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Heparin-binding EGF-Like Growth Factor Protects Mesenchymal Stem Cells

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Abstract

Background—We have previously demonstrated that mesenchymal stem cell (MSC) administration protects the intestines from injury in a mouse model of intestinal ischemia/reperfusion (I/R) injury. We have also shown that heparin-binding EGF-like growth factor (HB-EGF) is a potent intestinal cytoprotective agent *in vivo* that may protect the intestines via its effects on SC (SC). The goal of the current study was to examine the effects of HB-EGF on both amniotic fluid (AF)- and bone marrow (BM)-derived MSCs *in vitro*.

Materials and Methods—MSCs were isolated from the amniotic fluid and bone marrow of pan-EGFP mice, grown in MSC-specific culture medium, and purified by sequential passages based on their adherence properties. Pluripotency was confirmed by induced differentiation. After incubation of MSCs with HB-EGF, proliferation was quantified using the CyQuant cell proliferation assay kit under normoxic and anoxic conditions, chemotaxis was quantified using the CHEMICON QCM cell migration kit, and apoptosis was determined by caspase-3 immunohistochemistry after exposure of MSC to anoxic stress.

Results—AF-MSC and BM-MSC showed significantly increased proliferation and migration in response to HB-EGF. HB-EGF significantly protected AF-MSC and BM-MSC from anoxia-induced apoptosis. The proliferative and anti-apoptotic effects of HB-EGF were even more pronounced in AF-MSC compared with BM-MSC.

Conclusions—These results demonstrate that HB-EGF acts as a mitogenic and chemotactic agent for MSC that protects MSC from injury. These findings may have important implications for future experiments designed to utilize MSC to protect the intestines from injury.

Keywords

heparin-binding EGF-like growth factor; mesenchymal stem cells; bone marrow; amniotic fluid; proliferation; apoptosis; chemotaxis

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INTRODUCTION

Mesenchymal stem cells (MSC) are non-hematopoietic, pluripotent, self-renewing progenitor cells with a characteristic spindle-shaped morphology, that have been shown to contribute to the maintenance and regeneration of various connective tissues.¹ They are mobilized from bone marrow in response to tissue injury and aid in repair after a variety of end-organ injury-models including models of myocardial infarction,² spinal cord injury,³ renal ischemia/reperfusion injury,⁴ intestinal radiation injury⁵ and ischemic cerebral injury.⁶ Populations of MSC derived from bone-marrow (BM-MSC)⁷ and amniotic fluid (AF-MSC)⁸ have been isolated, but there is little data characterizing the differences between these two groups.

The robust, self-renewing, multilineage differentiation potential of MSC makes these cells very desirable candidates for possible clinical cellular therapy.⁹ Ongoing investigations are exploring ways to optimize MSC efficacy prior to therapeutic delivery, including preconditioning by exposure to hypoxia,¹⁰ growth factors,¹¹ and various cytokines.^{12,13,14}

Previous studies have focused on BM-MSC, but there is a paucity of data on optimization of AF-MSC for delivery. Much like BM-MSC, AF-MSC have dynamic differentiation potential and can be induced into cells of all three embryonic lineages.^{15, 16} AF-MSC may actually have more potent differentiation potential compared to BM-MSC.¹⁷ Furthermore, unlike embryonic stem cells, AF-MSC are not tumorigenic.¹⁶ These characteristics make AF-MSC a very attractive option for potential cellular therapy.

Heparin-binding EGF-like growth factor (HB-EGF) is a member of the epidermal growth factor (EGF) family¹⁸ that was initially identified as a secreted product of cultured human macrophages.¹⁹ We have previously demonstrated that HB-EGF promotes angiogenesis,²⁰ and acts as a potent intestinal cytoprotective agent in animal models of intestinal ischemia/reperfusion (I/R) injury,²¹ hemorrhagic shock and resuscitation (HS/R)²² and experimental necrotizing enterocolitis (NEC).²³ We have also demonstrated that HB-EGF can protect native intestinal stem cells (ISC) in a rat model of NEC,²⁴ however the effects of HB-EGF on transplanted MSC have not been characterized to date.

In separate experiments, we have shown that administration of HB-EGF in conjunction with MSC transplantation leads to improved efficacy compared to either therapy alone in animal models of intestinal I/R injury and NEC. The goal of the current study was to investigate the interactions between HB-EGF and BM- and AF-derived MSC *in vitro* in order to identify HB-EGF-based strategies that may be useful in improving MSC transplantation efficacy in the future.

METHODS

Isolation of BM-MSC

All procedures were performed with approval from the Institutional Animal Care and Use Committee of the Research Institute of Nationwide Children's Hospital (protocol #AR06-0002). BM-MSCs were harvested from adult pan-EGFP C57/BL6 mice following previously described protocols.⁷ Briefly, mice were euthanized by cervical dislocation, and the femurs and tibias were removed and dissected free of surrounding tissue using sterile technique. The marrow was flushed out with 2 ml of phosphate-buffered saline (PBS) using a sterile syringe and 20 gauge needle. The marrow pellet was dispersed by gentle pipetting and transferred to uncoated cell culture flasks.

Isolation of AF-MSC

Amniotic fluid was obtained via amniocentesis of pan-EGFP C57/BL6 mice using an adaptation of techniques previously described.⁸ Female mice at 12.5 days gestation were anesthetized with 2.5% tribromoethanol via intraperitoneal (IP) injection. The abdominal skin was shaved and scrubbed with 70% ethanol. A midline laparotomy was performed and the gravid uterus identified. The uterus was opened and amniocentesis performed under direct vision of the individual placentas using a 23 gauge needle. Amniotic fluid samples were transferred to uncoated cell culture flasks.

Cell Culture

After harvesting, AF-MSC and BM-MSC were cultured in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 with GlutaMax (DMEM/F12; Invitrogen, Carlsbad, CA) supplemented with 10% MSC Qualified Fetal Bovine Serum (FBS; Invitrogen, Carlsbad, CA) and gentamycin (5 µg/ml) (Invitrogen, Carlsbad, CA) in uncoated cell culture flasks at 37°C in a humidified atmosphere of 5% CO₂/95% Nitrogen. After 24 h, non-adherent cells were washed away with PBS and discarded. Adherent MSC were purified and expanded during successive passages. MSC were passaged once they achieved 80% confluence to expand the primary cultures. MSC from passages four through nine were used for all experiments. Subsequent differentiation assays were performed with cells at passage four and five, with proliferation, migration and apoptosis assays performed using cells no higher than passage nine.

MSC Differentiation Assays

Differentiation assays were used to confirm pluripotency of MSC using the STEMPRO Adipogenesis Differentiation Kit (Invitrogen, Carlsbad, CA) and the STEMPRO Osteogenesis Differentiation Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, MSC were incubated in 12-well plates at 37°C in a humidified atmosphere of 5% CO₂/95% air, and grown in adipogenic or osteogenic differentiation media for 14 days. MSC grown in adipogenic differentiation media were then stained with Oil Red O to confirm adipogenic differentiation, and MSC grown in osteogenic differentiation media were stained with alkaline phosphatase to confirm osteogenic differentiation. Visual microscopic evaluation confirmed terminal differentiation into the adipocyte and osteocyte lineages (Figure 1).

MSC Proliferation

MSC proliferation was measured using CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Briefly, MSC (4000 cells per well) were plated for 24 h in 96-well plates. HB-EGF was then added at various concentrations (0, 5, 10, 25, 50, 100 ng/ml) and 1 h later cells were cultured under either normoxic conditions or with exposure to anoxia (95% N₂/5% CO₂) for 24 h followed by re-oxygenation for 24 h at 37°C. Media was removed, dye binding solution was added to each well, and plates were incubated for 1 h at RT. Results were quantified using a fluorescence plate reader (Molecular Devices, Sunnyvale, CA) using a 485/530 nm filter set. Fluorescent counts for the HB-EGF (0 ng/ml) group was normalized to 100%, with counts in the HB-EGF-treated groups compared to this standard.

MSC Migration Assay

MSC migration was assessed using the CHEMICON QCM Cell Migration Assay Kit (Millipore, Billerica, MA) according to the manufacturer's instructions. Briefly, 0.5×10^6 MSC/ml in serum-free media were placed in the inner wells of the migration chambers and HB-EGF at various concentrations (0, 5, 10, 25, 50, 100 ng/ml) in serum-free media was

placed in the outer wells of the chambers, with incubation at 37°C for 24 h. Media was then removed, lysis buffer/dye solution was added to each well, and plates were incubated for 15 min at RT. Results were quantified using a fluorescence plate reader (Molecular Devices, Sunnyvale, CA) using a 490/520 nm filter set. Fluorescent counts for the HB-EGF (0 ng/ml) group was normalized to 100%, with counts in the HB-EGF-treated groups compared to this standard.

MSC Apoptosis Assay

MSC apoptosis was assessed using caspase-3 immunohistochemistry (IHC). MSC were seeded on cover slips in 12-well plates in the growth media described above and allowed to adhere for 24 h. MSC were treated with HB-EGF at various concentrations (0, 5, 10, 25, 50, 100 ng/ml), and 1 h later were stressed by exposure to anoxia (95% N₂/5% CO₂) for 24 h followed by 24 h of re-oxygenation at 37°C. MSC were then washed with sterile PBS with 0.1% Tween 20 (PBS/Tween), fixed with 4% paraformaldehyde in PBS at RT for 20 min, washed with PBS/Tween, and blocked with 10% goat serum in PBS/Tween for 1 h at RT. MSC were then incubated overnight at 4°C with rabbit anti-cleaved-caspase 3 antibody (Cell Signaling Technology, Danvers, MA) at a 1:100 dilution. After washing with PBS/Tween, MSC were incubated for 1 h at RT with Cy3 AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson Immuno Research, West Grove, PA) at a 1:500 dilution. MSC were then washed, counterstained with DAPI and visualized using a Zeiss Axioskop fluorescent microscope (Carl Zeiss, New York, NY). Five random fields were counted per well in two separate experiments to quantify the ratio of apoptotic cells to total cells.

Statistical Analyses

Experimental data were expressed as mean ± SD. Intragroup statistical differences were determined using ANOVA, with statistical significance between pairs of individual experimental groups determined using post-hoc Student's *t*-test. Differences were considered to be statistically significant if $p < 0.05$.

RESULTS

HB-EGF Promotes MSC Proliferation under Normoxic and Hypoxic Conditions

Under normoxic conditions HB-EGF significantly stimulated both AF-MSC and BM-MSC proliferation over a range of HB-EGF doses from 5-100 ng/ml (Figure 2). HB-EGF had a significantly greater proliferative effect on AF-MSC compared with BM-MSC over the entire range of HB-EGF doses. Upon exposure to anoxia/reoxygenation, HB-EGF stimulated AF-MSC proliferation at the same doses, and BM-MSC proliferation at slightly higher doses (10-100 ng/ml) (Figure 3). The proliferation observed for AF-MSC under normoxic conditions was the most robust of all conditions tested.

HB-EGF Induces MSC Migration

AF-MSC and BM-MSC had increased migration in response to HB-EGF over a range of HB-EGF doses from 5-100 ng/ml (Figure 4). The chemotactic effect of HB-EGF was comparable in AF-MSC and BM-MSC.

HB-EGF Protects MSC from Anoxia-Induced Apoptosis

Under normoxic conditions, the percentage of apoptotic cells was 2.1% for AF-MSC and 1.7% for BM-MSC (Figure 5). Following exposure to anoxia for 24 h followed by re-oxygenation for 24 h, AF-MSC apoptosis increased to 7.2% and BM-MSC apoptosis increased to 9.6%. Addition of HB-EGF protected both AF-MSC and BM-MSC from anoxia-induced apoptosis in a dose-dependent fashion. At lower doses of HB-EGF (5 ng/ml

and 10 ng/ml), there was significantly decreased apoptosis in the AF-MSC group compared with the BM-MSC group.

DISCUSSION

In recent years MSC have emerged as an attractive target for regenerative medicine. Their properties in cell culture and their *in vivo* behavior are becoming increasingly characterized. Various methods have been explored in order to optimize these cells for potential therapeutic use, and attempts to characterize differences between MSC subtypes are currently underway.

HB-EGF has potent mitogenic effects on a variety of cell types, including smooth muscle cells, epithelial cells, fibroblasts, keratinocytes and renal tubular cells,²⁵ and is a known chemotactic agent for various cell types.^{26,27} It exerts its effects by binding to cell-surface EGFR^{28,29} and to the HB-EGF-specific receptor Nardilysin (Nrhc).^{30,31} Our current data show that HB-EGF promotes proliferation of both AF-MSC and BM-MSC under normoxic conditions, and even after exposure to anoxia/reoxygenation. AF-MSC appear to be more sensitive to the trophic effects of HB-EGF than BM-MSC, especially under normoxic conditions. Although HB-EGF increases chemotaxis in both AF-MSC and BM-MSC, it more readily protects AF-MSC from anoxia-induced apoptosis compared with BM-MSC. The specific mechanisms underlying the effects of HB-EGF on MSC have yet to be determined. Previous observations have demonstrated that hypoxia activates many survival and stress pathways, including down regulation of p38MAPK,³² down regulation of PI3K and ERK 1-2,³³ and up regulation of the Akt inhibitor PTEN.³⁴ We have shown that HB-EGF activates these pathways in other cell types,³⁵ and future experiments will elucidate the exact mechanisms utilized by HB-EGF in its effects on MSC.

In separate experiments, we have administered HB-EGF with MSC transplantation in a mouse model of ischemia/reperfusion injury based on segmental mesenteric artery occlusion. We found that administration of HB-EGF in conjunction with MSC results in increased MSC engraftment into the injured intestine, and that the combined therapy leads to improved gut barrier function compared to either therapy alone (unpublished observations). Our *in vivo* ischemia/reperfusion studies suggest that AF-MSC may be more efficacious than BM-MSC in protection of the intestines from injury when administered in conjunction with HB-EGF. These *in vivo* findings, in combination with the *in vitro* results reported here, may support the use of HB-EGF-based therapies to optimize MSC efficacy.

One of the major benefits of MSC therapy is the high level of tolerance to transplantation of these cells. The hypo-immunogenicity of MSC,^{36,37} including fetal-derived AF-MSC,³⁸ prevents rejection after allogeneic transplantation.^{36,37} When used for regenerative medicine, including the treatment of intestinal injury, MSC are efficient at homing to the injury site and modulating local inflammatory factors, however little is known about the process of mesenchymal to epithelial transition (MET) needed to complete intestinal restitution.³⁹ How much of the MSC transplantation efficacy is due to inflammatory modulation and how much is a direct result of MET promoting intestinal restitution is also uncertain. Elucidating the details of these interactions will represent an important step towards fully utilizing MSC-based therapies.

In addition to AF-MSC, there are similar other fetal-derived progenitor cells located in the chorionic⁴⁰ and placental⁴¹ tissues. These cells have similar characteristics to other MSC populations, but there is little data directly comparing the cells derived from these different anatomical territories. This is an area of future research that may also be beneficial in maximizing MSC therapeutic efficacy.

In conclusion, we have demonstrated that HB-EGF promotes proliferation of AF-MSC and BM-MSC under normoxic and anoxic conditions, induces MSC chemotaxis, and protects MSC from anoxia-induced apoptosis. The observation that HB-EGF induces a more robust proliferative response as well as increased resistance to anoxic stress in AF-MSC compared with BM-MSC may explain why AF-MSC appear to be more effective in protection of the intestines from injury *in vivo* when administered in conjunction with HB-EGF. Our data support the use of HB-EGF as a potential method to improve the efficacy of MSC transplantation for therapeutic use in the future.

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Figure 1. MSC differentiation

Shown are representative images of A) AF-MSC and B) BM-MSC grown in *a*) regular MSC medium, *b*) adipogenic medium, and *c*) osteogenic medium. LipidTOX Neutral Red staining was used to identify adipocytes and Alizarin Red S staining was used to identify osteocytes.

Figure 2. Effect of HB-EGF on MSC proliferation under normoxic conditions

MSC were treated with varying concentrations of HB-EGF, cultured for 48 h at 37°C under normoxic conditions, and cell proliferation quantified. The experiment was performed twice, with each parameter assayed in duplicate. * $p < 0.01$ compared with no HB-EGF; ** $p < 0.01$ comparing AF-MSC with BM-MSC.

Figure 3. Effect of HB-EGF on MSC proliferation under anoxic conditions

MSC were treated with varying concentrations of HB-EGF, cultured for 24 h at 37°C under anoxic (95% N₂/5% CO₂) conditions followed by a 24 h incubation under normoxic conditions, and cell proliferation quantified. The experiment was performed twice, with each parameter assayed in duplicate. * $p < 0.05$ and ** $p < 0.01$ compared with no HB-EGF.

Figure 4. Effect of HB-EGF on MSC migration

MSC were placed in the inner wells and HB-EGF was placed in the outer wells of migration chambers, followed by incubation at 37°C for 24 h. The experiment was performed three times, with each parameter assayed in duplicate. * $p < 0.05$ and ** $p < 0.01$ compared with no HB-EGF.

Figure 5. Effect of HB-EGF on MSC apoptosis

MSC were exposed to 24 h of anoxia (95% N₂/5% CO₂) followed by 24 h of normoxia, and apoptotic cells identified by caspase-3 immunohistochemistry. Five random fields were counted per well for each experimental data point, and the ratio of apoptotic cells to total cells was quantified. The experiment was performed twice with each parameter assayed in duplicate. * $p < 0.05$ and ** $p < 0.01$ compared with no HB-EGF. *** $p < 0.01$ comparing AF-MSC with BM-MSC.