Abstract:—The possibility of evaluating the function of transgenes in platelets requires the generation of platelets from nucleated progenitor cells in vitro. In this article, we provide effective culture conditions for generating functional culture-derived (CD) human and mouse platelets from CD34⁺ progenitor cells that allow expression of any foreign protein of interest. We have evolved an effective cytokine cocktail (thrombopoietin, stem cell factor, interleukin [IL]-1β, IL-6) that induces a high yield of CD platelets and optimal shedding from cultivated megakaryocytes generated from CD34⁺ progenitor cells. CD platelets showed similar functional and morphological characteristics compared with isolated blood platelets, including surface expression of platelet antigens (CD41, CD42, CD62P), aggregation, release of granule constituents (P-selectin, platelet factor 4, serotonin). Moreover, transmission electron microscopy revealed the presence of typical α- and dense granules and dense tubular system in CD platelets. Additionally, we showed that stable transgene expression in CD platelets can be performed through infection of CD34⁺ progenitor cells using adenoviral vectors. Thus, we describe a methodology that enables studying functional consequences of transgenes of interest in the natural environment of platelets that may impose substantial impact on potential future platelet research and therapeutic target evaluation. The full text of this article is available online at http://circres.ahajournals.org.

Key Words: platelets ■ progenitor cells ■ megakaryopoiesis ■ thrombosis

Platelets play a central role in a variety of vascular diseases. Considerable interest has focused on the identification and characterization of pivotal signaling proteins in human platelets. The study of proteins that are localized in the cytosol of platelets has, however, been hampered by the fact that these proteins cannot be easily accessed. One approach consisted in the permeabilization of platelets and the subsequent administration of antibodies to inactivate various proteins. Unfortunately, this permeabilization process leads to a severe disturbance of platelet physiology; therefore, the results of these studies are often in conflict with results from gene knockout studies in mice.

A direct approach to study the physiological role of proteins would be to overexpress them in platelets or to inhibit native platelet proteins by overexpression of dominant negative mutants. To date, however, platelets cannot be used for gene transfer directly because of the absence of a nucleus in these cells.

Several studies have investigated the use of in vitro-generated, culture-derived (CD) platelets in hematological settings. The group of Choi and Hunt has shown that platelets can be generated from megakaryocytes in vitro and that they have some characteristic morphological features of mature platelets. Other groups have tried to express foreign transgenes in megakaryocytes by retroviral gene transfer or adenoviral gene transfer. The use of tissue-specific promoters has also been shown. Alternatively, cultured megakaryocytic Meg-01 cells have been used to induce shedding of proplatelets, which were subsequently used for morphological and fluorescence-activated cell sorting (FACS) studies.

In all of these studies, however, the use of gene transfer into the progenitor megakaryocytic cells and subsequent physiological measurements of the originated platelets have not been investigated in detail. Such recombinant platelets could, however, be used to study the functional importance of a variety of interesting intracellular and membrane platelet proteins.

The present study investigates the question of whether functional, active human, and mouse platelets can be gener-
ated from CD34⁺ progenitor cells in vitro, and it investigates the precise conditions under which they can be modified through transgene expression.

Materials and Methods

Isolation of Platelets

Human platelets were isolated from sodium citrate-anticoagulated whole blood collected from healthy volunteers as described. Similarly, mouse platelets were isolated from healthy black-six wild-type mice. Mice were delivered from Charles River (Oelching, Germany), following standard care requirements, and directly used in experiments.

Isolation of Human and Mouse CD34⁺ Progenitor Cells

Human peripheral blood mononuclear cells were obtained from leukocyte-rich buffy coats by density gradient centrifugation on Percoll (Biochrom, Berlin, Germany) at 400 g and 20°C for 30 minutes. After washing, the CD34⁺ cells were enriched by immunoaffinity selection (CD34 Progenitor Cell Isolation Kit; Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer’s instructions, using 300 μL of Macs buffer, 100 μL of Fc-blocking reagent, and 100 μL of anti-human CD34 microbead antibody per 10⁶ peripheral blood mononuclear cells.

For isolation of mouse progenitor cells, bone marrow cells were harvested by flushing the femurs and tibiae of mice with phosphate-buffered saline containing 0.6% CPD-A and 0.5% bovine serum albumin (Sigma, Taufkirchen, Germany) were performed according to manufacturers’ instructions. No adherent cells were collected and resuspended in specific growth medium.

Generation of Megakaryocytes and CD Platelets

In Vitro

The human CD34⁺ cells were cultured in IMDM medium with stable glutamine (Gibco; Wiesbaden, Germany), which was supplemented with 1.5% bovine serum albumin (Sigma, Taufkirchen, Germany), 300 μg/mL iron-saturated transferrin, 1 mM sodium pyruvate, 1× minimum essential medium vitamins, 1× minimum essential medium nonessential amino acids, 0.02 mg/mL L-asparagine, 0.01 mM/L monothioglycerol (all from Gibco). Our new cytokine combination included human thrombopoietin (TPO) (10 ng/mL) and interleukin (IL)-6 (10 ng/mL), both from Cell Systems, IL-1, SCF, and platelet factor-4 (PF-4) (Roche Diagnostics, Mannheim, Germany) were performed according to manufacturers’ instructions.

Adenoviral Infection of Megakaryocytes

Recombinant (E1/E3-deficient) adenoviruses were generated as described. The virus expressed green fluorescent protein (GFP). Megakaryocytes were harvested 7 days after infection and 4×10⁶ cells were infected with a multiplicity of infection of 400 plaque-forming units/cell.

Platelet Flow Cytometry

Human CD platelets were harvested by centrifugation, washed and, where indicated, activated for 10 minutes with thrombin receptor–activating peptide (TRAP, 25 μM/L) or ADP (5 μM/L). To induce conformational change of the glycoprotein (GP)IIb-IIIa receptor (ligand-induced binding site [LIBS] expression), CD platelets or isolated platelets were incubated with GRGDSP peptide (1 mM/L) or the biologically inactive control GRGESP peptide (1 mM/L). CD or freshly isolated platelets were then stained with fluorescein isothiocyanate–conjugated antibodies directed against CD34, CD41 (both Immunotech, Krefeld, Germany), CD42a, and CD62P (both from Dianova, Hamburg, Germany), PAC-1 (Becton Dickinson, Heidelberg, Germany), and LIBS-1 (kindly provided by Dr Mark Ginsberg, Research Institute of Scripps Clinic, La Jolla, Calif). The antibodies for mouse CD41, CD61, and CD62P were from Pharmingen (Leiden, The Netherlands). Platelet flow cytometry was performed as described.

Electron Microscopy

For scanning (SEM) and transmission electron microscopy (TEM), cells were fixed twice in a fixative buffer consisting of glutaraldehyde (2.5%) and tannin (0.02%) in a sodium cacodylate buffer (pH 7.4) and washed and plated on glass slides.

Platelet Aggregation

Where indicated, the aggregometer (Chrono-Log 500 VS) was filled with 250 μL of platelet-rich plasma or a mixture of 10⁷ CD platelets/mL in modified Tyrode buffer containing fibrinogen (300 μM/L) and CaCl₂ (2.5 mM/L). After adding the respective agonist, light transmission was recorded continuously for the following 20 minutes. In some experiments, washed CD platelets were incubated with the peptide GRGDSP or its negative control GRGESP (each 1 mM/L) in the presence of fibrinogen (300 μM/L), CaCl₂ (2 mM/L), or ADP (20 μM/L) under constant agitation using a light transmittance aggregometer. Thereafter, aliquots of CD platelets were analyzed by light microscopy.

Platelet Degranulation

CD platelets or freshly isolated platelets were stimulated with agonists where indicated. Thereafter, supernatant for determination of compounds released from platelets was prepared as described. ELISA assays for platelet factor-4 ( PF-4) (Roche Diagnostics, Mannheim, Germany) and serotonin (DLD Diagnostics, Hamburg, Germany) were performed according to manufacturers’ instructions.

Immunoblot Analysis

Lysates from control or adenovirus infected CD platelets were obtained by incubation platelets in a buffer containing 20 mM/L Tris, 300 mM/L NaCl, 2 mM/L EGTA, 2% Triton X-100, 2% sodium deoxycholate, 0.2% SDS, and protease inhibitors. After incubating 30 minutes on ice, the samples were centrifuged at 16000g to remove insoluble material. Total protein content was determined with standard colorimetric assays (DC Protein Assay, Bio-Rad, Munich, Germany), normalized aliquots of those samples have subsequently been used. Immunoblotting procedures and antibodies have been described previously.

Results

Generation of CD Platelets from CD34⁺ Hematopoietic Progenitor Cells

Human CD34⁺ progenitor cells were isolated from peripheral blood progenitor cells, which were obtained from human blood donors by means of apheresis. After isolation of CD34⁺ cells, we first determined in preliminary studies optimal culture conditions and cytokine combination for expansion and differentiation of CD platelets. After extensive testing of various culture protocols, we found that the combination of TPO (10 ng/mL), SCF (50 ng/mL), IL-6 (10 ng/mL), and IL-1β (10 ng/mL) resulted in significantly higher amounts of CD platelets. Starting from equal amounts of CD34⁺ progenitor cells (100 000 cells in each case), this novel protocol resulted in an ~10-fold higher platelet yield compared with the combination of TPO and SCF alone or TPO and IL-1β.
alone (P<0.05) (Figure 1A). Thus, the combination of TPO with IL-1β, IL-6, and the early acting cytokine SCF boosted platelet formation within 3 weeks of cell culture. Figure 1B shows that under these culture conditions, CD34⁺ progenitor cells differentiated into megakaryocytes within the first 2 weeks. CD platelets are formed from both human and mouse megakaryocytes, mostly in the third culture week (Figure 1B and 1C). Flow cytometric analysis verified that CD platelets exhibited a similar but broader size distribution than freshly isolated blood platelets (Figure 1D).

Whereas the isolated progenitor cells were homogeneously positive for the surface marker CD34 directly after isolation, they subsequently lost this antigen over time and developed expression of the platelet-specific antigen CD41 after the first week (Figure 1E). After 4 weeks, almost all cells were found to be negative for CD34, but intensely...
stained for CD41, indicating that almost all progenitor cells had differentiated into megakaryocytes/platelets (Figure 1E).

To generate mouse megakaryocytes, bone marrow was isolated from the long bones of mice and cultured under cytokine stimulation. Similar to human CD platelets, mouse CD platelets were shed from differentiated mouse stem cells over 4 weeks (Figure 1C). Also, the progressive generation of mouse megakaryocytes was characterized by a subsequent increase in the marker CD41, as assessed by FACS measurements (not shown).

SEM of in vitro generated platelets showed classic ultrastructural features of mature platelets. Figure 2A shows representative SEM images of human CD platelets. CD platelets exhibited morphologies typical for isolated platelets, including a discoid shape of resting platelets and formation of pseudopodia, spreading, and aggregation of activated platelets (Figure 2A). TEM demonstrated a typical platelet ultrastructure as characterized by absence of nuclei and presence of typical platelet-specific granules, corresponding to α-granules and dense granules (Figure 2B). In addition, CD platelets revealed morphological features typical for platelets, such as dense tubular and surface-connected systems.

Figure 2. CD platelets show identical morphology and cellular organelles as freshly isolated platelets. A, SEM of recombinant human CD platelets showing typical morphology at rest. B, TEM of freshly isolated mouse platelets (upper panel) and murine CD platelets (lower panel). CD platelets (lower panel) show identical ultrastructural characteristics than freshly isolated platelets. Pro indicates proplatelet; OCS, open canalicular system; DTS, dense tubular system; M, mitochondria; α, α-granule; dense, dense granule.

Figure 3. CD platelets show the same activation characteristics of surface markers as freshly isolated platelets. A, Representative histograms of immunofluorescence of freshly isolated and CD human platelets showing expression of the GPIb-IIIa complex (CD41) and GPIb (CD42a). B, Representative immunohisto-grams of surface expression of GPIb-IIIa complex (CD41) and of activated fibrinogen receptor (PAC-1 binding) on resting (dotted line) and activated (TRAP, 25 μmol/L) (solid line) human CD platelets (left and middle panel). Right, the effect of GRGDSP (500 μmol/L) (solid line) peptide or GRGESP control peptide (dotted line) on induction of LIBS-1 binding. LIBS-1 binding indicates correct conformational change of GPIb-IIIa on ligand binding.13
CD Platelets Show Functional Features Similar to Isolated Blood Platelets

We compared the physiological functions of CD platelets with those of isolated human and murine platelets by assessing their expression profile of platelet-specific antigens, activation response, and aggregation. Figure 3A demonstrates that the expression of platelet-specific antigens, such as CD41 (GPIIb-IIIa) and CD42a (GPIb), was similar on human CD platelets compared with isolated platelets. The average CD41 fluorescence signal was 88 ± 50 on CD platelets and 57 ± 13 on freshly isolated platelets, and the average CD42a signals were 49 ± 25 and 72 ± 27, respectively (means from 4 measurements each). Stimulation of CD platelets with the TRAP (25 μmol/L) (Figure 3B) or ADP (20 μmol/L; data not shown) induced enhanced surface binding of complex-specific anti-CD41 (GPIIb-IIIa) and of activation-specific PAC-1 mAb (activated fibrinogen receptor).

These results were well reproducible. In a series of 5 independent experiments, the average CD41 signal on CD platelets was shifted from 121 ± 50 (basal) to 300 ± 141 (after addition of 25 μmol/L TRAP) and 311 ± 146 (after addition of 5 μmol/L ADP) (P<0.05 agonist-stimulated versus basal).

In freshly isolated platelets, the corresponding values were 57 ± 13 (basal), 99 ± 25 (TRAP), and 92 ± 37 (ADP) (P<0.05 agonist-stimulated versus basal).

Figure 4. CD platelets show the same characteristics of aggregation as freshly isolated platelets. A, Direct light microscopy of the aggregation of human CD platelets. The aggregation could be specifically blocked by incubation with the antagonist peptide GRGDSP, whereas the control GREDSP did not inhibit aggregation. B, Representative original recordings of light transmittance aggregometry experiments of human CD platelets in response to agonists ristocetin (1.3 mg/mL) and thrombin (0.3 U/mL). For comparison, the corresponding curves of freshly isolated platelets are shown. C, Mean half-maximal (half-max) and maximal (max) activation times (t_{1/2max} and t_{max}) of CD platelets and freshly isolated platelets in response to thrombin. Shown are results from 5 independent experiments with means and SD. D, Concentration-dependent effect of thrombin on maximal aggregation of isolated and CD platelets. Shown are results from 5 independent experiments with means and SD. E, t_{1/2max} and t_{max} of CD platelets and freshly isolated platelets in response to ristocetin. Shown are means and SD of 5 independent experiments.
Similarly Comparable Values Were Also Obtained for Average PAC-1 Expression
To evaluate functional aspects of the GPIb-IIIa receptor complex, CD platelets were incubated with a GRGDSP peptide that induces neoepitopes (LIBS) on GPIb-IIIa (Figure 3B). As described for isolated platelets, GRGDSP, but not the inactive control peptide GRGESP, induced exposure of LIBS-1 epitopes on CD platelets (Figure 3B). Thus, CD-platelet surfaces express functional GPIb-IIIa receptor complexes that undergo conformational change on ligand binding and expose functional ligand binding sites (PAC-1 binding).

To evaluate further functional aspects of GPIb-IIIa on CD platelets, we evaluated aggregation of CD platelets using direct microscopy and light transmittance aggregometry. CD platelets were incubated in the presence of fibrinogen (300 μg/mL), CaCl$_2$ (2 mmol/L), and ADP (20 μmol/L) under agitation for 15 minutes. As shown in Figure 4, under these conditions, platelet aggregates were formed. Aggregation could be specifically inhibited in the presence of GRGDSP but not GRGESP (Figure 4A).

Furthermore, we found that when using light transmittance aggregometry, CD platelets aggregated after stimulation with thrombin or ristocetin, but not in the absence of an agonist (Figure 4B). The time course was similar to that of isolated platelets (Figure 4C and 4E). As expected, higher agonist concentrations resulted in shorter aggregation times. Figure 4D gives the maximum aggregation levels of CD platelets and freshly isolated platelets with 3 different concentrations of thrombin, underlining the reproducibility and comparability of these measurements.

**CD Platelets Degranulate and Release Platelet-Specific Compounds**
As shown above, by electron microscopy (TEM), CD platelets revealed typical platelet-specific granules (Figure 2B) that were secreted after activation. To further characterize the release reaction of α-granules, the surface expression of P-selectin (CD62P) on resting and activated CD platelets was determined by flow cytometry. After stimulation with TRAP (25 μmol/L) or ADP (20 μmol/L), translocation of P-selectin was significantly enhanced (Figure 5A), indicating that degranulation of α-granules occurred. In a series of 5 independent experiments, the mean fluorescence of the CD62P signal on CD platelets was shifted from 46±23 (basal) to 138±65 (TRAP) and to 121±61 (ADP), whereas it was shifted from 59±30 (basal) to 166±48 (TRAP) and 71±30 (ADP) in freshly isolated platelets (P<0.05 thrombin-stimulated versus basal in both groups).

Similarly, another α-granule compound, platelet-factor 4, was released after activation of CD platelets with thrombin or ristocetin to a similar degree when compared with isolated platelets (P<0.05 agonist-stimulated versus basal in both groups) (Figure 5B). To assess the release of dense granules, we evaluated the concentration of serotonin in the supernatant of nonstimulated and activated CD platelets. Similar to isolated platelets, release of serotonin was significantly enhanced in the presence of various platelet agonist (thrombin, ristocetin) (P<0.05 thrombin-stimulated versus basal in both groups) (Figure 5C). Taken together, the results indicate that CD platelets release platelet-specific granule constituents (P-selectin, PF-4, serotonin) after activation with a variety of soluble agonists. Thus, the release reaction of CD platelets is comparable to that observed in freshly isolated platelets.

**Stable Transgene Expression in CD Platelets**
To study the effect of transgenes in CD platelets, we infected human and mouse CD34$^+$ cells with an adenovirus-based vector. Infected the CD34$^+$ cells 7 days after isolation with a recombinant adenovirus resulted in robust expression of the transgenes, which was also maintained in the platelets shed from these megakaryocytes (Figure 6A). To test the infection efficacy, the CD34$^+$ cells were transduced with a GFP-expressing adenovirus, and GFP expression was determined by flow cytometry and immunoblotting (Figure 6B and 6C). We found that transgene expression occurred 48 hours after infection and persisted for >5 weeks. Approximately 40% of CD platelets were positive for GFP fluorescence and could be harvested continuously during that period (Figure 6B). These results indicate that transgenes can be efficiently expressed in
CD platelets by adenoviral infection of CD34⁺ progenitor cells.

**Discussion**

The major findings of this study are as follows. (1) Functional platelets can be generated in vitro from CD34⁺ progenitor cells. These CD platelets are characterized by similar morphological and functional features when compared with freshly isolated platelets. (2) We describe new culture conditions for the generation of CD platelets that optimize the yield of platelets derived from CD34⁺ progenitor cells in culture. (3) Viral gene transfer into CD34⁺ progenitor cells is feasible and causes substantial expression of the respective proteins in CD platelets. Thus, the findings of the present study show that platelets can be generated and genetically modified in vitro, which is an interesting method to study the functional consequences of transgenic proteins of interest in the environment of platelets.

The capacity to analyze platelets produced in culture from CD34⁺ progenitor cells enables the investigation of transgene expression on platelets. Previously, it has been reported that cultured megakaryocytes are capable of production of pro-platelets, which have similar characteristics as platelets. However, the function of these CD platelets has been poorly characterized mainly because of the low yield of CD platelets. Thus, we have evaluated a variety of culture conditions to improve the generation of CD platelets from CD34⁺ progenitor cells. Cytokines play a critical role for proplatelet formation and platelet shedding from megakaryocytes. However, the role of cytokines in generation of CD platelets is poorly understood. Megakaryocytopenesis is a unique process involving formation of polyploid platelet precursor cells with subsequent shedding of platelets via fragmentation of the megakaryocyte cytoplasm.²⁰

It has been suggested that TPO plays a crucial role in megakaryocyte maturation, but is not sufficient for platelet shedding.²¹ At high concentrations TPO might even inhibit proplatelet formation.²² Among the other cytokines, it has been shown in rodents that IL-6, IL-11, and erythropoietin can induce proplatelet formation.²³ The effect of IL-6 on proplatelet formation in humans is controversial.²⁴,²⁵ Others have shown that SCF in combination with promegapotin reveals favorable yields of megakaryocytic cells derived from CD34⁺ progenitor cells.²⁶

In the present study, we compared various cytokine cocktails and found that published culture protocols only allow for
the generation of a relatively low number of CD platelets. However, when human CD34+ progenitor cells were cultured in the presence of TPO, SCF, I IL-1β, and IL-6 in the indicated concentrations, we were able to enhance the yield of CD platelets 10-fold compared with published protocols,2,24 as tested by directly comparing this protocol with conventional ones that have been proposed in the existing literature: (1) the combination of SCF and TPO alone; or (2) a 6-factor combination of SCF, IL-3, FMS-like tyrosine kinase 3 ligand (Flt 3 ligand), IL-6, granulocyte macrophage-colony stimulating factor, and TPO; or (3) the different cytokine protocols investigated by Norol et al.24 In the latter study (which did not include the cytokine combination we used), TPO+SCF was regarded as the best combination. Comparing identical concentrations of cytokines on the same CD platelet preparations in parallel, our new combination was clearly more effective and resulted in markedly higher yields of CD platelets. Similarly, the cytokine cocktail was also optimized for the generation of mouse CD platelets.

This high yield of CD platelets enabled us to thoroughly evaluate various functional characteristics of CD platelets. We found that CD platelets expressed typical platelet-specific plasma membrane glycoproteins (eg, CD41, CD42, CD62P). Activation of CD platelets through soluble agonists, including thrombin and ADP, resulted in activation of the GPIIb-IIIa complex (PAC-1 binding) and ligand binding to GPIIb-IIIa induced induction of cryptic neoepitopes (LIPS epitopes) that indicate correct conformational change of this integrin receptor.13 Further evidence that GPIIb-IIIa is functional on CD platelets is documented by aggregation of CD platelets on stimulation with thrombin or ristocetin, as shown by direct microscopy and light transmittance aggregometry.

Electron microscopy studies showed that our CD platelets had typical morphological and ultrastructural features of isolated platelets. We show that α- and dense granules are present on CD platelets and that they release their content after activation. Stimulation of CD platelets with soluble agonists such as thrombin or ADP resulted in enhanced surface expression of P-selectin and liberation of PF-4 (α-granules) and release of serotonin (dense granules). Thus, we were able to show in the present study, that substantial amounts of platelets can be generated in vitro from CD34+ progenitor cells that are functional with respect to aggregation and degranulation.

The capacity to analyze platelets generated in vitro enables the investigation of transgene expression in the environment of platelets. Genetic modifications of platelet precursor cells might be used to investigate the function of specific genes in anuclear platelets. Previous studies have shown the feasibility of gene transfer in megakaryocyte progenitor cells.5 However, limited work is available whether efficient transgene expression is feasible in CD platelets. Here we show that using an adenosirus-based vector, CD34+ progenitor cells can be efficiently infected, resulting in substantial transgene expression in CD platelets. The method described herein is, thus, suitable for generating a high yield of genetically modified CD platelets with a potential future application for evaluating novel therapeutic target proteins.

Acknowledgments

This study was supported by grants from the Deutsche Forschungsgemeinschaft (Ga 481/4-2, Graduiertenkolleg “Vaskuläre Biologie” GRK 438) and Wilhelm Sander-Stiftung. We thank Kirsten Langenbrink, Sandra Kerstan, Helga Wehners, and Luise Jennen for their assistance.

References

1. Polgar J, Chung SH, Reed GL. Vesicle-associated membrane protein 3 (VAMP-3) and VAMP-8 are present in human platelets and are required for granule secretion. Blood. 2002;100:1081–1083.


Generation of Functional Culture–Derived Platelets From CD34⁺ Progenitor Cells to Study Transgenes in the Platelet Environment
Martin Ungerer, Mario Peluso, Angelika Gillitzer, Steffen Massberg, Ulrich Heinzmann, Christian Schulz, Görtz Münch and Meinrad Gawaz

_Circ Res._ 2004;95:e36-e44; originally published online August 5, 2004; doi: 10.1161/01.RES.0000141700.96085.2e

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/95/5/e36

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ 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 _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/