Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine significance of the lacZ-positive cells number and the marker protein produced in the medium using StatView 5.0 software (SAS Institute, Cary, NC). If a probability value of $p < 0.05$ was obtained, the Tukey test was then used for comparison for each group with the appropriate control. Values throughout the present study are given as a mean ± SD. Statistical differences in the value of hAAT serum were determined by Student's *t*-test. A probability value of $p < 0.05$ was considered statistically significant.

RESULTS

Establishment of Adenoviral-Mediated Gene Transduction Methods for Mouse Hepatocytes in Suspension

To determine the optimal conditions for adenoviral-mediated gene transduction into the isolated mouse hepatocytes, we altered the hepatocyte suspension medium as well as the incubation time with the adenoviral vectors. Hepatocytes were resuspended with either UW solution, DMEM, or DMEM with 10% FBS and infected with Ad-CA-lacZ for different incubation periods (from 4 min to 6 h) at a MOI of 1 at 4°C.

As shown in Figure 1A, hepatocytes suspended with UW solution demonstrated significant higher transduction efficiency than the other solutions, particularly during the shorter incubation periods from 4 min up to 1 h. Over 80% of transduction efficiency (83.2 ± 3.6%) could be achieved by using UW solution for 1-h infection. Viability and maintenance of the differentiated status of the Ad vector-treated hepatocytes were then determined by the hAAT and albumin production levels into the culture medium, respectively. No significant difference in the hAAT and albumin protein levels were detected in the culture medium, but the primary hepatocytes incubated in the UW solution had significantly higher hAAT and albumin at the longer incubation periods (i.e., 3 and 6 h) compared to the other two suspension media (Fig. 1B, C). For these reasons, we used the UW solution in all of the subsequent experiments.

Increasing doses of adenoviral vector were tested to determine the optimal amount necessary for efficient hepatocyte transduction using UW solution and the 1-h incubation period. As shown in Figure 2A and B, the hepatocytes showed a dose-dependent increase in the adenoviral transduction efficiency, reaching over 80% at MOI of 1. Because Ad vectors are known to produce cytotoxicity at higher dose of vector infection, we determined cell viability by hAAT production and maintenance of differentiated function by albumin production in the culture medium. Cell viability as well as function was stably maintained at MOIs between 0.04 and 1. Both the hAAT and albumin levels significantly decreased ($p < 0.01$) using adenoviral vector doses at MOIs higher than 5 or 25 (Fig. 2C, D). From these data, we have optimized efficient adenoviral-mediated transduction method using a MOI of 1 for an incubation period of 1 h in the UW solution as a cell suspension medium at 4°C. This optimized condition was used in the subsequent hepatocyte transplantation experiments.

Transplantation of Adenoviral Vector-Treated Hepatocytes

To determine the feasibility of the adenoviral vector-treated hepatocytes for cell-based therapies, we transplanted Ad-CA-lacZ-treated hepatocytes into the liver through the portal vein or into an ectopic site under the kidney capsule. At both transplantation sites, the transplanted hepatocytes genetically modified in a suspension as well as standard culture conditions with Ad-CA-lacZ were found to strongly expressed β-gal protein (Fig. 3A, C, E and Fig. 4A, C, and E) and demonstrated that the transduced gene was functionally transcribed and translated after transplantation in vivo. However, the survival characteristics of the hepatocytes differed depending on the site of transplantation. Under the kidney capsule, the Ad vector-treated hepatocytes were capable of surviving on a long-term basis regardless of the vector infection method (Fig. 4A–F). As measured by serum hAAT levels of mice following hepatocyte transplantation into the liver, cells infected in suspension demonstrated significantly higher survival than those infected in conventional adherent culture (Fig. 3I). We then determined the number of engrafted donor hepatocytes (hAAT-positive hepatocytes) and Ad infected hepatocytes (X-gal-positive hepatocytes) by counting 20,000 hepatocytes within the recipient liver at day 7. As shown in the Figure 3J, the number of X-gal-positive hepatocytes (82.8 ± 16.5) accounted for 81.3 ± 4.7% of the number of donor hepatocytes (101.6 ± 21.5), showing consistent percentage (83.2 ± 3.6%) of X-gal-positive hepatocytes used for transplantation. These data clearly demonstrated that Ad infection process itself did not affect on the engraftment rate in the liver.

The lower survival efficiency of the cultured hepatocytes in the liver is likely due to the difficulty of the hepatocytes to traverse from the portal vein into the liver plates via the sinusoidal pores. This would result in the cellular death as evidenced by our findings that nearly all of the cultured hepatocytes were aggregated in the portal pedicles at the day 2 time point, and that these hepatocytes had been eliminated at day 7 as evidenced by histological examination (Fig. 3E–H).

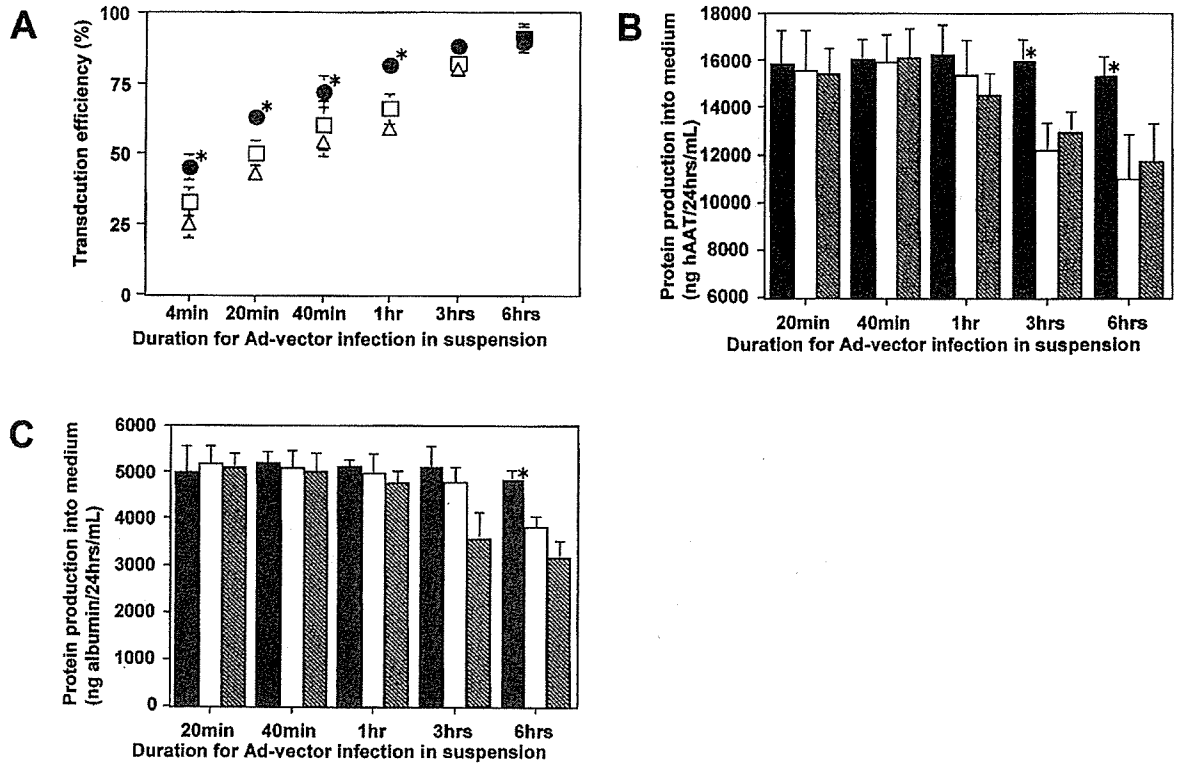

Figure 1. Optimization of Ad vector-mediated transduction into primary mouse hepatocytes in vitro. Hepatocytes in suspension with different medium (UW, DMEM with 10% FBS, DMEM) were infected with Ad-CA-lacZ at a MOI of 1 for different incubation times at 4°C. After the Ad vector incubation step, the hepatocytes were washed extensively and cultured for an additional 32 h on Primaria culture dishes. (A) Transduction efficiency was determined as a percentage by counting the X-gal-positive hepatocytes relative to the total number of hepatocytes. Filled circles: UW solution; open triangles: DMEM with 10% FBS; open squares: DMEM. Values are expressed as mean ± SD. (B) Viability of Ad vector-treated hepatocytes as determined by hAAT protein secretion into the culture medium. (C) Maintenance of the differentiated status of the Ad vector-treated hepatocytes as determined by the albumin production into the culture medium. Filled columns: UW solution; open columns: DMEM with 10% FBS; striped columns: DMEM. $*p < 0.05$ versus the other two groups ($n = 4$).

Ad-CA-lacZ-treated hepatocytes under kidney capsule were able to express the transduced marker gene (lacZ) as well as sustain the production of the serum marker protein (hAAT) for 7 days after the transplantation procedure in both suspension condition and culture conditions (Fig. 4A–D, G). In order to observe long-term survival of vector-treated hepatocytes in vivo, we infected hepatocytes with Ad-null or a mock (vehicle) solution under the suspension condition. Both groups showed stable survival under the kidney capsule for 26 weeks without showing intergroup differences (Fig. 4H). Hepatocytes infected in suspension were able to express the transduced marker gene (lacZ) under the kidney capsule for 21 days after the transplantation (Fig. 4E–F). No lacZ-positive cells were detected in other extra-renal organs, including liver, lung, intestine, and spleen.

Efficient Adenoviral Vector-Mediated Transduction of Isolated Primary Hepatocytes From Different Species

To determine if the established approach for the adenoviral-mediated gene transduction to primary hepatocytes is not specific to murine cells, similar experiments were tested in primary hepatocytes isolated from canine, porcine, and human liver. Hepatocytes were infected with Ad-CA-lacZ at increasing MOIs ranging from 0 to 100 for 1 h while cells are in suspended in UW solution. As shown in the Figure 5A and B, more than 80% transduction efficiency could be achieved at MOI of 1 at hepatocytes of any species. Among hepatocytes from four different species, human hepatocytes showed the highest transduction efficiency at MOI of 1 (94.5 ± 3.3%). Assessment of cellular function of the vector-treated hu-

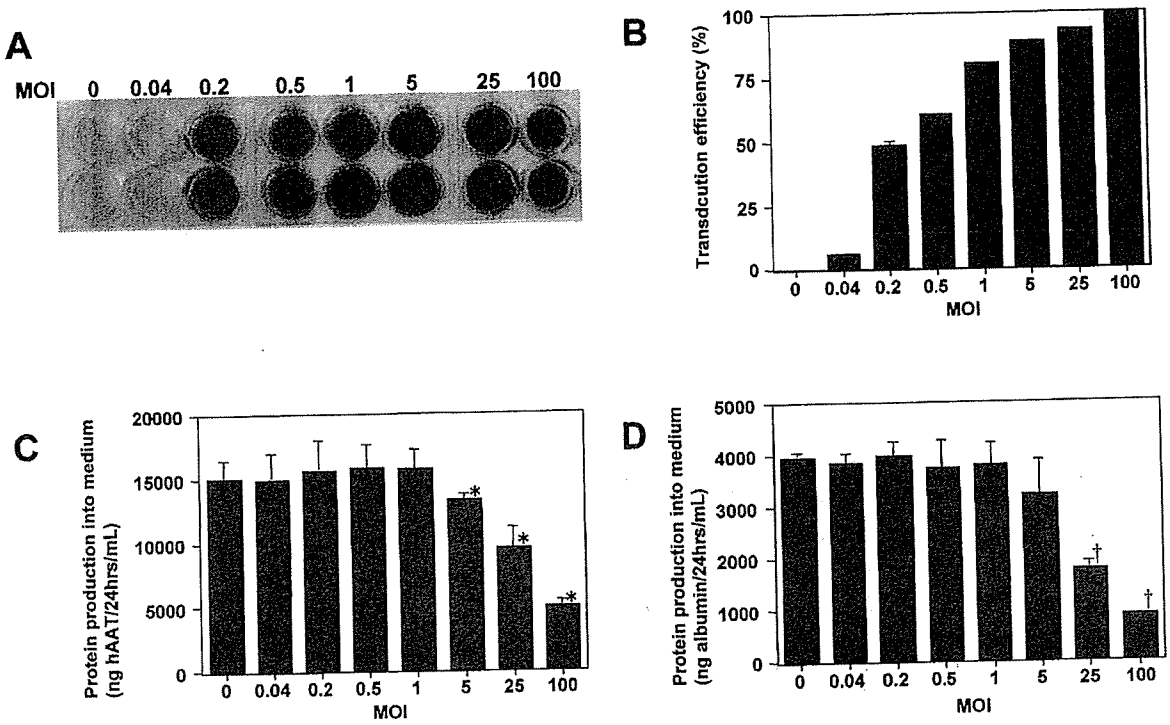

Figure 2. Efficient transduction using Ad vectors in a suspension solution of isolated hepatocytes. β-Galactosidase expression in hepatocytes following Ad vector-mediated transduction at increasing MOI from 0 to 100. Isolated hepatocytes were infected with Ad-CA-lacZ in suspension using UW solution at 4°C. After the Ad vector infection, cells were washed extensively and cultured for an additional 32 h on Primaria culture dishes with DMEM-medium. (A) Gross morphological appearance of X-gal-stained hepatocytes infected with Ad-CA-lacZ. Note that there was a dose-dependent increase in X-gal-positive cells from hepatocytes infected with a MOI of 0.04 to 1. (B) Transduction efficiency as determined as a percentage by counting the X-gal-positive hepatocytes relative to the total number of hepatocytes. (C) Viability of Ad vector-treated hepatocytes determined by hAAT protein secretion into the culture medium. (D) Maintenance of the differentiated status of the Ad vector-treated hepatocytes as determined by the albumin production into the culture medium. $*p < 0.05$ versus MOI of 1 or lower ($n = 4$). $\dagger p < 0.05$ versus MOI of 5 or lower ($n = 4$).

man hepatocytes in terms of the plating efficiency, a significant difference between MOI 1 compared to MOI 100 was observed ($p < 0.01$) (Fig. 5C). Functional assessment by measuring hAAT production into the medium showed stable level production at MOI of 0.04 to 5, but the production level decreased at increasing doses of adenoviral vector beginning at a MOI of 10 (Fig. 5D).

DISCUSSION

The present study describes a clinically relevant approach to sustain artificial liver function by transplanting isolated primary hepatocytes genetically modified using adenoviral (Ad) vectors under the kidney capsule. The primary hepatocytes were efficiently transduced using a low dose of Ad vector with minimal cell suspension time (i.e., 1 h). Functional preservation of the Ad vector-treated hepatocytes was confirmed by hAAT and albumin protein expression levels in vitro. Moreover, the hepatocytes were capable of maintaining their engraftment potential after transplantation into the liver and under the kidney capsule. The current protocol described in our study was able to be used for not only mouse primary hepatocytes, but from other large-animal species, including humans. These results provide tremendous potential for modifying cells, including hepatocytes, as a therapeutic method to promote hepatic tissue engineering following transplantation.

Genetic modification of hepatocytes as an ex vivo gene therapy has been a major paradigm in the advancement of hepatocyte-based therapies (10,18,23,35,44,48). In the transplantation setting of allogeneic liver grafts or hepatocytes, researchers have experimentally succeeded in achieving immune tolerance or immune system suppression effects by transducing immunosuppressive genes in an ex vivo fusion to the grafts (14,43,49). Ex vivo gene transduction to hepatocytes in culture condition has

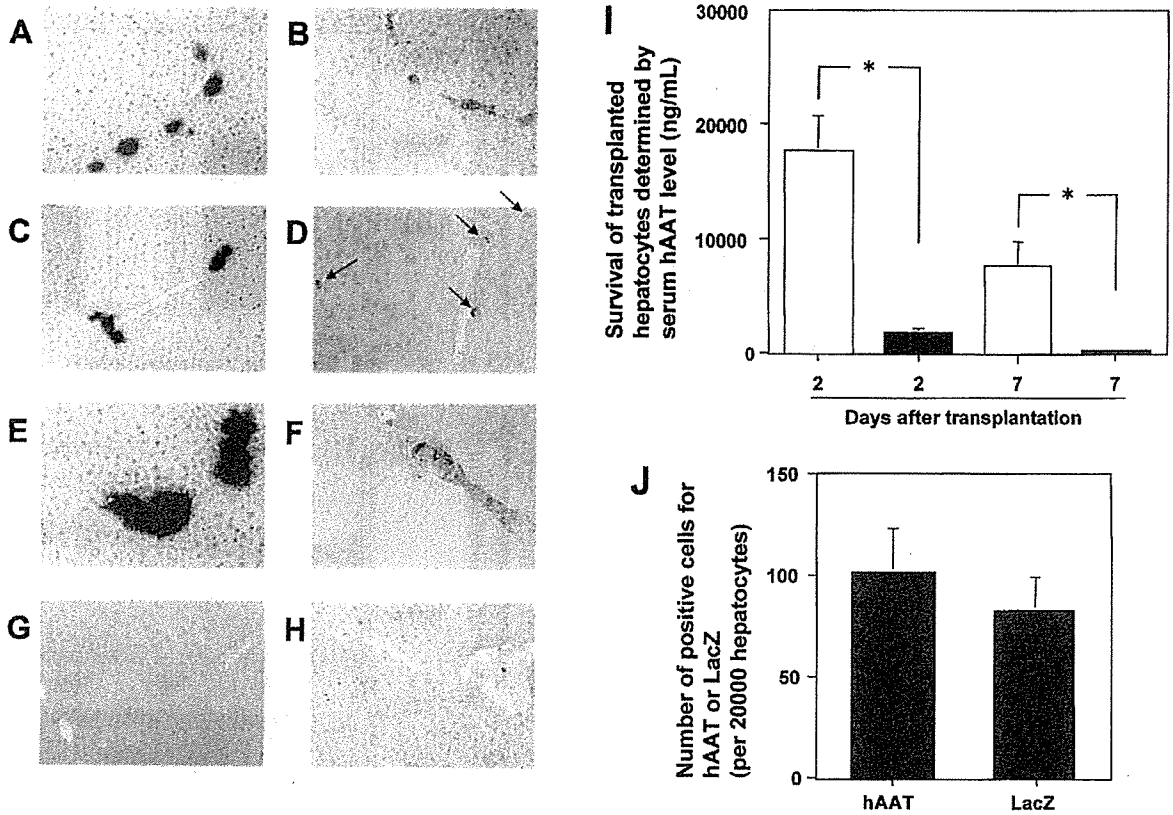

Figure 3. Demonstration of lacZ expression in Ad vector-transduced primary hepatocytes transplanted into the liver. Primary hepatocytes were infected with Ad-CA-lacZ in suspension under the condition of MOI of 1, UW solution for 1 h. After extensive washes, hepatocytes were transplanted (A–D) (see Materials and Methods section). Isolated hepatocytes were also infected with Ad-CA-lacZ in conventional (cell attached) culture conditions and then recovered from the culture dish for transplantation (E–H). The grafts were obtained at 2 days (A, B, E, F) or 7 days after transplantation (C, D, G, H). (A, C, E, G) β-Galactosidase expression of the transplanted hepatocytes; (B, D, F, H) immunohistochemical staining for hAAT, a marker protein produced in the transplanted hepatocytes. (A–H) Original magnification ×100. (I) The survival ability of transplanted hepatocyte as measured by the mouse serum hAAT level. Open columns: vector was infected in suspension; filled columns: vector was infected in culture condition. (J) Number of donor hepatocytes and gene-transduced donor hepatocytes engrafted in the liver determined by counting hAAT staining positive and X-gal staining positive hepatocytes, respectively. Values were expressed number of each staining positive hepatocytes per 20,000 hepatocytes counted. *$p < 0.01$ between groups.

also been experimentally applied to treat inherited genetic liver disorders (11,22). These ex vivo transduction methods followed by transplantation of autologus hepatocytes have been used clinically to treat patients with familial hypercholesterolemia (18). Although the procedures have been safely performed, minimal therapeutic effects have been observed (18,44). In these conventional ex vivo gene transduction approaches, hepatocyte cell culturing requires a minimum of 2–4 days to complete. During this time period, there will be at least two negative impacts that will decrease the efficiency for clinical applications: first, the hepatocytes will generally lose their function rapidly (45), and second, the length of time needed for the cell culture step will make it difficult to transport the cells to other hospitals in a timely fashion to treat critically ill patients.

In marked contrast, the benefit of our approach for ex vivo gene transduction of hepatocytes is highlighted by the facts that: 1) requires only an hour for the incubation of the vector; 2) can be performed as a cell suspension without the need for cell culture work; and 3) yields extremely high transduction efficiency with minimal adverse effects on cellular function was observed. Because we have confirmed that Ad vector infection could be performed at 4°C using UW solution, our approach is clinically applicable and could be performed during the

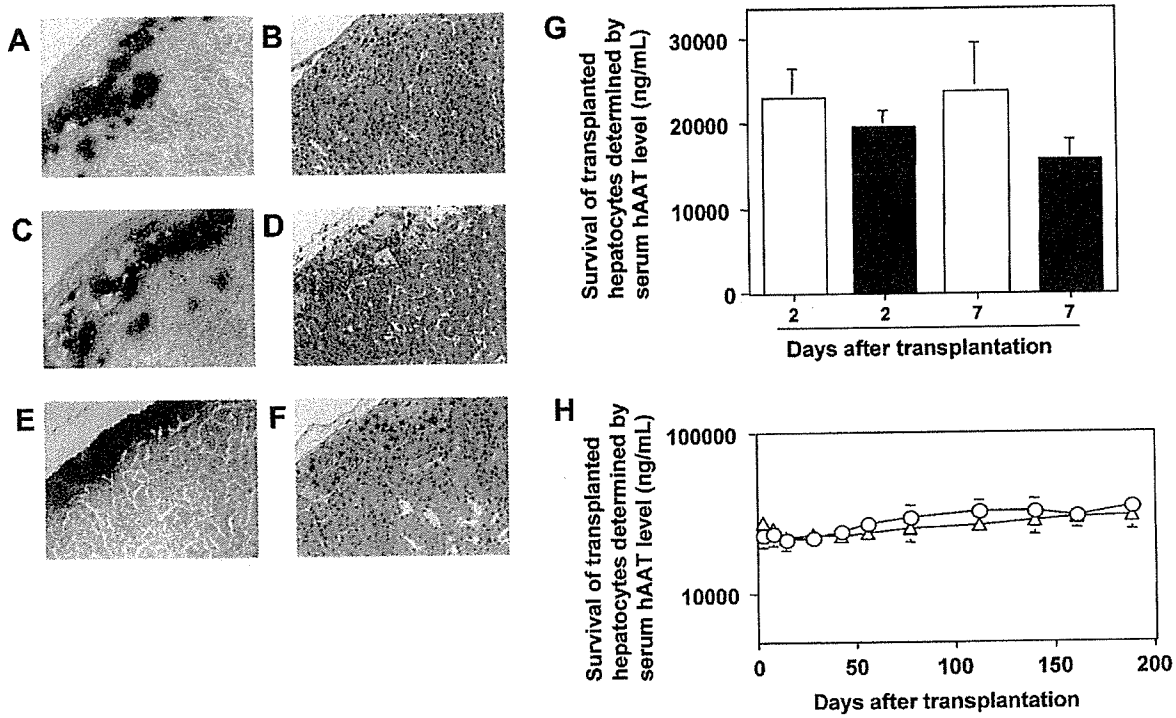

Figure 4. Hepatocyte transplantation under the mouse kidney capsule. Genetically modified primary hepatocytes (either in a suspension or standard culture conditions), which were genetically modified using Ad vectors, were transplanted under the mouse kidney capsule. Hepatocytes were infected with Ad-CA-lacZ in suspension under the condition of MOI of 1, UW solution for 1 h. After extensive washes, hepatocytes were transplanted (A, B, E, F) (see Materials and Methods section). Isolated hepatocytes were also infected with Ad-CA-lacZ in conventional (cell attached) culture conditions and then recovered from the culture dish for transplantation (C, D). The grafts were obtained at 7 days (A–D) or 21 days (E, F) after transplantation. (A, C, E) β-Galactosidase expression of the transplanted hepatocytes; (B, D, F) H&E staining. (G) Transplanted hepatocyte survival level determined by the mouse serum hAAT level at 2 or 7 days after transplantation. Open columns: hepatocytes were infected with Ad vector in suspension; filled columns: hepatocytes were infected with Ad vector after attachment to the dish. (H) Survival of transplanted hepatocyte determined by the mouse serum hAAT level for long term. Circles: mock-treated hepatocytes in suspension; triangles: hepatocytes infected with Ad-CA-null in suspension at MOI of 1.

transport process to other transplantation centers. Upon arrival at the center, only several wash steps would be needed to remove the Ad vector and the cells would be ready for transplantation within 30 min. This would prove to be a highly important and significant step in the utility of this ex vivo gene therapy approach.

To date, a variety of Ad vector systems have been developed to allow for differential periods of transgene expression, which would allow for our hepatocyte transplantation system to be tailored with these different episomal and integrating vector systems. Because early generation Ad vectors have a transient period of transgene expression (i.e., few weeks to months) (24), an ideal pathological disorder to treat would be fulminant liver failure. Transient therapeutic effect of cellular transplantation would fulfill the critical hepatic failure status to complete cure or bridge the patients to the timing receiving liver transplantation (16,35,48). In contrast, long-term effect would be necessary for the treatment of hereditary metabolic liver disorders. Ad vectors incorporated with a DNA transposon system or bacteriophage integrase may allow for genomic integration leading to the permanent transgene expression (42,52).

In terms of safety, major concerns have been described in the literature about the potential occurrence of Ad vector-mediated immunogenic responses following the infusion of the vector into the systemic circulation (9,24, 47). Because our approach includes several cycles of the washing prior to the transplantation step, the exposure to the Ad vector would be greatly minimized. Greber et al. (17) have shown that only a small fraction of the cell surface attached adenovirus released from cell surface. Furthermore, studies have shown that internalization process of the cell surface attached adenovirus was com-

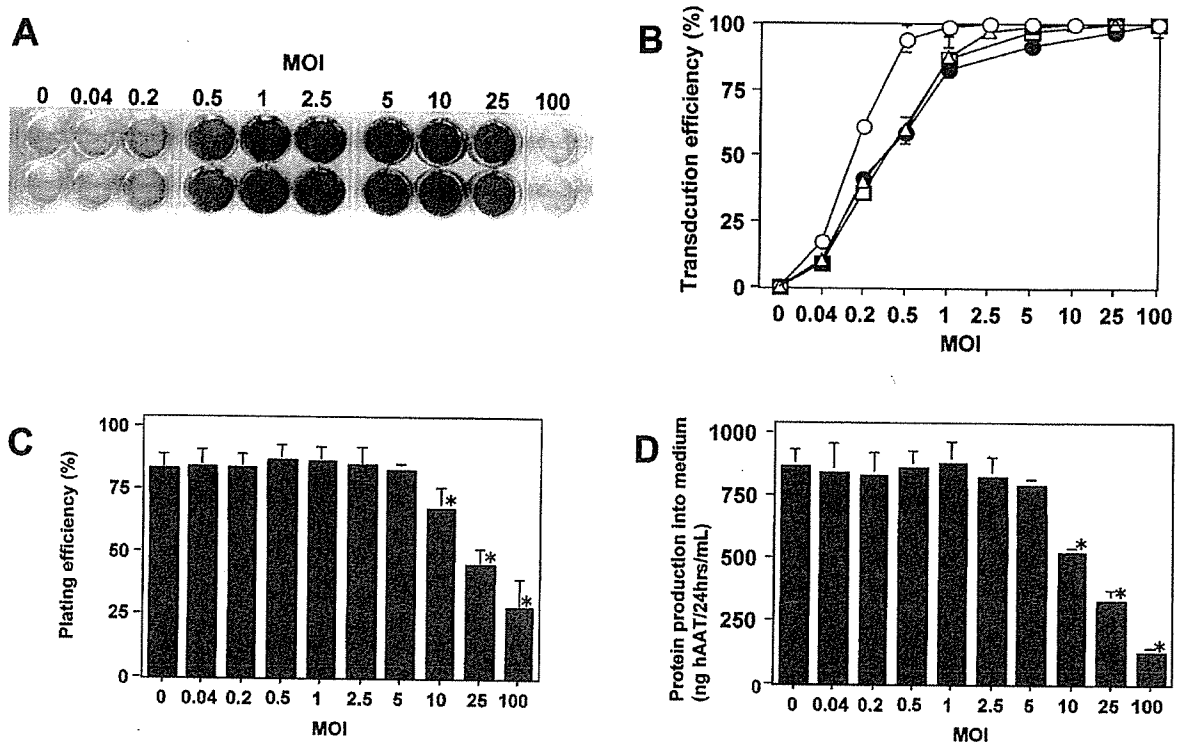

Figure 5. β-Galactosidase expression in canine, porcine, and human hepatocytes following adenoviral vector-mediated transduction into primary hepatocytes. Hepatocytes in suspension were infected with Ad-CA-lacZ at increasing MOI for 1 h at 4°C. After the Ad vector infection, hepatocytes were washed extensively and cultured for an additional 32 h on Primaria culture dishes using DMEM medium. (A) Gross morphological appearance of the X-gal-stained human hepatocytes that were infected with Ad-CA-lacZ in suspension. (B) Transduction efficiency determined as a percentage of X-gal-positive hepatocytes to the total number of hepatocytes. Open circles: human hepatocytes; open triangles: porcine hepatocytes; open squares: canine hepatocytes; filled circles: mouse hepatocytes (note that the mouse hepatocyte data were replotted from Fig. 2B). (C) Plating efficiency of the Ad vector-treated human hepatocytes. (D) Functional analyses of the Ad vector-treated human hepatocytes as measured by hAAT production in the culture medium. $*p < 0.05$ versus MOI of 5 or lower.

plete on the order of minutes once the cell temperature warmed up to 37°C (17,27). With these findings, it may reasonable to speculate that the presently described ex vivo gene transduction approach would allow for minimal virus exposure to the recipient compared to the vector injection approach. The evidence of the minimal vector shedding was confirmed by the lack of X-gal-positive cells in any of the other organs harvested in our study. Because the CAG promoter used in the present study is a strong and ubiquitous promoter (1), X-gal-positive cells should have been detected if unwanted transduction occurred via vector shedding into the general circulation. The localized transplantation coupled to our simple and safe approach clearly demonstrates an immediate benefit for the advancement of hepatocyte-based therapy in the clinics.

Because UW solution has been shown to have a role as an organ preservative (2), we used this solution to suspend our isolated hepatocytes to determine its effects on Ad vector-mediated transduction efficiency as well as on the preservation of the hepatocyte function. This solution was compared with other conventional cell culture media, including DMEM and DMEM with FBS. Our finding that the UW solution can be effectively used to support high transduction of Ad vectors into the suspended hepatocytes was consistent with a previous report by Takesue et al. (50) where suspended porcine hepatocytes were successfully cold preserved for 8 h using UW solution. Although the precise mechanism for the higher transduction efficiency of Ad vectors in the UW solution is not fully understood, it may be due to better preservation of the hepatocyte cell surface, which is rich in adenovirus receptors that are important for viral endocytosis and internalization (3,51). Because several inhibitory factors in the serum have been identified for adenoviral infection (4), it may not be a good idea to use FBS

or human serum as a supplement of the suspension medium. In fact, our present study clearly showed a lower transduction efficiency in the DMEM with FBS group compared with the DMEM group at a MOI of 0.5 or lower.

Previous work in our laboratory demonstrated that transplantation under the kidney capsule can offer higher hepatocyte survival compared to the transplantation of cells into the liver through the portal vein (34,40,41). When the hepatocytes were transplanted under the kidney capsule, the 2-day-old cultured hepatocytes did not show any observable differences in terms of cell engraftment rate and hepatocyte function compared with those of freshly isolated hepatocytes (34). Along with these data, our present study described the advantage of the ectopic kidney capsule site for tissue engineering purposes by achieving higher engraftment rates regardless of whether the Ad vector transduction was performed in suspension or in normal cell culture conditions. In marked contrast, engraftment in the liver could only be achieved when the hepatocytes had not been cultured prior to transplantation. Histological analyses at week 1 revealed that transduced hepatocytes in suspension translocated from the portal pedicles into the liver parenchyma. However, most of the transduced hepatocytes that underwent the normal culture condition failed to migrate into the liver parenchyma. The mechanism of the poor engraftment of the cultured hepatocytes transplanted into the liver has not been fully understood; however, one plausible explanation is the lack of integrin-dependent cellular signals on the cultured hepatocytes. The integrin family is key molecules promoting cellular adhesion and attachment (6,45), and the harvesting step during cell culture can routinely tear off some of the extracellular matrix components and integrins from the hepatocytes (45). Newsome et al. (31) have recently shown that the poor engraftment of hepatocytes transplanted into the liver could be overcome by activation of β1-integrin receptor of the grafts. The importance of β1-integrin-dependent cellular signals was also supported by our recent findings that providing type IV collagen, a potent ligand for β1-integrin, significantly increases the engraftment rate of transplanted hepatocytes (40). Regardless of the mechanisms, our approach for the hepatocyte gene transduction as a suspension solution resulted in higher engraftment under the kidney capsule as well as the liver.

In summary, the present studies demonstrated efficient gene transduction of isolated mouse hepatocytes using a short and simple approach. These genetically modified primary hepatocytes were capable of high engraftment efficiency following transplantation in vivo. These findings clearly represent an important step forward in advancing hepatocyte-based ex vivo gene therapies and hepatic tissue engineering for clinical applications to treat liver diseases.

ACKNOWLEDGMENTS: This work was supported in part by Grant-in-Aid from the Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, No. 15390832 (Y.N.) and No. 15691269 (K.O.).

REFERENCES

1. Araki, K.; Imaizumi, T.; Okuyama, K.; Oike, Y.; Yamamura, K. Efficiency of recombination by Cre transient expression in embryonic stem cells: Comparison of various promoters. J. Biochem. (Tokyo) 122:977–982; 1997.
2. Belzer, F. O.; Southard, J. H. Principles of solid-organ preservation by cold storage. Transplantation 45:673–676; 1988.
3. Bergelson, J. M.; Cunningham, J. A.; Droguett, G.; Kurt-Jones, E. A.; Krithivas, A.; Hong, J. S.; Horwitz, M. S.; Crowell, R. L.; Finberg, R. W. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275:1320–1323; 1997.
4. Bernal, R. M.; Sharma, S.; Gardner, B. K.; Douglas, J. T.; Bergelson, J. M.; Dubinett, S. M.; Batra, R. K. Soluble coxsackievirus adenovirus receptor is a putative inhibitor of adenoviral gene transfer in the tumor milieu. Clin. Cancer Res. 8:1915–1923; 2002.
5. Berry, M. N.; Friend, D. S. High-yield preparation of isolated rat liver parenchymal cells: A biochemical and fine structural study. J. Cell. Biol. 43:506–520; 1969.
6. Bissell, D. M.; Arenson, D. M.; Maher, J. J.; Roll, F. J. Support of cultured hepatocytes by a laminin-rich gel. Evidence for a functionally significant subendothelial matrix in normal rat liver. J. Clin. Invest. 79:801–812; 1987.
7. Bumgardner, G. L.; Li, J.; Heininger, M.; Ferguson, R. M.; Orosz, C. G. In vivo immunogenicity of purified allogeneic hepatocytes in a murine hepatocyte transplant model. Transplantation 65:47–52; 1998.
8. Cai, J.; Ito, M.; Nagata, H.; Westerman, K. A.; Lafleur, D.; Chowdhury, J. R.; Leboulch, P.; Fox, I. J. Treatment of liver failure in rats with end-stage cirrhosis by transplantation of immortalized hepatocytes. Hepatology 36: 386–394; 2002.
9. Chirmule, N.; Propert, K.; Magosin, S.; Qian, Y.; Qian, R.; Wilson, J. Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther. 6:1574–1583; 1999.
10. Csete, M. E.; Drazan, K. E.; van Bree, M.; McIntee, D. F.; McBride, W. H.; Bett, A.; Graham, F. L.; Busuttil, R. W.; Berk, A. J.; Shaked, A. Adenovirus-mediated gene transfer in the transplant setting. I. Conditions for expression of transferred genes in cold-preserved hepatocytes. Transplantation 57:1502–1507; 1994.
11. Chowdhury, J. R.; Grossman, M.; Gupta, S.; Chowdhury, N. R.; Baker, Jr., J. R.; Wilson, J. M. Long-term improvement of hypercholesterolemia after ex vivo gene therapy in LDLR-deficient rabbits. Science 254:1802–1805; 1991.
12. Demetriou, A. A.; Brown, Jr., R. S.; Busuttil, R. W.; Fair, J.; McGuire, B. M.; Rosenthal, P.; Am Esch, 2nd, J. S.; Lerut, J.; Nyberg, S. L.; Salizzoni, M.; Fagan, E. A.; de Hemptinne, B.; Broelsch, C. E.; Muraca, M.; Salmeron, J. M.; Rabkin, J. M.; Metselaar, H. J.; Pratt, D.; De La Mata, M.; McChesney, L. P.; Everson, G. T.; Lavin, P. T.; Stevens, A. C.; Pitkin, Z.; Solomon, B. A. Prospec-

tive, randomized, multicenter, controlled trial of a bioartificial liver in treating acute liver failure. Ann. Surg. 239: 660–667; 2004.
13. Eguchi, S.; Kamlot, A.; Ljubimova, J.; Hewitt, W. R.; Lebow, L. T.; Demetriou, A. A.; Rozga, J. Fulminant hepatic failure in rats: Survival and effect on blood chemistry and liver regeneration. Hepatology 24:1452–1459; 1996.
14. Fabrega, A. J.; Fasbender, A. J.; Struble, S.; Zabner, J. Cationic lipid-mediated transfer of the hIL-10 gene prolongs survival of allogeneic hepatocytes in Nagase an albuminemic rats. Transplantation 62:1866–1871; 1996.
15. Fox, I. J.; Chowdhury, J. R.; Kaufman, S. S.; Goertzen, T. C.; Chowdhury, N. R.; Warkentin, P. I.; Dorko, K.; Sauter, B. V.; Strom, S. C. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N. Engl. J. Med. 338:1422–1426; 1998.
16. Fox, I. J.; Chowdhury, J. R. Hepatocyte transplantation. J. Hepatol. 40:878–886; 2004.
17. Greber, U. F.; Willetts, M.; Webster, P.; Helenius, A. Stepwise dismantling of adenovirus 2 during entry into cells. Cell 75:477–486; 1993.
18. Grossman, M.; Raper, S. E.; Kozarsky, K.; Stein, E. A.; Engelhardt, F.; Muller, D.; Lupien, P. J.; Wilson, J. M. Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. Nat. Genet. 6: 335–341; 1994.
19. Gupta, S.; Rajvanshi, P.; Sokhi, R.; Slehria, S.; Yam, A.; Kerr, A.; Novikoff, P. M. Entry and integration of transplanted hepatocytes in rat liver plates occur by disruption of hepatic sinusoidal endothelium. Hepatology 29:509–519; 1999.
20. Hsu, S. M.; Raine, L. Protein A, avidin, and biotin in immunohistochemistry. J. Histochem. Cytochem. 29:1349–1353; 1981.
21. Kanegae, Y.; Lee, G.; Sato, Y.; Tanaka, M.; Nakai, M.; Sakaki, T.; Sugano, S.; Saito, I. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. Nucleic Acids Res. 23:3816–3821; 1995.
22. Kay, M. A.; Baley, P.; Rothenberg, S.; Leland, F.; Fleming, L.; Ponder, K. P.; Liu, T.; Finegold, M.; Darlington, G.; Pokorny, W. Expression of human alpha 1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. Proc. Natl. Acad. Sci. USA 89:89–93; 1992.
23. Kay, M. A.; Landen, C. N.; Rothenberg, S. R.; Taylor, L. A.; Leland, F.; Wiehle, S.; Fang, B.; Bellinger, D.; Finegold, M.; Thompson, A. R.; Read, M.; Brinkhous, K. M.; Woo, S. L. C. In vivo hepatic gene therapy: Complete albeit transient correction of factor IX deficiency in hemophilia B dogs. Proc. Natl. Acad. Sci. USA 91:2353–2357; 1994.
24. Kay, M. A.; Glorioso, J. C.; Naldini, L. Viral vectors for gene therapy: The art of turning infectious agents into vehicles of therapeutics. Nat. Med. 7:33–40; 2001.
25. Koyama, F.; Sawada, H.; Hirao, T.; Fujii, H.; Hamada, H.; Nakano, H. Adenoviral-mediated transfer of *Escherichia coli* uracil phosphoribosyltransferase (UPRT) gene to modulate the sensitivity of the human colon cancer cells to 5-fluorouracil. Eur. J. Cancer 36:2403–2410; 2000.
26. Kulig, K. M.; Vacanti, J. P. Hepatic tissue engineering. Transpl. Immunol. 12:303–310; 2004.
27. Leopold, P. L.; Ferris, B.; Grinberg, I.; Worgall, S.; Hackett, N. R.; Crystal, R. G. Fluorescent virions: Dynamic tracking of the pathway of adenoviral gene transfer vectors in living cells. Hum. Gene Ther. 10:367–378; 1998.
28. Malhi, H.; Annamaneni, P.; Slehria, S.; Joseph, B.; Bhargava, K. K.; Palestro, C. J.; Novikoff, P. M.; Gupta, S. Cyclophosphamide disrupts hepatic sinusoidal endothelium and improves transplanted cell engraftment in rat liver. Hepatology 36:112–121; 2002.
29. Muraca, M.; Gerunda, G.; Neri, D.; Vilei, M. T.; Granato, A.; Feltracco, P.; Meroni, M.; Giron, G.; Burlina, A. B. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. Lancet 359:317–318; 2002.
30. Nakamura, T.; Sato, K.; Hamada, H. Reduction of natural adenovirus tropism to the liver by both ablation of fiber-coxsackievirus and adenovirus receptor interaction and use of replaceable short fiber. J. Virol. 77:2512–2521; 2003.
31. Newsome, P. N.; Tsiaoussis, J.; Masson, S.; Buttery, R.; Livingston, C.; Ansell, I.; Ross, J. A.; Sethi, T.; Hayes, P. C.; Plevris, J. N. Serum from patients with fulminant hepatic failure causes hepatocyte detachment and apoptosis by a beta(1)-integrin pathway. Hepatology 40:636–645; 2004.
32. Nguyen, T. H.; Oberholzer, J.; Birraux, J.; Majno, P.; Morel, P.; Trono, D. Highly efficient lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes. Mol. Ther. 6:199–209; 2002.
33. Ohashi, K.; Tsutsumi, M.; Tsujiuchi, T.; Kobitsu, K.; Okajima, E.; Nakajima, Y.; Nakano, H.; Takahashi, M.; Mori, Y.; Konishi, Y. Enhancement of N-nitrosodiethylamine-initiated hepatocarcinogenesis caused by a colchicine-induced cell cycle disturbance in partially hepatectomized rats. Cancer Res. 56:3474–3479; 1996.
34. Ohashi, K.; Marion, P. L.; Nakai, H.; Meuse, F; Cullen, J. M.; Bordier, B. B.; Schwall, R.; Greenberg, H. B.; Glenn, J. S.; Kay, M. A. Sustained survival of human hepatocytes in mice: A model for in vivo infection with human hepatitis B and hepatitis delta viruses. Nat. Med. 6: 327–331; 2000.
35. Ohashi, K.; Park, F.; Kay, M. A. Hepatocyte transplantation: Clinical and experimental application. J. Mol. Med. 79:617–630; 2001.
36. Ohashi, K.; Meuse, L.; Schwall, R.; Kay, M. A. cMet activation allows persistent engraftment of ectopically transplanted xenogenic human hepatocytes in mice. Transplant. Proc. 33:587–588; 2001.
37. Ohashi, K.; Park, F.; Kay, M. A. Role of hepatocyte direct hyperplasia in lentivirus-mediated liver transduction in vivo. Hum. Gene Ther. 20:653–663; 2002.
38. Ohashi, K.; Park, F.; Schwall, R.; Kay, M. A. Efficient gene transduction to cultured hepatocytes by HIV-1 derived lentiviral vector. Transplant. Proc. 34:1431–1433; 2002.
39. Ohashi, K.; Kay, M. A. Extracellular matrix component cotransplantation prolongs survival of heterotopically transplanted human hepatocytes in mice. Transplant. Proc. 36:2469–2470; 2004.
40. Ohashi, K.; Waugh, J. M.; Dake, M. D.; Yokoyama, T.; Kuge, H.; Nakajima, Y.; Yamanouchi, M.; Naka, H.; Yoshioka, A.; Kay, M. A. Liver tissue engineering at extrahepatic sites in mice as a potential new therapy for genetic liver diseases. Hepatology 41:132–140; 2005.
41. Ohashi, K.; Kay, M. A.; Yokoyama, T.; Kuge, H.; Nakajima, Y. Stability and repeat regeneration potential of the

engineered liver tissue under the kidney capsule in mice. Cell Transplant. 14:621–627; 2005.
42. Olivares, E. C.; Hollis, R. P.; Chalberg, T. W.; Meuse, L.; Kay, M. A.; Calos, M. P. Site-specific genomic integration produces therapeutic factor IX levels in mice. Nat. Biotechnol. 20:1124–1128; 2002.
43. Olthoff, K. M.; Judge, T. A.; Gelman, A. E.; daShen, X.; Hancock, W. W.; Turka, L. A.; Shaked, A. Adenovirus-mediated gene transfer into cold-preserved liver allografts: Survival pattern and unresponsiveness following transduction with CTLA4Ig. Nat. Med. 4:194–200; 1998.
44. Raper, S. E.; Grossman, M.; Rader, D. J.; Thoene, J. G.; Clark, 3rd, B. J.; Kolansky, D. M.; Muller, D. W.; Wilson, J. M. Safety and feasibility of liver-directed ex vivo gene therapy for homozygous familial hypercholesterolemia. Ann. Surg. 223:116–126; 1996.
45. Runge, D.; Runge, D. M.; Drenning, S. D.; Bowen, Jr., W. C.; Grandis, J. R.; Michalopoulos, G. K. Growth and differentiation of rat hepatocytes: Changes in transcription factors HNF-3, HNF-4, STAT-3, and STAT-5. Biochem. Biophys. Res. Commun. 250:762–768; 1998.
46. Schneider, A.; Attaran, M.; Gratz, K. F.; Bleck, J. S.; Winkler, M.; Manns, M. P.; Ott, M. Intraportal infusion of 99mtechnetium-macro-aggregrated albumin particles and hepatocytes in rabbits: Assessment of shunting and portal hemodynamic changes. Transplantation 75:296–302; 2003.
47. Smaglik, P. Tighter watch urged on adenoviral vectors . . . with proposal to report all 'adverse events'. Nature 402:707; 1999.
48. Strom, S.; Fisher, R. Hepatocyte transplantation: New possibilities for therapy. Gastroenterology 124:568–571; 2003.
49. Takahashi, Y.; Geller, D. A.; Gambotto, A.; Watkins, S. C.; Fung, J. J.; Murase, N. Adenovirus-mediated gene therapy to liver grafts: Successful gene transfer by donor pretreatment. Surgery 128:345–352; 2000.
50. Takesue, M.; Maruyama, M.; Shibata, N.; Kunieda, T.; Okitsu, T.; Sakaguchi, M.; Totsugawa, T.; Kosaka, Y.; Arata, A.; Ikeda, H.; Matsuoka, J.; Oyama, T.; Kodama, M.; Ohmoto, K.; Yamamoto, S.; Kurabayashi, Y.; Yamamoto, I.; Tanaka, N.; Kobayashi, N. Maintenance of cold-preserved porcine hepatocyte function with UW solution and ascorbic acid-2 glucoside. Cell Transplant. 12:599–606; 2003.
51. Wickham, T. J.; Mathias, P.; Cheresh, D. A.; Nemerow, G. R. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 73:309–319; 1993.
52. Yant, S. R.; Ehrhardt, A.; Mikkelsen, J. G.; Meuse, L.; Pham, T.; Kay, M. A. Transposition from a gutless adenotrasposon vector stabilizes transgene expression in vivo. Nat. Biotechnol. 20:999–1005; 2002.

ELSEVIER

Journal of Hepatology 44 (2006) 1046–1054

Journal of Hepatology

www.elsevier.com/locate/jhep

In vivo hepatic HB-EGF gene transduction inhibits Fas-induced liver injury and induces liver regeneration in mice: A comparative study to HGF

Ngin Cin Khai[1,2], Tomoyuki Takahashi[1,3,5], Hiroaki Ushikoshi[1,2], Satoshi Nagano[1,4,5], Kentaro Yuge[1], Masayasu Esaki[1,2], Takao Kawai[1,2], Kazuko Goto[1,2], Yoshiteru Murofushi[1,5], Takako Fujiwara[6], Hisayoshi Fujiwara[2], Ken-ichiro Kosai[1,4,5,*]

[1]*Department of Gene Therapy and Regenerative Medicine, Gifu University School of Medicine, Gifu University Graduate School of Medicine, Gifu, Japan*
[2]*Department of Cardiology, Respiratory and Nephrology, Regeneration and Advanced Medical Science, Gifu University Graduate School of Medicine, Gifu, Japan*
[3]*Department of Advanced Therapeutics and Regenerative Medicine, Kurume University, Kurume, Japan*
[4]*Department of Pediatrics, Kurume University School of Medicine, Kurume University, Kurume, Japan*
[5]*Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute of Brain Diseases, Kurume University, 67 Asahi-machi, Kurume 830-0011, Japan*
[6]*Department of Food Science, Kyoto Women's University, Kyoto, Japan*

***Background/Aims*: It is unknown whether heparin-binding EGF-like growth factor (HB-EGF) can be a therapeutic agent, although previous studies suggested that HB-EGF might be a hepatotrophic factor. This study explores the potential of hepatic HB-EGF gene therapy in comparison with HGF.**

***Methods*: Mice received an intraperitoneal injection of the agonistic anti-Fas antibody 72 h after an intravenous injection of either adenoviral vector (1×10^{11} particles) expressing human HB-EGF (Ad.HB-EGF), human HGF (Ad.HGF) or no gene (Ad.dE1.3), and were sacrificed 24 or 36 h later to assess liver injury and regeneration.**

***Results*: Exogenous HB-EGF was predominantly localized on the membrane, suggesting the initial synthesis of proHB-EGF in hepatocytes. The control Ad.dE1.3-treated mice represented remarkable increases in serum ALT and AST levels and histopathologically severe liver injuries with numerous apoptosis, but a limited number of mitogenic hepatocytes. In contrast, the liver injuries and apoptotic changes were significantly inhibited, but the mitogenic hepatocytes remarkably increased, in both the Ad.HB-EGF- and Ad.HGF-treated mice. More mitogenic hepatocytes and milder injuries were observed in the Ad.HB-EGF-treated mice.**

***Conclusions*: HB-EGF has more potent protective and mitogenic effects for hepatocytes than HGF, at least for the present conditions. In vivo hepatic HB-EGF gene transduction is therapeutic for Fas-induced liver injury.**

***Keywords*: Heparin-binding epidermal growth factor-like growth factor; HB-EGF; Hepatocyte growth factor; HGF; Growth factor; Gene therapy; Apoptosis; Fulminant hepatic failure; Fas; Liver regeneration; Adenoviral vector**

Received 9 February 2005; received in revised form 20 September 2005; accepted 10 October 2005; available online 7 December 2005
* Corresponding author. Address: Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute of Brain Diseases, Kurume University, 67 Asahi-machi, Kurume 830-0011, Japan. Tel.: +81 942 31 7581; fax: +81 942 31 7911.
E-mail address: kosai@med.kurume-u.ac.jp (K.- Kosai).

0168-8278/$32.00
doi:10.1016/j.jhep.2005.10.027

1. Introduction

Certain growth factors act as hepatogenic and hepatotrophic factors and play essential roles in the development and homeostasis of the liver. The hepatotrophic factors that have been well studied are hepatocyte growth factor (HGF), epidermal growth factors (EGF) and transforming growth factor-α (TGF-α); their expressions are found in the adult liver under normal physiological conditions and are drastically upregulated during liver regeneration after a partial hepatectomy or liver injury [1–5]. The knockout of any of these genes in mice led to aplasia or dysmaturation of the liver [6,7], and the overexpression of any of them in the transgenic mice accelerated the proliferation of hepatocytes after a partial hepatectomy [8]. Moreover, several animal studies have shown that HGF can be a potent therapeutic agent for liver disorders by inhibiting hepatocyte apoptosis and/or inducing liver regeneration, regardless of administration of the recombinant protein or the gene therapy strategy used [3,9–16].

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), which was identified as a new member of the EGF-family of growth factors, is also expressed in the normal liver [17,18]. The biologically unique feature of this growth factor is that the membrane-anchored precursor form (proHB-EGF) is initially synthesized and subsequently cleaved at the juxtamembrane domain by a specific metalloproteinase [19–21], and the resultant soluble form (sHB-EGF) represents the potent mitogenic activity for a number of cell types [22,23]. The HB-EGF mRNA levels were rapidly increased in the nonparenchymal cells of the regenerating liver, mainly in Kupffer cells and sinusoidal endothelial cells, but not in hepatocytes, after a 70% partial hepatectomy [24] or after liver injury by hepatotoxins [25]. Interestingly, the increase in HB-EGF mRNA was more rapid than that of HGF mRNA, e.g. their maximal levels were reached at 6 and 24 h after partial hepatectomy, respectively [24], suggesting the distinct role and/or mechanism of HB-EGF in liver regeneration compared to HGF. Moreover, exogenous HB-EGF stimulated the DNA synthesis in rat hepatocytes in in vitro [25] and in vivo experiments [26], and the hepatocyte-specific overexpression of HB-EGF in the transgenic mice accelerated the in vivo proliferation of hepatocytes after a partial hepatectomy [27]. Thus, it is likely that HB-EGF may also be acting as the hepatotrophic factor, probably in the early phase of liver regeneration. However, the therapeutic potential of HB-EGF for liver disorders, e.g. the question of whether exogenous HB-EGF acts to inhibit liver injury, has not yet been studied. Other important points are that the possibility of HB-EGF gene therapy has not yet been studied for any diseases in any organs, including liver diseases, except for our recent study on heart disorders, and that the HB-EGF gene therapy trial for myocardial infarction in rabbits did not exert therapeutic effects but rather exacerbated remodeling [28]. Thus, it is biologically and clinically meaningful to investigate whether HB-EGF gene therapy can be potentially used to treat liver disorders.

In this study, the HB-EGF gene was adenovirally transduced into the mouse liver and its potential to inhibit liver injury and stimulate liver regeneration were investigated. Furthermore, these potentials were simultaneously compared to those of HGF, which is currently the best-studied hepatotrophic and therapeutic factor.

2. Materials and methods

2.1. Recombinant adenoviral vectors

Replication-defective recombinant adenoviral vectors (Ads), Ad.HB-EGF, Ad.HGF, Ad.LacZ and Ad.dE1.3, which express human HB-EGF, human HGF, LacZ and no gene under the transcriptional control of a Rous sarcoma virus long-terminal repeat, were prepared as described previously [28–31].

2.2. Animal studies

The schedule of the experiment on therapeutic potentials is shown in Fig. 2(A). Male 5 to 6 week-old C57BL/6J mice ($n=10$, each group) (Chubu Kagaku, Nagoya, Japan) were given an intravenous injection of 1×10^{11} particles of Ad via a tail vein. 72 h later, they were given an intraperitoneal injection of 4 μg of agonistic anti-mouse Fas monoclonal antibody (Jo-2, Beckton-Dickinson Biosciences, San Jose, CA) [14]. All mice were subsequently sacrificed 24 or 36 h later, and liver and blood samples were collected for examination. On the other hand, in vivo adenoviral gene transduction efficiency or the in situ detection of exogenous human HB-EGF was analyzed using the same dose (1×10^{11} particles) of either Ad.LacZ or Ad.HB-EGF, respectively, in intact mice. All animal studies were performed in accordance with the National Institute of Health guidelines as dictated by the Animal Care Facility at the Gifu University School of Medicine.

2.3. Histopathologic analysis

For histopathological observation, liver tissues were fixed in 10% formalin and embedded in paraffin, and 4-μm sections were cut and stained with hematoxylin and eosin (H-E). For assessing the in vivo adenoviral gene transduction efficiency, *O*-nitrophenyl-β-D-galactophyranoside (x-gal) staining was done using the frozen tissue as described previously [28–31]. For detecting apoptotic cells, a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) assay (ApopTag kit, Intergen Co., Purchase, NY) was done in accordance with the manufacturer's protocol.

Immunohistochemistry was carried out for in situ detection of the cells expressing the HB-EGF transgene or regenerating hepatocytes. In the former, 6-μm frozen sections were fixed in 4% paraformaldehyde and stained with primary goat anti-human HB-EGF antibody (R&D Systems Inc., Minneapolis, MN), secondary donkey anti-goat IgG Alexa 568 antibody (Molecular Probes, Inc., Eugene, OR), and Hoechst 33342 (Molecular Probes, Inc.). In the latter, the formalin-fixed and paraffin-embedded tissues were deparaffinized and rehydrated, and then heated in 10 mmol/L citrate buffer, pH 6.0 for 10 min for the antigen retrieval. Endogenous peroxidase activity and the non-specific binding of antibody were blocked by 0.3% H_2O_2 and normal rabbit serum. The anti-Ki67 (TEC-3, DakoCytomation, Denmark) primary antibody, biotinylated anti-rat IgG secondary antibody, avidin-peroxidase, diaminobenzidine in hydrogen peroxidase and chromogen were applied in order and were subsequently counterstained with hematoxylin.

Digital images observed with a laser-confocal microscope (LSM510, Carl Zeiss, Oberkochen, Germany) were employed for the morphometric and quantitative analyses using Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA). Quantitative analyses of the replicating and apoptotic hepatocytes were done as described previously with some modifications [13,15]. Briefly, Ki-67-positive, Ki-67-negative or TUNEL-positive hepatocytes were counted in 30 fields at random under 200×

magnification (approximately 2000 cells in 3 slides per mouse), and the percentages of the replicating hepatocytes and numbers of the apoptotic hepatocytes in a field were calculated.

2.4. Biochemical Analyses

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a standard clinical automatic analyzer (Hitachi 736) (Hitachi Co. Ltd., Tokyo, Japan) at 24 or 36 h after the anti-Fas antibody injection.

2.5. Statistical Analysis

Data were represented as the means +/− standard errors. Statistical significance was determined using the Student's *t*-test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Adenoviral gene transduction efficiency and HB-EGF transgene expression

In accordance with previous findings [16,32], Ad.LacZ injection and subsequent x-gal staining revealed that an intravenous injection of 1×10^{11} particles of Ad resulted in approximately between 60 and 90% gene transduction in hepatocytes (Fig. 1(A)). Ad.HB-EGF injection and the subsequent immunohistochemistry against the human HB-EGF confirmed this finding. Moreover, the exogenous HB-EGF protein was predominantly observed on the membrane, and the cytoplasm was faintly positive for exogenous HB-EGF. These findings suggest that the initially synthesized exogenous HB-EGF was membrane-anchored proHB-EGF, and that the resultant sHB-EGF by the shedding of proHB-EGF might bind to and activate hepatocytes in the autocrine fashion, followed by the endocytosis of exogenous HB-EGF in hepatocytes.

3.2. Liver enzymes after HB-EGF or HGF gene therapy

Recent studies have suggested that the initial and essential event in acute and/or chronic hepatitis, including fulminant hepatic failure, may be the excessive activation of the Fas system [33,34] and that the administration of the agonistic anti-Fas antibody in mice leads to fulminant hepatic failure [14,35]. As we clearly elucidated the therapeutic effect of HGF in this animal model [14], this model may be suitable to use for the initial examination of the in vivo anti-apoptotic effect on hepatocytes and the

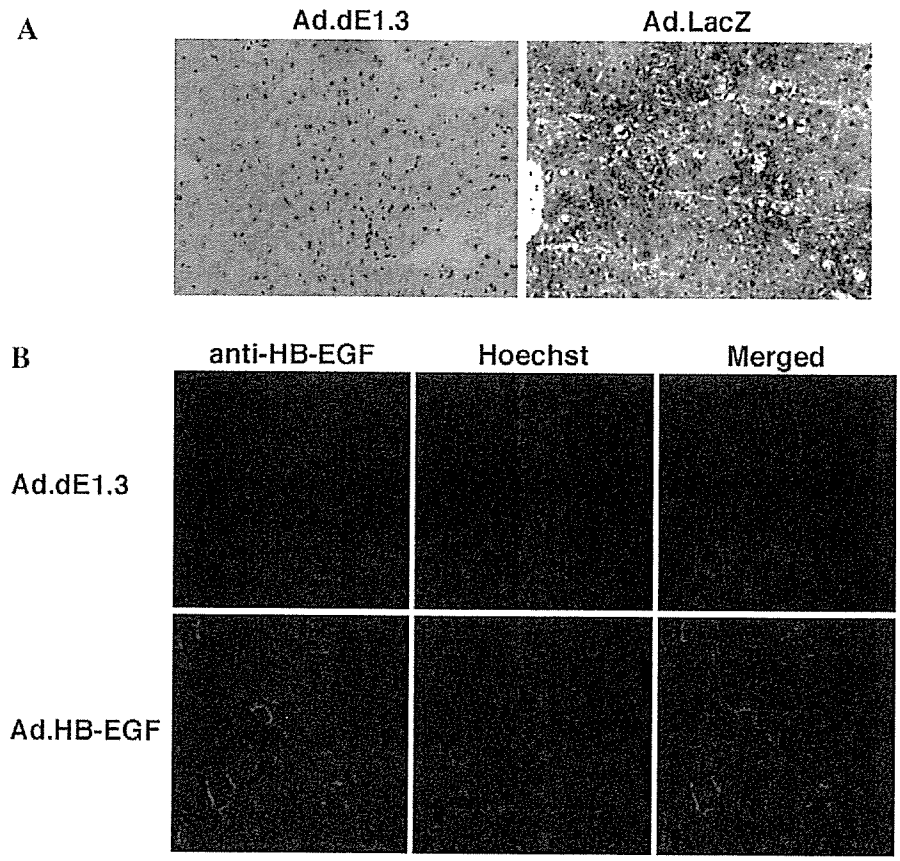

Fig. 1. Adenoviral gene transduction and expression in mice. (A) X-gal staining of the liver 48 h after an intravenous injection of either Ad.LacZ or control Ad.dE1.3 into an intact mouse. (B) Immunohistochemical staining for exogenous human HB-EGF of the liver 48 h after an intravenous injection of either Ad.HB-EGF or control Ad.dE1.3 into an intact mouse.

therapeutic potential of a certain agent for liver disorders. To perform this assessment, we initially injected Ad, and then subsequently injected the anti-Fas antibody 72 h later. We sacrificed the mice 24 or 36 h after the anti-Fas injection and examined the hepatic injuries (Fig. 2(A)).

The mice that received injections of the control Ad.dE1.3 and anti-Fas antibody represented a remarkable increase in the serum ALT and AST levels, which were up to 2240±450 and 1665±391 IU/L (62 and 21 times as high as the normal levels), respectively, 24 h after the anti-Fas injection (Fig. 2(B) and (C)). The increases in the serum ALT and AST levels were significantly attenuated by an injection of either Ad.HB-EGF or Ad.HGF before the anti-Fas injection ($P<0.01$); the serum ALT and AST levels were similar (i.e. no statistically significant differences between these two groups) and less than 230 IU/L in both groups. On the other hand, the serum ALT and AST levels 36 h after the anti-Fas injection in the Ad.HB-EGF-treated mice were remarkably lower than those in not only the control Ad.dE1.3-treated but also the Ad.HGF-treated mice. The serum ALT and AST levels in the Ad.HB-EGF-treated mice were 12 and 8.4 times (at 24 h) and 2.0 and 3.1 times (at 36 h) as low as those in the control Ad.dE1.3-treated mice, respectively. Those in the Ad.HGF-treated mice were 10 and 8.1 times (at 24 h) and 0.7 and 0.9 times (i.e. no protective phenotype) (at 36 h), as low as those in the control Ad.dE1.3-treated ones, respectively. Thus, these results indicate that HB-EGF has a more potent inhibitory effect on Fas-induced liver injuries than HGF, at least for the present condition.

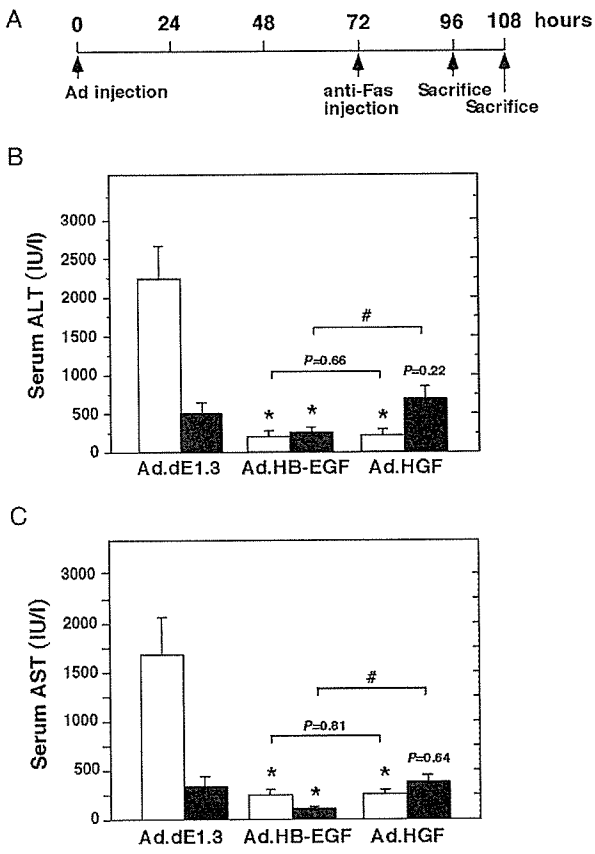

Fig. 2. Experimental schedule and liver enzymes after HB-EGF or HGF gene therapy for Fas-induced liver injury. (A) Experimental schedule of all the following therapeutic experiments. Mice ($n=10$, each group) received intraperitoneal injections of 4 μg of agonistic anti-Fas antibody 72 h after a tail vein injection of either Ad.hHB-EGF, Ad.hHGF or Ad.dE1.3 (1×10^{11} particles). The mice were then sacrificed, and liver and blood samples collected 24 or 36 h after injection of the anti-Fas antibody (i.e. 96 or 108 h after adenoviral injection). The serum ALT and AST levels at 24 (white bars) or 36 (black bars) hours after injection of the anti-Fas antibody are shown on (B) and (C), respectively (* $P<0.01$, either Ad.HB-EGF or Ad.HGF vs. control Ad.dE1.3: # $P<0.01$, Ad.HB-EGF vs. Ad.HGF).

3.3. Liver histopathology after HB-EGF or HGF gene therapy

The histological analysis of the livers at 24 and 36 h after the anti-Fas antibody injection in the control Ad.dE1.3-treated mice demonstrated severe liver injury with prominent apoptotic changes, as described previously [14,15] (Fig. 3). Briefly, apoptotic bodies characterized by nuclear and cell fragmentation within the shrunken and condensed cytoplasm were found, and there were a number of cells, consisting of spindle-shaped activated Kupffer cells, mononuclear cells and neutrophils, in the sinusoid. Such findings have often been observed in human patients with liver injuries as the apopto-necro-inflammatory reaction [33,36].

In contrast, there were minimal histopathological findings in the liver in the Ad.HB-EGF- or Ad.HGF-treated mice 24 and 36 h after the anti-Fas antibody injection; the inhibitory effect against liver injury as assessed by histopathologic observation on the H-E-stained slides in these mice was seemingly much more prominent than that assessed by the serum ALT and AST levels, as shown above. Apoptotic bodies were rarely seen, and the inflammatory reaction characterized by Kuppfer cell hyperplasia and accumulated inflammatory cells was not found. The difference in the histopathology of the liver between the Ad.HB-EGF- and Ad.HGF-treated mice was not clear at 24 h, but was remarkable at 36 h after the anti-Fas antibody injection. Notably, the pathological findings were still minimal in the Ad.HB-EGF-treated mice, but relatively prominent in the Ad.HGF-treated mice at 36 h after the anti-Fas antibody. These results indicated that HB-EGF has more potent cytoprotective and inhibitory effects against the Fas-induced apopto-necro-inflammatory reaction than HGF, at least for the present condition.

3.4. Potent anti-apoptotic effects of HB-EGF or HGF gene therapy

To obtain evidence that HB-EGF prevents hepatocyte apoptosis, apoptotic cells were detected by the in situ TUNEL assay (Fig. 4). In accordance with the

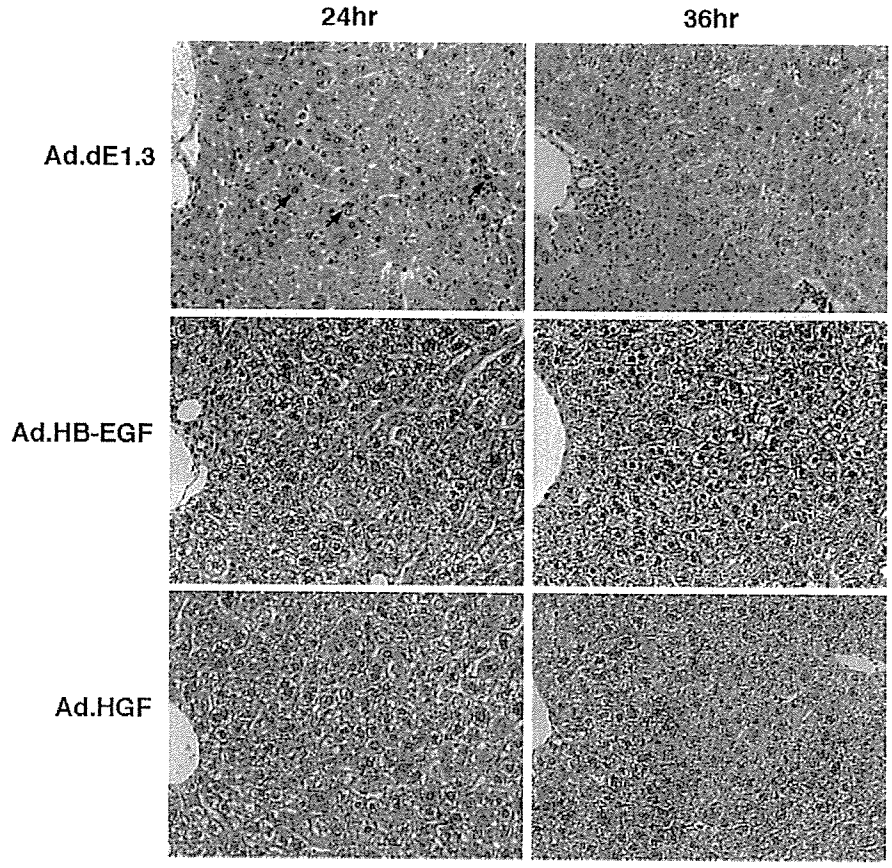

Fig. 3. Liver histopathology after HB-EGF or HGF gene therapy for Fas-induced liver injury. H-E stained liver slides of the mice that were given injections of the anti-Fas antibody and either Ad.dE1.3, Ad.HB-EGF or Ad.HGF on the protocol shown in Fig. 2(A) are shown. Arrows indicate typical acidophilic and apoptotic bodies. Original magnification, ×200. [This figure appears in colour on the web.]

histopathological findings on H-E slides, TUNEL-positive hepatocytes, approximately 10 or 6% of all hepatocytes, were observed throughout the liver parenchyma in the Ad.dE1.3-treated control mice 24 or 36 h after the anti-Fas antibody injection, respectively. Apoptotic bodies recognized in the disrupted hepatic cord or in the sinusoids were also TUNEL-positive. On the other hand, there were few (around 1%) TUNEL-positive hepatocytes in the liver of the mice treated with either Ad.HB-EGF or Ad.HGF at 24 and 36 h after the anti-Fas antibody injection. The morphometric and quantitative analysis of the TUNEL-positive cells revealed statistically significant differences between the control Ad.dE1.3- and either the Ad.HB-EGF- and Ad.HGF-treated groups ($P<0.01$), but no difference between the Ad.HB-EGF- and Ad.HGF-treated groups at 24 or 36 h after the anti-Fas antibody injection. These data suggest the anti-apoptotic effect of HB-EGF on hepatocytes is as potent as HGF.

3.5. HB-EGF induces liver regeneration more potently than HGF

We examined whether HB-EGF gene transduction and expression in the autocrine fashion may enhance liver regeneration after liver injury, and compared the degree of the inducible effect of HB-EGF to that of HGF by morphometric and quantitative analysis of Ki-67-positive cells (Fig. 5). In the control mice that received injections of Ad.dE1.3, Ki-67-positive replicating hepatocytes at 24 and 36 h after the anti-Fas antibody injection were 6.4 ± 0.7 and $12\pm2.2\%$, respectively, which were more than that of the intact liver without any treatment (less than 1%; data not shown). On the other hand, Ad.HGF injection significantly enhanced the hepatocyte replication at 24 and 36 h after the anti-Fas antibody up to 35.8 ± 2.8 and $28\pm3.0\%$, respectively ($P<0.01$, Ad.HGF vs. Ad.dE1.3). More promisingly, the Ad.HB-EGF injection induced the hepatocyte replication at 24 h after the anti-Fas antibody more efficiently up to $54.6\pm2.5\%$ ($P<0.01$, Ad.HB-EGF vs. Ad.HGF, and Ad.HB-EGF vs. Ad.dE1.3). The percentages of Ki-67-positive replicating hepatocytes in Ad.HB-EGF-treated mice at 36 h after the anti-Fas antibody were $29\pm2.9\%$, similar to those in the Ad.HGF-treated mice. Thus, HB-EGF gene transduction and expression in the hepatocytes induced the acute phase of liver regeneration after liver injury more potently than HGF.

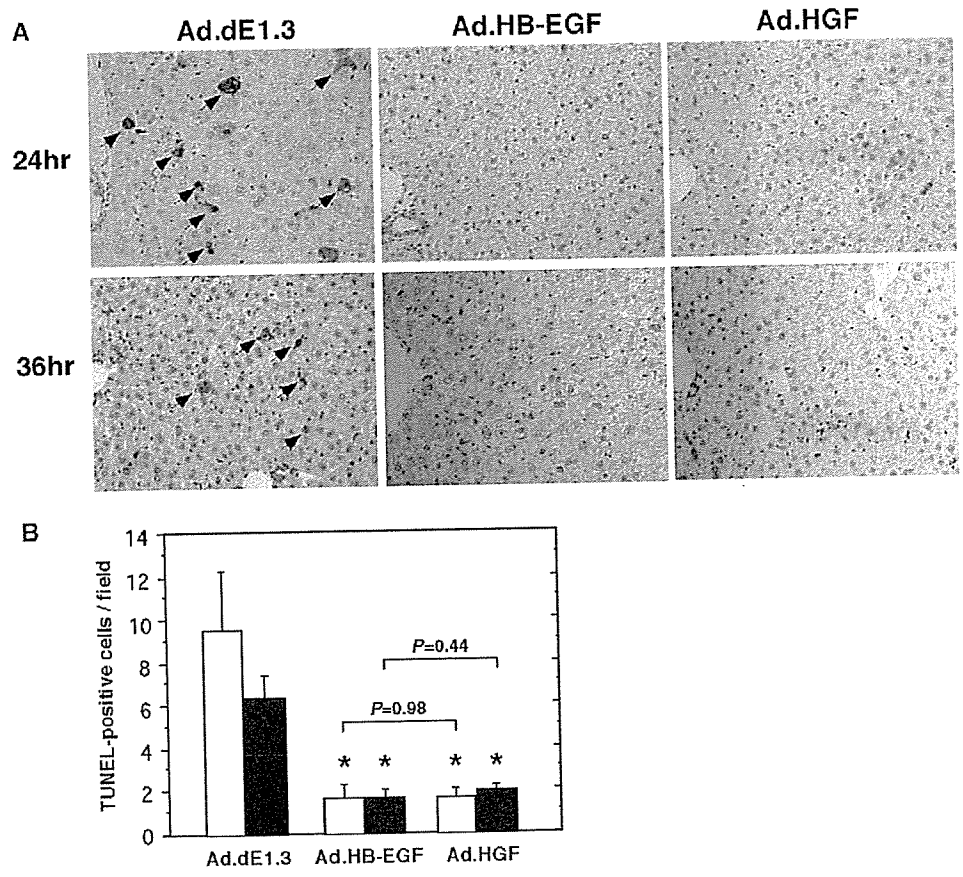

Fig. 4. TUNEL analysis of the liver after HB-EGF or HGF gene therapy for Fas-induced liver injury. (A) TUNEL staining using the liver tissue from the mice that were given injections of the anti-Fas antibody and either Ad.dE1.3, Ad.HB-EGF or Ad.HGF on the protocol shown in Fig. 2(A). Arrows indicate TUNEL-positive hepatocytes and apoptotic bodies. Original magnification, ×200. (B) The morphometric and quantitative analysis of TUNEL-positive cells at 24 (white bars) or 36 (black bars) hours after injection of the anti-Fas antibody in each group (* $P<0.01$, either Ad.HB-EGF or Ad.HGF vs. control Ad.dE1.3: # $P<0.01$, Ad.HB-EGF vs. Ad.HGF).

4. Discussion

It has been shown that exogenous HGF can be a potent therapeutic agent for liver disorders in terms of its beneficial effects of inhibiting liver injury and inducing liver regeneration, regardless of administration of the recombinant protein or the gene therapy strategy used [3,9–16]. In contrast to such extensive studies on HGF, the therapeutic potential of HB-EGF has not yet been explored. A few studies have shown the in vivo mitogenic effect of exogenous HB-EGF [26,27,37], but neither the anti-apoptotic effect on hepatocytes nor the inhibitory effect against liver injury have yet been studied. Thus, the present study revealed, for the first time, that HB-EGF might be a therapeutic agent for liver disorders in terms of both its anti-apoptotic and mitogenic effects on hepatocytes. On the other hand, we recently showed that in vivo HB-EGF gene transduction for myocardial infarction in the heart did not demonstrate therapeutic effects but instead exacerbated the remodeling, although HB-EGF is one of the essential cardiogenic and cardiotrophic factors [28]. Thus, the present results, taken together with our recent ones, are useful for understanding the physiological and pathological roles of HB-EGF. The phenotypic difference of HB-EGF may, at least in part, be due to potent and no cytoprotective effects of HB-EGF for hepatocytes and cardiomyocytes, respectively, although future extensive studies are necessary for overall elucidation.

Moreover, we obtained a further promising result in this study that the in vivo mitogenic and protective effects of HB-EGF on hepatocytes were more potent than those of HGF, at least in part and for the present condition. There are several possibilities why. The first possibility is the definitive difference between the HGF- and HB-EGF-dependent signal transduction pathways in hepatocytes related to the mitogenic phenotype; this is likely because their receptors, i.e. the c-met/HGF receptor and several EGF/erbB receptors, are different [3,20]. The second possibility is that this difference may result from the fact that HB-EGF plays more important roles in the early phase of liver regeneration than HGF. It was reported that the increase in the HB-EGF mRNA level occurred earlier than that of the HGF mRNA level during liver regeneration after partial hepatectomy or liver injury by hepatotoxins

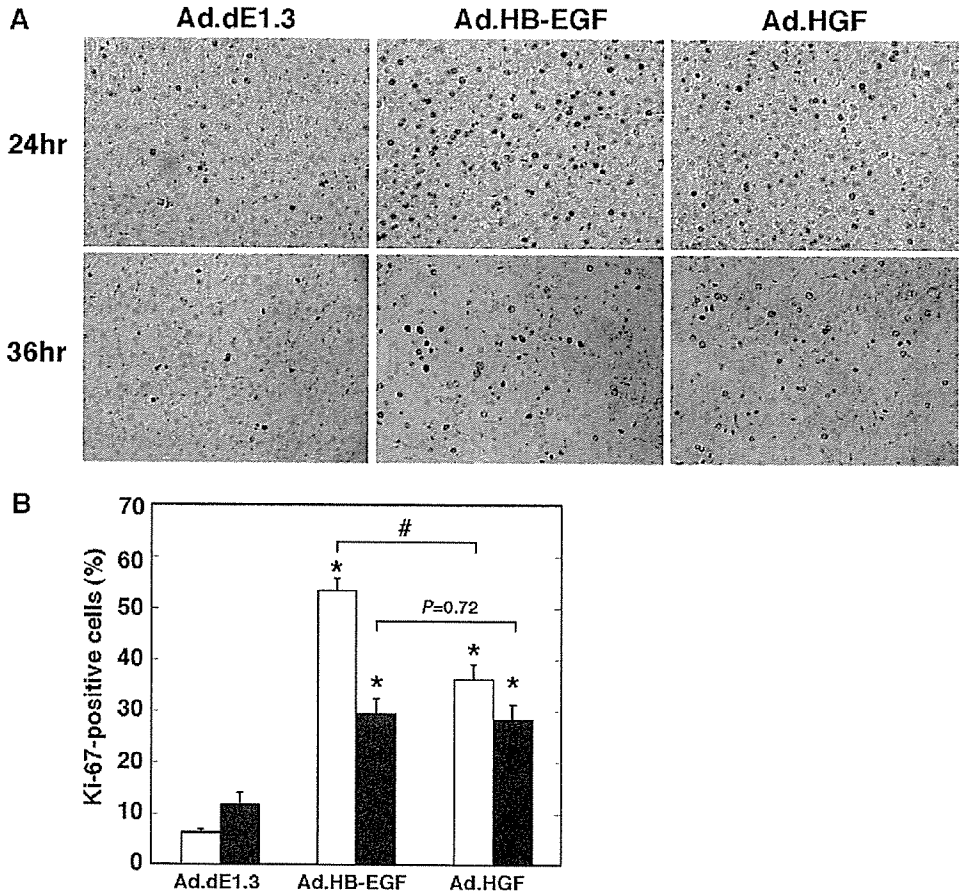

Fig. 5. Ki-67 immunohistochemistry of the liver after HB-EGF or HGF gene therapy for Fas-induced liver injury. (A) Immunohistochemistry against Ki-67 using the liver tissue from the mice that were given injections of the anti-Fas antibody and either Ad.dE1.3, Ad.HB-EGF or Ad.HGF on the protocol shown in Fig. 2(A). Original magnification, ×200. (B) The morphometric and quantitative analysis of Ki-67-positive cells at 24 (white bars) or 36 (black bars) hours after injection of the anti-Fas antibody in each group (* $P<0.01$, either Ad.HB-EGF or Ad.HGF vs. control Ad.dE1.3: # $P<0.01$, Ad.HB-EGF vs. Ad.HGF).

[24,25,38]. Another study showed that the additions of exogenous HB-EGF to nonparenchymal cells in the in vitro primary culture increased the HGF mRNA expressions in these cells, suggesting that HB-EGF may induce HGF production [24,26]. Thus, one hypothesis is that exogenous HB-EGF produced from the transduced human HB-EGF gene may sequentially activate several downstream factors, including HGF, and that these secondary effects together with the direct effect of HB-EGF may additively induce the higher mitogenic activity [39]. It would be interesting to investigate the detailed molecular mechanisms and the possibility of HB-EGF-induced liver regeneration in the case of a no priming event and the possible synergic or additive effects of HB-EGF and HGF combination gene therapy in future extensive studies.

In this study, we used an adenoviral vector and Fas-induced liver injury model solely for exploring the possibility and potential of HB-EGF gene therapy for liver diseases. A biologically important fact concerning the availability of hepatic HB-EGF gene therapy is that synthetic and proteolytic processes were reproduced even in hepatocytes, and that exogenous HB-EGF can efficiently activate hepatocytes in an artificial autocrine fashion. From the clinical viewpoint, more potent protective and mitogenic effects of HB-EGF for hepatocytes than those of HGF are apparently promising and potentially beneficial for treating liver disorders. However, it remains to be elucidated whether HB-EGF is a more clinically useful therapeutic agent for liver diseases than HGF. In actuality, some reports showed that HB-EGF was highly expressed in hepatocytes during hepatocarcinogenesis [40–42], and our recent study has shown that HB-EGF plays pathological roles in heart disorders [28]. In this regard, future studies are needed to investigate the clinical usefulness, including the possible adverse effects (e.g. fibrosis and hepatocarcinogenesis), of HB-EGF gene therapy for chronic hepatitis and liver cirrhosis, as we have carefully done in HGF gene therapy [29].

In conclusion, HB-EGF may have more potent protective and mitogenic activities for hepatocytes than HGF, at least

in part and for the present condition. In vivo HB-EGF gene transduction in the liver is therapeutic for Fas-induced liver injury.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and in part by the Suzuken Memorial Foundation. We would like to thank Hatsue Oshika for her technical assistance, and Dr E. Mekada for providing the human HB-EGF cDNA.

References

[1] Michalopoulos GK. Liver regeneration: molecular mechanisms of growth control. Faseb J 1990;4:176–187.
[2] Fausto N, Laird AD, Webber EM. Liver regeneration. 2. Role of growth factors and cytokines in hepatic regeneration. Faseb J 1995;9: 1527–1536.
[3] Matsumoto K, Nakamura T. Hepatocyte growth factor (HGF) as a tissue organizer for organogenesis and regeneration. Biochem Biophys Res Commun 1997;239:639–644.
[4] Roberts RA, James NH, Cosulich SC. The role of protein kinase B and mitogen-activated protein kinase in epidermal growth factor and tumor necrosis factor alpha-mediated rat hepatocyte survival and apoptosis. Hepatology 2000;31:420–427.
[5] Webber EM, FitzGerald MJ, Brown PI, Bartlett MH, Fausto N. Transforming growth factor-alpha expression during liver regeneration after partial hepatectomy and toxic injury, and potential interactions between transforming growth factor-alpha and hepatocyte growth factor. Hepatology 1993;18:1422–1431.
[6] Birchmeier C, Gherardi E. Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. Trends Cell Biol 1998;8:404–410.
[7] Fausto N. Liver regeneration. J Hepatol 2000;32:19–31.
[8] Pistoi S, Morello D. Liver regeneration 7. Prometheus' myth revisited: transgenic mice as a powerful tool to study liver regeneration. Fed Am Soc Exp Biol J 1996;10:819–828.
[9] Ishiki Y, Ohnishi H, Muto Y, Matsumoto K, Nakamura T. Direct evidence that hepatocyte growth factor is a hepatotrophic factor for liver regeneration and has a potent antihepatitis effect in vivo. Hepatology 1992;16:1227–1235.
[10] Matsuda Y, Matsumoto K, Ichida T, Nakamura T. Hepatocyte growth factor suppresses the onset of liver cirrhosis and abrogates lethal hepatic dysfunction in rats. J Biochem Tokyo 1995;118:643–649.
[11] Ueki T, Kaneda Y, Tsutsui H, Nakanishi K, Sawa Y, Morishita R, et al. Hepatocyte growth factor gene therapy of liver cirrhosis in rats. Nat Med 1999;5:226–230.
[12] Hwang TH, Yoon BC, Jeong JS, Seo SY, Lee HJ. A single administration of adenoviral-mediated HGF cDNA permits survival of mice from acute hepatic failure. Life Sci 2003;72:851–861.
[13] Kosai KI, Finegold MJ, Thi-Huynh BT, Tewson M, Ou CN, Bowles N, et al. Retrovirus-mediated in vivo gene transfer in the replicating liver using recombinant hepatocyte growth factor without liver injury or partial hepatectomy. Hum Gene Ther 1998;9: 1293–1301.
[14] Kosai K, Matsumoto K, Nagata S, Tsujimoto Y, Nakamura T. Abrogation of Fas-induced fulminant hepatic failure in mice by hepatocyte growth factor. Biochem Biophys Res Commun 1998;244: 683–690.
[15] Kosai K, Matsumoto K, Funakoshi H, Nakamura T. Hepatocyte growth factor prevents endotoxin-induced lethal hepatic failure in mice. Hepatology 1999;30:151–159.
[16] Phaneuf D, Chen SJ, Wilson JM. Intravenous injection of an adenovirus encoding hepatocyte growth factor results in liver growth and has a protective effect against apoptosis. Mol Med 2000;6: 96–103.
[17] Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science 1991;251:936–939.
[18] Raab G, Klagsbrun M. Heparin-binding EGF-like growth factor. Biochim Biophys Acta 1997;1333:F179–F199.
[19] Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, et al. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 1999;402: 884–888.
[20] Iwamoto R, Mekada E. Heparin-binding EGF-like growth factor: a juxtacrine growth factor. Cytokine Growth Factor Rev 2000;11: 335–344.
[21] Goishi K, Higashiyama S, Klagsbrun M, Nakano N, Umata T, Ishikawa M, et al. Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: conversion from juxtacrine to paracrine growth factor activity. Mol Biol Cell 1995;6: 967–980.
[22] Ito N, Kawata S, Tamura S, Kiso S, Tsushima H, Damm D, et al. Heparin-binding EGF-like growth factor is a potent mitogen for rat hepatocytes. Biochem Biophys Res Commun 1994;198:25–31.
[23] Higashiyama S, Abraham JA, Klagsbrun M. Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. J Cell Biol 1993;122:933–940.
[24] Kiso S, Kawata S, Tamura S, Higashiyama S, Ito N, Tsushima H, et al. Role of heparin-binding epidermal growth factor-like growth factor as a hepatotrophic factor in rat liver regeneration after partial hepatectomy. Hepatology 1995;22:1584–1590.
[25] Kiso S, Kawata S, Tamura S, Ito N, Tsushima H, Yamada A, et al. Expression of heparin-binding EGF-like growth factor in rat liver injured by carbon tetrachloride or D-galactosamine. Biochem Biophys Res Commun 1996;220:285–288.
[26] Kiso S, Kawata S, Tamura S, Umeki S, Ito N, Tsushima H, et al. Effects of exogenous human heparin-binding epidermal growth factor-like growth factor on DNA synthesis of hepatocytes in normal mouse liver. Biochem Biophys Res Commun 1999;259:683–687.
[27] Kiso S, Kawata S, Tamura S, Inui Y, Yoshida Y, Sawai Y, et al. Liver regeneration in heparin-binding EGF-like growth factor transgenic mice after partial hepatectomy. Gastroenterology 2003;124:701–707.
[28] Ushikoshi H, Takahashi T, Chen X, Khai NC, Esaki M, Goto K, et al. Local overexpression of HB-EGF exacerbates remodeling following myocardial infarction by activating noncardiomyocytes. Lab Invest 2005;85:862–873.
[29] Yuge K, Takahashi T, Nagano S, Terazaki Y, Murofushi Y, Ushikoshi H, et al. Adenoviral gene transduction of hepatocyte growth factor elicits inhibitory effects for hepatoma. Int J Oncol 2005; 27:77–85.
[30] Chen SH, Chen XH, Wang Y, Kosai K, Finegold MJ, Rich SS, et al. Combination gene therapy for liver metastasis of colon carcinoma in vivo. Proc Natl Acad Sci USA 1995;92:2577–2581.
[31] Terazaki Y, Yano S, Yuge K, Nagano S, Fukunaga M, Guo ZS, et al. An optimal therapeutic expression level is crucial for suicide gene therapy for hepatic metastatic cancer in mice. Hepatology 2003;37: 155–163.
[32] Peeters MJ, Patijn GA, Lieber A, Meuse L, Kay MA. Adenovirus-mediated hepatic gene transfer in mice: comparison of intravascular and biliary administration. Hum Gene Ther 1996;7: 1693–1699.

[33] Oksuz M, Akkiz H, Isiksal YF, Saydaogluc G, Serin M, Kayaselcuk F, et al. Expression of Fas antigen in liver tissue of patients with chronic hepatitis B and C. Eur J Gastroenterol Hepatol 2004;16:341–345.
[34] Kondo T, Suda T, Fukuyama H, Adachi M, Nagata S. Essential roles of the Fas ligand in the development of hepatitis. Nat Med 1997;3: 409–413.
[35] Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, et al. Lethal effect of the anti-Fas antibody in mice. Nature 1993;364:806–809.
[36] Ferrell L. Liver pathology: cirrhosis, hepatitis, and primary liver tumors. Update and diagnostic problems. Mod Pathol 2000;13: 679–704.
[37] Mitchell C, Nivison M, Jackson LF, Fox R, Lee DC, Campbell JS, et al. Heparin-binding epidermal growth factor-like growth factor links hepatocyte priming with cell cycle progression during liver regeneration. J Biol Chem 2005;280:2562–2568.
[38] Kinoshita T, Tashiro K, Nakamura T. Marked increase of HGF mRNA in non-parenchymal liver cells of rats treated with hepatotoxins. Biochem Biophys Res Commun 1989;165:1229–1234.
[39] Scheving LA, Stevenson MC, Taylormoore JM, Traxler P, Russell WE. Integral role of the EGF receptor in HGF-mediated hepatocyte proliferation. Biochem Biophys Res Commun 2002;290:197–203.
[40] Inui Y, Higashiyama S, Kawata S, Tamura S, Miyagawa J, Taniguchi N, et al. Expression of heparin-binding epidermal growth factor in human hepatocellular carcinoma. Gastroenterology 1994; 107:1799–1804.
[41] Miyoshi E, Higashiyama S, Nakagawa T, Suzuki K, Horimoto M, Hayashi N, et al. High expression of heparin-binding EGF-like growth factor in rat hepatocarcinogenesis. Int J Cancer 1996;68:215–218.
[42] Kiso S, Kawata S, Tamura S, Miyagawa J, Ito N, Tsushima H, et al. Expression of heparin-binding epidermal growth factor-like growth factor in the hepatocytes of fibrotic rat liver during hepatocarcinogenesis. J Gastroenterol Hepatol 1999;14:1203–1209.

ELSEVIER

Cardiovascular Research 69 (2006) 476 – 490

Cardiovascular Research

www.elsevier.com/locate/cardiores

Bone marrow-derived myocyte-like cells and regulation of repair-related cytokines after bone marrow cell transplantation

Yu Misao [a], Genzou Takemura [a], Masazumi Arai [a], Shigeru Sato [c], Koji Suzuki [a], Shusaku Miyata [a], Ken-ichiro Kosai [b], Shinya Minatoguchi [a], Takako Fujiwara [d], Hisayoshi Fujiwara [a,*]

[a] *Department of Cardiology, Regeneration Medicine and Bioethics, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu, 501-1194, Japan*
[b] *Gene Therapy and Regenerative Medicine, Gifu University Graduate School of Medicine, Gifu, Japan*
[c] *Central Institute for Electron Microscopic Research, Nippon Medical School, Tokyo, Japan*
[d] *Department of Food Science (T.F.), Kyoto Women's University, Kyoto, Japan*

Received 4 August 2005; received in revised form 19 October 2005; accepted 1 November 2005
Available online 20 December 2005
Time for primary review 27 days

Abstract

Objective: Whether bone marrow cells injected following acute myocardial infarction (MI) transdifferentiate into cardiomyocytes remains controversial, and how these cells affect repair-related cytokines is not known.
Methods: Autologous bone marrow-derived mononuclear cells (BM-MNCs) labeled with DiI, 1,1′-dioctadecyl-1 to 3,3,3′,3′-tetramethylindocarbocyanine perchlorate, or saline were intravenously injected into rabbits 5 h following a 30-min ischemia and reperfusion protocol, and cardiac function and the general pathology of the infarcted heart were followed up 1 and 3 months post-MI. To search for regenerated myocardium, electron microscopy as well as confocal microscopy were performed in the infarcted myocardium 7 days post-MI. Expression levels of repair-related cytokines were evaluated by immunohistochemistry and Western blotting.
Results: Improvements in cardiac function and reductions in infarct size were observed in the BM-MNC group 1 month and 3 months post-MI. Using electron microscopy 7 days after infarction, clusters of very immature (fetal) and relatively mature cardiomyocytes undergoing differentiation were identified in the infarcted anterior LV wall in the BM-MNC group, though their numbers were small. These cells contained many small and dense DiI particles (a BM-MNC marker), indicating that cardiomyocytes had regenerated from the injected BM-MNCs. The expression of both transforming growth factor-β, which stimulates collagen synthesis and matrix metalloproteinase-1, a collagenase, were both down-regulated 7 days and 1 month post-MI in the BM-MNC group. Stromal cell-derived factor-1, which is known to recruit BM-MNCs into target tissues, was overexpressed in the infarcted areas of BM-MNC hearts 7 days post-MI.
Conclusions: Intravenous transplantation of BM-MNCs leads to the development of BM-MNC-derived myocyte-like cells and regulates the expression of repair-related cytokines that facilitate repair following myocardial infarction.

Keywords: Myocardial regeneration; Ultrastructure; Cardiac repair; Bone marrow; Cytokines

1. Introduction

Transplantation of bone marrow-derived mononuclear cells (BM-MNCs), including hematopoietic and mesenchymal stem cells, following acute myocardial infarction (MI) diminishes left ventricular (LV) remodeling, improves LV function, and reduces the size of old infarcts in post-MI hearts [1–3]. Nevertheless, the presence of BM-derived cardiomyocytes in infarcted myocardium remains controversial because although they are readily detectable in some cases [1], they are undetectable in others [4–6]. Contributing to this discrepancy may be an overestimation or underestimation of the numbers of BM-derived cardiomyo-

* Corresponding author. Tel.: +81 58 230 6520; fax: +81 58 230 6521.
E-mail address: gifuim-gif@umin.ac.jp (H. Fujiwara).

0008-6363/$ - see front matter © 2005 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.cardiores.2005.11.001

cytes as a result of technical problems with the methods used. For instance, autofluorescence can interfere with the confocal microscopic analysis of immunofluorescence. In addition, earlier studies of post-MI regeneration were carried out using models that involved the permanent occlusion of coronary arteries, though clinical treatment for acute MI generally involves recanalization of infarct-related coronary arteries by thrombolysis or percutaneous angioplasty. Bearing these issues in mind, one of our aims in the present study was to use electron microscopy to determine whether intravenously injected autologous BM-MNCs can transdifferentiate into cardiomyocytes during post-MI repair in a rabbit ischemia–reperfusion model.

We also recently reported that the beneficial effects of granulocyte colony-stimulating factor are likely associated with the expression of repair-related cytokines as well as with cardiomyocyte regeneration [7], which suggests that BM-MNCs may also exert an effect on the expression of repair-related cytokines. Therefore, the second aim of this study was to determine whether expression levels of transforming growth factor (TGF)-β, a mediator stimulating collagen synthesis; matrix metalloproteinase (MMP)-1, a collagenase; or stromal cell-derived factor (SDF)-1, a chemoattractant known to recruit BM-MNCs into target tissues, correlate with post-MI repair after BM-MNC treatment.

2. Materials and methods

All the rabbits received humane care in accordance with the Guide for Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication 8523, revised 1985). The study protocol was approved by the Ethics Committee of Gifu University School of Medicine, Gifu, Japan.

2.1. Autologous BM-MNCs

Male Japanese white rabbits weighing 1.9–2.5 kg were anesthetized by intravenous injection with 30 mg/kg of sodium pentobarbital. Approximately 10 ml of iliac BM was aspirated and suspended in 20 ml of RPMI-1640 medium (Sigma) containing 2000 U of heparin sodium. BM-MNCs were then isolated by centrifugation on a Ficoll gradient (JIMRO, Takasaki, Japan), and approximately 1.0×10^8 BM-MNCs were suspended in 2 ml of phosphate-buffered saline.

2.2. Ischemia–reperfusion infarct model and injection of BM-MNCs

One hour after the aspiration of BM, a 30-min ischemia and reperfusion protocol was carried out as previously described [7]. The rabbits were randomly assigned to either a BM-MNC or saline group, which were respectively administered the autologous BM-MNCs or 2 ml of saline via an ear vein after 5 h of reperfusion.

2.3. Protocol I: cardiac function and pathology

2.3.1. Echocardiography

The 90 rabbits in the BM-MNC and saline groups received trans-thoracic echocardiography (Aloka SSD4000) using a 7.5-mHz sector scan probe 7 days, 1 month and 3 months after the 30-min ischemia ($n=15$ in each group). LV anterior wall thickness (AWT, mm), posterior wall thickness (PWT, mm), body weight-corrected LV end-diastolic dimension (EDD/BW, mm/kg), and LV ejection fraction (EF, %) were measured. All measurements were made by two examiners (Y.M. and M.A.) blinded to the conditions.

2.3.2. General pathology

Following the echocardiography, each of the 90 rabbits was sacrificed with an overdose of pentobarbital after heparinization (500 U/kg), after which body weight and LV weight were measured. The LV was then fixed in 10% buffered formalin and sliced into 7 transverse sections parallel to the atrio-ventricular ring from the apex to the base. The slices were embedded in paraffin, cut to a thickness of 4 μm, and stained with hematoxylin and eosin (HE) and Sirius red. For transversely sliced preparations with infarction, the LV wall areas, infarcted areas, non-infarcted areas, and Sirius red-positive collagen areas were calculated using an image analyzer connected to a light microscope (LUZEX-F, NIRECO, Tokyo) and expressed as mm^2/slice/body weight (kg). Comparisons were made by two persons (Y.M. and M.A.) blinded to the conditions.

2.3.3. Immunohistochemistry

Using the indirect immunoperoxidase method, immunohistochemical staining was carried out with the following monoclonal antibodies (mAbs) that all cross-react with rabbit tissues: mouse anti-rabbit sarcomeric actin mAb (1:75; DAKO), mouse anti-human endothelial cell CD31 mAb (1:100; DAKO), mouse anti-human α-smooth muscle actin mAb (1:250; DAKO, 1A4), mouse anti-macrophage mAb (1:100; DAKO, RAM11), mouse anti-human MMP-1 mAb (1:500; Daiichi Fine Chemical, F-67), mouse anti-human TGF-β mAb (1:2000; Oxford Biotechnology.) and mouse anti-human/mouse SDF-1 mAb (1:120; R&D Systems). Morphometric analyses were carried out by two persons (Y.M. and G.T.) blinded to the conditions.

2.4. Protocol II: DiI-labeling of BM-MNCs, electron microscopy, laser scanning confocal microscopy and Western blotting

BM-MNCs were isolated from 12 rabbits as described above and then incubated for 30 min at 37 °C. To label the