Regeneration of Infarcted Myocardium by Intramyocardial Implantation of Ex Vivo Transforming Growth Factor-β–Preprogrammed Bone Marrow Stem Cells

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Background—Recent studies have shown that bone marrow–derived stem cells differentiate into the phenotype of cardiomyocytes in vivo and in vitro. We tried to regenerate infarcted myocardium by implanting ex vivo transforming growth factor (TGF)-β–preprogrammed CD117 (c-kit)–positive (CD117+) stem cells intramyocardially.

Methods and Results—CD117+ cells were isolated from the bone marrow mononuclear cells of GFP-transgenic or normal C57/BL6 mice. The myogenic differentiation of CD117+ cells was achieved by cultivation with TGF-β. Using an acute myocardial infarction model, we also tried to regenerate infarcted myocardium by implanting untreated (newly isolated) or preprogrammed (24 hours of cultivation with 5 ng/mL TGF-β1) CD117+ cells intramyocardially. TGF-β increased the cellular expression of myosin, troponins, connexin-43, GATA-4, and NKx-2.5, which suggested that it induced the myogenic differentiation of CD117+ cells. Compared with the effects of PBS injection only, the microvessel density in the infarcted myocardium was increased significantly 3 months after the implantation of either TGF-β–preprogrammed or untreated CD117+ cells. Moreover, many of the TGF-β–preprogrammed CD117+ cells were stained positively for myosin, whereas few of the untreated CD117+ cells were. Histological analysis revealed newly regenerated myocardium in the left ventricular anterior wall after the implantation of TGF-β–preprogrammed cells but not untreated cells. Furthermore, the left ventricular percent fraction shortening was significantly higher after the implantation of TGF-β–preprogrammed cells than after the implantation of untreated CD117+ cells.

Conclusions—TGF-β conducted the myogenic differentiation of CD117+ stem cells by upregulating GATA-4 and NKx-2.5 expression. Therefore, the intramyocardial implantation of TGF-β–preprogrammed CD117+ cells effectively assisted the myocardial regeneration and induced therapeutic angiogenesis, contributing to functional cardiac regeneration. (Circulation. 2005;111:2438-2445.)

Key Words: stem cells • myocardial infarction • regeneration • transforming growth factors

E xperimental studies have shown that the intramyocardial implantation of various cells, including fetal cardiomyocytes, embryonic stem cell–derived cardiomyocytes, myoblasts, and bone marrow stem cells, offers a potentially effective method of repairing injured myocardium.1–7 Bone marrow stem cells have become one of the most focused upon cell sources for repairing injured myocardium, because bone marrow–derived mesenchymal stem cells can differentiate into the phenotype of cardiomyocytes in vivo and in vitro.7,8 Furthermore, recent studies have shown that ex vivo pretreatment of bone marrow–derived mesenchymal stem cells with 5-azacytidine induces myogenic differentiation and that the implantation of 5-azacytidine-treated cells has better potential for regenerating injured myocardium and improving cardiac function than the implantation of untreated cells.6 Despite these promising results, the molecular mechanisms underlying myogenic differentiation from bone marrow stem cells remain poorly understood. To achieve effective regeneration of injured myocardium, it is important to find a more physiological way of improving the in situ myogenic differentiation of implanted bone marrow stem cells.

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Transforming growth factor-β, (TGF-β), is a multifunctional cytokine involved in the differentiation, growth, and survival of various cells.9 Recent investigations have shown that bone morphogenetic proteins, a subset of the transforming growth factor superfamily, promote cardiogenesis in vertebrate embryos.10,11 One study demonstrated that exogenous TGF-β, or bone morphogenetic protein, increased the expression of cardiac transcription factors in embryonic stem cells and promoted the cardiac differentiation of embryonic...
stem cells. Therefore, we speculated that TGF-β can conduct the myogenic differentiation of bone marrow stem cells and that ex vivo TGF-β pretreatment of bone marrow stem cells will increase their potential for functional cardiac regeneration after implantation.

We conducted this study to investigate whether TGF-β could induce the myogenic differentiation of CD117+ bone marrow stem cells in vitro. Moreover, we tried to achieve functional cardiac regeneration by implanting ex vivo TGF-β–preprogrammed CD117+ stem cells using an acute myocardial infarction mouse model.

Methods

Animals
C57BL6/Tg14 (act-EGFP) OsbY01 mice were kindly provided by Masaru Okabe (Genome Research Center, Osaka University, Osaka) and bred in the Animal Center of Yamaguchi University. Male C57BL6 mice were purchased from Japan SLC (Shizuoka, Japan). All experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University.

Isolation and Culture of Bone Marrow–Derived CD117+ Stem Cells
CD117+ cells were obtained by sorting, using the magnetic cell sorting (MACS) system (Miltenyi Biotec) as described previously. Briefly, bone marrow mononuclear cells collected from the femur and tibia of mice were incubated with PE-conjugated anti-mouse CD117 (c-kit) antibody (Bioscience) for 30 minutes on ice. After washing with PBS, cells were incubated with anti-PE microbeads (Miltenyi Biotec) for 20 minutes. CD117+ cells were separated by passing a MACS column. The purity of the CD117+ cells collected by the MACS was ~90%, and the viability was >99%. The purified CD117+ cells expressed ~8% to 20% of the lineage markers (Gr-1, MAC-1, B220, and TER119), 60% CD34, and 40% Sca-1.

Newly isolated CD117+ cells were suspended in RPMI 1640 medium with a supplement of 15% FBS, then seeded in a 25-mm2 flask or on 4-well chamber culture slides (Nalge Nunc International) (Miltenyi Biotec) for 20 minutes. CD117+ cells were separated by passing a MACS column. The purity of the CD117+ cells collected by the MACS was ~90%, and the viability was >99%. The purified CD117+ cells expressed ~8% to 20% of the lineage markers (Gr-1, MAC-1, B220, and TER119), 60% CD34, and 40% Sca-1.

Reverse Transcription–Polymerase Chain Reaction
Total RNA was extracted from freshly isolated CD117+ cells or CD117− cells after 1, 3, and 7 days of culture by use of an RNA extraction kit (Omega Bio-Tek Inc). Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed with an RNA PCR Kit (Takara) according to the manufacturer’s instructions. The primers for RT-PCR were as follows: β-myosin heavy chain (528 bp); sense, 5′-GATCACAACACGACTCCCTACG-3′; antisense, 5′-ATGCGAGGCTGCTAAAAGC-3′; cardiac troponin I (167 bp); sense, 5′-TCTCTAACCCTGGATAAGCAATCCTGG-3′; antisense, 5′-GAAGTTTCCTGGAGCGGAG-3′; cardiac troponin T (416 bp); sense, 5′-AGGGCTCTGTAGGTAGGCTCA-3′; antisense, 5′-ATAGATGCTCTGCCACAGC-3′; connexin 43 (172 bp); sense, 5′-TTGACCTTGACCTCGCTCAAGG-3′; antisense, 5′-AATTGAATGCACGCGACGAC-3′; GAPDH (861 bp); sense, 5′-CGGATTGCTGGTGTGTTGGT-3′; antisense, 5′-TCAAGGTTGGAGGAGTGGG-3′. PCR reactions were performed under the following conditions: 1 cycle at 94°C for 5 minutes, followed by 35 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. The PCR products were size-fractionated by 2% agarose gel electrophoresis.

Western Blot Analysis
Total protein was collected from freshly isolated CD117+ cells or CD117− cells after 1 and 3 days of culture with or without the addition of 5 ng/mL TGF-β1. The expression of GATA-4 and Nkx2.5, 2 important cardiac transcription factors, was measured by Western blot analysis using polyclonal antibodies against GATA-4 and Nkx2.5 (Santa Cruz), as described previously.

Alkaline Phosphatase Activity Assay
To observe the osteoblast differentiation of CD117+ cells, alkaline phosphatase (ALP) activity was determined as described by Uchimura et al. Briefly, cells were collected after 1, 3, and 7 days of culture, then washed twice with PBS (−). The cell suspension was homogenized and sonicated, after which 20 μL of the sonicated cell suspension was used for DNA quantification. To measure the ALP activity, another sonicated cell suspension was centrifuged at 10 000 rpm for 1 minute at 4°C. An aliquot (20 μL) of the supernatant was assayed for ALP activity using p-nitrophenyl phosphate substrate (Zymed Laboratories Inc). The activity was represented by p-nitrophenol, which was released after incubation for 30 minutes at 37°C. ALP activity was compared with that of freshly collected cells and used for statistical analysis.

Myocardial Infarction Model and Intramyocardial Implantation of CD117+ Cells
A myocardial infarction model was established in C57/BL6 mice as described previously. After general anesthesia and tracheal intubation with a 20-gauge intravenous catheter, mice were artificially ventilated with room air (Harvard Apparatus Co) at 80 breaths per minute. We performed a left thoracotomy through the fourth intercostal space and ligated the left anterior descending artery with 9-0 Prolene under direct vision. The mice were then randomly given an intramyocardial injection with a 31-gauge needle in the infarction area at 4 points, with one of the following: 10 μL PBS (PBS group, n = 20), 5×104 newly isolated CD117+ cells (untreated group, n = 34), or 5×104 CD117+ cells preprogrammed with 5 ng/mL TGF-β for 24 hour (preprogrammed group, n = 34). The CD117+ cells used for implantation were taken from the bone marrow of OsbY01 mice. A left thoracotomy without ligation of the left anterior descending artery was performed as a control (sham group, n = 10).

Echocardiography
We assessed cardiac function before treatment and then 7, 14, 30, 60, and 90 days after treatment by echocardiography using a 7.5-MHz annular array transducer. After the induction of light general anesthesia, the hearts were imaged 2-dimensionally in long-axis views at the level of the greatest left ventricular (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 end-diastolic diameters (LVEDDs) and LV end-systolic diameters (LVEFSDs) were measured from M-mode recordings according to the leading-edge method. The LV fractional shortening (%SF) was calculated as (LVEDD−LVEFSD)/LVEDD×100.

Histological Analysis
Five of the surviving mice from the preprogrammed and untreated groups were euthanized 30 or 60 days after treatment, and the remaining mice were euthanized 90 days after treatment. The hearts were harvested, and frozen sections 5 μm thick were used for histological analysis. The survival of implanted cells was detected by immunostaining with rabbit anti-GFP antibody (Molecular Probes) followed by FITC-conjugated goat anti-rabbit secondary antibody. Myogenic differentiation of surviving CD117+ cells was identified by immunostaining with mouse anti-myosin, ventricular heavy chain αβ monoclonal antibody (Chemicon Inc) and goat anti-mouse osteocalcin (Chemicon Inc), followed by incubation with FITC or R–PE–conjugated secondary antibodies, after 3 and 7 days of cultivation.

To measure the microvessel density in the infarcted myocardium, sections were stained with anti-mouse CD31 antibody (Pharmingen). Capillaries were counted in 2 separate slides from at least 10 randomly selected fields under ×200 microscopy. The mean number...
of capillaries per field in the infarcted myocardium was used for statistical analysis.

Azan staining was also performed to determine the wall thickness and the degree of collagen fiber accumulation in the infarcted region. By use of the image analysis software NIH IMAGE (NIH, Research Service Branch), the mean wall thickness was measured from 3 equidistant points, and the area of fibrosis was calculated as the area of stained fibrotic tissue divided by the total area of tissue. The measurements of wall thickness and area of fibrosis were performed on 2 separated sections of each heart, and the averages were used for statistical analysis.

Statistical Analysis
Data are expressed as mean±SD. Survival of animals was assessed by Kaplan-Meier analysis. Multiple-group comparisons were performed by ANOVA and Scheffé’s test, using a significance level of P<0.05.

Results
TGF-β1 Induced Myogenic Differentiation of CD117+ Cells by Upregulating Expression of GATA-4 and Nkx2.5
After 7 days of culture with the supplement of 5 ng/mL TGF-β1, ~70% of the CD117+ stem cells had a myoblast-like morphology under phase microscopy and were stained positively for myosin (Figure 1A), although ~10% of these cells were also stained positively for myosin after 3 days of culture. However, they showed round and negative staining for myosin after 7 days of cultivation without the addition of TGF-β1 (Figure 1A). Furthermore, RT-PCR analysis showed that the expression of several cardiac genes, β-myosin heavy chain, troponin I, troponin-T, and connexin-43, were also observed distinctly in these CD117+ stem cells after 7 days of cultivation with 5 ng/mL TGF-β1, but not without the addition of TGF-β1 (Figure 1B).

Western blot analysis showed that the expression of GATA-4 and Nkx2.5 in these CD117+ stem cells increased significantly after 1 and 3 days of cultivation with 5 ng/mL TGF-β1, but not without the addition of TGF-β1 (Figure 2). These results indicated that TGF-β1 induced the myogenic differentiation of CD117+ stem cells by the upregulation of GATA-4 and Nkx2.5 expression. Because 1 day of TGF-β1 preprogramming was enough to induce myosin heavy chain expression after 7 days in vitro, we collected cells for implantation 1 day after cultivation with TGF-β1.

A Low Concentration of TGF-β1 Did Not Induce Osteoblast Differentiation of CD117+ Cells
The ALP activity of CD117+ stem cells increased ~1.5-fold that of newly isolated cells after 7 days of cultivation with 0, 1, and 5 ng/mL TGF-β1, but this did not differ significantly according to whether 0, 1, or 5 ng/mL TGF-β1 was added. However, the ALP activity increased to >10-fold after cultivation with 100 ng/mL TGF-β1 (Figure 3A). Furthermore, immunostaining for osteocalcin showed that these CD117+ stem cells were negative after 7 days of cultivation with 5 ng/mL TGF-β1, but ~10% were positive after 7 days of cultivation with 100 ng/mL TGF-β1 (Figure 3B).

Regeneration of Injured Myocardium and Induction of Angiogenesis by the Implantation of Ex Vivo TGF-β1-Preprogrammed CD117+ Cells
Histological examination revealed that the implanted CD117+ stem cells survived primarily in the scar and marginal region of infarcted myocardium in all mice from both the preprogrammed and untreated groups (Figure 4A). Most of the surviving CD117+ stem cells were stained positively for myosin in the preprogrammed group, indicating the myogenic differentiation of programmed CD117+ cells. However, myosin expression was rarely found in the untreated group. Quantitative analysis also showed that there were significantly more myosin-positive cells in the preprogrammed group than in the untreated group, although the total cell survival was similar in the 2 groups (Figure 4B).

A band of newly regenerated myocardium localized outside the scar tissue was observed in the preprogrammed group but not in the untreated group (Figure 5A). Although the wall thickness of the infarcted region was similar in the 2 groups, the area of fibrosis was significantly smaller in the preprogrammed group than in the untreated group (Figure 5B).

The mean microvessel count per field in the infarcted myocardium did not differ significantly between the preprogrammed and untreated groups (129.8±17.1 versus 138.2±20.5), but it was significantly higher in both the

![Figure 1. Myogenic differentiation of CD117+ cells.](image-url)
Enhanced Cardiac Function After the Implantation of Ex Vivo TGF-β1-Preprogrammed CD117+ Cells

Although the technique of left anterior descending coronary artery ligation and intramyocardial injection was successful, ≈10% of the mice died of cardiac dysfunction soon after treatment. Within the 90 days of follow-up, 5, 8, and 13 mice from the preprogrammed, untreated, and PBS groups, respectively, died before they were scheduled to be euthanized. Statistical analysis showed that the survival rate was significantly higher in the preprogrammed group than in the PBS group (P<0.01), but the difference between the untreated and PBS groups was not significant (P=0.052, Figure 7).

Echocardiography showed that the motion of the LV anterior wall was obviously better in the sham, preprogrammed, and untreated groups than in the PBS group (71.3±11.8, P<0.01; Figure 6). Although histological analysis was performed 30, 60, and 90 days after treatment, we presented the data only for 90 days after treatment, because the wall thickness and microvessel density did not change significantly within this time course.

Discussion

Bone marrow stem cells have the potential for both myogenic differentiation and the induction of therapeutic angiogenesis; therefore, bone marrow is an important somatic stem cell source for functional myocardial regeneration. This study revealed a new physiological method of inducing myogenic differentiation of CD117+ bone marrow stem cells by TGF-β preprogramming. Furthermore, functional regeneration of infarcted myocardium was effectively achieved by the intramyocardial implantation of TGF-β–preprogrammed CD117+ bone marrow stem cells, related to either the regeneration of new myocardium or the induction of therapeutic angiogenesis.

It is well established that embryonic stem cells can be directed to differentiate into cardiomyocytes by signaling...
through TGF-β/BMP2. TGF-β can also induce the differentiation of neural crest stem cells into functional smooth muscle cells by the activation of Smad2 and Smad3 signaling pathways. We found that TGF-β1 could induce the myogenic differentiation of CD117 bone marrow stem cells in vitro, by upregulating the expression of GATA-4 and NKx2.5 in CD117 bone marrow stem cells. This indicates that the signaling pathway mediated through TGF-β/BMP might play an important role in the myogenic differentiation of bone marrow stem cells. In fact, the myogenic differentiation in response to 5-azacytidine was also found to be partly dependent on Bmpr1a, a receptor for bone morphogenetic proteins. However, the molecular mechanism responsible for TGF-β mediating these stem cells into myocytes and smooth muscle cells is still unclear.

Considering that TGF-β can induce osteoblast differentiation of bone marrow mesenchymal stem cells by mediation through the TGF-β/BMP-smad pathway, it is possible that local calcification and bone formation within the myocardium might be induced by the intramyocardial injection of ex vivo TGF-β–preprogrammed CD117+ bone marrow stem cells. Fortunately, TGF-β preprogramming by a concentration of 5 ng/mL or less did not significantly increase ALP activity or induce osteoblast differentiation of the CD117+ cells in vitro or in vivo. However, osteoblast differentiation could be induced by a higher concentration of TGF-β.

Using the new physiological method of inducing myogenic differentiation of CD117+ cells by TGF-β, we investigated the potential of functional cardiac regeneration by the intramyocardial injection of TGF-β–preprogrammed CD117+ bone marrow stem cells. Although the intramyocardial implantation of CD117+/Lin- bone marrow stem cells can regenerate infarcted myocardium effectively, we separated the total CD117+ bone marrow stem cells, because more than 80% of the separated CD117+ cells are lineage-negative, and the remaining CD117+/Lin- cells are considered a valuable subpopulation for inducing angiogenesis to improve regional perfusion in the infarcted myocardium. In fact, the significant increase in microvessel density in our preprogrammed and untreated groups contributed, at least in part, to the functional cardiac regeneration by the intramyocardial injection of TGF-β–preprogrammed CD117+ bone marrow stem cells.
the improved cardiac function, which was also assisted by the injection of untreated CD117\(^+\) stem cells, even though myocardial regeneration was not observed.

The fact that the LV %FS was significantly better in the preprogrammed group than in the untreated group indicates that more effective functional cardiac regeneration could be achieved by the implantation of TGF-β–preprogrammed cells than untreated cells. Although the cell survival seemed similar in the preprogrammed and untreated groups, many of the surviving CD117\(^+\) cells in the preprogrammed group showed positive staining for myosin, whereas very few did in the untreated group. This indicates that 24 hours of TGF-β pretreatment improved the myogenic differentiation of CD117\(^+\) cells implanted into infarcted myocardium. Furthermore, a band of newly regenerated myocardium was observed outside the scar tissue in the preprogrammed group, whereas fibrotic tissue was observed in the untreated group. Because the microvessel density was similar in the preprogrammed

Figure 5. Azan staining of a cross section through infarcted myocardium 90 days after treatment. A, A band of newly regenerated myocardium (red line) was seen only outside scar tissue in preprogrammed group but not in untreated group. B, There was significantly more fibrous tissue in untreated group than in preprogrammed group, although wall thickness of infarcted region did not differ between 2 groups.

Figure 6. Microvessel density in infarcted myocardium 90 days after treatment. A, More microvessels were observed in preprogrammed and untreated groups than in PBS group. B, Quantitative analysis showed that microvessel density was significantly higher in preprogrammed and untreated groups than in PBS group, but it did not differ significantly between preprogrammed group and untreated group.

Figure 7. Cumulative percent survival of mice, plotted according to Kaplan-Meier method. Significance was analyzed with log-rank test.
and untreated groups, we speculate that the enhanced functional cardiac regeneration achieved by the implantation of TGF-β-preprogrammed CD117+ cells is related to the increased in situ myogenic differentiation from implanted cells and the decreased collagen fiber accumulation in the infarcted region.

A recent study by Beltrami and associates22 showed that the myocytes produced by cardiac CD117+ stem cells in infarcted myocardium are structurally and functionally competent, with normal contractile properties. Conversely, 2 very recent studies found that hematopoietic stem cells did not differentiate into cardiomyocytes in vivo.23,24 There is still no consensus that the bone marrow stem cells can differentiate into cardiomyocytes. Our data show that untreated CD117+ bone marrow stem cells rarely cause myogenic differentiation in vitro or in vivo. Although the myogenic differentiation of CD117+ cells was increased dramatically by TGF-β preprogramming, we were unable to demonstrate that these myogenic differentiated CD117+ bone marrow stem cells were really mature cardiomyocytes with normal contractile properties after implantation into infarcted myocardium.

Although TGF-β preprogramming favors the myogenic differentiation of CD117+ bone marrow stem cells, we must identify the molecular mechanism of how TGF-β mediates stem cell differentiation into cardiomyocytes. Further studies must be conducted to search for direct evidence that these myogenic differentiated CD117+ bone marrow stem cells were really mature cardiomyocytes with normal contractile properties after implantation into infarcted myocardium.

In conclusion, we found that adding a low concentration of TGF-β, induced the myogenic differentiation of CD117+ stem cells by upregulating GATA-4 and NKx-2.5 expression. Therefore, the intramyocardial implantation of ex vivo TGF-β-preprogrammed CD117+ bone marrow stem cells is a feasible method of functional cardiac regeneration, achieved by regenerating new myocardium through the in situ myogenic differentiation of implanted stem cells and by increasing regional perfusion through the induction of therapeutic angiogenesis.

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