Preferential expansion of human umbilical cord blood-derived CD34-positive cells on major histocompatibility complex-matched amnion-derived mesenchymal stem cells

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ABSTRACT

Background

We previously found in a murine hematopoietic system that hematopoietic stem cells show high differentiation and proliferation capacity on bone marrow-derived mesenchymal stem cells/stromal cells (microenvironment) with "self" major histocompatibility complex (MHC).

Design and Methods

We examined whether amnion-derived adherent cells have the characteristics of mesenchymal stem cells, and whether these adherent cells can support the proliferation of umbilical cord blood-derived lineage-negative and CD34-positive cells (Lin^-CD34^+ cells) obtained from the same fetus to a greater extent than those derived from other fetuses.

Results

Culture-expanded amnion-derived adherent cells expressed mesenchymal stem cell markers and HLA-ABC molecules and could differentiate into osteoblasts, adipocytes and chondrocyte-like cells, indicating that the cells have the characteristics of mesenchymal stem cells. The Lin^-CD34^+ cells purified from the frozen umbilical cord blood were strongly positive for HLA-ABC, and contained a large number of hematopoietic stem cells. When the Lin^-CD34^+ cells were cultured on the autologous (MHC-matched) or MHC-mismatched amnion-derived adherent cells in short-term assays (hematopoietic stem cell-proliferation) and long-term culture-initiating cell assays, greater expansion of the Lin^-CD34^+ cells was observed in the MHC-matched combination than in MHC-mismatched combinations. The concentration of granulocyte-macrophage colony-stimulating factor in the culture supernatants of the long-term culture-initiating cell assays was significantly higher in the MHC-matched combination than in MHC-mismatched combinations.

Conclusions

It is likely that a MHC restriction exists between hematopoietic stem cells and mesenchymal stem cells/stromal cells in the human hematopoietic system and that granulocute-macropage colony-stimulating factor contributes to some extent to the preferential hematopoiesis-supporting ability of the MHC-matched amnion-derived adherent cells.

Key words: mesenchymal stem cells, CD34-positive cells, umbilical cord blood, amnion, major histocompatibility complex.

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Introduction

Mesenchymal stem cells (MSC) are defined as cells capable of differentiating into multiple mesenchymal lineage cells.[1,2] MSC have the capacity to support the proliferation and differentiation of hematopoietic stem cells (HSC).[2-4] MSC have been isolated from various sources in the human body, including the bone marrow, peripheral blood, adipose tissue, umbilical cord blood (UCB),[3,5] placenta[6,7] and other fetal tissues.[8-11] Although human adult bone marrow is the most common source of MSC for clinical use, the frequency of MSC in this compartment is relatively low because the contents and differentiating potential of MSC in the adult bone marrow decrease significantly with age.[1,12] Moreover, the procedures for aspirating bone marrow are invasive and painful for the patients. In contrast, UCB is an attractive source of fetal hematopoietic cells, including MSC, because of easy access and availability, but MSC in UCB have been reported to be relatively infrequent[13] or even undetectable.[14] Although the use of fetal organs has some ethical limitations, the use of amnion in the placental tissue is free from ethical complications. Therefore, if MSC are contained in the amnion and have high expansion ability and hematopoiesis-supporting capacity, the amnion would be an attractive source of fetal MSC. Recent studies have shown that the amnion is indeed a rich source of fetal MSC and useful for regenerative medicine.[15-19] Unfortunately, most placentas are discarded as medical waste at birth.

More than 12 years ago, we demonstrated that cografting donor bones (bone marrow cells were flushed out but MSC/stromal cells remain) could facilitate the engraftment of donor HSC even in chimeric resistant combinations such as [normal mice → MRL/lpr mice][20] and [DBA/2 mice → B6 mice.][21] In the recipient mice, donor-type stromal cells were detected in the bone marrow and thymi, indicating that these cells had migrated into these tissues from the grafted bones, proliferated there, and provided a suitable environment for donor HSC. Moreover, we found a significant accumulation of donor bone marrow cells in the engrafted donor bone, whereas there were only a few donor bone marrow cells in the engrafted bone having a different major histocompatibility complex (MHC) phenotype from the donor bone marrow cells.[22] Thus, we proposed the concept of a MHC restriction between HSC and bone marrow stromal cells. The MHC restriction was further confirmed by cobblestone colony-forming assays; the formation of cobblestone colonies under MHC-compatible stromal cells was significantly greater than that under MHC-incompatible stromal cells.[23] These findings prompted us to examine whether the MHC restriction between HSC and MSC/stromal cells exists in the human hematopoietic system as well as in the murine hematopoietic system.

Many studies have shown that pluripotent HSC, having greater expansion and differentiation capacities, can be obtained from the UCB. It is well known that the amnion is of fetal origin, and it has recently been shown that amniotic tissues are a rich source of MSC.[15-19] In the present study, we, therefore, first attempted to obtain an HSC-enriched population (lineage-negative and CD34-positive cells: Lin^-CD34^+ cells) from the UCB and characterize the proliferation and differentiation capacities of the Lin^-CD34^+ cells. In addition, we attempted to obtain adherent cells from amnion (Am-Ad cells) and examined them to see whether they have the ability to support hematopoiesis, and whether they have the characteristics of MSC. Furthermore, we investigated whether the Lin^-CD34^+ cells show greater proliferation and differentiation on Am-Ad cells obtained from the same fetus than on those from other MHC-mismatched fetuses.

Design and Methods

Umbilical cord blood and amnion samples

After informed consent had been obtained under approval of the ethics committee of Kansai Medical University, UCB and amnion samples were obtained from patients who underwent selective Cesarean section in the third trimester of normal pregnancies.

Characterization of Lin^-CD34^+ cells derived from human umbilical cord blood

Collection of umbilical cord blood samples and processing

UCB was collected into bags containing citrate-phosphate-dextrose (Terumo, Japan) and processed within 24 h. Low-density mononuclear cells (MNC) were isolated by Ficoll-Paque density gradient centrifugation (1.077g/L, Amersham Biosciences). The low-density MNC were cryopreserved in Iscove's modified Dulbecco's medium (IMDM) containing 10% dimethyl sulfoxide and 20% fetal bovine serum (FBS) until use.

Purification of Lin^-CD34^+ cells from umbilical cord blood

There was a low percentage of viable cells (approximately 40-50%) in the frozen cells, and the dead cells were removed from the cryopreserved low-density MNC using Ficoll-Paque density gradient centrifugation. Lineage-positive cells were then depleted using a MACS lineage cell depletion kit (Miltenyi Biotec), and lineage-negative (Lin^-) cells were obtained. The Lin^- cells were double-stained with phycoerythrin-conjugated anti-human CD34 monoclonal antibody, and fluorescein isothiocyanate-conjugated anti-human CD45 monoclonal antibody, and then $CD34^+/45^{low/+}$ cells were sorted using a FACS EPICS ALTRA (Beckman Coulter). Approximately 40-50% of the Lin^- cell population was collected as $Lin^-/CD34^+/45^{low/+}$ cells (hereafter described as Lin^-CD34^+ cells). The Lin^-CD34^+ cells were considered to be HSC. The surface antigen expression and morphology of these cells were assessed by flow cytometry and May-Giemsa staining, respectively.

Clonal cell culture assay

The low density MNC, Lin^- cells, and Lin^-CD34^+ cells were plated in a 12-well plate containing 1 mL of semi-solid culture medium containing optimal doses of human cytokines (MethoCult GF+H4435, StemCell Technologies Inc.), and colony formation was assessed 12-14 days after the plating. The types of colonies were identified as granulocyte/macrophage colonies (CFU-

GM), granulocyte colonies (CFU-G), macrophage colonies (CFU-M), erythroid burst-forming units (BFU-E), and erythrocyte-containing mixed colonies (CFU-Mix) according to their typical morphological features.

Characterization of amnion-derived adherent cells

Collection of amnion samples and culture

Amnion samples were obtained from the same donors as the UCB. The amnion layer was peeled mechanically from the chorion of the placenta, and processed within 4 h. After washing, the amnion layer was minced into small pieces of less than 1 mm^3. Enzymatic digestion with 0.05% trypsin (40 min), 1 mg/mL collagenase type III (Worthington) (60 min) and 60 μg/mL deoxyribonuclease type I (Wako, Japan) (60 min) was then carried out by shaking the pieces in a water bath at 37°C. Single-cell suspensions were made from the digested amniotic tissue using a cell strainer (70 μm, BD Falcon). The amniotic MNC cells were obtained using Ficoll-Paque density gradient centrifugation, and subsequently cultured in M199 medium supplemented with 20% FBS, and 10 ng/mL recombinant human basic fibroblast growth factor (Peprotech).

The amniotic MNC were allowed to adhere for 2 days and non-adherent cells were removed as the medium was changed. The changes of the medium were carried out once or twice weekly thereafter. When the adherent cells reached 100% confluence, after 10-14 days, the cells were subcultured. The number of adherent cells was counted in each passage for the assessment of growth characteristics. First passage Am-Ad cells were used for the present study.

Flow cytometric analysis

The first passage Am-Ad cells were collected from a flask using EDTA treatment and stained with monoclonal antibodies against human CD14, CD29, CD34, CD44, CD45, CD56, CD62L, CD73, CD105, CD235a, HLA-ABC and HLA-DQ. The stained cells were analyzed using flow cytometry (FACScan, Becton Dickinson).

Osteogenic and adipogenic differentiation

The Am-Ad cells were cultured in osteogenic or adipogenic medium for 3 to 4 weeks in slide flasks until morphological changes could be seen. Osteogenic induction medium and adipogenic induction/maintenance medium were used following the manufacturer's instructions (Cambrex Bio Science). Osteogenic and adipogenic differentiation was visualized by von Kossa and oil-red O staining, respectively.

For transmission electron microscopic analysis, the samples were routinely processed and observed using an H- 7000 electron microscope (HITACHI, Japan) or a JEM-1400A electron microscope (JEOL, Japan).

Chondrogenic differentiation

A micromass of the Am-Ad cells was prepared at the bottom of tubes by centrifugation. The micromass was treated with chondrogenic medium for 4 weeks, according to the manufacturer's instructions (Cambrex Bio Science). Chondrogenic differentiation was evaluated by staining paraffin sections of the micromass with safranin-O. Human lumbar disc herniation tissue was used as a positive control.

For electron microscopic analysis, the samples were routinely processed and examined by transmission electron microscopy (JEM-1400A electron microscope).

Genomic typing of samples

The genetic profiles of the Am-Ad cells were compared with those of the UCB and maternal peripheral blood cells using short tandem repeats analysis. The procedures were as follows: (i) extraction of DNA, (ii) polymerase chain reaction (PCR) using an AmpFISTR SGM Plus™ PCR amplification kit (Applied Biosystems) and (iii) electrophoresis of the PCR products.[24] The short tandem repeat analyses were entrusted to SRL Laboratory (Tokyo, Japan).

Mixed lymphocyte reaction

The UCB-derived lymphocyte-enriched population (hereafter described as UCB-lymphocytes) was obtained using Lymphoprep (ρ=1.077, AXIS-SCHIELD) from UCB MNC. The mixed lymphocyte reaction was carried out in two combinations: (i) the UCB lymphocytes (responder: 4×10^5 cells/200 μL/well) versus the irradiated (15 Gy) MHC-matched or mismatched UCB lymphocytes (stimulator: 4×10^5 cells/200 μL/well); (ii) the UCB lymphocytes (responder: 3×10^5 cells/200 μL/well) versus the irradiated (15 Gy) MHC-matched or mismatched Am-Ad cells (stimulator: 4×10^5 cells/200 μL/well). These cells were incubated in 96-well plates for 5 days (three wells/sample). Culture medium (RPMI) was supplemented with 10% FBS and 50 μM 2-ME. As a control, wells containing only responder cells were prepared. The incubated cells were pulsed with ^{3}H-thymidine (TdR) for the last 18 h of the culture period. The uptake of ^{3}H-TdR was measured using 1450 MicroBeta TRILUX (PerkinElmer).

Co-culture of Lin^-CD34^+ cells on an amnion-derived adherent cell layer

Hematopoietic stem cell proliferation assay

The Am-Ad cells were subcultured in a 96-well plate in order to prepare the adherent cell layer. The Lin^-CD34^+ cells were suspended in IMDM supplemented with 20% FBS with or without various recombinant human cytokines, including 6 ng/mL stem cell factor (SCF;Kirin Brewery), 2 ng/mL thrombopoietin (TPO; Kirin Brewery), interleukin-3 (IL-3; PeproTech), and FLT-3L (PeproTech). The cells were then seeded on the MHC-matched or MHC-mismatched Am-Ad cell layers (1000 or 2000 cells/200 μL/well). As a control, the same number of Lin^-CD34^+ cells was cultured without the Am-Ad cells. Wells containing Am-Ad cells alone were also prepared. These cells were cultured for 7 days and ^{3}H-TdR was introduced during the last 24 h of the culture period.

Long-term culture-initiating cell assay

The culture-expanded Am-Ad cells were subcultured in flasks. When the Am-Ad cells had become subconfluent 3-5 days later, the Lin^-CD34^+ cells were seeded on the MHC-matched or MHC-mismatched Am-Ad cell layer at the concentration of 5×10^4 cells/8 mL/flask. The cells were cultured in 20% FBS/IMDM with or without

human cytokines (IL-3, TPO, and FLT-3L: 2 ng/mL, SCF: 6 ng/mL). As a control, the same number of Lin⁻CD34⁺ cells was cultured without the Am-Ad cell layer. Every week, half of the culture medium in the flasks (containing non-adherent cells) was removed and fresh medium was added to the flasks. The number of non-adherent cells per flask was counted and then the cells were used for methylcellulose assays. The non-adherent cells were also stained with monoclonal antibodies against human CD11b, CD14, CD34, CD38, CD41, CD45, CD133, CD235a and c-kit (CD117). The stained cells were analyzed using flow cytometry (FACScan).

Cytokine analyses of culture supernatants obtained from the long-term culture-initiating cell assay system

The culture supernatants collected from flasks of the long-term culture-initiating cell (LTC-IC) assay were measured to ascertain the concentrations of various cytokines using enzyme-linked immunosorbent assay (ELISA) kits. The analyzed cytokines were GM-CSF, M-CSF, SCF, LIF, IL-6 (R&D Systems), and G-CSF (BioSource).

Statistical analyses

All analyses were performed using Microsoft Excel. The significance of differences was determined using Mann-Whitney's U test. Data are expressed as mean ± standard deviation (SD). A p value <0.05 was considered to be statistically significant.

Results

Purification and characterization of hematopoietic stem cells derived from human umbilical cord blood

To enrich primitive HSC, low density MNC were fur-

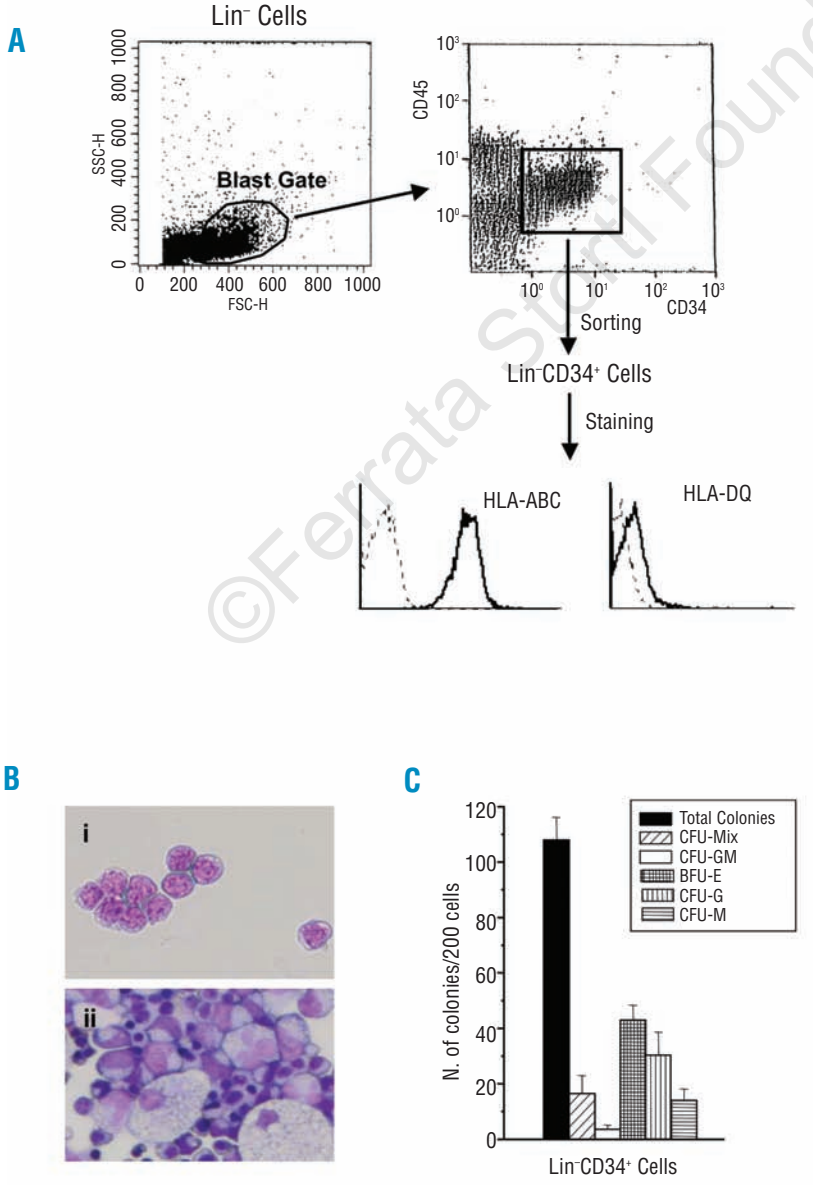

Figure 1. Morphological and functional characterization of Lin⁻CD34⁺ cells. (A) CD34 and CD45 expression pattern of Lin⁻ cells and HLA molecule expression pattern of sorted Lin⁻CD34⁺ cells. Lin⁻ cells were purified from MNC of UCB and then double-stained with phycoerythrin-conjugated anti-human CD34 monoclonal antibody and fluorescein isothiocyanate-conjugated-anti-human CD45 monoclonal antibody and then CD34⁺/45$^{low/+}$ cells (Lin⁻CD34⁺cells) were sorted. The sorted cells were positive for HLA-ABC. A representative pattern of more than ten independent experiments. (B) Morphology of Lin⁻CD34⁺ cells before and after clonal cell culture. The Lin⁻CD34⁺ cells (i) were stained with May-Giemsa reagents after cytospinning (x 400). The Lin⁻CD34⁺cells were incubated in semi-solid clonal cell culture medium (MethoCult GF+H4435) and a CFU-Mix colony (consisting of macrophages, granulocytes, and erythroblasts) was picked up 12 days later and stained with May-Giemsa reagents (ii) (x 400). Representative photographs of more than ten independent experiments. (C) Hematopoietic colony formation of Lin⁻CD34⁺ cells in clonal cell culture. The Lin⁻CD34⁺ cells were incubated in semi-solid clonal cell culture medium (MethoCult GF+H4435) and the numbers of colonies (CFU-Mix, CFU-GM, BFU-E, CFU-G and CFU-M) were counted 12-14 days later. The average number of colonies was calculated from triplicate wells in each experiment. Mean ± SD of five independent experiments.

ther purified, and an HSC-enriched fraction (Lin^- cell-fraction) was obtained. The Lin^- cells were double-stained with anti-CD34 and anti-CD45 monoclonal antibodies to avoid contamination with $CD45^-$ stromal cells, and the $CD34^+/CD45^{low/+}$ cells in the *blast* window on the SSC/FSC dot plot profile (hereafter described as Lin^-CD34^+ cells) were sorted as a highly-purified HSC population (Figure 1A). Figure 1B (i) shows the morphological assessment of the Lin^-CD34^+ cells: this population consisted of many immature hematopoietic cells showing HSC-like features (larger in size, lightly-stained nuclei with clear nucleolus, and narrow cytoplasm).

The colony-forming capacity of the Lin^-CD34^+ cells was examined using methylcellulose media containing optimal doses of cytokines (Figure 1C), and the plating efficiency was approximately 54%. Figure 1B (ii) shows the CFU-Mix colony of the Lin^-CD34^+ cells.

Analyses of adherent cells derived from human amnion

The culture-expanded Am-Ad cells proliferated rapidly with a doubling time of approximately 1.5-2 days, the growth rate reaching a plateau (a doubling time of 10-15 days) after the fourth passage. The cells retained a stable morphology for more than 20 passages (*data not shown*). Figure 2A shows the Am-Ad cells of the first passage, the cells displaying a fibroblast-like homogenous appearance. Next, the antigenic characteristics of cell surface markers on the first passage Am-Ad cells were assessed by flow cytometry (Figure 2B). The cells were positive for CD29, CD73, CD44 and HLA-ABC, but negative for CD14, CD34, CD45 and CD235a (*data not shown*) and HLA-DQ. The cells were stained weakly by monoclonal antibodies against CD56 and CD62L. These phenotypes were similar to those of MSC derived from adult bone marrow and UCB and other fetal tissues.[5,7,8]

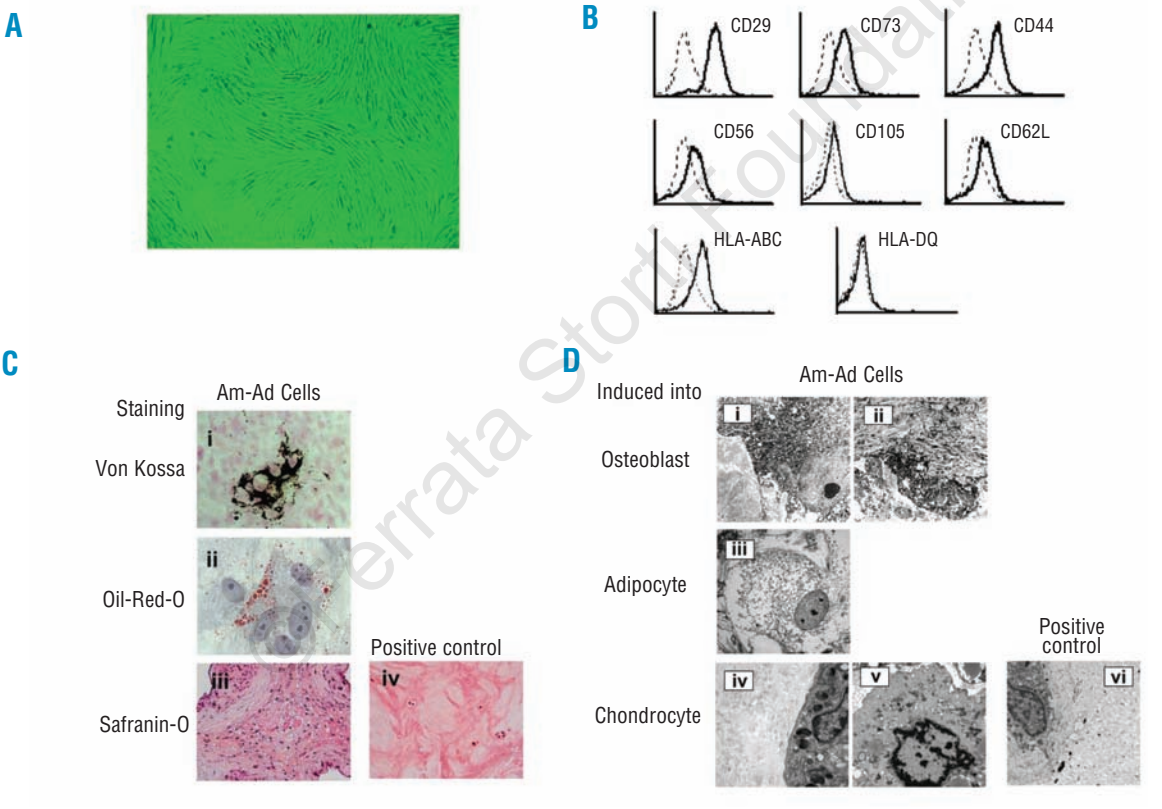

Figure 2. Morphological and functional characterization of Am-Ad cells. (A) Morphology of the Am-Ad cells. MNC derived from amniotic tissues were cultured in the medium supplemented with 20% FBS and 10 ng/mL recombinant human basic fibroblast growth factor. Non-adherent cells were removed by changes of the medium and adherent layers were formed. When the Am-Ad cells reached 100% confluence, 10-14 days later, the cells were subcultured. The Am-Ad cells showed a homogenous fibroblast-like morphology (slender cells with narrow, long cytoplasm) (x40). A representative photograph of more than ten independent experiments. (B) Flow cytometric analyses of Am-Ad cells. The Am-Ad cells were stained with a panel of monoclonal antibodies against surface antigens (solid lines). As controls, the cells were stained with isotype-matched control antibodies (dotted lines). The stained cells were then analyzed using a FACScan. Typical expression patterns of five independent experiments. (C) Capacity of Am-Ad cells to differentiate into osteoblasts, adipocytes and chondrocyte-like cells (histological analyses). The Am-Ad cells were incubated with the appropriate inductive medium for 3-4 weeks to induce differentiation into osteoblasts, adipocytes or chondrocytes. Their differentiation was confirmed by von Kossa (i), oil-red O (ii) and safranin-O (iii) staining. The untreated Am-Ad cells did not stain (*data not shown*). As a positive control of chondrocytes, a section of human lumbar disc herniation tissue was stained with safranin-O (iv). (i): x 200, (ii): x 1000, (iii and iv): x 40. Representative data of three independent experiments. (D) The capacity of Am-Ad cells to differentiate into osteoblasts, adipocytes and chondrocyte-like cells (ultrastructural analyses). The Am-Ad cells were incubated with the appropriate inductive medium for 3-4 weeks to induce differentiation into osteoblasts, adipocytes or chondrocytes and their differentiation was assessed by electron microscopic analyses. As a positive control of chondrocytes, a section of human lumbar disc herniation tissue is shown (vi). (i, ii and iii): x 2,000, (iv): x 20,000, (v): x 15,000 and (vi): x 12,000. Representative data of three independent experiments.

We next examined the osteogenic, adipogenic, and chondrogenic differentiation potentials of the first passage Am-Ad cells. When the cells were induced to differentiate into osteoblasts, mineralized matrix was detected by von Kossa staining (Figure 2C i). Electron microscopic analyses (Figure 2D i and ii) confirmed their differentiation into osteoblasts, because hydroxyapatite-like substances and ossification could be seen. In the induction into adipocytes, the formation of lipid vacuoles in the cytoplasm was visualized by oil-red O staining (Figure 2C ii), and electron microscopic analysis also confirmed the differentiation (Figure 2D iii). The accumulation of sulfated proteoglycans was found by safranin-O staining in pelleted micromass cultured under chondrogenic conditions (Figure 2C iii), similar to the positive control (human lumbar disc herniation tissue, Figure 2C iv). In electron microscopic analyses, cartilage matrix composed of fine proteoglycan granules was detected (Figure 2D iv), although most cells did not show the characteristics of chondrocytes (Figure 2D v); the structure and organelles were different from the positive control (Figure 2D vii). These data indicate that the Am-Ad cells have the ability to differentiate into mesenchymal lineages.

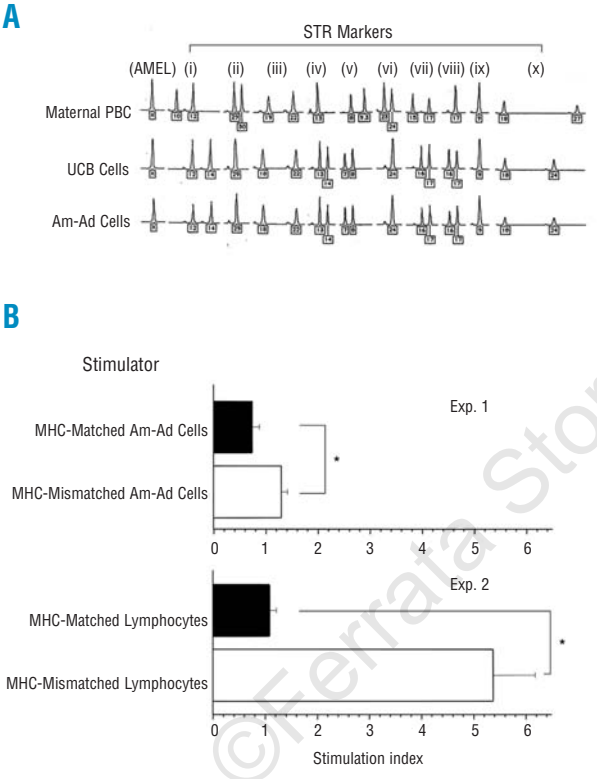

Figure 3. Analyses of the origin of Am-Ad cells. (A) Genetic typing of maternal peripheral blood cells (PBC), UCB and Am-Ad cells using multiplex PCR amplification of short tandem repeat (STR) markers. DNA was extracted from the maternal PBC, UCB cell and Am-Ad cells and ten kinds of STR were amplified from the DNA using an AmpFISTR profiler PCR amplification kit. The figure shows an illustration of electrophoresis patterns of the PCR products. Each peak indicates alleles detected by each STR marker. The value under each peak shows the repeat number of STR. The ten STR markers are as follows: (i) D8S1179, (ii) D21S11, (iii) D18S51, (iv) D19S433, (v) TH01, (vi) FGA, (vii) D3S1358, (viii) vWA, (ix) D16S539, (x) D2S1338. Analyses of the amelogenin (AMEL) locus showed that all three samples contained X chromosomes. (B) No proliferative response of UCB-derived lymphocytes against MHC-matched Am-Ad cells. Lymphocytes were enriched from UCB using Lymphoprep™ (responder) and incubated with 15 Gy-irradiated MHC-matched (obtained from the same fetus) or mismatched (from another fetus) Am-Ad cells (stimulator). The UCB lymphocytes (responder) were also incubated with 15 Gy-irradiated MHC-matched or mismatched UCB lymphocytes (stimulator) (three wells/sample). Five days later, ^{3}H-TdR uptake was measured. Mean ± SD of eight independent experiments. Stimulation index = ^{3}H-TdR uptake on sample well (responder + stimulator)/^{3}H-TdR uptake on control well (responder alone). *: $p<0.01$.

Evidence for the fetal origin of amnion-derived adherent cells

To confirm that the Am-Ad cells used in the present experiments were really derived from the fetus, short tandem repeat analyses were carried out. Figure 3A shows alleles of ten short tandem repeat markers in three samples: peripheral blood cells from the mother, UCB cells and first passage Am-Ad cells. The alleles of the Am-Ad cells are identical to those of the UCB cells in all the short tandem repeat markers, but mismatched in nine out of ten markers with those of peripheral blood cells from the mother. This result clearly indicates that the Am-Ad cells were of fetal origin. To further confirm the fetal origin of the Am-Ad cells, we used a mixed lymphocyte reaction assay; the UCB-derived lymphocyte-enriched population (UCB lymphocytes) was incubated with MHC-matched or MHC-mismatched Am-Ad cells, and the proliferation of the UCB lymphocytes was measured. As shown in Figure 3B Exp. 1, the UCB lymphocytes showed a proliferative response against the MHC-mismatched Am-Ad cells, but no response against the MHC-matched Am-Ad cells. This finding also indicates that the Am-Ad cells were of fetal origin. When the UCB lymphocytes were cultured with the MHC-mismatched UCB lymphocytes, significantly greater proliferation was observed than when they were cultured with the MHC-matched UCB lymphocytes (Figure 3B Exp. 2).

The response of the UCB lymphocytes against the MHC-mismatched Am-Ad cells (Exp. 1) was markedly lower than that against the MHC-mismatched UCB lymphocytes (Exp. 2). It is well known that MSC exert immunosuppressive effects on lymphocyte proliferation.[3,25,26] Accordingly, the lower response of the UCB lymphocytes against the MHC-mismatched Am-Ad cells might reflect this phenomenon.

Short-term co-culture of Lin^{-}CD34^{+} cells on the amnion-derived adherent cell layer (hematopoietic stem cell-proliferation assay)

We next investigated whether the Am-Ad cells had the capacity to support proliferation and differentiation of Lin^{-}CD34^{+} cells, and whether the Lin^{-}CD34^{+} cells expanded to a greater extent on MHC-matched Am-Ad cells obtained from the same fetus than on MHC-mismatched Am-Ad cells; namely, whether MHC restriction exists between Lin^{-}CD34^{+} cells and Am-Ad cells.

The expansion of the Lin^{-}CD34^{+} cells was examined in a stroma-based short-term co-culture system in which the Lin^{-}CD34^{+} cells were cultured on first passage MHC-matched or mismatched Am-Ad cells in the presence or absence of exogenous cytokines, and ^{3}H-TdR uptake was measured 7 days later (Figure 4A and B). The cytokines, even if added to the culture system at suboptimal con-

centrations, induced marked proliferation and differentiation of the Lin⁻CD34⁺ cells and, therefore, there was no significant difference in the expansion of the Lin⁻CD34⁺ cells between the cultures with or without Am-Ad cells (Figure 4A). In the absence of cytokines, however, the Lin⁻CD34⁺ cells alone showed very poor proliferation, whereas greater expansion of Lin⁻CD34⁺ cells was observed in the co-culture with Am-Ad cells (Figure 4B). This finding indicates that Am-Ad cells have the capacity to support hematopoiesis of Lin⁻CD34⁺ cells, even in the absence of exogenous cytokines. When the proliferation of the Lin⁻CD34⁺ cells was compared in the case of co-culture with either MHC-matched or MHC-mismatched Am-Ad cells, significantly greater proliferation was seen in the co-culture system with the MHC-matched combination (Figure 4B), indicating that the MHC-matched Am-Ad cells offered a more suitable environment for the proliferation of Lin⁻CD34⁺ cells.

Long-term co-culture of Lin⁻CD34⁺ cells on the amnion-derived adherent cell layer (long-term culture-initiating cell assay)

To further examine the facilitating effects of the MHC-matched Am-Ad cells on the proliferation of the Lin⁻CD34⁺ cells, LTC-IC assays were carried out. Figure 5A shows the number of non-adherent cells recovered from three different culture conditions: Lin⁻CD34⁺ cells alone and Lin⁻CD34⁺ cells co-cultured with first passage MHC-matched or mismatched Am-Ad cells. When exogenous cytokines were added to the culture, marked cell expansion occurred from 1 week of culture, and this high proliferation state was maintained thereafter in all the three culture conditions (Figure 5A). Many hematopoietic colonies, including adherent-type colonies or pseudoemperipolesis of the Am-Ad cells to the Lin⁻CD34⁺ cells, were observed from 1-2 weeks of culture in the co-culture systems with MHC-matched and mismatched Am-Ad cells (Figure 5B i and ii), suggesting that the Lin⁻CD34⁺ cells crawled under the stromal layer and then proliferated. In the MHC-matched co-culture system, the number and size of the colonies gradually increased, and the difference between the MHC-matched and mismatched co-culture system was evident at 8 weeks of culture (Figure 5 B iii and iv). In accordance with the enhancement of colony number and size, significantly greater expansion of non-adherent cells was observed in the co-culture system with the MHC-matched Am-Ad cells than in that with MHC-mismatched Am-Ad cells after 7 weeks of culture. Through the culture period, the number of non-adherent cells in the culture of the Lin⁻CD34⁺ cells alone was significantly lower than in the co-culture with the Am-Ad cells. Unfortunately, the Am-Ad cell layers began to detach from the flask surface at approximately 10 weeks of culture, and the cultures could not be continued. The detachment of the Am-Ad cell layer was caused by the overgrowth of the Am-Ad cells, because the cells were not irradiated before culture and therefore continued to proliferate during such a long culture period.

Non-adherent cells, recovered from the co-culture flasks with the MHC-matched and mismatched Am-Ad cells, contained immature and mature hematopoietic cells of all lineages: myelocytes, erythroblasts, granulocytes, and macrophages (*data not shown*). In contrast, the non-adherent cells, recovered from the culture of the Lin⁻CD34⁺ cells alone, contained mainly macrophages (*data not shown*). As shown in Figure 5C, significantly higher total colony formation was observed in the non-adherent cells recovered from the co-culture with MHC-matched Am-Ad cells than in those from the co-culture with MHC-mismatched Am-Ad cells at 5 weeks of culture. These colonies were composed of CFU-G, CFU-M, CFU-GM, and a few BFU-E. In contrast to the co-culture with MHC-matched or mismatched Am-Ad cells, very few hematopoietic colonies were observed in the control culture without Am-Ad cells. The differences in the total colony numbers between the MHC-matched and mismatched co-culture systems were more evident at 9 weeks of culture.

Next, we examined the cellular characteristics of the harvested cells from the co-culture system. The harvested cells from the MHC-matched combination (week 5 of

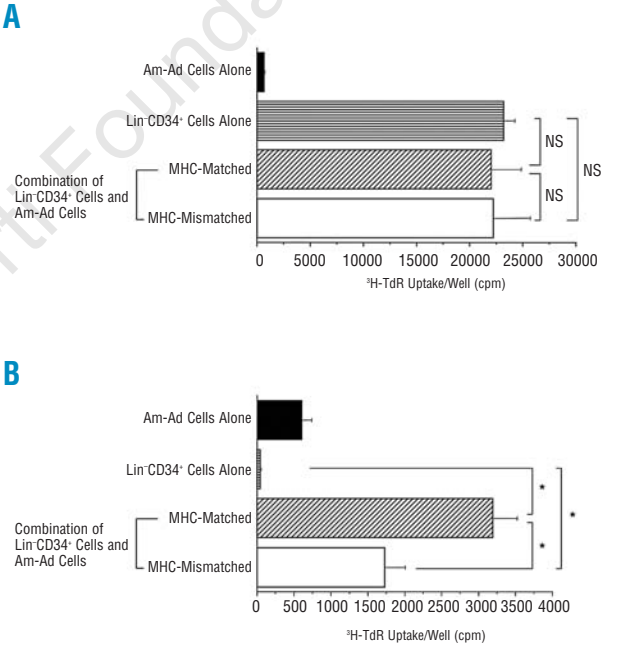

Figure 4. HSC- proliferation assay of Lin⁻CD34⁺ cells on Am-Ad cells. (A) Proliferation of Lin⁻CD34⁺ cells on Am-Ad cells in the presence of cytokines. Lin⁻CD34⁺ cells (1000 cells/200 μL/well) were cultured in 96-well plates in which a MHC-matched or mismatched Am-Ad cell layer had been prepared. The culture medium was supplemented with 20% FBS + recombinant human cytokines (6 ng/mL of SCF and 2 ng/mL of TPO, IL-3 and FLT-3L) (more than three wells/sample). As a control, the same number of Lin⁻CD34⁺ cells was cultured without the Am-Ad cells. Wells containing Am-Ad cells alone were also prepared. Seven days later, ³H-TdR was added during the last 24 h of the culture period and its uptake was measured. Data represent mean±SD of five independent experiments. NS: not significant. In each experiment, one kind of Am-Ad cells and two kinds of Lin⁻CD34⁺ cells were used; the Am-Ad cells were co-cultured with Lin⁻CD34⁺ cells derived from the same fetus (MHC-matched combination) or with Lin⁻CD34⁺ cells derived from another fetus (MHC-mismatched combination). (B) Proliferation of Lin⁻CD34⁺ cells on Am-Ad cells in the absence of cytokines. The Lin⁻CD34⁺ cells were cultured as described in (A), but without human cytokines (2000 cells/well). Data represent mean±SD of five independent experiments. *:$p<0.01$.

culture) contained 7.9±1.4% of $CD34^+CD45^{low}$ cells, 8.2±1.2% of $CD34^+CD38^+$ cells, 8.1±0.7% of $CD34^+CD38^-$ cells, 6.0±0.6 % of $CD34^+CD133^+$ cells and 4.9±0.3 % of $CD34^+$c-kit^+ cells. The percentages of these HSC-enriched populations were higher in the MHC-matched combination than in the MHC-mismatched combination; $CD34^+CD45^{low}$ cells: 1.82±0.34 times higher ($p<0.05$), $CD34^+CD38^+$ cells: 1.84±0.15 times higher ($p<0.05$), $CD34^+CD38^-$ cells: 1.52±0.20 times higher ($p=0.052$), $CD34^+CD133^+$ cells: 1.60±0.37 times higher ($p=0.095$), and $CD34^+$c-kit^+ cells: 1.58±0.48 times higher ($p=0.397$). In contrast, the percentages of lineage-positive cells ($CD11b^+$ cells, $CD14^+$ cells, $CD41^+$ cells and $CD235a^+$ cells) were similar or lower in the harvested cells from the MHC-matched combination than in those from the MHC-mismatched combination.

When the Lin^-CD34^+ cells were cultured in the absence of exogenous cytokines (Figure 5D), the expansion of the cells was much less than in the co-culture in the presence of exogenous cytokines. Moreover, in the clonal cell culture of non-adherent cells recovered from the culture flasks, only a few, very small hematopoietic colonies were formed after 4 weeks, and no colony formation was observed after 5 weeks, even in the MHC-matched co-culture system (*data not shown*). These results indicate that the addition of exogenous cytokines is necessary for

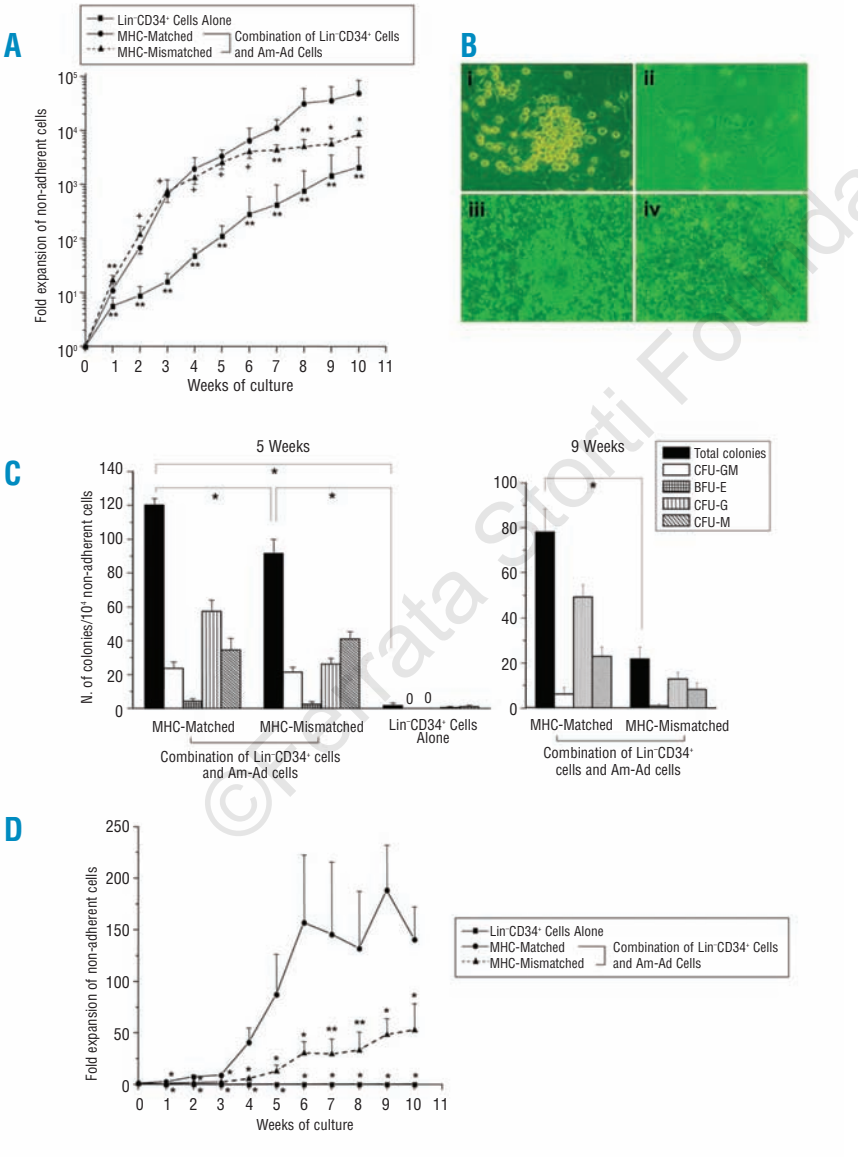

Figure 5. LTC-IC assay of Lin^-CD34^+ cells on Am-Ad cells. (A) Fold increase of non-adherent cells obtained from flasks of LTC-IC assay with cytokines. Lin^-CD34^+ cells ($5x10^4$ cells/8 mL/flask) were cultured in flasks in which a MHC-matched or mismatched Am-Ad cell layer had been prepared. The culture medium was supplemented with 20% FBS + recombinant human cytokines (6 ng/mL of SCF and 2 ng/mL of TPO, IL-3 and FLT-3L) (one or two flasks/sample). As a control, the same number of Lin^-CD34^+ cells was cultured without the Am-Ad cells. Every week, half of the culture medium in the flasks (containing non-adherent cells) was removed and fresh medium was added to the flasks. The number of non-adherent cells per flask was counted and the fold increase of non-adherent cells was calculated. Fold increase = the number of non-adherent cells per flask after culture / the number of non-adherent cells per flask before culture. Data represent mean ± SD of five independent experiments. +: not significant, *: $p<0.01$ and **: $p<0.05$ versus MHC-matched combination. In each experiment, one kind of Am-Ad cells and two kinds of Lin^-CD34^+ cells were used; the Am-Ad cells were co-cultured with Lin^-CD34^+ cells derived from the same fetus (MHC-matched combination) or with Lin^-CD34^+ cells derived from another fetus (MHC-mismatched combination). (B) Hematopoietic colonies in LTC-IC assay with cytokines. Representative photographs of an adherent colony (i) and cobblestone colony (ii) after 15 days of culture (x 200). Large adherent colonies were detected in the MHC-matched co-culture system (iii) at 8 weeks of culture, whereas only small adherent colonies could be seen in the MHC-mismatched co-culture system (iv) (× 40).

(C) Number of hematopoietic colony-forming cells in non-adherent cells recovered from culture flasks (after 5 and 9 weeks of culture). The non-adherent cells recovered from the LTC-IC culture were evaluated for their colony-forming ability using the methylcellulose assay (MethoCult GF+H4435). Mean±SD of five independent experiments. *: $p<0.01$. (D) Fold increase of non-adherent cells obtained from flasks of LTC-IC assay without cytokines. The Lin^-CD34^+ cells were cultured as described in (A), but without human cytokines. Every week, half of the culture medium in the flasks was removed and the numbers of non-adherent cells per flask were counted and the fold increase of non-adherent cells was calculated. Fold increase = the number of non-adherent cells per flask after culture / the number of non-adherent cells per flask before culture. Data represent mean±SD of five independent experiments. *$p<0.01$ and **$p<0.05$ versus MHC-matched combination.

Table 1. Concentrations of various cytokines in culture supernatants obtained from LTC-IC assays without cytokines (after 4 weeks of culture).

			Combination of Lin⁻CD34⁺ cells and Am-Ad cells	
Cytokines	Lin⁻CD34⁺ cells alone	Am-Ad cells alone	MHC-matched	MHC-mismatched
GM-CSF	0**	0.85±0.15**	2.28±0.22	1.39±0.18**
M-CSF	103±34**	2602±298*	2315±483	2737±320*
G-CSF	0	0	0	0
SCF	0.058±0.009**	6.2±1.3*	7.3±1.4	7.0±1.6*
LIF	0**	28±2.2*	38±5.6	32±6.6*
IL-6	1.7±0.26**	463±11*	468±4.8	468±9.8*

*Lin⁻CD34⁺ cells (5×10^4 cells/flask) were cultured in flasks containing 20% FBS/ IMDM, in which a MHC-matched or mismatched Am-Ad cell layer had been prepared (one or two flasks/sample). As a control, the same number of Lin⁻CD34⁺ cells was cultured without the Am-Ad cells, and the Am-Ad cells also cultured without Lin⁻CD34⁺ cells. Every week, half of the culture medium in the flasks (containing non-adherent cells) was removed and fresh medium was added to the flasks. The concentrations (pg/mL) of various cytokines in the culture supernatants collected at 4 weeks of culture were measured by ELISA. * NS and ** $p<0.01$ versus MHC-matched combination. The cytokine concentration was measured in five independent LTC-IC assays. Reproducible results were obtained in each experiment. Data represent mean ± SD of five independent experiments.*

the Am-Ad cells to induce long-lasting hematopoiesis of Lin⁻CD34⁺ cells, although such cytokines are unnecessary for the proliferation of Lin⁻CD34⁺ cells in short-term culture (Figure 4B). The data from the LTC-IC assays (Figure 5A and D) also show that MHC-matched Am-Ad cells have a greater capacity to support hematopoiesis of Lin⁻CD34⁺ cells than do MHC-mismatched Am-Ad cells.

Cytokine profile in supernatants obtained from a co-culture system of Lin⁻CD34⁺ cells and amnion-derived adherent cells

To assess the mechanisms by which the MHC-matched Am-Ad cells induce greater expansion of Lin⁻CD34⁺ cells than do MHC-mismatched Am-Ad cells, the cytokine profiles of LTC-IC culture supernatants, collected at 4 weeks of culture (Figure 5D), were examined using ELISA assays (Table 1). The concentration of GM-CSF was significantly higher in the MHC-matched combination than in the MHC-mismatched combination, but there were no significant differences in other cytokines (M-CSF, SCF, LIF and IL-6). Thus, higher production of GM-CSF is in part implicated in the greater hematopoiesis-supporting ability of the MHC-matched Am-Ad cells. The culture supernatants of the Am-Ad cells alone showed similar kinds and amounts of cytokine production to the MHC-matched and mismatched combinations, except for GM-CSF: the culture supernatants of the Am-Ad cells alone contained much lower amounts of GM-CSF than those of the Am-Ad cells co-cultured with Lin⁻CD34⁺ cells. Very low concentrations of cytokines were detected in the culture supernatants of the Lin⁻CD34⁺ cells alone. It is, therefore, conceivable that the cytokines found in the co-culture system of Am-Ad cells with Lin⁻CD34⁺ cells are mainly derived from the Am-Ad cells.

Discussion

In the present study, Lin⁻CD34⁺ cells showed a marked capacity for expansion and multilineage differentiation (Figure 1B ii and C), indicating that these cells contain a large amount of primitive HSC. Recently, there have been some reports claiming that human UCB-derived Lin⁻CD34⁻ cells have a higher SCID-repopulating cell activity than Lin⁻CD34⁺ cells, suggesting that Lin⁻CD34⁻ cells are more primitive than Lin⁻CD34⁺ cells.[27,28] *In vitro* expansion of Lin⁻CD34⁻ cells was markedly less than that of Lin⁻CD34⁺ cells, and the Lin⁻CD34⁻ cells could not form hematopoietic colonies in a semi-solid clonal cell culture assay.[28] Therefore, in the present *in vitro* study, we used Lin⁻CD34⁺ cells but not Lin⁻CD34⁻ cells in order to observe their *in vitro* expansion on Am-Ad cells. Lin⁻CD34⁺ cells are now widely applied for cord blood stem cell transplantations in clinical trials, and this is another of the reasons why we used these cells in our study.

Am-Ad cells can be considered as MSC, based on their morphology, phenotypes, and differentiation potential *in vitro* (Figure 2). Furthermore, it has been shown that Am-Ad cells have the ability to support the proliferation and maintenance of Lin⁻CD34⁺ cells in short-term cultures (Figure 4) as well as in long-term cultures (Figure 5). This ability was observed even without the addition of human cytokines to the cultures. Indeed, the Am-Ad cells produced a substantial amount of M-CSF and small amounts of GM-CSF, SCF, LIF and IL-6 (Table 1). Moreover, Am-Ad cells express some important adhesion molecules for hematopoiesis, such as CD29, CD44 and CD62L (Figure 2B). We have recently found that CD56 is also expressed on MSC and contributes greatly to hematopoiesis in mice[29] and in monkeys.[30] In the present study, expression of CD56 was shown in the Am-Ad cells (Figure 2B). Thus, Am-Ad cells appear to fulfill the criteria for MSC.

Am-Ad cells could support the Lin⁻CD34⁺ cells obtained from the same fetus to a greater extent than those derived from another fetus (Figures 4B, 5A and 5D). Since MHC class I molecules (but not MHC class II molecules) are expressed on both Lin⁻CD34⁺ cells and Am-Ad cells (Figures 1A and 2B), it is conceivable that the MHC class I molecules are related to the MHC preference, as shown in mice:[22,23] the MHC preference is restricted by MHC class Ia molecules (but not MHC class Ib and II molecules) according to the results of cobblestone colony-forming assays in a co-culture system of

B10 congenic mouse strains.[23] It can be speculated that putative unknown molecules reacting preferentially with *self* MHC class I molecules are expressed on HSC and/or MSC, and that the binding of MHC class I molecules and the putative MHC class I ligand induce stimulatory signal transductions and, as a result, induction of the expansion of the HSC. So far, CD8, class I receptors on natural killer cells (such as Ly49, p58 and NKB1) and PIR-A/B (paired immunoglobulin-like receptor)[31] are known ligands for MHC class I molecules. It is less likely that CD8 and Ly49 are expressed on HSC and MSC. Recent studies have indicated that PIR-A/B is expressed on B lymphocytes, macrophages and myeloid-lineage cells. Tun *et al.* suggested the possibility that PIR are expressed on hematopoietic progenitor cells.[32] However, it is uncertain whether PIR can discriminate polymorphic MHC class I molecules. We are currently attempting to detect previously unknown MHC class I-binding molecules.

The fates of HSC are controlled by many factors (including cell adhesion molecules, cytokines, and cell matrix molecules) produced by MSC/stromal cells. In the present study, the production of GM-CSF was enhanced significantly by the co-culture of the MHC-matched combination, clearly indicating the contribution of GM-CSF to the MHC restriction (Table 1). However, the mechanism enhancing the production of GM-CSF and to what extent GM-CSF contributes to the MHC restriction remain unclear. Our preliminary experiments revealed that the addition of anti-GM-CSF monoclonal antibody to a short-term co-culture (without cytokines) of Lin^-CD34^+ cells on MHC-matched Am-Ad cells markedly (65-86% of control) reduced the proliferation of the Lin^-CD34^+ cells (*data not shown*). We are now investigating whether anti-GM-CSF monoclonal antibody also inhibits the proliferation of the Lin^-CD34^+ cells in the LTC-IC assay. However, the possibility that other hematopoietic growth factors and cytokines also play important roles in MHC restriction remains to be elucidated. Indeed, our previous study in mice showed that cytokine messages (SCF, FLT-3L, and IL-6) are enhanced in the MHC-matched co-culture of HSC and fetal bone marrow-derived stromal cells.[23]

It is difficult to explain the presence of MHC restriction and the quite long-term expansion of Lin^-CD34^+ cells on MHC-matched Am-Ad cells only by the enhanced production of GM-CSF. The formation of hematopoietic colonies in the MHC-matched co-culture system was significantly greater than that in the MHC-mismatched one (Figure 5B). Although the growth and maintenance of colonies depend on hematopoietic cytokines to a certain extent, a contribution of adhesion molecules to MHC restriction cannot be ruled out; some important adhesion molecules might be induced to express on hematopoietic cells and/or Am-Ad cells in the MHC-matched combination. In fact, there is a study showing that human eosinophils, which do not constitutively express ICAM-1, are induced to express ICAM-1 molecules when stimulated with GM-CSF plus tumor necrosis factor-α.[33]

Cord blood stem cell transplantations have been used for clinical treatment frequently in the last two decades.[34,35] However, as a source of stem cells, UCB has some disadvantages, including limited cell numbers in a single UCB sample and delayed times to recovery of platelets and neutrophils, which expose recipients to the risk of infections for a longer time. If these problems could be overcome, UCB would become a better source of HSC. Here, we propose that the use of amniotic tissues provides a useful strategy for cord blood stem cell transplantation. First, the human amnion is a useful source of feeder cells for the expansion of UCB-derived HSC without the risk of zoonosis associated with the use of animal feeders. If methods of preserving amniotic tissue[36,37] can be promulgated, this clinical application would be exploited more conveniently. Second, there is the possibility that the co-transplantation of UCB with MHC-matched Am-Ad cells might induce earlier and more complete recovery of hematopoiesis and consequently reduce the incidence of cord blood transplantation-associated side effects. We previously found that a simultaneous injection of allogeneic bone marrow cells and bone marrow stromal cells into recipient mouse bone marrow cavity leads to significantly better engraftment than intravenous injection of bone marrow cells.[38-40] Very recently, we detected human $CD45^+$ cells in the bone marrow of SCID mice 4 weeks after bone marrow transplantation, when the Lin^-CD34^+ cells were transplanted into irradiated (3Gy) SCID mice in conjunction with Am-Ad cells via the intra-bone marrow route. In contrast, significantly less or no engraftment of human cells was observed in the SCID mice that received the Lin^-CD34^+ cells plus Am-Ad cells via the intravenous route or Lin^-CD34^+ cells alone via the intra-bone marrow route (*unpublished data*). If the safety of the injection method for the culture-expanded Am-Ad cells into the bone marrow cavity is confirmed in humans, this method would provide new insights for cord blood stem cell transplants.

Authorship and Disclosures

TM, HH and SI contributed to the conception and design of the study, and to the analysis and interpretation of data; TM provided the study materials, performed the majority of the experiments and drafted the article; HH revised the article; SI profoundly revised the article and obtained the necessary funding. HK contributed to the conception and design of the study, and provided the study materials. The other authors contributed to some of the experiments.

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