Role of Interleukin-1β in Acute Inflammation and Graft Death After Cell Transplantation to the Heart

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Background—Poor survival of grafted cells is a major factor hindering the therapeutic effect of cell transplantation; however, the causes of cell death remain unclear. We hypothesized that interleukin-1β (IL-1β) might play a role in the acute inflammatory response and graft death after cell transplantation and that inhibition of IL-1β might improve graft survival.

Methods and Results—14C-labeled male skeletal muscle precursor cells were implanted into female mouse hearts by direct intramuscular injection. The amount of 14C-label provides an estimate of the surviving cell number, whereas the amount of male-specific Smcy gene measured by polymerase chain reaction indicates the total (surviving + proliferated) number of donor-derived cells. At 10 minutes after implantation, 44.8±2.4% of the grafted cells survived and this steadily decreased to 14.6±1.1% by 24 hours, and to 7.9±0.6% by 72 hours (n=6 in each point). Proliferation of the surviving cells, which began after 24 hours, resulted in an increase in the total cell number from 15.5±0.8% at 24 hours to 24.4±1.6% at 72 hours. Acute inflammation was prominent at 24 hours and was reduced by 72 hours, in parallel with IL-1β expression. Administration of anti–IL-1β antibody improved graft survival at both 24 (25.6±1.6%) and 72 hours (14.8±1.1%) and resulted in a 2-fold increase in the total cell number at 72 hours (45.8±2.4%). The effects of IL-1β inhibition corresponded with a reduced inflammatory response.

Conclusion—IL-1β is involved in acute inflammation and graft death after direct intramyocardial cell transplantation. Targeted inhibition of IL-1β may be a useful strategy to improve graft survival. (Circulation. 2004;110[suppl II]:II-219–II-224.)

Key Words: cell transplantation ▪ skeletal myoblast ▪ interleukin-1β ▪ inflammation ▪ survival

Successful experimental studies have encouraged the clinical application of cell transplantation for treating heart disease using autologous bone marrow stem cells or skeletal muscle precursor cells (MPCs).1-4 Survival of grafted cells in the myocardium is an important determinant of final graft size and is likely to be critical to the therapeutic efficacy of the treatment. Several qualitative and quantitative studies have shown that the majority of cells grafted into the heart die during the first or second days.3-5-7 Identification of the mechanisms that underlie such a massive loss of donor cells will be useful for developing strategies for improving graft survival, which should result in enhanced therapeutic effects of cell transplantation to the heart.

It has been shown that direct intramuscular injection, a common means for cell implantation, causes mechanical damage and that this subsequently provokes an acute inflammatory response in the host organs.6-9 This acute inflammation and the associated cytokines are presumed to play a role in the death of cells grafted via this route.7-9 Interleukin-1β (IL-1β) is a key pro-inflammatory cytokine in acute myocardial inflammation and cardiac cell death after ischemia-reperfusion injury.10 IL-1β is known to cause dose-dependent apoptosis in various cells11,12 and is expressed by a variety of cells that could be important in cell transplantation to the heart, including MPCs, fibroblasts, and inflammatory cells.12,13 We therefore hypothesized that IL-1β might play a role in acute inflammation and graft death after direct intramuscular cell transplantation to the heart, and that inhibition of this cytokine might improve graft survival.

Proliferation of the surviving donor cells is another important factor in determining final graft size after cell transplantation, although this has not been examined in detail in the context of MPC implantation to the heart.14 In this study, we have analyzed the survival and proliferation of grafted MPCs using a modified dual-marker system, where [14C]-thymidine-labeled male MPCs were grafted into female myocardial...
The surviving proportion of the originally implanted MPCs was estimated by radiolabel measurement as validated previously. Through cell division, \(^{14}\)C radiolabel remaining in an originally grafted cell is shared between the 2 daughter cells, each of which containing 50% of the original radioactivity on average. Cell division is symmetrical and the divided cells are theoretically identical in all respects. It is thus most likely that there is an equal chance for either of the new cells to die. If 50% of originally implanted cells are dead, 50% of daughter cells are also assumed to die, correctly resulting in 50% survival. Therefore, the method gives a good estimation of surviving cells in a short-term experiment like in the current study. However, the total number of donor-derived MPCs (surviving plus newly proliferated) present within the myocardium was evaluated by measuring the amount of Y chromosome using polymerase chain reaction (PCR) for the male-specific Smcy gene. The extent of proliferation of the surviving MPCs can therefore be estimated by comparing the total donor-derived cell number with the surviving cell number.

**Methods**

**MPC Culture and \(^{14}\)C Radiolabeling**

Donor cells used were a conditionally immortalized MPC line, derived from a male \(H-2K^b\)-tsAS8/\(\beta\)-gal heterozygote mouse. These cells can be cultured over many passages in growth medium containing interferon (IFN)-\(\gamma\) (which drives expression of large T antigen via the \(H-2K^b\) promoter) at 33°C (a temperature at which the thermolabile T antigen is active) without losing myogenic differentiation, MPC suspension (5.0 \(\times\) \(10^5\) in 10 \(\mu\)L PBS) was injected into living organs. The in vitro differentiation capacity and nuclear \(\beta\)-galactosidase (\(\beta\)-gal) expression of the cells were confirmed (data not shown). At 24 hours before MPC harvesting, 54 nCi/mmol [methyl-\(^{14}\)C]thymidine (Nycomed Amersham, UK) was added to the culture to radiolabel the MPCs as described previously.

**Direct Intramuscular MPC Implantation to the Heart**

All animal studies were performed with the approval of the institutional ethics committee and the UK Home Office. The investigation conforms to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 85-23, 1996). Female C57BL/6J mice (17 to 20 grams; Charles River Laboratories, UK) were used as recipients. This is one of the parental strains of the \(H-2K^b\)-tsAS8/\(\beta\)-gal heterozygote and would therefore be expected to raise a minimal immune reaction against donor MPCs. Mice were anesthetized by subcutaneous injection of 0.005 mg/g body weight xylazine and 0.1 mg/g body weight ketamine. To analyze IL-1\(\beta\) expression after MPC implantation, MPC suspension (5.0 \(\times\) \(10^5\) in 10 \(\mu\)L PBS) was injected into the left ventricular (LV) anterior wall of recipient hearts via left thoracotomy under mechanical ventilation. In studies to investigate the role of IL-1\(\beta\) on acute graft death and inflammation, anti-IL-1\(\beta\) antibody or control IgG (10 \(\mu\)g in 5 \(\mu\)L PBS; R&D Systems) was added to the \(^{14}\)C-labeled MPC suspension (5.0 \(\times\) \(10^5\) in 5 \(\mu\)L PBS) immediately before MPC implantation into LV walls. Cell injection was performed carefully under continuous observation with a surgical microscope and, if leakage was suspected, the animal was excluded from the study. Surgical wounds were repaired and mice were extubated and returned to cages to recover.

**Reverse-Transcriptase PCR for Myocardial IL-1\(\beta\) Expression After MPC Implantation**

Total cellular RNA was extracted from sample hearts (\(n=4\) at each point) freed from right ventricular (RV) free wall using Trizol (Invitrogen, UK). IL-1\(\beta\) expression was assessed by reverse-transcriptase (RT)-PCR using the Cytoxpress kit (Biosource) according to the manufacturer's instructions. The PCR products were run on an agarose gel and bands visualized with an ultraviolet transilluminator. Quantitative analysis was performed using ImageJ software (National Institutes of Health).

**Generation of Standard Curves to Estimate MPC Numbers in Host Myocardium**

To obtain standard curves to calculate the MPC numbers from the amount of Smcy gene and \(^{14}\)C radioactivity, intact hearts were collected from female mice (\(n=3\) at each point) and freed of RV free wall. These were injected with known numbers (0, 5.0 \(\times\) \(10^4\), 1.0 \(\times\) \(10^5\), 5.0 \(\times\) \(10^5\), 1.0 \(\times\) \(10^6\), 2.5 \(\times\) \(10^6\), or 5.0 \(\times\) \(10^6\) in 10 \(\mu\)L PBS) of male \(H-2K^b\)-tsAS8/\(\beta\)-gal MPCs, which had been previously radio-labeled with \([^{14}\)C]-thymidine as described. Samples were analyzed for the amount of Smcy gene and \(^{14}\)C radiolabel as described.

**Measurement of \(^{14}\)C Radiolabel and Smcy Gene in Host Myocardium**

The amount of \(^{14}\)C radiolabel was measured to estimate the surviving MPC number, whereas the amount of Smcy gene was analyzed to evaluate the total number of donor-derived MPCs. As described previously, frozen LV samples (\(n=6\) at each point) were homogenized and digested with protease K. The radioactivity present in a 50-\(\mu\)L aliquot of lysate was measured using a Beckman LS6000SC counter. Genomic DNA was obtained from the remaining lysate by phenol/chloroform extraction and PCR was performed for Smcy using 500 ng DNA. Primers used were 5'-GCCCATG-TGCAGATGCTGGT-3' (sense) and 5'-TGTGCGAGGAGTGGAATCTCT-3' (antisense). Conditions used were: 94°C for 3 minutes followed by 40 cycles of 55°C for 1 minute, 72°C for 1 minute, and 94°C for 30 seconds. This was followed by a cycle of 55°C for 1 minute and 72°C for 10 minutes. The PCR products were analyzed as described.

**Measurement of Myocardial Myeloperoxidase Activity**

Myocardial myeloperoxidase (MPO) activity of the LVs (\(n=6\) at each point) was measured as an index of neutrophil accumulation (reflecting the severity of the acute inflammatory response), using o-dianisidine hydrochloride (Sigma, UK) as described previously.

**Histological Study**

Hearts (\(n=4\) at each point) were cut into 4 segments, and each segment was embedded in OCT compound, frozen, and cut into 10-\(\mu\)m cryosections. After fixation, sections were stained for \(\beta\)-gal as described previously with counterstaining using hematoxylin.

**Statistical Analysis**

All values are expressed as mean±SEM. Statistical comparison of the data were performed using ANOVA for repeated measures followed by Bonferroni test for individual significant difference. A value of \(P<0.05\) was considered statistically significant.

**Results**

**Surgical Outcome**

No mortality was encountered throughout the experiments. A total of 7 mice were excluded from the study because of suspected leakage of MPC digestion during implantation.
Myocardial IL-1β Expression After MPC Implantation

After MPC implantation, RT-PCR showed that IL-1β mRNA was strongly induced at 24 hours but reduced by 72 hours (Figure 1A). GAPDH mRNA levels showed no changes over the period. IL-1β expression, normalized to GAPDH levels, indicated a strong induction of IL-1β at 24 hours, which then decreased significantly by 72 hours (Figure 1B).

Standard Curves to Estimate MPC Number

To construct standard curves for estimating MPC numbers, intact female hearts were injected ex vivo with known numbers of 14C-labeled male MPCs and the amounts of Smcy gene and radiolabel were measured. There was a progressive increase in the amount of Smcy-PCR products corresponding to the number of MPCs injected (Figure 2A). High regressions were obtained between the number of grafted MPCs and the band intensity for Smcy (Figure 2C), as well as between the MPC number and the amount of radiolabel (Figure 2B).

Improved MPC Survival by IL-1β Inhibition

After implantation of 14C-labeled male MPCs into female hearts, with administration of anti–IL-1β antibody or control IgG, the surviving and total (surviving + proliferated) MPC numbers were calculated from the amounts of radiolabel and Smcy gene, respectively, using the standard curves. At 10 minutes, the surviving cell number in the control group was 44.8 ± 2.4% of the grafted MPC number, and this steadily decreased to 14.6 ± 1.1% by 24 hours and to 7.9 ± 0.6% by 72 hours (Figure 2D). The total MPC number was almost equal to the surviving number at 10 minutes and 24 hours, but 3-fold greater (24.4 ± 1.6%) at 72 hours, suggesting that proliferation of surviving population had occurred after 24 hours (Figure 2E). Both the surviving and total numbers were similar between the groups at 10 minutes, but increased at 24 hours (25.6 ± 1.6% survival and 25.8 ± 1.4% total) and 72 hours (14.8 ± 1.1% survival and 45.8 ± 2.4% total) in the anti-IL-1β antibody group compared with the controls. The number of proliferated (total minus surviving) cells at 72 hours was elevated 2-fold in the anti–IL-1β antibody group (45.8 to 14.8 [31.0%]) compared with the control (24.4 to 7.9 [16.5%]).

Attenuated Acute Inflammatory Response by IL-1β Inhibition

The inflammatory response after MPC implantation was evaluated by measuring MPO activity and by histological examination. MPO activity was not elevated at 10 minutes from the baseline (ie, intact hearts) but increased at 24 hours, followed by a decrease by 72 hours in both the anti–IL-1β antibody and control groups (Figure 3). The anti–IL-1β antibody group showed significantly reduced MPO activity at both 24 and 72 hours compared with the control. Histological study showed that numerous inflammatory cells, predominantly polymorphonuclear leukocytes, had infiltrated into the injection site with myocardial damage by 24 hours in the control group, although this appeared to be attenuated at 72 hours (Figure 4A through 4D). This response appeared to be reduced in the anti–IL-1β antibody group at both time points (Figure 4E to 4H), coincident with the change in MPO activity. At 24 hours, β-gal-positive cells were found around the injection sites in both groups, and the number of β-gal-positive cells appeared to be larger in the anti–IL-1β antibody group. Differentiated myotubes that had multiple β-gal-positive nuclei were observed only at 72 hours in both groups (Figure 4D, 4H).

Discussion

We have described the early dynamics of MPCs implanted into the myocardium by direct intramuscular injection and have shown that IL-1β inhibition can attenuate both the acute inflammatory response and graft loss. At 10 minutes after implantation, 45% of the grafted MPCs survived and the surviving cell number steadily decreased to 8% by 72 hours. Proliferation and myogenic differentiation of the surviving MPCs took place between 24 and 72 hours. Acute inflammation was most prominent at 24 hours and reduced by 72 hours as is indicated by MPO activity and histological findings, in parallel with myocardial IL-1β expression. Further, blockade of IL-1β by neutralizing antibody improved cell survival and resulted in an increase in the total number of donor-derived cells at 24 and 72 hours, corresponding to the attenuated inflammatory response. These data suggest that IL-1β plays an important role in acute inflammation and graft death after MPC transplantation to the heart. Although MPCs were used in this study, such nonspecific inflammatory responses are likely to occur, whichever cell types are implanted into the myocardium by direct intramuscular injection. Therefore, inhibition of IL-1β may prove useful to improve survival of cells implanted into the heart and is a promising approach to enhance therapeutic effects of cell transplantation. Furthermore, such a benefit might be especially significant when cell...
transplantation is applied to failing hearts such as postinfarction, in which IL-1β will have already been upregulated before cell delivery. The present study shows that administration of anti–IL-1β antibody resulted in increased MPC survival at 24 and 72 hours after implantation. Although it is unclear as to how long the antibody remains active after injection into the myocardium, the effect is unlikely to last for many days because of inactivation, degradation, or flush-out from the myocardium. Our RT-PCR results have shown that IL-1β was expressed strongly at 24 hours after MPC implantation and was reduced by 72 hours, in parallel with the change in the severity of acute inflammatory response. Together, these data suggest that the initial 24-hour period is vital for the IL-1β–associated mechanism of acute inflammation and graft death and therefore that even short-term IL-1β inhibition is effective in improving graft survival.

In contrast to the apparent effects on cell survival, transient inhibition of IL-1β did not appear to affect donor MPC proliferation. Proliferation of surviving MPCs was evident during the first 24 hours but was obvious at 72 hours after implantation in both groups. The number of MPC progeny at 72 hours was 2-fold greater in the anti–IL-1β antibody group (31.0%) compared with the control group (16.5%). Because it is unlikely that MPCs that disappeared by 72 hours could have proliferated, it is probable that only MPCs surviving at 72 hours (14.8% and 7.9%, respectively) could have divided. Judging from these numbers, proliferation rates of the surviving cells appeared to be similar between the groups. We therefore speculate that the enhanced number of donor-derived cells by anti–IL-1β antibody treat-
leakage, or myocardial contraction might cause a delayed squeeze of injected MPC suspension.

It is likely that many complicating factors, in addition to inflammation, are involved in the mechanisms of acute cell death after cell transplantation. These include mechanical injury, oxidative stress, and immune reaction. In this study, intact hearts were used to highlight the effects caused by cell injection itself. Although it is unlikely that grafted cells suffer a significant degree of ischemic injury after transplantation into the intact heart, such injury would be more relevant to cell transplantation into the infarcted/ischemic heart. When cells are implanted into ischemic/infarcted hearts, cell death, inflammation, and effects of IL-1β inhibition are likely to be affected by other pre-existing factors, such as poor blood supply, chronic inflammation, and modulated cytokine profiles including upregulation of IL-1β. In addition, the total volume, cell number, and cell concentration of the injected cell suspension are also likely to affect the graft survival and inflammation after cell transplantation. Further study is required to clarify these issues.

This study has demonstrated that IL-1β plays an important role in the inflammatory response after cell transplantation. It is likely that various complicating factors are involved in this IL-1β-related inflammation. IL-1β is known to upregulate ICAM-1 expression and activate NF-κB in the myocardium, which presumably result in stimulation of inflammation and cardiomyocyte death. IL-1β also directly induces cardiomyocyte apoptosis in vitro. We have shown that inhibition of IL-1β by overexpressing IL-1 receptor antagonist significantly attenuates acute inflammation and cardiomyocyte death (both necrosis and apoptosis) after myocardial ischemia-reperfusion injury. These mechanisms may be involved in the IL-1β-related inflammation after cell transplantation (both in grafted MPCs and the native myocardium), although further study is needed to clarify the details. Although a major role for apoptosis in rapid cell death during the acute phase of cell implantation is unlikely, clarification of the mode of cell death, whether by necrosis or apoptosis, would also be interesting as a future work.

In conclusion, we have described the acute dynamics of MPCs grafted by direct intramyocardial injection and have documented that IL-1β is involved in the mechanism of acute inflammation and graft death after cell transplantation. Targeted IL-1β inhibition by neutralizing antibody appears to be an effective strategy to improve early graft survival.

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