Master's thesis

The CD105^{+} cells in the blood of human lung cancer neighborhood contain mesenchymal progenitors

（ヒト肺がん近傍血液由来の CD105 陽性細胞画分には間葉系前駆細胞が存在する）

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Abstract

Mesenchymal progenitor cells (MPCs) derived from bone marrow are multipotent cells capable of differentiating into various mesenchymal tissues such as (myo)fibroblasts, bone, cartilage, fat, tendon, muscle and nerves. Recent data have suggested these MPCs migrate and contribute to tissue repair and fibrosis, such as tumor stroma formation, but the suggestion that circulating MPCs occur in adult human blood remains controversial. We found that MPCs can be isolated from adult human blood collected from the neighborhood of lung cancers. The MPCs have a fibroblast-like morphology and immunophenotype and have the ability to differentiate *in vitro* into at least two different tissues. These are bone and fat. Circulating MPCs were detected in pulmonary arterial blood harvests of 29/31 lung cancer patients. Furthermore, we found that MPCs derived from pulmonary arterial blood were contained within an abundant subset of $CD105^{+}$ cells. This work is the first clear proof that MPCs similar to those present in bone marrow are found in the adult human blood of lung cancer patients.

Introduction

It is now well accepted that mesenchymal progenitor cells (MPCs) share common characteristics, including adherence to culture plastic, fibroblast-like morphology, extensive expansion *ex vivo*, and the capacity to differentiate into various mesenchymal tissues such as (myo)fibroblasts, bone, cartilage, fat, tendon, muscle, and nerves [1, 2]. In adults, MPCs are prevalent in a variety of mesenchymal tissues, especially in the bone marrow. The bone marrow is the most extensively characterized source of MPCs [1-4].

Recent data from animal experiments [5] and a clinical trial [6] indicate that conditions characterized by increased cell turnover, such as wound healing or tissue remodeling, provide effective signals resulting in the recruitment, survival and proliferation of systemically delivered MPCs. Examples of such conditions would include multiple bone fractures in metabolic bone disease or circumstances in the rapidly growing embryo during prenatal development. In a related sense, tumor growth requires formation of supportive mesenchymal stroma [7]. The process of tumor stroma formation is similar to wound healing [8] and results in tissue remodeling with high proliferation of mesenchymal cells [9, 10]. Recently, investigators including our group reported that cancer-induced stromal tissues in mouse models contain various

proportions of fibroblasts derived from bone marrow [11, 12]. When MPCs derived from bone marrow play a role in repair and fibrosis of mesenchymal tissues, including tumor stroma formation, transit of these cells through the blood is to be expected. Although some reports of MPCs in blood have been published [3, 13-16], the results are debated and have not always been reproducible [17, 18]. We have recently shown that blood in the neighborhood of human lung cancers contains fibroblast progenitor cells with the capacity to migrate into cancer stromal cells and to differentiate into stromal fibroblasts. These results allow us to hypothesize that blood in the neighborhood of adult human lung cancers contains MPCs as well as fibroblast progenitor cells. Our aim in this study was to discover, isolate and characterize such cells.

Materials and Methods

Collection of blood mononuclear cells from the pulmonary arteries of study subjects

Dissected and ligated pulmonary arteries of surgically resected lungs contain large volumes (more than 10 ml) of blood. Blood from this source was obtained from lung cancer patients, and we refer to such samples as emanating from the neighborhood of the cancers. An 18-gauge needle was inserted into the artery and 10 ml of blood was collected. In all, we collected samples from 31 patients with histologically proven lung cancer who were not receiving chemotherapy.

All patients gave written informed consent to the procedure in a manner approved by International Review Boards.

Isolation and culture of mononuclear cells from PA blood

Mononuclear cells (MNCs) from pulmonary arterial blood were separated from buffy coat cells by Ficoll Paque (Amersham–Pharmacia, Piscataway, NJ) density gradient centrifugation. In this procedure, mononuclear cells banded at a density of 1.077 g/cm^3. Isolated cells were plated at a density of 5×10^6 cells/well in six-well tissue culture dishes (Becton Dickinson, Mountain View, CA). Mesenchymal progenitor cell growth medium (TM Cell Research, Tokyo, Japan) was used, supplemented with 1% (v/v) fetal

calf serum (FCS) and a growth factor supplement. Nonadherent cells were removed after 72 h of incubation, and adherent cells were further cultured. If fibroblastic colonies were found in culture dishes, cells were grown to subconfluency. Cells were detached with trypsin, washed, and cultured further after dilution with three parts of fresh medium. Every three days thereafter, medium containing floating cells was removed and fresh medium was added. Cells were cultured for up to eight weeks. Human bone marrow mesenchymal progenitor cells were obtained from Cambrex Bio Science (Walkersville, MD). Primary cultures of fibroblasts were obtained from both human lung cancer tissue and normal lung tissue. All cultures were maintained at 37 °C in a humidified atmosphere containing 95% (v/v) air and 5% (v/v) CO_2.

Immunocytochemistry

Cells were grown on eight-chambered slides (Lab-Tec, Poly Labo, Strasbourg, France) and fixed with formalin at room temperature. Endogenous peroxidases were inactivated with 3% (v/v) H_2O_2 in methanol. Nonspecific antibody binding was blocked by incubation with 3% (w/v) bovine serum albumin (BSA). Primary monoclonal antibodies (mAbs) against human CD14 (TUK4), CD31 (JC70A), CD34 (TUK3), CD105/endoglin (SN6h), CD117/c-kit (104D2), vimentin (v9) were from Dako, Glostrup, Denmark. Primary mAbs against CD44 (Novocastra, UK), CD45

(eBioscience, CA), collagen type I (Calbiochem, CA), smooth muscle actin (1A4; Sigma, St. Louis, MO) and STRO-1 (R&D Systems, Minneapolis, MN) were also purchased. Human bone marrow mesenchymal progenitor cells and primary cultured fibroblasts from human lung cancer tissue and normal lung tissue were examined by immunocytochemistry using the antibodies indicated above.

Osteogenic differentiation

Cells were seeded at a density of 3×10^3 cells/cm^2 in tissue culture dishes and cultured with mesenchymal stem cell growth medium containing 100 nM dexamethasone, 50 μM ascorbic acid, 2-phosphate, and β-glycerophosphate. Cultures were maintained for four weeks with replacement of the culture medium every three days.

Adipogenic differentiation

Cells were seeded at a density of 2×10^4 cells/cm^2 in tissue culture dishes. When cells were confluent, adipogenic differentiation was initiated by three cycles of induction/maintenance culture. Each cycle consisted of three days of culture in the induction medium (Dulbecco's modified Eagle's medium; DMEM) with 10% (v/v) FCS, 1 μM dexamethasone, 0.2 mM indomethacin, 10 μg/ml insulin and 0.5 mM

3-isobutyl-1-methylxanthine, followed by 24–72 h of culture in maintenance medium (DMEM with 10% (v/v) FBS and 10 μg/ml insulin).

Alkaline phosphatase staining

After 21 days of culture, cells were fixed with 10% (v/v) formalin and subsequently incubated in a solution containing 0.2 mg/ml naphthol AS-TR phosphate and 0.5 mg/ml Fast Red RC (all from Sigma) for 10 min.

Oil red O staining

After 21 days of culture, cells were fixed with 10% (v/v) formalin for 5 min, rinsed in 60% (v/v) isopropanol, and covered with 0.1% (w/v) oil red O (Sigma) for 10 min. After rinsed in 60% (v/v) isopropanol and subsequently in distilled water, the cells counterstained with hematoxylin.

Von Kossa staining

After 21 days of culture, cells were rinsed twice with PBS and then fixed with 10% (v/v) formalin for 10 min at room temperature. Fiwed cells were stained with 5% (w/v) silver nitrate (Wako, Osaka, Japan) for 60 min under UV light. After staining with silver nitrate, the sections were placed in 5% sodium (w/v) thiosulphate (Wako) for 1 min. The cells were counterstained with kernechtrot (Muto, Tokyo, Japan).

Cell isolation

To isolate $CD14^+$, $CD105^+$, $CD3^+$ or $CD20^+$ cells, MNCs were bound to Dynabeads according to the manufacturer's instructions. Briefly, MNCs (5×10^6 cells) were isolated, resuspended in PBS containing 0.1% (w/v) BSA and incubated with anti-CD14 (61D3), anti-CD20 (2H7) (both from eBioscience, CA), anti-CD105/endoglin (SN6h) or anti-CD3 (UCHT1) (both from Dako, Glostrup, Denmark) mAbs. Subsequently, goat antimouse IgG1 antibody coupled to magnetic beads (Dynal, Oslo, Norway) was added. Mock-treated MNCs incubated with isotype-matched mouse mAbs and bead-conjugated antimouse IgG antibody served as controls.

Results

Blood in the neighborhood of human lung cancers contains circulating MPCs:

We examined MNCs from pulmonary arterial blood of resected lungs of lung cancer patients for the presence of MPCs. In 29/31 cases, we found one or two isolated fibroblastic colonies per 5×10^6 MNCs after 3–27 days of culture (mean ± SEM: 8 ± 6 days) (Figure 1). On continued growth in culture, colonies became bigger and subsequently merged, resulting in the formation of a monolayer of fibroblast-like cells. In eight instances, we investigated the proliferative capacities of these fibroblast-like cells. After the mixed cultures had attained a subconfluent condition, the cells were passaged by harvest followed by dispersal between four new plates. The number of cell passages possible before onset of senescence was ≤ 8 in all cases and was not affected by the ages of the patients. In a single case, the fibroblast-like cells ceased to proliferate after only one passage (Table 1).

Protein expression profiles of the fibroblast-like cells derived from pulmonary arterial blood were examined by immunohistochemistry in three cases (Figure 2) and the data were compared with findings obtained from immunohistochemistry of adult human bone marrow stromal cells, lung cancer tissue and normal lung tissue (Table 2).

Fibroblast-like cells derived from three different pulmonary arterial blood samples stained positively for CD44, CD105, collagen type I, smooth muscle actin, vimentin and STRO-1. All these proteins are typically produced by cells of a mesenchymal origin. Fibroblast-like cells derived from pulmonary arterial blood were negative for the monocyte marker CD14, the hematopoietic cell maker CD45 and the stem cell markers CD34 and CD117. A small number of fibroblast-like cells stained positively for the endothelial cell maker CD31. These cells were intermingled with marker-negative cells. As shown (Table 2), the immunophenotypes of fibroblast-like cells from pulmonary arterial blood were similar to those of adult human stromal cells derived from bone marrow, lung cancer tissue and normal lung tissue.

MNCs were collected from the peripheral blood of six healthy volunteers and cultured under the same conditions that allowed fibroblast differentiation from cells in pulmonary arterial blood of lung cancer patients. No fibroblastic colonies were found.

These results indicate that MNCs derived from adult human pulmonary arterial blood of lung cancer patients can generate fibroblast-like cells with a typical mesenchymal immunophenotype and a capacity for extensive expansion *ex vivo*.

Ex vivo *outgrowth fibroblast-like cells derived from pulmonary arterial blood of human lung cancer patients potentially differentiate into osteogenic and adipogenic lineages:*

As *ex vivo* outgrowth fibroblast-like cells derived from pulmonary arterial blood did not differentiate spontaneously into mature mesenchymal cells, the fibroblast-like cells were cultured under conditions known to induce differentiation of MPCs into adipogenic or osteogenic cells. After three weeks growth in lineage-specific culture conditions, *ex vivo* outgrowth fibroblast-like cells from three donors were highly differentiated. The phenotypes of lineage-specific cell types were easily distinguished (Figure 3). Adipogenic differentiation was revealed by oil red O staining (Figure 3A). Differentiation was apparent from the accumulation of lipid-rich vacuoles within cells. The fibroblast-like cells subjected to the osteogenic induction procedure underwent a change in morphology from spindle-shaped to cuboidal. Almost every adherent cell formed calcium deposits, as indicated with von Kossa staining (Figure 3B). The cells also yielded positive staining reactions for alkaline phosphatase (Figure 3C). Earlier, we induced fibroblast-like cells to differentiate into a chondrogenic lineage [19]. Although MPCs derived from bone marrow differentiated into a chondrogenic lineage *in vitro,*

fibroblast-like cells from the pulmonary arterial blood did not differentiate into this lineage (data not shown).

Taken together, our data show that pulmonary arterial blood of lung cancer patients contains MPCs.

In addition, we tried, unsuccessfully, to induce primary cultured human fibroblast-like cells derived from lung cancer tissue and normal lung tissue to differentiate into osteogenic, adipogenic and chondrogenic lineages (data not shown).

CD105$^+$ cells in pulmonary arterial blood of lung cancer patients contain MPCs:

Next, we tried to identify the clonal origin of the *ex vivo* outgrowth MPCs in pulmonary arterial blood of patients. The MNCs derived from pulmonary arterial blood were exposed to anti-CD14, anti-CD105, anti-CD3, anti-CD20 mAbs and isotype-matched control IgG1 antibodies. Cells binding the antibodies were purified using a magnetic beads method similar to that described above. The selected cells were cultured. As shown (Table 3), cultures of whole cells without mAb selection generated fibroblastic cell colonies in 14/14 cases. The CD105$^+$ positive cell fraction also generated fibroblastic cell colonies in all eight cases. The CD14$^+$ and CD3$^+$ fractions generated fibroblastic cell colonies in 3/5 and 2/5 instances, respectively. No fibroblastic cell colonies were observed on culture of CD20$^+$ cells, or on culture of cells

selected by control IgG1 antibody. These results suggest that the $CD105^+$ fraction in the pulmonary arterial blood of lung cancer patients contains abundant MPCs.

We exposed $CD105^+$ cells to procedures (described above) encouraging differentiation into osteogenic and adipogenic lineages. Immunohistochemistry of the resulting cell lines revealed the same surface molecules as found (above) on osteogenic and adipogenic lines derived by *ex vivo* outgrowths of MPCs from whole MNC populations. The two colonies derived from a $CD105^+$ clonal cell underwent both osteogenic and adipogenic differentiation (Figure 4). The fact that the $CD105^+$ cell fraction of pulmonary arterial blood, grown and expanded as single colonies, possessed the capacity to differentiate into osteogenic and adipogenic lines supports our conclusion that positive selection using CD105 antibodies makes it possible to obtain a pure MPC population.

Discussion

In this study, we found that a $CD105^{+}$ subset of adult human pulmonary arterial blood cells from lung cancer patients has the ability to differentiate *in vitro* into at least two different tissues. These tissues are bone and fat. It is unlikely that MPCs circulate through the whole body as we could not find MPCs in cubital vein blood from any of six healthy volunteers. Mobilization of MPCs may occur as a consequence of stimuli from cancer cells and cancer-induced stromal tissue.

The lung cancer patients we examined ranged in age from 32 to 80 years. We found no evidence for any age-associated decline in the frequency of MPC production.

Several lines of evidence indicate that the adult human MPCs in pulmonary arterial blood of lung cancer patients are similar to those present in adult bone marrow. First, the morphology and immunophenotype of the pulmonary arterial blood cells were similar to those of the MPCs derived from bone marrow. Like MPCs derived from bone marrow, the pulmonary arterial blood cells were clearly negative for hematopoietic markers such as CD14, CD45, CD34 and CD117. The pulmonary arterial blood cells were positive for mesenchymal markers such as CD44, CD105, collagen type I, smooth muscle actin, vimentin and STRO-1 [1, 2]. Second, like MPCs derived from bone marrow, MPCs derived from pulmonary arterial blood had the ability to differentiate

into various mesenchymal cell types. On the other hands, unlike bone marrow-derived MPCs, chondrogenic differentiation was not observed in MPCs derived from the adult pulmonary arterial blood. This suggests that the differentiation potential of circulating MPCs does not completely correspond to that of the bone marrow-derived MPCs.

MPCs derived from adult human blood, like those derived from bone marrow, might be useful in autologous cell transplantation. Because of their limited lifespan, it is difficult to study such cells and to use them in transplantations. One of the reasons is that normal human cells undergo a limited number of cell divisions in culture and then enter a nondividing state called "senescence" [20-22]. In this study, the population of human MPCs derived from adult pulmonary arterial blood doubled, on average, only 20 times, before entering the senescent state. This means it would be difficult to obtain the numbers of cells required to restore the function of a failing human organ. Large numbers of cells must be injected into damaged tissues to restore function, and cells sometimes need to be injected throughout entire organs. To resolve these problems, the lifespan of human MPCs derived from adult pulmonary arterial blood may need to be extended by the retroviral transduction of human telomerase reverse transcriptase [23, 24] or human papillomavirus type 16 (HPV16) E6 and/or E7 [25-28].

Our data indicate that circulating MPCs can be isolated from pulmonary arterial blood of lung cancer patients at a very high frequency (29/31 cases). Some published papers have reported adult human MPCs in blood, but, in most instances, the blood MPCs were isolated after mobilization from the bone marrow by granulocyte-monocyte colony stimulating factor (GM-CSF) [3, 13, 16]. Fernandez et al. (1997) reported that MPCs (termed "peripheral blood progenitor cells" by these authors) capable of generating stromal cells were isolated from the blood of 11/14 breast cancer patients after chemotherapy and administration of granulocyte colony stimulating factor (G-CSF) or GM-CSF [13]. However, the MPCs derived from pulmonary arterial blood that we used were isolated from patients who were not receiving chemotherapy. Nor did we administer G-CSF or GM-CSF. On the other hand, Zvaifler et al. (2000) reported the isolation of adherent fibroblast-like cells from normal human blood without prior growth factor mobilization [14]. Aliquots of 500 ml of blood were used to isolate MPCs from the blood of normal individuals, indicating that such cells are uncommon. However, we found MPCs in much lower volumes (10 ml) of blood. In addition, we could not find any MPCs in cubital vein blood from healthy volunteers, using culture conditions identical to those used to find MPCs in pulmonary arterial blood of cancer patients. This suggests that blood in the neighborhood of lung cancers contains a high

level of MPCs. This is the first report to show that adult human MPCs can be isolated from a small amount of pulmonary arterial blood taken from adult human lung cancer patients, to whom cell growth factors have not been administered.

It is not yet clear whether the mesenchymal progenitor population contains multipotent cells or is a mixture of committed progenitors with a restricted potential. As only a subset of mesenchymal progenitors differentiated into induced lineages, mesenchymal progenitors are likely to be a heterogeneous cell population consisting of various committed progenitors. The cloning of mesenchymal progenitors at the single-cell level will be necessary to clarify this point. Presently, this is technically difficult.

To identify a subset of adult human blood MPCs, cells positive for the monocytic CD14 marker, the T lymphocytic CD3 marker, the B lymphocytic CD20 marker or the CD105 marker characteristic of mesenchymal or endothelial cells were selected and expanded in *ex vivo* outgrowths. The $CD105^{+}$ cells generated MPCs with highest efficiency (8/8 cases). The CD105 protein (endoglin) is an accessory receptor for transforming growth factor β and is expressed on human bone marrow stromal cells, endothelial cells, syncytiotrophoblasts, macrophages, and connective tissue stromal

cells. Our data suggest that CD105 may be a useful ligand in work aiming to purify MPCs from human blood.

The potential of MPCs as vehicles for gene delivery or protein production has been realized by many authors [29-35]. In particular, Studeny and colleagues demonstrated the potency of MPCs derived from bone marrow as vehicles for the delivery of biological agents into tumors [33]. We have shown that $CD105^+$ cells in adult human blood are rich in mesenchymal progenitors. The availability of pure MPCs may improve the efficiencies of a number of gene therapy protocols. Transplantation of MPCs may prove to be advantageous in clinical settings.

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References

1. Minguell, J.J., A. Erices, and P. Conget, *Mesenchymal stem cells.* Exp Biol Med (Maywood), 2001. **226**(6): p. 507-20.
2. Baksh, D., L. Song, and R.S. Tuan, *Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy.* J Cell Mol Med, 2004. **8**(3): p. 301-16.
3. Roufosse, C.A., et al., *Circulating mesenchymal stem cells.* Int J Biochem Cell Biol, 2004. **36**(4): p. 585-97.
4. Pittenger, M.F., et al., *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. **284**(5411): p. 143-7.
5. Liechty, K.W., et al., *Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep.* Nat Med, 2000. **6**(11): p. 1282-6.
6. Horwitz, E.M., et al., *Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta.* Nat Med, 1999. **5**(3): p. 309-13.
7. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
8. Dvorak, H.F., *Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing.* N Engl J Med, 1986. **315**(26): p. 1650-9.
9. Hasebe, T., et al., *Proliferative activities of tumor stromal cells play important roles in tumor thickness and progression of T3 ulcerative-type colorectal cancer.* Virchows Arch, 2003. **442**(6): p. 569-76.
10. Kuniyasu, H., et al., *Induction of ductal and stromal hyperplasia by basic fibroblast growth factor produced by human pancreatic carcinoma.* Int J Oncol, 2001. **19**(4): p. 681-5.
11. Ishii, G., et al., *Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction.* Biochem Biophys Res Commun, 2003. **309**(1): p. 232-40.
12. Direkze, N.C., et al., *Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts.* Cancer Res, 2004. **64**(23): p. 8492-5.
13. Fernandez, M., et al., *Detection of stromal cells in peripheral blood progenitor cell collections from breast cancer patients.* Bone Marrow Transplant, 1997. **20**(4): p. 265-71.

14. Zvaifler, N.J., et al., *Mesenchymal precursor cells in the blood of normal individuals.* Arthritis Res, 2000. **2**(6): p. 477-88.
15. Kuwana, M., et al., *Human circulating CD14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation.* J Leukoc Biol, 2003. **74**(5): p. 833-45.
16. Villaron, E.M., et al., *Mesenchymal stem cells are present in peripheral blood and can engraft after allogeneic hematopoietic stem cell transplantation.* Haematologica, 2004. **89**(12): p. 1421-7.
17. Lazarus, H.M., et al., *Human bone marrow-derived mesenchymal (stromal) progenitor cells (MPCs) cannot be recovered from peripheral blood progenitor cell collections.* J Hematother, 1997. **6**(5): p. 447-55.
18. Wexler, S.A., et al., *Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not.* Br J Haematol, 2003. **121**(2): p. 368-74.
19. Imabayashi, H., et al., *Redifferentiation of dedifferentiated chondrocytes and chondrogenesis of human bone marrow stromal cells via chondrosphere formation with expression profiling by large-scale cDNA analysis.* Exp Cell Res, 2003. **288**(1): p. 35-50.
20. Hayflick, L., *The cell biology of human aging.* N Engl J Med, 1976. **295**(23): p. 1302-8.
21. Hayflick, L., *Recent advances in the cell biology of aging.* Mech Ageing Dev, 1980. **14**(1-2): p. 59-79.
22. Campisi, J., *The biology of replicative senescence.* Eur J Cancer, 1997. **33**(5): p. 703-9.
23. Blackburn, E.H., *Telomere states and cell fates.* Nature, 2000. **408**(6808): p. 53-6.
24. Blackburn, E.H., *Switching and signaling at the telomere.* Cell, 2001. **106**(6): p. 661-73.
25. Sekiguchi, T. and T. Hunter, *Induction of growth arrest and cell death by overexpression of the cyclin-Cdk inhibitor p21 in hamster BHK21 cells.* Oncogene, 1998. **16**(3): p. 369-80.
26. Burk, R.D., et al., *Distribution of human papillomavirus types 16 and 18 variants in squamous cell carcinomas and adenocarcinomas of the cervix.* Cancer Res, 2003. **63**(21): p. 7215-20.

27. Takeda, Y., et al., *Can the life span of human marrow stromal cells be prolonged by bmi-1, E6, E7, and/or telomerase without affecting cardiomyogenic differentiation?* J Gene Med, 2004. **6**(8): p. 833-45.
28. Terai, M., et al., *Immortalization of human fetal cells: the life span of umbilical cord blood-derived cells can be prolonged without manipulating p16INK4a/RB braking pathway.* Mol Biol Cell, 2005. **16**(3): p. 1491-9.
29. Deans, R.J. and A.B. Moseley, *Mesenchymal stem cells: biology and potential clinical uses.* Exp Hematol, 2000. **28**(8): p. 875-84.
30. Gronthos, S. and P.J. Simmons, *The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro.* Blood, 1995. **85**(4): p. 929-40.
31. Clark, B.R. and A. Keating, *Biology of bone marrow stroma.* Ann N Y Acad Sci, 1995. **770**: p. 70-8.
32. Dao, M.A. and J.A. Nolta, *Use of the bnx/hu xenograft model of human hematopoiesis to optimize methods for retroviral-mediated stem cell transduction (Review).* Int J Mol Med, 1998. **1**(1): p. 257-64.
33. Studeny, M., et al., *Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents.* J Natl Cancer Inst, 2004. **96**(21): p. 1593-603.
34. Nakamizo, A., et al., *Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas.* Cancer Res, 2005. **65**(8): p. 3307-18.
35. Chan, J., et al., *Human fetal mesenchymal stem cells as vehicles for gene delivery.* Stem Cells, 2005. **23**(1): p. 93-102.

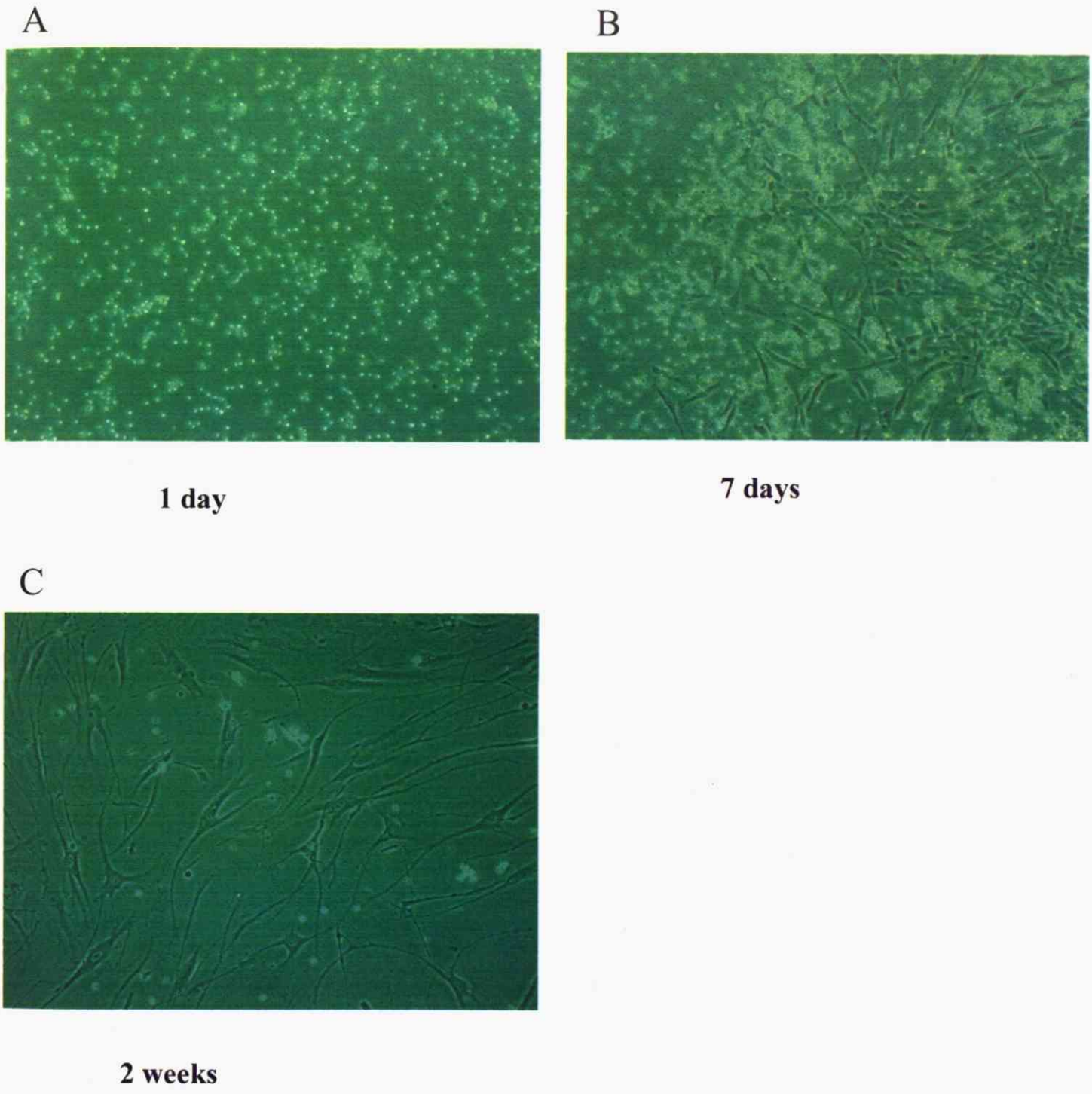

Figure 1. Morphology of fibroblast-like cells derived from pulmonary arterial blood.
Fibroblast-like cells were generated by culturing mononuclear cells derived from pulmonary arterial blood. Phase-contrast images at 1 day (A), 7 days (B), and 14 days (C) after the first passage are shown.

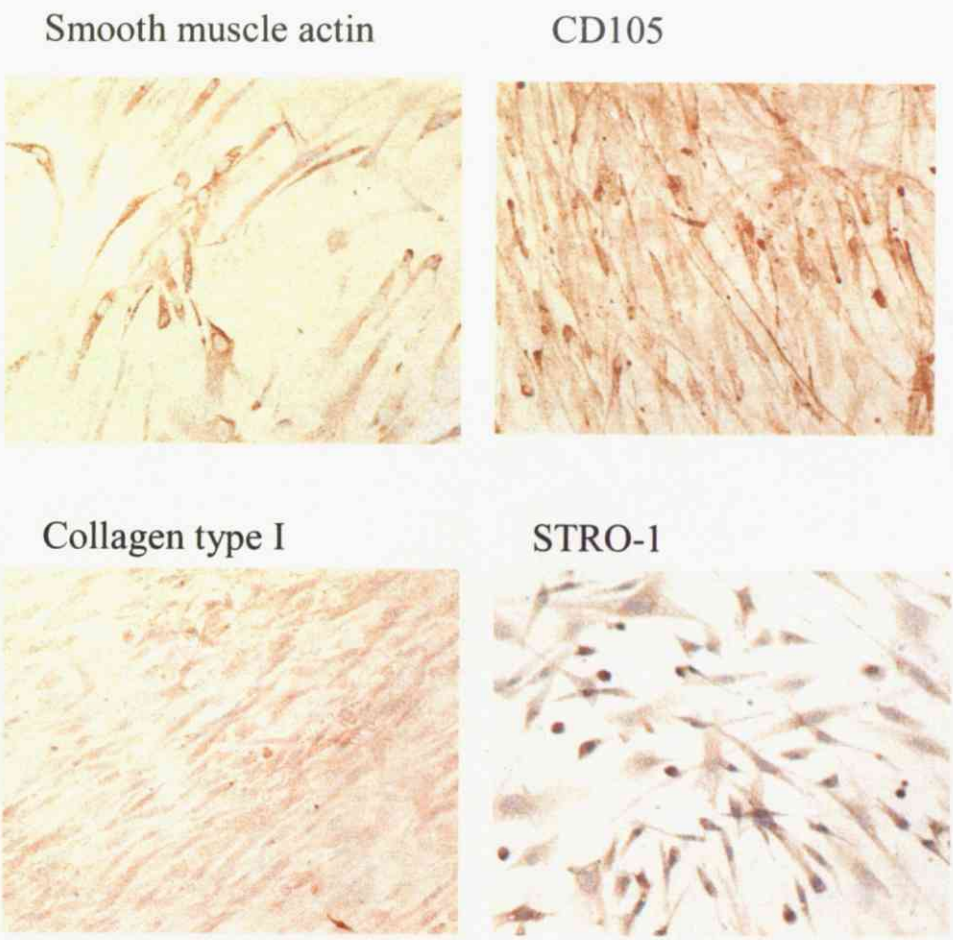

Figure 2. A representative immunophenotype of fibroblast-like cells derived from pulmonary arterial blood.
Fibroblast-like cells generated by culturing pulmonary arterial blood MNCs were passaged and replated onto eight-well chamber slides. After three days of culture, the slides were fixed with formalin and stained with mAbs as indicated. The nuclei were counterstained with hematoxylin. The results shown are representative of at least three independent experiments.

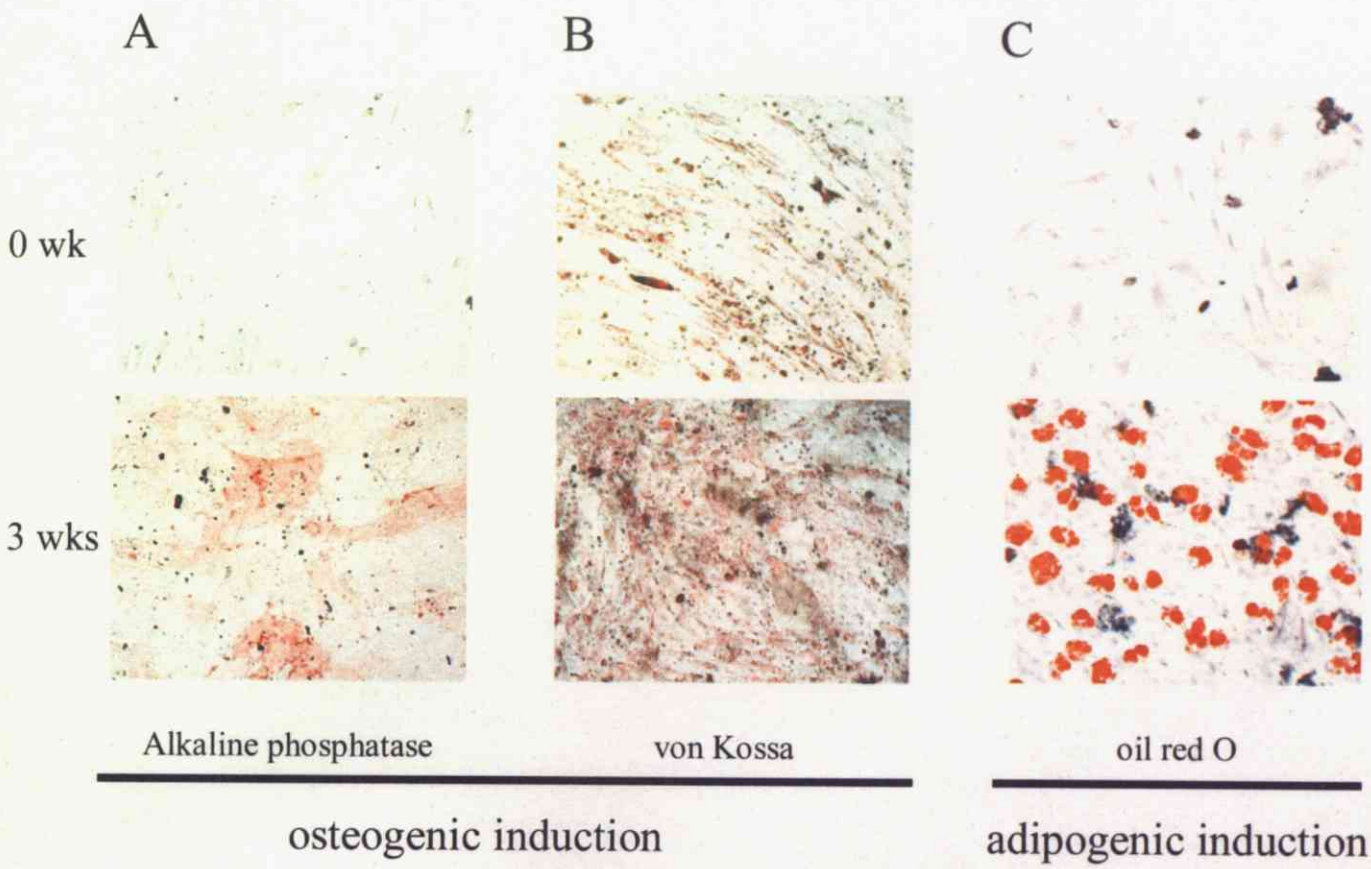

Figure 3. Osteogenic and adipogenic differentiation of fibroblast-like cells derived from pulmonary arterial blood.
MPCs derived from pulmonary arterial blood before and after four weeks of osteogenic induction were stained with the von Kossa (A) or to detect alkaline phosphatase (B). MPCs derived from PA blood before and after three weeks of adipogenic induction were stained with oil red O (C). The results shown are representative of at least three independent experiments.

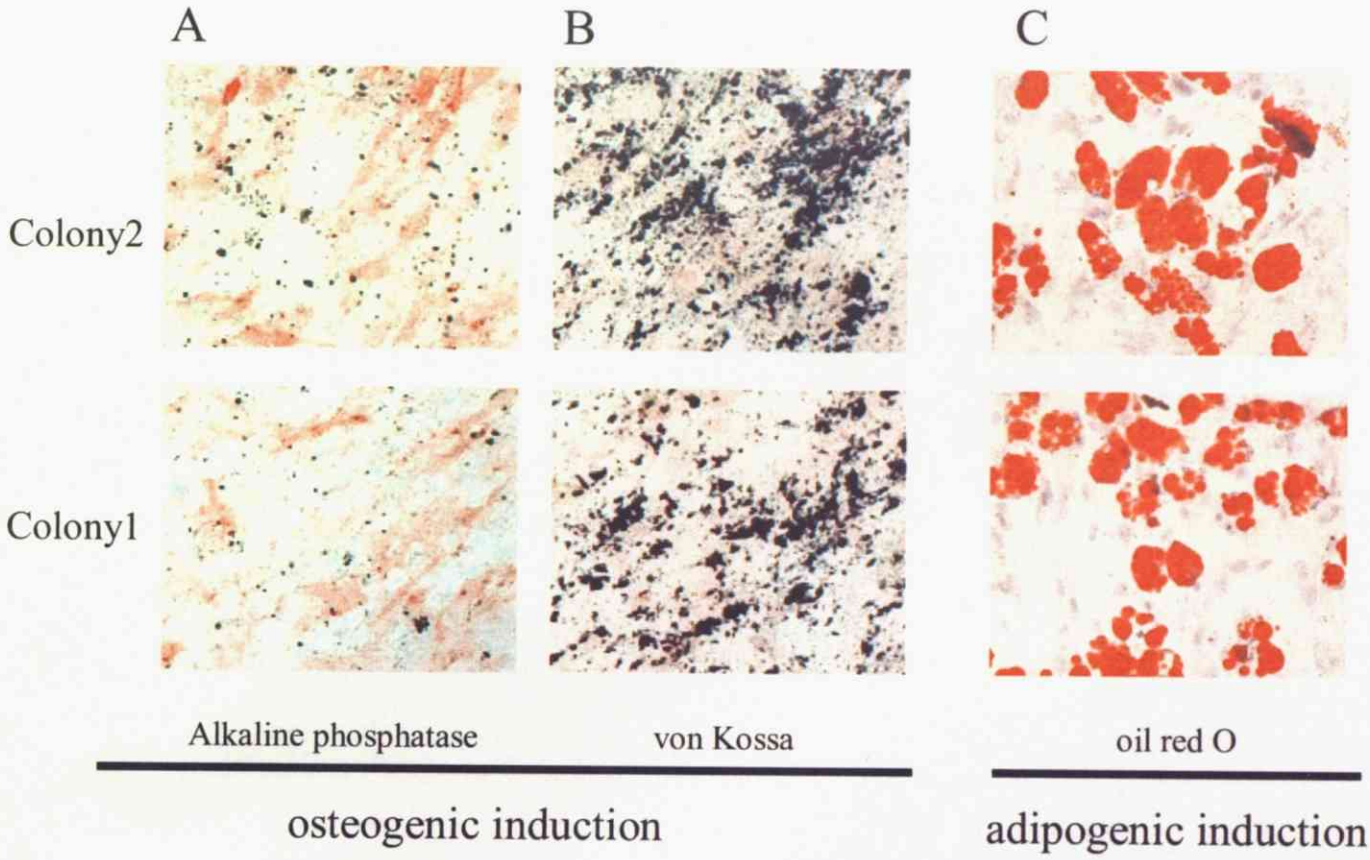

Figure 4. Osteogenic and adipogenic differentiation of fibroblast-like cells derived from the $CD105^+$ fraction of MPCs derived from pulmonary arterial blood.
The two colonies derived from the $CD105^+$ fraction of pulmonary arterial blood after three weeks of osteogenic induction were stained with the von Kossa (A) or to detect alkaline phosphatase (B). The two colonies derived from the $CD105^+$ fraction of pulmonary arterial blood after three weeks of adipogenic induction were stained with oil red O (C).

Table 1. Proliferative capacity of fibroblast-like cells derived from pulmonary arterial blood.

case	age	sex	Days to emerge fibroblast-like cells	passage
3	67	Male	7	P3
4	58	Female	5	P5
7	80	Male	5	P7
25	55	Female	3	P4
26	70	Male	6	P8
27	60	Male	4	P0
28	32	Male	3	P8
35	79	Male	5	P3

Table 2. Protein expression in pulmonary arterial blood, Bone marrow, Lung cancer tissue, and Normal lung tissue-derived fibroblast-like cells.

	PA blood	Bone marrow	Lung cancer	Normal lung
Monocyte marker				
CD14	—	—	—	—
Hematopoietic cell marker				
CD45	—	—	—	—
Stem cell / progenitor markers				
CD34	—	—	—	—
CD117/c-kit	—	—	—	—
Endothelial cell markers				
CD31	—/+	—/+	—/+	—/+
Mesenchymal cell markers				
CD44	+	+	+	+
CD105/endoglin	+	+	+	+
Collagen type I	+	+	+	+
vimentin	+	+	+	+
STRO-1	+	+	+	+
smooth muscle actin	+	+	+	+

Table 3. Isolation and determination of frequency of emerge of mesenchymal progenitor cells derived from pulmonary arterial blood.

selection	No. of donors	Frequency of emergence	Percent positive cells
None	14	14/14	-
CD14	5	3/5	6.6±3.7
CD105	8	8/8	3.9±1.6
CD3	5	2/5	13±5.5
CD20	3	0/3	9.4±5.3
Mouse IgG1	6	0/6	0.94±0.33