Establishment of Bone Marrow-Derived Endothelial Cell Lines From ts-SV40 T-antigen Gene Transgenic Rats

Kenji Hattori,[1,2] Mariko Muta,[1] Masakazu Toi,[3] Hisashi Iizasa,[1,2] Machiko Shinsei,[1] Tetsuya Terasaki,[2,4,5] Masuo Obinata,[2,6] Masatsugu Ueda,[7] and Emi Nakashima[1,2,8]

Received July 21, 2000; accepted October 10, 2000

Purpose. Postneonatal neovascularization is thought to result exclusively from the proliferation, migration, and remodeling of fully differentiated endothelial cells (ECs). Recently, it has been reported that bone marrow contains cells which can differentiate into ECs and contribute to neoangiogenesis in adult species. In this study, we tried to establish conditionally immortalized endothelial cell lines (TR-BME) derived from rat bone marrow.

Methods. Mononuclear cells were isolated and differentiated into ECs at 37°C from the bone marrow of a transgenic rat harboring temperature-sensitive SV40 large T-antigen (ts T-Ag) gene. Then, the cells were transferred and incubated at 33°C, a permissive temperature for ts T-Ag. Expression of vascular endothelial growth factor (VEGF) receptor (VEGFR)-1, 2, Tie-1, 2 and von Willebrand factor (VWF) were assayed by reverse transcriptase-mediated polymerase chain reaction (RT-PCR).

Results. We have established three cell lines incorporating 1,1′-dioctadecyl-3,3,3′,3-tetramethylindo-carbocyanine perchlorate (DiI-Ac-LDL) with a spindle shape. One of these, clone 2, strongly expressed VEGFR-2, and weakly expressed VEGFR-1 and VWF. In contrast, clone 8 showed strong expression of Tie-1, 2, and VWF, and weak expression of VEGFR-1,2. All markers were expressed strongly in clone 3.

Conclusions. These data confirm that the above three TR-BME cells are novel ECs derived from bone marrow progenitors.

KEY WORDS: SV40 large T antigen; bone marrow; endothelial cell; endothelial progenitor cell; cell line; rat.

[1] Department of Pharmaceutics, Kyoritsu College of Pharmacy, Tokyo, Japan
[2] CREST of Japan Science and Technology Corporation (JST), Japan
[3] Tokyo Metropolitan Komagome Hospital, Tokyo, Japan
[4] Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan
[5] NICHe, Tohoku University, Sendai, Japan
[6] Institute of Development, Aging and Cancer, Tohoku University, Japan
[7] YSNT, Tochigi, Japan
[8] To whom correspondence should be addressed. (E-mail: nakashima-em@kyoritsu-ph.ac.jp)

ABBREVIATIONS: BMEPC, bone marrow endothelial progenitor cell; DiI-Ac-LDL, acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate; EC, endothelial cell; EPC, endothelial progenitor cell; G3PDH, Glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate buffered saline; ts T-Ag, temperature sensitive SV40 large T antigen; VWF; von Willebrand Factor.

INTRODUCTION

In the pharmaceutical area, many cells are widely used for the screening of drugs. For example, Caco-2 cells are employed to predict intestinal drug permeability. On the other hand, a variety of cell lines with differentiated functions are still required for the detailed study of tissue functions at cellular and molecular levels. Many cell lines have been generated using transgenic mice harboring the ubiquitously expressing temperature-sensitive T-antigen (ts T-Ag) gene of simian virus 40 (SV40) (1). It has been suggested that cell line from muscles, endothelial cells or hematopoietic stem cells could be used as vehicles for gene therapy. Recently, the usefulness of these cell lines has been demonstrated, and it has been shown that a bone marrow stromal cell line, established from the bone marrow of transgenic mice harboring the ts T-Ag gene, has the potential to differentiate into adipocytic, osteoblastic, chondrocytic, and muscle cells (2).

Bone marrow endothelial cells (BMEC) are likely to play an important role in the "homing" of hematopoietic progenitor cells (3). However, Asahara *et al.* have isolated putative endothelial progenitor cells (EPCs) or angioblasts from human peripheral blood (4). These cells differentiated into endothelial cells (EC) *in vitro,* and the EPC was incorporated into sites of active angiogenesis in ischemic animal models (5). Moreover, it has been shown that there is an increase in circulating EPCs following vascular endothelial growth factor (VEGF) administration *in vivo,* and VEGF-induced mobilization of bone marrow-derived endothelial progenitor cells (BMEPCs) results in an increased differentiation of EPCs *in vitro,* and augmented corneal neovascularization *in vivo* (6). Takahashi *et al.* reported that ciruclating EPCs are mobilized endogenously in response to tissue ischemia, or exogenously by cytokine therapy and thereby increase the degree of neovascularization of ischemic tissues (7). The above reports indicate that the postnatal neovascularization does not rely exclusively on sprouting from preexisting blood vessels (angiogenesis). Instead, EPCs circulate from the bone marrow to incorporate into, and thus contribute to, postnatal physiological and pathological neovascularization, which is consistent with postnatal vasculogenesis.

However, the functions of EPCs and/or BMEPCs are complex and poorly understood. Determining the role of the individual cell types in the heterogeneous population is impossible without being able to isolate a purified cell population. It is desirable to establish the cell lines individually to offer a level of reproducibility, and thus standardization, which cannot normally be achieved using primary cells in complex culture systems. Until now, no BMEPC cell line has been established because of the lack of suitable methods to isolate BMEPC from all the other types of progenitor cells (PCs) in long-term bone marrow cultures. However, various (PCs) with morphology of a different type have already been reported to have been obtained from the bone marrow of ts T-Ag transgenic mice (8).

Using ts T-Ag gene transgenic animals, it may be possible to obtain immortalized BMEC and/or BMEPC lines from functional cell types of bone marrow (1). Moreover, ts T-Ag gene transgenic rats have been established recently (9). By choosing the rat as an animal model instead of the mouse, pharmacokinetic research involving the use of living cells as

0724-8741/01/0100-0009$19.50/0

drugs becomes a possibility. Here, we describe the establishment of conditionally immortalized endothelial cell lines (TR-BME) derived from rat bone marrow.

MATERIALS AND METHODS

Animals

The origin and characteristics of the ts SV40 large T-antigen transgenic rat have been previously described (9). In the present study, a female rat of line 1507-2 was used. All animal experiments were approved by the Institutional Animal Care and Use Committee, and complied with the standards set out in the Guideline for the Care and Use of Laboratory Animals in the Kyoritsu College of Pharmacy.

Preparation of Culture Dish

Forty μg/ml human plasma fibronectin (FN) (Gibco BRL) was put into a collagen type I-coated dish (IWAKI, Japan) for 1 hr at 37°C. The dish was washed once with phosphate buffered saline (PBS).

Establishment of Bone Marrow Endothelial Cells

Bone marrow was obtained from a transgenic rat, dissociated from the bone by flushing with PBSE (PBS with 1 mM EDTA [ethylenediaminetetraacetic acid]) using a 26-gauge needle (4). Bone marrow was collected by centrifugation at 1500 rpm at 4°C. The supernatant was discarded, and 3 ml 0.13 M sodium citrate was added. The suspended bone marrow cells were carefully added to Histopaque-1077 (Sigma, St. Louis, MO), and density gradient centrifugation was performed at 2150 rpm for 20 min at room temperature. Cells were washed with PBSE and cultured on a collagen- and fibronectin-coated 100 mm dish with EBM-2 medium (Clonetics, San Diego, CA) at 37°C.

Cells were harvested with 0.25% trypsin-EDTA (Gibco BRL) at 37°C for 5 min, and cells (8000 cells/dish) were then transferred to a new dish. During the first 12 hr, cells were incubated at 37°C to promote adhesion of cells to the substrata. Then, the cells were incubated in a humidified atmosphere of 5% CO_2 in air at 33°C, an active temperature for SV40 ts T-antigen. The cells were cloned and each colony was isolated using cloning rings (8).

Incorporation of DiI-Ac-LDL

A confluent culture of the cells on poly-L-lysine-coated glass was washed with RPMI 1640 serum-free medium and incubated with 10 μg/ml acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL) for 4 hr at 37°C. The cells were washed three times with RPMI 1640 serum-free medium and fixed with 3% formaldehyde for 20 min. Those incorporating DiI-Ac-LDL were then visualized by fluorescence microscopy (10).

RT-PCR Analysis

Expression of VEGFR-1, VEGFR-2, Tie-1, Tie-2, and von Willebrand Factor (VWF) by the endothelial cells was assayed by reverse transcriptase-mediated polymerase chain reaction (RT-PCR). Total RNA was isolated by the acid phenol procedure using ISOGEN (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) according to the manufacturer's protocol. The first-strand cDNA reaction was performed using ReverTra Ace M-MLV reverse Transcriptase (TOYOBO, Japan). One microgram of total RNA was incubated with 10 U RT, 20 U ribonuclease inhibitor, and 0.5 μg oligo(dT) in a 20 μl reaction vial. Polymerase chain reaction (PCR) amplification was performed using 10% of the first-strand reaction products with 1 U Taq polymerase (Ex Taq; Takara Shuzo Co., Ltd, Shiga, Japan) and a set of primers. The sequence for each primer was as follows: VEGFR-1; the sense primer 5′-CAGAAGAGGATGAGGGTGTCTA-3′ and antisense primer 5′-CCTAATGCCAAATGCCGAAGCC-3′ (386 bp). VEGFR-2; the sense primer 5′-GGTGATCCCATGCCGAGG-3′ and the antisense primer 5′-TTTGAGGACGGGAATTGCCAG-3′ (432 bp). Tie-1; the sense primer 5′-TCTGCCACCTGCCTCACCAT-3′ and the antisense primer 5′-CCTGCAAAGTCTCGATGGTCA-3′ (350 bp). Tie-2; the sense primer 5′-GGGCAAAAATGAAGACCAGCAC-3′ and the antisense primer 5′-GCATCCATCCGTAACCCATCCT-3′ (525 bp). VWF; the sense primer 5′-GCCTCTACCAGTGAGGTTTTGAAG-3′ and the antisense primer 5′-ATCTCATCTCTTCTCTGCTCCAGC-3′ (297 bp). The PCR was carried out using the following protocol. After an initial melting temperature of 94°C for 3 min, there was 30 sec of denaturation at 94°C, 30 sec of annealing at 57°C for VEGFR-2, Tie-1; 59°C for VEGFR-1, Tie-2; 65°C for VWF; and 1 min of extension at 72°C for repeated cycles of amplification, followed by a final extension at 72°C for 10 min. The PCR product was analyzed on a 5% acrylamide gel, stained with ethidium bromide and visualized under ultraviolet light.

Tube Formation Assay

Matrigel (Becton Dickinson, Bedford, MA) was used to coat a 24-well culture plate (0.25 ml/well). After polymerization of the matrigel at 37°C for 1 hr, the cells suspended in 0.5 ml EBM-2 medium (40000 cells/well) were transferred to the gel and incubated at 37°C for 18 hr (11).

Western Blotting

Cells were washed with PBS and lysed with lysis buffer (10% SDS, 10 mM Tris-HCl, 1 mM EDTA, 10% glycerol), followed by heating at 100°C for 10 min, and centrifugation at 12000 rpm for 10 min at 4°C. Supernatants were separated and used as whole cell extracts. Total protein concentrations were determined using albumin as a standard (BCA protein assay kit, Pierce, Rockford, IL). Samples (3 μg protein) were separated on 7.5% denaturing SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was incubated with Tween-PBS (0.05% Tween 20 in PBS) containing 5% nonfat dry milk overnight to block nonspecific antibody binding. The membrane was then incubated with a 1:100 dilution of rat anti-SV40 monoclonal antibody (Oncogen, Seattle, WA), followed by peroxidase-conjugated anti-mouse Ig (Amersham Pharmacia Biotech, UK). Immunoreactive blots were identified using a chemiluminescence detection kit (ECL kit, Amersham Pharmacia Biotech, UK).

RESULTS

Establishment of TR-BME

TR-BME cells were established from the primary culture of adherent marrow cells from a ts T-Ag gene transgenic rat. About 1×10^7 mononuclear cells were isolated from bone marrow of a ts T-Ag gene transgenic rat, and cultured on a collagen- and fibronectin-coated dish with EBM-2 medium in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells differentiated into rounded shapes in three days. After six days of culturing, about 5% of isolated mononuclear cells attached to the dish and differentiated into endothelial cells (Fig. 1).

After the first passage of primary cell culture, cells were plated on a collagen- and fibronectin-coated dish, then cultured for 12 hr at 37°C to promote adhesion of the cells to the substrata. Then, the cells were incubated at 33°C, an active temperature for SV40 ts T-Ag. After the first passage of the primary culture, cells were cloned by colony formation for eight weeks. Ten weeks after primary culture, 16 clones were obtained in an experiment from a single rat, and their morphological phenotypes and growth were examined. Then, we selected three clones; clone 2, clone 3, and clone 8.

TR-BME Differentiation Assay

Endothelial cells are known to incorporate DiI-Ac-LDL (7,8). Almost all primary cultured cells which were incubated for 14 days at 37°C incorporated DiI-Ac-LDL (Fig. 2). The TR-BME cell lines, clone 2 and clone 8, incorporated DiI-Ac-LDL strongly (Fig. 3). Clone 3 showed weaker incorporation of DiI-Ac-LDL than clone 2 and clone 8 (Fig. 3).

Although primary cultured cells had a single "cobblestone" morphology, the established cell lines exhibited a variety of different morphologies. While clone 2 had a typical spindle shape (Fig. 3), clone 3 showed dendritic cytoplasmic processes with a refractive cell edge (Fig. 3). It has been reported that an endothelial cell line has a similar morphological phenotype (8). Clone 8 had a similar shape to clone 2 and was about twice as larger than clone 2 (Fig. 3).

Expression Patterns of Endothelial-Specific-Marker by RT-PCR

Expression patterns of several endothelial-specific-markers,—VEGFR-1 (12), VEGFR-2 (13,14), Tie-1 (15), Tie-2 (4,16), and VWF (3),—were assayed by RT-PCR. VEGFR-1, VEGFR-2, Tie-2, and VWF were expressed in clone 2 but Tie-1 was not. However, all were expressed in clones 3 and 8 and primary endothelial cells (Fig. 4).

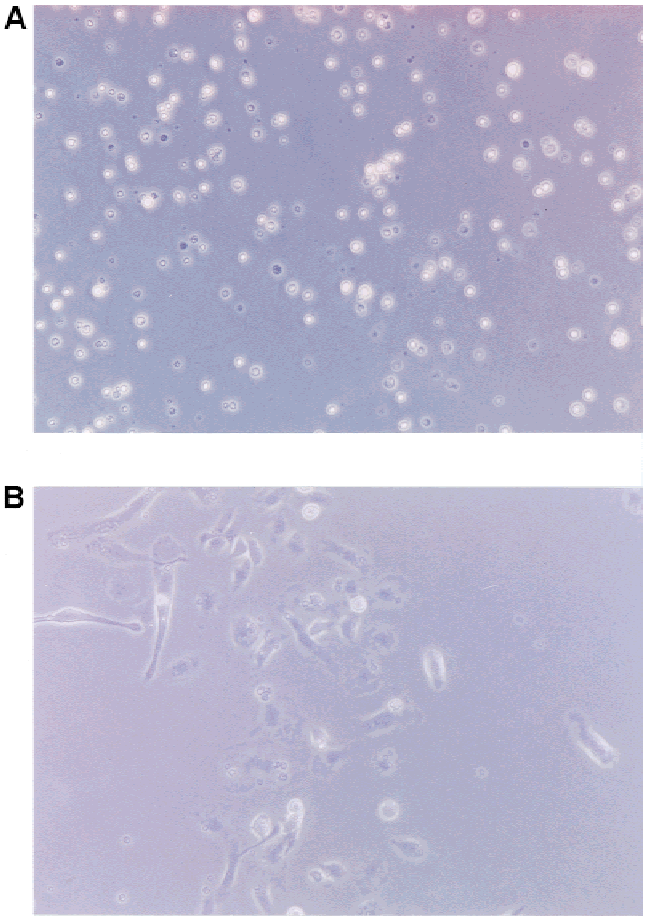

Fig. 1. Attachment of endothelial progenitor cells *in vitro*. The low-density mononuclear cells were spherical and nonadhesive at day 0 (A). About 5% of cells attached and their morphology became round-shaped within 6 days (B).

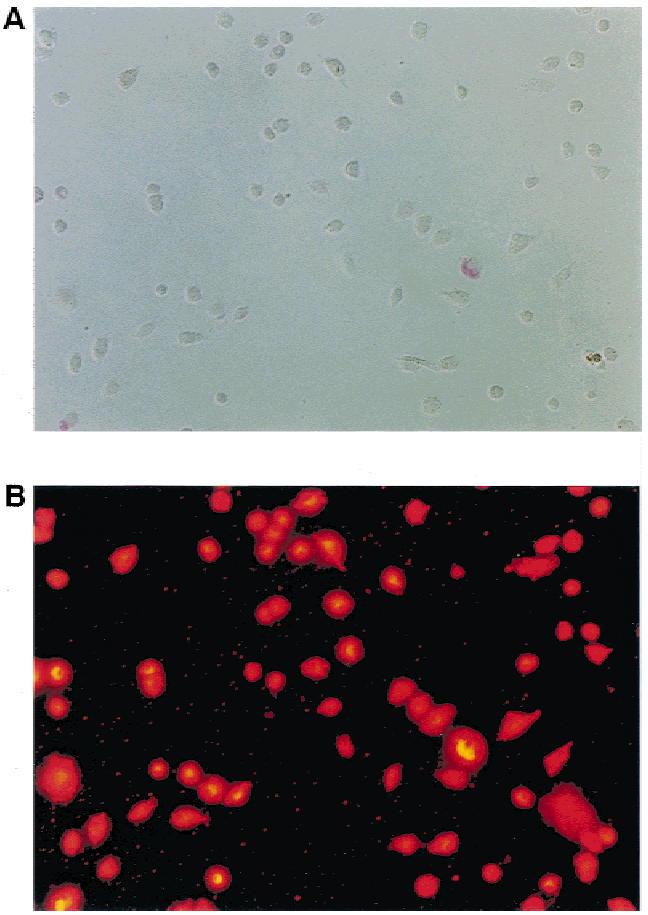

Fig. 2. Incorporation of DiI-Ac-LDL into primary cultured endothelial progenitor cells. The endothelial progenitor cells were cultured on collagen- and fibronectin-coated dishes at 37°C for 14 days and differentiated into endothelial cells. The cultured cells were incubated with 10 μg/ml DiI-Ac-LDL for 4 hr at 37°C. The cells were visualized under a bright field (A) or by incorporation of DiI-Ac-LDL using standard rhodamine excitation (B). Original magnification, ×100 (A–F).

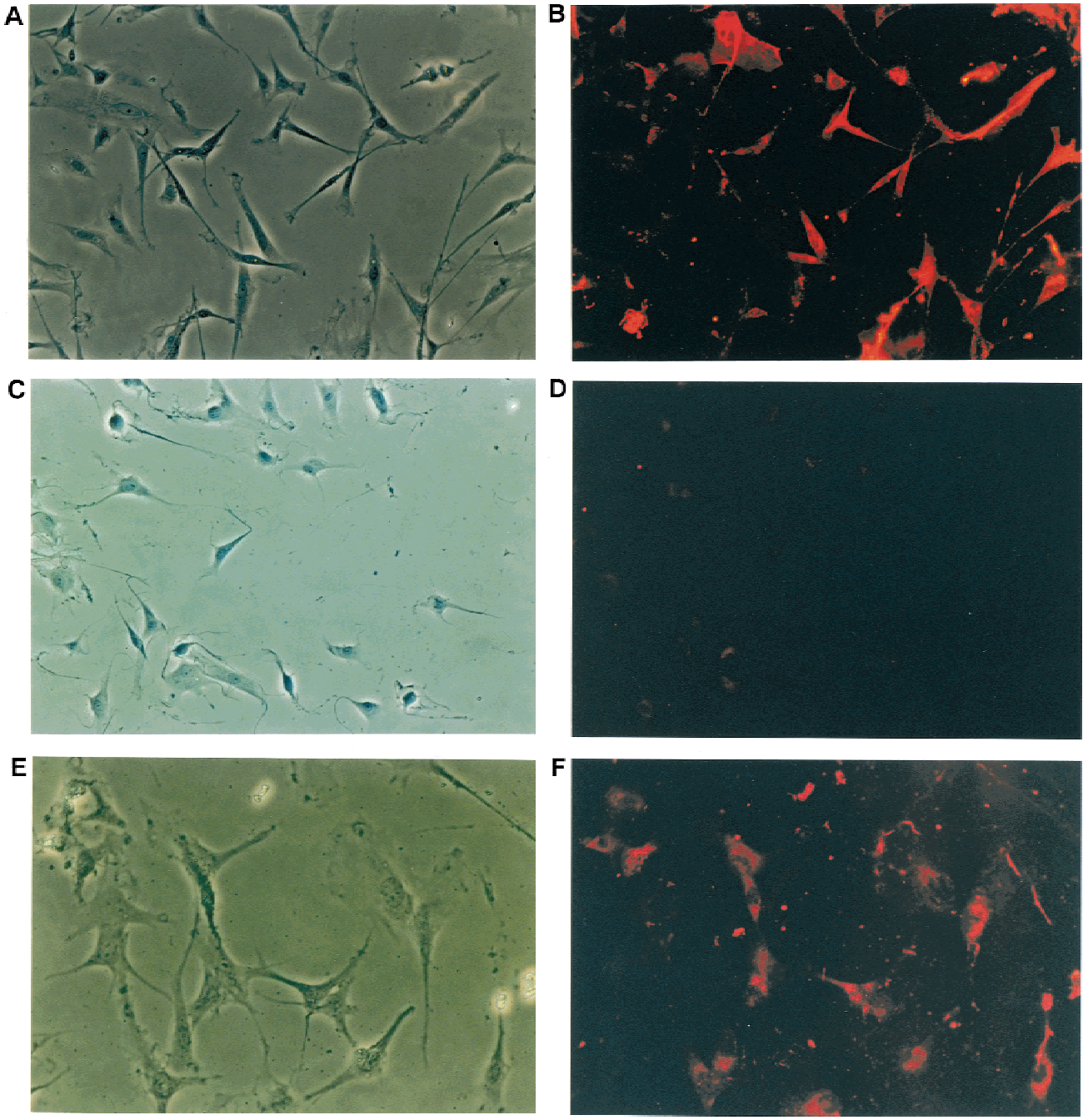

Fig. 3. Incorporation of DiI-Ac-LDL into TR-BME cell lines. Clone 2 (A,B), clone 3 (C,D), and clone 8 (E,F) were incubated with 10 μg/ml of DiI-Ac-LDL for 4 hr at 37°C. The cells were visualized in bright field (A,C,E) or by the incorporation of the DiI-Ac-LDL using a standard rhodamine excitation (B,D,F). Original magnification, ×100 (A–F).

Ability of Tube Formation *in vitro*

The tube formation appeared to be relatively specific for endothelial cells. We have examined tube formation in clone 2, clone 3, and clone 8 (10). Each clone was cultured on Matrigel at 37°C. All began to align themselves end to end and became elongated in Matrigel within 1 hr. After 8 hr, these cell cultures showed an extensive network of branching and anastomoising cords of cells (data not shown). By 18 hr, these cells had formed an interconnected network of anastomoising cells that had a honeycomb appearance under low power light microscopy (Fig. 5). While the tube structure of clone 3 and clone 8 showed similar results, the network of clone 2 was finer than that of clone 3 and clone 8.

Expresson of Temperature-Dependent T-Antigen

TR-BME cell lines from a large T-antigen bearing transgenic rat, clone 2, clone 3, and clone 8, were cultured at the active temperature of 33°C and immortalized (9). We detected SV40 T-antigen by Western blotting. T-antigen was expressed strongly in clone 2, clone 3, and clone 8 incubated at 33°C. When these cells were incubated at 37°C for 4 days,

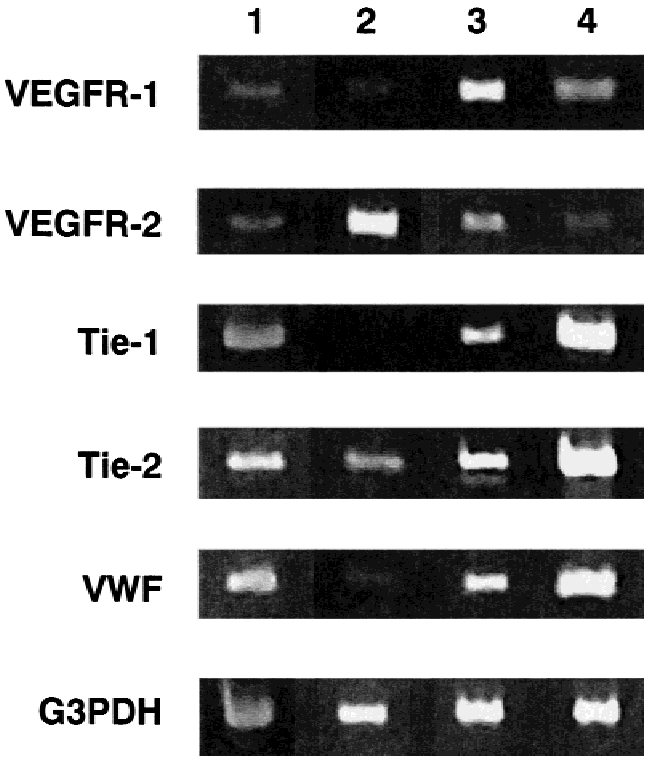

Fig. 4. Expression of endothelial markers in TR-BME cell lines by RT-PCR. Reverse transcriptase-mediated polymerase chain reaction (RT-PCR) with specific primer for VEGFR-1, VEGFR-2, Tie-1, Tie-2, and VWF was performed with total cellular RNAs extracted from clone 2, clone 3, and clone 8 and primary cultured endothelial progenitor cells. RT-PCR with primer for G3PDH was performed as RNA quality and quantity control. Lane 1, primary cultured progenitor cells; lane 2, clone 2; lane 3, clone 3; lane 4, clone 8.

the expression of T-antigen decreased in clone 2, and was undetectable in clone 3 and clone 8 (Fig. 6).

DISCUSSION

In this study, we have established three cell lines, TR-BMEs, derived from the bone marrow of SV40 ts T-Ag transgenic rats. These cells exhibited a variety of characteristics, such as expression of endothelium-specific-marker (Fig. 4), incorporation of DiI-Ac-LDL (Fig. 3), and tube formation (Fig. 5).

The main finding of this study was that putative EPC could be collected from bone marrow by the method used to collect EPC from peripheral blood described by Asahara *et al.* (4). Another finding is the ability to immortalize EPC by using ts T-Ag gene transgenic rats. Even in primary culture of EPC, ts T-Ag increased the viability of cells so it was essential to use transgenic rats for establishing the TR-BMEs.

Much of our knowledge regarding the importance of endothelial cells in tissue remodeling processes has been made possible by the isolation and culturing of endothelial cells derived from large vessels such as human umbilical vein endothelial cell (HUVEC). However, there are several problems associated with these models, such as their limited growth potential, relatively short lifespan, and lot-to-lot variability in functional assays. Growing evidence supports the concept that EPCs are involved in postnatal vasculogenesis in

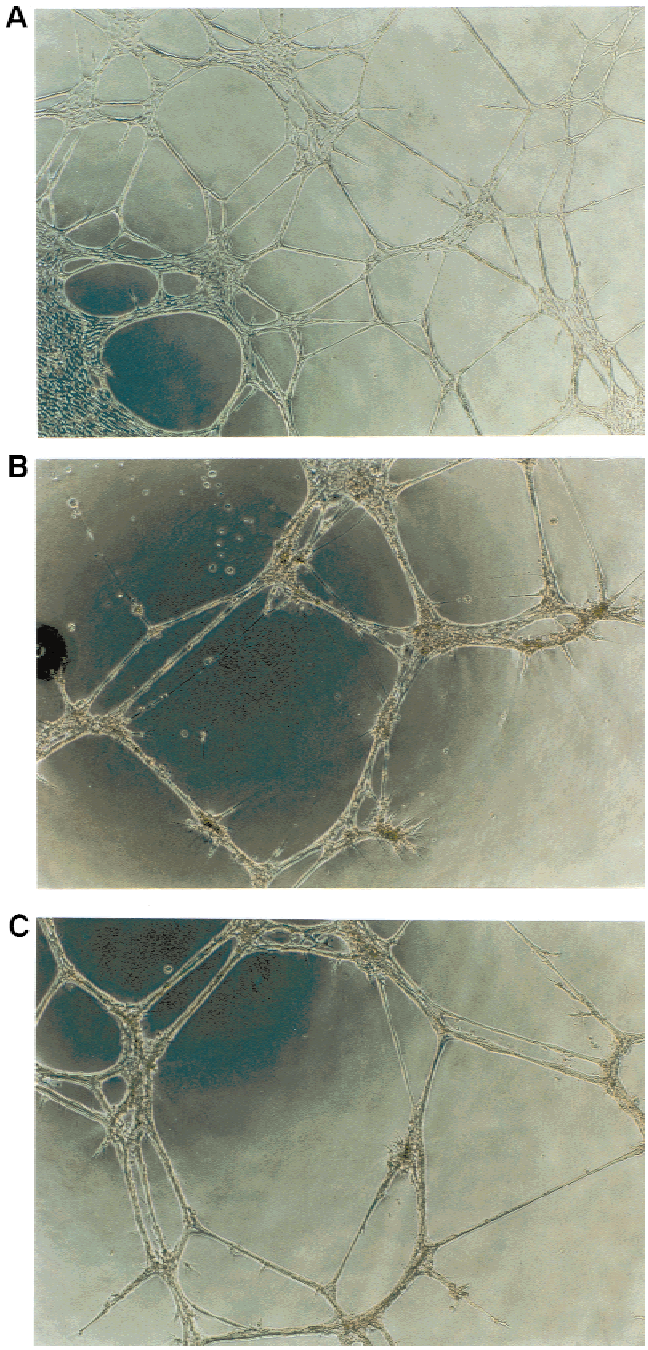

Fig. 5. Tube formation of TR-BME cell lines on Matrigel. Clone 2, clone 3, and clone 8 were cultured on Matrigel for 18 hr. A, clone 2; B, clone 3; C, clone 8. Original magnification, ×40.

physiological and pathological neovascularization (4–7). This indicates the usefulness of the cell lines established in the present study.

Unfortunately, the differences in cell-specific-markers between EC and EPC are still unclear. Asahara *et al.* (4) reported that CD34-positive mononuclear blood cells isolated from human peripheral blood were attached to the substrata and became spindle-shaped when they were cultured on fibronectin-coated dishes. The spindle-shaped cells incorporated DiI-Ac-LDL, and expressed Flk-1 (VEGFR-2) and Tie-2 endothelium-specific-marker.

Our results are similar to theirs except for the morphol-

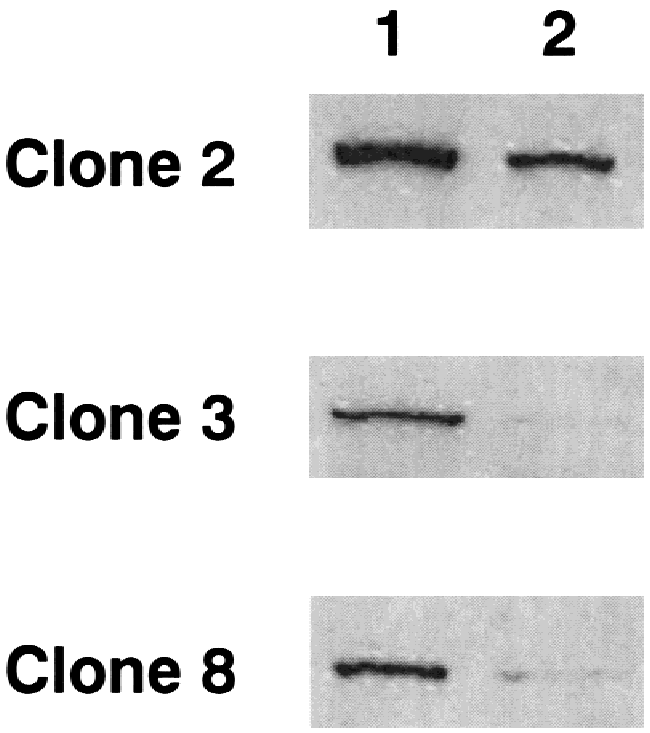

Fig. 6. Detection of SV-40 T-Ag by Western blotting. Western blot analysis was performed with anti SV40-T-Ag antibody. Each clone was cultured at 33°C (lane 1) and 37°C (lane 2) for 4 days.

ogy (Figs. 1 and 2). The initial morphological difference may be due to the fact that our primary cultured cells were isolated from bone marrow and cultured on collagen- and fibronectin-coated dishes. However, as shown in Fig. 3, after cloning, both clone 2 and clone 8 adopted a spindle shape.

Clones 2, 3, and 8 all exhibited endothelial cell characteristics, but these cell lines showed a number of differences. While the morphology of clone 2 and clone 8 was spindle-shaped, clone 3 had dendritic cytoplasmic processes with a refractive cell edge (8). Clone 3 showed weaker incorporation of DiI-Ac-LDL than clone 2 and clone 8. Clone 2 formed a finer network than that of clone 3 and clone 8 in Matrigel (Fig. 5). The doubling time of TR-BMEs is 30 hr, 50 hr, and 40 hr for clone 2, clone 3, and clone 8, respectively. Growth of the established cell lines is positively correlated with the amount of ts T-Ag (Fig. 6).

There have been many discussions involving the specific markers related to the differentiation of EPC. Shalaby *et al.* reported that Flk-1 (VEGFR-2) is essential for yolk sac blood-island formation and vasculogenesis in mouse embryo (14). Nishikawa *et al.* reported Flk-1+ VE-cadherin+ cells as a diverging point of hematopoietic and endothelial cell lineage (13). The TEK (Tie-2)-positive fraction of primary aorta–gonad–mesonephros cells is a candidate for hemangioblasts, which can differentiate into both hematopoietic and endothelial cells (16). Although the process of differentiation was unclear, the degree of differentiation may not be the same in clones 2, 3, and 8.

In conclusion, we have established three novel endothelial cell lines, TR-BMEs, which were differentiated from bone marrow endothelial progenitor cells *in vitro.* These cell lines represent a unique model of angiogenesis. In the future, using various cells established from SV40 ts T-Ag gene transgenic rats, it will be possible to examine the interactions between endothelial cells and organ/tissue cells. And we are investigating the pharmacokinetics of the cells after intravenous injection of the cells. In clinical use, it may become possible to inhibit or activate the angiogenesis *in situ* by using these cells as vehicles for gene therapy.

ACKNOWLEDGMENT

This work is supported in part by funds from the Private School Promotion Foundation and CREST. The authors thank Dr. Asahara for his kind suggestions.

REFERENCES

1. M. Obinata. Conditionally immortalized cell lines with differentiated functions established from temperature-sensitive T-antigen transgenic mice. *Genes Cells* **2**:235–244 (1997).
2. Y. Negishi, A. Kudo, A. Obinata, K. Kawashima, H. Hirano, N. Yanai, M. Obinata, and H. Endo. Multipotency of a bone marrow stromal cell line, TBR31-2, established from ts-SV40 T antigen gene transgenic mice. *Biochem. Biophys. Res. Commun.* **268**:450–455 (2000).
3. C. M. Schweitzer, C. van der Schoot, A. M. Drager, P. van der Valk, A. Zevenbergen, B. Hooibrink, A. H. Westra, and M. M. Langenhuijsen. Isolation and culture of human bone marrow endothelial cells. *Exp. Hematol.* **23**:41–48 (1995).
4. T. Asahara, T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, and J. M. Isner. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**:964–967 (1997).
5. T. Asahara, H. Masuda, T. Takahashi, C. Kalka, C. Pastore, M. Silver, M. Kearne, M. Magner, and J. M. Isner. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ. Res.* **85**:221–228 (1999).
6. T. Asahara, T. Takahashi, H. Masuda, C. Kalka, D. Chen, H. Iwaguro, Y. Inai, M. Silver, and J. M. Isner. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *Embo. J.* **18**:3964–3972 (1999).
7. T. Takahashi, C. Kalka, H. Masuda, D. Chen, M. Silver, M. Kearney, M. Magner, J. M. Isner, and T. Asahara. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat. Med.* **5**:434–438 (1999).
8. J. Kameoka, N. Yanai, and M. Obinata. Bone marrow stromal cells selectively stimulate the rapid expansion of lineage-restricted myeloid progenitors. *J. Cell Physiol.* **164**:55–64 (1995).
9. R. Takahashi, M. Hirabayashi, N. Yanai, M. Obinata, and M. Ueda. Establishment of SV40-tsA58 transgenic rats as a source of conditionally immortalized cell lines. *Exp. Anim.* **48**:255–261 (1999).
10. K. M. Schweitzer, P. Vicart, C. Delouis, D. Paulin, A. M. Drager, M. M. Langenhuijsen, and B. B. Weksler. Characterization of a newly established human bone marrow endothelial cell line: distinct adhesive properties for hematopoietic progenitors compared with human umbilical vein endothelial cells. *Lab Invest.* **76**:25–36 (1997).
11. G. C. Haralabopoulos, D. S. Grant, H. K. Kleinman, and M. E. Maragoudakis. Thrombin promotes endothelial cell alignment in Matrigel in vitro and angiogenesis in vivo. *Am. J. Physiol.* **273**:c239–c245 (1997).
12. B. Witzenbichler, T. Asahara, T. Mourohara, M. Silver, I. Spyridopoulos, M. Magner, N. Principe, M. Kearney, J. S. Hu, and J. M. Isner. Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am. J. Pathol.* **153**:381–394 (1998).

13. S. I. Nishikawa, S. Nishikawa, M. Hirashima, N. Matsuyoshi, and H. Kodama. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development* **125**:1747–1757 (1998).
14. F. Shalaby, J. Rossant, T. P. Yamaguchi, M. Gertsenstein, X. F. Wu, M. L. Breitman, and A. C. Schuh. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376**: 62–66 (1995).
15. M. J. McCarthy, R. Burrows, S. C. Bell, G. Christie, P. R. Bell, and N. P. Brindle. Potential roles of metalloprotease mediated ectodomain cleavage in signaling by the endothelial receptor tyrosine kinase Tie-1. *Lab Invest.* **79**:889–895 (1999).
16. I. Hamaguchi, X. L. Huang, N. Takakura, J. Tada, Y. Yamaguchi, H. Kodama, and T. Suda. In vitro hematopoietic and endothelial cell development from cells expressing TEK receptor in murine aorta-gonad-mesonephros region. *Blood* **93**:1549–1556 (1999).