Niche-Dependent Translineage Commitment of Endothelial Progenitor Cells, Not Cell Fusion in General, Into Myocardial Lineage Cells

Satoshi Murasawa, Atsuhiko Kawamoto, Miki Horii, Shuko Nakamori, Takayuki Asahara

Objective—Previous studies from our laboratory have shown therapeutic potential of ex vivo expanded endothelial progenitor cells (EPCs) for myocardial ischemia. Our purpose was to investigate the mechanisms regulating EPC contribution to myocardial regeneration.

Methods and Results—To evaluate niche-dependent expression profiles of EPCs in vitro, we performed coculture using cultured EPCs derived from human peripheral blood and rat cardiac myoblast cell line (H9C2). Reverse-transcription polymerase chain reaction (PCR) disclosed the expression of human-specific cardiac markers as well as human-specific smooth muscle markers. Cytoimmunochemistry presented several cocultured cells stained with human specific cardiac antibody. To prove this translineage differentiation in vivo, human cultured EPCs were injected into nude rat myocardial infarction model. Reverse-transcription PCR as well as immunohistochemistry of rat myocardial samples demonstrated the expression of human specific cardiac, vascular smooth muscle, and endothelial markers. We observed the distribution of colors (Qtracker; Quantum Dot Corp) in coculture to detect the fused cells, and the frequency of cell fusion was <1%.

Conclusions—EPCs can contribute to not only vasculogenesis but also myogenesis in the ischemic myocardium in vivo. Transdifferentiation, not cell fusion, is dominant for EPCs commitment to myocardial lineage cells. Ex vivo expanded EPCs transplantation might have enhanced therapeutic potential for myocardial regeneration. (Arterioscler Thromb Vasc Biol. 2005;25:1388-1394.)

Key Words: cardiovascular diseases ■ endothelial ■ myocardium ■ regeneration ■ stem cells ■ vasculogenesis

Somatic stem and progenitor cells have recently demonstrated the flexibility in lineage commitment for tissue regeneration. Although bone marrow cells presented multiple lineage potential, hematopoietic stem cell demonstrated translineage commitment into other lineage cells, such as vascular cell,1,2 neural cell,3,4 hepatic cell,5,6, and mesenchymal cell lineages.2 Neural stem cell has also shown the adaptability for another lineages.7,8 These were followed by reports that differentiated endothelial cells, either freshly isolated from mouse dorsal aorta at embryonic day 9 or established as homogenous cells in culture, differentiate into cardiomyocytes, and express cardiac markers when cocultured with neonatal rat cardiomyocytes or when injected into postischemic adult mouse heart. They also demonstrated that human umbilical vein endothelial cells also differentiate into cardiomyocytes.9

Bone marrow-derived endothelial progenitor cells (EPCs)10,11 have shown the potential in myocardial ischemic animal model1,12 via ex vivo expansion and incorporation into foci of neovascularization. The study from our laboratory1 has demonstrated ex vivo expanded EPCs transplantation into ischemic hearts resulted in enhanced myocardial neovascularization, as well as improved cardiac function (such as reduction in left ventricular dilatation). Histological findings supported that there occurred not only vascular regeneration but also myocardial regeneration, contributing to favorable effects of EPCs on cardiac function. Given these results, we believe EPC, which is considered the cell source for vascular regeneration, might reveal favorable potential in heart tissue regeneration, such as cardiomyocyte and vascular smooth muscle cell lineages.

Recently, Badorff et al have reported transdifferentiation of EPC into cardiomyocytes.13 The results encourage the possibility of EPC translineage commitment into cardiomyocyte for the treatment of myocardial ischemic disease. However, they lack the in vivo evidence of this translineage commitment for organogenesis by EPC transplantation to ischemic disease patients. Furthermore, we consider the necessity of

Original received January 24, 2005; final version accepted March 17, 2005.
From the Department of Regenerative Medicine (S.M., A.K., M.H., S.N., T.A.), Institute of Biomedical Research and Innovation/Stem Cell Translational Research, RIKEN Center for Developmental Biology, Kobe, Japan; and the Department of Physiology (T.A.), Tokai University School of Medicine, Japan.
Correspondence to Dr Takayuki Asahara, Department of Regenerative Medicine, Institute of Biomedical Research and Innovation/Stem Cell Translational Research, RIKEN Center for Developmental Biology, Japan 2-2 Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047, Japan. E-mail asa777@aol.com
© 2005 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org DOI: 10.1161/01.ATV.0000168409.69960.e9

1388
pursuing not only myocardial but smooth muscle lineage commitment, which is required for the stabilization of newly formed vasculatures by EPCs themselves. Very recently, Yeh et al have reported transdifferentiation of CD34+ -enriched cell into cardiomyocyte and smooth muscle cell in vivo.14 Their results have shown transdifferentiation of human peripheral blood CD34+ cell into cardiomyocyte was enhanced in the injured heart compared with in the heart without injury, although they did not indicate any functional significance of transdifferentiation.

In this regard, to evaluate niche-dependent expression profiles of EPCs in vitro, we performed coculture of EPCs derived from human peripheral blood and rat cardiac myoblast cell line (H9C2). We also evaluated the frequency of cell fusion phenomenon in the coculture system. Furthermore, to prove equivalent translineage commitment in vivo, human cultured EPCs were transplanted into nude rat myocardial infarction model to sample for transcriptional and expressional evidences.

Methods

Coculture With EPC and H9C2 Cell Line
Total peripheral blood mononuclear cells were isolated from human volunteers by density gradient centrifugation. All procedures were in accordance with the institutional committee. After 4 days in culture, nonadherent cells were removed by washing with phosphate-buffered saline (PBS), new media was applied, and the culture was maintained through day 7 or later. In the culture of EPC after day 7, reseeding was performed once per week.15 Rat cardiac myoblast cell line (H9C2) was cultivated in DMEM with 10% fetal bovine serum and 5% horse serum. EPC was detached at day 7 and re-seeded onto semi-confluent H9C2 monolayer. Coculture was maintained in the feeding medium of H9C2 for 7 days with 1-time application of new suspension (RT-PCR). Detection of specific expression of each target.

Other than GAPDH and mGAPDH were designed to identify human cultured EPCs. For RT-PCR were designed as shown in the Table. These primers were synthesized by Clontech (Palo Alto, Calif). Briefly, each primer was amplified for 35 cycles. In every case, each cycle consisted of heating at 95°C for 30 seconds, followed by 65°C for 3 minutes. The primers for RT-PCR were designed as shown in the Table. These primers other than GAPDH and mGAPDH were designed to identify human specific expression of each target.

Cytoimmunohistochemistry

Day 7 coculture (human EPCs plus H9C2) on a 4-chamber slide was fixed with ice-cold 100% methanol for 7 minutes and washed with PBS 3 times. Cytoimmunohistochemistry was performed using cardiac antibodies, αβ-ventricular myosin heavy chain (Chemicon, Temecula, Calif), brain natriuretic peptide (kindly provided from Dr Itoh, Kyoto University, Japan), cTn-I (Chemicon), smooth muscle lineage antibody, α-SMA (clone 1A4) (Sigma, Saint Louis, Mo), endothelial lineage antibody, CD31 (DAKO, Carpenteria, Calif), and human leukocyte antigen (HLA)-ABC (BD Biosciences Pharmingen) for detecting human cells. Antibodies except for cTn-I and α-SMA are reactive only for humans. We used human-specific αβ-ventricular MHC antibody to evaluate cardiac lineage commitment in coculture with sorted subpopulation of EPCs and H9C2. Proportion of cardiac lineage commitment was evaluated by counting αβ-ventricular MHC-positive cells per total seeded sorting cells in each chamber slide.

Rat Myocardial Infarction Model

Athymic nude rats (Harlan, Indianapolis, Ind) aged 7 weeks and weighing 135 to 140 grams were anesthetized with ketamine and xylazine intraperitoneally. After operatively induced myocardial ischemia,1 the arrhythmic nude rats each received systemic (1×106) or intramuscular injection of 2.5×106 culture-expanded human EPCs in 2 sites of myocardial ischemic lesions; 2.5×106 EPCs were suspended in 25 μL of PBS, and only 25 μL of PBS was injected in control group. We performed EPC transplantation in 10 rats (5 for systemic injection, 5 for intramuscular injection), and injected PBS in 5 rats (control). Three weeks after operation and injection, these rats were euthanized and myocardial samples were put into OCT compound (Sakura, Torrance, Calif) for frozen tissue section (immunohistochemistry) or directly frozen in liquid nitrogen for RNA extraction (RT-PCR).

Immunohistochemistry

Frozen slides were prepared by Criostat (Microm, HM505E; Waldorf, Germany) and stained with cardiac antibodies (αβ-ventricular MHC) (Biocytex, Marseille, France), BNP (kindly provided by Dr Itoh, Kyoto University, Japan), cTn-I (Biomed, Foster City, Calif), or smooth muscle lineage antibody, Calponin (DAKO), or endothelial lineage antibody, CD31 (DAKO). Both αβ-ventricular MHC and cTn-I are different antibodies used in cytoimmunohistochemistry. Connexin43 (BD Biosciences Pharmingen) was used for experiment of gap junction and double-stained with HLA-ABC (BD Biosciences Pharmingen) for detecting human cells. Antibodies except for connexin 43 were active only for humans. We used DAB system (brown) for single antibody staining for visualizing the signals. Double staining was performed using DAB system for HLA antibody and VIP system (purple) for connexin 43 antibody.

Evaluation of Frequency of Cell Fusion in Coculture System

We performed coculture using Qtracker (Quantum Dot Corp, Hayward, Calif), which is a nanocrystal labeling marker incorporated in the vesicles of the cytoplasm. EPCs were labeled with the Qtracker 565 and H9C2 cells were labeled with Qtracker 655. We observed the distribution of colors to detect the fused cells by fluorescent microscopy (Olympus IX71; Tokyo, Japan). When the cells fuse, the fused cell has both colors. Because the nanocrystals are larger than organic dyes, they are not transferred between cells, so each cell type would maintain the single color until they fuse. EPC fusion ratio was detected by counting the number of fused cells that indicated yellow fused cell has both colors. EPC fusion ratio was measured by counting the number of fused cells that indicated yellow color. The data were shown as the mean±SD.
signed 3 types of GAPDH primers, human-specific, mouse-/
cardiac and smooth muscle lineage-specific genes. We de-

Results

Coculture of Human EPCs and Rat Cardiac Myoblasts (H9C2) Expressed Human-Specific Cardiac and Smooth Muscle Markers

We performed RT-PCR to evaluate the human specificity of the primers. Cardiac-specific markers such as cTn-T, MLC-2V, and α-MHC were expressed in human heart RNA (Clontech) but not expressed in RNA from H9C2 and human EPCs. Smooth muscle markers such as sm22α and α-SMA were expressed in RNA from human smooth muscle cells, but not expressed in RNA from rat smooth muscle cells and human EPCs. Endothelial marker such as CD31 was expressed in RNA from human umbilical vein endothelial cells and human EPCs, but not expressed in RNA from rat endothelial cells (data not shown). Also, these primers except for CD31 were not expressed in RNA from human EPCs alone. RT-PCR was performed using these primers 7 days after initiating coculture of human EPCs and H9C2. RT-PCR from coculture samples disclosed the expression of cardiac markers (cTn-T, MLC-2V, α-MHC) and smooth muscle markers (sm22α, α-SMA) (Figure 1a). These data suggested that coculture condition induced human EPCs to express cardiac and smooth muscle lineage-specific genes. We designed 3 types of GAPDH primers, human-specific, mouse-/rat-specific, and both human and rat cross-reactive, to standardize the amount of DNA in each lane.

Cocultured Cells Stained With Cardiac and Smooth Muscle Antibody

Cytoimmunochemistry was performed after fixation of cocultured cells. Human-specific cardiac antibody (α/β-ventricular MHC) stained human EPCs 7 days after initiating coculture with H9C2. The morphology of α/β-ventricular MHC-positive cells was round or spindle, and the frequency was ~0.1% (Figure 2a). Several cocultured EPCs double-stained with both human-specific cardiac antibody (α/β-ventricular MHC) and α-SMA antibodies were observed (Figure 2b). The morphology of these double-stained cells was spindle and the frequency was <0.08%. We observed positive cells for human cardiac antibody besides human endothelial lineage-positive cells (Figure 2c and 2d). Based on the identification of human-derived cells by HLA antibody, Tn-I and HLA double-stained cardiac lineage-positive cells derived from human cells (Figure 2e), and α-SMA and HLA double-stained human-derived smooth muscle lineage cell (Figure 2f). These in vitro data suggested that coculture condition induced human EPCs to express both cardiac and smooth muscle lineage-specific proteins. Furthermore, we
designed coculture with subpopulation of cultured EPCs and H9C2 to define the endothelial marker subpopulation that will mainly contribute to translineage commitment. We sorted CD31 positive fraction and negative fraction, or CD34 positive fraction and negative fraction, and then cocultured with H9C2 in each fraction (Figure 3a). Cytoimmunocytochemistry disclosed the frequency of cardiac lineage commitment in each sorting fraction, and no difference was observed among the coculture for cardiac lineage commitment in coculture with both CD31 and CD34 fractioning (Figure 3b).

**RT-PCR of EPC-Injected Myocardial Samples Demonstrated the Expression of Human-Specific Cardiac and Vascular Smooth Muscle Markers**

RT-PCR using human EPC-injected rat myocardial samples disclosed the expression of cardiac (cTn-T, MLC-2V, α-MHC) and smooth muscle-specific (sm22α, α-SMA) genes. However, RT-PCR using PBS-injected rat myocardial samples did not express any cardiac and smooth muscle genes (Figure 1b). The data confirmed RT-PCR using coculture samples in vitro. We designed human-specific GAPDH to standardize the DNA amount derived from transplanted human cells.

**Immunohistochemistry of EPC-Injected Myocardial Samples Demonstrated the Expression of Cardiac and Vascular Smooth Muscle Markers**

Frozen sections of EPC-injected myocardial samples were stained with human-specific cardiac antibodies (αβ-ventricular MHC, cTn-I, BNP) (Figure 4a-1, 4b-1, 4c-1, respectively), human-specific smooth muscle cell antibody (Calponin) (Figure 4d-1), and human-specific endothelial marker (CD31) (Figure 4e-1). For gap junction experiment, connexin 43 and HLA antibodies were used for double staining because connexin 43 antibody had cross-reactivity with human and rat. In PBS-injected rat myocardium, connexin 43 stained gap

staining by single antibody) were performed using chemical (DAB) method. Nuclei were stained by hematoxylin staining. Photos from (a to e) ×400 magnification. Photos (f-1 and f-2) ×1000 magnification.
junctions in cardiomyocytes (Figure 4f-2). However, double-stained cells disclosed the connection between rat cardiomyocyte and human-derived cell (Figure 4f-1, arrowhead). To test the human specificity of the antibodies, immunohistochemistry was performed using PBS-injected rat myocardium as the negative control. Each antibody did not react with rat cardiomyocytes (Figure 4a-2, 4b-2, 4c-2), rat smooth muscle cells (Figure 4d-2), and rat endothelial cells (Figure 4e-2). These in vivo data suggested that human EPCs transplantation caused multi-lineage differentiation into cardiac, smooth muscle, and endothelial lineages in the ischemic myocardium. Immunostaining by using connexin 43 and HLA revealed that transdifferentiated EPCs connected to other surviving rat derived cardiomyocytes (Figure 4f-1).

### Evaluation of Frequency of Cell Fusion in Coculture System

We performed coculture using Qtracker (Quantum Dot Corp), which is a nanocrystal labeling marker incorporated in the vesicles of the cytoplasm. EPCs were labeled with the Qtracker 565 (red) and H9C2 cells were labeled with Qtracker 655 (green) (Figure 5c). Only EPCs attached to H9C2 incorporated both green and red dye markers (white square in Figure 5c and yellow arrow in Figure 5a and 5b). However, the frequency of cell fusion was very low from these data. We evaluated EPC fusion ratio by counting fusion cells out of total human-derived cells and indicated the frequency as mean ± SD (0.50 ± 0.23%) (Figure 5d). These data were equivalent to the result demonstrated by Badorff et al.15

### Discussion

Previous studies from our and another laboratories showed therapeutic potential of ex vivo expanded EPCs for myocardial ischemia.1,12 We hypothesized that EPCs can contribute to not only vasculogenesis but also myogenesis in the ischemic myocardium. Although differentiated endothelial cells were the candidate for therapeutic application of ischemic disease, EPCs proved themselves as much more effective by animal model experiment16.

Considering the flexibility of somatic stem and progenitor cells for lineage commitments,1,8,13 we investigated whether the translineage commitment of EPCs contribute to cardiomyogenesis and vasculogenesis for functional improvement after EPC transplantation. To elucidate the mechanism of translineage commitment, we developed the detection system to differentiate target cell transcription and expression. Established coculture system detected human myocardial and smooth muscle lineage profiles from the cell population derived from human EPCs and rat cardiomyocytes without cross-reactivity between species. Rat cardiac myoblast cell line (H9C2) was cocultured with human EPCs for RT-PCR to distinguish species-specific markers. The RT-PCR system detected only human-specific cardiac and smooth muscle markers but not rat cardiac and smooth muscle markers. Using this system, translineage commitment from EPC to cardiac and smooth muscle lineages was detected precisely.

Using human-specific cardiac antibody (αβ-ventricular MHC), the percentage of positively stained EPCs was ≈0.1% among incubated EPCs by immunohistochemical determination. In addition, using both human-specific cardiac and smooth muscle antibodies, the percentage of double-positively stained EPCs was <0.08%. This indicates the phenomenon of EPC translineage commitment is not a common differentiation cascade during in vitro condition cocultured with myocardial lineage cells. Despite that we have already found the therapeutic potential of cultured EPCs in ischemic animal models, it still remains the issue which subpopulation of EPCs mainly contributes to cardiac lineage commitment. To address this point, we performed the sorting of cultured EPCs using CD34 or CD31 surface marker as one of the candidate markers for EPC and also established markers for endothelial cells. It should be noted that no specific markers are available for purifying EPCs yet, although a lot of challenges have been reported from various laboratories around the world. However, it could be possible...
to compare positive and negative fractions and evaluate the
tendency regarding cardiac lineage commitment. Our find-
ings suggested that coculture in positive or negative fractions
with cardiac lineage cells (H9C2) revealed no difference in
cardiac lineage commitment in the case of both CD31 and
CD34 fractioning as shown in Figure 3b. In this experiment,
we conclude that at least both CD31 and CD34 are not key
markers to determine the contribution of cardiac lineage
commitment, and that the chance of contamination of mes-
enchymal stem cells is excluded because negative fraction
that is supposed to include mesenchymal stem cells is
incompetent in cardiac lineage commitment compared with
positive fraction of CD31 or CD34. We will make effort to
identify the precise marker for purifying EPCs in our next
research endeavors.

Although we are interested in the emergence of double-
lineage marker expressing (α/β-ventricular MHC and
α-SMA) cell in vitro as the process of translineage commit-
ment, in early heart development multiple smooth muscle
lineage genes are reported to be expressed as regulators of
muscle differentiation. α-SMA as well as sm22-α, a
calponin-related protein, is expressed in cell lines derived
from embryonic and adult hearts.17 These protein detections
might reflect early phase of myocardial lineage differentia-
tion in this coculture system.

As discussed for years, we are still clueless regarding the
mechanism of translineage differentiation. Along with for-
merly discussed transdifferentiation and de-differentiation,
several groups have recently reported spontaneous cell fusion
occurring in coculture between embryonic stem cells and
bone marrow cells,18 or between embryonic stem cells and
brain-derived cells.19 Cell fusion has long been known to
achieve effective reprogramming of cells. Terada et al have
reported that the frequency of spontaneous cell fusion was
very low. Nevertheless, Lagasse et al have reported robust
(30% to 50%) levels of transdifferentiation.6 To define the
frequency of cell fusion in this coculture condition, we used
Qtracker system to determine the population of cell fusion.
The frequency of cell fusion was rarely seen (0.50±0.23%)
though Qtracker system clearly disclosed the phenomenon of
cell fusion. Transdifferentiation, but not cell fusion, is the
main mechanism in our coculture system. Our finding regard-
ning cell fusion is compatible with the data reported by
Badorff et al.13 They have concluded that cell-to-cell contact,
but not cellular fusion, mediated EPC transdifferentiation.
Although our data indicated lower proportion of cardiac
lineage commitment, it could be the difference in methods,
for example, EPC culture method, evaluation method, and
antibodies used for the evaluation. Yeh et al have not
investigated the cell fusion issue in their article; however,
they have also suggested that phenotypic conversion of the
injected CD34+ cells may occur predominantly through
transdifferentiation.14

We have expanded in vitro experiments to deduce whether
this is a pathophysiological phenomenon observed in vivo.
After transplantation of human EPCs to rat ischemic heart
models, myocardial samples disclosed both human cardiac
and smooth muscle, as well as endothelial lineage gene
expressions detected by RT-PCR and immunohistochemistry.

We also performed the experiment to confirm the cross-talk
between ischemic rat cardiomyocyte and transplanted human-
derived EPC by immunohistological staining with connexin
43, one of the gap junctional molecules. The functional
connection was observed between rat cardiomyocyte and
human EPC in ischemic region.

The evidence that translineage commitment of EPCs into
cardiomyocyte and smooth muscle cell lineages in vivo
encourages therapeutic application of EPCs for myocardial
ischemic diseases. The results indicate the occurrence of
niche-dependent translineage differentiation of EPCs for
vasculogenesis and cardiomyogenesis for heart regeneration.
Because the severely damaged myocardium requires signifi-
cant heart organogenesis, the potency of EPCs to supplement
myocardial and smooth muscle lineage cells is very reason-
able to regenerate heart tissues. The emergence of newly
formed cardiomyocyte may reconstitute destroyed myocardi-
um and provide cross-talk signaling toward vasculogenesis.
Furthermore, the occurrence of smooth muscle lineage sup-
ports the maturation and maintenance of newly formed blood
vessels by original endothelial lineage cells derived from
EPCs. Recent publication suggested CD34 transdifferentia-
tion into cardiomyocytes, smooth muscle cells, and endothe-
rial cells in ischemic rat heart.14 These generated systemic
biological cross-talk between lineages are proceleusmatic for
the ischemic heart disease treatment. These data suggest that
EPC transplantation therapy has beneficial effects via both
blood flow improvement and myogenesis in myocardial
regeneration.

However, the frequency of myogenesis observed in this
study is not enough to encourage functional improvement by
transdifferentiation of EPCs themselves. Further
mechanistic investigation is necessary to improve the trans-
differentiation ratio and apply for clinical trial.

Acknowledgments
We appreciate the technical assistance of H. Takano, H. Iwasaki, A.
Oyamada, M. Ishikawa, and K. Sadamoto.

References
1. Kawamoto A, Gwon HC, Yamaguchi JJ, Uchida S, Masuda
H, Silver M, Ma H, Kearney M, Isner JM, Ashara T. Therapeutic
potential of ex vivo expanded endothelial progenitor cells for myocar-
marrow cells regenerate infarcted myocardium. Nature. 2001;410:
701–705.
3. Brazelton TR, Rossi FM, Keshet GI, Blau HM. From marrow to brain:
expression of neuronal phenotypes in adult mice. Science. 2000;290:
1775–1779.
blood into brain: cells bearing neuronal antigens generated in vivo from
5. Petersen BE, Bowen WC, Patrone KD, Mars WM, Sullivan AK, Murase
N, Boga SS, Greenberger JS, Goff JP. Bone marrow as a potential source
6. Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L,
Wang X, Finegold M, Weissman IL, Grompe M. Purified hematopoietic
stem cells can differentiate into hepatocytes in vivo. Nat Med. 2000;6:
1229–1234.
Lendahl U, Friesen J. Generalized potential of adult neural stem cells.


Niche-Dependent Translineage Commitment of Endothelial Progenitor Cells, Not Cell Fusion in General, Into Myocardial Lineage Cells
Satoshi Murasawa, Atsuhiko Kawamoto, Miki Horii, Shuko Nakamori and Takayuki Asahara

Arterioscler Thromb Vasc Biol. 2005;25:1388-1394; originally published online April 28, 2005; doi: 10.1161/01.ATV.0000168409.69960.e9
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/7/1388

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/