Transplantability of Bone Marrow Microenvironment Components \textit{In Vitro} \\
Analysis with Long-Term Cultured Fibroblastic Stromal Cells

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Abstract The bone marrow microenvironment components following allogeneic bone marrow transplantation (BMT) are reported to be of the recipient origin, implying that the marrow components can not be transplantable \textit{in vivo}. To investigate the transplantability of the marrow microenvironment components, we have constructed an \textit{in vitro} model of transplantation using 12 pairs of long-term cultured fibroblastic stromal cells obtained from different individuals. In our \textit{in vitro} model of transplantation, after ablation of all hemopoietic cells, we simultaneously established a pair of confluent layers of fibroblastic stromal cells from different individuals and transplanted one of them on the other confluent layer cells. The overlaid stromal cells grow on the allogeneic confluent layer as observed by phase-contrast microscopy. The immunostaining showed that the stromal cells were vimentin-positive fibroblasts. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) showed mixed chimerism in all 12 pairs. The chimerism was found if the dose of overlaid stromal cells was more than one-tenth that of the confluent layer cells. These data indicate that fibroblastic stromal cells are transplantable \textit{in vitro} even if the surface of the flask is completely covered with the allogeneic stromal cells, and their dose may be largely related to their transplantability following allogeneic BMT in humans.

Key words: bone marrow transplantation, bone marrow microenvironment, bone marrow fibroblastic stromal cells, transplantability of bone marrow microenvironment
I. Introduction

Bone marrow transplantation (BMT) is an established and important clinical procedure for the treatment of many life-threatening human diseases. Both the differentiation and the proliferation of transplanted hemopoietic stem cells are regulated by the marrow microenvironment, consisting of a variety of cells collectively termed the marrow stroma. Therefore, successful engraftment of transplanted hemopoietic stem cells requires an adequate marrow microenvironment. In spite of recent advances in BMT procedures, the delayed hemopoietic recovery is observed in some patients following allogeneic BMT. One of the reasons may be inadequate support of damaged marrow microenvironment due to myeloablative treatment against original diseases and/or pre-transplant conditioning regimen.

The reports concerning the origin of bone marrow microenvironment components following allogeneic bone marrow transplantation demonstrated that bone marrow-derived fibroblastic stromal cells which can proliferate in long-term cultures are of the recipient origin and concluded that fibroblastic stromal cells are not transplantable in humans. It remains to be solved why donor fibroblastic stromal cells can not be engrafted even though the transplanted donor bone marrow cells which contain a considerable amount of fibroblastic stromal precursors.

The long-term bone marrow culture system, first described by Dexter et al. in the mouse, is widely used as the in vitro model of the bone marrow microenvironment. In our present study, using this long-term bone marrow culture system, we developed a pair of bone marrow-derived fibroblastic stromal cell populations from different individuals and performed in vitro transplantation of the fibroblastic stromal cells on the allogeneic stromal cell layer. Then, we examined the origin and transplantability of the fibroblastic stromal cell by phase-contrast inverted microscopy, immunostaining and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), a technique based on the detection of DNA polymorphic sequences of individual markers.

II. Materials and Methods

1. Bone Marrow Cell Processing and Culture

Bone marrow samples were obtained from healthy volunteers by aspiration from the anterior iliac crest. The mononuclear cells (MNCs) were separated by centrifugation on Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden), and $5 \times 10^8$ of the cells were plated in 25-cm$^2$ tissue culture flask (Falcon, Franklin Lakes, NJ, U.S.A) in 8 ml of RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A) supplemented with 10% fetal calf serum (FCS) and 8 $\mu$l/ml gentamycin (Gibco). The cultures were maintained in a humidified atmosphere at 37°C with 5% CO$_2$, and fed at weekly intervals by complete replacement of the medium.

2. Establishment of Cultured Stromal Cells

After an initial culture of BM MNCs for 3 days, all nonadherent cells were removed by discarding the medium. After washing twice with phosphate-buffered saline (PBS), the flask was replenished with fresh medium. The primary culture had been carried for 1 to 2 weeks until a confluent layer was formed. At confluence, the adherent cells were separated with 0.25% trypsin containing 0.01% EDTA. After three washings with PBS, $5 \times 10^5$ of the suspended fibroblastic stromal cells were re-seeded in a 25-cm$^2$ tissue culture flask (Falcon). After four passages, we used these adherent cells as stromal cells.

To obtain pure fibroblastic stromal cells, after two passages, the adherent cells were irradiated at a dose of 10 Gy using a Linac to ablate the residual hemopoietic cells, and then cultured for 2 weeks in Chang medium (Irvine Scientific, Santa Ana, CA, U.S.A) to eliminate the macrophages and endothelial cells.

3. In Vitro Transplantation of Stromal Cells

As an in vitro model of transplantation of fibroblastic stromal cells, we used a pair of cultured stromal cell populations derived from different individuals. After four passages, the stromal cells were re-seeded at a dose of $2 \times 10^5$ cells per 35-mm$^2$ tissue culture dish (Falcon). After the adherent layer became confluent, the suspended stromal cells derived from a different individual were overlaid on the confluent stromal layer. To see the manner of engraftment and growth of the overlaid fibroblastic stromal cells, the suspended fibroblastic stromal cells were overlaid on the allogeneic fibroblastic stromal confluent layer as well as on the empty surface of fresh culture flask. The adherence and growth of the cells were observed and recorded under a phase-contrast inverted microscope (Olympus, Tokyo, Japan).

In all of our experiments, we used almost the same dose of fibroblastic stromal cells in each pair. We
also examined the influence of the dose of transplanted fibroblastic stromal cells on the in vitro transplantation. Namely, we overlaid the fibroblastic stromal cells at different ratios of the cell dose from 1/20 to 20/1 those used for the allogeneic confluent layers.

4. Immunohistochemistry of Adherent Stromal Cells Layer

Aliquots of the stromal cells after four passages were grown on Lab-Teck chamber glass slides (Nunc, Neperville, IL, U.S.A.) at a dose of $1 \times 10^4$ cells per chamber. The cultured stromal cells were fixed on the chamber glass slides by cold acetone. A direct immunoperoxidase technique was performed using monoclonal antibodies (mAb) against vimentin (Dako, Kyoto, Japan) expressed on fibroblasts, CD 68 (Dako) expressed on macrophages, and von Willebrand's factor (Dako) expressed on endothelial cells. The glass slides were incubated with the 1:100 diluted primary antibody at 4°C overnight. After washing with PBS, the slides were incubated with horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin diluted 1:200 in PBS containing 1% bovine serum albumin for 1h at room temperature. After rinsing with PBS, the slides were incubated with 3, 3'-diaminobenzidine $\text{H}_2\text{O}_2$ solution for 2-8 min. After a further rinse with PBS, counterstaining was done with 1% methyl green solution.

5. Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from the cultured pellets of all experiments by a rapid extraction technique using Centricon 30 dialysis/concentration tubes (Amicon, Beverly, MA, U.S.A.). The D1S80 gene was amplified by using the variable number tandem repeats (VNTR) MCT 118 primers pair. The sequence of the MCT 118 primers were:

$$5' - \text{GAAACTGGCCCTCCAACACTGCCC} \text{GCG} - \text{CG}-3'$$
$$5' - \text{GTCTTGTGGAGATGCACGTGCCC} \text{CTT-GC}-3'$$

for the 5' and 3' primers, respectively. DNA templates were amplified by the PCR procedure with 2.5 units of Taq DNA polymerase (Takara Co. Kyoto, Japan). The reaction mixture was subjected to 30 cycles of 1 min at 90°C, 1 min at 62°C and 2 min at 72°C. The final amplification was terminated by a 4-min supplementary extension step at 72°C. After amplification, polymorphic bands were detected by ethidium bromide staining after electrophoresis on 4% polyacrylamide gels.

III. Results

1. Characterization of Long-Term Cultured Fibroblastic Stromal Cells

Since bone marrow stromal cells comprise various kinds of cells such as fibroblasts, endothelial cells, adipocytes and macrophages, we characterized the nature of long-term cultured bone marrow adherent cells by immunohistochemistry. After four passages, most of the adherent cells were spindle-shaped cells and a small percentage of them were stellate cells. Results of immunohistochemistry showed that most of the adherent cells strongly
expressed vimentin (Fig. 1A), and a few stellate cells expressed CD68 molecule (Fig. 1B). The percentage of CD68-positive cells in the adherent layer was less than 0.5%. The absence of endothelial cells was confirmed by the negativity of expression of von Willebrand's factor in the adherent layer (data not shown). These results indicate that the marrow-derived adherent cells are predominantly fibroblasts in our long-term culture system.

**Fig. 2** A series of phase-contrast photomicrographs of fibroblastic stromal cells
These photographs show the manner of the engraftment and growth of fibroblastic stromal cells on an empty surface of the culture flask (a,b,c,d) and on the allogeneic confluent layer (e,f,g,h). Photomicrographs were taken soon (a,e) 1 h (b,f) 7 h (c,g) and 22 h (d,h) after the seeding of the suspended fibroblastic stromal cells. The suspended fibroblastic stromal cells behaved similarly on the empty surface of the culture flask and on the allogeneic fibroblastic stromal layer; they adhered within 1 h (b,f), became flattened within 7 h (c,g), and became spindle-shaped and made interlocking network within 22 h (d,h). The overlaid fibroblastic cells produced a second layer of fibroblastic stromal cells on the allogeneic fibroblastic stromal layer, which is not distinguishable from the underlaid confluent layer of fibroblastic stromal cells (h).
2. In Vitro Transplantation of Fibroblastic Stromal Cells

Since the long-term cultured bone marrow fibroblastic stromal cells are supposed to originate from the recipient at any period following allogeneic BMT,\textsuperscript{14,15} we further investigated whether fibroblastic stromal cells can be transplantable on the confluent layer of allogeneic fibroblastic stromal cells. We seeded the fibroblastic stromal cells on the allogeneic confluent layer and on the empty surface of tissue culture flask as a control. The adherence and growth of the fibroblastic stromal cells on the empty surface of the culture flask as well as on the allogeneic confluent layer were visually estimated under a phase-contrast inverted microscope. As shown in Fig. 2, the fibroblastic stromal cells seeded on the empty surface (Fig. 2a) adhered on the surface of the culture flask within 1 h (Fig. 2b), gradually became flattened within 7 h (Fig. 2c), and finally became spindle-shaped and made an interlocking network within 22 h (Fig. 2d). The morphological change of the overlaid cells on the allogeneic confluent layer during the initial period of engraftment was the same as that of the control. Namely, the suspended fibroblastic stromal cells overlaid on the allogeneic confluent layer of fibroblastic stromal cells (Fig. 2e) adhered on the confluent layer (Fig. 2f), gradually became flattened (Fig. 2g), and finally became spindle-shaped and made interlocking network (Fig. 2h). These overlaid cells produced a second layer of fibroblastic stromal cells on the allogeneic fibroblastic stromal layer, which were not distinguishable from the underlaid confluent layer of fibroblastic stromal cells on photomicrographs.

We further performed immunostaining of the engrafted overlaid cells to confirm their fibroblastic nature. Immunostaining was performed after culturing the fibroblastic stromal cells on the confluent layer for 24 h. The result showed that almost all of the stromal cells were spindle-shaped and positive for vimentin (Fig. 3A), and a very few cells were positive for CD 68 (Fig. 3B), indicating that the second layer cells were also fibroblasts. These results demonstrate that the fibroblastic stromal cells can be transplantable and can grow on the allogeneic stromal cell layer.

3. Polymerase Chain Reaction (PCR)

As described above, by the microscopic observation, we could confirm the engraftment of fibroblastic stromal cells on the allogeneic confluent layer, forming a second layer. For further confirmation, we performed PCR with the DNA obtained from the fibroblastic stromal cells comprising both the underlaid confluent layer and the overlaid engrafted cells after culturing for 1-3 weeks. The PCR amplification of the D1S80 locus-specific sequence demonstrated engraftment in all 12 pairs. In Fig. 4A (one of the 12 pairs), lanes 1, 2 to 5, and 6 showed the restriction fragment patterns of the confluent layer cells, mixtures of the confluent layer and overlaid cells, and overlaid cells, respectively. To examine the influence of the culture period on the in vitro transplantation, the fibroblastic stromal cells were cultured for 1 week (lane 2) and 3 weeks (lane 3) after seeding on the allogeneic confluent layer. Both lanes 2 and 3 have three bands, in-
indicating the mixed chimerism independently of the culture period examined. In some experiments, we irradiated the confluent layer before overlaying the allogeneic suspended stromal cells and cultured them for 1 week (lane 4) and 3 weeks (lane 5). There was no change in the restriction fragment patterns of the confluent layer and overlaid cells, respectively. The suspended stromal cells were overlaid on the non-irradiated confluent layer and cultured for 1 week (lane 2) and 3 weeks (lane 3). In some experiments, the confluent layers were irradiated before overlaying the suspended stromal cells, and the overlaid cells were cultured for 1 week (lane 4) and 3 weeks (lane 5). On several complete confluent fibroblastic stromal layers the allogeneic fibroblastic stromal cells were overlaid at different ratios of the cell dose, cultured for 10 days, and processed for PCR (B). Lanes 1, 2 to 10, and 11 in B show the restriction fragment patterns of the confluent layer cells, mixtures of the confluent layer and overlaid cells, and overlaid cells, respectively. The ratios of the dose of the overlaid cells to that of the confluent layer cells were 1/20 (lane 2), 1/10 (lane 3), 1/5 (lane 4), 1/2 (lane 5), 1/1 (lane 6), 2/1 (lane 7), 5/1 (lane 8), 10/1 (lane 9), and 20/1 (lane 10).

There is evidence that the detection of engrafted fibroblastic stromal cells depends on the dose of transplanted cells in the BMT. In conventional bone marrow transplantation in humans, the proportion of transplanted donor stromal cells was extremely small compared to the recipient's stromal cells. In this context, we studied the influence of the dose of transplanted fibroblastic stromal cells on the detection of their engraftment on the allogeneic stromal cell layer in our in vitro model of transplantation using the PCR-RFLP method. In Fig. 4B, lanes 1, 2 to 10, and 11 showed the restriction fragment patterns of the confluent layer cells, mixtures of the confluent layer and overlaid cells, and overlaid cells, respectively. When the dose of the overlaid cells was 1/20 that of confluent layer cells, there were no bands of the overlaid cells (lane 2), indicating no mixed chimerism. When the ratios of the dose of overlaid cells to that of the confluent layer cells were increased from 1/10 to 10/1, the mixed chimeric bands became detectable (lanes 3 to 9). The bands of the confluent layer cells were almost lacking at the ratio of 20/1 (lane 10). These results indicate that when the cell dose of either of the cell populations was less than one-tenth of the other in the transplantation, the conventional PCR-RFLP method using VNTR MCT 118 primer pair may not detect the growth of the cell population.

IV. Discussion

During the last few years, several investigators studied the origin of bone marrow microenvironment components in patients following allogeneic BMT. The engraftment of donor stromal cells in mice has been reported by Marshall et al. The transplantability of the bone marrow microenvironment in humans was also reported by Keating et al.: they reported that in vitro long-term cultured stromal cells following allogeneic BMT were of the donor origin. More recent studies, however, have demonstrated that fibroblastic stromal cells, a main component of the bone marrow microenvironment, are of recipient origin following allogeneic BMT, which indicates that the bone marrow fibroblasts are not transplantable in humans. Our findings of the similar examination are in good agreement with these recent reports demonstrating that the fibroblastic stromal cells originate from the recipient (data not shown). So, after allogeneic BMT if only hemopoietic progenitors are engrafted, the question arises of why stromal progenitors are
not transplantable or why they are rejected. One of the speculations is that even after the pre-transplant conditioning relatively radio-resistant recipient's stromal cells may cover the surface of bone marrow space and inhibit the engraftment of donor’s precursors of the fibroblastic stromal cells. The goal of this study was to examine whether the stromal progenitors can be engrafted in allogeneic BMT.

In our in vitro system of bone marrow transplantation, phase contrast photomicrography demonstrated the growth of the fibroblastic stromal cells on the allogeneic confluent layer cells. The immunostaining confirmed that they are fibroblasts. The engraftment of those overlaid cells was also confirmed by PCR. Currently PCR is the most sensitive method for DNA analysis. With regard to the sensitivity of PCR using VNTR MCT 118 primers, a minor cell population of 1% can routinely be detectable. Our results thus demonstrate that the fibroblastic component of bone marrow stroma can be engrafted on the allogeneic stromal cells in vitro.

In the conventional human allogeneic BMT it appears that the donor fibroblastic progenitors may be rejected. Several explanations are considered for this discrepancy between the in vivo and in vitro models. First, in vivo, the donor’s bone marrow and recipient’s peripheral blood include T-cells. So, it may be possible that residual immune system and/or structure of marrow may inhibit engraftment and growth of donor stromal cells. Secondly, in vivo, during conventional allogeneic BMT the transplanted donor’s bone marrow contain the precursors of fibroblastic stromal cells, but in our in vitro model of transplantation we transplanted only the matured fibroblasts and not the precursors of the fibroblastic stromal cells. We have also performed in vitro transplantation of bone marrow MNCs containing the precursors on the allogeneic confluent layer; we failed to estimate the transplantability of precursors of fibroblastic stromal cells, as we could not remove all hemopoietic cells from the allogeneic confluent layer because of the invasion of considerable numbers of hemopoietic cells into the stromal cell layer (data not shown). Thirdly, there is the possibility that the number of transplanted donor stromal progenitors is too small compared with that of the recipient’s stromal cells to establish detectable stroma chimerism. In our in vitro experiment, when almost the same dose of suspended stromal cells as that of the allogeneic cells used for the confluent layer were transplanted, clear bands of their mixed chimerism was demonstrable by PCR-RFLP, but when the dose of transplanted stromal cells was less than one-tenth that of the allogeneic confluent layer cells the growth of the donor stromal cells was not detectable by PCR-RFLP. These data certainly support the second possibility. In this regard, Friedenstein et al and Piersma et al studied whether intravenously infused fibroblastic colony-forming cells (CFU-F) are transplantable in mice. It is very interesting that Friedenstein et al failed to transplant donor stromal cells, while Piersma et. al succeed in the transplantation of stromal cells via the intravenous route in mice. In respect to this discrepancy between these studies, Friedenstein et al transplanted a relatively low dose of donor cells but Piersma et al transplanted an apparently high dose of donor bone marrow cells. Thus, it is likely that the dose of transplanted stromal cells is largely related to their transplantability following allogeneic BMT in humans.

We conclude from our study, although further studies are required for clinical application, that transplantation of mature fibroblastic stromal cells might be possible in humans if an adequate number of fibroblastic stromal cells could be transplanted.

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References


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