Cytokines produced by bone marrow cells can contribute to functional improvement of the infarcted heart by protecting cardiomyocytes from ischemic injury

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The implantation of bone marrow mononuclear cells (BM-MNCs) into ischemic heart diseases (1, 7, 13, 27–31). Although these results have been confirmed by definitive studies, the precise mechanisms of cell-based therapy in myocardial repair are still unclear.

It has been reported that the implanted stem cells of BM-MNCs can survive and be proliferated and differentiated into endothelial cells or cardiomyocyte in situ, contributing to functional improvement of the injured heart by rebuilding the damaged myocardium and cardiac vessels (14, 18, 26). However, there is no consensus on the plasticity of bone marrow cells, and most studies have found that myogenic or endothelial differentiation rarely occurs, if at all (2, 4, 22, 23, 25). Therefore, it is doubtful that the in situ differentiation or proliferation of implanted cells contributes remarkably to functional improvement of the injured heart.

Conversely, it is speculated that the cytokines produced by these implanted BM-MNCs play a more important role than their differentiation and proliferation (15). Experimental studies have shown that BM-MNCs produce many angiogenic factors, known as cytokines, in vitro and in vivo, including VEGF, basic fibroblast growth factor (bFGF), and angiopoietin-1 (11, 17, 16, 24). The implantation of BM-MNCs increases microvessel density and blood flow to the injured heart significantly (10, 11, 14, 17, 18, 24), and the resulting improvement in cardiac function is thought to be attributed to the induction of therapeutic angiogenesis by the angiogenic cytokines released from the implanted BM-MNCs. Beyond the effect of enhancing therapeutic angiogenesis, the cytokines produced by BM-MNCs may also directly improve the survival and contractile capacity of ischemic myocardium. Thus we investigated the role and mechanisms of the secretory pathway of BM-MNC implantation for myocardial repair by in vitro assessments and by using a rat model of acute myocardial infarction.

METHODS

Animals. Inbred male Wister rats, 13–16 wk old and weighing 250–300 g, were used in this experiment. All animals were obtained from Japan SLC (Hamamatsu, Japan) and housed under clean conditions at the Institute of Laboratory Animals, Yamaguchi University School of Medicine. All experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University School of Medicine.

Collection and cultivation of BM-MNCs. The BM-MNCs were collected and cultivated as described previously (24). Briefly, bone marrow mononuclear cell; cytokine; apoptosis

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Marinuclear cells were separated by gradient centrifugation. Freshly collected cells were suspended at a density of 5.0 × 10^6 cells/ml in MEM supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. Cells were seeded on a 24-well culture plate (5.0 × 10^6 cells/well) and incubated under normoxia or hypoxia (1% O_2) at 37°C. After 24 h of cultivation, the total number of surviving cells was counted after staining with 0.4% trypan blue solution (Sigma), and the supernatant was harvested for the following studies.

Detection of cytokine release from BM-MNCs by ELISA and Western blot analysis. We measured the concentrations of VEGF and IL-1β in the culture supernatant with a rat IL-1β ELISA kit and a rat VEGF ELISA kit (R&D Systems), according to the manufacturer’s instructions (11, 17). The expressions of IGF-1, bFGF, and transforming growth factor-β (TGF-β) in the culture supernatant were measured by the Western blotting method using monoclonal or polyclonal antibodies against rat IGF-1, bFGF, PDGF, and TGF-β, as described previously (24).

Endothelial cell proliferation assay. To evaluate the effect of cytokines produced by BM-MNCs on the proliferation of endothelial cells, coronary endothelial cells were harvested from the Wistar rat hearts by collagenase digestion, as described previously (12). Purified endothelial cells were seeded on collagen I-coated dish and expanded for 7–10 days (2 passages) by culture with Endothelial Medium Kit Liquid (Sigma). Expanded endothelial cells were then seeded in a collagen I-coated 96-well plate (1 × 10^4 cells/well) and cultured in DMEM containing 10% fetal calf serum and 1% penicillin-streptomycin, by the addition of 0%, 20%, or 40% of the supernatants of BM-MNCs. After 3 days of cultivation, the proliferation of endothelial cells was measured by the uptake of bromodeoxyuridine, by using a cell proliferation ELISA system (Amersham Life Science, Little Chalfont, UK) (12).

Estimation of apoptosis of isolated adult rat cardiomyocytes. To observe if cytokines produced by BM-MNCs protected cardiomyocytes against apoptosis, adult cardiomyocytes were isolated from 15- to 17-day-old rats by using a modified protocol described elsewhere (32). Briefly, the hearts were excised quickly and cannulated via the ascending aorta. The hearts were perfused for 2 min with Ca^2+ -free minimal essential medium (MEM; Sigma) containing 5 mM taurine, 3.8 mM creatine, and 10 mM 2,3-butanedione monoxime at 37°C and then with MEM containing 50 mM of CaCl_2 and 0.8 mg/ml of collagenase B (Roche Applied Science, Indianapolis, IN). After 30 min of digestion, the left ventricle (LV) was removed quickly and then cut into several chunks and digested further in a shaker (60–70 rpm) for 10 min in the same enzyme solution at 37°C. Isolated cardiomyocytes were suspended in control medium, or in the supernatants of BM-MNCs collected beforehand, and then seeded on laminin-coated culture slides. After 72 h of cultivation, the apoptosis of cardiomyocytes was detected by terminal dUTP nick-end labeling (TUNEL) assay with the use of Apoptosis Detection Kits (R&D System, Minneapolis, MN). The apoptosis index was calculated as the number of apoptotic cells per 100 nucleated cells.

Measurement of contraction and Ca^2+ transient of isolated adult cardiomyocytes. Adult rat cardiomyocytes were isolated and cultured on laminin-coated tissue culture dishes, as described above. After 8 h (baseline) and 72 h of cultivation, the contraction and Ca^2+ transient were measured by loading with 1 mM of fura-2 ( Molecular Probes, Eugene, OR) and stimulated electrically by using bipolar pulses (5-ms duration, 1 Hz). The electrical stimulation was done in HEPES buffer (37°C) containing 1.08 mM Ca^2+. Only cells that remained rod shaped, without blebs or other visible morphological alterations, and responded adequately to stimulation at 1 Hz were included in the testing protocol. Fura-2 fluorescence was measured by using a dual-fluorescence, calcium ion-sensing system (Ion Optix, Milton, MA) at a sampling rate of 500 Hz. At the same time, cell length and shortening were measured by a video-based edge-detection system. The cell shortening and Ca^2+ ratio after isolation were identified as the baseline.

Rat myocardial infarction model and supernatant injection. The rat myocardial infarction model was established as described previously (17). After the left anterior descending artery was ligated, rats were randomly selected and given an intramyocardial injection immediately, with control medium (control group, n = 8), supernatant of BM-MNCs cultured in normoxic condition (normoxia group, n = 9), or supernatant of BM-MNCs cultured in hypoxic condition (hypoxia group, n = 9). With the use of a 31-gauge needle and 100-µl syringe, each rat was given four point injections of 10 µl/injection) in the border zone of infarction, ~2 mm apart. As our preliminary studies showed that a single intramyocardial injection failed to improve the cardiac function, the rats in each group were also matched for an additional intraperitoneal injection of 500 µl control medium or supernatants of BM-MNCs cultured in normoxic and hypoxic condition, respectively, on days 2, 4, and 6 after infarction.

Echocardiography. Cardiac function was measured before treatment and then 7, 14, and 28 days after treatment by a single observer blind to the treatment regimen, with echocardiography by using a 10-MHz annular array transducer (20, 21, 24). After the induction of light general anesthesia, the hearts were first imaged two dimensionally in long-axis views at the level of the largest LV diameter. The systolic and diastolic LV areas were measured at the same time. This view was used to position the M-mode cursor perpendicular to the LV anterior and posterior walls. The LV dimension was measured at the end-diastolic dimension (LVDD) and at the end-systolic dimension (LVESD). The dimensions were calculated from the average measurements of three selected beats. The LV percent fractional shortening (LVFS%) was calculated as (LVEDD − LVESD)/LVEDD × 100 (%).

Histological evaluation. After the echocardiography on postinfarction day 28, the rats were killed by an overdose of anesthesia, and the hearts were harvested. Samples were frozen in liquid nitrogen and stored at −80°C until use. To detect the development of microvessels, 5-µm-thick frozen sections were stained with alkaline phosphatase by using an indoxyl tetrazolium method and then counterstained with eosin (12, 24). The number of microvessels was counted under a microscope with ×200 magnification, by a single observer blind to the treatment regimen. At least 10 different fields were selected randomly from the central infarct zone, the infarct border zone, and the remote noninfarcted zone on three independent slides of each sample, and the mean number of microvessels per field in each zone was calculated for statistical analysis.

Azan staining was also done to determine the wall thickness and the degree of collagen fiber accumulation in the infarcted region (20). By using the image analysis software NIH Image (NIH, Research Service Branch), the mean wall thickness was measured from three equidistant points of the central infarct area, and the area of fibrosis was calculated as the area of stained fibrotic tissue divided by the total area of LV wall. Measurements were done on at least two separate sections from each heart, and the averages were used for statistical analysis.

Statistical analysis. All data are expressed as means ± SD. Echocardiographical data were compared by two-way ANOVAs for repeated measures, and group differences at specific time points were then assessed by a Bonferroni t-test. Other data were assessed by one-way ANOVAs, followed by a Bonferroni t-test. A value of P < 0.05 was considered significant.

RESULTS

Various cytokines were produced by BM-MNCs, and their production was enhanced under conditions of hypoxia. About 90% of the BM-MNCs survived after 24-h cultivation, and there was no difference in survival between cells subjected to hypoxia and those subjected to normoxia. Many cytokines and
growth factors, including VEGF, IL-1β, bFGF, PDGF, IGF-1, and TGF-β, were detected in the supernatants of BM-MNCs after 24-h cultivation under conditions of normoxia and hypoxia (Fig. 1). Quantitative measurement by ELISA showed that the concentrations of VEGF and IL-1β in the supernatants were significantly higher when cultured under hypoxia than normoxia, the respective values for VEGF being 18.9 ± 5.81 vs. 61.54 ± 21.2 pg/ml (P < 0.001; Fig. 1A) and for IL-1β, 178.2 ± 27.7 vs. 231.9 ± 17.5 pg/ml (P < 0.01; Fig. 1B). Similarly, Western blot analysis detected the production of bFGF, PDGF, IGF-1, and TGF-β from the BM-MNCs, which also seemed to be enhanced under hypoxic conditions (Fig. 1C).

**Supernatants of BM-MNCs increased the proliferation of endothelial cells and inhibited the apoptosis of isolated cardiomyocytes.** We observed that the supernatants of BM-MNCs increased the proliferation of coronary endothelial cells in vitro by a dose-dependent method (P < 0.001, Fig. 2A). Furthermore, when compared with the control medium, the supernatants of BM-MNCs under conditions of normoxia and hypoxia inhibited the apoptosis of isolated adult cardiomyocytes significantly in vitro (P < 0.05, Fig. 2B).

**Supernatants of BM-MNCs preserved the contractile capacity of isolated adult cardiomyocytes.** We evaluated the ability of cytokines produced by BM-MNCs to preserve the contractile capacity of cardiomyocytes by measuring the percent fractional shortening (FS%) and Ca2+ transient in isolated adult rat cardiomyocytes (Fig. 3). The FS% of isolated cardiomyocytes after 72 h of cultivation in control medium was significantly lower than the baseline value, at 5.60 ± 1.49% vs. 7.81 ± 2.12%, respectively (P < 0.05), but it did not decrease significantly when cardiomyocytes were cultured in the normoxic and hypoxic supernatants of BM-MNCs, with respective values of 7.21 ± 1.58% and 7.32 ± 1.53% (Fig. 3B). Although the maximal rate of relengthening (Fig. 3C) and the maximal rate of shortening (Fig. 3D) of isolated cardiomyocytes decreased significantly from the baseline values after 72 h of cultivation, they were significantly better when cultured in the normoxic and hypoxic supernatants of BM-MNCs than in the control medium.

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**Fig. 1.** Detection of cytokines and growth factors in the supernatants of bone marrow mononuclear cells (BM-MNCs) after 24-h cultivation under conditions of normoxia and hypoxia. ELISA measurement showed that the BM-MNCs produced VEGF (A) and IL-1β (B), which were both enhanced significantly under conditions of hypoxia (*P < 0.05 vs. normoxia; data derived from 6 independent experiments). C: production of basic fibroblast growth factor (bFGF), PDGF, IGF-1, and transforming growth factor-β (TGF-β) by BM-MNCs was also detected distinctly by Western blot analysis, and some of these cytokines seemed to be enhanced by hypoxia stimulation.

**Fig. 2.** A: assessment of endothelial cell proliferation in vitro. Supernatants of BM-MNCs showed a dose-dependent increase in the proliferation of endothelial cells. *P < 0.001 vs. 0%; †P < 0.001 vs. 20%. B: detection of apoptosis of isolated adult cardiomyocytes by terminal dUTP nick-end labeling (TUNEL). B, left: representative photo, stained by 4,6-diamidino-2-phenylindole (DAPI; top) and TUNEL (bottom), of isolated cardiomyocytes after 72-h cultivation in control medium. Arrowheads, apoptotic cardiomyocytes; arrows, necrotic cardiomyocytes. Quantitative analysis revealed that the apoptosis index (%apoptotic cells) of isolated cardiomyocytes was significantly higher after 72 h of cultivation in control medium than that after 72 h of cultivation in the supernatants of BM-MNCs under both normoxic and hypoxic conditions (*P < 0.05 vs. control; data derived from 6 independent experiments).
The assessment of Ca\textsuperscript{2+} transient in isolated adult cardiomyocytes (Fig. 3, E–G) showed that the peak Ca\textsuperscript{2+} ratio decreased significantly from the baseline values of 1.09 ± 0.11 after 72-h cultivation in control medium (0.89 ± 0.12), the normoxic supernatants (0.94 ± 0.12), and the hypoxic supernatants (0.99 ± 0.10). However, the amplitude of the Ca\textsuperscript{2+} ratio decreased significantly after 72-h cultivation in the control medium to 0.21 ± 0.10 vs. the baseline value of 0.35 ± 0.06 (P < 0.001). This decrease was less significant in the normoxic supernatants of the BM-MNCs, at 0.28 ± 0.010 (P = 0.038 vs. baseline) and was not significant in the hypoxic supernatants of the BM-MNCs, at 0.33 ± 0.15 (P = 0.54 vs. baseline).

** Supernatants of BM-MNCs improved function of the infarcted heart.** Cardiac function was assessed by echocardiography before and then 7, 14, and 28 days after treatment (Fig. 4). The LVFS% decreased after ligation of the left anterior descending artery in all the rats, without significant differences among the groups. The LVFS% increased obviously within 7 days of treatment in the groups given both normoxic and hypoxic supernatants of the BM-MNCs, but not in the group given control medium. Thereafter, the LVFS% decreased gradually when the supernatants of the BM-MNCs were stopped. However, the LVFS% was significantly higher in the hypoxia group (P < 0.01 on day 7 and P < 0.05 on day 14 after treatment) and in the normoxia group (P < 0.01 on day 7 after treatment) than in the group given control medium. Although the LVEDD did not differ significantly among the groups, the LVESD was significantly lower in the hypoxia and normoxia groups than in the control group (P < 0.05 by group × time interactions analysis).

**Supernatants of BM-MNCs induced angiogenesis and improved remodeling of the infarcted heart.** Histological staining revealed more microvessels in the border zone of infarcted myocardium of the rats injected with supernatant from the BM-MNCs under hypoxia and normoxia than in the rats given control medium (Fig. 5A). Quantitative analysis indicated that the microvessel density of the infarct border zone in the normoxia and hypoxia groups was significantly higher than that in the control group (Fig. 5B). However, the microvessel density in the central infarct zone and the remote noninfarcted zone did not differ significantly among the groups. Conversely, Azan staining showed less fibrosis tissue and more surviving myocardium in the infarcted area of the LV in the normoxia and hypoxia groups than in the control group (Fig. 6A). Quantitative analysis also indicated that the fibrotic area of the...
infarcted myocardium was significantly smaller in the normoxia and hypoxia groups than in the control group (Fig. 6B). However, the wall thickness of the LV wall did not differ significantly among the groups (Fig. 6C).

**DISCUSSION**

Clinical trials have been carried out worldwide on cell-based therapy, especially the delivery of autologous cells derived from bone marrow, to treat ischemic heart diseases (1, 7, 13, 15, 27–31). If the effectiveness and safety are confirmed, it is expected that cell-based therapy will eventually replace cardiac transplantation for myocardial repair in patients with advanced heart failure. Although some positive effects of cell-based therapy using BM-MNCs, such as improved blood flow and cardiac function, have been reported in preliminary clinical trials and experimental studies, the precise mechanism remains unclear.

Recent experimental evidence suggests that the improvement of blood flow and cardiac function achieved by the delivery of BM-MNCs may be attributed mainly to the release of multiple cytokines and chemokines rather than to endothelial or myogenic differentiation (2, 15, 16, 23, 25). Thus it is now thought that therapeutic angiogenesis induced by the release of cytokines by the delivered BM-MNCs plays a major role in improving the function of the ischemic heart. However, there is little evidence documented on the effect of cytokine release from delivered BM-MNCs for directly protecting cardiomyocytes against ischemic injury. Using the supernatants of BM-MNCs and a rat model of acute myocardial infarction, we investigated the mechanisms of the implantation of BM-MNCs for myocardial repair through the secretory pathway.

First, we examined the production of several important cytokines from BM-MNCs in vitro, by cultivation under hypoxia and normoxia. In accordance with previous reports, many important cytokines for regulating cell proliferation, survival, and the angiogenic process were clearly detected in the supernatants of BM-MNCs after 24 h of cultivation. These cytokines included VEGF, bFGF, IL-1β, PDGF, IGF-1, and TGF-β, some of which were enhanced under conditions of hypoxia. We also found that the supernatants of BM-MNCs increased the proliferation of endothelial cells and inhibited the apoptosis of isolated adult cardiomyocytes in vitro. This suggests that the cytokines produced by BM-MNCs have the potential to induce therapeutic angiogenesis and inhibit cardiomyocytes apoptosis.

Second, we measured the contractile capacity and Ca²⁺ transient of isolated adult cardiomyocytes after cultivation in the supernatants of BM-MNCs or in the control medium. We found that the fractional shortening and amplitude of the Ca²⁺ ratio in isolated adult cardiomyocytes were significantly damaged after 72 h of cultivation in the control medium but that they were very well preserved when cultured in the supernatant of the BM-MNCs. This provided the first direct evidence that...
cytokines released by BM-MNCs have a protective effect on the contractile capacity of cardiomyocytes, providing new insight into the mechanism of how BM-MNCs improve cardiac function.

Third, using a rat model of acute myocardial infarction, we investigated the potency of cytokines produced by BM-MNCs for myocardial repair. Initially, we gave a single intramyocardial injection of supernatants after infarction. Contradictory to our in vitro findings, the single intramyocardial injection of supernatants did not increase the LVFS% significantly. It was reported previously that significant decrease in infarct size and myocyte apoptosis could be achieved by a single intramyocardial injection of the conditioned medium from mesenchymal stem cells overexpressing Akt gene but not by conditioned medium from mesenchymal stem cells without Akt overexpression (9). We speculated that the contradictory findings might be related to the insufficient dose of cytokines delivered by the single intramyocardial injection of supernatants, and we changed our in vivo protocol by giving an additional injection of supernatants. As expected, we found that the additional intraperitoneal injection of the supernatants of BM-MNCs improved the LVFS% significantly, but the administration of control medium did not. Histological analysis revealed that the administration of the supernatants of BM-MNCs increased the microvessel density in the border zone of infarcted myocardium and decreased collagen deposition significantly in the LV wall. Although we did not measure the levels of local and systemic cytokines, we speculated that the intraperitoneal injections of 500 μl of supernatants increased the levels of systemic cytokines and helped to repair the injured heart. These findings provided further evidence that cytokines produced by BM-MNCs have the capacity to protect the injured heart, which should be attributed to the augmentation of

Fig. 5. Microvessel density in the infarcted myocardium 28 days after treatment. A: more microvessels in the infarct border zone were observed in the normoxia and hypoxia groups than in the control group. B: quantitative analysis showed that the microvessel density in the infarct border zone was significantly higher in the normoxia and hypoxia groups than in the control group, but there is not significantly different of microvessel density in the central infarct zone and the remote noninfarcted zone among these groups.

Fig. 6. Azan staining of a cross section through the infarcted myocardium 28 days after treatment. A: more surviving myocardium, but less fibrous tissue, was observed in the normoxia and hypoxia groups than in the control group. B: quantitative analysis revealed significantly less fibrotic areas in the normoxia and hypoxia groups than in the control group. C: thickness of the left ventricular wall, however, did not differ significantly among the groups.
neovascularization and the improvement of remodeling after myocardial infarction.

Although we observed an increase in microvessel density and a decrease in collagen deposition in the infarcted myocardium 28 days after treatment, the LVFS% did not differ significantly among the groups 28 days after treatment. Thus regional improvements in myocardial perfusion and a decrease in collagen deposition are not enough to increase global cardiac function. According to our data, the LVFS% began to deteriorate gradually after we stopped giving the supernatants of BM-MNCs on day 7. This suggests that the continuous administration of supernatants may be necessary to maintain functional improvement of the injured heart. Our previous studies showed that BM-MNCs survived well after intramyocardial implantation and that the cytokines VEGF, IL-1β, and angiopoietin were increased in the infarcted heart for 1–2 wk after cell therapy (17, 24). These findings indicated that the cytokines were released continuously from the implanted cells after cell delivery. If so, cell-based therapy will become a revolutionary method for repairing the injured heart. However, recent investigations on cell-based myocardial repair found functional improvement 4 wk but not 6 mo after the implantation of mesenchymal stem cells (3). If further studies confirm that these therapeutic benefits are transient, we must find ways of extending and maintaining them.

Interestingly, the supernatants of BM-MNCs cultivated under conditions of hypoxia were more effective than those cultivated under conditions of normoxia, for preserving the contractile capacity of isolated cardiomyocytes and for improving the cardiac function of injured hearts. This difference might explain the higher concentration of cytokines in the hypoxic supernatant because cytokine production by BM-MNCs is enhanced under conditions of hypoxia. Although we could not conclude that the functional improvement of infarcted hearts is dependent on the quantity of cytokines produced by BM-MNCs, the mild potency of supernatant for repairing infarcted myocardium might be attributed to the fact that we used “thin” supernatant (collected after 24 h cultivation of 5 × 10^6/ml BM-MNCs) in this study.

Our findings showed that various cytokines are produced by BM-MNCs. Although many of these cytokines, including IGF-1 and PDGF, have been reported to prevent apoptosis of myocytes and improve cardiac function (5, 6, 8, 19), it is also possible that some of the inflammatory cytokines produced by BM-MNCs, such as TNF-α and IL-6, are detrimental to myocardial repair. However, it is clear that the mixed cytokines produced by BM-MNCs can increase the proliferation of endothelial cells, inhibit apoptosis of cardiomyocytes, preserve contractile function of isolated cardiomyocytes, and help repair the infarcted heart in vivo. We tried to identify the key cytokine in the medium that protected the heart and found that antibody perturbation to IGF-1 impaired partly (~30%) the inhibitive effect of cardiomyocytes on apoptosis, induced by the supernatant of BM-MNCs (data not shown). This indicates that multiple cytokines contribute to the protective effect of the heart. However, we do not know the functional role of each cytokine and growth factor and which cytokines or factors play the most important roles in myocardial repair. Further studies must be done to investigate the roles of each cytokine and evaluate their molecular mechanisms in myocardial repair.

In conclusion, we found that BM-MNCs produce many cytokines, which contribute to functional improvement of the infarcted heart by directly preserving the contractile capacity of the myocardium, by inhibiting apoptosis of cardiomyocytes and by inducing therapeutic angiogenesis of the infarcted heart. Our data also suggest that a secretory pathway might play the key role, albeit a complicated mechanism, in myocardial repair by cell-based therapy, such as the implantation of BM-MNCs into the infarcted heart.

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