Effect of Paclitaxel and Mesenchymal Stem Cells Seeding on Ex Vivo Vascular Endothelial Repair and Smooth Muscle Cells Growth

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Abstract: Late thrombosis and neointima proliferation after paclitaxel-eluting stents implanting may be related to delayed endothelial cells (ECs) regeneration. This study was to investigate whether mesenchymal stem cells (MSCs) seeding can accelerate endothelial repair and attenuate late smooth muscle cells (SMCs) proliferation after paclitaxel intervention. An ex vivo model of endothelium repair was developed in which rabbit smooth muscle cells were inoculated in the upper chamber and rabbit endothelial cells/human mesenchymal stem cells in the lower chamber of a co-culture system. Paclitaxel (10 nmol/L, 20 min) inhibited smooth muscle cell growth of the confluent endothelial cell group during the observed period. However, increased smooth muscle cells growth was observed in the proliferative endothelial cells group 10 days after paclitaxel intervention. Mesenchymal stem cell seeding inhibited late smooth muscle cell growth incompatible with the effect of proliferative endothelial cells. However, no inhibition on smooth muscle cell growth was observed with mesenchymal stem cell seeding in comparison to the effect of confluent endothelial cells. No vWF but Flk-1 protein was observed in the 25.71% of mesenchymal stem cells after having been co-cultured with rabbit endothelial cells for 5 days. These results indicate that late smooth muscle cell proliferation is closely related to the delayed endothelial cells regeneration after paclitaxel application. Mesenchymal stem cell seeding partly attenuates the late smooth muscle cell proliferation. Mesenchymal stem cells co-cultured with mature endothelial cells have the ability to differentiate toward endothelial cells.

Key Words: co-culture, endothelial cell, growth, paclitaxel, smooth muscle cell, stem cell

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Despite the fact that percutaneous coronary intervention (PCI) has revolutionized the treatment of coronary artery disease, restenosis is still troublesome to interventional cardiologists.[1,2] The proliferation and migration of vascular smooth muscle cells (SMCs) is the major pathophysiological change of in-stent restenosis and intense effort has been applied to develop therapies to inhibit SMCs overgrowth. Paclitaxel, the potential antineoplasty drug, can stabilize polymerized microtubules and significantly inhibit SMCs overgrowth. Many clinical trials have indicated that paclitaxel-eluting stents promise a striking benefit in interventional treatment of coronary lesions because of the significant low restenosis rate.[3,4] However, some clinical data also show that late neointima proliferation and thrombosis occur, and anti-thrombosis therapy must be enhanced in clinical practice.[5,6]

Endothelial integrity is critical to the prevention of thrombosis and neointima proliferation.[7] The late thrombosis and neointima proliferation may be related to the direct inhibitory effect of paclitaxel on vascular endothelial cells (ECs) regeneration. Repopulating the damaged endothelial layer by stem cell transplantation is a novel method in endothelium reconstruction. Mesenchymal stem cells (MSCs) are self-renewing and multipotential adult stem cells in the bone marrow.[8] Although previous studies have shown that MSCs transplantation can promote neovascularization and regenerate damaged myocardium,[9] it remains unknown whether MSCs can be used to repair injured cellular components of the vessel wall after paclitaxel intervention. We hypothesized that late SMCs proliferation be related to the delayed ECs regeneration after paclitaxel intervention. MSCs seeding could accelerate endothelial repair and attenuate late SMCs proliferation. To explore the feasibility of applying MSCs to endothelium reconstruction after paclitaxel application, an ex vivo model of endothelium repair in a co-culture system was developed. The effects of paclitaxel or paclitaxel and MSCs seeding on the quantitative growth of rabbit vascular SMCs and ECs as well as the plasticity of the seeded MSCs toward endothelial lineage were investigated.

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METHODS

Drug

Paclitaxel (Sigma, St. Louis, MO) was dissolved in 100% ethanol and sterile filtered. Then a stock solution (1 mmol/L) was prepared with Dulbecco Modified Eagle medium (DMEM, Hyclone, Logan, UT). The stock solution was diluted to different test concentrations between 1 nmol/L

and 1 μmol/L before use. The final concentration of ethanol was less than 0.05%.

Cell Culture

Animals

The male New Zealand White rabbits (250–500 g) were purchased from the Experimental Animal Center of Xin Qiao Hospital. The investigation conforms to the European Community guiding principles for the care and use of experimental animals.

Rabbit Endothelial Cells Primary Culture

Endothelial cells were enzymatically isolated from the rabbit aorta and cultured as previously described.[10] The cells of passage 3-5 were used for experiments. More than 95% of cells were positive by immunocytochemical staining with the antibody against vWF-related antigen.

Rabbit Smooth Muscle Cells Primary Culture

Smooth muscle cells were obtained from the rabbit aorta by the explant method.[11] The cells of passage 3–5 were used for experiments. The cells cultured by this method contained at least 95% of SMCs, which were confirmed by positive staining with the antibody against α-SM-actin.

Isolation and Culture of Human Mesenchymal Stem Cells

Mesenchymal stem cells were isolated from bone marrows of volunteers.[8] Informed consent was obtained from each volunteer and the study protocol was approved by the Local Internal Review Board. Nucleated cells were isolated with a density gradient (Ficoll-Paque, 1.077, Pharmacia, USA) and re-suspended in 20 mL DMEM (Hyclone) with the supplements of 10% fetal bovine serum (FBS, Hyclone), 100 U/mL penicillin, and 100 μg/mL streptomycin. All of the cells were plated in plastic culture dishes and incubated at 37°C in a humidified atmosphere with 5% CO_2. Forty-eight hours later, non-adherent cells were discarded and the adherent cells were further incubated for 5 to 7 days. The cells were subcultured, harvested, and re-plated at about 3 cells/cm^2 in 6-well plates (Corning, NY). Then, the single-cell-derived colonies were further expanded and used for experiments.

Co-Culture Protocol

The rabbit SMCs were seeded in the transwell insert membrane (with pores of 0.4 μm in diameter, Corning), and rabbit ECs and/or human MSCs were cultured in the lower chamber of the co-culture system. Paclitaxel of different concentrations (0, 1, 10, 100, and 1000 nmol/L) was added to the lower chamber. The ECs and SMCs were respectively harvested for cell proliferation assay after 24 hours or 20 minutes of paclitaxel application. (See online supplement data).

Cell Proliferation Assay

Cell proliferation was assessed by ^{3}H thymidine (^{3}H-TdR, Shanghai Atomic Nucleus Research Institute, China) incorporation and cell counting as previously described.[12] Cell viability was examined using trypan blue exclusion. Data were expressed by percentage of control or counts per minute per well (cpm/well).

Western Blot Analysis

The expression of SMCs proliferating cell nuclear antigen (PCNA) was analyzed by Western blot as previously described.[13] Briefly, the SMCs were harvested and lysed. Identical amount of protein extract was separated by electrophoresis through SDS-polyacrylamide gel and then transferred onto the PVDF membrane (Roche, Basel, Switzerland). Nonspecific binding was blocked by preincubating the PVDF membrane in phosphate buffered saline (PBS) solution containing 5% nonfat dry milk and 0.1% Tween20 at room temperature for 1 hour. Blots were then incubated with the antibody against PCNA or actin (1:1000, Santa Cruz, CA) at 4°C overnight and subsequently with HRP-conjugated goat anti-mouse IgG (1:1000, Zymed, South San Francisco, CA) for 1 hour at 37°C. Immunoreactivity was revealed by chemiluminescence. Actin reprobes served as a loading control. PCNA protein expression was quantified with scanning densitometry and normalized to actin. Data were expressed by relative optical density (OD) value.

Migration Assay

The migration assay of vascular ECs and SMCs in the co-culture system was performed as previously described by Trochon[14] and Gorog[15] with modifications. First, 1.2% agarose was dissolved in the culture medium to make a gel in the well of 24-well culture plates (Corning). Half of the gel was then cut off for each well. ECs were inoculated into the empty part of the wells and cultured until confluence. Then the remaining agarose gel filling the other half of the well was removed to allow the cells migrating toward the resulting space. Simultaneously, the transwell insert was inserted into the 24-well culture plate and SMCs were seeded in the transwell insert membrane with pores of 8 μm in diameter (Corning). Paclitaxel was then added into the lower chamber. After the cells were co-cultured for 6 hours at 37°C in a humidified atmosphere with 5% CO_2, the insert was removed and non-migrated SMCs were scraped off the membrane with a rubber. The SMCs migrated to the lower surface of the membrane were then stained with hematoxylin and counted in 5 randomly selected optical fields (×400). Twenty-four hours later, the ECs migrated to the other half of each well were counted in 5 randomly selected optical fields (×100). The average number of migrated cells was calculated. The data were expressed by percentage of control.

Fluorescence-Activated Cell Sorter (FACS) Analysis

Using flow cytometry, the surface markers of MSCs were evaluated based on the expression of CD105, CD166, CD34, Flk-1, and vWF. The cells were stained respectively with primary antibodies (mouse anti-human CD105, CD166, and CD34, Pharmingen, San Diego, CA; rabbit anti-human Flk-1 and vWF, Santa Cruz). Negative control was incubated in 0.01 mol/L PBS in the same manner. The cells were subsequently reacted with an FITC-conjugated goat anti mouse or rabbit secondary antibody (Zymed). The stained

cells were then fixed with 1% paraformaldehyde and immediately analyzed with flow cytometry (FACSCalibur, Becton Dickinson, USA).

Fluorescence Immunocytochemistry

To investigate the role of vascular endothelial growth factor (VEGF) in MSCs differentiation, human MSCs were inoculated onto the coverlips. When the MSCs grew to about 60% confluence, the medium was changed with DMEM containing 50 ng/mL VEGF (Sigma). To evaluate the milieu-dependent plasticity of MSCs, rabbit ECs were inoculated onto the coverlips. When the ECs grew to about 60% confluence, MSCs were seeded onto the coverlips with ECs. Six hours later, the non-adherent MSCs were discarded and the adherent cells were further cultured in fresh DMEM with different concentrations of neutralizing anti-VEGF antibody (0, 50, 100, and 500 ng/ml, R&D Systems, Minneapolis, MN). MSCs were either cultured in DMEM with VEGF or co-cultured with mature ECs for 5 days. Then, the coverlips were taken out. The cells were fixed in 4% paraformaldehyde for 5 minutes at room temperature and incubated with the primary antibody (rabbit anti-human Flk-1 1:50, vWF 1:100, Santa Cruz; mouse anti-human CD105 and CD166,1:100, Pharmingen) overnight at 4°C. Negative control was incubated with 0.01 mol/L PBS in the same manner. Subsequently, the samples were stained with goat anti rabbit or mouse secondary antibody (1:100, Zymed) for 30 minutes at room temperature. After the cell nuclei were stained with 4' 6'-diamidino-2-phenylindole (DAPI, 5 μg/mL, Sigma) for another 30 minutes, the samples were examined under a fluorescence microscope (Leica DMIRB, Germany).

Statistical Analysis

Experiments were performed at least 6 times. Data were expressed as mean ± SD. Multiple comparisons were evaluated by one-way ANOVA analysis and significance of the difference between 2 groups was analyzed by post hoc test. The half-inhibitory concentration (IC_{50}) of paclitaxel on the ^{3}H-TdR incorporation and migration of SMCs and ECs was calculated by linear regression. Values of $P < 0.05$ were considered significant.

RESULTS

Effects of Paclitaxel on the Quantitative Growth of Rabbit Smooth Muscle Cells and Endothelial Cells

The ^{3}H-TdR incorporation, cell counting, and migration of rabbit vascular SMCs were inhibited by paclitaxel of 1 nmol/L to 1 μmol/L in a concentration-dependent manner (n = 6, $P < 0.001$). The ^{3}H-TdR incorporation and cell counting of rabbit vascular ECs were inhibited by paclitaxel of 10 nmol/L to 1 μmol/L and migration by paclitaxel of 1 nmol/L to 1 μmol/L in a concentration-dependent manner (n = 6, $P < 0.001$) (Fig. 1). The ^{3}H-TdR incorporation assay resulted in the IC_{50} of 10.09 ± 0.47 nmol/L on SMCs and 19.06 ± 0.35 nmol/L on ECs proliferation. The migration

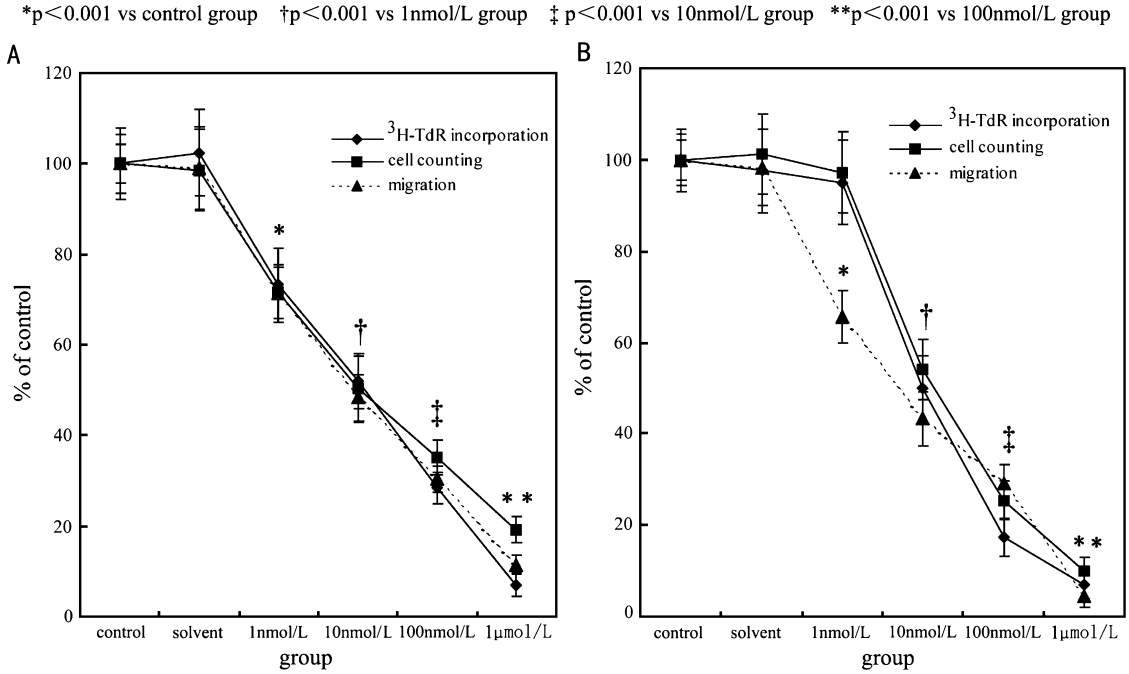

FIGURE 1. Effects of paclitaxel of 1 nmol/L to 1μmol/L on the growth of rabbit vascular smooth muscle cells (SMC) (A) and endothelial cells (EC) (B). A, The ^{3}H-TdR incorporation, cell counting, and migration of rabbit vascular SMCs were inhibited by paclitaxel of 1 nmol/L to 1 μmol/L in a concentration-dependent manner. B, The ^{3}H-TdR incorporation and cell counting of rabbit vascular ECs were inhibited by paclitaxel of 10 nmol/L to 1 μmol/L and migration by paclitaxel of 1 nmol/L to 1 μmol/L in a concentration-dependent manner.

assay resulted in the IC_{50} of 9.16 ± 0.54 nmol/L on SMCs and 5.37 ± 0.51 nmol/L on ECs migration.

The time course of ECs and SMCs growth was investigated after paclitaxel of 10 nmol/L and 20 minutes application. The results showed that the inhibitory effect of paclitaxel on the ^{3}H-TdR incorporation and cell counting of ECs was continued for 3 days. No inhibition on ECs was observed 7 days after paclitaxel application when compared with the control group (Fig. 2). Paclitaxel of 10 nmol/L continuously inhibited the ^{3}H-TdR incorporation and cell counting of SMCs in the confluent ECs group during the observed 10 days. However, no inhibition on SMCs growth was observed 7 days after paclitaxel application and increased SMCs growth was observed 10 days after paclitaxel application in the proliferative ECs group (Fig. 2).

Characterization of Mesenchymal Stem Cells

The mononuclear cells were isolated from human bone marrow and the plastic adherent cells were cultured. The cell colonies were selected and further expanded. The expanded cells initially generated heterogeneous morphologic character. After 2 or 3 passages, they began to appear uniformly spindle shaped. FACS analysis showed that compared with the negative control (4.06 ± 0.23%), these bone marrow cells did not express CD34, Flk-1, or vWF (CD34 expressing cells 4.16 ± 0.16% versus negative control n = 6, P = 0.986; Flk-1 expressing cells 3.86 ± 0.25% versus negative control n = 6, P = 0.999; vWF expressing cells 3.39 ± 0.28% versus negative control n = 6, P = 0.964). However, the cells expressed CD105 and CD166 (CD105 expressing cells 90.20 ± 2.35%; CD166 expressing cells 82.30 ± 3.22% versus negative control, n = 6, $P < 0.001$) (Fig. 3). About 33.42 ± 3.11% of cells began to express vWF protein after culture in DMEM with VEGF (50 ng/ml) for 5 days (Figs. 3 and 4). CD34 is a surface epitope of hematopoietic progenitors. Flk-1 and vWF are the surface markers of ECs. CD105 and CD166 are the major phenotypic molecules of mesenchymal cells. These findings indicate that these cells originate from bone marrow stroma and have the differentiation potential toward ECs.

Although MSCs have been studied extensively for over 30 years, the precise criteria for characterizing the cells have not been developed. The characterization of MSCs is often confirmed by the phenotypic feature and differentiation potential. In this study, the phenotypic characteristics and differentiation potential of the bone marrow cells obtained reveal the nature of MSCs.

Effects of Mesenchymal Stem Cells Seeding on the Proliferation of Smooth Muscle Cells After Paclitaxel Application

After the ECs in the lower chamber were intervened by paclitaxel (1,10, and 100 nmol/L) for 20 minutes, MSCs were seeded in the proliferative ECs layer. The SMCs were harvested 10 days later for cell proliferation assay. (See online supplement data.) The results showed that compared with the control group, the proliferative ECs strongly stimulated but the confluent ECs inhibited the ^{3}H-TdR incorporation of SMCs. MSCs alone did not affect SMCs ^{3}H-TdR incorporation (data not shown). The SMCs ^{3}H-TdR incorporation of the MSCs seeding group was significantly lower than that of the proliferative ECs group (1 nmol/L:12265 ± 991 versus 14505 ± 1013 cpm/well; 10 nmol/L:8401 ± 783 versus 10511 ± 934 cpm/well; 100 nmol/L:5880 ± 569 versus 7457 ± 768 cpm/well, n = 6, $P < 0.001$) but was still higher than that of the confluent ECs group (1 nmol/L: 12265 ± 991 versus 8671 ± 642 cpm/well; 10 nmol/L:8401 ± 783 versus 6175 ± 743 cpm/well; 100 nmol/L:5880 ± 569 versus 4423 ± 406 cpm/well, n = 6, $P < 0.001$) (Fig. 5). The results of PCNA protein expression coincided with the findings of ^{3}H-TdR incorporation. The SMCs PCNA protein expression of the MSCs seeding group was lower than that of the proliferative ECs group(1nmol/L:0.92 ± 0.06 versus 1.15 ± 0.07; 10 nmol/L:0.97 ± 0.07 versus 1.07 ± 0.08; 100 nmol/L:0.91 ± 0.05 versus 1.18 ± 0.11, n = 6, $P < 0.001$) but was still higher than that of the confluent ECs group (1 nmol/L:0.92 ± 0.06 versus 0.74 ± 0.07; 10 nmol/L:0.97 ± 0.07 versus 0.78 ± 0.06; 100 nmol/L:0.91 ± 0.05 versus 0.71 ± 0.05, n = 6, $P < 0.001$) (Fig. 5).

Plasticity of the Seeded Mesenchymal Stem Cells Toward Endothelial Lineage

We seeded MSCs into the mature ECs layer and further investigated whether mature ECs can induce MSCs to differentiate toward ECs. To distinguish the differentiating MSCs in

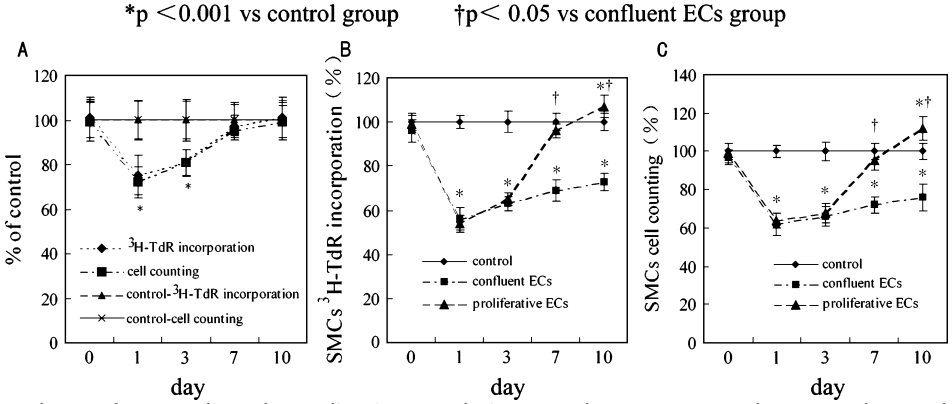

FIGURE 2. Time course of endothelial cells (EC)(A) and smooth muscle cells (SMC)(B,C) growth after paclitaxel of 10 nmol/L and 20 minutes application. A, The inhibitory effect of paclitaxel on the ^{3}H-TdR incorporation and cell counting of ECs was continued for 3 days. No inhibition on ECs was observed 7 days after paclitaxel application. B and C, Paclitaxel inhibited the ^{3}H-TdR incorporation (B) and cell counting (C) of SMCs in the confluent ECs group during the observed 10 days. No inhibition on SMCs growth was observed 7 days after paclitaxel application and increased SMCs growth was observed 10 days after paclitaxel application in the proliferative ECs group.

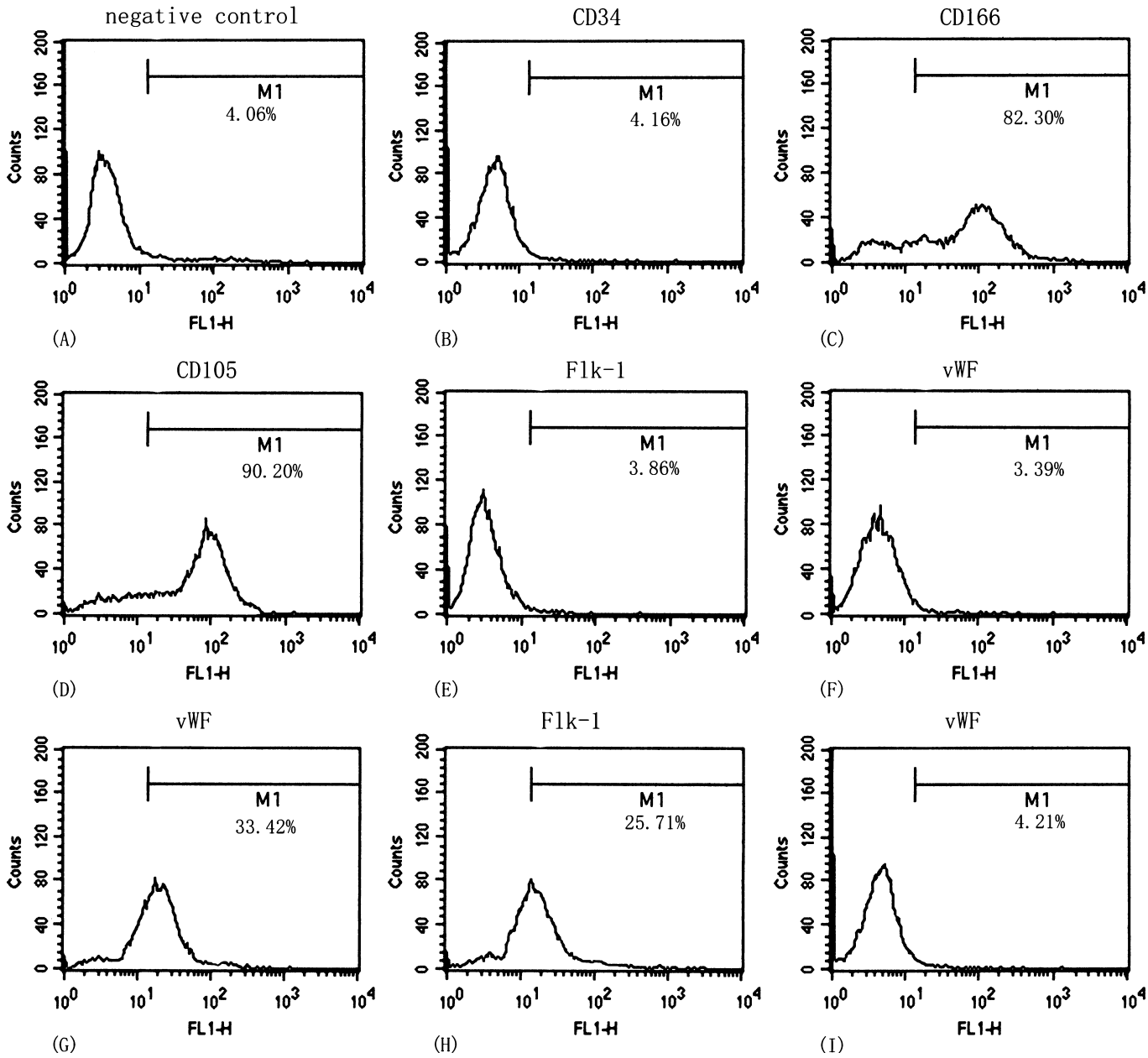

FIGURE 3. The phenotypic characterization of mesenchymal stem cells. A, negative control; B–F, the CD34,CD166,CD105,Flk-1, and vWF expression of the freshly isolated MSCs; G, the vWF expression of MSCs after culture with VEGF (50 ng/mL) for 5 days; H and I, the Flk-1 and vWF expression of MSCs after co-culture with mature rabbit ECs for 5 days. M1 indicated the positively stained cells that are sorted.

the process expressing certain ECs phenotypic characteristics from the mature ECs, human MSCs and rabbit ECs were selected for co-culture. The design is based on the consideration of the low expression of MHCII antigen on MSCs and the weak immunogenicity.[16,17] Furthermore, the rabbit anti-human Flk-1 and vWF antibody can only bind Flk-1 and vWF proteins of human origin specifically without cross-reaction with rabbit ECs. Therefore, the positive stained cells must have been derived from human MSCs. The results showed that the freshly isolated MSCs did not express Flk-1 or vWF protein. After co-culture with mature ECs for 5 days, about 25.71 ± 3.08% of MSCs began to express Flk-1 protein (Figs. 3 and 6), though none of the cells expressed vWF. However, the Flk-1 positive cells did not coexpress CD105 or CD166. Anti-VEGF antibody decreased the counts of Flk-1 positive MSCs-derived cells in a dose-dependent manner (Fig. 7).

DISCUSSION

The novel finding of the present study is that paclitaxel inhibits not only SMCs growth but also ECs growth and delays

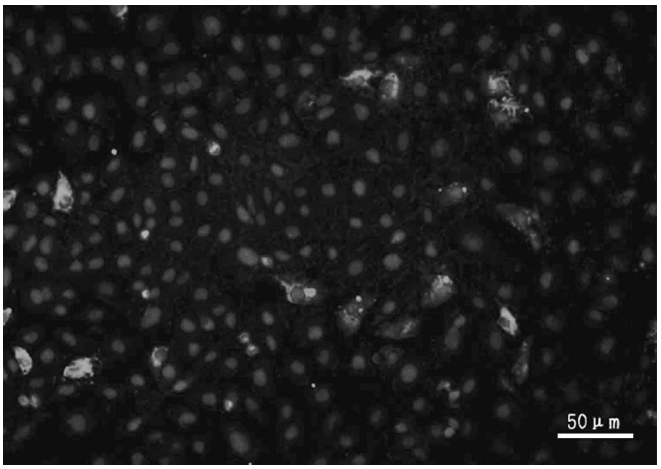

FIGURE 4. Representative photomicrograph of vWF protein expression of the differentiating mesenchymal stem cells. Some of the MSCs began to express vWF protein after culture in DMEM containing VEGF (50 ng/mL) for 5 days. The cell nuclei of MSCs were counterstained with DAPI and expressed blue fluorescence. The cells expressing red fluorescence were vWF-positive cells derived from MSCs.

intact endothelial repair, thereby inducing late SMCs proliferation. MSCs seeding can attenuate the late SMCs proliferation after paclitaxel intervention. The seeded MSCs have the milieu-dependent differentiation potential along endothelial lineage.

Paclitaxel can cause the formation of numerous decentralized and unorganized microtubules and enhance the assembly of extraordinarily stable microtubules, thereby inducing cellular modifications that result in reduced proliferation, migration, and signal transduction.[18] However, microtubules, the major component of cytoskeleton proteins, generally exist in eukaryotic cells.[19] Therefore, the antiproliferative effect of paclitaxel on eukaryotic cells is non-selective. Our findings also confirmed that paclitaxel inhibits not only SMCs proliferation but also ECs growth. The results of IC_{50} indicated that although the inhibitory intensity of equal dose paclitaxel on SMCs proliferation is stronger than that on ECs, the response of ECs migration to paclitaxel is more sensitive than that of SMCs. Paclitaxel is highly lipophilic and can exert long-lasting antiproliferative action after single-dose application.[20] Our findings also indicated that paclitaxel of 10 nmol/L and 20 minutes application exerts continuous inhibitory effect on SMCs growth. However, the response of SMCs growth in the upper chamber is not only related to paclitaxel but also closely related to the growth states of ECs in the lower chamber. Paclitaxel of 10 nmol/L continuously inhibited SMCs growth of the confluent ECs group during the observed 10 days. However, increased SMCs growth was observed 10 days after paclitaxel application in the proliferative ECs group. Previous studies have shown that ECs influence the proliferative features of SMCs depending on their growth states. The proliferative ECs are strong sources of growth factors and can significantly promote SMCs proliferation.[21,22] In this study, the late SMCs proliferation may be related to the new, untreated ECs in the proliferative ECs group. These new ECs would generate growth factors and induce SMCs growth. Therefore, it can be inferred from these findings that the late SMCs proliferation is closely related to the delayed ECs growth.

The limitations should be mentioned for the present study. The effects of drug eluting stents are not only determined by the drug itself but also related to stent design and drug-eluting model, which both can influence the drug releasing speed and concentration in tissues.[23] In addition, the effect of paclitaxel on cultured SMCs and ECs in vitro is different from the drug releasing kinetics of paclitaxel-eluting stents in vivo and the dose of paclitaxel used for stent coating is given in $\mu g/mm^2$. However, the approach can at least be compared on the basis of their biologic effects. These results still demonstrate that paclitaxel can inhibit not only SMCs growth but also ECs growth and delay intact endothelial repair, thereby inducing late SMCs proliferation.

Alternatively, the results indicate that paclitaxel continuously inhibits SMCs proliferation of the confluent ECs group during the observed period. The intact endothelium is essential in the prevention of SMCs proliferation. These results implied that we should not consider only the "anti-tumor" approach to restenosis prevention. The more optimal revascularization results would be achieved if the endothelial regeneration were simultaneously accelerated.

Accelerating intact endothelial repair by stem cell transplantation is a novel method in restenosis prevention.

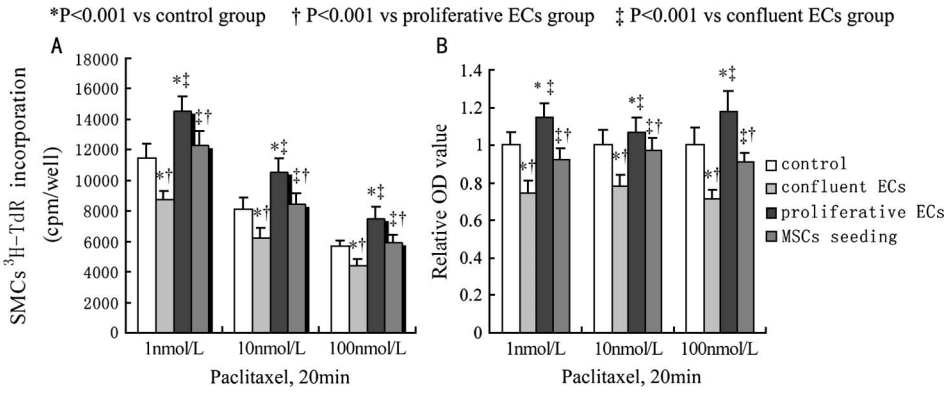

FIGURE 5. Effects of mesenchymal stem cells (MSC) seeding on the proliferation of smooth muscle cells (SMC) after paclitaxel of 20 minutes application. A, The proliferative endothelial cell (EC) stimulated but the confluent ECs inhibited the ^{3}H-TdR incorporation of SMCs. The SMCs ^{3}H-TdR incorporation of the MSCs seeding group was significantly lower than that of the proliferative ECs group but was still higher than that of the confluent ECs group. B, The proliferative ECs stimulated but the confluent ECs inhibited the PCNA protein expression of SMCs. The SMCs PCNA protein expression of the MSCs seeding group was significantly lower than that of the proliferative ECs group but was still higher than that of the confluent ECs group.

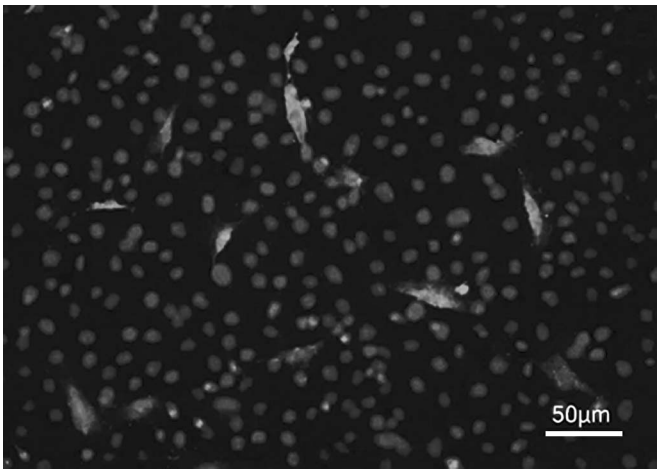

FIGURE 6. Representative photomicrograph of Flk-1 protein expression of the differentiating mesenchymal stem cells. Some of the MSCs began to express Flk-1 protein after coculture with mature endothelial cells (ECs) for 5 days. The cell nuclei of ECs and MSCs were counterstained with DAPI and expressed blue fluorescence. The cells expressing green fluorescence were Flk-1 positive cells derived from MSCs.

Evidence suggests that ex vivo expanded endothelial progenitor cells (EPCs) contribute to reendothelialization after endothelial injury.[9,24] However, EPCs show some phenotypic overlaps with hematopoietic cells and mature ECs, and controversy still exists with respect to the identification and origin of EPCs.[25,26] MSCs are also self-renewing and multipotential adult stem cells in the bone marrow. Recent studies have shown that MSCs can acquire several features of mature ECs when cultured under optimal condition. MSCs may therefore be an alternative source for EPCs.[27] Studies in animal models and clinical trials have shown that MSCs can prevent deleterious remodeling and improve left ventricular function when introduced into the infarct heart.[9,28,29] However, it remains unknown whether MSCs can accelerate endothelial repair and inhibit late SMCs proliferation of the vessel wall after paclitaxel intervention.

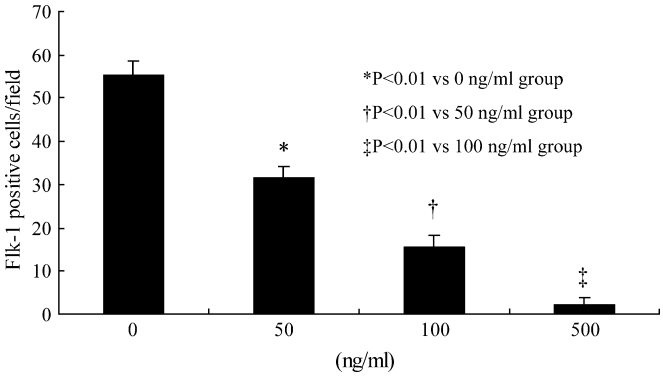

FIGURE 7. Effect of anti-VEGF antibody on the counts of Flk-1 positive MSCs-derived cells. Anti-VEGF antibody decreased the counts of Flk-1 positive MSCs-derived cells in a dose-dependent manner.

The findings of this study indicate that MSCs seeding can inhibit the late SMCs proliferation when compared with the effect of proliferative ECs. However, the seeding cannot inhibit SMCs proliferation just like the confluent ECs because the seeded MSCs cannot act completely as mature ECs. The proliferative ECs strongly stimulate SMCs proliferation. Because the growth pattern of ECs is density dependent, the strong growth ability of proliferative ECs decreases after MSCs are seeded. Accordingly, its growth-stimulating effect exerted on SMCs is also reduced. Therefore, the inhibitory effect of MSCs seeding on SMCs growth may be partly related with the reduced proliferation of ECs. Spees et al[30] developed an ex vivo model of epithelial repair by co-culture MSCs with epithelial cells and found that fusion between MSCs and epithelial cells was a frequent biologic event. The reduced SMCs proliferation in this study may also be related with the fusion between MSCs and ECs.

Studies have shown that adult stem cells from bone marrow undergo milieu-dependent differentiation to express phenotypes that are similar to the cells in the local microenvironment.[31,32] The results of this study also showed that about 25.71% of MSCs began to express Flk-1 protein after co-culture with the mature ECs for 5 days, though none of the cells expressed vWF and the morphologic appearance was also different from that of the adjacent mature ECs. Previous studies have shown that ECs go through different stages of differentiation. Initially, they express Flk-1/KDR, which is one of the earliest differentiation markers for ECs and plays an important role in ECs growth, followed in sequence by Tie-2, Tie-1, VE-cadherin, CD34, and vWF.[33–35] Therefore, these results indicate that MSCs have the milieu-dependent differentiation potential along endothelial lineage when co-culture with mature ECs and they are in the early stage of differentiation toward endothelial lineage. However, the Flk-1 positive cells did not coexpress CD105 or CD166, which indicates that MSCs began to lose the phenotypic characteristics of mesenchymal cells when they started differentiating toward ECs. Anti-VEGF antibody potently inhibits the differentiation of MSCs, indicating the important role of VEGF in the differentiation of MSCs toward ECs. However, it is still unclear why MSCs do not rapidly differentiate into the same morphologic and molecular phenotype of the adjacent ECs. In this study, we did not introduce any damage effect to the rabbit ECs. It is possible that certain injuries on mature ECs play the important role in the differentiation of MSCs.

In summary, this study was able to demonstrate that MSCs seeding can accelerate endothelial repair and partly attenuate late SMCs proliferation after paclitaxel intervention. With the further elucidation of the differentiating and regulating mechanisms of MSCs, drug-eluting stents implanting and stem cell transplantation might become a new potent treatment of coronary artery disease in the future.

CONCLUSION

Our aim was to investigate whether MSCs seeding can accelerate endothelial repair and attenuate late SMCs proliferation after paclitaxel intervention. We conclude that late SMCs proliferation is closely related to the delayed ECs

regeneration after paclitaxel application. MSCs seeding partly attenuates the late SMCs proliferation. MSCs co-cultured with mature ECs have the ability to differentiate toward ECs. However, the validity of the results must be confined to the experimental protocol.

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