Therapeutic Angiogenesis of Human Early Endothelial Progenitor Cells Is Enhanced by Thrombomodulin


Objective—We examined the effect of thrombomodulin (TM) domains 2 and 3 (TMD23) on human early endothelial progenitor cells (EPCs).

Methods and Results—TM was expressed and released by human EPCs cultured from peripheral blood mononuclear cells (PBMCs). Addition of TMD23 (100 ng/mL) to the cultured PBMCs increased the colony-forming units, chemotactic motility, matrix metalloproteinase activity, and interleukin-8 secretion but decreased tumor necrosis factor-α (TNF-α) release. Analysis of the signal pathway showed that TMD23 activated Akt. Inhibition of phosphatidylinositol-3 kinase–Akt blocked the effects of TMD23 on chemotactic motility, matrix metalloproteinase-9, interleukin-8, and TNF-α. In hindlimb ischemia mice, laser Doppler perfusion imaging of the ischemic limb during the 21 days after arterial ligation showed that the perfusion recovered best with intraperitoneal infusion of TMD23 plus local injection of early EPCs, followed by either infusion of TMD23 or injection of the cells. Animals without either treatment had the worst results. Animals treated with TMD23 also had lower circulating and tissue levels of TNF-α.

Conclusion—TM is expressed and released by human circulating EPCs. Exogenous TMD23 enhances the angiogenic potential of early EPCs in vitro through activation of phosphatidylinositol-3 kinase-Akt pathway. Coadministration of TMD23 plus early EPCs augments therapeutic angiogenesis of the EPCs in ischemic tissues. (Arterioscler Thromb Vasc Biol. 2011;31:2518-2525.)

Key Words: angiogenesis ■ ischemia ■ peripheral arterial disease ■ signal transduction

The discovery of endothelial progenitor cells (EPCs) has highlighted the importance of these cells in vascular repair and vascular regeneration.1–3 Accumulated data showed that circulating EPCs contain distinct populations, of which, in humans, those cells appearing early from culture of peripheral blood mononuclear cells (PBMCs), named early EPCs, were reported to possess therapeutic potential for alleviating tissue ischemia in occlusive vascular diseases.4 Laboratory investigation has demonstrated that secretion of angiogenic molecules, such as interleukin (IL)-8 and vascular endothelial growth factor (VEGF), is a key mechanism underlying the contribution of the early EPCs to angiogenesis/vasculogenesis.5 These findings suggest that supply of exogenous angiogenic molecules may enhance the therapeutic potential of the early EPCs.

Thrombomodulin (TM) is an endothelial-cell-membrane glycoprotein containing 5 distinct domains: an NH2-terminal lectin-like region (designated D1), a domain with 6 epidermal growth factor–like structures (D2), an O-glycosylation site–rich domain (D3), a transmembrane domain (D4), and a cytoplasmic tail domain (D5).6 Initial studies of TM considered this molecule to be an anticoagulant. Subsequent reports showed that TM has various biological roles, including contribution to cell adhesion, migration, proliferation, and inflammation.7 Recent studies examining recombinant TM domains indicated that the TM domains 2 and 3 (TMD23) contributes to angiogenesis.8 Because EPCs also participate in angiogenesis, this raised the possibility that human EPCs may express and release TM to facilitate angiogenesis. In addition, the angiogenic response to TMD23 shown in animal experiments8 may be attributed to the action of TMD23 on EPCs.

To clarify these issues, in the present study, we examined the expression of TM in human EPCs derived from the PBMCs and evaluated the effects of TMD23 on the early EPCs. We further compared the therapeutic potential of TMD23, the EPCs, and the combined treatment of both in a murine model of hindlimb ischemia. Previous studies had shown that in this model, intramuscular injection of the early EPCs improves subcutaneous perfusion and enhances neovascularization.9
Materials and Methods
Isolation and Cultivation of PBMCs
Ethical approval was granted by the Institutional Review Board of the Mackay Memorial Hospital, Taipei, Taiwan. Informed consent was obtained from healthy donors before the collection of peripheral blood (80 mL). The mononuclear cells were fractionated from other blood components by centrifugation on Ficoll-Paque plus (Amer- sham Biosciences, Uppsala, Sweden) according to the manufactur- er’s instructions. Isolated PBMCs were resuspended in EGM-2 BulletKit system (Lonza, Walkersville, MD) consisting of endothe- lial basalmedium, 2% fetal bovine serum (FBS), human epidermal growth factor, VEGF, human fibroblast growth factor-B, insulin-like growth factor-1, ascorbic acid, and heparin. Cells (1×10^6 cells/cm^2) were seeded on fibronectin-coated dishes or coverslips (both from BD Biosciences, Bedford, MA) supplemented with the EGM-2 BulletKit system and incubated in a 5% CO_2 incubator at 37°C. Under daily observation, the first medium change was performed 3 days after plating. Thereafter, medium was changed every 3 days. In addition, CD34^+ progenitor cells were obtained from the isolated PBMCs using the CD34 MicroBead kit and MACSTIM Cell Sepa- ration System (all from Miltenyi Biotec, Bergisch Gladbach, Ger- many). CD34^+ EPCs were maintained in the EGM-2 BulletKit system supplemented with 20% FBS. Cells (1×10^6 cells/cm^2) were seeded on fibronectin-coated dishes and maintained in a 37°C incubator under a humidified atmosphere of 95% air and 5% CO_2. Under daily observation, the first medium change was performed 3 days after plating. Thereafter, medium was changed every 3 days. A cobblestone-like cell colony could be seen after 2 weeks. The colony seeding contained 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA) for passage.

ELISA
For TM measurement, at 7, 14, and 21 days of PBMCs culture, the medium was collected, centrifuged (4000g, 5 minutes, 4°C) to remove particulates and aggregates, and concentrated using 30 000 MW Amicon Ultra centrifugal filter devices (Millipore, Billerica, MA). Thereafter, TM in the concentration solution was measured using a CD141 ELISA kit (Diaclone, Besancon, France).

For tumor necrosis factor-α (TNF-α), IL-6, IL-8, and VEGF measurement in the cultured EPCs and for measurement of TNF-α and IL-6 in nude mouse serum, see the supplemental material, available online at http://atvb.ahajournals.org.

 Colony-Forming Assay
Isolated PBMCs were resuspended in EndoCulture medium (StemCell Technologies, Vancouver, British Columbia, Canada) or plus different amounts of TMD23 (0.01, 1, or 100 ng/mL) and plated in fibronectin-coated 6-well plates. After 2 days, the nonadherent cells were collected, and 1×10^6 cells were replated on a fibronectin-coated 24-well plate. Five days later, the number of colony-forming units (CFUs) per well was counted. A CFU of EPCs was defined as a central core of round cells with elongated sprouting cells at the periphery (see Supplemental Figure 1A).

Early EPC Chemotaxis Assay
The chemotactic motility of early EPCs was evaluated using a Transwell (Costar, Cambridge, MA) with 6.5-mm-diameter polycar- bonate filters (8 μm pore size). The lower filter surface was coated with 10 μg of gelatin (Merck, Darmstadt, Germany). TMD23 (50 ng), mouse monoclonal anti-TM antibody (1 μg; American Diagnos- tica Inc., Stamford, CT), goat anti-human IL-8 antibody (1 ng/mL; R&D Systems), or TMD23 plus either antibody in endothel- ial basalmedium-2 plus 0.2% FBS (0.5 mL) was placed in the lower wells. Cell suspension (100 μL) containing 5×10^5 cells was loaded into each upper well. The chamber was incubated at 37°C for 4 hours. Cells were fixed with ice-cold methanol and stained with bisbenzamide (1 μg/mL) for 15 minutes. Chemotaxis was quantified with optical microscopy (Leica, Heidelberg, Germany) at ×40 magnification using QWIN image analysis software (Leica) by counting the cells that migrated to the lower side of the filter. Four randomly selected fields in each well were counted. Each test was performed in triplicate, and assays were repeated 3 times. To determine the role of extracellular signal–regulated kinase 1/2 (ERK1/2), phosphatidylinositol 3-kinase (PI3K), and Akt in TMD23-mediated cell migration, assays were performed in the presence of PD98059, LY294002 (a PI3K inhibitor, 10 μmol/L, Sigma-Aldrich), or the Akt specific inhibition peptide.

In Vivo Angiogenesis Model
The work was conducted in accordance with the Republic of China Animal Protection Law (Scientific Application of Animals) of 1998. Forty BALB/c female athymic nude mice, aged 8 weeks and weighing 18 to 22 g, were used. The experiment was a 2×2 factorial design. The animals were anesthetized with intraperitoneal injection of pentobarbital (80 mg/kg) and given a continuous intravenous infusion of either TMD23 (84.5 μg/kg per day; n=20) or phosphate- buffered saline (PBS) (n=20) using an osmotic pump (Alzet, model 2ML4). To create the hindlimb ischemia model, the right femoral artery and vein were ligated and cut from just above the deep femoral arteries to the popliteal artery and vein.24 Twenty-four hours after surgery, 10 animals given TMD23 infusion and 10 animals given PBS were injected intramuscularly with 2×10^3 human early EPCs in 50 μL of saline into the right thigh and calf. The remaining 10 animals given TMD23 infusion and 10 animals given PBS were injected intramuscularly only saline 50 μL. Twenty-one days later, animals were euthanized by intraperitoneal injection of an overdose of pentobarbital. Calf muscles were dissected and prepared for immunohistochemical microscopy and histology examination.

 Laser Doppler Perfusion Imaging
Detection of hindlimb subcutaneous blood flow was performed using a Laser Doppler imager (Moor Instruments, Milwey, United King- dom). Mice were anesthetized with intraperitoneal injection of pentobarbital (160 mg/kg) and placed on a heater at 37°C before scanning. The region of interest included the leg and foot.14 Perfu- sion analyses were performed sequentially at 24 hours after surgery (just before the injection of EPCs) and at 7 and 21 days after the injection. The calculated perfusion was expressed as a ratio of left (ischemic) to right (normal) hindlimb.

Immunofluorescence, Western Blot, Gelatin Zymography, and Real-Time Polymerase Chain Reaction
For immunofluorescence, Western blot, gelatin zymography, and real-time polymerase chain reaction, see supplemental material.

Statistical Analysis
The number of cell cultures or animals used in each experiment is given in the figure legends. Densitometric scanning and analysis were performed on immunoblots and gelatin gel using Totalab software (Nonlinear Dynamics, Newcastle upon Tyne, United King- dom). Data, expressed as mean±SD, were compared by Student t test or ANOVA test followed by post hoc analysis using the Tukey method for the cell culture experiments and the least significant difference method for animal experiments. P<0.05 was considered statistically significant.

Results
 Cultured PBMCs from venous blood exhibited CFUs after 3 to 5 days of culture. The cells were elongated and had a spindle shape, with the typical uptake of acetylated low-density lipoprotein binding of Ulex europeus agglutinin-1 lectin (see Supplemental Figure I for the char- acterization data and cell surface markers).
Figure 1. Detection of thrombomodulin (TM) by immunoconfocal microscopy (A) and ELISA (B). A, presence of TM (red labels) in a colony-forming unit (CFU) of early endothelial progenitor cells (EPCs) (right) but not in freshly isolated peripheral blood mononuclear cells (PBMCs) (left). Nuclei were counterstained with bisbenzamide (blue). Images are of the same magnification. Scale bar=50 μm. B, Time-dependent increase of TM in the medium of cultured early EPCs. At least 12 experiments were done for each bar. *P<0.05 compared with control, #P<0.05 compared with day 7.

TM Was Expressed and Released by Cultured PBMCs

Imunoconfocal microscopy showed that TM was absent in freshly isolated PBMCs (Figure 1A). However, by the time when CFUs appeared, TM was detected in the cells, both inside and outside the CFUs (Figure 1A). Consistent with the immunolabeling findings, TM was found in the medium, and the amount gradually increased during the 21-day period of time (Figure 1B).

TMD23 Increased the CFU Number of Human Early EPCs and Stimulated Chemotactic Motility

To understand the effects of TMD23 on the growth of human early EPCs, 0.01, 1, and 100 ng/mL TMD23 were added to the PBMCs cultured in the EndoCult medium. The results showed that TMD23 increased the CFU number in a dose-dependent manner; at 100 ng/mL, the number increased by 218% (Figure 2A). Similarly, TMD23 enhanced the chemotactic motility of human early EPCs in a dose-dependent manner; at 100 ng/mL, the number of Transwell-migrating cells increased by 105% (Figure 2B). The chemotactic effect of TMD23 was abolished by anti-TM antibody (Figure 2B) and minimally contributed to by the proliferation of EPCs (Supplemental Figure II). Because 100 ng/mL TMD23 had an apparent effect on the EPCs, the dose was used for the following experiments.

Augmentation of Subcutaneous Perfusion and Neovascularization in Hindlimb Ischemia by Combined Administration of TMD23 and Human Early EPCs

In hindlimb ischemia nude mice, infusion of TMD23 by an abdominal osmotic pump enhanced the perfusion of the ischemic limb. Such an effect is equivalent to that of local intramuscular injection of the early EPCs (Figure 3A). Combined infusion of TMD23 and injection of the early EPCs further improved the perfusion (Figure 3A). Analysis also showed that calf muscle of mice given combined administration of TMD23 plus EPCs had the highest value of capillary density, followed by those treated with either TMD23 alone or EPCs alone, leaving the PBS infusion/saline injection group the lowest value (Figure 3B and Supplemental Figure III). Consistent with this, the hindlimb ischemia mice treated with both TMD23 and the early EPCs all preserved the limbs, whereas 20% of the mice treated with TMD23 alone and 20% of those treated with the EPCs alone had toe necrosis. Animals with PBS infusion/saline injection had the worst outcomes (40% had amputation, toe necrosis, or both; see Figure 3C). In addition, analysis of the calf muscle weight showed that animals treated with the EPCs alone, TMD23 alone, or both EPCs and TMD23 had heavier muscle mass compared with the animals treated with PBS infusion/saline injection (Figure 3D).

To clarify the fate of injected human EPCs, the calf muscle was sectioned at the end of animal experiments, and the injected human cells, identified using anti-human nuclear antigen antibody, were counted. In addition, the number of human nuclei antigen+ cells colocalized with CD31 labels, indicative of localization of the EPCs to the vascular wall, were also counted. Analysis showed that in mice with ligation of femoral vessels, those treated with TMD23 had more EPCs per unit area and a trend toward a higher percentage of EPCs colocalized with endothelial cells compared with those without TMD23 treatment (Supplemental Figure IV).

To further understand the response of mice to the experiment procedures, the cell composition of peripheral blood and bone marrow was examined at 24 hours and at 7 days after ligation of femoral vessels. The results showed that at 24 hours after the ligation, the composition of both peripheral blood and bone marrow did not change significantly (Supplemental Figure V). In contrast, the composition changed markedly at 7 days after the ligation. In the peripheral blood, compared with the control mice, intraperitoneal placement of an osmotic pump containing PBS with or without ligation of femoral vessels led to a significant increase of CD14+ CD45+ cells. However, the increase became insignificant when PBS in the pump was replaced by TMD23 (Supplemental Figure VIA). In addition, placement of a pump containing PBS with or without ligation of femoral vessels led to a significant increase of circulating CD34+ cells. However, no increase was seen in mice implanted with a pump containing TMD23, unless femoral vessels were ligated.
On the other hand, in the marrow, both CD34^+ cells and CD34^+/KDR^+ cells were increased in the various treatment groups compared with the control mice (Supplemental Figure VIC and VID).

**TMD23 Enhanced the Secretion of IL-8 and Activity of Matrix Metalloproteinase-9**

An ELISA test showed that addition of 100 ng/mL TMD23 to the cultured cells for 2 hours substantially increased the level of IL-8 in the supernatant of early EPCs (untreated cells, 102.9±10.7 pg/mL; treated cells, 123.3±22.4 pg/mL; untreated versus treated, *P*<0.05; see Figure 4A) but decreased the level of IL-8 in the supernatant of CD34^+ cells (untreated cells, 749.9±60.0 pg/mL; treated cells, 635.0±41.0 pg/mL; untreated versus treated, *P*<0.05), whereas IL-8 was undetectable in the medium made of endothelial basal medium-2 plus 0.2% FBS. Real-time polymerase chain reaction showed that in early EPCs treated with TMD23 for 72 hours, the transcript levels of IL-8 did not significantly change (treated cells/untreated cells, 81.0±3.0%, *P* = 0.38). In contrast, VEGF was present in the medium made of endothelial basal medium-2 plus 0.2% FBS; however, the level of VEGF in the supernatant of the cultured cells was only minimally changed by TMD23 (medium made of endothelial basal medium-2 plus 0.2% FBS, 2.8±0.7 pg/mL; untreated cells, 7.9±3.6 pg/mL; treated cells, 8.4±2.5 pg/mL; untreated cells versus treated cells, *P* = 0.81). Gelatin zymography demonstrated that the activity of matrix metalloproteinase (MMP)-9, but not that of MMP-2, was elevated in the cells treated with 100 ng/mL TMD23 by 40% compared with those without the treatment (Figure 4B).

**TMD23 Induced the Activation of Akt and ERK but Not p38 Mitogen-Activated Protein Kinase**

To further investigate whether the presence of TMD23 induces activation of signal pathways, activation of ERK, AKT, and endothelial nitric oxide synthase was determined by Western blot analysis. Akt and ERK pathways were found activated soon after the addition of 100 ng/mL TMD23. In contrast, there was no change of phospho-p38 mitogen-activated protein kinase (Figure 5A). Endothelial nitric oxide synthase was not detectable (see Supplemental Figure VII).

To understand whether the enhanced chemotaxis by TMD23 is attributed to the enhanced secretion of IL-8 and activation of PI3K-Akt or ERK pathway, in a chemotaxis assay, the lower chamber was loaded with TMD23 plus anti-IL-8 antibody, LY294002, Akt inhibition peptide, or PD98059. The results showed that the enhanced migration by TMD23 was completely abolished by each of anti-IL-8 antibody, LY294002, and Akt inhibition peptide, whereas PD98059 did not significantly attenuate the enhanced migration (Figure 5B and 5C). To further clarify the pathways underlying the increased secretion of IL-8 and enhanced...
activity of MMP-9, the cells were treated for 1 hour with either LY294002 or Akt inhibition peptide followed by addition of TMD23 to the cells for 2 hours. The results showed that the enhanced activity of MMP-9 and the increased secretion of IL-8 were both completely abolished by LY294002 and Akt inhibition peptide (Figure 4).

TMD23 Attenuated the Expression of Inflammation Markers

The levels of both TNF-α and IL-6 in the media of human early EPCs were lower than those of freshly isolated PBMCs. Treatment with TMD23 for 2 hours further reduced the levels of TNF-α but not IL-6 (Figure 5D and 5E). The TMD23-

Figure 3. Animal studies using hindlimb ischemia model to evaluate the effect of thrombomodulin domains 2 and 3 (TMD23), early endothelial progenitor cells (EPCs), and the combination of both. A, Laser Doppler perfusion images and computer-assisted analyses. PBS, intraperitoneal osmotic pump loaded with PBS. NS indicates local intramuscular injection with saline. *P<0.05 between any 2 of the 4 groups of the same time point, except that between PBS/EPC and TMD23/NS, #P<0.05 between PBS/NS and any 1 of the remaining 3 groups of the same time point. **P<0.05 between PBS/NS and TMD23/EPC of the same time point. B, Capillary density of the ligated limb calf muscle. Red labels are CD31-positive endothelial cells. Blue labels are laminin outlining individual myocytes. Scale bar=50 μm. *P<0.05 compared with PBS/NS (leftmost bar), #P<0.05 compared with TMD23/EPC (rightmost bar). C, Outcome of the ligated hindlimbs. D, Muscle weight. *P<0.05 compared with unligated control (Ctrl) hindlimbs, #P<0.05 compared with those without TMD23 or EPCs. See text for details. Ten mice were used for each group.
induced reduction of TNF-α was inhibited by the Akt inhibition peptide but not PD98059 (Figure 5D). In the animal experiments, at 24 hours after ligation of femoral vessels, whereas the circulating levels of TNF-α remained low, the levels of IL-6 were all elevated (Supplemental Figure VIII). At 7 days after the ligation, the circulating levels of both TNF-α and IL-6 increased in animals implanted an osmotic pump containing PBS, regardless of the ligation of femoral vessels. However, when the PBS was replaced by TMD23, both TNF-α and IL-6 levels were significantly decreased or had a trend to decrease (Figure 6A and 6B). Consistently, immunolabeling of calf muscle at the end of experiment showed that in both groups (one group with EPC alone, the other with both EPC and TMD23) of mice with EPCs treatment and the remaining 2 groups (one group with TMD23 alone, the other group without any treatment) without EPCs treatment, the expression of TNF-α and IL-6 was less in the groups given TMD23 compared with the corresponding groups given PBS (Figure 6C).

Discussion

This study demonstrated that TM was expressed and released by human early EPCs. In addition, TMD23 possessed multiple effects on the EPCs, including (1) stimulation of the growth, (2) enhancement of the chemotactic motility, (3) upregulation of MMP-9 activity and IL-8 secretion, (4) augmentation of the subcutaneous perfusion and neovascularization in murine ischemic hindlimb, and (5) attenuation of the expression of inflammation markers. The beneficial effects on the hindlimb ischemia were not only present when TMD23 was administrated alone, but also additive to the effects of the intramuscular injection of the EPCs. In addition, effects of TMD23 on early EPCs involved activation of the PI3K-Akt signal pathway. These novel findings expand the existing knowledge of TMD23 and have clinical implications.

Mechanisms Underlying the Role of TMD23 in Human EPCs

So far, EPCs lack a specific marker or groups of markers for clear identification. Although the cell population under investigation in the present study represents a heterogeneous population of vascular and blood cells/progenitors, the findings presented here should represent the effects of TMD23 on a majority of cells, if not all cells, collected by this standard protocol for isolation and culture of early EPCs. The finding that TMD23 had multiple positive effects on the angiogenic potential of EPCs indicated that the enhanced angiogenesis in response to TMD23 seen in animal experiments was at least partly attributable to the action of TMD23 on the EPCs. Comparing the findings of cell culture experiments from that study with those of the present study, although TMD23 enhanced the angiogenic potential of both human umbilical
had even lower expression. Scale bar = 100 μm.

Figure 6. Effects of thrombomodulin domains 2 and 3 (TMD23) on inflammation markers in vivo at 7 days after ligation of femoral vessels. A, Circulating tumor necrosis factor-α (TNF-α) levels. B, Circulating interleukin (IL)-6 levels. See text for details. *P<0.05 compared with leftmost bar, #P<0.05 compared with the adjacent left bar. At least 6 animals were used for each bar. C, Tissue expression of TNF-α and IL-6 (red labels) at 21 days after the ligation. See text for details. Note that although animals given endothelial progenitor cells (EPCs) plus PBS had low expression of TNF-α and IL-6, animals given EPCs plus TMD23 had even lower expression. Scale bar = 50 μm.

Avascular tissue repair progresses in two phases: inflammation and angiogenesis. Inflammation is a central process involved in the repair of tissue injury, whereas angiogenesis is a process that results in new vessel formation. The hematopoietic progenitor cells isolated from the bone marrow and the peripheral blood (human early EPCs) are a source of the tissue repair activity. Tissue repair involves both local cell interactions and chemotactic factors. In the first phase of inflammation, chemotactic factors attract the cells, followed by angiogenesis and vessel remodeling in the second phase. Inflammation is a central process involved in the repair of tissue injury, and angiogenesis is a process that results in new vessel formation. The hematopoietic progenitor cells isolated from the bone marrow and the peripheral blood (human early EPCs) are a source of the tissue repair activity. Tissue repair involves both local cell interactions and chemotactic factors. In the first phase of inflammation, chemotactic factors attract the cells, followed by angiogenesis and vessel remodeling in the second phase.

However, the present study showed that p38 mitogen-activated protein kinase, and the PI3K-Akt–endothelial nitric oxide synthase pathway was 1000 times higher than the released TM amount detected in the medium, the endogenous TM, mainly located at the cell membrane, the endogenous TM, mainly located at the cell membrane, can still work in an autocrine manner and does not need a large amount for achieving such an action. However, this may require conversion of the membrane-bound TM into the media of EPCs treated with TMD23) and in vivo (evidenced for both TNF-α and IL-6 by reduced blood levels and tissue expression in the ischemic hindlimb in mice treated with TMD23). The lower increment of CD14+CD45− cells in mice treated with TMD23 also supported an effect of TMD23 on the EPCs. Here, the increased secretion of IL-8 by the human early EPCs occurred as early as 2 hours after exposure to TMD23. Based on the effects of TMD23 on the EPCs and the blockade of TMD23’s chemotactic effect by anti-TM antibody shown in the present study, endogenous TM in EPCs may be an important cytokine responsible for the angiogenic activity of the cells. Although in the present study, the amount of TMD23 showing significant effects on the cells was 1000 times higher than the released TM amount detected in the medium, the endogenous TM, mainly located at the cell membrane, can still work in an autocrine manner and does not need a large amount for achieving such an action. However, this may require conversion of the membrane-bound TM into the media of EPCs treated with TMD23.

Another novel finding in the present study is that TMD23 attenuated the expression of proinflammatory mediators. This effect existed in vitro (demonstrated by a reduced TNF-α level in the media of EPCs treated with TMD23) and in vivo (evidenced for both TNF-α and IL-6 by reduced blood levels and tissue expression in the ischemic hindlimb in mice treated with TMD23). The lower increment of CD14+CD45− cells in the blood after pump implantation or ligation of vessels in mice treated with TMD23 also supported an effect of TMD23 against inflammation. Although proinflammatory mediators, such as TNF-α and IL-6, are known to stimulate angiogenesis, overwhelming inflammation leads to severe damage of the tissues, as well as, in the case of cell therapy, the survival of transplanted cells. Taking the damage by inflammation into consideration, the attenuated expression of TNF-α and IL-6 by TMD23 may contribute to the beneficial effects of TMD23 on the recovery of ischemic hindlimb treated either with TMD23 alone or with EPCs plus TMD23. This view was supported by the immunoconfocal finding in the present study that the injected EPCs had a better survival and incorporation into the vascular wall, as evidenced by an increased number of human nuclei antigen+ cells and a trend toward a higher percentage of human nuclei antigen+ cells colocalized with endothelial cells in the ischemic hindlimb of animals treated with TMD23.
The beneficial effects of TMD23 on the murine ischemic hindlimb may not be attributed to enhanced recruitment of progenitor cells from bone marrow into circulation, because either in the marrow or blood, the increased number of CD34+ cells after pump implantation and ligation of vessels was not further increased by administration of TMD23.

Clinical Implication and Future Direction
In human peripheral artery disease, several clinical trials have demonstrated the effects of stem cell of various sources, including early EPCs as used in the present study, to relieve tissue ischemia.4 Because, in the present study, combined administration of TMD23 and early EPCs was superior to early EPCs alone in improving subcutaneous perfusion and preventing tissue necrosis, such a combination therapy seemed promising for human peripheral artery disease. However, peripheral blood derived EPCs have to be expanded ex vivo using specific culture media, which nowadays is not permitted by local authorities in most cases. In contrast, bone marrow-derived progenitor cells are available in adequate amounts without ex vivo culture and have been used in clinical trials. Additional studies are required to approve the use of TMD23 in humans and to examine the effects of combined TMD23 with bone marrow-derived progenitor cells. On the other hand, a previous animal study had shown that mixed transplantation of early EPCs and late EPCs had an synergistic effect on therapeutic angiogenesis.9 The finding of the present study that TMD23 augments the angiogenic effect of early EPCs indicates that the effect of TMD23 is comparable to that of the late EPCs. In addition, IL-8, shown here to be increased by TMD23 in the early EPCs, was also reported to increase the proliferation of late EPCs.10 These findings suggested that TMD23 may also enhance the angiogenic potential of late EPCs, which required additional studies to prove.

In conclusion, TM is expressed and released by human EPCs cultured from PBMCs. Recombinant human TMD23 augments the angiogenic potential of the early EPCs both in vitro and in vivo. The enhancement is mediated via activation of PI3K-Akt pathway. Coadministration of TMD23 plus the progenitor cells has therapeutic potential in human peripheral artery disease.

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Disclosures
None.

References
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Therapeutic angiogenesis of human early endothelial progenitor cells is enhanced by thrombomodulin

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METHODS

Characterization of early EPCs

After 7 days of culture, acetylated-low density lipoprotein labeled with the fluorescent probe 1,1'-dioctadecyl-3,3',3',3'-tetramethyl-indocarbocyanine perchlorate (Dil-acLDL; 1:100; Molecular Probes, Paisley, UK) was added to the medium. Two hours later, the early EPCs were washed with PBS and fixed using 4% paraformadehyde (Sigma) for 10 minutes. After 3 times of PBS wash, the early EPCs were treated with fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin-1 lectin (UEA-1; Sigma) in 0.5% BSA (1:100) at 37°C for 1 hour. After 3 times of PBS wash, the cells were then counterstained with bisbenzamide (1 μg/ml; Sigma) for 15 minutes and mounted. More than 4 images were
taken at high power fields using a Leica TCS SP confocal laser scanning microscope. Data of
the percentage of cells double positive for both UEA-1 and Dil-acLDL were obtained.

**Flow cytometry analysis**

**Early EPCs**

After 5~7 days of culture, the early EPCs were collected and applied to flow cytometry
analysis. 2x10^5 cells were incubated with the antibodies specific to KDR (CD309) conjugated
with Alexa Fluor® 647, CD31 conjugated with FITC, CD14 conjugated with R-phycoerythrin,
and CD45 conjugated with FITC. Isotype-identical antibodies were used as controls. All
antibodies were obtained from BD (San Jose, CA, USA). After 30 minutes of incubation
under 4°C in dark, cells were washed twice and resuspended in HBSS buffer. A total of
20,000 events were collected on FACSCalibur™ flowcytometer (BD, San Jose, CA, USA)
and analyzed using FCS3 express software (De novo software, Los Angeles, CA, USA).

**Peripheral blood and bone marrow**

Bone marrow cells were harvested followed by flushing tibias and femurs, centrifugation, and
resuspend in 500 μL PBS with 1% BSA seven days post ischemia surgery. Peripheral bloods
were collected from the right ventricle. 50 μL peripheral blood or 100 μL bone marrow
cells were stained with the antibodies PE-conjugated CD34, FITC-conjugated CD45 (both from BD, San Jose, CA, USA), PE-conjugated CD14 (eBioscience, San Diego, CA, USA), and Rabbit antibody against mouse KDR (Cell signaling, Danvers, Massachusetts, USA) followed by FITC-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Control isotype IgG2a antibodies were obtained from BD. After incubation, cells were lysed with FACS Lysing solution (BD), washed with PBS and fixed in 1% paraformaldehyde. Total events of 100,000 and 10,000 leukocytes were collected for samples of bone marrow and peripheral blood, respectively. Low SSC cells, including monocytes and lymphocytes, were applied to CD34+ and CD34+/KDR+ cells analysis and all the leukocytes were gated for CD14+/CD45+ cells analysis.

Enzyme-link immunosorbent assay (ELISA)

For tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), and VEGF measurement, at 7 days early EPCs culture medium was changed to EBM-2 plus 0.2% FBS and 100 ng/mL of recombinant human TMD23, the generation and preparation of which had been previously reported.¹ In selected experiments, TAT-AKT-in (an Akt specific inhibition peptide (H-YGRKKRRQRRR-AVTDHPDRLWAWAEKFOH; TAT-TCL110-24); 25 μM; Calbiochem, Merk, Darmstadt, Germany) or PD98059 (an ERK1/2 inhibitor; 10 μM;
Sigma-Aldrich) was added. Two hours later, the supernatant was collected and centrifuged (4000 g, 5 minutes, 4°C) to remove particulates and aggregates. TNF-α, IL-6, IL-8, and VEGF were respectively measured by using human TNF-α, IL-6, IL-8, and VEGF ELISA kit (R&D systems, Minneapolis, USA).

For measurement of TNF-α and IL-6 in nude mice serum, peripheral bloods were collected from the right ventricle at 24 hours and 7 days after vascular ligation surgery. Serum was collected after centrifugation (3000 g, 10 minutes, 25°C). TNF-α and IL-6 were measured by using mouse TNF-α and IL-6 ELISA kit (R&D systems).

**Immunofluorescence**

PBMCs grown on coverslips were fixed with methanol at -20°C for 5 minutes. After blocking with 0.5% bovine serum albumin (BSA), the cells were incubated with a mouse monoclonal anti-TM antibody (1:100; Dako, Glostrup, Denmark) at 37°C for 2 hours followed by incubation with a CY3-conjugated donkey anti-mouse antibody (1:500; Chemicon). The cells were then counterstained with bisbenzamide (1 μg/mL; Sigma) for 15 minutes.
Tissue samples collected from the calf muscles were placed in 30% sucrose-PBS for 24 hours, bisected at the middle level, mounted in OCT compound (Leica), and snap-frozen in liquid nitrogen. For capillary density measurement, two sections taken approximately 3 mm apart were used. One of the sections was from the upper half and the other section from the lower half of the muscles. Both sections were perpendicular to the long axis of the muscles and taken approximately 1.5 mm distant from the bisecting plane (i.e., the 2 sections were taken approximately 3 mm apart). The sections, fixed with methanol for 10 min and washed briefly with PBS, were stained with a mixture of a monoclonal rat anti-murine platelet-endothelial cell adhesion molecule-1 (CD31) antibody (1:200; B&D Pharmingen, San Diego, CA) and a polyclonal rabbit anti-laminin antibody (1:100; Chemicon) at 37°C for 2 hours followed by incubation with a mixture of CY3-conjugated anti-rat antibody and CY5-conjugated anti-rabbit antibody (both from Chemicon). In parallel, methanol-fixed frozen sections were stained with TRITC-conjugated murine EC-specific Bandeiraea simplicifolia lectin 1 (1:50 Sigma) and a polyclonal rabbit anti-laminin antibody (1:100; Chemicon) at 4°C for overnight followed by incubation with a CY5-conjugated anti-rabbit antibody. Capillaries were counted per 30 randomly chosen high-power fields on the 2 sections per animal without amputation of the hindlimb (7 animals in the untreated group and 10 animals in each of the remaining 3 groups). The results were calculated as capillaries per myocyte.
For detection of TNF-α and IL-6 in the calf muscles, frozen sections were fixed with 4% paraformaldehyde for 10 minutes. After blocking with 10 % FBS, the sections were stained with a mixture of goat anti-murine TNF-α or goat anti-murine IL-6 antibody (1:50 for each; R&D systems, Minneapolis, USA) and a rabbit anti-laminin antibody (1:100; Chemicon) at 37°C for 2 hours followed by incubation with a mixture of CY3-conjugated anti-goat antibody (Chemicon) and CY5-conjugated anti-rabbit antibody for 45 minutes.

For localization of human EPCs, 80 consecutive frozen sections, each 10 μm thick, were collected from the muscle tissues of the same anatomic level. Sections of odd number were used for analysis. The section were fixed in 4 % paraformaldehyde for 10 min, washed briefly with PBS, and incubated with MOM kit (Vector, Burlingame, CA, USA) to blocked tissue nonspecific antigen, then stained with a mixture of a monoclonal rat anti-murine CD31 antibody (1:200; B&D Pharmingen, San Diego, CA, USA) and a monoclonal anti-human-specific nuclear antigen (1:50; Chemicon) at 37°C for 2 hours followed by incubation with a mixture of CY3-conjugated anti-rat antibody (Chemicon) and biotin-conjugated anti-mouse antibody (Vector, Burlingame, CA, USA).Finally sections were stained with avdin-conjugated fluorescein (Vector) and counterstained with Hochest 33258 (Sigma).
All stained samples were examined using a Leica TCS SP confocal laser scanning microscope.

**Western blot**

Early EPCs treated with TMD23 (100 ng/mL) for 10, 30 and 120 minutes were lysed with SB-20 buffer (0.69 mol/L SDS, 10 mmol/L EDTA, 100 mmol/L Tris-HCl, pH 6.8). Protein concentrations were determined with modified Lowry’s method (Bio-Rad DC protein assay kit, California, USA). Aliquot of cell lysates were loaded into 10% SDS-polyacrylamide gels, electrophoresed, and transblotted onto PVDF membranes (Amersham, Zaventem, Belgium). The blots were blocked with 0.1 g/mL BSA for 1 hour and detected with primary antibodies specific for phosphorylated ERK1/2, total ERK1/2, phosphorylated p38 mitogen-activated protein kinase (MAPK), total p38 MAPK, phosphorylated Akt and total Akt, and eNOS (all from Cell signaling, Danvers, Massachusetts, USA) with the same dilution 1:1000). The blots were further incubated with alkaline phosphatase-conjugated secondary antibodies for 1 hour at room temperature. Immunoreactivity was visualized using CDP-star system (Roche, Mannheim, Germany) according to the manufacturer’s instruction. To normalize the expression level, blots were stripped with stripping buffer (69 mmol/L SDS, 100 mmol/L 2-mercaptoethanol, 93.75 mmol/L Tris-HCl, pH 6.8) at 56°C, and incubated with
anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:30000; Chemicon) as internal control.

Bromodeoxyuridine (BrdU) incorporation assay

Cell proliferation was determined using bromodeoxyuridine (BrdU) labeling kit (Calbiochem, California, USA). To examine whether the chemotactic motility of TMD23 was attributed to cell proliferation, human EPCs were treated with TMD23 (100 ng/mL) and BrdU (10 mg/mL) for 4 hours and fixed, according to the manufacturer’s instruction. Labeled cells were subsequently identified with a primary antibody against BrdU, a horseradish peroxidase conjugated secondary antibody and visualized by substrate tetra-methylbenzidine. The absorbance was carried out at 450 nm with 540 nm reference.

Gelatin zymography

Early EPCs culture medium at day 7 was changed to EBM-2 plus 0.2% FBS and TMD23 (100 ng/mL) or in the presence of inhibitors. After 2 hours, the condition medium was collected and centrifuged (4000 g, 5 minutes, 4°C) to remove particulates and aggregates. Supernatant was mixed 1:1 with non-reducing buffer (0.0625 M Tris-HCL, PH6.8; 10%
glycerol; 4% SDS; 0.005% bromophenol blue) and then incubated for 30 minutes at room temperature. Samples were loaded onto a gelatin containing gel (7.5% polyacrylamide gel containing 2 mg/mL gelatin) and then electrophoresed at 90V for 3 hour in Tris-glycine SDS running buffer. After electrophoresis SDS was removed from the gel by washing in 2.5% Triton X-100 at room temperature for 30 minutes. And then the gel was placed in incubation buffer (50 mM Tris-HCL, PH7.6; 10 mM CaCl₂·2H₂O; 50 mM NaCl; 0.05% Brij35) overnight at 37 ℃. The gel was stained with 0.25% Coomassie Blue (Sigma) solution of methanol/acetic acid/water (40:10:50 vol/vol) for 1 hour at room temperature and then destained with methanol/acetic acid/water (40:10:50 vol/vol) for 30 minutes at room temperature.

**Real time PCR**

Total RNA from human early EPCs were extracted using RNeasy Plus mini kit (Qiagen). One μg of RNA was applied to the reaction of reverse transcription using SuperScript II First-Strand Synthesis System kit (Invitrogen). One μl of the RT products was used for PCR amplification in a final volume of 10 μl containing specific primers for IL-8 (sense: CCA CTG TGC CTT GGT TTC; antisense : TCT TGC ACA AAT ATT TGA TGC) and β-actin. Amplified products were analyzed by running through 2% (w/v) agarose gels stained with
ethidium bromide. The images were captured and quantified using ImageMaster TotalLab software (AmershamPharmacia Biotech). To conduct real-time PCR, endothelial cDNA were amplified using iQ™ SYBR Green Supermix reagent and detected with iQ™ Single Color Real-Time PCR Detector System (all from Bio-Rad). Relative mRNA levels were normalized with the corresponding levels of β-actin. At least three independent experiments were conducted for analysis.

REFERENCE


Supplemental Figure I

A, cultured PBMCs displayed colony-forming units (CFU), uptake of Dil-acLDL, and binding of UEA-1 lectin. Image analysis showed that 89.9±8.0% of the cultured cells were positive for both Dil-acLDL and lectin UEA-1. B, representative flow cytometry analysis showed that the early EPCs were positive for KDR, CD31, CD14, and CD45. Images in A are of the same magnification. Bar, 100 μm.
Supplemental Figure II. Determination of the proliferation of EPCs treated with and without TMD23 by using BrdU incorporation assay. Note that, compared to untreated EPCs, the BrdU incorporation had a 4% increase in EPCs treated with TMD23 (100 ng/ml) for 4 hours, which are exactly the same dose of TMD23 and exposure time used in chemotaxis assay. Eight experiments were conducted for each bar and Student’s t test was applied.
Supplemental Figure III

A

B

Supplemental Figure III. Capillary density of calf muscle of the ligated limb. Red labels are lectin BS-1-positive endothelial cells, except right image of A, in which red labels are CD31-positive endothelial cells. Blue labels are laminin outlining individual myocytes. A, in
control animals, comparison between lectin BS-1 and anti-CD31 antibody on consecutive
sections of calf muscle showed that cells recognized by lectin BS-1 (left) were less, compared
to anti-CD31 antibody (right). B, analysis of capillary density showed that results using lectin
BS-1 were very much similar to those using anti-CD31 antibody, i.e., combined
administration of TMD23 plus EPCs had the highest value of capillary density, followed by
those treated with either TMD23 alone or EPCs alone, leaving the PBS infusion/saline
injection group the lowest value (see Figure 3B). Annotations, *, #, and number of animals
used are as in Figure 3B. Bar, 50 μm.
Supplemental Figure IV

Supplemental Figure IV. Fate of human EPCs in the ischemic hindlimb post 21 days of vascular ligation. A, double labeling of human nuclear antigen (HNA; green) and CD31 (red) showed that some of the injected human EPCs (positive for HNA) were co-localized with mouse endothelial cells (positive for CD31; see arrows of upper 2 images), while others were
not co-localized (arrowheads of lower 2 images). Nuclei were counterstained with bisbenzamide (blue). Bar, 50 μm. B, image analysis showed that HNA$^+$ cells are increased in mice given TMD23 plus human EPCs, compared to mice given EPCs only. C, image analysis showed a trend that of all HNA$^+$ cells the percentage of HNA$^+$ cells co-localized with mouse endothelial cells are increased in animals given TMD23 plus human EPCs, compared to those given EPCs only. Four mice were used for each bar. Four mice were used for each bar and Student’s $t$ test was applied. *, p<0.05, compared to leftmost bar.
Supplemental Figure V

**A** Peripheral blood

<table>
<thead>
<tr>
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<th>CD14+CD45+ cells (%)</th>
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<td>PBS</td>
<td>-</td>
</tr>
<tr>
<td>TMD23</td>
<td>+</td>
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<tr>
<td>Ligation</td>
<td>+</td>
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**B** Peripheral blood

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<th>CD34+ cells (%)</th>
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<tr>
<td>TMD23</td>
<td>+</td>
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<tr>
<td>Ligation</td>
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**C** Bone marrow

<table>
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<tr>
<td>TMD23</td>
<td>+</td>
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<td>Ligation</td>
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**D** Bone marrow

<table>
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<tr>
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<td>TMD23</td>
<td>+</td>
</tr>
<tr>
<td>Ligation</td>
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Supplemental Figure V. Peripheral blood and bone marrow cell composition in mice at **24 hours post vascular ligation**. No significant difference was seen between the groups.

Four mice were used for each bar.
Supplemental Figure VI. Peripheral blood and bone marrow cell composition in mice at 7 days post vascular ligation. See text for details. Six mice were used for each bar. *, p<0.05, compared to leftmost bar. #, p<0.05, compared to the adjacent left bar.
Supplemental Figure VII

Supplemental Figure VII. Time course of eNOS expression detected by Western blotting.

Note that eNOS was not detectable in the early EPCs without TMD23 treatment (0 minute) or after the treatment (100 ng/mL) for up to 4320 minutes (72 hours). Human aortic endothelial cells (HAEC) were used as a positive control.
Supplemental Figure VIII

Supplemental Figure VIII. Effects of TMD23 on inflammation markers in vivo at 24 hours post ligation of femoral vessels. A, circulating TNF-α levels. B, circulating IL-6 levels. Four mice were used for each bar. *, p<0.05, compared to leftmost bar. #, p<0.05, compared to animals with PBS only.