ng/ml) are associated with fewer episodes of acute cellular rejection in patients who have undergone heart transplantation,[10] and that acute cellular rejection should be suspected when the C_2 level is below 600 ng/ml.[6,11] At present, at the NCVC, the AUC_{0-4h} is predicted from C_0 and C_2, which are monitored in outpatients to determine the dose of CsA. However, the appropriate target value for either the AUC_{0-4h} or C_2 of CsA in heart transplant recipients is not fixed.

On the other hand, the target AUC_{0-12h} value for MPA after heart transplantation has been reported to be 30–60 $\mu g \cdot h \cdot ml^{-1}$.[13] In addition, the 3-point monitoring of C_0, $C_{0.5}$, and C_2 has been reported to be highly correlated with the AUC_{0-12h}.[24]

We demonstrated that a high CsA AUC_{0-4h} may help prevent cardiac allograft rejection in patients who temporarily stop MMF treatment. When MMF is stopped or drastically reduced, the dose of CsA should be increased to maintain the high CsA AUC_{0-4h} (>4,000 $ng \cdot h \cdot ml^{-1}$). Although our study had a limited number of patients, it is the first to characterize the relationship between acute rejection and either the CsA or MPA level in heart transplant recipients. Further studies should be conducted to investigate the relationship between the CsA AUC_{0-4h} or MPA AUC_{0-12h} and the risk of rejection, and the effectiveness of CsA C_2 monitoring in heart transplant patients should be confirmed.

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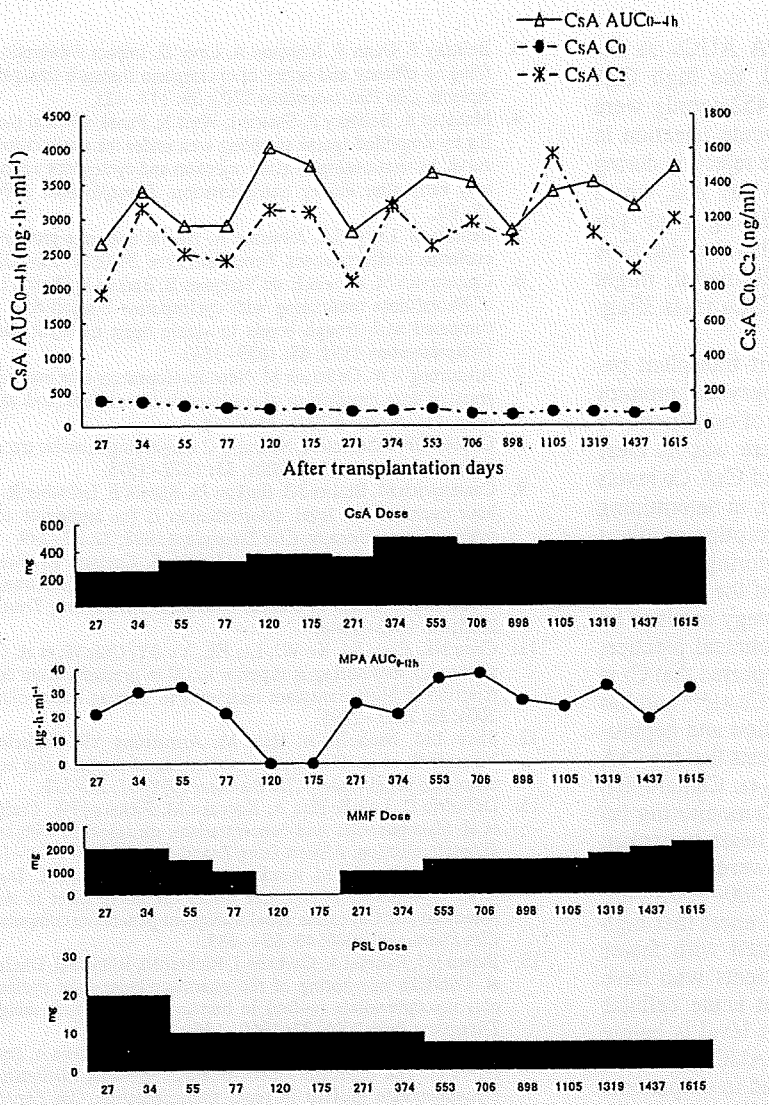

Fig3. The blood concentration profiles of CsA and MPA, and the doses of CsA, MMF and PSL in patient 3.

cyte count recovered to the normal value, so MMF was gradually increased to 2.5 g/day. Thereafter, the dose of CsA was set according to the monitoring of C_2 and AUC_{0-4h}, and no further episodes of acute rejection occurred.

Patient 3 (Without Acute Rejection)

A man in his 30s with DCM as the underlying disease, underwent cardiac transplantation under the support of LVAS. HLA (A, B, DR) compatibility was 0/6, CMV antibody was donor (+) and recipient (+). After the transplant, the patient's serum creatinine level increased to 2.2 mg/dl so immunosuppressive therapy was initiated with OKT-3, followed by the 3-drug combination therapy.

The blood concentration profiles of CsA and MPA, and the doses of CsA, MMF and PSL are showed in Fig 3. Up to day 75 post-transplant, the MMF dose was at 1.5 g/day, but the leukocyte count decreased to 3,770/μl, so the MMF dose was decreased from 1.5 to 1 g/day. On day 99 post-transplant, the leukocyte count decreased further to 3,270/μl, MMF was stopped and the CsA dose was increased from 320 to 380 mg/day. When the CsA dose was at 320 mg/day, C_0 was at 267 ng/ml, C_2 at 954 ng/ml, and AUC_{0-4h} at 2,897 ng·h·ml^{-1}. When the CsA dose was at 380 mg/day, C_0 was at 247 ng/ml, C_2 at 1,249 ng/ml, and AUC_{0-4h} at 4,019 ng·h·ml^{-1}. On day 262 post-transplant, the leukocyte count recovered to 8,000/μl, which is within the normal range, so MMF treatment was reinstated at 0.5 g/day. During the washout of MMF, myocardial biopsy was performed twice, but acute rejection was not seen.

Discussion

Our experience with the 3 heart transplant patients presented here suggests that monitoring of the CsA AUC_{0-4h} or C_2 may be useful in preventing acute rejection, as may a high AUC_{0-4h} or C_2, even if MMF is stopped or drastically decreased.

In patient 1, the CsA AUC_{0-4h} and C_2 were greatly decreased, with a low MPA AUC_{0-12h} (20.8 ng·h·ml^{-1}) on day 361 post-transplant (ISHLT grade 3a). In patient 2, the CsA AUC_{0-4h} and C_2 greatly decreased on day 550 post-transplant (ISHLT grade 3a). Although the MPA AUC_{0-12h} value on day 550 was not calculated, approximately 15 ng·h·ml^{-1} could be predicted because the MPA AUC_{0-12h} values on days 370 and 556 were 15.3 μg·h·ml^{-1} and 14.3 μg·h·ml^{-1}, respectively. The MPA dose remained unchanged from day 370 to day 556. Low CsA AUC_{0-4h} and MPA AUC_{0-12h} might have been the cause of acute rejection on

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Drug interactions between tacrolimus and phenytoin in Japanese heart transplant recipients: 2 case reports

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Key words
phenytoin – tacrolimus – drug interactions – Japanese heart transplant recipients

Abstract. Objective: The purpose of the study was to demonstrate how the interaction between phenytoin and tacrolimus (FK 506) can be managed clinically and to characterize the change in FK 506 levels after discontinuation of phenytoin in two Japanese heart transplant recipients with different dosing periods of phenytoin. Methods: A drug interaction between phenytoin and FK 506 was investigated in 2 patients. The concentration-dose ratios (CDR: trough blood FK 506 level (ng/ml)/FK 506 dose (mg/day) on the previous day) were calculated as an index of the induction of the CYP3A4 enzyme during and after phenytoin therapy. Results: About 2- to 3-fold dosages of FK 506 were required to maintain the required blood level when phenytoin was used concomitantly in the two cases examined. The FK 506 dose was constant within 21 days after discontinuing phenytoin in Patient 1 who had 36 days of phenytoin therapy. In Patient 2 with 21-day phenytoin therapy, the FK 506 doses and CDR varied for 10 days after discontinuing phenytoin, and expected FK 506 C_0 levels were achieved within 11 days. Conclusions: The persistence of CYP induction after discontinuing phenytoin is dependent on the history of administration and, perhaps, on the dosing period in particular.

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Introduction

Tacrolimus (FK 506), a neutral macrolide antibiotic isolated from Streptomyces tsukubaensis, is widely used in solid organ transplantation [Kino et al. 1987]. It is a potent immunosuppressive agent that inhibits calcineurin to prevent the production of interleukin-2 by T cells, inhibiting their maturation and proliferation [Ochiai et al. 1987]. Therapeutic drug monitoring (TDM) is required for FK 506 because of a narrow therapeutic window. Metabolism of the drug largely occurs through the cytochrome P450 (CYP) 3A4 enzyme system, which is present in the liver and gut [Lamba et al. 2002]. Agents that affect the metabolism of FK 506 by inducing or inhibiting CYP3A4 enzymes may change blood levels of FK 506, resulting in transplant rejection or side effects. Inhibitors of CYP3A4, such as clarithromycin, diltiazem, fluconazole, indinavir and grapefruit juice, have been reported to increase FK 506 levels [Fireman et al. 2004]. In contrast, inducers of CYP3A4, such as rifampicin, phenobarbital and St. John's wort have been shown to decrease FK 506 levels [Fireman et al. 2004].

Phenytoin is a hydantoin anticonvulsant for the control of generalized tonic-clonic and complex partial seizures. Phenytoin accelerates CYP1A-, CYP2B-, CYP2C- and CYP-3A-mediated oxidative metabolism of antiepileptic drugs (e.g. zonisamide, carbamazepin, valproate), cardioactive drugs (e.g. nifedipine, disopyramide, procainamide), oral anticoagulants (e.g. warfarin) and immunosuppressants (e.g. cyclosporine (CsA), FK 506) [Anderson 1998]. A drug interaction between phenytoin and FK 506 has been previously reported [Formea et al. 2005, Karasu et al. 2001, Moreno et al. 1999]. However, changes in FK 506 blood levels after discontinuation of phenytoin have not been characterized in previous reports. In this report, we present two cases of a drug interaction between phenytoin and FK 506 in Japanese heart

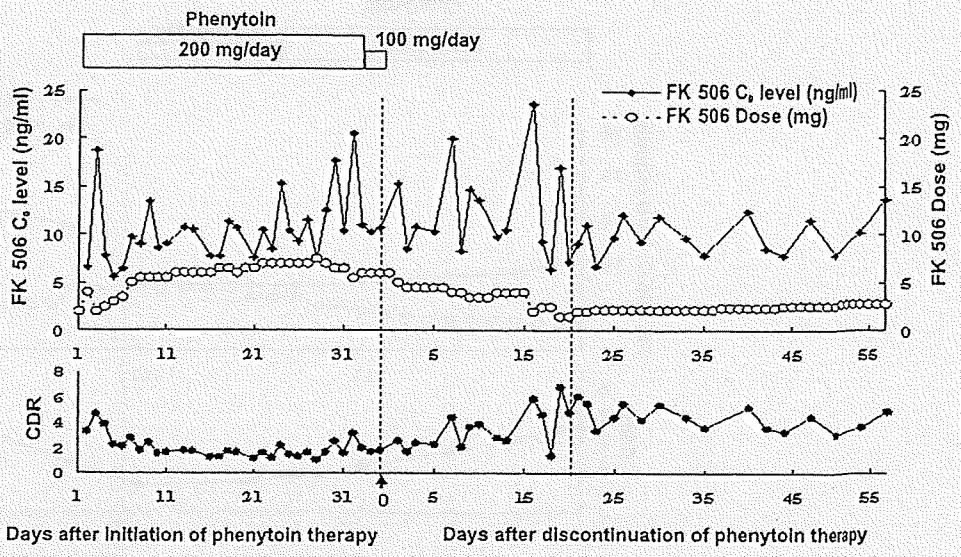

Figure 1. The effect of phenytoin treatment on trough serum FK 506 levels in Case 1 patient.

transplant recipients. The purpose of the study was to show how the drug interaction between phenytoin and FK 506 can be managed clinically and to characterize the changes in FK 506 levels after discontinuation of phenytoin in Japanese heart transplant recipients.

Methods

Among the 38 patients who had received immunosuppression therapy with CsA or FK 506, mycophenolate mofetil (MMF) and prednisone (PSL) between May 1999 and November 2006, there were 2 patients who received FK 506 and phenytoin concomitantly. One patient was a woman in her 40's who underwent heart transplantation at the National Cardiovascular Center (NCVC). The primary disease of the patient was dilated cardiomyopathy. The other patient was a woman in her 20's with dilated cardiomyopathy as a primary disease, who underwent heart transplantation at an overseas hospital. These 2 patients were investigated to evaluate a drug interaction between phenytoin and FK 506. Blood levels of FK 506 and CsA were measured by fluorescent polarization immunoassay (TDx, Abbott Japan CO., Tokyo, LTDA). The concentration-dose ratios (CDR: trough blood FK 506 level) (ng/ml)/FK 506 dose (mg/day) on the previous day were calculated as an index for the induction of the CYP3A4 enzyme during and after phenytoin therapy [Keown et al. 1984]. The changes in CDR for FK 506 between combination periods and FK 506-only periods were investigated. All research procedures were conducted according to the clinical research guidelines in our institute. All patients gave their written informed consent concerning the disclosure of their clinical data.

Results

Patient 1

This patient received triple-drug immunosuppression with CsA, MMF and PSL after surgery. The patient developed an impaired level of consciousness and orientation on the 7th day after surgery. For the next 7 days, she had narrowed visual fields, and on the 16th day after surgery she developed a convulsive seizure. The CsA trough (C_0) levels were measured routinely. The maximum CsA C_0 level throughout the neurological events was 439 ng/ml, which was within the target range. The magnesium level was 0.44 mmol/l. Magnetic resonance imaging (MRI) of the head demonstrated multifocal areas of increased signal in the white matter of the right mater lobus occipitalis. Oral phenytoin was started for treatment of a convulsive seizure at a dose of 200 mg/day, and CsA was changed to FK 506 on the next day because of potential CsA neurotoxicity. Abnormal MRI signals signifi-

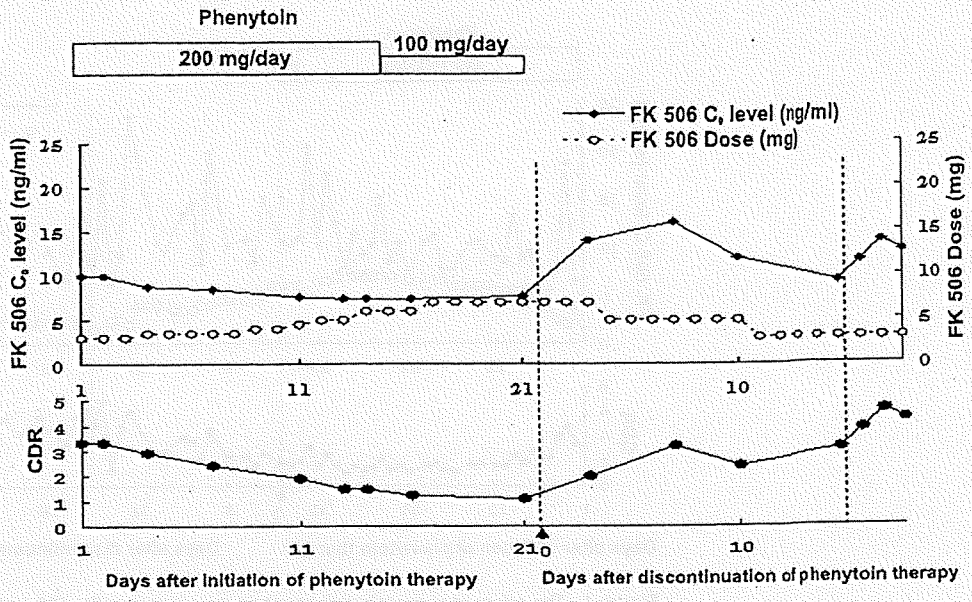

Figure 2. The effect of phenytoin treatment on trough serum FK 506 levels in Case 2 patient.

cantly diminished, and narrowed visual fields disappeared on the 6th day after discontinuing CsA. The abnormal MRI signals disappeared on the 28th day after CsA was stopped. Phenytoin was decreased to 100 mg/day on the 34th day after the initiation of therapy and was stopped within 2 days.

Changes of phenytoin dose, FK 506 dose, blood FK 506 C_0 levels and the CDR are shown in Figure 1.

FK 506 was started at an initial daily dose of 2 mg. FK 506 C_0 levels were measured routinely. The dose of FK 506 was gradually increased and a C_0 level of 10.5 ng/ml was obtained with a daily dose of 6.0 mg on the 14th day after initiation of phenytoin therapy. Thereafter, 5.5 – 7.5 mg/day of FK 506 gave C_0 levels of 7.7 – 20.5 ng/ml. The CDR was 4.7 on the 3rd day after initiation of FK 506 therapy and gradually decreased.

On the 7th day after phenytoin was stopped, 4 mg/day of FK 506 resulted in a C_0 level of 20 ng/ml. Although the FK 506 dose was finely adjusted thereafter, the C_0 levels varied widely. After discontinuing phenytoin therapy, the FK 506 dose was gradually reduced. Consequently, C_0 levels of 10 – 13 ng/ml were maintained with daily doses of 2.4 – 2.8 mg for 3 weeks after stopping phenytoin. The mean value of CDR for 3 weeks after stopping phenytoin was 3.63 ± 1.67. The mean value of CDR for the next 21 days (22nd – 42nd day after stopping phenytoin) was 4.52 ± 0.84. The coefficient of variation of the CDR for 3 weeks after discontinuing phenytoin was 46%. The coefficient of variation of the CDR for the next 21 days was 18.7%. These observations suggest that enzyme induction by phenytoin persisted for 21 days. Concomitant drugs were not changed during phenytoin therapy, and there was no hepatic disease.

Patient 2

This patient received triple-drug immunosuppression with FK 506, MMF and PSL. She developed a convulsive seizure on the 16th and 20th day after surgery and was treated with oral levetiracetam (unapproved in Japan). The convulsive seizure immediately disappeared. On the 70th day after surgery, the patient returned to Japan to enter the NCVC. On the day after admission, oral levetiracetam was changed to 200 mg/day of oral phenytoin. The phenytoin daily dose was decreased to 100 mg on the 15th day and was stopped on the 21st day after the initiation of therapy. Changes of phenytoin dose, FK 506 dose, blood FK 506 C_0 levels and CDR are shown in Figure 2. The FK 506 C_0 levels were measured routinely. Before the start of phenytoin dosing, the FK 506 C_0 level was 10 ng/ml with a daily dose of 3 mg. Thereafter, there was a decrease in the FK 506 C_0 level

(8.8 ng/ml) on the 4th day after the start of phenytoin. Despite increasing the daily dose of FK 506 to 6 mg, a C_0 level of 7.4 ng/ml and a CDR value of 1.5 were obtained on the 14th day after the start of phenytoin. Phenytoin was discontinued on the 21st day after it was initiated, and the FK 506 C_0 level reached 14 ng/ml within 3 days of a daily dose of 7 mg. For the next 7 days, the FK 506 dose was reduced to 5 mg/day, however, the C_0 level did not decrease. Thereafter, the FK 506 dose was reduced to 3 mg/day and C_0 levels of 9 – 14 ng/ml were obtained. Fixed dosing of FK 506 was achieved within 11 days after discontinuing phenytoin.

The mean CDR was 2.53 ± 0.61 for 10 days after discontinuing phenytoin. The mean CDR for the next 8 days (11th – 18th day after discontinuing phenytoin) was 3.95 ± 0.64. A CDR value of 3.9 on the 16th day after discontinuing phenytoin was about the same as obtained at the onset of phenytoin dosing. The coefficient of variation of the CDR for 10 days after stopping phenytoin was 24.1%. The coefficient of variation of the CDR for the next 8 days was 16.2%. This suggests that the effect of enzyme induction by phenytoin disappeared in 11 days. Other concomitant drugs were not changed during phenytoin therapy, and there was no hepatic dysfunction.

Discussion

The present study suggested that the difference of the persistence of CYP induction after discontinuing phenytoin may partly be explained by variation of the dosing period. Drug interactions associated with CYP induction are expected to occur gradually because CYP induction requires increased production of metabolic protein. In general, the effects of CYP induction occur and dissipate slowly in a few weeks after the start and discontinuation of inducers. In the case of phenytoin, it has been reported that induction of the CYP3A4 enzyme occurs within 2 – 5 days, reaching a maximum 10 – 14 days after the initiation of therapy [Karasu et al. 2001]. In our patients, the CDR was stable within 14 – 21 days after initiation of phenytoin. About a 2- to 3-fold higher dose of FK 506 is required to maintain expected blood levels when phenytoin is given concomitantly.

The impact of CYP3A4 enzyme induction on blood levels of FK 506 disappeared slowly within 21 and 11 days after stopping phenytoin in Patients 1 and 2, respectively. There were differences in the dosing period and total dosage of phenytoin between these 2 patients. The dosing period of phenytoin in Patient 2 was shorter than in Patient 1. The half-life of CYP enzyme turnover ranges from 1 – 6 days. In addition, the time course of induction is dependent on the time period required for enzyme degradation and new enzyme production [Michalets 1998]. The persistence of CYP induction after discontinuing inducers may change depending on the history of administration. It was reported that the time period of CYP induction depended on the half-life of inducers, the half-life of the CYP enzyme, the patient's hepatic function and the patient's age [Michalets 1998]. The effect of CYP induction by phenytoin could depend on these factors, and the persistence of CYP induction after discontinuing phenytoin disappears slowly and is not constant. In our 2 patients, the persistence of CYP induction varied with the dosing period of phenytoin.

In conclusion, the 2 cases in the present study suggest that the persistence of CYP induction after discontinuing phenytoin depends on the history of administration and, perhaps, on the dosing period in particular. When FK 506 is used concomitantly with phenytoin, the dose of FK 506 after discontinuing phenytoin should be carefully adjusted taking into account the dosing period of phenytoin, and FK 506 blood levels should be closely monitored during and after phenytoin therapy. Further study is required to clarify the relationship between the dosing period of phenytoin and the persistence of CYP induction.

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Reduction of Antigenicity and Risk of Infection in Regenerative Tissue Transplantation by Cold Isostatic Pressing

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Abstract

Tissue engineered heart valves based on acellular tissue have been studied to have more durability and bio-functionality with growth potential and less immunogenicity. Whereas they have still several problems to be solved such as complete cell removal and transfer of unknown animal related infectious diseases. In this paper, our novel tissue processing for decellularization using ultrahigh pressure for the safe tissue transplantation was reported. Porcine cardiac tissues were isolated and treated by a cold isostatic pressing for a disruption of donor cells. The cell debris was then washed out by washing solution at 4°C. The tissues treated were completely cell free when they were applied to 980 MPa for 10 min. There was no porcine endogeneous retrovirus detected. There were no significant changes in biomechanical properties of the breaking strength and elastic modulus. The acellular grafts of pulmonary valve were transplanted to allogeneic miniature pigs. The explanted grafts showed remarkable cell infiltration and endothelialization. This processing may provide more durable and safe scaffold for the regenerative tissue transplantation.

Keywords: tissue engineering, tissue transplantation, acellular, scaffold

1. Introduction

The implantable cardiovascular medical devices have been clinically used for more than 30 years as substitution for the patient's deficient tissues. The artificial heart valve is one of the most clinically used medical devices applied to about 300,000 patients per year worldwide. There are two kinds of artificial heart valves currently used. A xenograft heart valve is made of the chemically crosslinked porcine valve or bovine pericardium to reduce antigenicity of the xenogeneic tissue. A mechanical heart valve is made of pyrolytic carbon or titanium. The former has good biocompatibility, hemodynamics, and resistant to infections compared with the latter. However, the durability of the xenograft valve is relatively short especially in pediatric patients for about 5-10 years by the calcification of the gltaraldehyde-fixed animal tissue. Recent establishment of the human tissue bank has made it easy to use allogeneic tissues for the transplantation that are superior to the current artificial devices. However, since they are donated from the cadavers, the supply is very limited and some donated tissues may not be applicable due to infection. In addition to the above issues, all the devices and tissues lack the growth potential and they may be replaced repeatedly through the patients' growth process.

All of the current medical devices remain as foreign bodies even after the implantation. If a device accepts host cell impregnation and is replaced by the host tissue after the implantation,

it may acquire perfect biocompatibility and growth ability. An ideal candidate for such a regenerative scaffold is a decellularized allogeneic or xenogeneic tissue since it does not require tissue fixation for removal of antigenicity. Detergents and/or enzymes such as Triton® X-100, sodium dodecyl sulphate, deoxycholate, trypsine, DNase, and RNase have been commonly used for the cell removal media from the tissue [1-4]. However, the decellularization depends on their permeation in the tissue and may not be achieved completely in large or hard tissues. And furthermore, since the detergents are generally cytotoxic and it takes time for their removal, it may lead denature of biological properties and contamination in the process. Recent BSE (Bovine Spongiform Encephalopathy) and vCJD (variant Creutzfeldt-Jakob disease) issues have been affecting to the tissue transplantation from the point of view of safety. In this paper, a cold isostatic pressing (CIP) was applied for removal of the cells and inactivation of viruses in the cardiovascular tissues to have scaffold for the safe regenerative tissue transplantation.

2. Material and methods

The porcine heart valves were isolated from 4 month-old Clawn miniature pigs (Japan Farm Co. Ltd, Kagoshima, Japan) weighing about 10 kg under the sterile condition. The harvested tissues were packed immediately in sterile bags filled with phosphate buffered saline (PBS) and treated by ultrahigh pressure of 980 MPa for 10 min using a CIP apparatus (Dr. Chef, Kobe Steel Ltd, Kobe, Japan) for cell demolition (Fig. 1). The range of temperature in the process is about 5 to 30°C. They were then rinsed by PBS for 2 weeks under gentle stirring at 4°C for removal of the residues of the broken cells. They were subjected to the histological observation by the light and electron microscopy, DNA and phospholipids assay, detection of porcine endogeneous retrovirus (PERV) by the PCR, and biomechanical study by the tensile strength measurement.

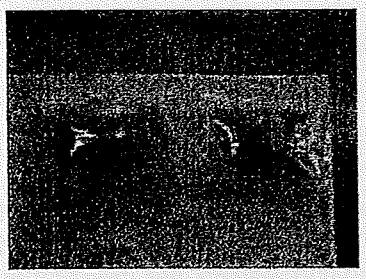

Fig. 1 Packed porcine heart valves for CIP treatment.

The acellualr tissues were transplanted orthotopically into nine allogeneic miniature pigs. The pulmonary valves were transplanted at right ventricular outflow tract through a median sternotomy with extracorporeal circulation without blood oxygenation [5]. The postoperative anticoagulation or anti-platelet therapy was not instigated. They were explanted 4, 12, and 24 weeks (n=3) after the transplantation and examined histologically and immunohistologically. All animals were carefully reared in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH publication No.85-23, revised in 1985).

3. Results and discussion

The tissues were completely cell free when they were treated by the CIP for 10 min followed by washing for 2 weeks from the H-E staining (Fig. 2). The amount of DNA and phospholipids were lower than 1 μg/ml and 0.5 mg/wet g, respectively and those were less than 10% in the native tissue (Fig. 3).

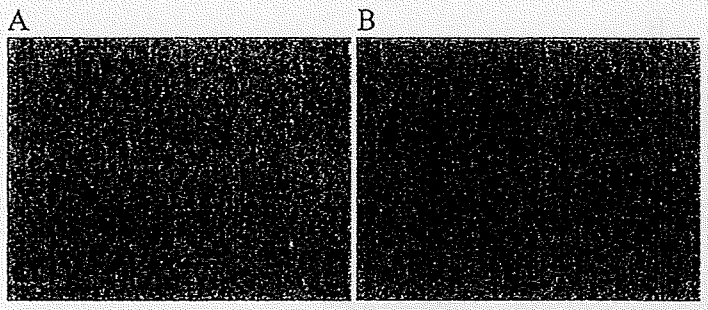

Fig. 2 Cross sections of (A) native and (B) treated tissues (H-E staining).

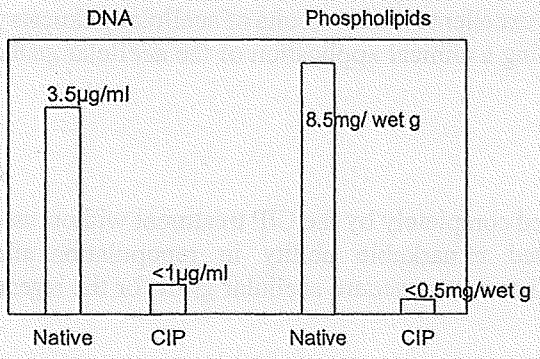

Fig. 3 Residual amounts of DNA and phospholipids in native and treated tissues.

The collagen and elastin fibers were well maintained in the acellular tissue and there were no significant changes in biomechanical properties of the breaking strength and elastic modulus. We have already found that this process could be successfully applied to cartilage tissues for decellularization (not shown). More effectively, it has been reported that the most of viruses including HIV are inactivated by the CIP only of more than 600 MPa without washing [6]. This means the treatment is able to sterilize the tissue in addition to the decellularization. The Clawn miniature pig was chosen as a donor animal since its size adapts human tissues well and its genome has been well studied in order to develop a human gene induced transgenic animal for the organ transplantation. There was no PERV detected in PCR assay from the tissue treated (Fig. 4).

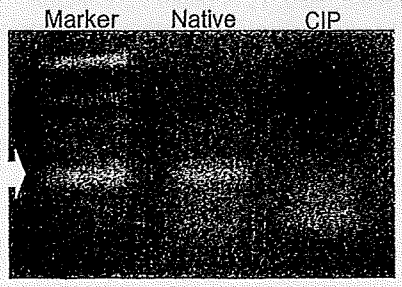

Fig. 4 PCR products of PERV (arrow) in native and treated tissues.

The animals survived after the transplantation in the all cases. The explanted grafts showed no macroscopical abnormality and no dilatation and aneurysmal changes including their anastomosis. The inner surface was completely covered with endothelial cells and the inside was infiltrated by cells from both sides of endothelium and outer tissue after 12 weeks. It was dominant in the latter. Almost of the tissue including cusps were filled by the cells at 24 weeks, mainly by smooth muscle cells (Fig. 5). There was no inflammation and calcification observed in the tissue.

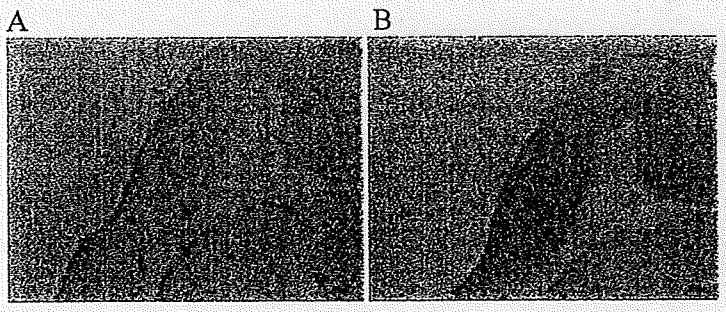

Fig. 5 Cross sections of (A) anti-vWF (endothelial cells) and (B) anti-αSMA (smooth muscle cells) immunostained treated tissues 24 weeks after the transplantation.

Recently, some groups have reported excellent clinical results of acellular pulmpnary heart valve transplantation [7-9]. We are planning a clinical application of the acellular grafts made by this process in the near future.

4. Conclusion

Porcine cells and PERV were removed completely by the CIP treatment without using any detergents. The acellular grafts showed remarkable ability in repopulation after the transplantation. This CIP treatment may have more secure acellular graft for the regenerative tissue transplantation.

5. Acknowledgement

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ORIGINAL ARTICLE

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Preparation of poly(vinyl alcohol)/DNA hydrogels via hydrogen bonds formed on ultra-high pressurization and controlled release of DNA from the hydrogels for gene delivery

Abstract Poly(vinyl alcohol) (PVA) hydrogels interacting with DNA mediated by hydrogen bonds (PVA/DNA hydrogel) were developed using ultra-high pressure (UHP) technology. The goal was to create a new method of gene delivery by controlled release of DNA. Mixed solutions of DNA and PVA at various concentrations were pressurized at 10000 atmospheres at 37°C for 10 min. PVA/DNA hydrogels with good formability were produced at PVA concentrations of more than 5% w/v. The presence of DNA in the obtained hydrogels was confirmed by spectroscopic analysis and nucleic acid dye staining. DNA release from the hydrogels was investigated using PVA/DNA hydrogel samples of 5% and 10% w/v formed by UHP treatment or by conventional freeze–thaw methods. The DNA release curves from both types of samples showed a rapid phase in the initial 15 h followed by a sustained release phase. However, there was a difference in the amount of DNA released. Less DNA was released by the pressurized hydrogels than by the freeze–thaw hydrogels. Also, the cumulative amount of DNA released decreased as the PVA content in the hydrogels increased. These results indicate that DNA release from the hydrogels can be modulated by changing the preparation method and the PVA content. Furthermore, it was demonstrated that DNA release could be controlled by varying the amount and duration of pressurizing used to form the hydrogels. Intact fractions of plasmid DNA released from the hydrogels were separated by agarose gel electrophoretic analysis. These results suggest that, using controlled release, DNA from PVA/DNA hydrogels formed by UHP treatment can be transfected into cells.

Key words Controlled release · Ultra-high pressure · DNA · Hydrogel · Poly(vinyl alcohol)

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Introduction

Safe and biocompatible synthetic materials have been developed as biomaterials.[1] In gene therapy, nonviral synthetic gene carriers have been the focus of attention due to their biological safety advantages over viruses.[2] In many cases, cationic synthetic materials, such as cationic lipids, liposomes,[3] polyethyleneimine,[4] polyamideamine dendrimer,[5] poly-L-lysine (PLL), PLL derivatives,[6] and other cationic peptides,[7] have been used as nonviral vectors. It is possible to form complexes between these materials and DNA using the electrostatic interaction between their cationic groups and the anionic groups of DNA, making the DNA robust against nuclease degradation and enabling effective transfection into mammalian cells.[8,9] However, the cytotoxicity of cationic materials was reported to be a significant problem.[10,11] For safer and more efficient gene delivery, it is necessary to develop a noncationic or less cationic gene carrier through nonelectrostatic interaction with DNA. Sakurai et al. reported that a triple helical complex of single-strand DNA and double-strand schizophyllan, which is a kind of polysaccharide (β-1,3 glucan), was formed through hydrogen bonding.[12] In addition, we previously reported that nanoparticles of poly(vinyl alcohol) (PVA) bonded to DNA via hydrogen bonds were obtained when mixed solutions of PVA (less than 0.01% w/v) and DNA were treated under ultra-high pressure (UHP) at

10000 atmospheres (980MPa) and 40°C for 10min.[13] It is well known that intra- and intermolecular hydrogen bonding increases in these conditions.[14] The PVA/DNA nanoparticles could be internalized into mammalian cells, suggesting that they have utility as a novel nonviral vector that uses nonelectronic interactions.

Recently, controlled release of DNA was also investigated as a possible method of enhancing transfection efficiency using various biomaterials such as poly (lactide-co-glycolide) (PLGA),[15] hyaluronic acid,[16] atelocollagen,[17] and gelatin.[18,19] Shea et al. reported that the sustained delivery of DNA from PLGA led to effective transfection of a large number of cells in vitro and in vivo.[15] However, it was difficult to regulate the release of DNA owing to the lack of interaction forces, such as covalent, electrostatic, and hydrogen bonding, with which DNA molecules are loaded into PLGA with polymer molecules. Tabata et al. reported enhancement and prolongation of gene expression using a cationized gelatin hydrogel interacting with DNA electrostatically.[18,19] The controlled release of DNA depended on hydrogel degradation, but the cationized gelatin hydrogel was crosslinked by glutaraldehyde, which has generally cytotoxic properties, to obtain different degrees of cationization.

In the present study, we report the preparation of a novel PVA hydrogel with DNA crosslinked physically by hydrogen bonds using UHP technology and its application to the controlled release of DNA. The goal is to develop an effective, low-cytotoxic and gene-releasable biomaterial. PVA/DNA hydrogels were obtained for various pressurization conditions, temperatures, and processing times. DNA release from the hydrogels was investigated in vitro. PVA is widely used for biomedical applications because of its biocompatibility and neutrally charged nature.[20] It is also known that PVA hydrogel is formed by physical crosslinking with hydrogen bonds when PVA solution is frozen and thawed several times, which is called the freeze–thaw method.[21]

Materials and methods

Materials

In our experiments, we used PVA samples with an average molecular weight of 74800 and a degree of saponification of 99.8%, as supplied by Kuraray (Osaka, Japan). We also used salmon sperm DNA purchased from Wako (Osaka, Japan), plasmid DNA encoding enhanced green fluorescence protein under a cytomegalovirus promoter (pEGFP-N1, BD Science, Palo Alto, CA, USA), and nucleic acid staining dye solution (Mupid Blue) obtained from Advance (Tokyo, Japan).

Preparation of PVA/DNA hydrogels by UHP

Aqueous PVA solutions of 6%, 8%, 10%, 14%, and 20% w/v were prepared by autoclaving three times for 30min at 121°C. Salmon sperm DNA was dissolved in a Tris-EDTA buffer (TE, pH = 7.8) at a concentration of 16.3mg/ml. The DNA solution was mixed with PVA solutions of 10%, 14%, and 20% w/v at a ratio of 1:1. The 0.7-ml samples were transferred in silicon tubes (9 × 25mm) with both ends capped by silicon plugs. The tubes were pressurized under various UHP conditions, using different pressures, temperatures, and durations, in a high-pressure machine (Kobe Steel, Kobe, Japan).

Confirmation of the presence of DNA in the PVA/DNA hydrogels

The presence of DNA in the PVA/DNA hydrogels produced by UHP treatment was confirmed by nucleic acid dye staining and UV-visible spectroscopy. For the former method, the PVA/DNA hydrogels were immersed in nucleic acid dye solution for 1min and then transferred to 70% ethanol. After 1min, they were immersed in ion-exchanged water for 1min. For the latter method, after the PVA/DNA hydrogels were melted at 90°C for 10min, their DNA concentration was measured by a spectrophotometer (V-560, JASC, Tokyo, Japan).

DNA release from hydrogels

The PVA/DNA hydrogels prepared by UHP were immersed in 5ml of phosphate-buffered saline (PBS) for 144h at 37°C. At 0.25, 0.5, 2, 3, 15, 27, 48, 111, and 144h, 20μl of the samples in the outer part of the PBS solution was collected and the DNA concentration was measured spectrophotometrically at 260nm (Gene Quant Pro S, Amersham, Tokyo, Japan).

Stability of plasmid DNA released from hydrogels

Plasmid DNA (pDNA) was used instead of salmon sperm DNA and the mixed solutions of pDNA (100μg/ml) and PVA (5% or 10% w/v) were treated by UHP under the conditions described above. The obtained PVA/pDNA hydrogels were immersed in PBS for 12 and 48h, and then the samples in the outer part of the solution were collected and analyzed by agarose gel electrophoresis at 100V for 45min.

Results and discussion

Aqueous solutions of PVA at concentrations ranging from 3% to 10% w/v were hydrostatically pressurized at 10000 atm at 37°C for 10min. With a PVA solution of 3% w/v, the clear solution was transformed into a turbid and viscous solution by pressurization (Fig. 1A). An aggregation of PVA particles with an average diameter of 1μm was observed in the PVA solution on scanning electron microscopy (SEM, data not shown). For PVA concentrations of more than 4% w/v, hydrogels were produced on pressuriza-

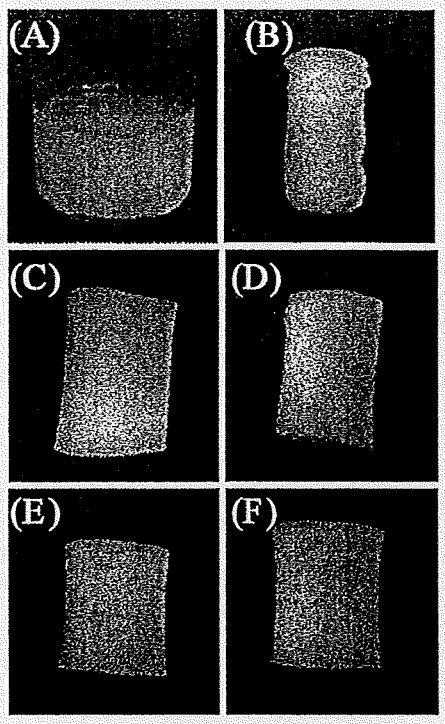

Fig. 1. Photographs of poly(vinyl alcohol) (PVA) hydrogels (**A–D**) and PVA/DNA (**E,F**) hydrogels at concentrations of **A** 3% w/v, **B** 4% w/v, **C,E** 5% w/v, and **D,F** 10% w/v obtained by ultra-high pressure treatment

tion (Fig. 1B–D). The PVA hydrogel of 4% w/v was fragile (Fig. 1B), but increasing the PVA concentration enhanced hydrogel formability, and hard hydrogels were obtained at a PVA concentration of 10% w/v (Fig. 1D). These results indicate that pressurization induced physical cross-linking of PVA molecules and that the degree of cross-linking increased as the PVA concentration increased. To investigate whether the PVA molecules were physically cross-linked by hydrogen bonding, a PVA solution of 5% w/v with urea (3.3 M), which was used as a hydrogen bond inhibitor, was treated under the above pressurizing conditions. The solution remained translucent (data not shown), indicating that the PVA hydrogel obtained by pressurization was mediated by hydrogen bonding.

The gelation of mixed solutions of DNA and PVA (5% and 10% w/v) was achieved by pressurization in the conditions described above (Fig. 1E,F). To confirm the presence of DNA in the hydrogels obtained, they were heat treated at 90°C for 10 min and then the DNA concentration of the solutions obtained was measured spectrophotometrically at 260 nm. Roughly equal amounts of DNA were contained in each hydrogel (Fig. 2A). Also, when the hydrogels were immersed in nucleic acid dye solution, which interacts electrostatically with the phosphate groups of DNA, the PVA hydrogel with DNA was stained, whereas the PVA hydrogel without DNA was not (Fig. 2B). These results indicate that a PVA hydrogel that sustains DNA (PVA/DNA hydrogel) was formed on pressurization. On the other hand,

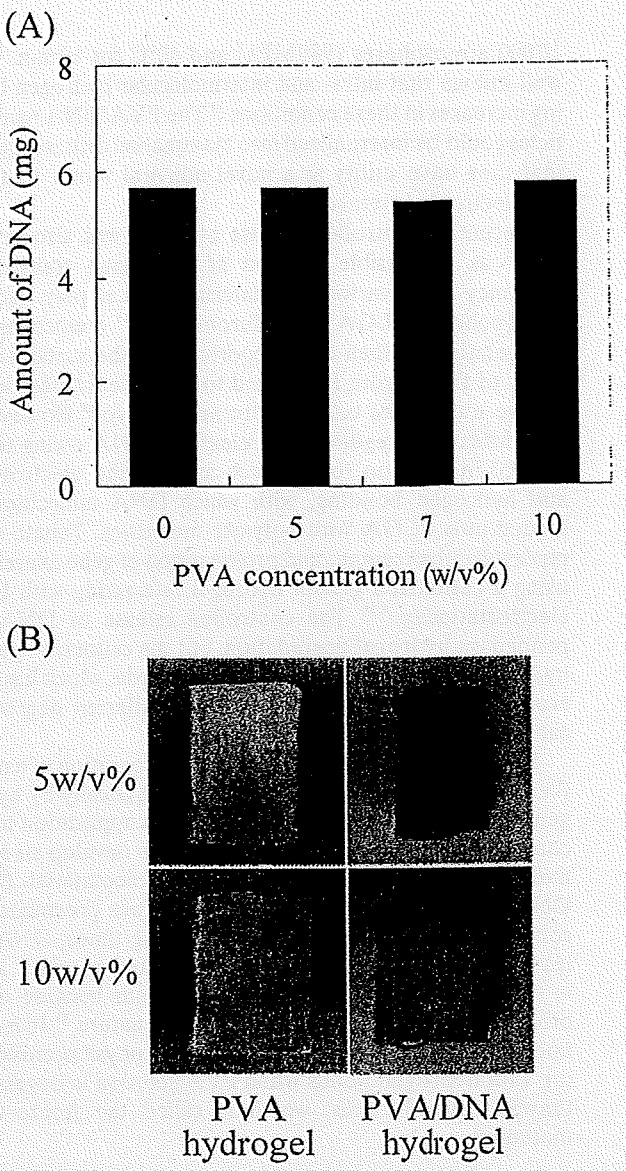

Fig. 2A,B. Presence of DNA in PVA/DNA hydrogels. **A** Amount of DNA in solution obtained by melting PVA/DNA hydrogels prepared using ultra-high pressure processing. **B** Photographs of PVA hydrogels and PVA/DNA hydrogels stained with nucleic acid dye

when urea was introduced, PVA/DNA hydrogel was not obtained on pressure treatment. This result suggests that hydrogen bonding between PVA and DNA took place in the pressurized PVA/DNA hydrogel.

DNA release from the PVA/DNA hydrogel formed by pressurization at 10000 atm at 37°C for 10 min was investigated. PVA/DNA hydrogels produced by the freeze–thaw method, a common method of forming PVA hydrogels,[21] were used as control samples. Figure 3A shows DNA release profiles from the PVA/DNA hydrogels at PVA concentrations of 5% and 10% w/v obtained by pressurization and the freeze–thaw method. Each release curve of DNA from a hydrogel consisted of a rapid phase in the initial 15 h followed by a sustained release phase. However, the amount

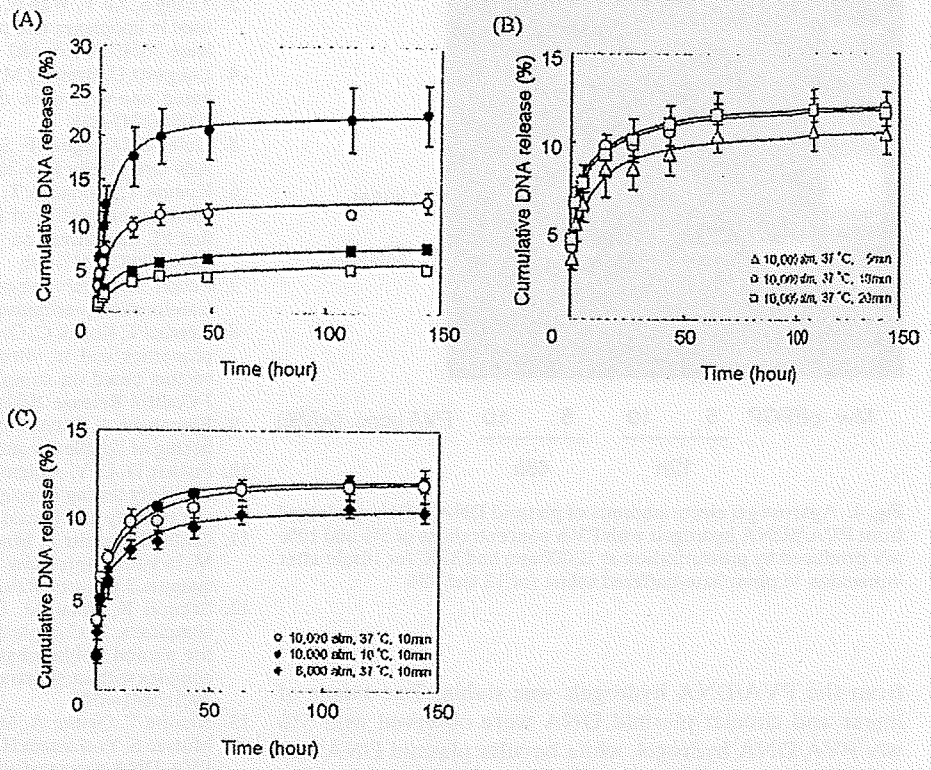

Fig. 3A–C. DNA release test from PVA/DNA hydrogels produced by pressurization under various conditions or by the freeze–thaw method. **A** Release profiles of DNA from hydrogels at PVA concentrations of 5% w/v (○, ●) and 10% w/v (□, ■) PVA concentration. *Open* and *solid* symbols indicate DNA from hydrogels obtained by pressurization (at 10000 atm and 37°C, 10 min) and the freeze–thaw method, respectively. **B** Release profiles of DNA from hydrogels of 5% w/v obtained by pressurization at 10000 atm and 37°C for 5 min (□), 10 min (○), and 20 min (□). **C** Release profiles of DNA from hydrogels of 5% w/v obtained by pressurization at 10000 atm and 37°C (○), 10000 atm and 10°C (●), and 8000 atm and 37°C (□) for 10 min

of DNA released was dependent on PVA content and on which procedure was used to prepare the hydrogels. The DNA release from the 10% w/v PVA/DNA hydrogels was lower than that from the 5% w/v PVA/DNA hydrogels, irrespective of the preparation methods. This is consistent with the fact that the 5% w/v samples were more easily stained by nucleic acid dye than the 10% w/v samples. We suppose that the increased crosslinking in the hydrogel caused by the increase in the PVA content contributed to the reduction of DNA released from the hydrogel. On the other hand, at the same PVA concentrations, DNA was more effectively released from the freeze–thaw hydrogels than from the pressurized hydrogels. Fibrous structures with large spaces (larger than 1 μm) were observed on SEM in the hydrogels made from 5% w/v PVA obtained by the freeze–thaw method, while many porous structures with diameters of 300 μm were observed in the pressurized hydrogels (data not shown). We believe that this difference in internal structure between sample types affected the interaction of PVA and DNA, resulting in the larger release of DNA from the freeze–thaw hydrogels.

To investigate the influence of the pressure conditions used to form hydrogels on DNA release, PVA/DNA hydrogels of 5% w/v were prepared by different levels of pressurization at different temperatures and for different durations. First, with pressure processing periods varying from 5 to 20 min at 10000 atm and 37°C, similar DNA release profiles were exhibited for the hydrogels obtained at pressurizing times of 10 and 20 min, but the amount of DNA released by hydrogel samples pressurized for 5 min (Fig. 3B) was less than that released by samples with longer pressurizing times. Second, the DNA release curves of the PVA/DNA hydrogel produced on pressurization at 10000 atm and 10°C for 10 min were the same as those for hydrogels produced on pressurization at 10000 atm and 37°C for 10 min. However, less DNA was released by hydrogels produced at pressures of 8000 atm and 37°C for 10 min than by hydrogels produced at 10000 atm and 37°C for 10 min (Fig. 3C). These results indicate that DNA release from pressurized hydrogels is dependent on the level and duration of pressure used in the hydrogel formation process. We previously reported that PVA gelation was promoted by increasing the pressure and by prolonging the pressurization time, by which close hydrogen bonds between PVA molecules are formed.[22] It seems that DNA was easily released from PVA/DNA hydrogels pressurized under conditions of more than 10000 atm for longer than 10 min because the hydrogen bonding interaction between PVA and DNA was more unstable than that between PVA molecules under more intense pressure conditions.

It is important for DNA to be released from hydrogels without structural change or degradation.[2,23] Plasmid DNA (pDNA), which is generally used as the DNA delivered by a nonviral vector, was used instead of salmon sperm DNA. PVA/pDNA hydrogels at PVA concentrations of 5% and 10% w/v were obtained by pressurization at 10000 atm at 37°C for 10 min and then immersed in 5 ml PBS. After 12 and 48 h of immersion, the outer part of the solution was collected and analyzed by agarose gel electrophoresis at 100 V for 30 min to investigate the stability of released pDNA from the hydrogels (Fig. 4). No degradation of DNA was observed, indicating that the plasmid DNA released

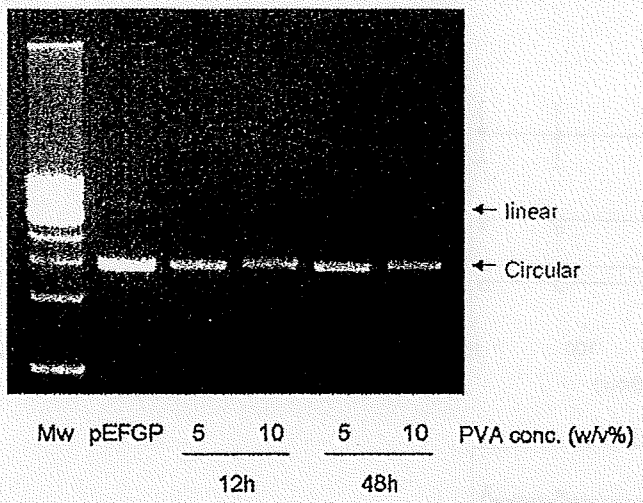

Fig. 4. Agarose gel electrophoresis of plasmid DNA (pDNA) released from PVA/pDNA hydrogels with PVA concentrations of 5% and 10% w/v produced by pressurization at 10000 atm and 37°C for 10 min after immersion in phosphate-buffered saline for 12 and 48 h

from the PVA/DNA hydrogels was stable. Two bands of linear and circular plasmid DNA were observed with 5% w/v PVA/DNA hydrogel, while circular plasmid DNA was released from the 10% w/v PVA/DNA hydrogel, indicating that the linear form of plasmid DNA tends to interact more strongly with PVA than the circular plasmid DNA.

Conclusions

Novel PVA/DNA hydrogels crosslinked physically by hydrogen bonds were developed using UHP technology. DNA released from the hydrogels was controlled by varying the PVA concentration and pressurization conditions, such as the level and duration of pressure used to form the hydrogels. The demonstrated stability of the DNA released from the hydrogels suggests that PVA/DNA hydrogels have potential as a candidate for gene delivery.

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解　説

繊維と線維(生体線維の洗浄と再生医療への展開)

Textile Fiber and Medical Fiber

澤田和也・寺田堂彦・藤里俊哉

1. はじめに

衣料用繊維素材は、植物や動物等の天然由来のものから、ナイロンやポリエステル等に代表される人工的なものまで、その種類が極めて多岐に渡っている。製造・加工技術の著しい進歩により、高機能化された繊維製品も多く登場し、我々の衣生活スタイルも大きく変化してきた。近年、繊維製品は工業・産業資材等にも広く応用化されていることは周知の通りであるが、それでも"繊維=衣料品"のイメージが強いことに変わりない。ここでは視点を少し変え、一般に殆ど認識されていない動物由来繊維の一種を紹介したい。動物由来繊維として連想できるものは、獣毛や絹糸等の体外で採取される蛋白質繊維であろう。しかし実際には、体内にも同様の線維が存在している。ここで、"繊維"と"線維"の単語を使い分けたが、その差に大きな意味は無い。慣例的に、衣料分野では"繊維"が、医療分野では"線維"が用いられている。英語に訳せば何れもfiberであり、「細くて長いもの」という定義で本質的に同じである。さて、体内に存在する線維として、最も理解し易い例として血管を挙げることが出来る。実際、繊維状の生体適合性高分子材料を用い、人工血管を造形することも多い。図1に血管の概略図を示した。血管組織を大雑把に見ると、遺伝情報を含む生物体の構成単位である細胞、それを支持している細胞外マトリックスから成る。さらに、細胞外マトリックスは、グリコサミノグリカン、プロテオグリカン、フィブロネクチン、ラミニン等の細胞接着性蛋白質と、コラーゲン及びエラスチンを主とする線維性蛋白質から構成されている。従って、血管組織から細胞や接着因子を除去すれば、最終的にはコラーゲン線維およびエラスチン線維が残ることになる。上述した生体内の線維とは、これらを意味しており、体の部位により組成は異なるが、脊椎動物の身体の構造要素の主体である。ここで紹介させて頂く線維のテーマは、これら生体内線維を再生医療で応用化しようとする研究例である。

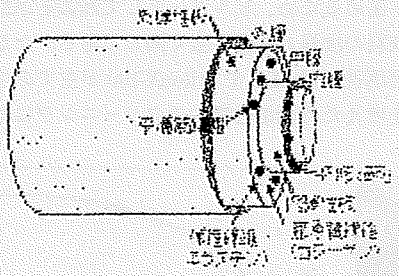
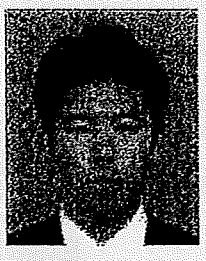


図1　血管構造の概略図

2. 研究背景

我々の体内に疾病組織が生じた場合、その回復を図る手段として最も望ましいことは、自己治癒により組織そのものを治癒化させることである。しかし、欠損もしくは機能不全に陥った組織に対しては、代替物との置換、つまり"移植"という手段も適応される。筆者らの研究チームが対象とする組織は心臓弁であるが、これについても同様である。現在、心臓弁置換の代替物には、パイロライト製の機械弁を用いるのが主である。最近では、一生の使用に耐え得る強度を有する製品も開発されている。しかし、これらの機械弁にも多くの問題が含まれている。例えば、生涯にわたる抗血栓剤の服用は、安全性や経済面において問題が残る。



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