Sato K, Nakaoka T, Yamashima N, Yagita H, Kawasaki H, Morimoto C, Baba M and Matsuyama T	TRAIL-transduced dendritic cells protect mice from acute graft-versus-host disease and leukemia relapse	*Journal of Immunology*		In press	2005
Iwata T, Fujita T, Hirao N, Matsuzaki Y, Okada T, Mochimaru H, Susumu N, Matsumoto E, Sugano K, Yamashita N, Nozawa S, and Kawakami Y	Frequent immune responses to a cancer/testis antigen CAGE in patients with microsatellite instability positive endometrial cancer	*Clinical Cancer Research*		In press	2005

phosphatases with the membrane (Cuppen et al., 1998; Kuroda et al., 1996). Therefore, it can be hypothesized that enigma would mediate the effects of estrogen such as growth inhibition in VSMC through the binding with some phosphatases such as SHP-1 or MKP-1 which could be induced by estrogen (Takeda-Matsubara et al., 2002).

Downstream of the estrogen-ER signaling pathway has not been clarified in the vasculature as much as in reproductive organs. Estrogen augmented the promoter activity of caveolin-1, which did not contain any palindrome estrogen responsive elements in the 3 kb promoter region (Razandi et al., 2002). The sequences of the promoter region of SmLIM, enigma, and Id3 genes have not been reported. Analysis of the promoter of these genes may provide some hints to understand the downstream signals of ER in the vasculature. Also, in this study, we could not check all of the genes expressed differentially between the OVX + E group and OVX + V group obtained from the high-oligonucleotide microarray analysis. Thus, further study should be done to identify other estrogen-regulated genes that might play more important roles in the vasculature.

Acknowledgements

This work was supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (13557062) and a Grant-in-Aid for Science Research from the Ministry of Health, Labor and Welfare of Japan (H13-Choju-016).

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A potential pro-angiogenic cell therapy with human placenta-derived mesenchymal cells

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Received 22 September 2004

Abstract

Recently several strategies to treat ischemic diseases have been proposed but the ideal way has to be determined. We explored whether human placenta-derived mesenchymal cells (hPDMCs) can be used for this purpose because placenta is very rich in vessels. First, production of human vascular endothelial growth factor (hVEGF) from hPDMCs was examined. The amount of hVEGF secreted by hPDMCs was similar to the amount produced by HeLa cells. hVEGF was barely detected in human umbilical vein endothelial cells (hUVECs) or human peripheral blood mononuclear cells. hVEGF secreted from hPDMCs stimulated the proliferation of hUVECs, indicating its biological activity. Transplantation of hPDMCs to the ischemic limbs of NOD/Shi-scid mice significantly improved the blood flow of the affected limbs. Blood vessel formation was more prominently observed in the limbs of treated mice as compared to the control mice. Real-time RT-PCR revealed that hPDMCs produced hVEGF for at least 7 days after transplantation. Thus, transplantation of hPDMCs could potentially be a promising treatment for human ischemic diseases.

Keywords: Angiogenesis; Vascular endothelial growth factor; Cell Therapy; Atherosclerosis

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The discovery of potent angiogenic regulators has led to the development of pro-angiogenic therapies for ischemic diseases [1]. Angiogenic factors can be applied to ischemic lesions by direct injection or expression of the gene by plasmid or virus vectors [2–6]. However, the efficacy of these therapies is not always satisfactory. For example, vascular endothelial growth factor (VEGF) gene therapy causes edema and hemangioma due to unregulated gene expression [7,8]. To overcome the shortcomings of single angiogenic factor therapies, the combination of two angiogenic factors has been tested as a treatment for ischemic diseases. These combination therapies are effective in inducing functional and stable vascular networks [9–11]. Alternatively, delivery of VEGF, when maintained below a threshold microenvironmental level, can lead to normal angiogenesis without other exogenous growth factors [12]. Along with the application of angiogenic factors, cell therapies for ischemic diseases using peripheral blood-derived endothelial progenitor cells [13,14], cord blood-derived endothelial precursor cells [15], peripheral blood mononuclear cells [16], or bone marrow mononuclear cells [17,18] have been reported. Clinical studies of the transplantation of bone marrow mononuclear cells or peripheral blood mononuclear cells have also been performed and the effectiveness in patients has been demonstrated [19]. VEGF and basic fibroblast growth factor (bFGF) produced by mononuclear cells or the transplanted cells are implicated for clinical efficacy, although the exact

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doi:10.1016/j.bbrc.2004.10.003

mechanism has not been fully elucidated. The collection procedures used for these cell therapies do put the patient at some risk. To collect an adequate number of bone marrow cells, systemic anesthesia is required. In the case of sampling peripheral blood mononuclear cells, leukapheresis is necessary.

Since the establishment of cord blood banks, umbilical cord blood is being frequently used for hematopoietic stem cell transplantation [20]. When cord blood is collected, the adjacent placenta is discarded as medical waste. The placenta contains a large amount of vessels, which are created in a short period of time. Placental cells can be obtained from normal full-term deliveries and banked with HLA typing. It is possible that placenta cells may become a promising resource for cell therapy.

In this study, we used human placenta-derived mesenchymal cells (hPDMCs), which were $CD34^-CD45^-SH2^+$ $SH3^+$ [21]. We examined whether hPDMCs produced angiogenic factors and whether transplantation of these cells could improve blood flow in a hindlimb ischemia model of NOD/Shi-scid mice.

Methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin–amphotericin B solution, trypsin, and EDTA were purchased from Invitrogen (Carlsbad, CA). RPMI 1640 medium was purchased from Sigma–Aldrich (Munich, Germany), phosphate-buffered saline (PBS) was purchased from Nissui (Tokyo, Japan), and fetal bovine serum (FBS) was purchased from Thermo Trace (Melbourne, Australia). Human umbilical vein endothelial cells (hUVECs) and endothelial growth medium (EGM) were purchased from Clonetics (San Diego, CA). HeLa cells were provided by Riken (Tokyo, Japan). Recombinant hVEGF and anti-human VEGF blocking antibody was purchased from R&D Systems (Minneapolis, MN). Ficoll–Hypaque was purchased from Amersham (Uppsala, Sweden). Biotinylated anti-rabbit IgG was purchased from Biosource International (Camarillo, CA). Anti-factor VIII polyclonal antibody was purchased from DakoCytomation (Copenhagen, Denmark).

Isolation of hPDMCs. Human placentas were harvested from full-term deliveries (38–40 weeks of pregnancy) and cell processing was started within 4 h of delivery. The use of human placenta was approved by the Ethics Committee of the Institute of Medical Science, University of Tokyo. After removal of the umbilical cord and amnion from the placenta, small pieces of tissues were cut out of maternal part of placenta (decidua). Tissues were chopped with scissors, washed in PBS, and then stirred in PBS with 0.05% trypsin and 0.53 mmol/L EDTA at ambient temperature for 10 min. After being filtered through nylon mesh, FBS (15 mL) was added and the fractions containing the released cells were pooled. The tissue sections were digested 2 more times, and the 3 fractions were mixed. The cells were centrifuged at low speed (1000 rpm for 10 min) and were suspended in PBS. Following flotation on Ficoll–Hypaque, the mononuclear cell fraction was isolated and washed in PBS. Then, the collected cells were seeded at a density of 3.0×10^6 cells/cm^2 in DMEM with 10% FBS. Ten days after trypsin digestion, adherent and non-adherent cells were harvested with 1 mmol/L EDTA solution. $CD34^-CD45^-SH2^+$ cells were sorted as hPDMCs using a FACS (Vantage SE, Becton–Dickinson). hPDMCs used in this study were within 25 population doubling levels. Surface marker analysis of hPDMCs revealed that they were positive to CD105 and CD73 antibodies and negative to CD14, CD21, CD23, and CD86 antibodies. Chimerism assay showed that hPDMCs are of maternal origin (data not shown).

Cell culture. The cells were grown at 37 °C in an atmosphere of 95% air and 5% CO_2. Antibiotic–antimycotic (1%) was added to all media. hPDMCs (PL26, PL54C, and PL78C) isolated from 3 independent placenta donors were used in this study. hPDMCs were maintained in DMEM with 10% FBS. hPDMCs were routinely passaged just before reaching confluence by brief exposure to 0.05% trypsin and 0.53 mmol/L EDTA at a ratio of 1:8. hUVECs were grown in EGM containing 2% FBS. HeLa cells were maintained in DMEM with 10% FBS. PBMCs were isolated from human peripheral blood using Ficoll–Hypaque and cultured in RPMI 1640 with 10% FBS. PBMCs-1 and PBMCs-2 were obtained from 2 healthy volunteers (a 40-year-old male and a 42-year-old male).

Assays for hVGEF and other angiogenic factors. To study hVEGF production, fresh culture medium was added when cells reached 80% confluence in 10-cm culture dishes. After 24 h the culture medium was collected and centrifuged at 1000 rpm for 10 min. The supernatant was frozen until assayed. Cultured cells were detached from culture dishes using 0.05% trypsin and 0.53 mmol/L EDTA. Cells were resuspended in 1 mL PBS and the cell number was counted. Recovered cells were subjected to 3 cycles of freeze and thaw. After centrifugation at 2000 rpm for 10 min the supernatant was collected and frozen until intracellular hVEGF content was assayed. hVEGF concentration was measured by ELISA using the Quantikine hVEGF immunoassay kit (R&D Systems, Minneapolis, MN). The detection range of this kit was 5–2000 pg/mL hVEGF. hVEGF concentration was normalized by dividing by the cell number. Triplicate experiments were performed. Data are expressed as means ± SE. TNF-α (QuantiGlo human TNF-α immunoassay), PlGF (Quantikine human PlGF immunoassay), EGF (Quantikine human EGF immunoassay), b-FGF (Quantikine human basic FGF, R&D Systems, Minneapolis, MN), and HGF (Ohtsuka ELISA kit, Ohtsuka Japan) were also measured.

Thymidine uptake. Thymidine uptake by hUVECs was examined as described by Conn et al. [22]. hPDMCs were cultured in serum-free DMEM for 48 h. Then the hPDMC culture medium was collected and condensed (by 25 times) using Centriplus YM-10 (Millipore, Bedford, MA). hUVECs (1×10^6 cells/mL) were seeded into 24-well culture dishes. After 48 h, condensed hPDMC conditioned medium or recombinant hVEGF was added to the culture medium of hUVECs. Uptake of ^3H was measured after 48 h. Triplicate experiments were performed. Data are expressed as means ± SE.

Mouse hindlimb ischemia model and cell transplantation. NOD/Shi-scid mice were supplied by Nihon Clare (Tokyo, Japan). All animal experiments were performed in the animal research center, observing the guidelines for animal experiments of the Institute of Medical Science, University of Tokyo.

Mice were anesthetized with xylazine 15 mg/kg (i.m.) and ketamine 90 mg/kg (i.p.). Ischemia was induced by ligating the femoral artery and vein of one hind limb beneath the inguinal ligament [23]. Seven days after surgery, ischemia of the limb was evaluated. Mice were anesthetized, and the blood flow in each limb was measured on the sole of the foot using a laser doppler imaging system (Moor Instruments, Sussex UK). Mice with incomplete ischemia (blood flow greater than 30% of the normal limb) were discarded. Mice with decreased blood flow (less than 750 arbitrary flow units) in the normal hind limb were also discarded. Selected mice were systemically irradiated with 1 Gy of gamma ray and received anti-asialo GM1 antiserum to reduce the rejection of transplanted cells [24]. After this procedure 1×10^6 hPDMCs suspended in 0.1 mL medium were injected into ischemic limbs. Injection was divided into 5 or 6 sites on the ischemic limb.

Seven days after and 14 days after hPDMC transplantation we measured the blood flow of the ischemic and non-ischemic limbs. After blood flow was scanned, stored images were subjected to computer-assisted quantification, and the ratios of average flows of ischemic and non-ischemic limbs were calculated.

Immunohistochemistry. To study the new vessel formation after hPDMC transplantation, ischemic adductor muscles were dissected after measurement of blood flow. They were fixed with formalin and embedded in paraffin. Paraffin-embedded sections were then stained with anti-factor VIII antibody [25], followed by secondary antibody reaction and visualized using Dako EnVision plus and Dako DAB substrate kit (DakoCytomation, Copenhagen, Denmark). The capillaries in randomly chosen fields were counted. The number of capillaries was divided by the number of muscle fibers, and this ratio was compared.

Real-time RT-PCR. Primers were designed to amplify the 3′ untranslated region of hVEGF cDNA (GenBank:AF022375) so that only VEGF-A mRNA would be detected by RT-PCR. The sequences of the primers were hVL1: GGTCCCTCTTGGAATTGGAT and hVR1: TGTATGTGGGTGGGTGTGTC. The expected PCR fragment length was 115 base pairs. Total RNA was extracted from the adductor muscles of NOD/Shi-scid mice with Tri-zol (Invitrogen, Carlsbad, CA). Five micrograms of total RNA was reverse transcribed with ThermoScript (Invitrogen, Carlsbad, CA) for 1 h at 50 °C. The product was then subjected to the following PCR conditions (QuantiTect SYBR Green PCR kit, Qiagen, Hilden, Germany): 95 °C for 15 min, 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s (34 cycles), followed by 72 °C for 7 min (iCycler, Bio-RAD, Hercules, CA).

Statistical analysis. The non-parametric Mann–Whitney *U* test was used to evaluate the statistical significance of differences between two groups. *P* values less than 0.05 were considered statistically significant.

Results

hVEGF production by hPDMCs

hVEGF production by various types of cells is presented in Table 1. Data were normalized by dividing the hVEGF concentration by the cell number. HeLa cells were used as a positive control [26]. hPDMCs (PL26, PL54C, and PL78C) and HeLa cells produced hVEGF, and hVEGF concentrations in the media of hPDMCs were similar to those of HeLa cells. In contrast, the hVEGF concentration was very low in the culture media of PBMCs and it was undetectable in the culture media of hUVECs. Considerable amounts of intracellular hVEGF were detected in hPDMCs and HeLa cells. However, only a very small amount of intracellular hVEGF was detected in PBMCs, and intracellular hVEGF was not detected in hUVECs. These results were confirmed in 3 other experiments. Other angiogenic factors (HGF, EGF, PlGF, and TNF-α) were not detected in the supernatant of hPDMCs (data not shown).

Table 1
ELISA of hVEGF

Cell	hVEGF/per 10^4 cells (pg/mL)
hVEGF concentration in the medium	
HeLa	2.50 ± 0.24 (mean ± SE)
PL26	3.58 ± 0.26
PL54C	1.52 ± 0.20
PL78C	0.47 ± 0.05
PBMCs-1	0.0194 ± 0.0022
PBMCs-2	0.0008 ± 0.0004
hUVECs	n.d.[a]
EGM (medium alone)	n.d.[a]
DMEM + 10%FBS	n.d.[a]
Intracellular hVEGF content	
HeLa	1.00 ± 0.13
PL26	0.36 ± 0.06
PL54C	0.33 ± 0.06
PL78C	0.22 ± 0.02
PBMCs-1	0.0022 ± 0.0013
PBMCs-2	0.0048 ± 0.0005
hUVECs	n.d.[a]

[a] Not detected.

Biological activity of hVEGF produced by hPDMCs

To test the biological activity of hVEGF produced by hPDMCs, the proliferation of hUVECs was examined. Application of recombinant hVEGF increased the thymidine incorporation in hUVECs in a dose-dependent manner. Twenty-five times condensed conditioned medium from hPDMCs also increased thymidine uptake, whereas condensed DMEM did not increase thymidine uptake (Fig. 1). The hVEGF concentration in the condensed conditioned medium was 5400 pg/mL (measured by ELISA). The conditioned medium (50 μL) was added

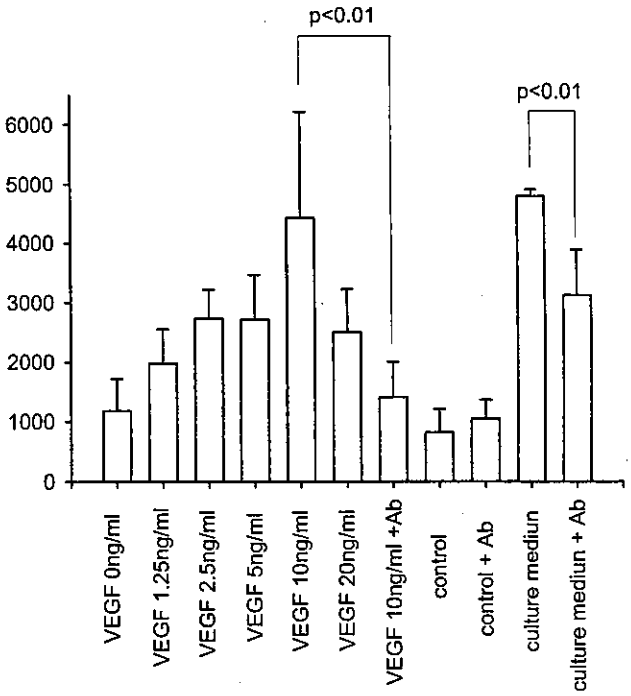

Fig. 1. Thymidine uptake of hUVECs stimulated by recombinant hVEGF. The ordinate indicates the counts of thymidine uptake by hUVECs. Data are expressed as means ± SE ($n = 4$). Left six bars show the counts of thymidine uptake, at different concentrations of recombinant hVEGF. "Culture medium" indicates thymidine uptake of hUVECs stimulated by the condensed hPDMC culture medium. "Control" indicates the condensed DMEM. "Ab" indicates that anti-human VEGF blocking antibody was added to the conditioned medium.

to the hUVEC culture medium (450 μL), so that the final hVEGF concentration was approximately 0.54 ng/mL. Judging from Fig. 1, the thymidine incorporation induced by the hPDMC conditioned medium was the same as the level induced by 10 ng/mL recombinant hVEGF. A similar result was obtained in 2 other experiments. Thus, the biological activity of hPDMC conditioned medium appeared to be stronger than that predicted by the hVEGF concentration determined by ELISA. When 0.4 μg/mL anti-VEGF blocking antibody was added with 10 ng/mL recombinant VEGF, it completely blocked the bioactivity. Whereas anti-VEGF blocking antibody only partially blocked the bioactivity of condensed hPDMC conditioned medium.

Improvement of hindlimb ischemia by hPDMC transplantation in NOD/Shi-scid mice

We transplanted hPDMCs into ischemic hindlimbs of NOD/Shi-scid mice to examine whether hPDMCs improved ischemia in vivo. Representative results of blood flow changes in ischemic limbs are shown in Fig. 2. The upper panels show the blood flow of the limbs in the control (untransplanted) mouse. The lower panels show the blood flow in a mouse that received hPDMCs. The blood flow of ischemic limbs improved after hPDMC transplantation, whereas such improvement was hardly observed in the control mouse. Fig. 3 summarizes the blood flow changes of ischemic limbs after hPDMC transplantation. Before transplantation the blood flow of ischemic limbs was 0.211 ± 0.008 ($n = 15$) (mean ± SE) of the blood flow of non-ischemic limbs in the hPDMC-transplanted group. It was 0.228 ± 0.012 ($n = 13$) in the untransplanted group, 0.220 ± 0.013 ($n = 14$) in the medium-injected group, and 0.207 ± 0.009 ($n = 15$) in the PBMC-transplanted group. Seven days after treatment, the blood flow of ischemic limbs increased to 0.511 ± 0.056 of the blood flow of the non-ischemic limbs in the hPDMC-transplanted group. It was 0.346 ± 0.028 in the untransplanted group, 0.313 ± 0.017 in the medium-injected group, and 0.400 ± 0.045 in the PBMC-transplanted group. Fourteen days after treatment, the blood flow of ischemic limbs was 0.495 ± 0.048 of the blood flow of the non-ischemic limbs in the hPDMC-transplanted group. It was 0.367 ± 0.050 in the untransplanted group, 0.376 ± 0.048 in the medium-injected group, and 0.450 ± 0.054 in the PBMC-transplanted group. The ischemia in the hPDMC-transplanted group was significantly improved at day 7 and day 14 ($p < 0.05$ as compared to untransplanted group and medium-injected group). These results indicate that hPDMC transplantation is an effective treatment in the hindlimb

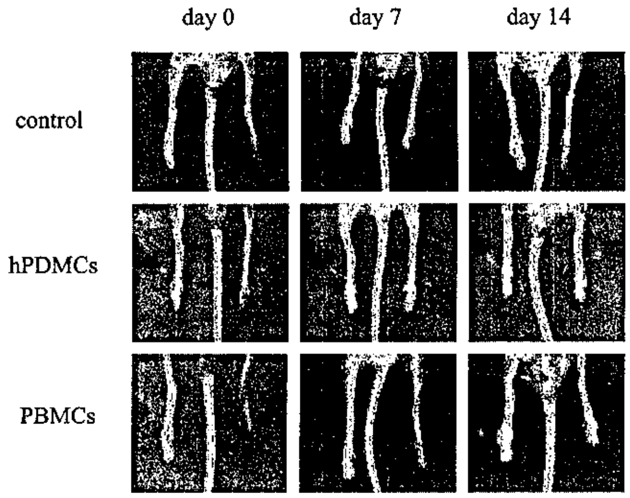

Fig. 2. Improvement of hindlimb ischemia by hPDMC transplantation in NOD/Shi-scid mice. The upper panels (control) show the blood flow changes of limbs in a control NOD/Shi-scid mouse. The middle panels (hPDMCs) show the blood flow changes in a mouse that received transplanted hPDMCs in the ischemic limb. The lower panels (PBMCs) show the blood flow changes in a mouse that received transplanted PBMCs in the ischemic limb. Data at days 0, 7, and 14 are shown.

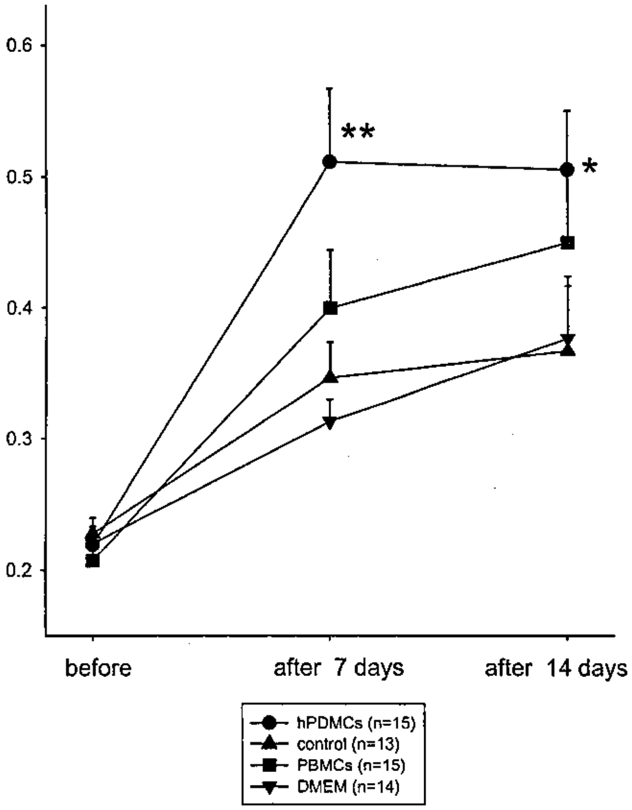

Fig. 3. The blood flows of ischemic limbs before and after hPDMC transplantation are shown (mean ± SE). The ordinate indicates the blood flow of the ischemic limb as compared to that of the unaffected limb. "hPDMCs" (circles) indicates data from mice transplanted with hPDMCs, "control" (triangles) indicates data from mice without any injection, "DMEM" (inverted triangles) indicates data from mice injected only with DMEM, and "PBMCs" (squares) indicates data from mice injected with PBMCs. Data are expressed as means ± SE. $*p < 0.05$ and $**p < 0.05$ as compared to "control" and "DMEM" group.

ischemia model of NOD/Shi-scid mice. The ischemia in the PBMC-transplanted group appeared to be improved but the increase of the blood flow was not statistically significant.

Immunohistochemical analysis of new vessel formation

Upper panels of Fig. 4 show representative photographs of ischemic muscles stained with hematoxylin and eosin (HE). Lower panels show the immunohistochemical staining using anti-factor VIII antibody. Endothelial cells and capillaries are clearly observed with this staining (arrows). The hPDMC-transplanted group (left) showed a larger number of endothelial cells and capillary formation than the control group and the DMEM group (middle and right). The numbers of capillaries and muscle fibers were counted in 10 randomly selected sections of muscles from three mice in each group (under 200× magnification) and the mean was calculated. Capillary/muscle fiber ratios for the hPDMC-transplanted mice, medium-injected mice, and uninjected mice were 0.136 ± 0.008 (mean ± SE) ($n = 3$), 0.052 ± 0.001, and 0.053 ± 0.003, respectively (Fig. 5). The capillary/muscle fiber ratio of the hPDMC-transplanted mice was significantly greater than those of the medium-injected mice ($p < 0.01$) and the uninjected mice ($p < 0.01$), indicating that the angiogenesis was promoted by hPDMC transplantation. These results correspond to the results of the blood flow analysis described above.

Detection of hVEGF by real-time RT-PCR

To determine the length of time that transplanted hPDMCs produce hVEGF in NOD/Shi-scid mice, we used real-time RT-PCR to detect hVEGF mRNA expression in muscle (Fig. 6). hVEGF mRNA expression was normalized by setting the mean mRNA expression 3 h after hPDMC transplantation to be 1.0 (1.0 ± 0.40) (mean ± SE, $n = 3$). After 2 days, hVEGF mRNA expression was about 0.11 ± 0.017 of the expression at day 0. hVEGF mRNA expression was still observed after 7 days (0.013 ± 0.00086 of the expression at day 0). Only very weak signal was detected in the control muscle without hPDMC transplantation ($3.56 \times 10^{-4} \pm 5.4 \times 10^{-5}$). These results indicate that transplanted hPDMCs were alive and kept producing hVEGF at least for 7 days at the site of injection.

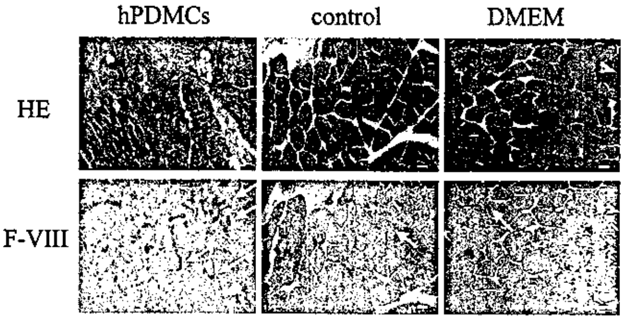

Fig. 4. Immunohistochemical analysis of new vessel formation. Representative photographs of limb muscle stained with HE (upper panels) and with anti-factor VIII antibody (F-VIII) (lower panels) are shown. Arrows indicate the capillary or endothelium. "hPDMCs" indicates a mouse transplanted with hPDMCs, "control" indicates a mouse without any injection, and "DMEM" indicates a mouse injected only with DMEM. Bar = 50 μm.

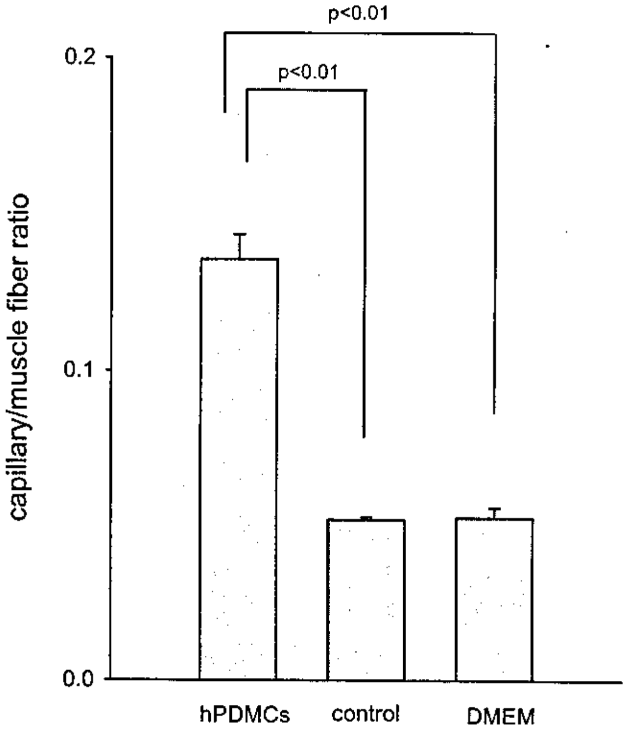

Fig. 5. Capillary/muscle fiber ratio of ischemic muscles. Ten samples in each animal were randomly selected, and the capillaries and muscle fibers were counted. The ordinate indicates the capillary/muscle fiber ratio. "hPDMCs" indicates a mouse transplanted with hPDMCs, "control" indicates a mouse without any injection, and "DMEM" indicates a mouse injected only with DMEM. Data are expressed as means ± SE.

Discussion

The results of the present study revealed that hPDMCs produced hVEGF. The amount of hVEGF secreted from hPDMCs was similar to the amount produced by HeLa cells, which are malignant cells that form tumor vessels. hVEGF production was not detected in hUVECs. hVEGF production in PBMCs was very small, consistent with a previous report by Salven et al. [27]. We detected VEGF-A mRNA in this study. However, further study is necessary to confirm the expression pattern of other VEGF isoforms. A hUVEC proliferation assay confirmed that the hVEGF secreted by hPDMCs was biologically active. The fact that the biological activity of hPDMC conditioned medium

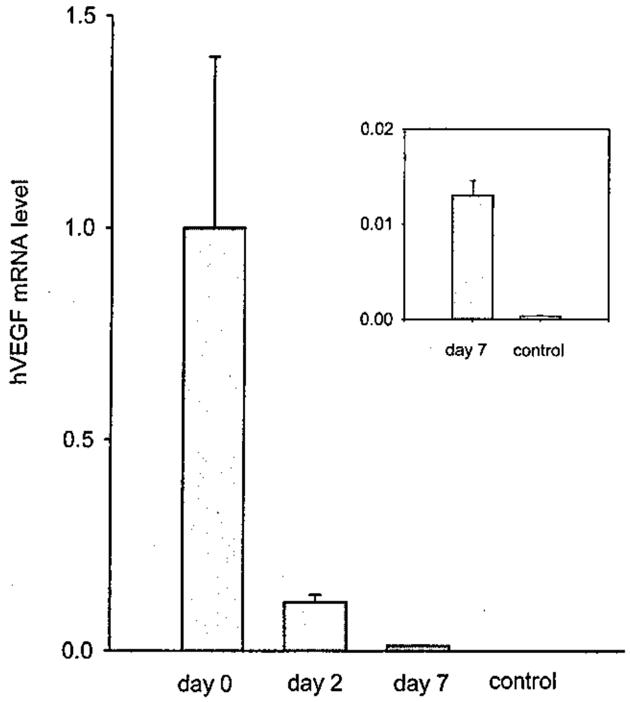

Fig. 6. Detection of hVEGF in mouse muscle by real-time RT-PCR. The ordinate indicates the hVEGF mRNA expression level in the limb muscle of NOD/Shi-scid mouse. The mean hVEGF expression level 3 h after hPDMCs transplantation was determined as 1.0, and the other expression levels were normalized. Data are expressed as means ± SE ($n = 3$). The inset in the figure shows the data at day 7 and those of the control in an amplified vertical scale.

was stronger than that predicted by ELISA and that anti-VEGF blocking antibody only partially blocked the bioactivity of hPDMC conditioned medium suggests other factor(s) than hVEGF are also involved in the stimulation of hUVEC proliferation. Further study is needed to identify the factor(s). In an animal model of hindlimb ischemia, transplantation of hPDMCs significantly improved the blood flow of affected limbs. Histological examination demonstrated that the new blood vessel formation in treated mice was more abundant than that in control mice. Real-time RT-PCR showed that transplanted hPDMCs produced hVEGF for at least 7 days in NOD/Shi-scid mice. These results suggest that cell therapy using hPDMCs may be a useful treatment for ischemic diseases. We postulated that the mechanism of angiogenesis enhanced by hPDMC-transplantation was the local production of pro-angiogenic growth factors or cytokines. However, there is also a possibility that transplanted hPDMCs differentiated into endothelial cells. Further studies are necessary to elucidate it.

Recently much attention has been paid to the benefits of using placenta as a source of cells for tissue engineering [28]. There is no risk to donors, and the placenta is a large tissue from which many cells can be harvested. The risk of abnormal transformation or proliferation can be minimized if placentae from full-term deliveries are used. Immature placenta or hydatidiform moles should not be used due to the risk of abnormal transformation or proliferation. hPDMCs used in this study were positive to CD105 and CD73 antibodies, and negative to CD21 and CD23 antibodies. Therefore, hPDMCs are different from fibroblasts, which are CD105 and CD73 negative, and they are also different from human decidual stroma cells described by Oliver et al. [29], which are positive to CD21 and CD23. hPDMCs are also different from CD86-positive Hofbauer cells, which are derived from macrophage and produce VEGF [30], because CD86 and CD14 were negative in hPDMCs. Clark et al. [31] found hVEGF mRNA expression in placenta using in situ hybridization. They also reported that PlGF is detected in villous and extravillous trophoblasts, whereas hVEGF is solely detected in maternal glands. This finding coincides with that the hPDMCs used in this study are of maternal origin. Further characterization of hPDMCs remains to be seen.

Pro-angiogenic therapies previously reported are direct injection of angiogenic factors, expression of angiogenic genes by plasmid or virus vectors, and cell therapy [2–5,11,13–18]. Administration of angiogenic factors, such as VEGF or FGF-2, has been reported to be an effective treatment for ischemia. However, there are controversial reports about its efficacy, and there are also reports about serious adverse events [7,8]. In animal models angiogenic factors are applied using slow-release polymers [9,10], but the safety of these beads has not been proven in humans. Without a slow-release device, angiogenic factors injected into skin or muscle are easily absorbed into systemic circulation and it is difficult to stably apply angiogenic factors at local lesions. Gene expression of angiogenic factors in ischemic muscles can overcome the problem of local administration. However, gene expression is not currently well regulated, which causes unfavorable adverse events [7,8]. Additionally, a death has been reported after in vivo application of virus vectors into a human [32]. The clinical efficacy of cell therapy for ischemic diseases using autologous endothelial progenitor cells or mononuclear cells has been reported [19]. Because apheresis is required, facilities in which these cell therapies can be performed are limited. There are also patient safety problems with the cell collection procedures. Thus, an ideal therapy for ischemic diseases has not yet been established.

hPDMCs used in this study are derived from normal placentae at full-term deliveries. Abundant vessels with normal architecture are formed in the placenta to exchange nutrients between mother and fetus. The present study shows that hPDMCs produce a large amount of bioactive hVEGF without any gene modification. Transplantation of hPDMCs to NOD/Shi-scid mice significantly improved the blood flow of ischemic limbs. If a human clinical study is considered, HLA information may be necessary for allogeneic hPDMC transplantation. Real-time RT-PCR revealed that hVEGF was expressed in NOD/Shi-scid mice for at least 7 days after transplantation. Although the length of time that transplanted hPDMCs would survive in humans is unknown, it is improbable that they would be acutely rejected if HLA matching is performed. Because immunosuppressants would not be used in human clinical studies, transplanted hPDMCs would eventually be rejected after local administration of hVEGF, which supports the safety of this therapy.

Iba et al. [16] have reported that transplantation of peripheral blood mononuclear cells improved blood flow in ischemic limbs. However, we did not observe significant improvement of ischemia in the PBMC-transplanted group, which may be ascribed to the difference of experimental conditions although the exact reason is unknown. VEGF in combination with angiopoietin induces angiogenesis more effectively and with fewer adverse events than VEGF alone [11]. Similarly, the combination of platelet-derived growth factor (PDGF)-BB and FGF-2 induces functional and stable vessels much more effectively than a single angiogenic factor [10]. We previously reported that adenovirus and adeno-associated virus efficiently mediate gene expression in hPDMCs [33]. By transducing the cells with a gene (other than hVEGF) which promotes angiogenesis, hPDMCs may become more potent inducers of angiogenesis. Safety is a very important issue in gene therapy. Because the transduction of hPDMCs is done ex vivo, there is little possibility of vector contaminating patients. The safety of this treatment may be enhanced by irradiating the transduced cells before transplantation. Thus, it is possible that cell therapy for ischemia using hPDMCs can be improved in the future.

Acknowledgments

We are grateful to Dr. Hirofumi Hamada (Sapporo Medical University) for advice on animal experiments. We thank Ms. Kaori Sato for technical assistance.

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TRAIL Protects Mice from Acute Graft-Versus-Host Disease and Leukemia Relapse Mediated Through the Peripheral Deletion of Pathogenic T Cells and Leukemia Cells

Running head: Regulatory role of TRAIL in immunity.

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Abstract

We report here the potential usefulness of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) for the treatment of lethal acute graft-versus-host disease (GVHD) and leukemia relapse. Dendritic cells (DCs) genetically modified to express TRAIL showed more potent cytotoxicity than soluble TRAIL against both alloreactive T cells and leukemic cells mediated through TRAIL/death receptor (DR) pathway. In addition, cell gene therapy with genetically modified DCs expressing TRAIL was more effective than in vivo gene transfer of TRAIL for the protection against acute GVHD and leukemia relapse. Thus, gene transfer of TRAIL involving DCs is useful for the treatment of acute GVHD and leukemia relapse by selective targeting of the pathogenic T cells and leukemia relapse.

Key Words: Gene Transfer·TRAIL·Dendritic Cells·T Cells·Leukemia Cells.

INTRODUCTION

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) also known as Apo2 ligand, is a type-II transmembrane protein belonging to the TNF family[1]. TRAIL can potentially interact with five different receptors. These include death receptor (DR)4 (TRAIL-R1), DR5 (TRAIL-R2), decoy receptor (DcR)1 (TRAIL-R3, TRAIL receptor without an extracellular domain [TRID]), DcR2 (TRAIL-R4, TRAIL receptor with a truncated death domain [TRUNDD]), and a soluble receptor called osteoprotegerin[2]. Receptors for TRAIL are constitutively expressed in a variety of cell types [2]. On the other hand, the constitutive expression of TRAIL was observed in liver NK cells, whereas the levels of TRAIL expression in T cells as well as NK cells can be markedly upregulated following cell activation [3-5]. In addition, TRAIL preferentially induces apoptotic cell death in a wide variety of transformed cells whereas it induces no apoptosis but inhibits activation of Ag-specific peripheral T cells via blockade of cell cycle progression in humans and animals [6, 7].

The presence of multiple receptors for TRAIL strongly suggest that TRAIL is involved in the maintenance of immunological homeostasis under steady state conditions as well as in the initiation and progression of immunopathogenesis. Previous studies have shown that TRAIL plays a crucial role in the surveillance of tumor initiation and metastasis in mice [4]. Although the role of TRAIL in the negative selection of thymocytes remains to be controversial[8, 9], TRAIL plays a crucial role in the initiation and the progression of autoimmune diseases [6, 8, 10]. However, the potential regulatory effect of TRAIL on immune responses and its therapeutic potential in immunopathogenic diseases remains unclear.

Dendritic cells (DCs) are antigen (Ag)-presenting cells (APCs), which consist of heterogeneous subsets with different lineages and maturity, and they not only initiate immunity but are also involved in the induction of tolerance in vivo[11-13]. Therefore, in addition to their original application for the therapy of cancer and infectious diseases, strategies involving immunoregulatory DCs are thought likely to be effective for the prevention and treatment of autoimmune diseases and allergic diseases, and in allogeneic organ transplantation.

Genetic modification of DCs with genes encoding immunoregulatory molecules provides a potential approach for Ag-specific regulation of T-cell-mediated immunity by selectively targeting Ag-restricted T cells. The use of these genetically modified DCs was reportedly effective for the prevention of experimental autoimmune and allergic diseases as well as allogeneic organ transplantation in animals, possibly mediated through the downregulation of the activation of Ag-specific T cells[14-17].

Allogeneic bone marrow (BM) transplantation (BMT) is an effective treatment for hematologic malignancies as well as genetic disorders[18-21]. However, acute GVHD, which is caused by alloreactive T cells in donor BM inocula, is a major cause of morbidity and mortality in patients undergoing allogeneic BMT[18-21]. Although the incidence and the severity of acute GVHD can be dramatically improved by T-cell depletion or the combination of immunosuppressive agents, the risk of leukemia relapse may be increased in turn, possibly due to the lack of antileukemia effect of allogeneic T cells infused, the so-called graft-versus-leukemia (GVL) effect[19-21]. Therefore, there is an increasing interest in the development of strategies that suppress acute GVHD but exert a GVL effect.

Here, we report that adenoviral gene transfer of TRAIL involving DCs is effective for the protection of the recipients from the lethality caused by acute GVHD and leukemia relapse mediated through the deletion of pathogenic alloreactive T cells and leukemia cells.

RESULTS

Regulatory function of human DCs genetically modified to express TRAIL

To clarify the regulatory role of TRAIL in T-cell activation, we generated adenovirus vector encoding the human TRAIL gene (hTRAIL-Ad). Introduction of hTRAIL-Ad into 293 cells resulted in specific and functional expression of TRAIL **(Fig. 1a-b)**.

To test the potential use of TRAIL for selectively targeting Ag-specific T cells, we examined the conditions for the generation of human DCs genetically engineered to express TRAIL. Although stimulation of immature DCs (iDCs) with interferon (IFN)-γ or lipopolysaccharide (LPS) induced slight expression of TRAIL[22, 23], adenoviral gene transduction of TRAIL into DCs resulted in more potent expression of TRAIL. Of note, introduction of hTRAIL-Ad into iDCs followed by stimulation with LPS resulted in the generation of mature DCs (mDCs) with higher levels of dose-dependent expression of TRAIL compared with adenoviral infection of the hTRAIL gene into iDCs or mDCs **(Fig. 1c-e)**.

To determine the functional expression of hTRAIL in DCs genetically modified to express TRAIL (designated as hTRAIL-Ad/DCs), we examined the cytotoxicity of these cells against various cell types. As shown in Figure 2A, adenoviral infection had little or no effect on the expression of MHC and costimulatory molecules **(Fig. 2a)**. hTRAIL-Ad/DCs showed more potent killing activity against Jurkat cells known to be hTRAIL-sensitive target cells than mDCs, control Ad-transduced mDCs (control Ad/mDCs) and soluble TRAIL. In addition, their cytotoxicity was blocked by anti-hTRAIL mAb **(Fig. 2b)**, indicating that hTRAIL is functionally expressed on genetically modified DCs. In contrast, concanavalin A (Con A)-blasts **(Fig. 2b)** and DCs (data not shown) were relatively resistant to TRAIL-mediated cytotoxicity.

To determine the mechanism responsible for the difference in the sensitivity against TRAIL-mediated cytotoxicity, we examined the expression levels of the receptors for TRAIL in various cell types **(Fig. 2c)**. iDCs constitutively expressed DR4, DR5, RcR1 and DcR2 at similar levels, and the expression of these receptors did not changed following maturation. Unlike Jurkat cells, which predominantly expressed DR5, little or no expression of these receptors was observed on unstimulated CD4$^+$T cells and Con A-blasts. Interestingly, cognate interaction of CD4$^+$T cells with allogeneic DCs induced specific upregulation of DR5 **(Fig. 2c)** whereas the stimulation with mAbs to CD3 and CD28 upregulated its expression to a lesser degree (data not shown). These results indicate that the sensitivity to TRAIL is associated with the expression levels of the receptors for TRAIL.

We also examined the T-cell regulatory function of hTRAIL-Ad/DCs. Soluble TRAIL showed a minimal inhibition of activation of CD4$^+$T cells when the cells were cultured with mAbs to CD3 plus CD28 or allogeneic mDCs **(Fig. 2d)**. In contrast, hTRAIL-Ad/DCs, but not mDCs or control-Ad/mDCs, displayed a potent suppressive effect on activation of alloreactive CD4$^+$T cells **(Fig. 2d-e)** although all of these effector cell types showed similar expression of MHC and costimulatory molecules **(Fig. 2a)**. In addition, this suppression was abrogated by anti-hTRAIL monoclonal antibody (mAb) and anti-hDR5 mAb **(Fig. 2d)**. These results indicate that DCs genetically modified to express TRAIL inhibit the activation of CD4$^+$T cells through the interaction of TRAIL-DR5.

Previous studies have shown that soluble TRAIL induces no apoptosis but inhibits activation of T cells through blockeage of cell cycle progression in humans and animals [6, 7]. To clarify the mechanism underlying T-cell regulatory function of DCs genetically modified to express TRAIL, we characterized the CD4$^+$T cells primed with hTRAIL-Ad/DCs. The proportion of dividing cells was significantly reduced in allogeneic CD4$^+$T cells primed with hTRAIL-Ad/DCs when compared with those primed with mDCs and control-Ad/mDCs **(Fig. 2F)**. On the other hand, hTRAIL-Ad/DCs induced more potent apoptosis in allogeneic CD4$^+$T cells than mDCs and control-Ad/mDCs **(Fig. 2G)**. Similarly, numerous apoptotic cells were detected in allogeneic CD4$^+$T cells primed with hTRAIL-Ad/DCs, whereas the productive cells (S phase and G2+M phase) were increased in allogeneic CD4$^+$T cells primed with mDCs and control-Ad/mDCs compared with unprimed CD4$^+$T cells **(Fig. 2h)**. These results indicate that DCs genetically modified to express TRAIL induce apoptosis rather than cell cycle arrest.

Expression of TRAIL in adenoviral gene transferred mice

To determine the regulatory role of TRAIL on immunnopathogenesis, we generated adenovirus vector encoding the murine TRAIL gene (mTRAIL-Ad). Infection of 293 cells with mTRAIL-Ad induced cytotoxicity against L929 cells expressing DR5 (**Fig. 3a-b**), indicating that the adenoviral gene transfer of mTRAIL could induce its functional expression of mTRAIL in target cells.

We next examined the effect of adenoviral infection on the numbers of spleen leukocytes and their constitution (**Fig. 3d-e**). A injection of control-Ad into mice increased the number of leukocytes and the rate of T cells compared to normal mice, indicating that these changes involve the immune response against adenoviral infection. On the other hand, the mice injected with mTRAIL-Ad showed a reduced number of leukocytes and constituency of T cells compared to mice injected with control-Ad. In addition, the introduced TRAIL was specifically detected in F4/80$^+$cells (tissue macrophages) and CD11c$^+$cells (resident DCs). These results indicate that adenoviral transduction of the TRAIL gene suppresses the immune response against adenovirus, and transduced TRAIL was mainly expressed in both macrophages and DCs.

Gene transfer of mTRAIL prevents murine acute GVHD

Previous studies have shown the dominant role of the Fas/Fas ligand (FasL) pathway in acute GVHD, whereas TRAIL has a minimal contribution to this pathogenesis[19, 20]. We therefore tested the effect of in vivo gene therapy with adenovirus vector encoding TRAIL on acute GVHD in murine allogeneic BMT (**Fig. 4a**). All recipients of allogeneic BMT died on day 8 following transplantation, and a single injection of control-Ad into mice before allogeneic BMT had no effect on the incidence and the severity of acute GVHD. In contrast, a single injection of mTRAIL-Ad protected mice from acute GVHD-induced lethality in a dose-dependent manner. However, a single injection of mTRAIL-Ad after allogeneic transplantation failed to cause a therapeutic