Donor-Derived, Liver-Specific Protein Expression after Bone Marrow Transplantation

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Background. Bone marrow transplantation (BMT) may represent a novel mechanism to deliver a functional gene to a deficient liver. Bone marrow-derived hepatocytes are rare and without a defined contribution to liver function. Consequently, the clinical significance of BMT to treat liver disease is unclear. We sought to quantify bone marrow-derived hepatocyte protein expression after BMT and determine whether the process is inducible with liver injury.

Methods. Mice transgenic for *human* alpha-1 antitrypsin (hAAT) under a hepatocyte-specific promoter were used as bone marrow donors. Adenoviral transduction of modified urokinase plasminogen activator (Ad-muPA) was used to induce liver injury. Eight weeks after lethal irradiation and BMT, recipients were stratified into two groups: BMT alone (n=5) and BMT + Ad-muPA (n=10). Both groups of animals were bled before (t=0) and at 2, 4, 8, and 16 weeks after Ad-muPA administration, and the serum samples were assessed for hAAT by enzyme-linked immunosorbent assay.

Results. Transgenic donor mice expressed 5 to 10 mg/mL of hAAT. Recipients of BMT alone expressed less than 80 ng/mL of hAAT over all time periods. Animals receiving BMT + Ad-muPA showed sustained and stable hAAT expression of approximately 200 ng/mL. Differences were statistically significant at each time point.

Conclusion. Serum protein levels from liver-specific transgene expression are detectable and persist after BMT. Expression is low, but inducible with liver injury. We are currently developing strategies to augment donor-derived, liver-specific protein expression after BMT.

Keywords: Bone marrow transplantation, Cell therapy, Hepatocyte, Liver reconstitution.

(*Transplantation* 2004;78: 530–536)

Many of the genetic and metabolic diseases of the liver are a manifestation of a single gene deficiency and require lifelong medical therapy or orthotopic liver transplantation. For example, hemophilia, ornithine transcarbamoylase deficiency, and hereditary tyrosinemia each result from a single gene deficit. These conditions are potentially amenable to treatment with cell transplantation, because the successful engraftment of functional cells could serve as a replacement for the deficit in hepatocyte function.

In an early proof-of-principle experiment of the therapeutic potential of cellular transplantation, hepatocyte injection corrected a lethal liver deficiency in mice (*1*). The experiments in that study were based on a model of hereditary tyrosinemia type I, a metabolic deficiency causing severe and chronic hepatocellular injury, and thus creating significant positive selection for transplanted cells. Bone marrow transplantation (BMT) also rescued mice in the tyrosinemia model (*2*), initiating a debate over the potential clinical significance of BMT in the treatment of liver disease. A potential advantage of BMT in the treatment of liver deficiency, relative to other investigational cell-based strategies, is that BMT may induce graft tolerance to an allogeneic replacement cell source.

Cell fusion of donor bone marrow-derived cells with recipient hepatocytes is the likely mechanism that corrected the enzyme deficiency in the hereditary tyrosinemia model (*3*, *4*). However, transdifferentiation of bone marrow-derived cells into hepatocytes could not be excluded (*4*). A variety of other acute selection processes in other organs have produced rare engraftment of bone marrow-derived cells (*5–7*). These and other reports have looked for evidence of donor-derived cells histologically, a labor-intensive process that does not independently demonstrate functional engraftment and that requires the recipient animals to be euthanized, reducing the capability to analyze the kinetics of engraftment. Therefore, the functional contribution of bone marrow-derived hepatocytes over time remains an unanswered question. Furthermore, although transplanted hepatocytes do not need to "fuse or transdifferentiate" to establish function, they will not necessarily achieve stable long-term engraftment without chronic selection or tolerance.

In this series of experiments, donor-derived, liver-specific protein expression after BMT was defined by using a serum-based assay. Hepatocyte transplantation was studied first to establish a basis of comparison. Our aims were (1) to quantify the baseline contribution of bone marrow cells to liver function, (2) to clarify whether the process is inducible through liver injury, and (3) to determine whether BMT could yield persistent and measurable levels of liver-specific protein expression.

MATERIALS AND METHODS

Animals

Male mice transgenic for human alpha-1 antitrypsin (hAAT) under the alpha-1-antitrypsin promoter were used for donor cells, as previously described (*8*). Recipient animals

This research was funded by intramural support from Stanford University, the American College of Surgeons Faculty Research Fellowship (K.G.S.), the Oak Foundation (K.G.S.), and the National Institutes of Health AI 41320 (M.A.K.).

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Received 21 January 2004. Revised 17 February 2004. Accepted 24 March 2004.

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ISSN 0041-1337/04/7804-530

DOI: 10.1097/01.TP.0000130180.42573.B1

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were syngeneic FVB/n female mice between 6 and 10 weeks old (Taconic Farms; Germantown, NY). Transgenic animals constitutively expressing green fluorescent protein (GFP) through the β-actin promoter (Jackson Laboratories; Bar Harbor, ME) supplied unfractionated bone marrow as a surrogate to determine the level of hematopoietic chimerism achieved with our BMT protocol. Mice were housed in 12-hr light and dark cycles, with free access to food and water, and were cared for in accordance with Stanford University's Administrative Panels on Laboratory Animal Care.

Bone Marrow Isolation

Bone marrow isolation was performed as described (*7*). Briefly, donor mice were euthanized, and the lower extremities were prepared sterilely. After removing the soft tissue, the femurs were excised and placed into ice-cold Betadine solution. Under a laminar flow hood, the femurs were washed once in Betadine and transferred into ice-cold Hank's balanced salt solution supplemented with 2% fetal bovine serum. The ends of the femurs were removed, and the bone marrow was flushed using a 25-gauge needle and ice-cold media. The cells were triturated, centrifuged, and suspended to a dilution of 1×10^6 unfractionated bone marrow cells per 200 μL in ice-cold media.

Hepatocyte Isolation

Hepatocytes were harvested and purified as described (*8*). Briefly, transgenic hAAT mice were anesthetized with ketamine, xylazine, and acepromazine. A laparotomy was performed, and the inferior vena cava was cannulated. A calcium-free buffer was infused at 2 mL/min, and after the liver had blanched, the portal vein was cut. After 5 min, 0.1% collagenase with 5 mM $CaCl_2$ was infused for an additional 5 min at 2 mL/min. The liver was excised, and the cells were dispersed in William's Eagle Media supplemented with glutamine and antibiotics. The suspension was filtered through 80-μm gauze, and centrifuged for 5 min. The supernatant was removed, and the cells were resuspended in media. After a second centrifugation, the cells were counted, viability was checked by trypan blue exclusion, and the cells were diluted to a concentration of 1×10^6 cells per 200 μL.

Bone Marrow Transplantation

BMT was performed as described (*7*). Briefly, recipient mice were exposed to a lethal radiation dose of 9.6 Gy, split in two sessions separated by 3 hr. One million unfractionated bone marrow cells diluted into 200 μL of media were injected through the tail vein within 2 hr of the second dose. After BMT, animals were housed in autoclaved cages with antibiotic-supplemented water for 4 weeks. Two months after hAAT BMT, animals received adenoviral transduction of modified urokinase plasminogen activator (Ad-muPA) through the tail vein (n=10) or were uninjured (n=5). Both groups of animals were bled before (t=0) and at 2, 4, 8, and 16 weeks after Ad-muPA administration.

Analysis of Hematopoietic Chimerism

Animals previously lethally irradiated and reconstituted with GFP^+ unfractionated bone marrow were retro-orbitally bled 8 weeks after BMT (n=10). An amount of 300 μL of 2% dextran/Hank's balanced salt solution was added to each sample of whole blood to sediment the red blood cells. After incubation, the supernatant was removed, and any remaining red blood cells were lysed using ACK solution. Cells were centrifuged and subsequently labeled with three separate markers of hematopoietic lineage: Mac-1 (macrophages), B220 (B cells), and CD3 (T cells). The percentage of each of these hematopoietic cells expressing GFP was then analyzed by using fluorescence-activated cell sorting to estimate the level of hematopoietic chimerism achieved with our BMT protocol.

Hepatocyte Transplantation

Hepatocyte transplantation was performed as described (*8*). Briefly, recipient mice were anesthetized with ketamine, xylazine, and acepromazine. The abdominal wall was shaved and prepared sterilely. A small laparotomy was performed in the left upper quadrant, and the spleen was gently eviscerated. With a 27-g needle, 1×10^6 hAAT hepatocytes diluted into 200 μL of media were intrasplenically injected. After visual assurance of hemostasis, the spleen was placed carefully back into the abdomen, and the skin was closed with 4-0 Vicryl suture. Two groups were studied: (1) animals injected with Ad-muPA 6 days before hAAT hepatocyte transplantation (n=4) and (2) mice receiving hAAT hepatocytes alone, that is, without prior liver injury (n=4). Both groups of animals were bled immediately before (t=0) and at 2, 4, and 8 weeks after hAAT hepatocyte transplantation.

Liver Injury

An adenoviral construct expressing Ad-muPA under the phosphoglycerate kinase promoter was used to selectively injure hepatocytes, as described previously (*9*). To induce liver injury, 3×10^9 plaque-forming units of Ad-muPA were given through the tail vein. Carbon tetrachloride (CCl_4) was used as a comparison model of acute liver injury after BMT; a single dose of CCl_4 (1 mL/kg, diluted to a total volume of 200 μL in mineral oil) was injected intraperitoneally. Finally, Ad-lacZ was used as an adenoviral control of nonspecific liver injury; 3×10^9 plaque-forming units of Ad-lacZ were given through the tail vein.

RNA Extraction

Transgenic donors and wild-type FVB/n mice were euthanized, and liver, spleen, and bone marrow tissue were harvested. Samples were individually snap-frozen in liquid nitrogen and stored at −80°C. Tissues were homogenized in 0.6 mL of Trizol (Invitrogen, Carlsbad, CA) by using a pistol and mechanical vibration. RNA was extracted and precipitated by using chloroform and 2-propanol. RNA was further washed with diethylpyrocarbonate-treated 70% ethanol.

Transgene Detection by Reverse-Transcriptase Polymerase Chain Reaction

Screening for transgene expression was performed as described (*8*). Briefly, washed and quantified RNA was treated with DNAase I (Ambion, Austin, TX) to clear genomic DNA, and 5 μg of total RNA from each sample was reverse transcribed to cDNA by using random primer hexamers (Invitrogen, Carlsbad, CA). Polymerase chain reaction (PCR) was performed using the following hAAT primers: sense, 5'-TGGAGAGACCCTTTGAAGTC-3', and antisense, 5'-TCCAATTCCGAACCTTCC-3' (Genemed Biotechnologies, S. San Francisco, CA) to produce a

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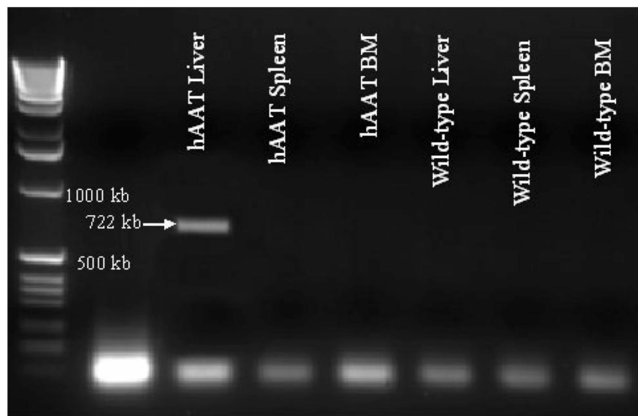

FIGURE 1. Human alpha-1 antitrypsin (hAAT) transgenics and wild-type FVB/n mice were killed, and mRNA was extracted from liver, spleen, and bone marrow. After reverse transcriptase-polymerase chain reaction (PCR), hAAT expression was detected only in transgenic liver (722-base pair band) and not in any of the wild-type tissues, confirming promoter and tissue specificity of the transgene.

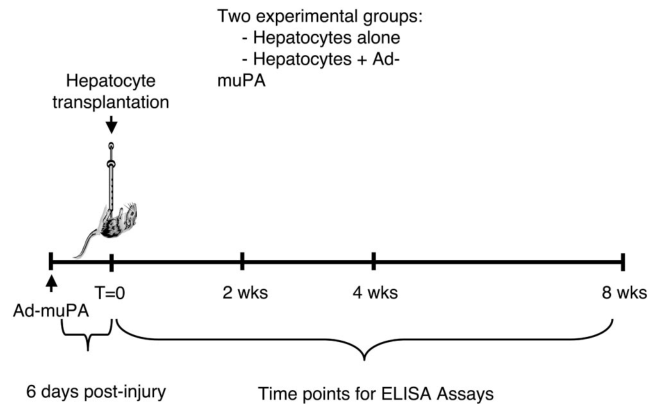

FIGURE 2. Hepatocyte transplantation experimental protocol. Two experimental groups were compared: intrasplenic injection of 1×10^6 hAAT hepatocytes alone and intrasplenic transplantation of 1×10^6 hAAT hepatocytes 6 days after tail-vein injection of adenoviral transduction of modified urokinase plasminogen activator (Ad-muPA). Animals in each group were bled before (t=0) and 2, 4, 6, and 8 weeks after hepatocyte transplantation.

722-base pair amplicon. For hAAT, PCR was run 30 cycles; each cycle was 1 min at 94°C, 1 min at 57°C, and 1.5 min at 72°C. The appropriate band was identified in 1.5% agarose gel. A GAPDH was used as internal control using the following primers: sense, 5'-GGGGTGAGGCCGGTGCTGAGTAT-3', and antisense, 5'-CATTGGGGGTAGGAACAC-GGAAGG-3' (Genemed Biotechnologies) to produce a 459-base pair amplicon. For GAPDH, PCR was run 35 cycles; each cycle was 1 min at 94°C, 2 min at 60°C, and 1 min at 72°C.

Bleeding

Mice were anesthetized with isoflurane and retro-orbitally bled at specific time intervals, as outlined previously. The samples were spun at 5,000 rpm for 10 min, and the serum was collected. The samples were stored at −20°C until processing by enzyme-linked immunosorbent assay (ELISA).

Serum Enzyme-Linked Immunosorbent Assay

Serum was analyzed for hAAT expression by using a standard sandwich ELISA, as described (*8*). Briefly, plates were coated with anti-human alpha-1-antitrypsin antibody (DiaSorin; Stillwater, MN). The samples were incubated overnight at 4°C after blocking with 5% milk powder in TBS-tween. An antigen-specific indicator antibody (Research Diagnostics, Inc.; Flanders, NJ) linked to horseradish peroxidase was used to detect bound antigen. After applying the substrate TMBD (Sigma, St. Louis, MO) and termination of the substrate reaction with sulfuric acid, the absorbance was measured in a fluorescent plate reader at a wavelength of 490 nm. Absorbance values were converted to nanograms/milliliter by comparison with a standard curve using standardized human serum.

Statistical Analysis

Data from each experimental group were compared at each time point by using the Student *t* test. Statistical significance was determined by a *P* value less than 0.05.

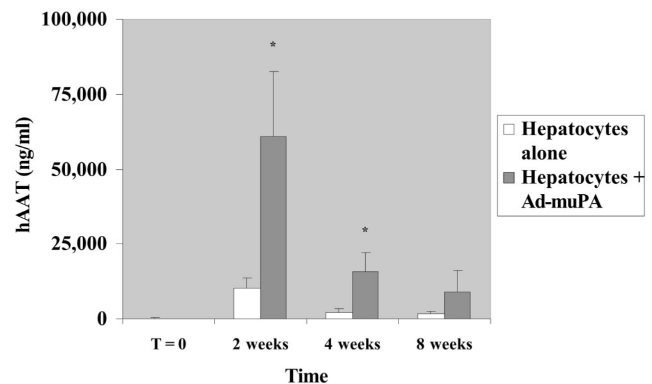

FIGURE 3. Comparison of protein expression data between hepatocyte transplantation alone and hepatocyte transplantation 6 days after liver-specific injury with Ad-muPA. Recipients were bled before (t=0) and 2, 4, and 8 weeks after hepatocyte transplantation, and their serum was analyzed for hAAT expression by enzyme-linked immunosorbent assay (ELISA). At each time point, values were compared by using the Student *t* test; statistical significance (*) was determined by a *P* value less than 0.05.

RESULTS

To establish baseline serum hAAT expression in donor animals, ELISAs were performed at random in these mice, revealing hAAT levels from 5 to 10 mg/mL (data not shown). To test the specificity of the transgene, mRNA from liver, spleen, and bone marrow from hAAT transgenics and wild-type FVB/n mice was extracted and analyzed for hAAT by reverse transcriptase-PCR. Only liver samples from transgenic mice demonstrated hAAT mRNA, thus confirming promoter and transgene liver specificity (Fig. 1). To validate liver injury after Ad-muPA administration, transaminase levels were studied. Transaminase levels were elevated in mice injected with Ad-muPA in a dose-dependent manner, confirming hepatocellular necrosis (data not shown). All animals

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injected with the highest dose, 1×10^{10} plaque-forming units of Ad-muPA, died of fulminant liver failure within 8 days; a dose of 3×10^9 plaque-forming units per animal of Ad-muPA was used in all subsequent experiments.

To establish a basis of comparison for liver-specific protein expression using this model, hepatocyte transplantation was studied before BMT. Hepatocyte viability after donor liver collagenase perfusion was greater than 95%, as determined by trypan blue exclusion (data not shown). The timing of hepatocyte transplantation after Ad-muPA injection was determined to coincide with the period of maximal liver damage. Four days after Ad-muPA administration, intrasplenic injection of hepatocytes produced lethal hemorrhage from the injection site in recipient mice and 100% mortality. Six days after Ad-muPA administration, in excess of 85% of mice receiving 1×10^6 hAAT-positive hepatocytes through intrasplenic injection survived (data not shown). Figure 2 provides a schematic representation of the hepatocyte transplantation experimental protocol.

Two groups of mice received hepatocytes: animals receiving Ad-muPA 6 days before transplantation (n=4) and animals injected with hAAT hepatocytes alone, without prior liver injury (n=4). Mice receiving Ad-muPA 6 days before hepatocyte transplantation expressed 60,000 ng/mL, 15,000 ng/mL, and 9,000 ng/mL of hAAT at 2, 4, and 8 weeks, respectively. Mice receiving hepatocytes alone demonstrated 10,000 ng/mL, 2,300 ng/mL, and 1,700 ng/mL of hAAT at 2, 4, weeks and 8 weeks, respectively. Statistically significant differences in hAAT expression between the two experimental groups were seen at 2 and 4 weeks. These data support the hypothesis that liver injury before transplantation led to compensatory repopulation of the recipient liver with donor hepatocytes (Fig. 3). Two negative control groups were also studied: sham intrasplenic injection of normal saline (n=5) and Ad-muPA without hepatocyte transplantation (n=4). None of these mice demonstrated any evidence of hAAT expression (data not shown).

The next series of experiments studied the contribution of bone marrow-derived cells to liver function after BMT. More than 95% of lethally irradiated mice that underwent BMT survived; animals that did not undergo BMT died within 2 weeks, as expected (data not shown). A surrogate assay was used to determine the level of hematopoietic chimerism achieved with the BMT protocol. Two months after

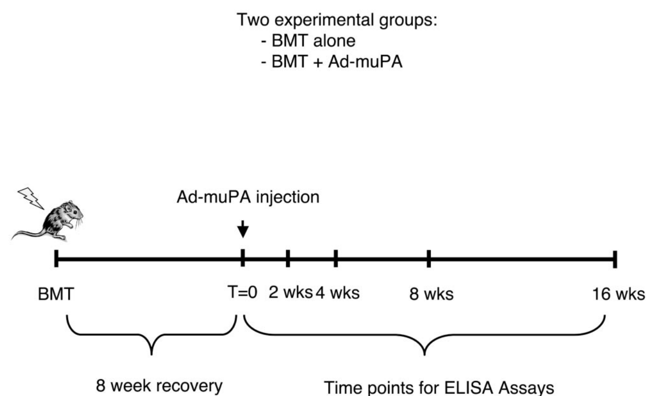

FIGURE 4. Bone marrow transplantation (BMT) experimental protocol. After an 8-week recovery period (t=0), two groups of animals were studied: those that underwent BMT alone and those that received Ad-muPA to induce liver-specific injury. Animals in each group were bled before (t=0) and 2, 4, 8, and 16 weeks after Ad-muPA injection, if given.

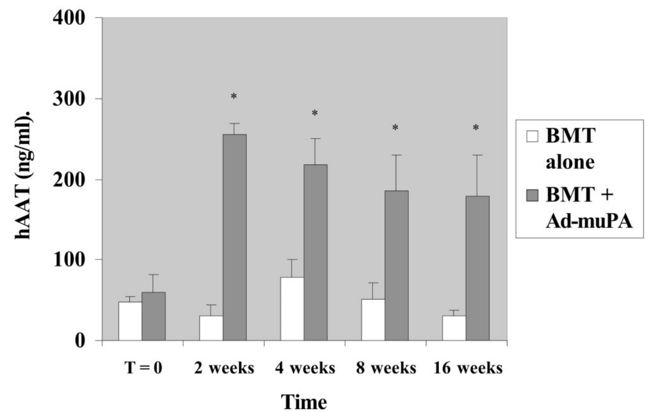

FIGURE 5. Protein expression data comparing mice that underwent BMT alone with those that underwent BMT with subsequent liver-specific injury. Syngeneic FVB/n mice were lethally irradiated and received BMTs from hAAT transgenic donors. After an 8-week recovery, animals were stratified into two groups: mice that received Ad-muPA through the tail vein (BMT + Ad-muPA) and mice that did not receive any liver-specific injury (BMT alone). Animals in each group were bled before (t=0) and at 2, 4, 8, and 16 weeks after Ad-muPA injection, if given. Serum hAAT concentration was determined by ELISA. At each time point, protein expression after injury (BMT + Ad-muPA) was compared with uninjured values (BMT alone) by using the Student *t* test. Statistical significance (*) was determined by a *P* value less than 0.05.

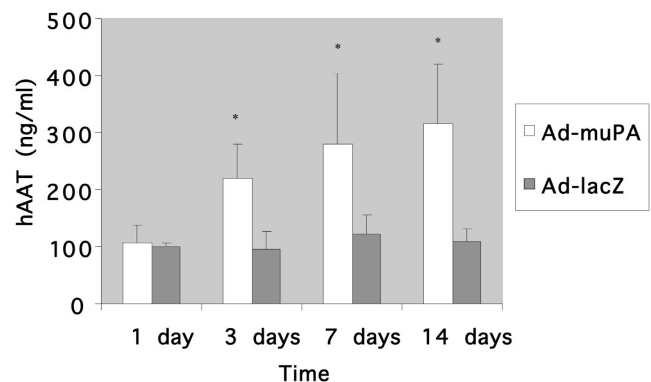

FIGURE 6. Comparison of Ad-muPA with a nonspecific adenoviral transfection. Syngeneic FVB/n mice were lethally irradiated, with subsequent BMT from hAAT transgenic donors. After recovery and hematopoietic engraftment, recipients were injected with Ad-muPA or Ad-lacZ. Animals were bled 1, 3, 7, and 14 days after adenoviral injection, and their serum hAAT expression was determined by ELISA. At each time point, the serum concentration of hAAT from animals injected with Ad-muPA was compared with the serum concentration of hAAT in animals injected with Ad-lacZ by using the Student *t* test. Statistical significance (*) was represented by a *P* value less than 0.05.

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lethal irradiation and transplantation of GFP-labeled unfractionated bone marrow, fluorescence-activated cell sorting analysis of whole blood samples taken from the retroorbital plexus of recipient mice demonstrated hematopoietic chimerism ranging from 30% to 85% (n=10; data not shown).

The protocol for experiments studying liver-specific protein expression after BMT is listed in Figure 4. Eight weeks after hAAT BMT (t=0), recipients demonstrated approximately 50 ng/mL of hAAT in their serum. To determine whether bone marrow-derived liver-specific protein expression is inducible with liver injury, animals were divided into two groups: BMT alone (n=5) and BMT + Ad-muPA (n=10). Both groups of animals were bled before (t=0) and at 2, 4, 8, and 16 weeks after Ad-muPA administration, if given. The BMT alone group expressed less than 80 ng/mL of hAAT throughout the study period. After injection with Ad-muPA, mean serum hAAT expression was 255 ng/mL at 2 weeks, 218 ng/mL at 4 weeks, 185 ng/mL at 8 weeks, and 188 ng/mL at 16 weeks. Statistically significant differences were seen between the two experimental groups at each time point (Figure 5). As a comparison model of liver injury after BMT, mice were injected with a single dose of 1 mL/kg of CCl_4. These mice stably expressed approximately 110 ng/mL of hAAT at 4, 8, and 16 weeks after injury, achieving statistical significance relative to BMT alone in the last two time points (data not shown). As an additional control, unfractionated bone marrow from hAAT donors was injected into animals without prior lethal irradiation. Serum from these animals did not demonstrate any detectable expression of hAAT (data not shown).

To determine whether the adenovirus or uPA was responsible for the increase in hAAT expression after Ad-muPA administration, Ad-muPA was compared with a nonspecific adenovirus (Ad-lacZ) after hAAT BMT. Eight weeks after lethal irradiation and hAAT BMT, animals were stratified into two groups: one group received Ad-muPA (n=5), and the other group received Ad-lacZ (n=5). Each animal received 3×10^9 plaque-forming units. Mice were bled 1, 3, 7, and 14 days after adenoviral injection. The mice injected with Ad-muPA demonstrated a gradual increase of hAAT expression to 300 ng/mL. Animals injected with Ad-lacZ stably expressed 100 ng/mL of hAAT. Statistically significant differences between the two experimental groups were seen at 3, 7, and 14 days (Figure 6). These data establish that transduction of modified uPA leads to the increase in hAAT expression, and not the adenovirus itself. Furthermore, these data corroborate that donor-derived, liver-specific protein expression after BMT is inducible with liver injury.

DISCUSSION

BMT may offer a mechanism to deliver a functional cell to a deficient tissue. The liver is an attractive target, because many genetic and metabolic diseases of the liver manifest from a single gene deficiency. To this end, hepatocytes derived from bone marrow have been demonstrated histologically after gender discordant transplantation (*3–6*, *10–12*), and BMT has been shown to reconstitute hepatocyte function in the setting of severe, chronic injury (*2*). Despite these findings, the clinical significance of bone marrow-derived hepatocytes has been questioned (*5*), largely a result of its rarity in most models (*5*, *6*, *13*).

The precise threshold to achieve a therapeutic response in the treatment of liver disease is uncertain and will depend on the disease, the percentage engraftment of the transplanted cells, and the level of expression of the target gene. However, it has been estimated that as little as 2% expression of normal factor VIII or IX levels would be beneficial to patients with hemophilia (*14*). Other genetic, metabolic, and acquired diseases of the liver would likely require higher levels of engraftment and expression for metabolic correction.

The therapeutic potential of BMT in liver reconstitution is based largely on evidence from an experimental model of tyrosinemia (*2–4*, *13*). The enzyme deficiency causing tyrosinemia in that model confers continuous toxicity to the deficient hepatocytes and thus creates an unparalleled level of positive selection and survival advantage for metabolically corrected cells. Even with this unique positive selection, hepatocyte replacement after BMT was estimated at only 1 in 150,000, or 0.001%, and the authors concluded that the transition of bone marrow to hepatocytes is a stochastic, low-frequency process, and not a response to injury (*13*). In another study, evidence of bone marrow-derived hepatocytes was demonstrated histologically in only 5 of 4.1×10^5 hepatocytes, or 0.0012% (*5*). In the latter study, histology specimens were analyzed after BMT in three separate models of chronic liver injury: albumin-uPA transgenic recipients, hepatitis B transgenic recipients, and after chronic CCl_4 administration (*5*). Fluorescence or gender mismatch analysis, however, does independently establish functional engraftment. To address these issues, an assay to quantify donor-derived, liver-specific protein expression after BMT may prove useful in clarifying the functional contribution of bone marrow-derived hepatocytes. Furthermore, these data would establish a baseline from which manipulations of liver injury or hepatic regeneration could then be studied.

In this series of experiments, transgenic mice expressing a distinct secreted protein (hAAT) driven by a liver-specific promoter (AAT) were used as donors. Analysis of mRNA extracted from liver, spleen, and bone marrow from transgenic and wild-type mice confirmed the specificity of the transgene in this model (Fig. 1). Because the AAT promoter is uniquely active in parenchymal liver cells (*8*, *15*), only hepatocytes synthesize hAAT, which is subsequently secreted into the blood stream (*8*). The serum half-life of hAAT is less than 2 hr (*8*); consequently, the protein does not accumulate over time. Therefore, after transplantation of hAAT-positive cells, the analysis of serum samples taken from recipient animals permits the real-time quantification of functional engraftment without relying on histologic analysis of recipient tissue (*8*).

To create a stimulus for regeneration and increase the positive selection of donor cells, the livers of recipient animals were injured. Two injury models were used. uPA, in addition to its role in hemostasis and extracellular matrix degradation during liver remodeling (*16*, *17*), causes hepatocellular damage when overexpressed intracellularly (*5*, *18–20*). The adenoviral construct used in our experiments, Ad-muPA, expresses a variant of uPA that is not secreted by liver cells (*9*). Systemic injection of Ad-muPA leads to increased intracellular protease activity and the degradation of intracellular proteins; cell death subsequently occurs, with resultant hepatocellular mitosis and hyperplasia of remnant hepatocytes (*9*). In an early experiment, the injection of increasing doses of Ad-muPA leads to elevated serum transaminases in a dose-dependent fashion, confirming hepatocellular necrosis in re-

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cipient mice (data not shown). CCl_4 injection was also used to induce liver injury, because it causes acute centrilobular inflammation and chemical necrosis in liver parenchyma (*21–23*). Furthermore, CCl_4 has been reported to increase the engraftment of transplanted hepatocytes (*22*).

To establish a basis of comparison for hAAT expression after BMT, hepatocyte transplantations with and without prior Ad-muPA injection were performed first. Two weeks after liver injury and hepatocyte transplantation, 60,000 ng/mL of hAAT was expressed, representing between 0.25% and 0.5% of donor expression of 5 to 10 mg/mL. Mice receiving hepatocytes alone without prior liver injury demonstrated a maximum of 10,000 ng/mL of hAAT 2 weeks after transplantation. These data concur with previously reported evidence for hepatocyte transplantation, in which less than 1% of recipient hepatocytes were identified as donor derived (*8*, *15*, *22*, *24*). Furthermore, these data also agree with other reports in which hepatocyte engraftment was improved in the setting of acute liver injury. Notably, hAAT expression after hepatocyte transplantation both with and without prior liver injury was not sustained, decreasing rapidly over time (Fig. 3).

The transplantation of hAAT bone marrow into lethally irradiated syngeneic recipients permits a mechanism to calculate the functional contribution of bone marrow-derived hepatocytes. Eight weeks after BMT and hematopoietic recovery ($t=0$), recipients expressed approximately 50 ng/mL of hAAT. After induction of liver injury through the administration of Ad-muPA, maximal hAAT expression was 255 ng/mL. Because the transgenic donors express between 5 and 10 mg/mL of hAAT, bone marrow-derived hepatocyte function after liver-specific injury was at best 0.005% of the original donor hAAT expression. In the BMT alone group (i.e., without liver injury), hAAT expression at the same time point was approximately 0.0016% of donor expression. Although expression was low, injection of Ad-muPA yielded statistically significant higher levels of hAAT expression at each time point, relative to BMT alone (Fig. 5). Furthermore, Ad-muPA administration yielded statistically significantly increased hAAT expression after BMT relative to recipient mice receiving a nonspecific adenovirus (Figure 6). Therefore, although rare, bone marrow-derived hepatocyte protein expression in this model is inducible with liver injury.

The rapid decline of hAAT expression after hepatocyte transplantation, relative to the stable although low expression of hAAT after BMT, highlights a potential advantage of BMT over other investigational cell-based strategies to treat liver disease. As the source of the functional cell, BMT may induce tolerance to donor-derived hepatocytes, potentially yielding long-term graft function. Conversely, T cells remove transplanted hepatocytes (*8*), and thus cell transplant recipients would likely require long-term immunosuppression for long-term graft function. Although minor disparities in major histocompatibility complex expression between the FVB/n recipient mice and the syngeneic hAAT-transgenic donor mice cannot be excluded, these data still highlight the impact of immune surveillance on graft function. Furthermore, these data indicate that engraftment in these models occurs through a separate process, and thus may permit an exploration into, and exploitation of, their respective mechanisms. Although cell fusion is the likely process that generates bone marrow-derived hepatocytes (*3*, *4*, *6*), the responsible cell has not been conclusively identified.

The detection of hAAT expression after bone marrow reconstitution in the BMT alone group raises the question of engraftment in the absence of liver-specific injury. Recipient animals received lethal, whole-body irradiation, and consequently incurred a level of liver injury at the time of BMT. A possible explanation is that a chemokine facilitating the homing of transplanted hematopoietic progenitors to bone marrow is also up-regulated in liver after whole-body irradiation (*25*). Whether the level of hAAT expression is a consequence of engraftment of transplanted cells after lethal irradiation, or whether the level of hAAT expression represents the stochastic engraftment of bone marrow-derived cells into the liver parenchyma, cannot be determined with the data provided. Notably, animals undergoing transplantation with hAAT bone marrow without prior irradiation did not demonstrate any detectable level of hAAT. Overall, these data are in agreement with other reports demonstrating the rarity of bone marrow-derived hepatocytes.

Finally, alpha-1-antitrypsin is an acute phase protein and therefore is induced after injury. However, if hAAT expression was up-regulated only during the acute phase reaction to injury, gene expression would be short-term, and not the long-term expression of hAAT seen in this series of experiments. Earlier work previously demonstrated long-term hepatocyte expansion after Ad-muPA injection (*26*). Consequently, the acute phase reaction is an unlikely significant long-term contributor to hAAT expression.

In this series of experiments, BMT does not yield sufficient liver-specific protein expression to be used as a method to treat liver disease. However, although donor-derived, liver-specific protein expression is low, it is inducible with injury. These data will serve as a baseline to evaluate the impact of various strategies (e.g., limiting native hepatocyte regeneration, inducing more severe liver injury, increasing bone marrow cell fusion within the liver, or combining bone marrow and hepatocyte transplantation) in hopes of developing cell-replacement and gene-transfer strategies toward clinical significance.

ACKNOWLEDGMENTS

We thank Rich Sherwood for his assistance in the analysis of level of chimerism, and the entire staff at the Veterinary Service Center for the excellent care of research animals.

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