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Stem cell therapy with skeletal myoblasts accelerates neointima formation in a mouse model of vein graft disease

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ABSTRACT

Although still a matter of controversial discussion, skeletal myoblasts are one of the options for stem cell transplantation improving cardiac function after myocardial infarction, exhibiting several advantages including the availability, the ability of self-renewal and differentiation, and the lack of ethical and immunological problems. The aim of this study was to investigate the impact of stem cell therapy with skeletal myoblasts on experimental venous bypass grafts in a mouse model of vein graft disease.

Forty C57BL/6J mice underwent bypass grafting interposing a venous bypass graft of the donor mouse into the carotid artery of the recipient mouse.

Twenty mice received periadventitially treatment with 1 million fluorescence labeled skeletal myoblasts suspended in culture medium (treatment group), the other twenty mice received only culture medium without myoblasts (control group).

Two weeks after bypass surgery, the vein grafts of all 40 mice were harvested, stained and histologically investigated under light and immunofluorescence microscope.

Against our expectations, skeletal myoblasts stayed in place and were still located in the adventitia after bypass grafting. Additionally, vein grafts of the myoblast group revealed a 2fold increased neoneointima formation, a decreased media thickness, a slightly increased neovascularization, a higher percentage of re-endothelialization and also a slightly higher percentage of PDGFR α, PDGFR ß, MMP-7 and MMP-9 positive cells, suggesting a paracrine mechanism responsible for accelerated neointima formation.

In conclusion, the results of our study do not support the use of skeletal myoblast for the treatment of vein graft disease after coronary artery bypass surgery.

1. Introduction

Cardiovascular disease is the leading cause of death and disease globally, with over 17 million people dying of cardiovascular disease each year.

Coronary artery bypass grafting (CABG) and percutaneous coronary intervention (PCI) are the two invasive treatment options of choice. The success of CABG performed with saphenous vein grafts is often limited by restenosis or vein graft failure.

Neointimal formation is central to both vein graft failure and restenosis (Bennett and O'Sullivan, 2001; Komatsu et al., 1998; Lefkovits and Topol, 1997; Schwartz et al., 1992). The neointima is predominantly formed of smooth muscle cells (SMCs) and extracellular matrix, but other components, such as inflammatory cells and thrombus remnants, may also be present (Chung et al., 2002; Gallo et al., 1998; Moreno et al., 1996; Steele et al., 1985). Many approaches have been explored to prevent neointimal formation. These strategies include pharmacological (e.g., cytostatic drugs, anti-inflammatory agents), mechanical (sheaths, internal stents), genetic (gene transfer, stem/progenitor cells), and combination therapies (drugeluting stents, antibody-directed treatments, nanoparticlebased platforms) (Bult, 2000; Lefkovits and Topol, 1997; Mehta et al., 1998), but the complete therapeutic prevention of neointimal formation is yet to be achieved.

Since the early 1990s, stem cell therapy has been explored as a potential new therapy to alleviate the consequences of myocardial infarction (MI) and chronic ischemic heart failure. The approach is based

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on the notion that transplantation of cardiomyocytes, or alternatively stem cells, will result in the formation of new cardiac muscle in the recipient heart.

Autologous skeletal myoblasts, which are mononucleated unipotent precursors to skeletal myofibers, are a potential resource for cardiac repair because of their biological properties and lack of ethical problems.

The great advantage of using myoblasts are the availability, the lack of immunologic barriers to the transplantation process, which precludes the need for immunosuppression to allow donor cell acceptance by the host, the diminished risk of tumorigenesis, and the high resistance to tissue ischemia. Another advantage lies in the fact that these cells can be isolated from the patient's own skeletal muscle, expanded in vitro, and transplanted back into the patient's heart (Minami et al., 2003).

Skeletal myoblasts are either injected directly into the scar area or in the sourrounding tissue. Most of previous animal and clinical studies evaluated the effects of stem cells on neoangiogenesis of the periinfarct area and did not investigate the effects of stem cells on already existing coronary artery bypass grafts, although more and more patients with a medical history of ischemic heart disease and myocardial infarction have already undergone coronary artery bypass surgery.

Our study aimed to investigate the effect of skeletal myoblasts on experimental vein grafts interposed into a carotid artery in a murine mouse model of vein graft disease.

2. Material and methods

2.1. Myoblast cell culture and cell labelling

For cell culture a fragment of the vastus lateralis muscle of a C57BL/6J mouse was used (equivalent to 5–8 g). All non muscular tissues of the fragment were removed, and skeletal myoblasts were isolated according to the technique of Delaporte et al. (1984). In brief, the skeletal tissue was digested with 0.075% type II collagenase (Sigma-Aldrich, St. Louis, MO, USA) in a 5% CO_2 incubator for 1 h at 37 °C. This mixture was filtered through a 140 μm nylon mesh and centrifuged at 1,200 × *g* for 10 min at 4 °C. The assays were performed in 25 cm^2 flasks. The resulting interphase was washed and cultured in Dulbecco's modified Eagle medium-low glucose (DMEM-LG; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (MDgenics, St. Louis, MO, USA) in a 5% CO_2 incubator at 37 °C for four to five passages before transplantation. For cell labeling of vital cells PKH26 Red Fluorescent Cell Linker Kit For General Cell Membrane Labelling (Sigma-Aldrich, St. Louis, MO, USA) was used according to the manufacturer's instructions.

2.2. Animals and operative procedure

C57BL/6J mice were purchased from Harlan-Winkelmann (Borchen, Germany). They were maintained at 24 °C and received food and water ad libitum. All procedures were performed in accordance to protocols approved by the Austrian Ministry of Science (§ 8 of the law on animal experiments). All animals were treated according to the *Guide for the Use and Care of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Donor and recipient mice were anesthetized with pentobarbital sodium (50 mg/kg body weight, intraperitoneally). Additionally, atropine sulfate (1 mg/kg body weight) was administered for cardiorespiratory stabilization. The anterior thoracic cage of the donor mouse was removed by two lateral thoracotomies. The inferior vena cava with a length of 1 cm was separated from surrounding tissue, resected and stored in cold Ringer's lactate solution until grafting.

In the recipient mice the right common carotid artery was mobilized from the bifurcation towards the proximal and distal ends. The vessel was ligated on both ends with two 8-0 silk sutures and dissected in the middle. The proximal and distal stumps of the carotid artery were passed through polyethylene cuffs with a length of 1 mm and an inside diameter of 0.5 mm (Portex LTD, London, UK). The vessel was fixed to the extension of the cuff with Yasargil micro clamps. After removing the ligature the artery was everted over the cuff and fixed to the cuff with an 8-0 silk suture. The inferior vena cava from the donor mouse was interposed between the carotid artery cuffs by pulling the end of the vein over the everted and cuffed parts of the artery with 8-0 silk sutures. Finally, the Yasargil clamps were removed and the skin incision was closed with a 6-0 running suture.

2.3. Administration of skeletal myoblasts

In the treatment group (n = 20) 1 million skeletal myoblasts were applied periadventially in 100 μl culture medium. Control animals (n = 20) received culture medium without skeletal myoblasts. After two weeks of follow up vein grafts were harvested and the animals were sacrificed under terminal anesthesia.

2.4. Histologic examination

After harvesting of the vein grafts, 20 vessels (10 of the control group and 10 of the treatment group) were embedded in Tissue-Tek® O.C.T. compound, 4 μm thick frozen sections were performed and stained with hematoxylin and eosin. The presence and location of the myoblasts was examined by immunofluorescence microscopy.

For further staining procedures and immunohistochemistry, the remaining 20 vessels (10 of the control group and 10 of the treatment group) were embedded in mouse liver pieces, fixed in 4% phosphate buffered formaldehyde, dehydrated and wax embedded. Sections of 2 μm thickness were stained with hematoxylin and eosin, elastica staining for verifying elastic fibers, alcian blue staining for the visualization of acid mucopolysaccharids, Masson's trichrome stain for assessment of vessel wall fibrosis and Gordon & Sweet's staining for verification of reticulin fibers in the vein graft layers.

For immunohistochemistry, the following rabbit polyclonal antibodies (all Abcam, Cambridge, UK) were used: smooth muscle actin antibody (dilution 1:50, ab5694), metalloproteinase-7 (MMP-7) antibody (dilution 1:100, ab5706), metalloproteinase-9 (MMP-9) antibody (dilution 1:100, ab38898), PDGF Receptor alpha (PDGFR α) antibody (dilution 1:100, ab124392), PDGF Receptor beta (PDGFR ß) antibody (dilution 1:50, ab32570). All antibodies needed microwave antigen retrieval, smooth muscle actin antibody microwave antigen retrieval with 10 mM citrate buffer (ph 6.0).

All slides were photographed by an Olympus camera at 10-fold magnification. The luminal area, intima and media thickness was measured (4 measurements in all quarters of the vein grafts) using the ImageJ software for Java (National Institutes of Health, USA).

The median value of all measurements was regarded as representative for the neointimal and media thickness.

For the inflammation grading the following semiquantitive score was used: 0 – no inflammation, 1 – mild inflammation/inflammation of 1/3 of the circumference of the vein graft wall, 2 – moderate inflammation/inflammation of 2/3 of the circumference, 3 – intense inflammation/inflammation of the whole circumference.

The occurrence of acid mucopolysaccharids, elastic fibers, reticulin fibers, vessel wall fibrosis and the reendothelialization was given as percentage of area; MMP-7, MMP-9, It should be PDGFR α and PDGFR β positive cells as percentage of the total amount of cells in the vessel wall layers. The number of capillaries in intima, media and adventitia was counted in 10 high-power fields (HPF) at 40-fold magnification. The number of positively stained vascular smooth muscle cells were counted in high-power fields and extrapolated to 1 mm^2.

2.5. Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.).