Cell cycle-specific changes in hTERT promoter activity in normal and cancerous cells in adenoviral gene therapy: A promising implication of telomerase-dependent targeted cancer gene therapy

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Abstract. Based on the finding that telomerase is reactivated solely in cancer cells, the human telomerase reverse transcriptase (hTERT) promoter has recently been used to target cancer cells by gene therapy. The recent, surprising observation that telomerase is physiologically activated even in normal somatic cells during S-phase has raised concerns as to the safety of this methodology. To clarify this issue, the present study carefully examined the changes in endogenous telomerase activities, hTERT mRNA expression, and hTERT promoter-based transgene expression in normal and cancer cells at synchronized phases of the cell cycle. Telomerase activity and hTERT expression were detected at variable, but relatively high, levels in all 12 cancer cell lines, while both were undetectable in the 11 normal cell lines. In HepG2 cancer cells, the highest levels of hTERT expression and telomerase activity, seen in the G_1/S- and S-phases, were 2-3-fold higher than the lowest levels of both, observed in G_0-phase and during asynchronization. No hTERT expression or telomerase activitiy could be detected in normal WI-38 fibroblasts at any phase of the cell cycle, including S-phase. Consequently, activity of the shorter hTERT promoter, which was transferred into HepG2 cancer cells via adenovirus transduction, was stronger than that of the longer hTERT promoter at all phases and that of two representatives of ubiquitously strong promoters, at both S-phase and asynchronization, but not at G_0-phase. In contrast, neither of hTERT promoters induced detectable transgene expressions in normal WI-38 cells at any cell cycle phase, including S-phase. These results, particularly the lack of problematic levels of S-phase-specific activation of hTERT promoters in normal cells, have promising implications for hTERT promoter-based targeted gene therapy of cancer.

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Abbreviations: hTERT, human telomerase reverse transcriptase; CRA, conditionally replicating adenovirus; HPRT, hypoxanthine guanine phosphoribosyl transferase; TRAP, telomeric repeat amplification protocol; RSV, Rous sarcoma virus long-terminal repeat; CMV promoter, cytomegalovirus immediate-early gene enhancer/promoter; MOI, multiplicity of infection

Key words: adenovirus, cancer gene therapy, cancer-specificity, hTERT, targeting cancer, promoter, telomerase, telomere

Introduction

Telomeres are the distal ends of human chromosomes composed of tandem repeats of the sequence TTAGGG. These sites may function to stabilize chromosomal ends and prevent chromosome degradation, end-to-end fusion, rearrangement, and loss (1-4). Telomeres in somatic cells undergo progressive shortening with each successive cell division; it has been hypothesized that the reduction in telomere length may function as an intrinsic clock involved in the onset of cellular senescence (1,2,5). In immortal cells, telomeres are resynthesized and maintained by telomerase, a specialized DNA polymerase responsible for replication of chromosomal ends (6,7). Human telomerase reverse transcriptase (hTERT), the catalytic subunit of human telomerase, is the major determinant of telomerase activity; ectopic expression of hTERT is sufficient to reconstitute telomerase activity in telomerase-negative cells

(1,2,8,9). Human telomerase activity and hTERT expression are detected in the majority (>90%) of human cancer cells, but are typically absent from normal cells (1,2,10,11). In conjunction with the fact that immortalization correlates well with a stabilization of telomere length, it has been proposed that human cancer cells achieve immortalization through illegitimate activation of telomerase expression (1,2,6).

One of the major challenges of cancer gene therapy is the restriction of transgene expression to cancer cells, because non-specific, extratumoral expression of therapeutic genes would result in the destruction of normal tissues (12,13). Tissue-specific promoters, such as the carcinoembryonic antigen promoter (14), have been used as a treatment for adenocarcinoma to achieve tumor-specific transgene expression. These tissue-specific promoters, however, have the disadvantage of targeting only limited cancer types. In addition, they may exhibit insufficient cancer specificity (leaky activation in normal cells) and/or weak activity, even in cancer cells. To increase the potential efficacy of gene therapy, hTERT promoters have been utilized for cancer gene therapy (13,15,16). Although the lengths of the promoter used differed between reports, hTERT promoter-based transgene regulation should be able to target a broad range of cancers with little effect on mature somatic cells. Recently, the hTERT promoter was also used to generate a conditionally replicating adenovirus (CRA); several studies have demonstrated that hTERT promoter-based CRA can selectively replicate in and kill a panel of cancer cells (17-20).

The majority of previous studies examining hTERT promoter-based cancer gene therapy focused on achieving efficacy in particular cancer models (13,15,16,21,22). The potential adverse effects in normal cells have not yet been thoroughly investigated, although some concerns have been implicated by the fact that telomerase activity is observed at low levels in certain normal cells, such as bone marrow and peripheral blood mononuclear cells (23,24). Relatively high levels of telomerase activity have been reported in hematopoietic cells, the basal layer of the epidermis, endometrial tissues during the menstrual cycle, fetal tissues, and the proliferative zone of intestinal crypts (1,24,25). Surprisingly and unexpectedly, endogenous telomerase activity and hTERT expression could also be detected in an S-phase-specific manner in common normal somatic fibroblasts, which were previously thought to lack both; the upregulated telomerase played a physiological role in the proliferation of normal cells (26). These results strongly suggest a need to evaluate carefully the potential adverse effects of such treatment, specifically determining if leaky expression of a transgene under the control of the hTERT promoter would occur in normal cycling cells to achieve harmful levels. This study carefully examined the changes in telomerase activity, hTERT expression, and hTERT promoter-based transgene expression in normal and cancer cells at specific phases of the cell cycle. The obtained results provide important general implications for hTERT promoter-based targeted cancer gene therapy.

Materials and methods

Cell culture. The human cancer cell lines MKN-1, -28, and -45 (gastric cancer), HCT-15, LoVo, and colo-205 (colon cancer), HepG2 and Hep3B (hepatoma), HeLa (cervical cancer), and HOS-MNNG, KHOS-NP, and SaOS-2 (osteosarcoma) were obtained and maintained as described previously (20). Normal human cell lines, WI-38 and IMR-90 (lung fibroblasts), were obtained from the RIKEN Cell Bank (Tsukuba, Japan), while MRC-5 (lung fibroblasts) and HUV-EC (human umbilical vein endothelial cells) were obtained from the Health Science Research Resources Bank (Osaka, Japan). Primary cultured human cells, NHDF (dermal fibroblasts), NHOst (osteoblasts), HMVEC-d (dermal-derived microvascular endothelial cells), HMEC (mammary epithelial cells), PrEC (prostate epithelial cells), HRE (renal epithelial cells), and SAEC (small airway epithelial cells) were obtained from Cambrex Bio Science Walkersville (Walkersville, MD, USA). All normal cells were maintained according to the manufacturer's protocol.

Reverse transcription-polymerase chain reaction analysis (RT-PCR). Extraction of total RNA from cells and semi-quantitative RT-PCR analysis of hTERT and hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA levels were performed as described previously (27,28). For nested RT-PCR analysis, cDNA was subjected to initial PCR amplification with 20 cycles of 94°C for 30 sec, 59°C for 60 sec and 74°C for 60 sec in the presence of Taq DNA polymerase (Promega, Madison, WI, USA) and primer set 1 (P1; sense, 5'-TTCCTGCACTGGCTGATGAGTGT-3', and antisense, 5'-CGCTCGGCCCTCTTTTCTCTG-3') (29). Subsequently, 1/25 of the amplified cDNA was subjected to a second PCR amplification of 35 cycles of 94°C for 45 sec, 60°C for 45 sec and 72°C for 90 sec with Taq DNA polymerase and primer set 2 (P2; sense, 5'-CCTGCTGGATTACATTAA AGCACTG-3', and antisense, 5'-AAGGGCATATCCAACA ACAA-3') (7).

Endogenous telomerase activity. Endogenous telomerase activity in cells was examined using the telomeric repeat amplification protocol (TRAP) with *T*elo *TAGGG* Telomerase PCR ELISA^{PLUS} kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol.

Cell cycle analysis. Cell cycle synchronization was induced as described (30), with the following modifications. Briefly, synchronization of cells in G_0-, S-, or G_2/M-phases was achieved by treatment with serum starvation (0.5%) for 72 h, 0.3 mM hydroxyurea (Sigma-Aldrich, St. Louis, MO, USA) for 32 h, or 0.4 μg/ml nocodazole (Sigma-Aldrich) for 20 h, respectively. Cells were synchronized in G_1/S phase by treatment with 5 mM thymidine (Sigma-Aldrich) for 20 h, followed by treatment with 5 μg/ml aphidicolin (Sigma-Aldrich) for 16 h (30). The percentage of cells entering each phase of the cell cycle was determined by flow cytometric analysis of propidium iodide-stained cells using a FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA) and ModFit software (Verity, Topsham, ME, USA).

Generation of adenoviral vectors. The short (260-bp; -181 to +79) and long (1454-bp; -1375 to +79) hTERT promoters [hTERT(S) and hTERT(L)] were isolated by *Mlu*I/*Bgl*II digestion from the pGL3-181 and pGL3-1375 plasmids (the kind gift of Dr S. Kyo, Kanazawa University, Kanazawa,

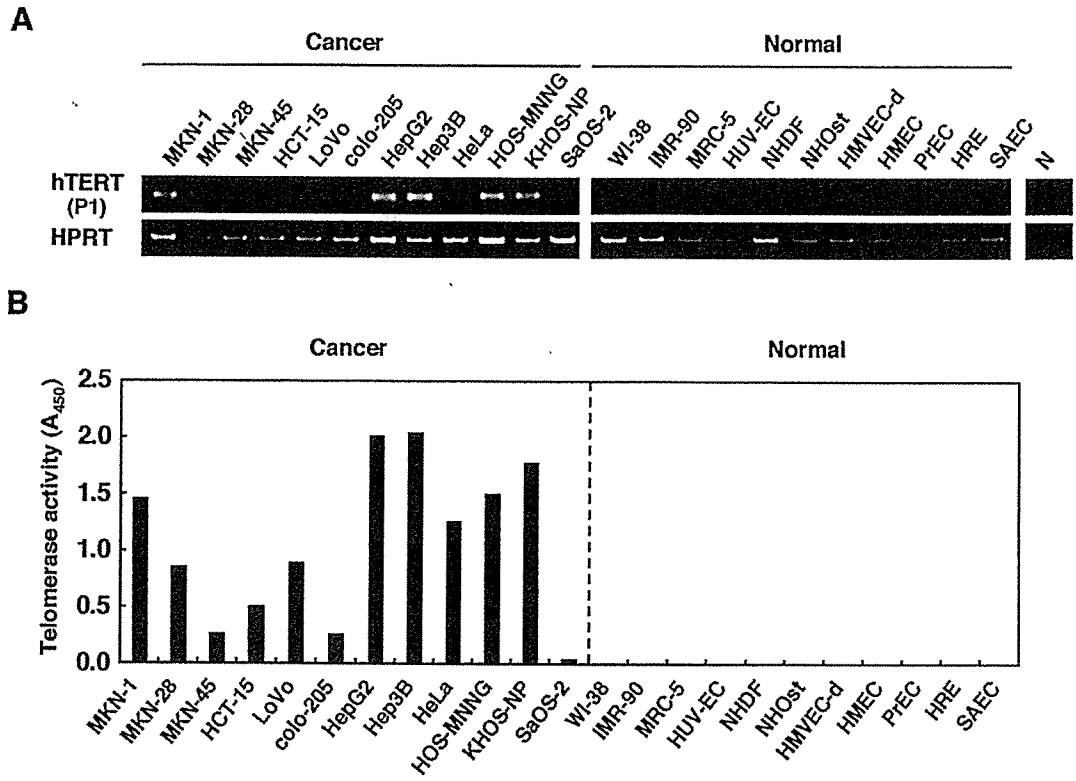

Figure 1. Endogenous hTERT expression and telomerase activity in a variety of cancer and normal cells. (A) Endogenous hTERT mRNA was detected by standard RT-PCR using the primer set 1 (P1) in all of 12 cancer cell lines, but was undetectable in 11 normal cells. Amplification of the HPRT gene served as an internal control. N, no template served as a negative control. (B) Endogenous telomerase activity was detected by TRAP assay at varying levels in all cancer cell lines, but could not be detected in any of the normal cells. Data are represented as the levels at A_{450}.

Japan), respectively (31). We generated E1-deleted replication-defective adenoviral vectors, Ad.hTERT(S)-LacZ, Ad.hTERT(L)-LacZ, Ad.RSV-LacZ, and Ad.CMV-LacZ, which express the LacZ gene under the control of the hTERT(S) promoter, the hTERT(L) promoter, the Rous sarcoma virus long-term repeat (RSV) promoter, and the cytomegalovirus immediate-early gene enhancer/promoter (CMV promoter), respectively (20,32). Adenoviral stocks were prepared and titered as described previously (33,34).

Promoter activity. HepG2 ($4x10^5$ cells/well) and WI-38 ($1.2x10^5$ cells/well) cells in 6-well plates were infected with each adenoviral vector at a multiplicity of infection (MOI) of 10 for 1 h and at MOI of 30 for 24 h, respectively, conditions which provided almost similar gene transduction efficiencies in both cell types (about 30%) without apparent cytotoxicity. The cells were synchronized at each phase of the cell cycle as described above and subsequently harvested. ß-galactosidase activity was measured using a ß-Galactosidase Enzyme Assay System (Promega) as described previously (14,20).

Results

Endogenous hTERT mRNA levels and telomerase activities in various human cancerous and normal cell types. Consistent with our previous results, hTERT mRNA expression was readily detected by standard semi-quantitative RT-PCR analysis in all of the examined cancer cells, which were derived from a variety of tissue origins; a more sensitive nested RT-PCR was not necessary for detection. The expression levels varied considerably among cell types (Fig. 1A); notably, both hepatoma cell lines (HepG2 and Hep3B) and two of the three osteosarcoma cell lines (HOS-MNNG and KHOS-NP) exhibited high expression levels of hTERT mRNA. Endogenous telomerase activity was detected by TRAP assays in all 12 cancer cell lines at varying levels; eleven cancer cell lines exhibited relatively high activity, while SaOS-2 cells, which have been reported as a telomerase-negative osteosarcoma (35,36), displayed only low levels of telomerase activity (Fig. 1B). The telomerase activity in each cancer cell line correlated well with the expression levels of hTERT mRNA. In contrast, neither hTERT mRNA expression nor telomerase activity could be detected in the 11 normal cell lines derived from a variety of tissues.

Thus, these findings support the widely accepted notion that hTERT is the telomerase catalytic subunit and is reactivated specifically in cancer cells (6,7,37), although the diversity of levels between individual cell lines is relatively large.

Expressions of endogenous hTERT mRNA in cancer and normal cells at each phase of cell cycle. Previous studies demonstrated that telomerase activity in cancer cells changed throughout the cell cycle (30,38). A recent study revealed that hTERT was also expressed in normal human fibroblasts, which were previously thought to lack hTERT expression and telomerase activity. This expression, however, was restricted to the S-phase (26). These results suggest that hTERT

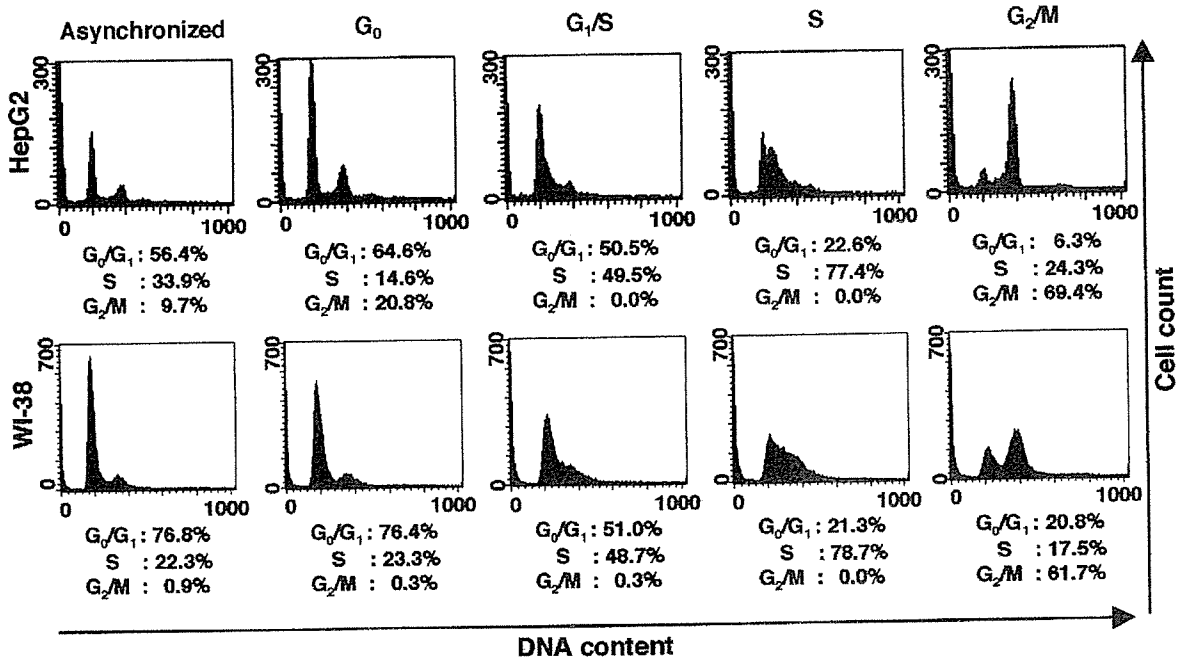

Figure 2. Synchronization of cancer and normal cells at specific phases of the cell cycle. HepG2 cancer and WI-38 normal cells were synchronized at G_0-, G_1/S-, S-, and G_2/M-phases by treatment with 0.5% serum starvation for 72 h, 5 mM thymidine for 20 h with 5 μg/ml aphidicolin for additional 16 h, 0.3 mM hydroxyurea for 32 h, and 0.4 μg/ml nocodazole for 20 h, respectively. The percentage of the cells entering the specific phase of the cell cycle was determined by flow cytometric analysis of propidium iodide-stained cells.

expression and/or telomerase activity might be detected in many normal cell types during S-phase of the cell cycle. We next compared the maximal levels of both hTERT expression and telomerase activity in normal cells with those in cancer cells. After synchronizing HepG2 hepatoma cells and WI-38 normal fibroblasts, we examined hTERT mRNA expression and telomerase activity. We chose the HepG2 and WI-38 cell lines for these experiments, because they have been widely used for both telomerase (26,30,37) and adenoviral gene therapy studies (20,34,39).

Through several pilot studies, we determined the optimal conditions for cell synchronization and adenoviral gene delivery/expression, as shown in Materials and methods. HepG2 and WI-38 cells were successfully synchronized and/or arrested at G_0-, G_1/S-, S-, or G_2/M-phases by treatment with serum starvation, thymidine/aphidicolin, hydroxyurea, or nocodazole, respectively. Assessment of DNA content by flow cytometry demonstrated that the percentages of cells in the desired phases of the cell cycle were high and similar between HepG2 and WI-38 cells (Fig. 2).

We examined the expression of endogenous hTERT mRNA in HepG2 and WI-38 cells by RT-PCR analysis at each phase of the cell cycle. HepG2 cancer cells exhibited high levels of hTERT mRNA at all phases of the cell cycle, including G_0-phase, as well as under conditions of asynchronization (Fig. 3A). In contrast, hTERT mRNA expression could not be detected in WI-38 normal fibroblasts at any phase of cell cycle, either by standard RT-PCR analysis or the more sensitive nested RT-PCR analysis (Fig. 3B), although a recent report noted that hTERT mRNA could be detected in WI-38 cells during S-phase by standard PCR using the same primers (P2) (26).

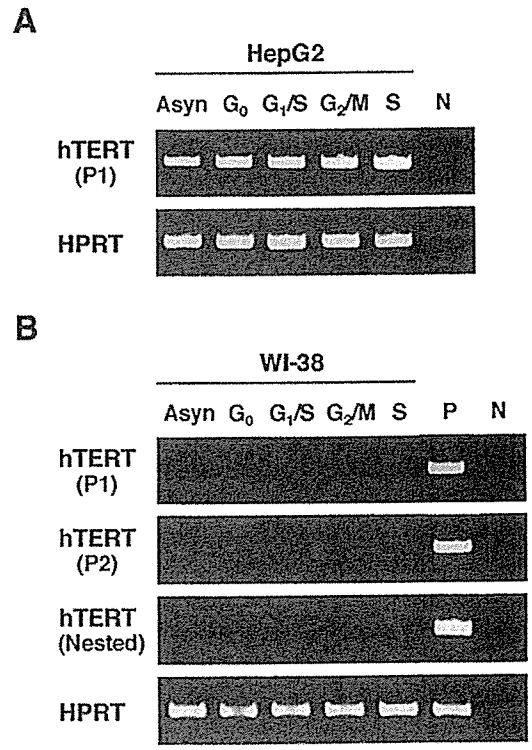

Figure 3. Endogenous hTERT mRNA expression at specific phases of the cell cycle in cancer and normal cells. HepG2 (A) and WI-38 (B) cells were synchronized at each phase of cell cycle, as described in Fig. 2. hTERT mRNA expression was then examined by RT-PCR. (A) hTERT mRNA expression was prominent in HepG2 cancer cells at all phases by standard RT-PCR analysis using the primer set 1 (P1). (B) In contrast, hTERT mRNA expression could not be detected by either standard RT-PCR using primer sets 1 or 2 (P2) or by nested RT-PCR. The HPRT gene was amplified as an internal control. Asyn, asynchronized cells; N, no template served as a negative control; P, template from HepG2 served as a positive control.

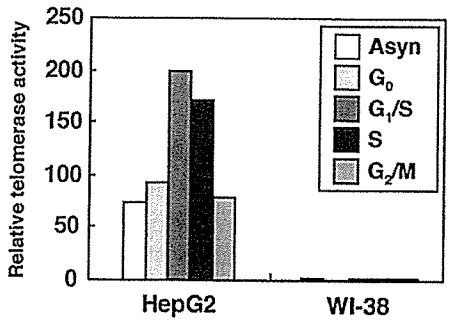

Figure 4. Endogenous telomerase activities at specific phases of the cell cycle in cancerous and normal cells. HepG2 and WI-38 cells were synchronized at each phase of the cell cycle, as described in Fig. 2. Telomerase activities were examined by TRAP assay. Data were represented as telomerase activity normalized to the supplied standard control.

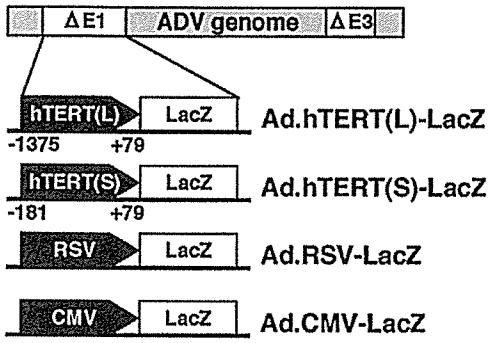

Figure 5. Schematic representation of replication-defective adenoviral vectors. A LacZ gene, downstream of the hTERT(S), hTERT(L), RSV, or CMV promoters was inserted into E1-deleted replication-defective adenoviral vectors.

Endogenous telomerase activities in cancer and normal cells at each phase of cell cycle. Next, we examined endogenous telomerase activity in HepG2 and WI-38 cells by TRAP assay at each phase of cell cycle. High levels of telomerase activity were observed in HepG2 hepatoma cells throughout the cell cycle, including in G_0-phase, although cell cycle-dependent changes of telomerase activity levels were prominent (Fig. 4). Telomerase activity at G_1/S- and S-phases were 2-3-fold higher than that seen under conditions of asynchronization. Despite the S-phase-specific upregulation (30,38), the fact that telomerase activities in G_0 and G_2/M (i.e., out of S-phase of cell cycle) remain relatively high is surprising. In contrast, no telomerase activity was detected in WI-38 normal fibroblasts at any phase of the cell cycle. These results suggest that both baseline hTERT expression and the resulting telomerase activity are significantly higher in cancer cells than the maximal levels seen in normal cells, despite the tight regulation of hTERT expression in both cell types in a cell cycle-dependent manner.

Cell cycle-dependent transgene regulation in cancer and normal cells using the hTERT promoters carried in an adenoviral vector. Although several studies have identified hTERT promoters of different lengths, a recent report demonstrated that two hTERT promoters [hTERT(L): -1375 to +79 and hTERT(S): -181 to +79] had similar, potent activities in several cancer cell lines (31). We therefore constructed four E1-deleted replication-defective adenoviral vectors, Ad.hTERT(L)-LacZ, Ad.hTERT(S)-LacZ, Ad.RSV-LacZ, and Ad.CMV-LacZ, which expressed LacZ under the control of the hTERT(L), hTERT(S), RSV, and CMV promoters; the latter two, both strong promoters functioning in all cell types, served as positive controls (Fig. 5). We initially infected either HepG2 or WI-38 cells with each adenoviral vector. After either synchronizing the cells at G_0- or S-phase or leaving the cells without any synchronization, we measured ß-galactosidase activity, as described in the Materials and methods.

Highly S-phase-specific transgene expression was observed in HepG2 cancer cells after adenoviral delivery of transgenes controlled by either hTERT promoter; the activities of both hTERT promoters in HepG2 cells at S-phase were 5-7-fold higher than those seen at G_0-phase (Fig. 6). Unexpectedly, the activity of hTERT(S) was significantly higher than that of

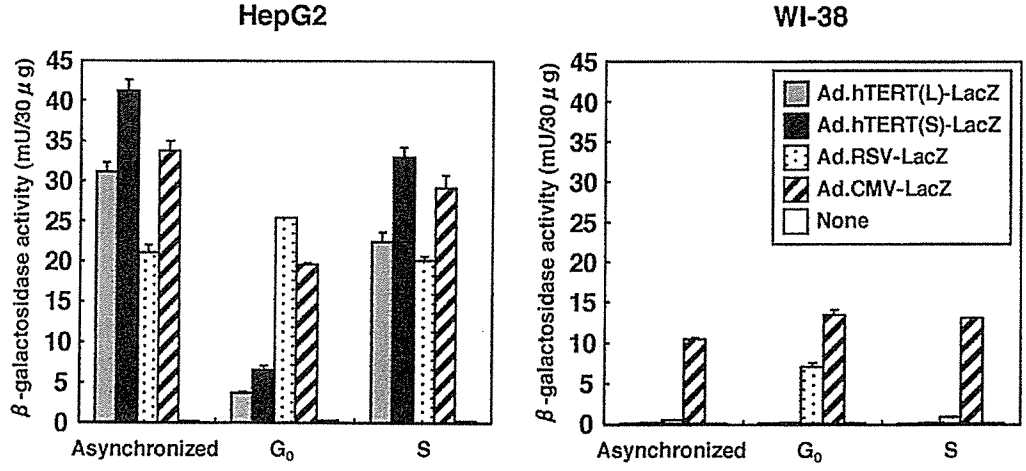

Figure 6. hTERT promoter activity at specific phases of the cell cycle in cancerous and normal cells. HepG2 and WI-38 cells were infected with either adenoviral vector shown in Fig. 5, then synchronized at each phase of the cell cycle as described in Fig. 3. We then measured ß-galactosidase activities; each bar represents the mean ± the standard error.

hTERT(L) in HepG2 cells under all conditions, including G_0- and S-phases. Notably, hTERT(S) activity at S-phase and under conditions of asynchronization, but not during G_0-phase, was higher than RSV and CMV promoter activities. In contrast, no hTERT(S) or hTERT(L) activity could be detected in WI-38 normal fibroblasts at any phase of the cell cycle or under conditions of asynchronization.

Discussion

Although the anti-cancer effects and cancer-selectivity of hTERT promoter-based cancer gene therapy look promising (13,15,16,21,22), the potential safety issues have remained concerning, because of both the potential telomerase activity in subsets of normal cells and the physiological upregulation of telomerase activity specifically during S-phase of the cell cycle, recently reported even in normal somatic cells, which were previously believed to lack both hTERT expression and telomerase activity (1,23-25). Through careful analysis of telomerase throughout the cell cycle, we elucidated the critical potential safety issues of hTERT promoter-based gene therapy. The results were promising, supporting the safe clinical applicability of hTERT promoter-based gene therapy. We observed high cancer-specificity of endogenous telomerase activity and hTERT expression (Fig. 1); the generality of this finding was verified using 12 cancerous and 11 normal cell types, including WI-38 normal fibroblasts previously used by Masutomi *et al* (26). In addition, endogenous hTERT expression, endogenous telomerase activity, and hTERT promoter activity in WI-38 normal fibroblasts were undetectable at all phases of the cell cycle, including S-phase. This result sharply contrasted the high levels of all three seen in HepG2 cancer cells even at G_0-phase. The discrepancy of our results with the previous study reporting detectable telomerase activity in normal cells may result from the different experimental systems and methods used; these methods included the release after synchronization in contrast to direct synchronization to obtain specific phases of the cell cycle, conventional transfection in contrast to adenoviral gene transduction, and the sensitive immunoprecipitation-TRAP as opposed to the standard TRAP assay in the previous and current studies, respectively (26).

The recently-proposed theory that S-phase-specific activation of telomerase is physiologically necessary for proliferation, but not the maintenance of telomere length, of normal cells remains a possibility (26). The levels of telomerase activity in normal cells, however, are significantly lower than those in cancer cells, being almost undetectable by the standard TRAP assay. The abnormally high telomerase activities in cancer cells may play a different, pathological role in the maintenance of the telomere length, leading to cancer cell immortalization (1,2,6). The differences in hTERT expression levels and hTERT promoter activities between cancer and normal cells should be much larger than the fluctuations seen in each cell subset throughout the cell cycle, regardless of parent cell type. Consequently, leaky transgene expression in normal cells remains close to undetectable levels, which likely does not approach a problematic level for the majority of hTERT promoter-based targeting cancer gene therapies.

Insufficient activity of tissue-specific promoters, as well as insufficient cancer-specificity has remained the critical problem with tissue-specific promoter-based targeting cancer gene therapy (40-42). These drawbacks significantly diminish the clinical utility of this technique, as weak anti-cancer effects often result in little or no benefit. The activity of hTERT(S) promoters were stronger than that of the RSV and CMV promoters, representative strong promoters, in HepG2 cancer cells under conditions of asynchronization and during S-phase, although it was weaker than the RSV and CMV promoters at G_0-phase. While stronger promoter activity than that of RSV and CMV does not guarantee an increased efficacy in all cancer gene therapy strategies, previous studies suggested that stronger therapeutic gene expression resulted in more beneficial outcomes using a variety of cancer gene therapy approaches (12,13). In previous studies, the RSV promoter was the best of three representative strong promoters for achieving optimal therapeutic expression levels of a suicide gene, providing maximal anti-cancer effects without conspicuous adverse side effects in the treatment of metastatic liver cancer (14). Therefore, a good quality for a useful cancer-specific promoter should be stronger activity than the RSV promoter in targeted cancer cells (14). Thus, stronger activity of the hTERT(S) promoter than those of RSV and CMV promoters is clearly beneficial, supporting the usefulness of hTERT(S) in cancer gene therapy. It would be interesting to compare hTERT(S) activity with therapeutic potential using specific therapeutic genes and cancer models in future studies that focus on potential clinical applications.

Previous studies have utilized several different hTERT promoter lengths of 1720-bp (-1543 to +77) (43,44), 457-bp (-378 to +79) (13,22), 204-bp (-239 to -36) (16,17), and 260-bp (-181 to +79) (15,21) for gene therapy strategies. Each hTERT promoter region worked well when examined individually, but these have not been compared with each other in gene therapy strategies. We therefore carefully evaluated the activities and cancer specificities of the longest [hTERT(L); -1375 to +79] and the shortest [hTERT(S); -181 to +79] hTERT promoters in specific phases of the cell cycle. These studies elucidated that hTERT(S) exerted stronger activity in cancer cells at all phases of the cell cycle and under conditions of asynchronization than the hTERT(L) promoter. The differential promoter activities may be explained by negative regulatory elements between -578 and -378, upstream of the transcriptional start site of the hTERT gene; a previous study suggested that MZF-2 (myeloid-specific zinc finger protein 2) bound to this site, potentially playing a role in the transcriptional repression of hTERT (45). The most important finding, however, for the application of gene therapy technology to clinical medicine is that neither hTERT(L) nor hTERT(S) exhibited any detectable promoter activity in normal cells at any phase of the cell cycle, including S-phase. Taken together, this study indicates that hTERT(S) will be effective and safe for future targeted cancer gene therapy, at least in combination with adenoviral gene therapy.

In conclusion, hTERT(S), the suitable hTERT promoter, carried by an adenoviral vector conferred strong transgene expression in a strictly cancer- and S-phase-specific manner. The levels of S-phase-specific hTERT promoter activity in

normal cells were virtually undetectable, which will likely not be problematic for targeted cancer gene therapy.

Acknowledgments

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Genetic Modification of Hepatocytes Towards Hepatocyte Transplantation and Liver Tissue Engineering

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Cell-based therapies, including liver tissue engineering following hepatocyte transplantation, have therapeutic potential for several types of liver diseases. Modifications in the methodology to manipulate the donor hepatocytes in a more simple and timely manner prior to transplantation would enhance the therapeutic efficacy of this procedure. Conventional approach for vector-mediated gene transduction to the isolated hepatocytes has been performed under primary culture conditions that routinely require several days to complete. In our study, we have established a clinically feasible approach that requires only 1 h of infection time with an adenoviral vector system that results in an extremely efficient transduction efficiency (>80%). To optimize transduction efficiency and sustain normal cellular function, we determined that the isolated hepatocytes should be maintained in UW solution as a suspension medium and infected with adenoviral vectors (Ad) for no more than 1 h at a MOI of 1. To establish if the isolated hepatocytes could be used as a source for cell-based therapies, we transplanted the Ad-transduced hepatocytes into the liver or under the kidney capsule. When the cells were transplanted into the liver, Ad-transduced hepatocytes cultured in suspension conditions were found to have a significantly higher survival rate ($p < 0.01$) than Ad-transduced hepatocytes cultured under standard conditions. We also confirmed that these Ad-transduced hepatocytes have ability to survive long term and were able to engineer a biologically active hepatic tissue under the kidney capsule. Finally, we obtained high level of transduction into canine, porcine, and human isolated hepatocytes in a suspension solution mixed with Ad. In conclusion, the present studies demonstrate that isolated hepatocytes could be genetically modified using Ad when kept in a suspension solution. For this reason, this cell-modified technique could be used for the treatment of liver-targeted diseases and/or disorders.

Key words: Hepatocyte transplantation; Gene modification; Ex vivo gene therapy; Adenoviral vector; Liver tissue engineering

INTRODUCTION

The use of primary or genetically modified primary hepatocytes offers new-generation therapeutic approaches, including hepatocyte transplantation, in the treatment of various types of liver diseases (8,12,13,35,48). Clinically, isolated hepatocytes have been transplanted into the livers of more than 60 patients to provide a temporal bridge until an orthotopic liver transplantation can be performed, in cases such as fulminant liver failure or as an improvement of hepatic metabolic deficiency (48). Recent clinical hepatocyte transplantation trials have reported encouraging results, providing significant therapeutic efficacy that persisted for a long-term period of time (15,29). In most of the cases, hepatocyte transplantation had been conducted by infusing cells into the liver through the portal vein or via the splenic circulation. However, one of the major limiting factors has been the low number of hepatocytes that can be efficiently infused into the liver without causing complications (i.e., 2–5% of the total number of viable hepatocytes of the host liver) (15,35,46). Moreover, researchers have shown that only a fraction (10–20%) of the transplanted hepatocytes into the liver is able to be engrafted in the liver parenchyma from the portal pedicles (19,28). In order to facilitate hepatocyte transplantation as a more clinically effective therapeutic approach, it would be important to increase the engraftment rate of the hepatocytes and to develop a

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method to maintain hepatocytes at a more optimal functioning status.

Another innovative way to develop new therapies towards the treatment of liver diseases would be engineering new hepatic tissue using isolated hepatocytes, which would be transplanted into ectopic sites (16,26,35). To address this issue, a number of studies have been conducted to determine whether hepatocyte transplantation is possible in ectopic sites outside of the liver, but the results have been largely unsuccessful in terms of ineffective engraftment as well as poor survival rates. In our lab, we have targeted the space under the kidney capsule and other subcutaneous sites as possible ectopic delivery of the isolated hepatocytes, which have recently been shown to provide higher hepatocyte engraftment efficiencies and persistent survival rates (34,36,39–41). It is conceivable that additional modifications would enhance the likelihood of effective hepatic tissue engineering in these sites, and would allow for this application to be a viable therapeutic modality.

Genetic modification of the isolated hepatocytes followed by transplantation, called ex vivo gene therapy, could be a valuable approach to enhance hepatocyte engraftment and survivability. An important factor required for the ex vivo gene therapy is to achieve a high level of transduction efficiency without cellular function loss causing by toxicity issues. Among currently available viral vectors for hepatocyte ex vivo gene manipulation, many of the studies have used simple retroviral vectors (22,23). One clinical trial has been conducted by transplanting genetically modified hepatocytes using retroviral vectors (18). Unfortunately, the therapeutic efficacy of these experimental and clinical studies has been limited, due in large part to the extremely low transduction efficiency into the hepatocytes by the retroviral vectors. Another potential problem with retroviral vectors is the length of time that is required (i.e., several days) to produce the minimally effective transduction into the hepatocytes, but this extended period of time can result in hepatocyte dedifferentiation, leading to a functional loss and loss of engraftment ability (22,23,38). In marked contrast, recombinant adenoviral (Ad) vectors have shown remarkable transduction in the liver (mainly hepatocytes) when administered into an intravenous route (24). Predominant expression of a receptor for adenovirus, CAR, in the hepatocytes contributes to the high adenovirus affinity to the hepatocytes (30).

In the present study, we attempted to exploit the liver-targeting advantage displayed by the Ad vectors to effectively promote high transduction into the isolated hepatocytes in a short period after isolation (i.e., hours rather than days). Vector infection conditions in terms of suspension media selection, Ad vector dose, as well as incubation period were examined in the isolated hepatocytes to optimize transduction efficiencies. Subsequently, the genetically modified hepatocytes were transplanted into either the liver or under the kidney capsule in order to assess their engraftment and long-term survivability. This study demonstrated the potential of the Ad vector-manipulated hepatocytes to act as a cell source for transplantation or tissue engineering purposes in the treatment of liver diseases.

MATERIALS AND METHODS

Animals

Transgenic mice expressing human alpha-1 antitrypsin (hAAT) under the hepatocyte-specific promoter (hA1AT-FVB/N, H-2^q, 12–15 weeks old) (7) were used as a donor of hepatocytes for vector infection experiments and transplantation experiments. Wild type FVB/N mice (10–12 weeks old) were maintained at the Animal Center at Nara Medical University and used as the recipient mice for the hepatocyte transplantation. All animal procedures were conducted in accordance with the institutional guidelines set forth by Nara Medical University Animal Care Committee. Mice were placed in cages within a temperature-controlled room with a 12-h light/dark cycle and ad libitum access to food and water.

Hepatocyte Isolation and Purification

Hepatocytes were isolated from hAAT transgenic mice using a modified two-step collagenase perfusion method (5) with slight modification as previously described (7,34,38,40). Briefly, the liver was directly perfused using a catheter placed into the inferior vena cava with Hank's balanced salt solution (Sigma, St. Louis, MO) containing 0.09% EGTA followed by a second solution containing 0.03% collagenase type I (Sigma) and $CaCl_2$ (5 mM). Isolated cells were filtered through a nylon mesh membrane and hepatocytes were then purified by centrifuging at $50 \times g$ for 5 min. Cells were resuspended with DMEM medium (Sigma), and the cell viability was determined by trypan blue exclusion test. In our experiments, we only used isolated hepatocytes from a particular isolation if the viability of the cells exceeded 90%. In some experiments, hepatocytes were isolated from a piece of canine, porcine, and human liver pieces as described previously (15,29,34). Human liver samples were surgically obtained from a noncancerous portion of benign liver tumors. Written informed consent was given to the patient prior to the surgery. After isolation, the same purification steps as the mouse hepatocytes were followed.

Vector Preparation

Replication-defective recombinant adenoviral (Ad5) vector expressing either lacZ (Ad-CA-lacZ) or a non-

functional β-galactosidase-neomycin phosphotransferase (Ad-CA-null) driven by the CAG promoter (cytomegalovirus immediate-early enhancer, a modified chicken β-actin promoter) were generated using homologous recombination as previously described (21,25). These recombinant Ad5 vectors were propagated in 293 cells and recovered after 3 days by sonication (Bioruptor, Tosho Electric Co., Ltd. Kanagawa, Japan). The vector solutions were concentrated using Virakit according to the manufacturer's protocol (Virapur, LLC, San Diego, CA). Viral titers were determined by a plaque assay using 293 cells and the titer was expressed as plaque forming unit (PFU)/ml (21).

Adenoviral Vector Transduction to Primary Hepatocytes in Suspension

Isolated hAAT transgenic mouse hepatocytes were infected with Ad-CA-lacZ in suspension (at a density of 8×10^6 hepatocytes in 6 ml of suspension medium) in a 10-cm tissue culture polystyrene dish (Becton Dickinson Labware, Franklin Lakes, NJ) at 4°C. Adherence to the dish was avoided by shaking the dish gently at 20-min intervals. In order to optimize conditions, the following parameters were tested: vector dose [multiplicity of infection (MOI) from 0.04 to 100], duration of vector infection (4 min to 6 h), and different suspension media (UW solution, ViaSpan, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan; DMEM without FBS; or DMEM with 10% FBS). After the infection, hepatocytes were extensively washed with DMEM medium. The Ad vector-treated hepatocytes were cultured to either determine hepatocyte transduction efficiency or for transplantation purposes. For the determination of the transduction efficiency, cells were plated onto a culture dish (Primaria, Becton Dickinson). Eight hours later, hepatocyte plating efficiency was determined and the medium was changed with Williams E medium (WE) (Sigma). After a 24-h incubation period with WE, culture medium was collected and assayed for protein analysis of hAAT and albumin production to determine the hepatocyte function, and the hepatocytes were stained with X-gal to determine the transduction efficiency as described previously (32,38). Briefly, cultured hepatocytes were fixed with ice-cold 0.5% glutaraldehyde for 5 min and stained overnight using 5-bromo-4chloro-3indolyl-β-D-galactosidase (X-gal, Wako Pure Chemical Ltd, Osaka, Japan). Transduction efficiency was determined by counting at least 2000 hepatocytes and the data were expressed as a percentage of the X-gal-positive hepatocytes. Morphological structure of the hepatocytes was also determined. Hepatocyte gene transduction using Ad-CA-null was performed using the same protocol based on the optimized condition from the Ad-CA-lacZ experiment.

Hepatocyte Transplantation

Hepatocytes were prepared for transplantation under the kidney capsule space as previously described (34,36, 38,40). In brief, vector-treated or mock-treated hA1AT-FVB/N hepatocytes were resuspended with the mixture of serum-free DMEM and an equal volume of EHS-gel (Matrigel, BD Biosciences, Bedford, MA), which is an extracellular matrix component extracted from Engelbreth-Holm-Swarm cells, to a final ratio of 1.5×10^6 cells per 100 µl. A total of 3×10^6 hepatocytes were transplanted bilaterally under the kidney capsule space, respectively. Because EHS-gel can quickly polymerize into a three-dimensional gel at room temperature, all the procedures were done at 4°C. For hepatocyte transplantation into the liver, 1.5×10^6 hepatocytes were resuspended with 200 µl of serum-free DMEM and slowly infused directly into the portal vein for 5 min. All the surgical procedures were performed under inhalation anesthesia using isofluorane (Forane, Abbott Laboratories, Abbott Park, IL).

Enzyme-Linked Immunosorbent Assay (ELISA)

hAAT concentration in the culture medium and recipient mouse serum were measured by ELISA using a primary antibody against hAAT (DiaSorin, Stillwater, MN) and a secondary goat anti-hAAT-HRP antibody (Reseacrch Diagnostics Inc., Flander, NJ) as previously described (7,34,38,40). Albumin concentration in the culture medium was measured by ELISA using antibody against mouse albumin (Bethyl Laboratories, Montgomery, TX).

Histological Examination

The detection of the transplanted lacZ-transduced hepatocytes was performed using snap-frozen tissues in Tissue Tek O.C.T. compound (Sakura, Torrance, CA) (37). Sections (10 µm) were fixed with 0.5% glutaraldehyde and stained overnight for β-gal (X-gal, Wako Pure Chemical Ltd.). Cells were lightly counterstained with hematoxylin. For H&E staining and immunohistochemical staining against hAAT, the transplanted hepatocytes, including kidney tissues, were harvested and fixed in 10% buffered formalin. Specimens in paraffin-embedded blocks were sectioned at a thickness of 5 µm. Immunohistochemical staining for hAAT was performed using a rabbit anti-hAAT antibody (1:300, YLEM, Roma, Italy) and HRP-conjugated anti-rabbit antibody based on the Avidin-Biotin-Complex method (20,33,34) in the Vectastain ABC Elite Kit (Vector Laboratories, Inc., Burlingame, CA). Visualization of the immune complexes was performed by incubation with 3,3′-diaminobenzidine (DAB, Sigma). The sections were lightly counterstained with hematoxylin.

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).

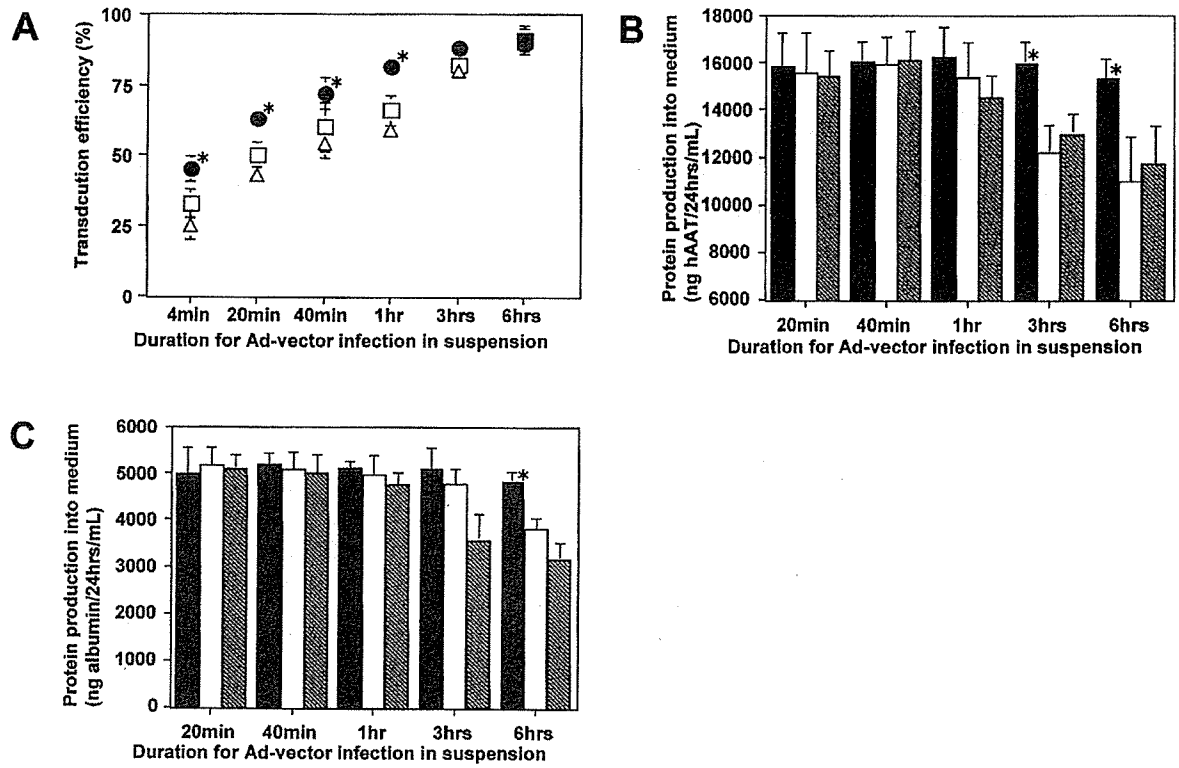

Figure 1. Optimization of Ad vector-mediated transduction into primary mouse hepatocytes in vitro. Hepatocytes in suspension with different medium (UW, DMEM with 10% FBS, DMEM) were infected with Ad-CA-lacZ at a MOI of 1 for different incubation times at 4°C. After the Ad vector incubation step, the 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-

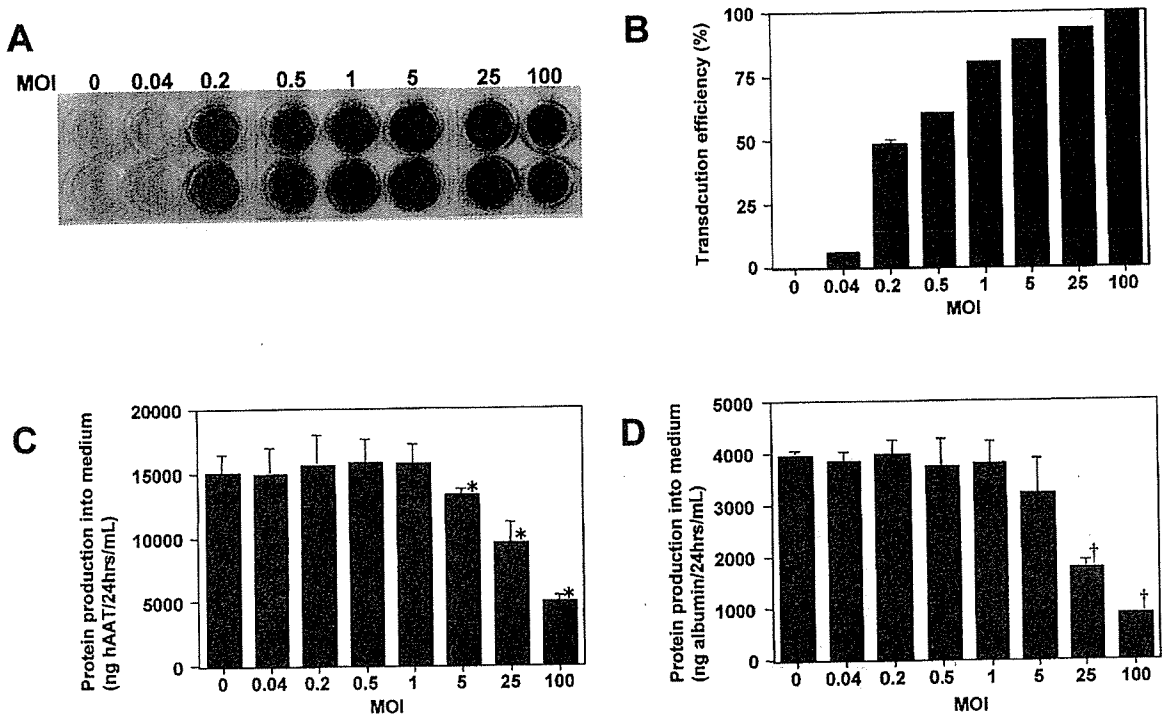

Figure 2. Efficient transduction using Ad vectors in a suspension solution of isolated hepatocytes. β-Galactosidase expression in hepatocytes following Ad vector-mediated transduction at increasing MOI from 0 to 100. Isolated hepatocytes were infected with Ad-CA-lacZ in suspension using UW solution at 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

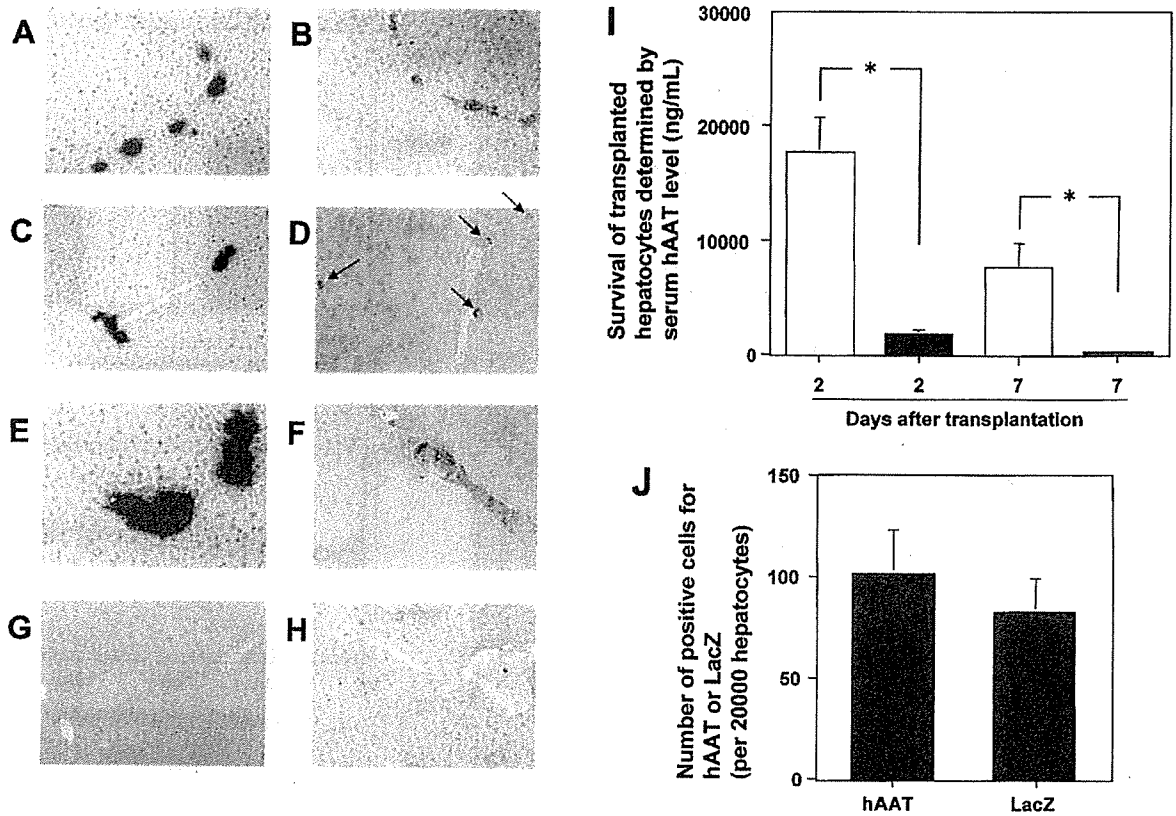

Figure 3. Demonstration of lacZ expression in Ad vector-transduced primary hepatocytes transplanted into the liver. Primary hepatocytes were infected with Ad-CA-lacZ in suspension under the condition of MOI of 1, UW solution for 1 h. After extensive washes, hepatocytes were transplanted (A–D) (see Materials 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

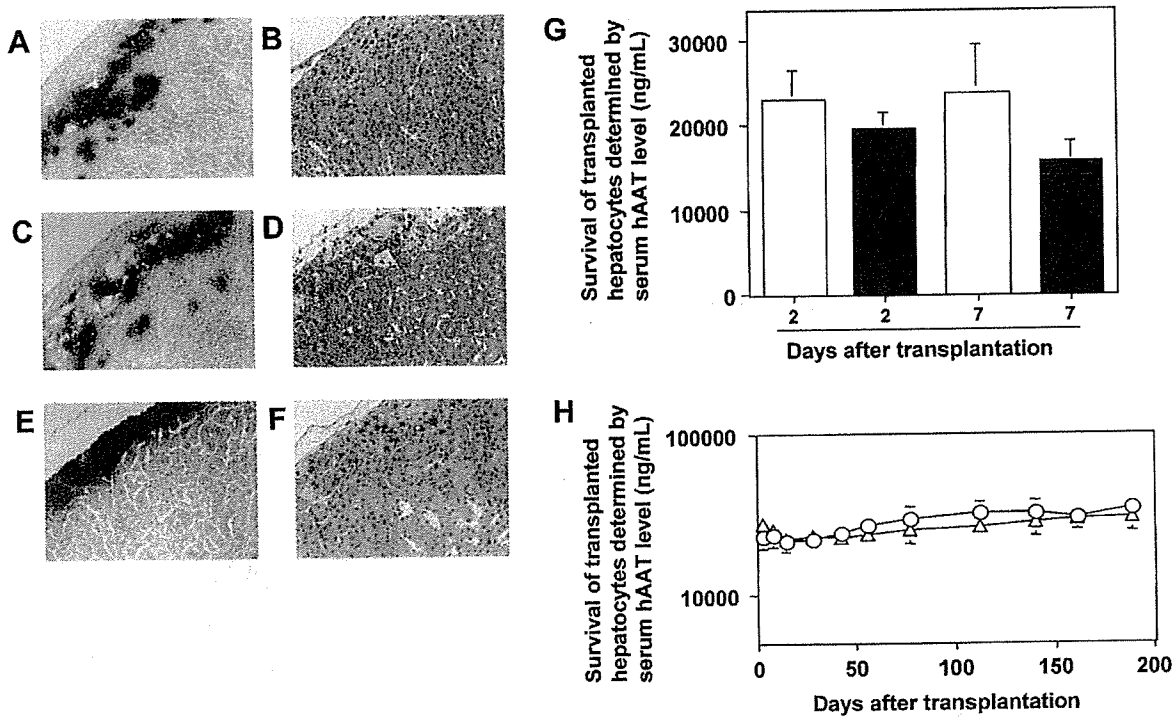

Figure 4. Hepatocyte transplantation under the mouse kidney capsule. Genetically modified primary hepatocytes (either in a suspension or standard culture conditions), which were genetically modified using Ad vectors, were transplanted under the mouse kidney capsule. Hepatocytes were infected with Ad-CA-lacZ 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-

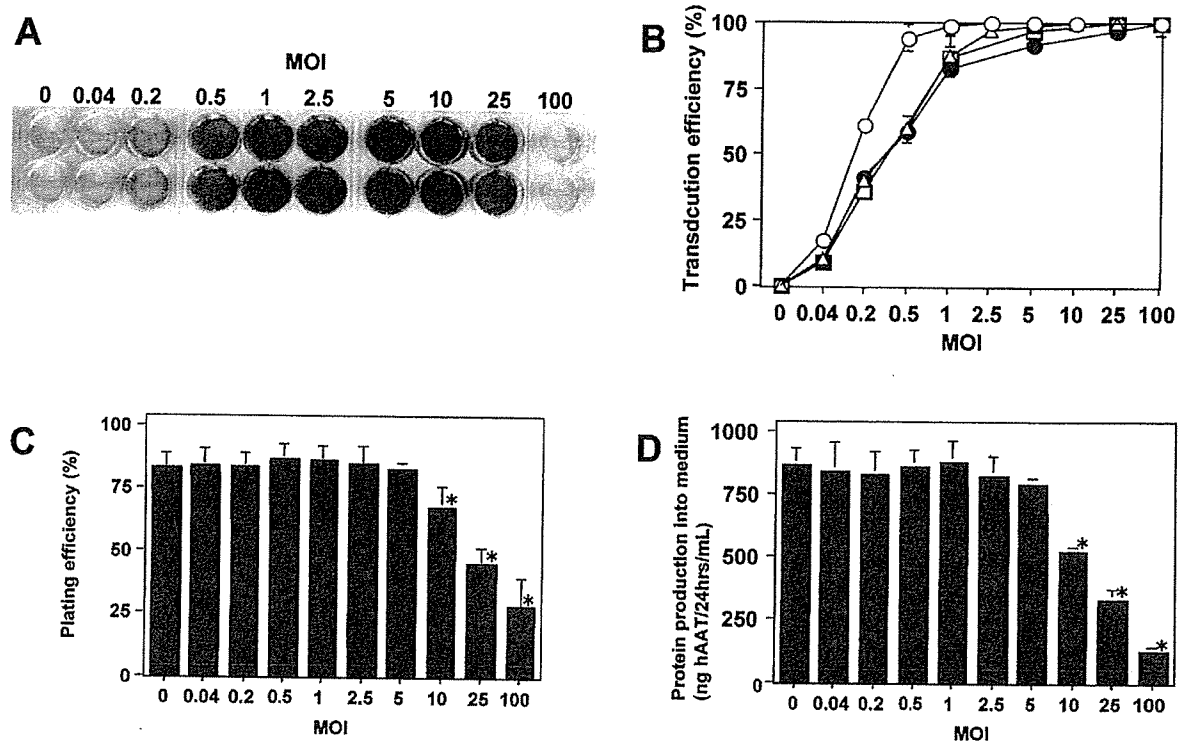

Figure 5. β-Galactosidase expression in canine, porcine, and human hepatocytes following adenoviral vector-mediated transduction into primary hepatocytes. Hepatocytes in suspension were infected with Ad-CA-lacZ at increasing MOI for 1 h at 4°C. After the Ad vector infection, hepatocytes were washed extensively 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.

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