Significance and Therapeutic Potential of Endothelial Progenitor Cell Transplantation in a Cirrhotic Liver Rat Model

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Background & Aims: **We investigated whether endothelial progenitor cell (EPC) transplantation could reduce established liver fibrosis and promote hepatic regeneration by isolating rat EPCs from bone marrow cells.** ***Methods:*** **Recipient rats were injected intraperitoneally with carbon tetrachloride (CCl_4) twice weekly for 6 weeks before initial administration of EPCs. CCl_4 was then readministered twice weekly for 4 more weeks, and EPC transplantation was carried out for these same 4 weeks.** ***Results:*** **At 7 days in culture, the cells expressed Thy-1, CD31, CD133, Flt-1, Flk-1, and Tie-2, suggesting an immature endothelial lineage. Immunohistochemical analyses showed fluorescent-labeled, transplantation EPCs were incorporated into the portal tracts and fibrous septa. Single and multiple EPC transplantation rats had reduced liver fibrosis, with decreased α2-(I)-procollagen, fibronectin, transforming growth factor-β, and α-smooth muscle actin-positive cells. Film in situ zymographic analysis revealed strong gelatinolytic activity in the periportal area, in accordance with EPC location. Real-time polymerase chain reaction analysis of multiple EPC-transplantation livers showed significantly increased messenger RNA levels of matrix metalloproteinase (MMP)-2, -9 and -13, whereas tissue inhibitor of metalloproteinase-1 expression was significantly reduced. Expression of hepatocyte growth factor, transforming growth factor-α, epidermal growth factor, and vascular endothelial growth factor was increased in multiple EPC-transplantation livers, while hepatocyte proliferation increased. Transaminase, total bilirubin, total protein, and albumin levels were maintained in EPC-transplantation rats, significantly improving survival rates.** ***Conclusions:*** **We conclude that single or repeated EPC transplantation halts established liver fibrosis in rats by suppressing activated hepatic stellate cells, increasing matrix metalloproteinase activity, and regulating hepatocyte proliferation.**

It is widely recognized that the development of liver fibrosis is intimately associated with the progression of chronic liver disease caused by agents such as chronic hepatitis virus B and hepatitis virus C infection, alcohol abuse, or nonalcoholic steatohepatitis.[1–3] Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension, and this condition often requires liver transplantation. Liver transplantation may be the only alternative to treat patients with a heavily damaged liver, as in cases of severe cirrhosis. Liver transplantation improves both survival and quality of life. However, there are only a limited number of donor livers available for patients.[1,4] Therefore, it is very important to investigate appropriate new therapies.

Several recent promising studies have shown that hematopoietic stem cells from bone marrow (BM) have the capacity to differentiate into a variety of nonhematopoietic cell tissues such as liver, heart, and brain.[5–11] Even multiorgan differentiation was demonstrated from hematopoietic stem cells and from the recently described multipotent adult progenitor cells.[12,13] Perfusion of these cells is one potential approach to promoting fibrosis resolution and hepatic regeneration.

Endothelial progenitor cells (EPCs) are mobilized from BM and incorporated into sites of vascular disorders, at which they aid in neovascularization.[14–17] Neovascularization involves angiogenesis, ie, formation of new blood vessels via sprouting of preexisting mature endothelial cells, and also vasculogenesis, which is formation of blood vessels by differentiation of EPCs.[14,18,19] We recently reported that EPC transplantation significantly enhanced vascularization and improved survival rates after acute liver injury in mice.[20] However, it remains unclear whether EPCs can inhibit fibrogenesis in vivo and

Abbreviations used in this paper: α-SMA, α-smooth muscle actin; BM, bone marrow; CCl_4, carbon tetrachloride; ECM, extracellular matrix; EGF, epithelial growth factor; EPC, endothelium progenitor cell; Flk-1, fetal liver kinase-1; Flt-1, fms-like tyrosine kinase-1; HGF, hepatocyte growth factor; HSC, hepatic stellate cell; MMP, matrix metalloproteinase; RAECs, rat aortic endothelial cells; TAA, thioacetamide; TGF, transforming growth factor; Tie-2, tyrosine kinase with Ig and EGF homology domains-2; TIMP, tissue inhibitor of metalloproteinase; VEGF, vascular endothelial growth factor.

0016-5085/07/$32.00
doi:10.1053/j.gastro.2007.03.110

whether EPC transplantation could be effective against established liver fibrosis. We therefore investigated the antifibrogenic and regenerative effects of EPC transplantation in carbon tetrachloride (CCl_4)- and thioacetamide (TAA)-induced cirrhosis.

Materials and Methods

Animals

The study was conducted using 6-week-old male Wistar rats having an approximate body weight (BW) of 120–150 g. Wistar rats were purchased from CLEA Japan Inc. (Shizuoka, Japan). Animals were maintained in temperature-controlled rooms (21°C ± 2°C) under a 12/12-hour dark/light cycle and allowed food (standard laboratory chow) and water ad libitum.

Isolation of Mononuclear Cells

EPC-like mononuclear cells (MNCs) in the BM were quantified. Donor rats were killed to collect BM cells from the right and left femurs by flushing the BM cavity with heparinized saline.[21] The resulting cell suspension was centrifuged over a Ficoll step gradient (density, 1.077 g/mL) (Ficoll-Histopaque 1077; Sigma-Aldrich, Steinheim, Germany) at 2000 rpm for 30 minutes. The interface fraction was collected, and mononuclear cells were then resuspended in cell culture medium as described below. They were then plated on 2% gelatin-coated 100-mm plastic dishes.

Culture of EPCs

Medium 199 containing 20% fetal bovine serum (FBS; Biowest, Caille, France), bovine pituitary extracts (Biomedical Tech. Inc., Stoughton, MA) to stimulate cell growth, heparin (100 μg/mL), and antibiotics (GIBCO, Auckland, NZ) were used for cell culture. Attached (AT) cells were allowed to develop into EPC for 7 days of culture, at which time culture dishes were washed with phosphate-buffered saline (PBS), and flow cytometry was performed as described previously.[14,20,22] EPCs attached to dishes were treated with trypsin-EDTA solution (Sigma-Aldrich) for 5 minutes, and detached cells were collected. After washing with PBS, EPCs were suspended in PBS at a density of 3.0×10^6/1 mL and injected into rats as described below.

Primary Culture of Rat Aortic Endothelial Cells

Rat aortic endothelial cells (RAECs) were isolated from rat aorta and then subcultured as reported previously[23] and were then grown in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, Ayershire, United Kingdom) containing 10% fetal bovine serum (FBS) and P-S solution (GIBCO).

Cell Labeling

Experiments were carried out to detect EPCs as follows: the cultured cells were labeled with red fluorescent marker PKH26-red (Sigma Chemical Co., St. Louis, MO) following the manufacturer's instructions before EPC transplantation into rats, as described previously.[20]

Experimental Conditions and Transplantation of EPCs

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kurume University School of Medicine. Cirrhosis was induced by 2 different methods. For the CCl_4 (Wako, Osaka, Japan)-treated model, phenobarbital sodium (Wako; 35 mg/dL) was added to the drinking water for 1 week. The rats then received intraperitoneal injections of 50% CCl_4 (10 mg/kg body weight) twice weekly for 6 weeks, as described previously.[24] After 6 weeks of CCl_4 injections, the rats were divided randomly into 3 treatment groups: (1) saline infused (n = 26); (2) EPC treated (n = 20) once weekly; and (3) EPC treated (n = 18) on day 43 only. EPC (recipient) rats received EPCs via the tail vein (3.0×10^6 cells/rat). Following EPC transplantation, CCl_4 administration was continued twice weekly for another 4 weeks. Rats also received EPCs or a saline injection once weekly over this same 4-week period. The rats were killed after this 10-week period of CCl_4 injection. For the TAA (Wako)-treated model, rats were injected intraperitoneally with TAA (200 mg/kg body weight) twice weekly for 6 weeks, as described previously.[25] After 6 weeks of TAA injections, the rats were divided randomly into 2 treatment groups: (1) saline infused (n = 15) and (2) EPC treated once weekly (n = 15). EPC (recipient) rats received EPCs via the tail vein (3.0×10^6 cells/rat). Following EPC transplantation, TAA administration continued twice weekly for another 3 weeks. Rats also received EPCs or a saline injection once weekly over this same 3-week period. The rats were killed after this 9-week period of TAA injection.

To determine whether the living EPCs were effective, we performed irradiated EPC transplantation. EPCs were irradiated with 100 mJ/cm^2 ultraviolet (UV) radiation for 3 hours in a Spectrolinker XL-1000 UV crosslinker (Spectronics Corporation, Westbury, NY), and the cells were collected and underwent transplantation using the same experimental methods as for EPCs.

To clarify whether transplanted ($PKH26^+$) cells were tracked in cirrhotic liver, we performed sex-mismatched EPC transplantations from male donor rats into female recipients; female rats treated for 6 weeks with CCl_4 received male EPCs (3.0×10^6 cells/rat). The rats were killed 1 week after this EPC transplantation, and liver sections were analyzed using fluorescent in situ hybridization (FISH) to detect the Y chromosome.

Flow Cytometry

EPCs on day 7 of culture (n = 5) were analyzed by fluorescence-activated cell sorting (FACS; FACS SCAN