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Adenovirus-mediated viral IL-10 gene transfer prolongs survival of xenogeneic spheroidal aggregate-cultured hepatocytes

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Abstract Xenotransplantation of hepatocytes appears to be a novel promising therapy for some forms of liver disease, and may well overcome the problem of donor shortage. We have previously reported that hepatocytes with a spheroidal shape (spheroids) are ideal for cell transplantation. The application of gene transfer techniques to this hepatocyte transplantation could possibly regulate the xenogeneic rejection reaction and, therefore, result in prolongation of the survival of the transplanted hepatocytes. In this study, we chose the adenovirus as a vector and an immunosuppressive cytokine named viral IL-10 (vIL-10) for transfection. A series of experiments was performed to elucidate the efficacy of transfection to the spheroids with adenovirus vectors and the effect of transfected vIL-10 on the survival of xenogeneic hepatocytes. We examined the cell survival quantitatively by evaluating β-galactosidase (β-gal) activity, which was transfected into the hepatocytes in the xenogeneic spleen, and semi-quantitatively by the histological findings. The results of in-vitro studies identified an efficient expression of the β-gal gene within the spheroids infected with Ad-CMVLacZ (LacZ-encoding adenovirus vector with CMV promotor) and the presence of BCRF1 mRNA within the spheroids transfected with AdCMVvIL-10 (vIL-10-expressing adenovirus vector with CMV promotor) under the condition of 1 MOI, for 1 h. Xenogeneic hepatocytes with a spheroidal shape showed comparable survival to syngeneic hepatocytes for up to 4 days after transplantation with co-transplantation of the vIL-10-transfected hepatocytes. From this study, we concluded that adenovirus-mediated vIL-10 gene transfer prolongs the survival of xenogeneic hepatocyte spheroids. Furthermore, spheroids possess ideal properties for gene transfection, as well as cell transplantation.

Key words Hepatocyte spheroid · Xenotransplantation · Gene engineering · Viral IL-10

Abbreviations *CMV* cytomegalovirus. *ELISA* enzyme-linked immunosorbant assay. *H-E* Hematoxyline and Eosin. *HRF20* homologous restriction factor 20. *MHC* major histocompatibility complex. *MOI* multiplicities of infection. *pfu* plaque-forming unit. *PMSF* phenylmethylsurfonylfluoride. *PBS* phosphate-buffered saline. *RT-PCR* reverse transcriptase-polymerase chain reaction. *Th1* helper 1 – T cell. *UW* university of Wisconsion. *vIL-10* viral interleukin-10

Introduction

Hepatocyte transplantation is an attractive modality for treating acute liver failure or hepatic enzyme deficiency [1]. We have previously reported that syngeneic hepatocytes with a unique spheroidal shape (spheroids) show longer and greater survival than primarily isolated hepa-

tocytes [2, 3]. Furthermore, xenogeneic spheroids escape from immediate rejection and survive for a few days after transplantation [4]. However, little is known about the immune response of the xenogeneic host against transplanted hepatocytes [5]. It would be necessary to control the xenogeneic rejection reaction if this cell transplantation method is to be applied to clinical use because of the limitation of the allogeneic donor.

According to previous reports concerning immunosuppression, the rejection of xenogeneic hepatocytes may well involve a cellular response, since conventional immunosuppressive agents such as cyclosporin A and FK506 can prolong survival [6–8]. In spite of this, systemic administration of such immunosuppressive agents can cause general adverse effects. Instead of the current immunosuppressive method, we aimed at local immunosuppression by transfecting some genes that produced a protein that had an immunosuppressive property. Such a candidate is BCRF1 gene product viral IL-10 (vIL-10). VIL-10 is a secreted 17-kD protein homologous to human and murine IL-10 and sharing primarily those properties that down-regulate the function of antigen-presenting cells (APC) and Th1 activation [9–12]. On the other hand, it has been reported to show various ways of transfection. The adenovirus vector can be purified and highly concentrated in large quantities. In addition, this vector can transduce genes into nondividing cells and achieve highly efficient transfection. The adenovirus vector is rendered replication-defective by deleting the E1a and E1b sequences, making recombinant adenovirus a suitable vector for gene therapy [13]. Therefore, in this study, we selected the adenovirus vector for gene transfer into the hepatocyte spheroids.

The purpose of this study was to achieve local immunosuppression around transplanted cells using the gene transfer technique. We examined the following: (1) efficacy of gene transfection using the adenovirus vector in vitro; (2) detection of the presence of vIL-10 mRNA expression within the spheroids; (3) prolongation of survival of xenogeneic spheroids with co-transplantation of the hepatocytes expressing the vIL-10 gene.

Materials and methods

Animals

We used 6- to 8-week old male WKA rats weighing 200 ~ 250 g as hepatocyte donors; 8- to 10-week old male WKA rats weighing 250 ~ 300 g and 10- to 12-week old male hamsters weighing 150 ~ 200 g served as recipients.

Virus vectors

The adenovirus vectors, AdCMVLacZ, AdCMVvIL-10 and DL312 were used for this study. AdCMVLacZ contains an expression cassette encoding the *Escherichia coli* LacZ gene, under control of the human cytomegalovirus (CMV) promotor, and a polyadenylation signal in exchange for the deleted E1a/b region. AdCMVvIL-10 contains an expression cassette encoding the CMV promotor, Epstein-Barr virus BCRF1 cDNA and a polyadenylation signal. AdCMVvIL-10 was provided by Dr. H. Tahara (University of Pittsburgh). The vector DL312 is similarly deleted of E1a/b, but does not have an insertion of foreign DNA. All vectors were adjusted to 1×10^8 pfu/ml.

Isolation of hepatocytes

The hepatocytes were isolated from WKA rats by a modified technique of the two-step collagenase perfusion originally described by Berry and Friend and adapted by Seglen [14, 15]. The yield of hepatocytes was $2–3 \times 10^8$ per liver, and the viability ranged from 85 to 90%, as assessed by the trypan blue exclusion test.

Hepatocyte culture

The basic culture medium was Williams'E with penicillin added at 100 units/ml and streptomycin, at 100 μg/ml. This medium was supplemented with a defined mixture of hormones, growth factors and trace elements to produce HDM (hormonally defined medium). Spheroid cultures were initiated by seeding a total of 3×10^6 isolated hepatocytes in 8 ml of HDM onto 100-mm hydrophobic dishes. The cultures were kept at 37 °C in a humidified 95% O_2-5% CO_2 mixture incubator.

Transfection of hepatocytes and formation of spheroids

At 8 h after the start of spheroid culture, the adenovirus vectors, AdCMVLacZ, AdCMVvIL-10 and DL312 were added to the culture medium under the condition of 1 MOI (multiplicities of infection; virus : cell ratio = 1 : 1). Infected hepatocytes were kept at 37 °C for 1 h. The culture medium was then changed to HDM medium containing no adenovirus vectors. The hepatocytes aggregate, form spheroidal shapes and float in the medium during the 48-h culture period after the initial infection of the vector. The spheroids thus formed were collected and then were used for further experiments.

Transplantation of spheroids

The spleen of the recipient was exteriorized through a left subcostal incision under ether anaesthesia. The spheroids were suspended in 0.2 ml HBSS (Hanks' balanced salt solution) and injected directly into the spleen of the recipient using a 1-ml syringe with a 23G needle. Temporary clamping of the splenic vessels at the hilum was used to prevent the spheroids from running off into the portal vein. Haemostasis was obtain by ligation at the site of injection.

X-gal staining of spheroids

The AdCMVLacZ-infected spheroids were fixed in 2% formaldehyde/0.2% glutaraldehyde solution for 10 min, washed in phosphate-buffered saline (PBS) three times, and incubated in the solution containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 5 m*M* potassium ferricyanide, 5 m*M* potassium ferrocyanide and 2 m*M* $MgCl_2$ in PBS at 37 °C for 8 h.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Successful gene transfer was determined by the presence of BCRF1 mRNA within the spheroids using RT-PCR. RNA was extracted using a standard RNAzol method [16]. cDNA was obtained by random primer reverse transcription of RNA and amplified by PCR using two primers specific for the vIL-10 gene, 5' - -ATG-GAGCGAAGGTTAGTGGTC - 3' (sense) and 5' - -ACT-CTTGTTCTCACACGGCAG - 3' (anti sense), which yield a 387-bp fragment. The PCR cycles were 96 °C for 15 s, 60 °C for 30 s and 72 °C for 2 min. After 30 cycles, the PCR products were run on 1 % agarose gels. The positive control was AdCMVvIL-10-producing 293 cells and the negative control was AdCMVLacZ-infected spheroids.

β-galactosidase levels in spleen

The enzyme-linked immunosorbant assay (ELISA; 5 prime → 3 prime inc.) was performed to determine β-galactosidase (β-gal) levels within transplanted tissue at 0 and 12 h, and at 1, 2, 3, 4, 5, 6 and 7 days after transplantation. The spleen was removed and homogenized, and 3 ml of cold 0.25 *M* Tris-Cl, pH 7.8, containing 1.0 m*M* phenylmethylsufonyl-fluoride (PMSF) per gram of tissue was added. Tissue was homogenized at 4 °C until it was disrupted as much as possible, and centrifuged at 7500 rpm/s at 4 °C for 30 min. The supernatant was recovered and quantitation of *E. coli* β-gal protein was determined by double-antibody ELISA.

Serum VIL-10 levels

Recipients were sacrificed at 12 h and at 1, 3, 5, and 7 days after transplantation. For the determination of serum vIL-10 levels, blood samples were collected from the portal vein and tail vein. Serum vIL-10 levels were quantitated by double-antibody ELISA (Pharmingen, San Diego, Calif.).

X-gal staining of spleen

Recipients were sacrificed for histological studies at 12 h and at 1, 2, 3, 4, 5, 6 and 7 days after transplantation. The removed spleens were quickly frozen and 5 μm thin sections were fixed in a 2% formaldehyde/0.2% glutalaldehyde solution for 10 min, washed three times in PBS and stained by X-gal solution for 8 h.

H & E staining

Splenic specimens were fixed in 10% formalin and stained by H & E (hematoxylin and eosin).

Statistics

Statistical significance was analysed using Student's *t*-test.

Experimental design

Animals were divided into four groups; group 1 comprised AdCMVLacZ-transfected spheroid transplantation (LacZ-STx, 15×10^6 hepatocytes) + non-transfected STx (15×10^6 hepatocytes) in the syngeneic combination ($n = 5$); group 2 comprised LacZ-STx + non-transfected STx in the xenogeneic combination ($n = 5$); group 3 comprised LacZ-STx + AdCMVvIL-10-transfected STx (15×10^6 hepatocytes) in the xenogeneic combination ($n = 5$); group 4 comprised LacZ STx + DL312-transfected STx (15×10^6 hepatocytes) in the xenogeneic combination ($n = 5$).

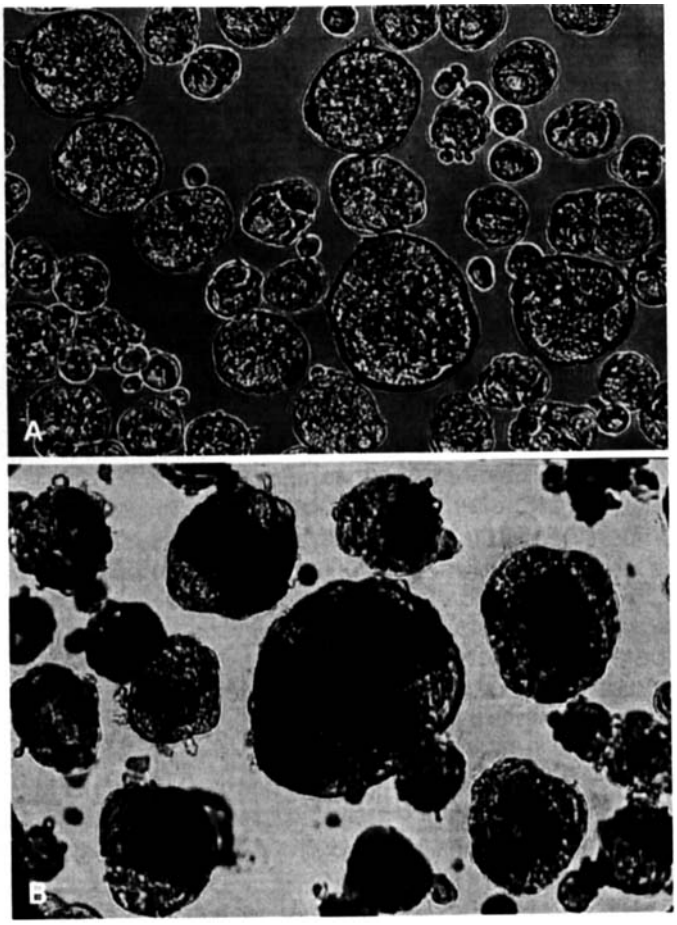

Fig. 1 X-gal staining of spheroidal aggregate hepatocytes (spheroids) (× 400). **(A)** Non-infected: no cells stained blue for β-gal. **(B)** AdCMVLacZ-infected spheroids: the expression of β-gal was observed in 65–70 % of the hepatocytes in the spheroids

Results

Expression of β-gal gene in spheroids

According to a preliminary study, the optimal infection condition of adenovirus vectors was set at 1 MOI for 1 h. In order to examine the efficacy of gene transfection, the number of X-gal-stained hepatocytes was counted. The expression of β-gal was observed in 65–70 % of the transfected hepatocytes in the spheroids (Fig. 1 B). In contrast, control spheroids were not stained (Fig. 1 A). On the other hand, the expression rate of β-gal was very low (10–15 %) in the primarily isolated hepatocytes (data not shown).

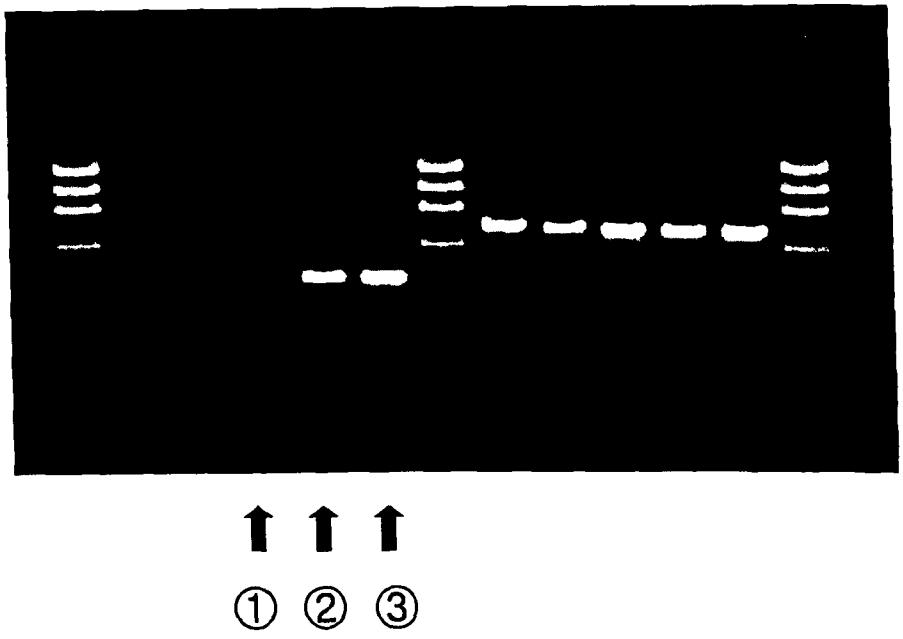

Fig. 2 Detection of vIL-10 gene expression in spheroids transfected with AdCMVvIL-10 by RT-PCR. Lane 1, AdCMVLacZ-infected spheroids (negative control); lane 2, AdCMVvIL-10-transfected spheroids; lane 3, AdCMVvIL-10-infected 293 cells (positive control). vIL-10 mRNA was detected in AdCMVvIL-10-transfected spheroids

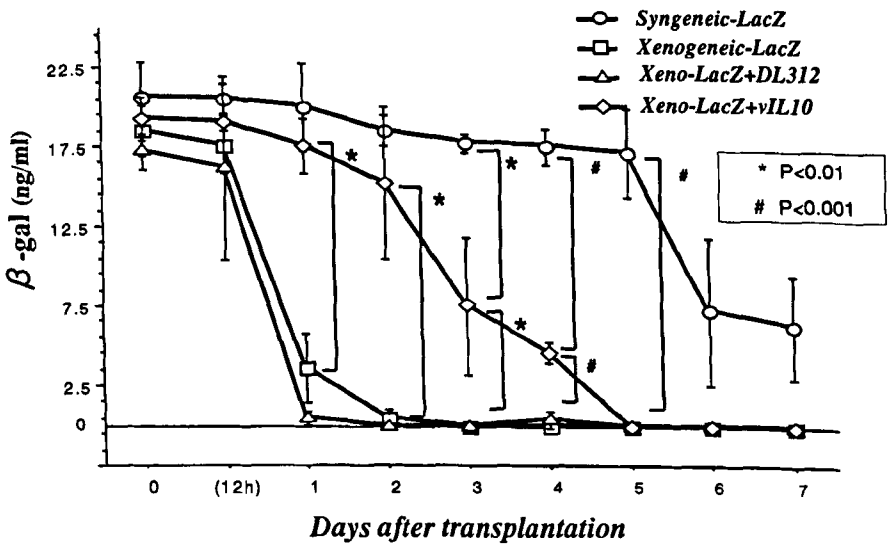

Fig. 3 Quantity of β-galactidase (β-gal) in the spleen after LacZ-transfected spheroids transplantation (LacZ-STx) by ELISA. In syngeneic-LacZ group, the quantity of β-gal showed a continuous peak up to 5 days after LacZ-STX, then reached the baseline by day 7. In the xenogeneic-LacZ and DL312 groups, the quantity of β-gal abruptly declined at 1 day after LacZ-STx. In contrast, in the xenogeneic vIL-10 group, β-gal expression lasted up to 4 days after LacZ-Stx

Expression of vIL-10 gene in spheroids

Successful vIL-10 transfection of the spheroids was confirmed by RT-PCR. The detection of the vIL-10 gene was manifested by a 500-bp sequence (Fig. 2). The vIL-10 gene expression was detectable in the spheroids transfected under the condition of 1 MOI for 1 h with ADCMVvIL-10. No vIL-10 mRNA was detected in AdCMVLacZ-infected spheroids.

Quantity of β-gal in spleen after transplantation of spheroids (Fig. 3)

Syngeneic rats showed a continuous peak in β-gal levels in the spleen from days 1 to 5 after transplantation, then they gradually reached baseline by day 7. On the 1st day after transplantation, the quantity of β-gal in the spleen declined abruptly in groups 2 and 4. Xenogeneic rats with vIL-10-transfected spheroids exhibited a high quantity of β-gal comparable to that in the syngeneic group up to 2 days after transplantation, and, finally β-gal expression lasted up to 4 days after transplantation.

Serum vIL-10 after transplantation

Serum vIL-10 levels were undetectable in the blood samples from the portal vein or tail vein throughout the experiment (data not shown).

Histological findings of transplanted spheroids (Figs. 4–6)

In the syngeneic group, substantial numbers of the X-gal-stained hepatocytes were observed as clusters within the splenic parenchyma for 3 days after transplantation. A few X-gal-positive hepatocytes were seen in groups 2 and 4 during the 1st day after transplantation, but then disappeared rapidly. On the other hand, a number of X-gal-stained hepatocytes comparable to that of the syngeneic group, was observed in xenografts with vIL-10 transfection at 3 days after transplantation.

Discussion

In the past decade interest has been raised in hepatocyte transplantation as a potential treatment for a variety of liver diseases [1]. Transplantation of hepatocytes in a syngeneic combination has been reported to improve the survival rate in rats with acute liver failure [17], and to correct hepatic encephalopathy [18]. Attempting to apply this method to a clinical situation, there is little possibility to obtain syngeneic donor hepatocytes. Donor shortage limits the successes of using allogeneic hepatocytes. To solve this problem, a xenogeneic donor source should be considered. Fortunately, it has been reported that transplanted cells between species do not suffer from hyperacute rejection, which is inevitable in vascularized organ transplantation in a xenogeneic combination [19]. We might, therefore, overcome the xenogeneic rejection reaction in cell transplantation by using a similar technique to control the rejection of the allografts.

Gunsalus et al. have reported that porcine hepatocytes transplanted into Watanabe rabbits with inherited hyperlipidemia can effectively reduce serum cholesterol levels by using cyclosporin A [6]. FK506 has also been reported to prolong the survival of xenogeneic hepatocytes transplantated into Nagase's analbuminemic rats [7, 8]. On the other hand, Hayashi et al. have shown that xenogeneic hepatocytes transfected with homologous restriction factor (HRF20, CD59) escape complement-dependent cytolysis by human serum [20]. The role of complement in the xenogeneic hyperacute reaction is well known, but it has been suggested that rejection of transplanted cells might be related to a complement-dependent reaction. Taking this possibility into consideration, the main target for the control of the xenogeneic rejection in hepatocyte transplantation should be a cellular immunity.

The concept of local immunosuppression aiming at local delivery of an immunosuppressive agent to the site of the transplanted organs or cells is attractive, since systemic immunosuppression causes several adverse effects that are usually experienced in the clinical setting. Previous attempts to achieve this local immunosuppression by using micro-osmolar pumps have been reported.

Recently, an immunosuppressive cytokine called IL-10 has attracted attention, because of its broad action of immunosuppression [9, 10]. Viral IL-10, a product encoded by Epstein-Barr virus BCRF1 open reading frame, is homologous to both murine and human IL-10, especially in the coding region of mature protein sequence. Viral IL-10 shares many biological properties with murine and human IL-10, including inhibition of inflammatory cytokine synthesis and antigen-presenting cell, and down-regulation of MHC class II antigen on the monocytes [11, 12]. Viral IL-10, however, lacks T-cell co-stimulatory activity that authentic cellular IL-10 possesses [21]. This makes vIL-10 a more potent candidate for a biological immunosuppressive agent to control xenogeneic cellular rejection.

In this study, we investigated the effect of genetically transfected vIL-10 on the survival of transplanted xenogeneic hepatocytes in the spleen. Syngeneic rats showed a continuous peak in β-gal levels in the spleen from days 1 to 5 after transplantation, which gradually reached baseline by day 7. In comparison, 1 day after transplantation, the quantity of β-gal in the spleen had abruptly declined in the xenogeneic combination without any immunosuppression. Xenogeneic rats with vIL-10-transfected hepatocytes exhibited a high quantity of β-gal comparable to that in the syngeneic group up to 4 days after transplantation. These results indicated that the survival of the transplanted heptocytes was prolonged up to 4 days after transplantation with the co-transplantation of vIL-10-transfected hepatocytes. It is suggested that prolonged survival of the hepatocytes in the xenogeneic host might be attributed to a local immunosuppressive effect induced by continuous secretion of vIL-10 from vIL-10-transfected hepatocytes. In all recipients, serum vIL-10 was not detected throughout the experiment, which might sustain the concept of local immunosuppression. Xenogeneic hepatocytes, however, were almost all rejected within 4 days even under local immunosuppression with vIL-10. These findings indicated that other various mechanisms, including humoral factors, might be involved in the rejection reaction in xenogeneic cell transplantation.

Hepatocytes isolated from newborn rats have been reported to form a spheroidally aggregated shape [22]. Koide et al. have reported that adult rat hepatocytes also form spheroids in primary culture with hormonally defined medium containing some growth factors, hor-

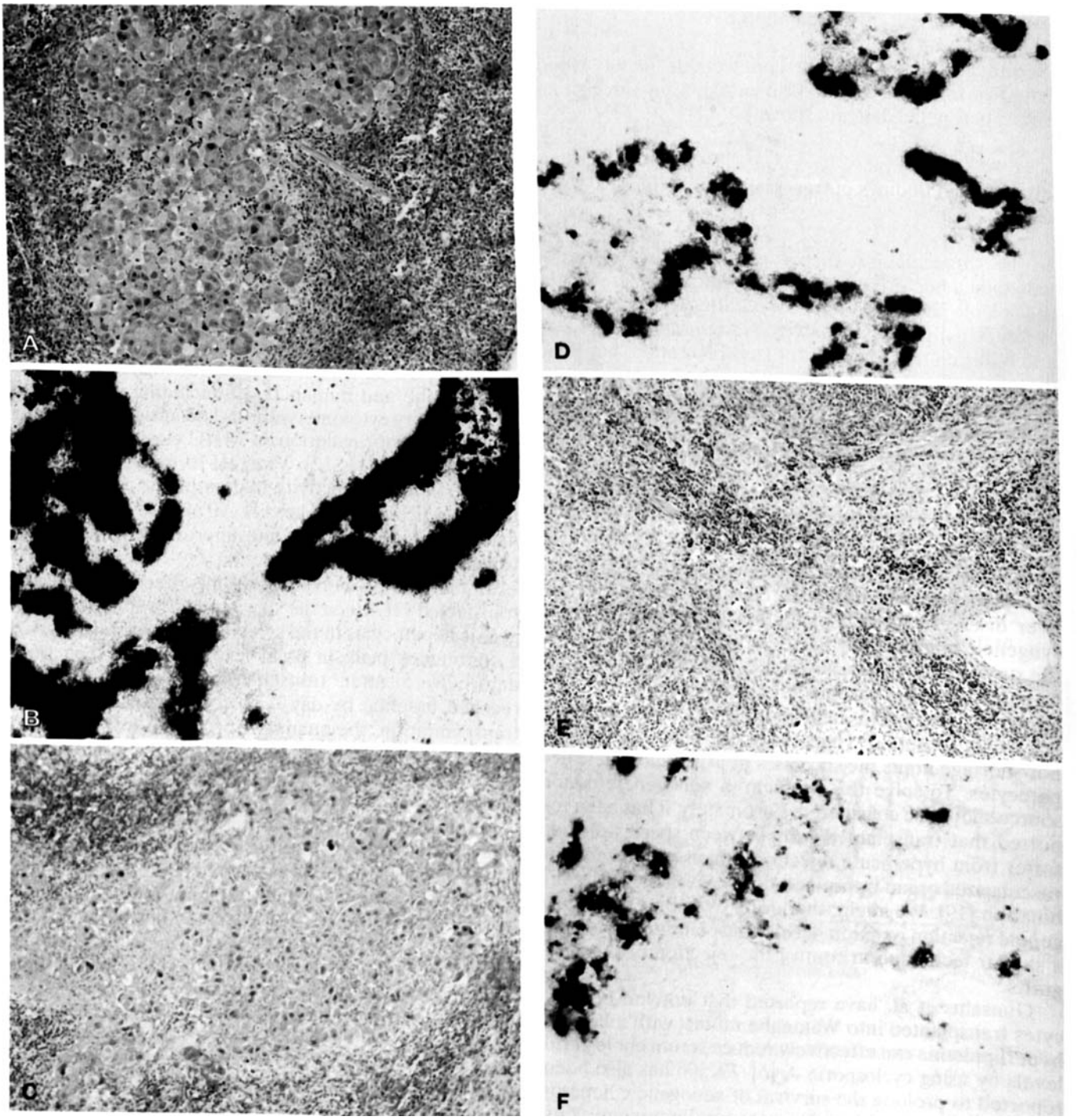

Fig. 4 Histological findings of the spleen after LacZ-STx in a syngeneic combination (× 100). **(A)** 1 day (H-E). **(B)** 1 day (X-gal). **(C)** 3 days (H-E). **(D)** 3 days (X-gal). **(E)** 7 days (H-E). **(F)** 7 days (X-gal). Substantial numbers of the X-gal-stained hepatocytes were observed as clusters within splenic parenchyma at 1 and 3 days after STx

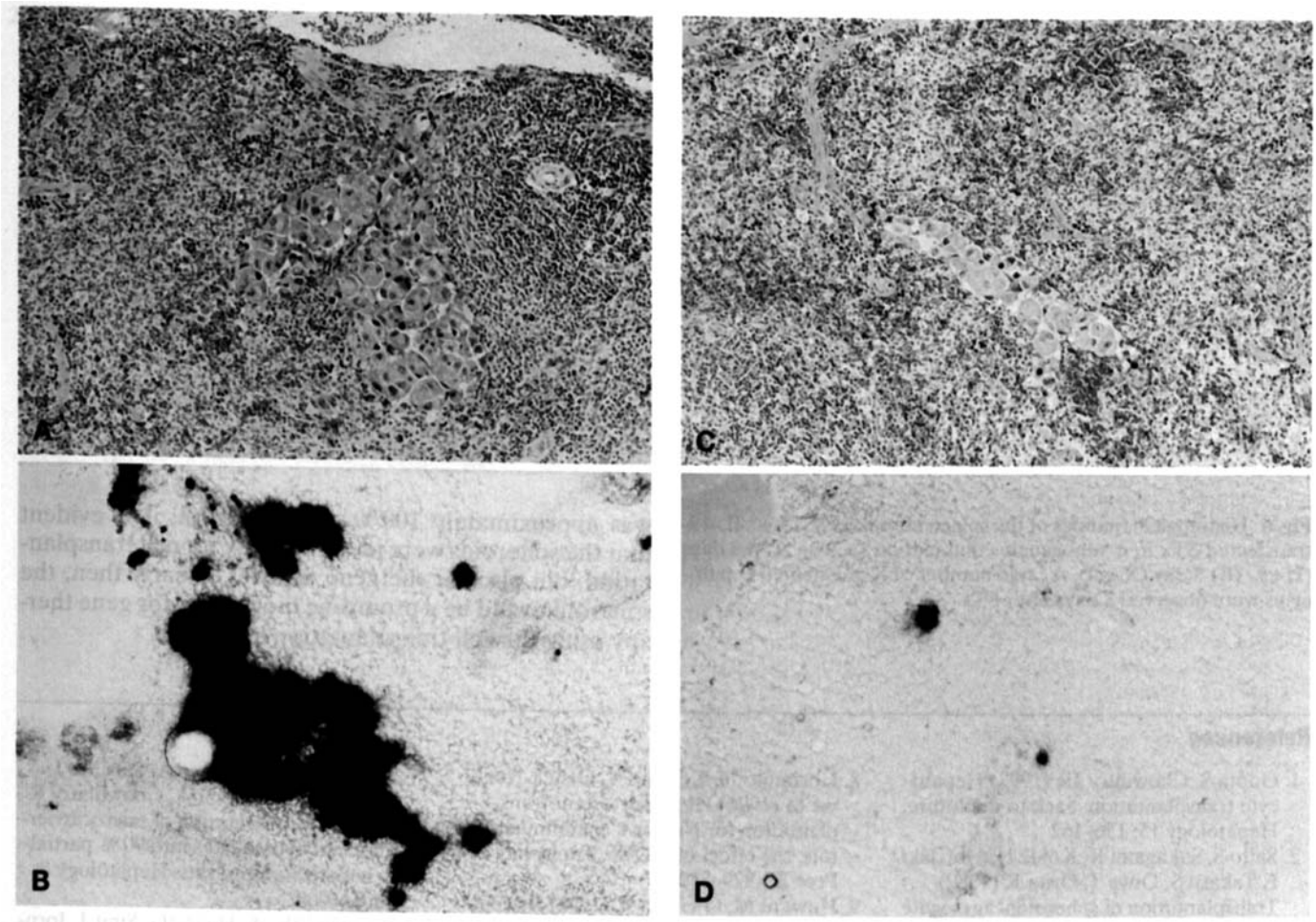

Fig. 5 Histological findings of the spleen after LacZ-STx in a xenogeneic combination (×200). **(A)** 12 h (H-E). **(B)** 12 h (X-gal). **(C)** 1 day (H-E). **(D)** 1 day (X-gal). A few X-gal-positive hepatocytes were seen at 1 day after STx

mones and trace elements, but no serum [23]. These hepatocytes with a spheroidal shape (spheroids) preserved more liver-specific function in vitro than hepatocytes with a conventional shape [24]. We have transplanted these hepatic spheroids into syngeneic rat spleen, and have revealed that a greater number of hepatocytes survive in rats receiving spheroids than in those transplanted with primarily isolated hepatocytes [2, 3]. These advantages of the spheroids are thought to be attributed to their size and property for attachment in the spleen. The spheroids consist of 4–5 layers of hepatocytes and their size is about 5–10 times that of single hepatocytes. Primarily isolated hepatocytes are reported to easily surge from the spleen to the portal vein after direct injection in spite of temporally clumping in the splenic hilum. Spheroids are thought to be of such a size that they remain in the hepatic parenchyma. It is also suggested that the deposition of the extracellular matrix covering the spheroids might play some role in interacting with the fiber network in the spleen. In addition to these favourable properties for transplantation, the spheroids showed another advantage in gene transfer with an adenovirus vector in this experiment.

Csete et al. have reported that successful gene transfer to freshly isolated hepatocytes can be achieved during cold storage in UW solution using an adenovirus vector [25]. This procedure, however, requires long-term cold preservation before transplantation and, moreover, shows insufficient efficacy for gene transfection compared with that in cultured hepatocytes with conventional monolayer cultivation. Conventional primary culture of isolated hepatocytes is suitable for gene transfer with an adenovirus vector because the hepatocytes have a broad and flat surface facilitating efficient contact with the adenovirus [26], but it would be difficult to obtain free-floating hepatocytes from these hepatocytes spread on the dishes. For the spheroid culture, hepatocytes spontaneously aggregate after initiation of conventional monolayer culture. In our study, hepatocytes were infected with an adenovirus vector for 1 h at the beginning of this primary monolayer culture, and efficacy of gene transfection with this method

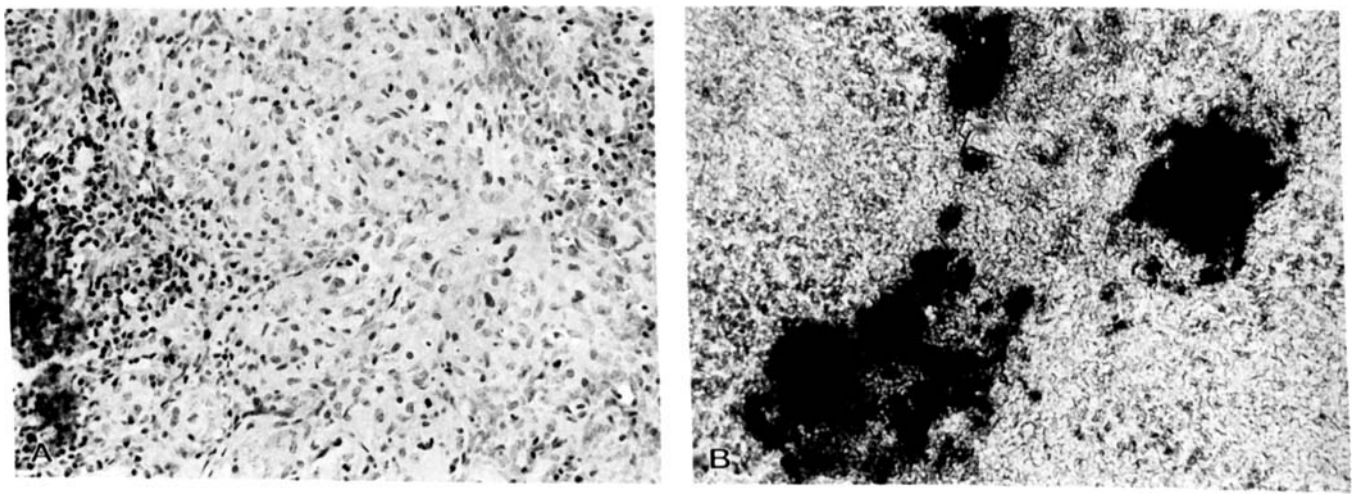

Fig. 6 Histological findings of the spleen after LacZ-STx + vIL-10-transfected-STx in a xenogeneic combination (×200). **(A)** 3 days (H-e). **(B)** 3 day (X-gal). A large number of X-gal-stained hepatocytes were observed 3 days after STx

was approximately 100%. In conclusion, it is evident that the spheroids were ideal, not only for cell transplantation, but also for the gene transfer. Clearly then, the spheroid would be a promising bioparticle for gene therapy using the cell transplantation technique.

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