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Delivery of Hepatotrophic Factors Fails to Enhance Longer-Term Survival of Subcutaneously Transplanted Hepatocytes

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ABSTRACT

Tissue engineering approaches have been investigated as a strategy for hepatocyte transplantation; however the death of a majority of transplanted cells critically limits success of these approaches. In a previous study, a transient increase in hepatocyte survival was achieved through delivery of vascular endothelial growth factor (VEGF) from the porous polymer scaffold utilized for cell delivery. To enhance longer-term survival of the hepatocytes, this delivery system was modified to additionally deliver epidermal growth factor (EGF) and hepatocyte growth factor (HGF) in a sustained manner. Hepatocytes were subcutaneously implanted in SCID mice on scaffolds containing EGF and/or HGF, in addition to VEGF, and survival was monitored for two weeks. A short-term enhancement of hepatocyte survival was observed after one week and is attributed to VEGF-enhanced vascularization, which was not altered by EGF or HGF. Surprisingly, long-term hepatocyte engraftment was not improved, as survival declined to the level of control conditions for all growth factor combinations after two weeks. This investigation indicates that the survival of hepatocytes transplanted into heterotopic locations is dependent on multiple signals. The delivery system developed for the current study may be useful in elucidating the specific factors controlling this process, and bring therapeutic transplantation of hepatocytes closer to implementation.

INTRODUCTION

While hepatocyte transplantation has been suggested as a promising strategy to treat some liver diseases and alleviate organ donor shortage, the success of this approach has been severely limited by the death of a majority of the transplanted cells.[1,2] Previous work has indicated that insufficient vascularization of the construct utilized for hepatocyte delivery is an important factor contributing to the loss of transplanted hepatocyte populations.[3] However, speeding vascularization of the implants via vascular endothelial growth factor (VEGF) delivery resulted in only a transient increase in hepatocyte survival, suggesting that the cells require additional factors to promote longer-term survival.

Factors present in the portal circulation have a positive impact on the survival of transplanted hepatocytes, as hepatocytes exhibit significantly increased survival rates when transplanted into animals in which the portal vein is shunted to the vena cava (portal caval shunt) such that blood intended for the liver is redirected to the systemic circulation.[4–7] A combination of portal caval shunting and partial hepatectomy has also been demonstrated to enhance transplanted hepatocyte engraftment,[6] as signals promoting hepatocyte proliferation are likely present in higher concentrations following partial hepatectomy.[8] While some of the factors present in the portal circulation that promote hepatocyte survival and/or proliferation likely remain to be discovered, several growth factors and hormones important in these processes have

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been identified. Two growth factors that have been demonstrated to play a key role in liver regeneration are epidermal growth factor (EGF) and hepatocyte growth factor (HGF).[9–12] Both EGF and HGF are also known to promote the survival and proliferation of hepatocytes in culture[13,14] and have been shown to effect transplanted hepatocyte survival.[4,15,16]

It was therefore hypothesized that sustained delivery of EGF and HGF might promote longer-term survival of transplanted hepatocytes. To investigate this hypothesis, a polylactide-co-glycolide scaffold system previously developed to deliver VEGF was used to deliver EGF and HGF additionally. The scaffold fabrication procedure was first modified to release EGF and HGF in a sustained manner throughout the planned study timeframe. *In vitro* characterization of the scaffolds was then performed to confirm that the released growth factors retained bioactivity. Finally, hepatocytes were transplanted into subcutaneous tissue using scaffolds containing various combinations of VEGF, EGF, and HGF to determine whether inclusion of the hepatotrophic factors would promote longer-term survival of the transplanted cells.

MATERIALS AND METHODS

Scaffold fabrication

Scaffolds were formed using a modification of a previously published process,[17,18] which involves fabricating polylactide-co-glycolide (PLG) into microspheres prior to forming scaffolds from the microspheres. Microspheres were fabricated from 85:15 (Medisorb, Alkermes, Cambridge, MA) or 75:25 PLG (RG752, Boehringer-Ingleheim, Ingleheim, Germany) via a standard double emulsion process.[19] In brief, a 5% (w/v) solution of PLG in ethyl acetate (EtAc) (Sigma, St. Louis, MO) was combined with distilled, deionized water in a test tube. In conditions using 75:25 PLG microspheres to encapsulate EGF, lyophilized murine EGF (Peprotech, Rocky Hill, NJ) was reconstituted in the water used as the aqueous phase. This two-phase mixture was then sonicated to yield a single emulsion. An equal volume of an aqueous solution containing 1% polyvinyl alcohol (PVA, MW 25,000, 88% hydrolyzed; Polysciences, Warrington, PA) and 7% EtAc was added to the single emulsion, and the resulting solution was vortexed (Vortex Genie, VWR International, West Chester, PA) to yield the double emulsion. This double emulsion was immediately transferred to a rapidly stirring aqueous solution of 0.3% PVA/7% EtAc. After allowing the EtAc to evaporate for 3 h, the microsphere-containing solution was filtered to retrieve the microspheres, which were then rinsed with distilled water, flash frozen, and lyophilized.

For each scaffold, 4 mg of 75:25 PLG microspheres and 16 mg of 85:15 PLG microspheres were combined with 100 μL of 1% MVM alginate (FMC Biopolymer, Philadelphia, PA) solution. When VEGF and/or HGF were included in the scaffolds, 40 μg recombinant human VEGF (R&D Systems, Minneapolis, MN) and/or 40 μg of recombinant human HGF (Research Diagnostics, Flanders, NJ) were dissolved in the alginate solution. In conditions where EGF was included, the 75:25 PLG microspheres were fabricated to contain this growth factor, as described above. All growth factors were omitted from the fabrication process for conditions requiring blank scaffolds. The desired microsphere/alginate/growth factor mixture was next lyophilized to form a powder, combined with 380 mg NaCl (sieved to yield a particle diameter between 250 and 425 μm), and pressed into a 13 mm diameter die to create a 1.5 mm thick disk. The PLG was gas foamed by subjecting the disks to 800 psi CO_2 for 24 h, followed by a rapid reduction of pressure to ambient. The NaCl was leached from scaffolds by incubating in 0.1 M $CaCl_2$ for 16 h prior to use in experiments. Upon removal from the leach solution, each scaffold was flash-frozen in liquid nitrogen and cut into quarters using a razor blade, and these quarter scaffolds were used in all experiments. Scaffolds for determining growth factor release kinetics and bioactivity were fabricated to contain 0.8 μg EGF or 3.6 μg HGF each, and scaffolds for *in vivo* experiments were fabricated to contain 3.6 μg VEGF and 1.3 μg EGF and/or 3.6 μg HGF. In studies where scaffolds were incubated with cells or implanted into mice, they were soaked in 100% ethanol for 20 min, followed by five 5 min washes with sterile phosphate buffered saline (PBS) prior to cell seeding.

EGF and HGF release kinetics

To monitor the incorporation efficiency and release kinetics of EGF from the porous scaffolds, receptor grade ^{125}I-labeled recombinant human EGF (ICN Biomedical, Irvine, CA) was used as a tracer. When fabricating the EGF-containing microspheres for this portion of the study, 3 μCi radiolabeled EGF and 300 μg unlabeled EGF were dissolved in the aqueous phase added to the polymer solution, as described above. A sample of this aqueous solution was measured using a gamma counter to determine the radioactivity added to each batch of microspheres. After microsphere fabrication and lyophilization were complete, the radioactivity of a known mass of microspheres was measured in the gamma counter and compared to the radioactivity initially added to the total polymer mass for the batch to assess incorporation efficiency into the microspheres. Scaffolds were then made according to the method described previously. After completing the gas foaming process but before leaching the NaCl from scaffolds, the radioactivity present in each scaffold was measured. After leaching, each scaffold was again analyzed with the gamma counter to determine the amount of protein remaining in the scaffolds. Next, scaf-

folds were cut into quarters, which were each placed in 3 mL PBS (Gibco, Carlsbad, CA) with 0.132 g/L $CaCl_2$-$2H_2O$ and 0.10 g/L $MgCl_2$-$6H_2O$ added. Scaffolds were maintained in PBS at 37°C and at various times, a 1 mL sample of the PBS was removed for measurement with the gamma counter, and the remaining PBS was aspirated and replaced with 3 mL of fresh PBS. The quantity of EGF that had been released at each time point was determined by comparing the radioactivity of the PBS samples to the total incorporation, as measured prior to initiating the release study.

The incorporation efficiency and release kinetics of HGF were determined using a human HGF ELISA kit (R&D Systems). Scaffolds for this part of the study were fabricated to contain only HGF, as specified in the previous section, and samples of the leached solution were reserved to measure the amount of growth factor lost in this step and determine the incorporation efficiency. To monitor HGF release, scaffolds were incubated in 2 mL of PBS solution, as described above, and maintained at 37°C. At various times the PBS was removed, reserved for analysis, and replaced with 2 mL of fresh PBS. The ELISA was performed according to the kit instructions in 96-well plates using 50 μL of each sample or HGF standard. Immediately following completion of the final step of the ELISA, the optical density of each well was measured using a microplate reader to determine the amount of HGF in the samples. Finally, calculations were performed to determine the amount of HGF released from the scaffolds at each time point.

Bioactivity of released EGF and HGF

To assess the mitogenic potential of EGF and HGF that had been incorporated into scaffolds, a fibroblast cell line (NIH-3T3) and lung endothelial cells (4MBr-5) (both from ATCC, Manassas, VA) were incubated with scaffolds releasing only EGF or HGF, respectively. EGF-containing scaffolds were fabricated as described using microspheres of 75:25 PLG, with 300 μg EGF added to the aqueous phase during processing. Scaffolds containing only HGF were also made according to the method outlined above.

The biological activity of released EGF or HGF was confirmed by placing the scaffolds ($n = 5$), supported in transwells (12 mm, 3 μm pore diameter), directly over NIH-3T3 or 4MBr-5 cells growing in culture wells of a 12-well plate (Corning, Cambridge, MA) with 2 mL of medium. NIH-3T3 cells were allowed to attach to culture wells for 4 h in DMEM (Gibco) containing 2% FBS (Gibco). Following the attachment period, the culture medium was exchanged for serum-free DMEM, and transwells containing scaffolds were placed in each well. 4MBr-5 cells were allowed to attach to the culture plate overnight in Ham's F12K medium (ATCC) with 10% FBS, and serum was reduced to 2% prior to adding scaffolds. In both studies, the cells were cultured in the presence of scaffolds for 72 h before cell number was determined using a Coulter counter (Beckman-Coulter, Fullerton, CA). The scaffolds in transwells were then moved to wells freshly seeded, as described for each condition, and allowed to incubate for an additional 72 h. The magnitude of growth stimulation was compared to control conditions where cells were cultured in the presence of scaffolds containing no growth factor and with known concentrations of EGF (0.1–10 ng/mL) or HGF (0.5–20 ng/mL).

Hepatocyte isolation

Hepatocytes were isolated from adult male Lewis rats (150–200 g, Charles River Breeding Laboratories, Wilmington, MA) with a modification of the original Seglen two-step collagenase perfusion procedure.[20] Briefly, the liver was perfused via the portal vein, first with calcium-free buffer (142 mM NaCl, 7 mM KCl, 20 mM Hepes, pH 7.4, 37°C) for 10 min, followed immediately by collagenase-containing buffer (142 mM NaCl, 7 mM KCl, 20 mM CaCl2, 20 mM Hepes, 0.5 mg/mL collagenase type 2 [Worthington Biochemical, Lakewood, NJ] pH 7.4, 37°C). Perfusion with the collagenase-containing buffer was continued for 7–10 min. The perfused liver was then placed in ice-cold William's Medium E (Gibco), and cells were dissociated by removing the liver capsule and gently shaking the hepatocytes free from the remaining liver matrix. This cell suspension was filtered through a 400 μm Nytex mesh (Tetko, Elmsford, NY) to remove large debris. The cell yield per liver was $1–2 \times 10^8$, and the initial percentage of viable cells was 80–90%. To further purify the cell population and remove cellular debris, density gradient centrifugation using Percoll (Sigma) was performed. The collected hepatocytes, which had a total cell viability of >90%, were then placed on ice and immediately used in experiments.

SCID mouse implants

For the implant study, scaffolds were fabricated with three different growth factor combinations: 1) VEGF and EGF, 2) VEGF and HGF, and 3) VEGF, EGF, and HGF. The EGF-containing microspheres for this study were fabricated with a larger quantity of EGF (500 μg per batch) in an effort to better promote hepatocyte survival. To make scaffolds, 4 mg of these EGF-microspheres or blank 75:25 PLG microspheres were combined with 16 mg of 85:15 PLG microspheres, and VEGF and HGF were incorporated as described previously. Immediately before scaffold implantation, 1×10^6 hepatocytes were suspended in 30 μL of 1:1 mixture of William's Medium E and growth factor reduced Matrigel (BD Biosciences, Bedford, MA), which was included to help retain cells within the scaffolds, and allowed to absorb into the polymer scaffold for 5 min. The cell-seeding efficiency was

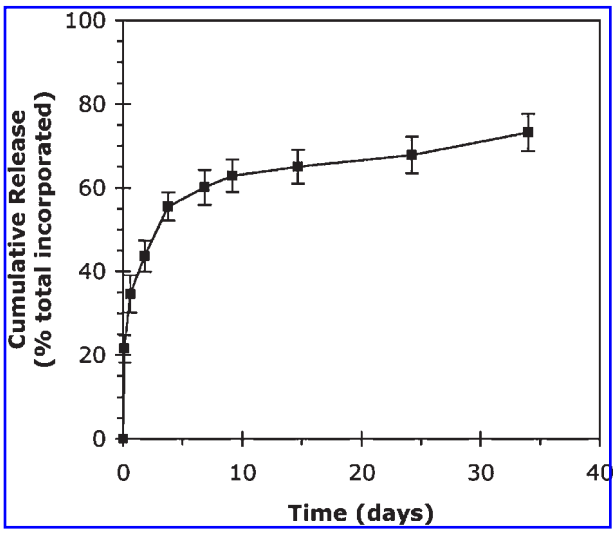

FIG. 1. EGF release from PLG scaffolds. Values represent mean and standard deviation ($n = 6$).

determined by counting the cells remaining in the dish after this absorption period.

Scaffolds ($n = 4$) from each condition were then implanted in subcutaneous pockets (2 per animal) on the dorsal region of 7- to 9-week-old male SCID mice (cb17/SCID, Taconic Labs, Germantown, NY). A 1.5 cm longitudinal incision was made on the animal's back, and one implant was then placed on either side of the incision. The incision was then closed with VetBond tissue adhesive (3M, St. Paul, MN). All NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23, rev. 1985) were observed.

Mice were sacrificed and scaffolds retrieved at 3, 7, and 14 days following implantation. The scaffolds were dissected out of the tissue and immediately placed into 10 mL of buffered zinc-formalin, where they remained for 24 h at 4°C. The zinc-formalin was then exchanged for 70% ethanol, and samples were stored at 4°C until they were embedded in paraffin by the University of Michigan School of Dentistry Histology Core.

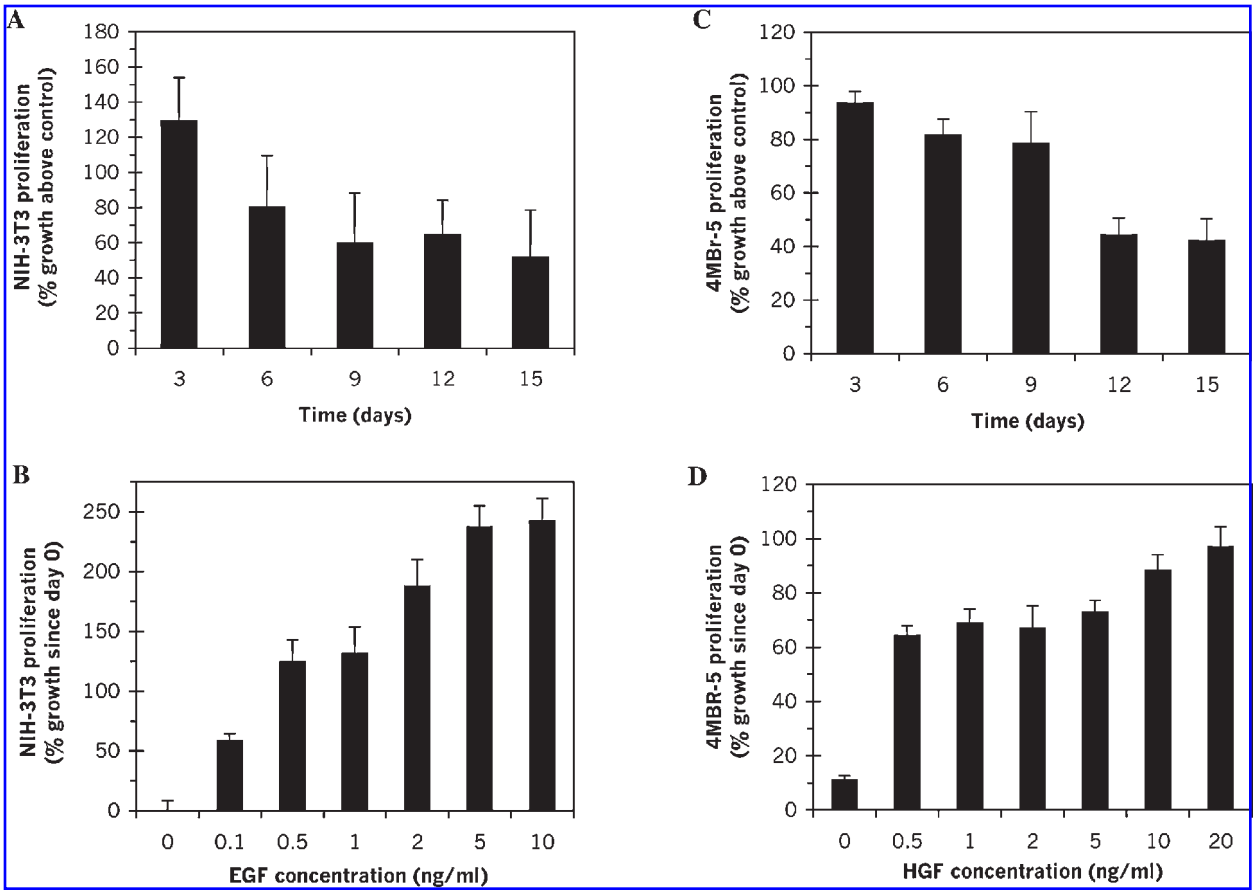

FIG. 2. EGF **(A)** and HGF **(C)** released from scaffolds exhibit maintenance of biological activity *in vitro*, as compared with cells' response to known quantities of EGF **(B)** and HGF **(D). (A and C)** Cells were incubated with scaffolds releasing either EGF or HGF and their proliferation was compared to that of cells incubated with blank scaffolds ($n = 5$). **(B and D)** Cells were cultured with a range of growth factor concentrations for 3 days ($n = 3$).

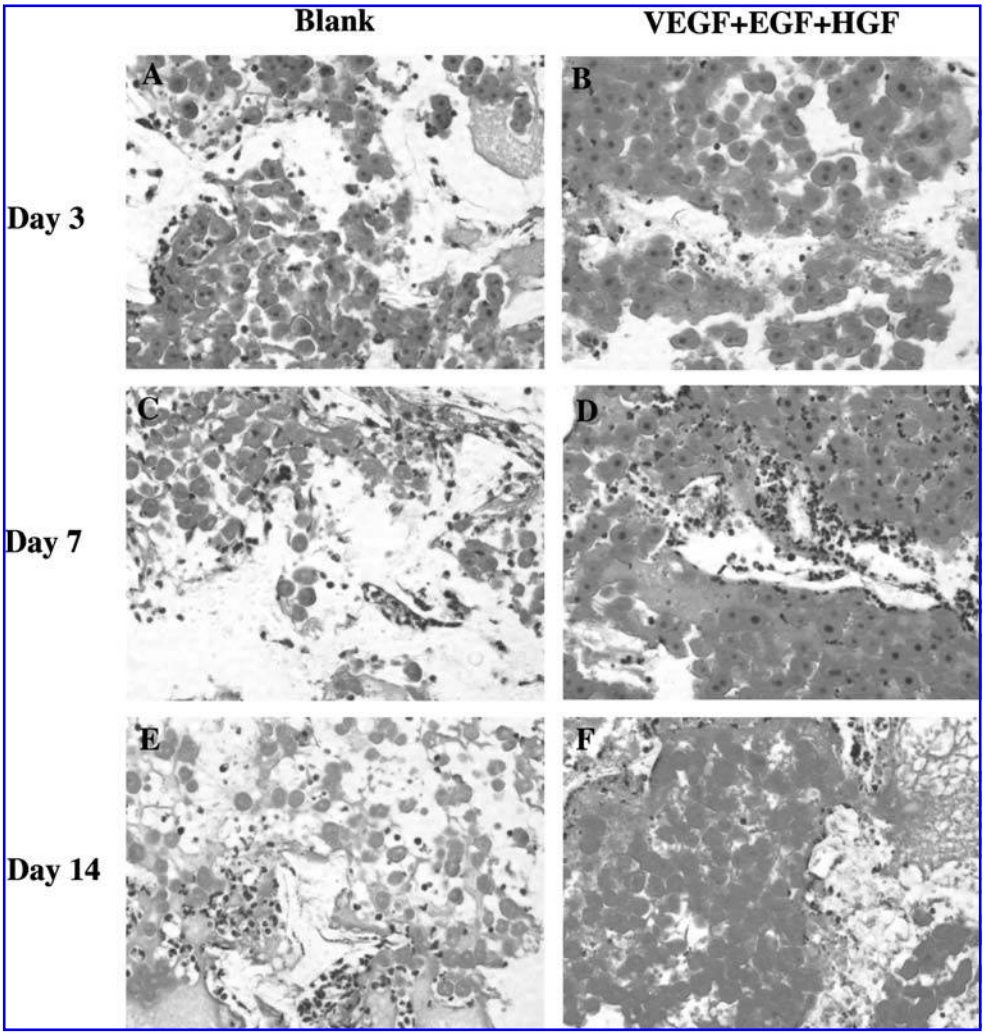

FIG. 3. H&E-stained sections of transplanted scaffolds containing hepatocytes. Hepatocytes were seeded onto matrices, implanted into SCID mice, and retrieved after 3, 7, and 14 days. Scaffold conditions not shown (VEGF + EGF and VEGF + HGF) were both similar in appearance to the condition including all three factors. original magnification × 400

Histological staining and analysis

The paraffin-embedded scaffolds were cut into 5 μm thick sections and placed on glass slides for histological analysis. The University of Michigan Comprehensive Cancer Center Histology Core performed the immunohistochemical staining for mouse CD31 antigen to identify endothelial cells present in the vasculature. To visualize overall tissue morphology and distinguish surviving hepatocytes, other sections were stained with hematoxylin and eosin.

Tissue sections were visualized and photographed with an E-800 light microscope (Nikon, Melville, NY). Four samples from each condition, with 3 sections from each sample, were analyzed manually and digitally with ImagePro Plus software (Media Cybernetics, San Diego CA). Blood vessels present in implants retrieved at 7 and 14 days were analyzed for their total number and were identified, at 200 × magnification, by their defined lumens in sections stained for mouse CD31 antigen. For implants retrieved at 3, 7, and 14 days, the total number of engrafted hepatocytes in each section was manually counted at 200 × magnification. Engrafted hepatocytes were identified by their large size, distinct cytoplasmic staining, and large, spherical nuclei, while dead hepatocytes were identified by their lack of a nucleus, as described in a number of published reports.[2,21,22]

Statistical analysis

The Student's *t* test (one-tailed) was employed to determine the statistical significance of differences between data from growth factor and control conditions for both engrafted hepatocyte and blood vessel quantification, with $p \leq 0.05$ considered significant. InStat software (Version 2.01, GraphPad Software, San Diego, CA) was used to perform this analysis.

RESULTS

Growth factor incorporation and release

The incorporation efficiency and release kinetics of EGF from constructs were monitored using ^{125}I labeled EGF as a tracer. Analysis revealed that 25 ± 2% of the EGF added during processing remained in the microspheres when fabrication was complete, which yielded 1.5 μg EGF/mg microspheres. When scaffolds were fabricated using these microspheres to monitor overall EGF incorporation and release, 55 ± 3% of the EGF present in the microspheres became incorporated into the scaffolds to provide 0.8 μg EGF/scaffold. These scaffolds were then incubated in PBS, and the release of EGF was monitored over the course of 35 days. The release profile was characterized by an initial burst in which about 50% of the incorporated growth factor was released over the first 4 days, followed by slow sustained release of about 0.7% per day (Fig. 1). A similar incorporation efficiency and release rate were observed for HGF (data not shown).

Bioactivity of released EGF and HGF

To confirm that incorporated growth factors retained biological activity after scaffold fabrication, scaffolds containing either EGF or HGF were incubated with an appropriate cell type and cell proliferation was monitored. Scaffolds containing approximately 0.8 μg EGF were allowed to release directly into tissue culture medium over NIH-3T3 cells. The released EGF induced significant proliferation of the cells as compared to control cell cultures that were incubated with blank scaffolds (Fig. 2A). Knowledge of the release kinetics of EGF from these scaffolds, together with the response of NIH-3T3 cells to known concentrations of EGF (Fig. 2B), revealed that the released growth factor retained 60–80% of its bioactivity over this release period. Results obtained by incubating 4MBr-5 cells with HGF-containing scaffolds were similar, as the cells demonstrated a significant proliferative response to the released HGF (Fig. 2C). Again, by comparing the magnitude of the response to values obtained for known HGF concentrations (Fig. 2D) and taking into account the HGF release rate, it was determined that the growth factor also remained between 60 and 80% bioactive for the 15 day release period.

Hepatocyte survival

To determine whether the inclusion of EGF and HGF would promote the survival of transplanted hepatocytes beyond 1 week, scaffolds containing VEGF (3.6 μg/scaffold) and EGF (1.3 μg/scaffold), VEGF and HGF (3.6 μg/scaffold), or all three growth factors were seeded with hepatocytes and transplanted subcutaneously in SCID mice for 3, 7, or 14 days. The seeding efficiency of hepatocytes was determined to be 62 ± 5%. Observation of H&E-stained tissue sections indicated that a majority of the transplanted hepatocytes had survived the initial 3 days *in vivo*, and differences between conditions were not apparent (Fig. 3A and B). After 7 days, hepatocytes delivered in scaffolds containing any combination of growth factors demonstrated enhanced survival as compared to those delivered in blank scaffolds (Fig. 3C and D). In samples containing no growth factor, the overall number of hepatocytes had decreased, and many of the remaining cells lacked evidence of a nucleus, indicating death. In contrast, the hepatocytes in growth factor-releasing scaffolds appeared similar to those at day 3 with respect to overall number and morphology. However, differences in growth factor combinations did not have an obvious effect on hepatocyte survival at this point. The addition of EGF and/or HGF to the delivery system did not lead to long-term transplanted hepatocyte survival, as by day 14 the vast majority of hepatocytes remaining in scaffolds from all conditions were dead (Fig. 3 E and F).

Quantification of hepatocyte survival confirmed the observed trends, as there was no measurable difference between any of the conditions at day 3, where approximately 70% of cells in blank and growth factor–containing scaffolds remained alive. To clearly illustrate the trend for hepatocyte survival over the course of the implant study, the numbers of living hepatocytes have been normalized to the number living at day 3 (Fig. 4). The number of hepatocytes surviving in conditions including growth factors remained nearly constant from days 3 to 7, with no significant difference between growth factor combinations. Survival of the cells transplanted in blank

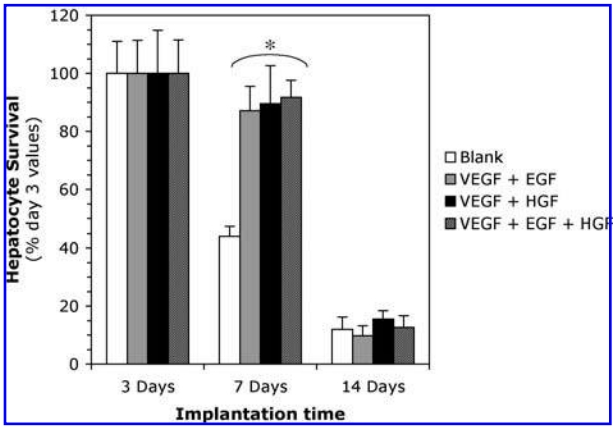

FIG. 4. Quantification of hepatocyte survival following transplantation. Hepatocytes were counted in histological sections at 200 × magnification after 3, 7, and 14 days *in vivo*. Data presented are normalized to the number of live cells present in each condition at day 3. Results are expressed as mean values and SEM with $n = 4$.

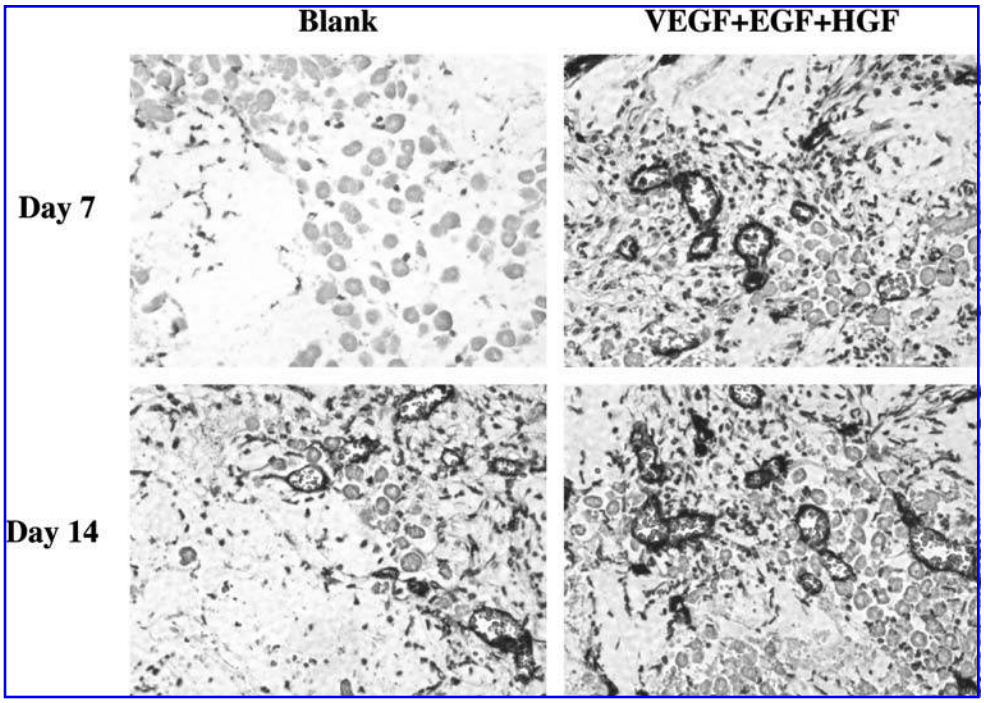

FIG. 5. Growth factor delivery enhances blood vessel formation. Blood vessels were identified in tissue sections from implanted scaffolds immunostained for mouse CD31 antigen. Experimental conditions not shown (VEGF + EGF and VEGF + HGF) were similar in appearance to the condition including all three factors. Note that hepatocytes present in the scaffolds are visible as large, round cells. original magnification × 400

scaffolds declined to less than 50% of the day 3 value during the same time interval. However, there was a dramatic decrease in hepatocyte survival from days 7 to 14 in all conditions, with the number of surviving cells at 10–20% of day 3 values.

Implant vascularization

The extent of vascularization within scaffolds from all conditions was assessed at 7 and 14 days to determine whether the addition of EGF and/or HGF to the delivery system had an effect on previously observed vascularization induced by VEGF alone.[3] Scaffold sections were immunostained with antibodies against CD31 for identification of endothelial cells present in blood vessels. Microscopic visualization of the tissues revealed that a greater blood vessel density was present in growth factor-containing scaffolds than in blank scaffolds at both time points (Fig. 5). There was no observable difference between the different growth factor combinations, and in these conditions the enhanced vascularity was more pronounced at day 7 than at day 14. Quantification confirmed these observations, as the values of vessel density for growth factor-releasing scaffolds were almost an order of magnitude greater than those for blank scaffolds at day 7, with no significant difference between the different growth factor combinations (Fig. 6). At 14 days, the three growth factor conditions were again similar to each other, and had over twice the number of blood vessels as control scaffolds without growth factor. Qualitative differences in vessel diameter and maturity were not observed between conditions containing VEGF alone and those containing EGF and/or HGF, in addition to VEGF.

DISCUSSION

In this investigation, scaffolds previously developed to deliver a single growth factor (VEGF) were modified to deliver additionally bioactive EGF and/or HGF in a sustained manner. However, inclusion of these hepatotrophic factors in the system did not enhance long-term hepatocyte survival as anticipated. The transient improvement in hepatocyte survival observed at the 1 week time point was expected from previous work and is likely attributed to VEGF-induced vascularization of the implants, which was not affected by the presence of EGF or HGF.

Time-appropriate release of bioactive EGF and HGF was achieved via the methods developed for incorporating these growth factors into scaffolds. The delivery system described in this report was optimized to provide sustained release of both EGF and HGF throughout the 2 week implant studies, such that a significant portion of the included growth factors were released in a sustained manner over this time period. Different methods of in-

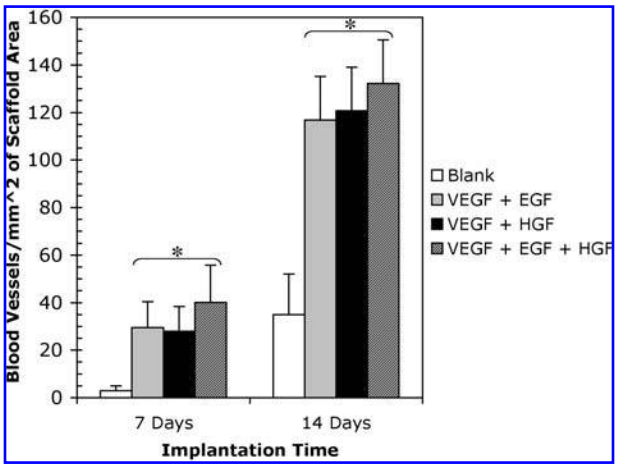

FIG. 6. Blood vessel density in scaffolds utilized for delivery of hepatocytes and combinations of VEGF, EGF, and HGF. Blood vessels were manually counted at 200 × magnification in tissue sections stained with antibodies for the presence of mouse CD31. The number of blood vessels counted in the growth factor samples was significantly greater than ($p < 0.05$) that observed in blank scaffolds after both 7 and 14 days. There was no measurable difference between any of the growth factor combinations at either time point. Results are expressed as mean values and SEM ($n = 3$).

corporating the two growth factors were required to achieve similar release kinetics for each. This was expected, and is likely due to significant differences in the molecular weights of EGF (6 kDa) and HGF (80 kDa) since smaller molecules are able to diffuse out of the scaffolds more quickly. Thus, EGF was first incorporated into PLG microspheres used for scaffold fabrication, as this method has previously been shown to greatly attenuate protein release from this system.[23] The adaptability of this scaffold system for independently controlled release of multiple factors and delivery of a variety of cell types suggest that it will find utility in future tissue engineering applications.

Long-term hepatocyte survival was not enhanced when the cells were transplanted on scaffolds containing EGF and/or HGF, in addition to VEGF. The trend observed at the earlier time points was similar to that previously observed for delivery of VEGF alone: approximately 70% of the transplanted hepatocytes survived until day 3, with no significant decline in survival by day 7 for conditions where VEGF was included to enhance implant vascularization. This trend was not surprising since these survival rates are already quite high and would have been difficult to improve upon with the addition of EGF and HGF. Inclusion of these hepatotrophic factors also did not appear to provide longer-term benefit to the transplanted hepatocytes, as the number of surviving cells declined to the level observed in blank scaffolds after 14 days *in vivo*. This result was surprising, as other experimental models in which transplanted hepatocytes have been provided with EGF or HGF have demonstrated that these factors do enhance survival of the transplanted cells.[4,15,16] An important difference between these other models of hepatocyte transplantation and the model employed here is the location chosen for delivery of the cell population. Scaffolds containing hepatocytes have most often been transplanted to the mesentery of the small intestine,[2,4–6,15,16] instead of to a subcutaneous site. One previous study suggested that EGF delivery only enhanced transplanted hepatocyte survival when an appropriate background of soluble signals existed at the transplant site.[4] Together with these previous investigations, the results presented here suggest that a mesenteric site is superior to a subcutaneous one for applications of hepatocyte transplantation, perhaps because the vasculature in this area carries nutrient-rich blood that supplies a better approximation of the soluble signals present in the liver.

Other potential reasons for the failure of this system to prolong hepatocyte survival might be that insufficient quantities of EGF and HGF were delivered to the cells, or that the factors were delivered with inappropriate release kinetics. Growth factor release kinetics may have been affected by the use of Matrigel for *in vivo* experiments. However, the potential effect of Matrigel is likely small, as a comparison of studies where VEGF has been delivered using this system with and without the inclusion of Matrigel does not reveal a significant effect on the speed or extent of induced vascularization.[3,23] There is a possibility that EGF and HGF interact in a negative fashion to reduce their bioactivities when simultaneously released, although this seems unlikely. The factors are present in low quantities in the scaffolds (<0.03 wt% each) and are known to have an additive effect on hepatocyte proliferation. In addition, previous work with this delivery system has indicated that multiple factors are able to retain bioactivity *in vivo* when simultaneously released from a single scaffold.[23]

The blood vessel density present in scaffolds at 7 and 14 days was significantly enhanced with delivery of VEGF and EGF and/or HGF as compared to control scaffolds containing no growth factor. However, there was no measurable difference among the growth factor-containing conditions, and the inclusion of EGF and/or HGF did not significantly alter the extent of vascularization induced by VEGF alone.[3] It was anticipated that these growth factors would further enhance the VEGF response, as they have also been implicated as promoters of angiogenesis.[24–27] One of the mechanisms by which HGF promotes angiogenesis is to induce VEGF production by a variety of cell types.[28–30] The VEGF delivered from the scaffolds may have already saturated the local environment, minimizing any effect due to enhanced cell

secretion of VEGF in the transplant area. An impact of EGF upon vascularization may not have been realized since its small size and non-heparin binding character likely allow it to diffuse away from the implanted construct more rapidly than VEGF or HGF. There were also no qualitative differences in vessel diameter observed between conditions containing VEGF alone or in combination with EGF and/or HGF, indicating that these factors did not appear to promote vessel maturation over this timeframe.

The system developed in this work for simultaneous delivery of multiple factors from a porous polymer scaffold, which may also be utilized for delivery of a cell population, has potential utility in future investigations of cell-based therapies. Further studies of transplanted hepatocyte survival could be performed by implanting the same constructs to a different location, and alternate or greater quantities of the same growth factors could be tested for their ability to enhance survival. The effect of this system might also be enhanced by altering the release kinetics of EGF and HGF; for example, a greater release rate of these factors at later times may better support long-term survival. If one is interested in determining the effect of a single or a few factors upon hepatocyte survival, subcutaneous transplantation may provide an appropriately isolated environment. This delivery system could also be modified for transplantation of other cell types by including factors known to promote their survival and/or differentiation. Overall, the results of this study confirm that significant obstacles exist for hepatocyte transplantation to become a clinically relevant therapy, as suggested by many previous investigations. However, the delivery system developed here may help to increase understanding of these obstacles and bring therapeutic transplantation of hepatocytes, or other cell types, closer to reality.

ACKNOWLEDGMENTS

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