that human natural preformed antibodies could bind to hADSCs/MSCs after exposure to fresh NHS. The subtype of natural preformed antibodies was mainly IgG, not IgM. This human IgG binding was related to the amount of Neu5Gc on the hADSCs/MSCs, because hADSCs/MSCs cultured with heat-inactivated NHS which expressed negligible levels of Neu5Gc showed negligible levels of IgG binding when exposed to fresh NHS. This result is also consistent with the previous study that anti-Neu5Gc antibodies constitute the majority of natural preformed xenoreactive antibodies besides anti-galactose-alpha 1,3-galactose (Gal) antibodies, particularly in the IgG subclass.[20,29] In effect, hADSCs/MSCs cultured with FBS may seem like xenogeneic cells to the human immune systems.

When xenogeneic grafts are transplanted into humans, binding of natural preformed antibodies that recognize xenoantigens, including Gal and Neu5Gc, mediates two types of rejection response, hyperacute rejection (HAR) and acute humoral xenograft rejection (AHXR).[30] HAR begins with binding of natural preformed antibodies to the xenogeneic epitopes on donor endothelial cells, including Gal and Neu5Gc xenoantigens, leading to complement activation by mainly classical pathway.[30] The graft is rejected within minutes to hours. Therefore, we analyzed the CMC of hADSCs/MSCs cultured with FBS that express Neu5Gc xenoantigen, using fresh NHS. However, we could not confirm the existence of CMC. The deposition of C4 and C3 fragments on hADSCs/MSCs after a short incubation with fresh NHS could not also be detected. In this issue, there are no reports describing the CMC of hADSCs/MSCs cultured with FBS that express Neu5Gc xenoantigen. Martin *et al.* reported that binding of natural preformed antibodies to Neu5Gc on hESCs mediated complement activation leading to cell death.[13] In contrast, Cerdan *et al.* reported that complement activation by anti-Neu5Gc antibody does not mediate killing of hESCs.[23] Several reasons for this discrepancy have been supposed. One is the difference of procedures used for testing cell cytotoxicity. Previous two reports detected cell cytotoxicity by propidium iodide or 7-AAD exclusion using flow cytometry. Single-cell suspension required for this procedure may cause extensive cell death even under controlled conditions. We detected cell cytotoxicity by conventional LDH release assay, which is often used in cytotoxicity assays.[32,33] The other and more possible reason is the biological difference among the human stem cells, including hESCs and hMSCs. We assessed the expression of complement regulatory proteins such as CD46, CD55, and CD59 on hADSCs/MSCs. hADSCs/MSCs were weakly positive for both CD46 and CD55 and highly positive for CD59. It is reported that HAR could be prevented by inhibiting complement activation, using transgenic animals bearing transgenes encoding human complement regulatory proteins.[34–36] Thus, it is supported that hADSCs/MSCs express complement regulatory proteins and may be largely resistant to killing by CMC mechanism. However, the expression of complement regulatory proteins on other human stem cells such as hESCs remains uncertain and further investigation is needed.

AHXR occurs when HAR is prevented, and it can be induced by low levels of natural preformed antibodies.[30] The binding of natural preformed antibodies to xenogeneic endothelial cells results in ADCC by natural killer cells, macrophages, and neutrophils, endothelial cell activation, thrombosis, and vasoconstriction.[30] It is reported that AHXR could be mediated by natural preformed antibodies against non-Gal xenoantigen,[37,38] particularly Neu5Gc xenoantigen.[39] Therefore, we analyzed the ADCC of hADSCs/MSCs cultured with FBS that express Neu5Gc xenoantigen. Our data indicated the clear existence of ADCC of hADSCs/MSCs cultured with FBS. This ADCC is supposed to be mediated by preformed natural antibodies that recognize Neu5Gc because ADCC of hADSCs/MSCs cultured with heat-inactivated NHS which expressed negligible levels of Neu5Gc could not be detected. We also analyzed the antibody-mediated cell phagocytosis of hADSCs/MSCs cultured with FBS by monocyte-derived macrophage because macrophages can target opsonized cells. However, in our study, a low level of phagocytic activity of hADSCs/MSCs cultured with FBS even in the absence of NHS was detected and this phagocytic activity clearly increased in the presence of NHS. Ide *et al.* reported that human macrophages could phagocytose porcine cells in an antibody- and complement-independent manner and elimination of Gal on porcine cells that expressed Neu5Gc did not prevent this phagocytic activity.[40] Our data indicated that hADSCs/MSCs cultured with heat-inactivated NHS which expressed negligible levels of Neu5Gc were resistant to phagocytosis mediated by human macrophages in the presence or absence of fresh NHS. Accordingly, human macrophages may be able to recognize Neu5Gc xenoantigen and phagocytose hADSCs/MSCs.

We showed here that hADSCs/MSCs cultured with FBS expressed Neu5Gc xenoantigen and that binding of natural preformed antibodies led to immune response. Based on current data, it is clear that hADSCs/MSCs should be chased without animal materials. Yamaguchi *et al.* have tried xeno-free techniques on hematopoietic stem cells by growing them on human stromal cells and using medium containing NHS.[41] To eliminate Neu5Gc on hADSCs/MSCs, we cultured them in a medium in which FBS was replaced by heat-inactivated NHS for a week after culturing with FBS. The expression of Neu5Gc on these hADSCs/MSCs was extremely reduced. Heiskanen *et al.* described that BM-derived hMSCs became decontaminated after 2 weeks of culture in a medium in which FBS was replaced by NHS, but complete decontamination was difficult to achieve by changing culture conditions.[14] Therefore, hADSCs/MSCs may not be completely decontaminated with Neu5Gc by culturing with heat-inactivated NHS for a week. However, our data suggested that human immune responses mediated by Neu5Gc on hADSCs/MSCs, such as ADCC and phagocytosis, were nearly completely prevented by this culture condition. Adipogenic and osteogenic differentiation potentials of hADSCs/MSCs cultured with heat-inactivated NHS were not less than that of those cultured with FBS. This work implies that the culture conditions avoiding renewed exposure to animal materials can reduce the expression of Neu5Gc on hADSCs/MSCs and consequently prevent human immune responses against hADSCs/MSCs. Although major complications have not been reported in the clinical trials with hMSCs cultured with FBS, human immune responses mediated by Neu5Gc may potentially influence the survival and efficacy of the transplanted cells and thus bias the published results. For clinical application of stem cell therapies based on hADSCs/MSCs, hADSCs/MSCs that presented Neu5Gc on their cell surfaces after

exposure to FBS should be cleaned up by chasing without Neu5Gc condition and thus might be rescued from xenogeneic rejection.

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AU6 ▶ ### Disclosure Statement

No competing financial interests exist.

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Bcl-2 Expression Enhances Myoblast Sheet Transplantation Therapy for Acute Myocardial Infarction

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Running title: Bcl-2 enhances myoblast sheet transplantation

ABSTRACT

Myoblast sheet transplantation is a promising novel treatment modality for heart failure after an ischemic insult. Low supply of blood and nutrients may, however, compromise sheet survival. The aim of this study was to investigate the effect of mitochondria-protective Bcl-2-modified myoblasts in cell sheet transplantation therapy. In the Bcl-2-expressing rat L6 myoblast sheets (L6-Bcl2), increased expression of myocyte markers and angiogenic mediators was evident as compared to wild type (L6-WT) sheets. The L6-Bcl2 sheets demonstrated significant resistance to apoptotic stimuli, and their differentiation capacity *in vitro* was increased. We evaluated the therapeutic effect of Bcl-2-modified myoblast sheets in a rat model of acute myocardial infarction (AMI). 64 Wistar rats were divided into 4 groups. One group underwent AMI (n=22), another AMI and L6-WT sheet transplantation (n=17), and a third AMI and L6-Bcl2 sheet transplantation (n=20). 5 rats underwent a sham operation. Echocardiography was performed after 3, 10, and 28 days. Samples for histological analysis were collected at the end of the study. After AMI, the Bcl-2-expressing sheets survived longer on the infarcted myocardium, and significantly improved cardiac function. L6-Bcl2 sheet transplantation reduced myocardial fibrosis and increased vascular density in infarct and border areas. Moreover, the number of c-kit positive and proliferating cells in the myocardium was increased in the L6-Bcl2 group. In conclusion,

Bcl-2 prolongs survival of myoblast sheets, increases production of proangiogenic paracrine mediators, and enhances the therapeutic efficacy of cell sheet transplantation.

Key Words: Apoptosis, Bcl-2, Cell Sheet Therapy, Myoblast, Myocardial Infarction

INTRODUCTION

Although the first myoblast transplantation therapies were administered more than a decade ago for the treatment of heart failure (29,34), problems associated with cell injections, such as the massive loss of donor cells, inadequate cell proliferation in the host myocardium, and arrhythmogenicity, remain to be solved. To overcome some of these problems, epicardial transplantation of cell sheets provides a conceptual alternative and a minimally invasive method for cell delivery. In this system, a tissue-engineered multicellular patch or cell sheet is made with a temperature-responsive cell culture dish (22,31). With this technique freely transplantable cell sheets consisting of 3 to 6 million cells can easily be manufactured with no added scaffold material. Such sheets can then be implanted on top of the injured myocardium to which they adhere immediately. Moreover, therapy can be enhanced by piling two or more layers of sheets on top of each other. The superiority of sheet transplantation over intramyocardial injections for cell therapy of heart failure has been demonstrated in both small and large animal studies (12,15,20). The therapeutic effect of cell sheets is considered to be mediated via finite production of paracrine effectors that locally stimulate the underlying injured myocardium. In models of cardiac ischemia and infarction, myoblast sheet therapy has been shown to inhibit fibrosis and to stimulate angiogenesis (20).

These previous studies have shown that multiple myoblast sheets are required for ischemic heart failure therapy. The sheets on the injured myocardium are exposed to apoptotic stress and nutrient deprivation, and thus an increased number of cells are required for therapeutic benefit. In order to reduce the amount of transplanted sheets and to increase their tolerance of the death-promoting host environment, we investigated the effect of *bcl2* gene expression in myoblast sheets and their therapeutic efficacy in a rat model of acute myocardial infarction (AMI).

The family of Bcl-2 proteins comprises several members with anti- or pro-apoptotic functions. Bcl-2 itself is anti-apoptotic, functioning in the mitochondrial pathway by counteracting functions of the pro-apoptotic Bax and Bak, and inhibiting cytochrome c release (26). Bcl-2 overexpression has been shown to promote cell survival and to inhibit cell death induced by such apoptosis-inducers as staurosporine, or by nutrient deprivation (6). Bcl-2 gene therapy has been used in cardiac cell therapy to prevent apoptosis upon cell injection into the myocardium (17,19).

In this study, we evaluated the Bcl-2 expression-mediated effects on L6 myoblast sheets both *in vitro* and *in vivo*. We first compared the apoptosis resistance and gene expression profiles of wild type and Bcl-2-modified L6 myoblast monolayers and sheets. We then expanded these results to an *in vivo* setting, and evaluated the effects of wild type and Bcl-2-overexpressing myoblast sheet transplantation in a rat model of AMI.

MATERIAL AND METHODS

Cell Culture and Sheets

The L6 rat skeletal myoblast cell line was obtained from the American Type Culture Collection (CRL-1458, Manassas, VA). Cells were cultured at 37°C with 5% CO_2 in growth medium (DMEM supplemented with 10% FCS and antibiotics), passaged three times weekly, and kept at 60% confluency to retain their differentiation potential. Passages 5 to 15 were used for the experiments. Cell sheets were formed by plating 6×10^6 myoblasts on thermoreactive culture dishes (CellSeed, Tokyo, Japan) to achieve sheet thickness of approximately 5 cell layers. Cells were incubated for 16 hours at 37 C° to induce sheet formation. The sheets detached spontaneously at room temperature within 45 minutes, and were then washed once with growth medium.

Measurement of proliferation, apoptosis and differentiation

Myoblast viability after 48-hour serum deprivation or staurosporine treatment was assessed by the mitochondria-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyltetrazolium bromide (MTT) to formazan (Roche, Mannheim, Germany) as described previously (33). Briefly, NADPH produced by the cells was visualized by adding MTT (10 µl, 5 mg/ml in PBS) to 100 µl of cell suspension. Following a 4-hour incubation period at 37°C, the samples were dissolved in DMSO, and the amount of formazan dye generated was quantified (A540 nm/A650 nm). Cell adherence was determined 48 hours after treatment. Nuclei were stained with DAPI, and cells were washed thoroughly with PBS. Cell nuclei were counted with CellC software version 1.11 (Selinummi and Seppälä, Tampere University of Technology, www.cs.tut.fi/sgn/csb/cellc). The early phase of apoptosis, characterized by phosphatidyl serine translocation, was determined 24 hours after serum deprivation or staurosporine treatment by cell surface annexin V binding (Invitrogen, Carlsbad, CA). Fluorescence (340 nm/460 nm) was measured with Wallac Victor2 (Perkin-Elmer,

Wellesley, MA). Myoblast sheet apoptosis was determined by measuring caspase-3 activity after the induction of apoptosis by either serum deprivation or staurosporine for 24 hours. Caspase-3 activity was measured using EnzChek® caspase-3 assay kit (Invitrogen). For differentiation, sheets were deprived of serum to induce myoblast differentiation into myotubes. Western blotting samples were then collected for evaluation of troponin T and myogenin expression.

VEGF-A Measurements

VEGF-A protein secretion from myoblast sheets was determined from growth medium conditioned for 48 h using a rat VEGF DuoSet™ ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. VEGF-A expression was determined from staurosporine-treated or nutrient-deprived sheets.

Bcl-2 Overexpression and Transfection

pBabepuro-*bcl2* retroviral vector was a kind gift from Dr. Juha Klefström, University of Helsinki, Finland (14). L6 myoblasts were transfected with incubation for 48 hours in the presence of retroviral vector and 8 μg/ml polybrene (Sigma-Aldrich, Saint Louis, MO). Transfected cells were then selected with 2 μg/ml puromycin (Sigma) for 48 hours.

Western Blotting

Western blotting samples were prepared in Laemmli sample buffer (Bio-Rad Laboratories, Redmond, WA). Detection was carried out as previously described (3) using appropriate alkaline phosphatase-conjugated secondary antibodies. The primary antibodies used were mouse monoclonal anti-Bcl-2 (610539, clone 7, BD Biosciences, San Jose, CA), mouse monoclonal anti-myogenin (sc-12732, clone F5D, Santa Cruz Biotechnology Inc, Santa Cruz, CA), and mouse monoclonal anti-troponin T (T6277, clone JLT-12, Sigma-Aldrich).

Immunofluorescence Imaging

Cells were grown on coverslips for 48 hours prior to fixation with 4% paraformaldehyde and permeabilization with 0.2% Triton X-100. Coverslips were incubated with anti-Bcl-2 antibody, washed with PBS, and incubated with an appropriate Alexa-Fluor fluorophore-conjugated (Invitrogen) secondary antibody. Nuclei were stained with DAPI, and coverslips were mounted on microscopy slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Samples were visualized with an Olympus IX70 microscope (Olympus Finland, Vantaa, Finland).

RNA Extraction and Microarray

Myoblast sheets were prepared as described. After a 40-hour incubation period to enable three-dimensional culture-induced gene expression *in vitro*, total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. Further purification was carried out with an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quality was verified with an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Purified RNA was synthesized into double-stranded cDNA with a Superscript Double-stranded cDNA Synthesis Kit (Invitrogen). A Bioarray High Yield RNA Transcript Labelling Kit (Enzo LifeSciences, Faimingdale, NY) was then used to synthesize cRNA. cDNA and cRNA products were purified with a GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA). cRNA products were then used for fragmentation reaction and hybridized into the Affymetrix Rat Genome 230 2.0 GeneChip Array. After 18 hours, chips were washed and stained with a GeneChip Fluidics Station 400 (Affymetrix), followed by chip scanning with a GeneChip Scanner 3000 7G (Affymetrix). Data analysis was performed with GeneSpring GX software, version 7.3.1 (Agilent Technologies), normalized to the median 50 percentile, and presented as gene expression fold changes.

Animals

This study used 92 male Wistar rats (250-400g) of which 64 rats survived surgery and follow-up. The rats were divided into four groups: Group 1 - control, left anterior descending coronary artery (LAD) ligation (n=22); Group 2 - LAD ligation and L6-WT sheet transplantation (n=17); Group 3 - LAD ligation and L6-Bcl2 sheet transplantation (n=20); and Group 4 - Sham operation (n=5). All rats were euthanized 28 days after ligation. Experimental procedures were conducted according to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were evaluated and approved by the ethical committee of the Hospital District of Helsinki and Uusimaa, Meilahti Hospital, Department of Surgery.

LAD Ligation and Cell Sheet Transplantation

AMI was induced by ligation of LAD as described (23). Briefly, animals were anesthetized subcutaneously using 0.05 mg/kg of medetomidine (Orion Pharma Inc, Turku, Finland) and 5 mg/kg i.p. ketamine (Parke-Davis, Barcelona, Spain). They were then intubated, and a respirator served to maintain ventilation during surgery. The heart was exteriorized through a left thoracotomy and pericardiotomy. LAD was ligated 3 mm from its origin. Immediately after LAD ligation, two circular myoblast sheets (total 1.2×10^7 cells), approximately 25 mm in diameter, were placed on the left ventricular anterior wall for each rat in Groups 2 and 3. Group 2 received L6-WT sheets, and Group 3 L6-Bcl2 sheets. After sheet transplantation, the heart was returned to its normal position and covered with pericardium to avoid adhesion to the lung and to the chest wall, and to prevent movement of the sheets. Throughout surgery, normal body temperature was maintained with a thermal plate. After surgery, anesthesia was antagonized with atipamezole hydrochloride (1.0 mg/kg s.c., Orion Pharma Inc). Buprenorphine hydrocholoride (0.05 mg/kg s.c., Reckitt and Colman Ltd, Hull, UK) was administered for post-operative analgesia.

Echocardiography

All rats underwent echocardiography under anesthesia 3 (baseline), 10, and 28 days after surgery. The animals were sedated with 0.5 mg/kg medetomidine and placed on a thermal plate. Measurements were performed with a 7.5 MHz transducer (MyLab®25, Esaote SpA, Genoa, Italy). Anterior wall thickness, posterior wall thickness at diastolic (AWTd, PWTd) and systolic (AWTs, PWTs) phases, and left ventricular diameter at diastolic (Dd) and systolic (Ds) phases were measured in the short-axis right parasternal projection just below the mitral valves. Units are presented in millimeters. Dd and Ds were used to calculate fraction shortening (LVFS) and ejection fraction (LVEF) percentage:

$LVFS\ (\%) = (Dd-Ds)/Dd$

$LVEF\ (\%) = (Dd^3-Ds^3)/Dd^3$

Histology and Immunostaining

At 28 days after surgery, the rats were euthanized after echocardiography. The heart was then excised and cut into four equal transverse parts. Two middle parts (next apex and next basal) were fixed in 4% neutral-buffered formalin for 48 hours. The samples were then embedded in paraffin, and were cut into 4-μm-thick sections for histology and immunostaining. Sirius Red stain served to analyze fibrosis. Immunostaining was performed using a Ventana Discovery Automate (Ventana Medical Systems Inc, Tuczon, AZ). To demonstrate vascular density, endothelial cells were stained with antibody against von Willebrand Factor (vWF, RB-281, Labvision Inc, Fremont, CA). Cell proliferation was evaluated using anti-Ki67 antibody (RM-9106, Labvision Inc), and apoptosis was detected with antibody specifically recognizing active cleaved caspase-3 (CST #9664, Cell Signaling Technology Inc, Danvers, MA). The stem cell antigen, c-kit, was detected with an anti-c-kit antibody (RA14132, Neuromics, Northfield, MN). Sections stained for Ki67 proliferation-associated antigen were double-stained for myocytes using anti-tropomyosin antibody (MS-1256, Labvision Inc).

Analysis of fibrosis

Fibrosis was evaluated from scanned images of Sirius Red-stained sections. Percentage of fibrosis was calculated as Sirius Red-stained area divided by whole section area as evaluated using Photoshop 7.0 (Adobe Inc, San Jose, CA).

Analysis of Vascular Density, Cell Proliferation, Apoptosis and c-kit-positive Cells

The vWF- and Ki67-immunostained sections were photographed with a microscope (×100 magnification) from six fields (2 from infarcted area, 2 from border area, 2 from remote area). Vascular density and proliferating cell number were calculated with ImageJ (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij). Briefly, RGB images were background-subtracted and divided into Hue-Saturation-Brightness channels from which the Brightness channel was further analysed by thresholding for the staining intensities of vWF or Ki67. All images were processed with the same parameters using a pre-programmed macro. Data were collected separately for infarcted, border, and remote areas. Apoptotic cells were manually counted as the number of positive cells for cleaved caspase-3 from six fields per slide. From c-kit-stained sections, the c-kit-positive cells were counted manually from the entire section.

Analysis of Sheet Survival in vivo

A substudy was designed to specifically evaluate sheet survival on top of the infracted area. Six rats were used. L6-WT and L6-Bcl2 myoblasts were labelled with green fluorescent protein (GFP) by incubation with a lentiviral vector carrying the *gfp* gene and 8 μg/ml polybrene for 24 hours. The lentiviral vector was a kind gift from Professor Seppo Ylä-Herttuala (AIV Institute, Kuopio, Finland). AMI, myoblast cell sheet formation and transplantation were done as described earlier. Animals, each receiving either L6-WT-GFP (n=6) or L6-Bcl2-GFP (n=6) sheets, were euthanized and hearts excised at three weeks after the surgery. Bright field and fluorescence images of the excised heart surface were acquired from the site of transplantation using the Leica

MZ FLIII stereomicroscope (Leica Microsystems, Wetzlar, Germany). From these images, the intensity of GFP signal was assessed with the ImageJ software. Images were background-subtracted and the fluorescence intensity was measured from green channels of the RGB-images.

Statistical analysis

Data are presented as mean ± SEM. Differences between groups were compared using the student *t* test. The Bonferroni post-test was applied for correction with multiple comparisons. Statistical analyses were performed with Graph Pad Prism 4.0 (GraphPad Software Inc., San Diego, CA).

RESULTS

Bcl-2 overexpression and function in myoblasts and myoblast sheets

Bcl-2 expression was introduced to the L6 rat myoblast cell line with pBabepuro retroviral vector carrying the *bcl-2* gene. Bcl-2 protein expression was verified with Western blotting (Figure 1A), and was 28-fold higher in transfected cells than in wild type cells. Immunofluorescence detection of Bcl-2 demonstrated a granular cytosolic pattern, suggesting a mitochondrial localization (Figure 1B). The resistance to apoptosis of transfected myoblasts was evaluated with MTT assay. After serum deprivation, L6-Bcl-2 cells showed 33% higher viability than did L6-WT cells (0.371 ± 0.002 and 0.279 ± 0.004, $p<0.001$); the baseline values before treatment were 0.420 ± 0.004 and 0.44 3± 0.006 for L6-WT and L6-Bcl2, respectively. After staurosporine treatment, L6-Bcl2 cells showed 2.4-fold higher viability than did L6-WT cells (0.244 ± 0.012 and 0.102 ± 0.006, $p<0.001$) (Figure 1C); the baseline values were 0.481 ± 0.010 and 0.469 ± 0.015.

After 48-hour serum deprivation or staurosporine treatment the number of adherent L6-Bcl2 cells was 2-fold and 1.6-fold higher than that of L6-WT cells, respectively ($p<0.001$ for both treatments) (Figure 1D). Overexpression of Bcl-2 also prevented the induction of apoptosis after serum deprivation or staurosporine treatment ($p<0.001$ each) as detected by the binding of annexin V to cell surface phosphatidyl serine, an early marker for cellular apoptosis (Figure 1E).

Both cell types efficiently formed cell sheets, and the introduced overexpression of Bcl-2 was retained. Both types of sheets showed similar proliferative activity as evaluated by Ki67 expression. L6-Bcl2 sheets showed fewer cells positive for active cleaved caspase-3 (Figure 2A).

To study the functionality of Bcl-2, we measured the activity of caspase-3 in unstimulated, serum-deprived, or staurosporine-treated myoblast sheets after 24 hours. We found that the introduction of Bcl-2 to myoblast sheets prevented the activation of

effector caspase-3, whereas prolonged culture (p=0.041) and cellular stress induced by serum deprivation (p=0.0097) and staurosporine (p=0.0003) led to caspase-3 activation in L6-WT sheets (Figure 2B).

Since culture in reduced serum medium is standard practice for inducing differentiation of myoblasts (21), we used this method to investigate the differentiation of myoblast sheets. L6-Bcl2 myoblasts differentiated into myotubes as evaluated by the expression of differentiation markers troponin T and myogenin, whereas L6-WT myoblasts underwent cell death instead of differentiation under serum starvation. After prolonged culture, L6-WT sheets showed signs of disruption whereas cell survival in L6-Bcl2 sheets was predominant, and myotubes were visible (Figure 2C).

Bcl-2 induces expression of proangiogenic mediators in myoblast sheets

Since Bcl-2 expression has been shown to affect several cellular processes (5), we used whole-genome microarrays to investigate the effect of *bcl2* gene transduction on gene expression in myoblasts. Interestingly, in monolayer cultures no differences in gene expression, other than increased *bcl2*, were observed between L6-WT and L6-Bcl2 cells (data not shown). When myoblast sheets were formed, however, distinct differences emerged (Table 1). Interestingly, the most striking induction emerged in genethonin, thus suggesting a specific increase in muscle-related gene expression (4). Of significance to myoblast sheet transplantation and its paracrine effect, we observed induction in angiogenesis-associated genes, such as *vegf* and *plgf*, suggesting that the pro-angiogenic potential of myoblast sheet therapy is incremented by Bcl-2 (Table 1). For verification of the microarray results, we evaluated the production of VEGF from myoblast sheets. L6-Bcl2 sheets produced greater amounts of VEGF in the culture medium under serum deprivation (p=0.0014) or staurosporine-induced stress (p=0.0383) than did L6-WT sheets during the 48-hour incubation period (Figure 2D).

Bcl-2-expressing myoblast sheets enhance ventricular function after AMI

To study the increased therapeutic efficacy with L6-Bcl2 vs. L6-WT sheet transplantation, we used the rat LAD ligation model. Table 2 shows cardiac performance data assessed with echocardiography at 3, 10, and 28 days after ligation and sheet transplantation.

The function of the left ventricle, as measured by LVEF and LVFS, improved only in the L6-Bcl2 sheet-treated group. These parameters were significantly enhanced at day 10, and remained elevated until the end of the study at day 28. At day 10, the LVEF of both the L6-WT and L6-Bcl2 groups recovered from baseline, whereas the LVEF of the control group decreased (L6-WT: 30.3 ± 2.3 to 32.2 ± 2.1%, L6-Bcl2 31.4 ± 2.3 to 37.8 0 ± 1.9%, control: 32.6 ± 1.7 to 29.1 ± 2.0%). Even at day 28, LVEF remained significantly higher in the L6-Bcl2 group than in the L6-WT and control groups (L6 Bcl2: 33.2 ± 2.0%, vs. control: 27.3 ± 2.2%, p=0.035, and L6-WT: 29.1 ± 1.4%, p=0.036) (Figure 3). The AWTd of LAD ligated groups was decreased significantly from baseline and was thinner than that of the sham group at day 10 ($p<0.001$), and remained as such until day 28. PWTd was significantly higher in the L6-Bcl2 group than in the control and L6-WT groups at day 28.

Myoblast sheets expressing Bcl-2 exert therapeutic effects on injured myocardium

Because Bcl-2 expression improved the myoblast sheet production of angiogenic growth factors, such as VEGF and PlGF, we evaluated the end-point vessel density after sheet transplantation. Higher vessel density was evident with vWF immunostaining at the infarct area on which the sheets were placed (Figure 4). This area thus received maximal concentrations of growth factors and paracrine stimulation deriving from the sheets. Paracrine effects of myoblast sheet transplantation were also observed in the border area, and to a lesser extent, in the remote area, suggesting an enhanced effect of L6-Bcl2 sheets. The higher vascular density was associated with decreased fibrosis. The

L6-Bcl2 group showed a significantly lower percentage of fibrosis than did the control and L6-WT groups (L6-Bcl2: 21.2 ± 0.8%, vs. control: 25.6 ± 1.8%, p=0.001, and L6-WT: 24.6 ± 1.6%, p=0.035) (Figure 5).

Cell death in AMI has been shown to occupy various forms ranging from overt necrosis to programmed apoptosis (27). To understand how myoblast sheet transplantation influences apoptosis and proliferation, we evaluated the expression of active caspase-3 as well as that of the proliferation-associated Ki67 antigen. No differences between groups in caspase-3-positive cells were found at the end of the study period (data not shown). In contrast, the number of proliferating cells within the myocardium was increased in groups receiving myoblast sheet transplantation (Figure 6). In the L6-Bcl2 group, a significant number of proliferating cells was also found in the remote area (L6-Bcl2: 47.5 ± 3.7 vs. control: 30.9 ± 3.5, p=0.002 or L6-WT: 31.9 ± 3.3, p=0.003), further suggesting an increased paracrine effect. Since the higher number of proliferating cells, taken together with enhanced cardiac function, suggests activation of a regenerative response, we evaluated the amount of c-kit-positive cells in the myocardium. We found the number of cells positive for stem cell antigen c-kit to be significantly higher in the L6-Bcl2 group than in either the control group or the L6-WT group (L6-Bcl2: 52.3 ± 5.4 cells vs. control: 35.7 ± 6.1, p=0.029 or L6-WT: 36.8 ± 5.4 cells, p=0.027) (Figure 7A). All analyses of immunostainings were performed in a blinded fashion using computer-assisted automated evaluation.

Bcl-2 enhances myoblast sheets survival in vivo

To demonstrate the enhanced survival of sheets by Bcl-2 expression *in vivo,* we generated GFP-expressing L6-WT or L6-Bcl2 myoblast sheets. These sheets were then transplanted onto the infarcted hearts as in previous experiments. We found that after three weeks, animals receiving L6-Bcl2 (n=6) sheets showed 2.1-fold higher green fluorescence intensity in the heart than did the L6-WT (n=6) group (p=0.006) (Figure 8A), suggesting that the Bcl-2-expressing myoblast sheets outlive their wild type