Tetrapeptide AcSDKP Induces Postischemic Neovascularization Through Monocyte Chemoattractant Protein-1 Signaling

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Background—We investigated the putative proangiogenic activity and molecular pathway(s) of the tetrapeptide acetyl-N-Ser-Asp-Lys-Pro (AcSDKP) in a model of surgically induced hindlimb ischemia.

Methods and Results—Hindlimb ischemia was induced by femoral artery ligature and an osmotic minipump was implanted subcutaneously to deliver low (0.12 mg/kg per day) or high (1.2 mg/kg per day) doses of AcSDKP, for 7 or 21 days. Angiography scores, arteriole density, capillary number, and foot perfusion were increased at day 21 in the high-dose AcSDKP-treated mice (by 1.9-, 1.8-, 1.3-, and 1.6-fold, respectively) compared with control animals (P<0.05, P<0.01, P<0.01, respectively). AcSDKP treatment for 24 hours upregulated the monocyte chemoattractant protein-1 (MCP-1) mRNA and protein levels by 1.5-fold in cultured endothelial cells (P<0.01). In the ischemic hindlimb model, administration of AcSDKP also enhanced MCP-1 mRNA levels by 90-fold in ischemic leg (P<0.001) and MCP-1 plasma levels by 3-fold (P<0.001 versus untreated ischemic control mice). MCP-1 levels upregulation were associated with a 2.3-fold increase in the number of Mac3-positive cells in ischemic area of AcSDKP-treated mice (P<0.001 versus untreated animals). Interestingly, AcSDKP-induced monocyte/macrophage infiltration and postischemic neovascularization was fully blunted in MCP-1-deficient animals.

Conclusion—AcSDKP stimulates postischemic neovascularization through activation of a proinflammatory MCP-1-related pathway. (Arterioscler Thromb Vasc Biol. 2006;26:773-779.)

Key Words: angiogenesis ■ ischemia ■ inflammation ■ AcSDKP ■ MCP-1

Postnatal neovascularization involves 3 principal processes, ie, vasculogenesis, angiogenesis, and collateral growth.1 Inflammation is one of the key events implicated in blood vessel growth, especially in the setting of ischemia. Both monocytes/macrophages and lymphocytes have been shown to adhere to the vascular wall, infiltrate in the ischemic tissue and increase production of cytokines such as IL-10 and IL-18,2,3 and growth factors like bFGF, tumor necrosis factor-α, granulocyte macrophage colony-stimulating factor and vascular endothelial growth factor.4-6 Accumulation of leukocytes at inflammatory sites is regulated by chemotactic low-molecular-weight proteins called chemokines. One of the chemokines, the monocyte chemoattractant protein-1 (MCP-1), promotes monocytes/macrophages recruitment through activation of its receptor CCR2.7,8 and subsequently accelerates ischemia-induced blood vessel growth.4,5,9 MCP-1 can also act as a direct mediator of angiogenesis.10 Similarly, CXCR3 and its ligand CXCL-10 positively modulate ischemia-induced neovascularization, likely through modulation of inflammatory cell infiltration.11 Therefore, modulators of inflammation play a critical role in determining the extent of neovascularization after ischemic injury.

The tetrapeptide Acetyl-Ser-Asp-Lys-Pro (AcSDKP), purified from bone marrow was originally described as a physiological negative regulator of hematopoiesis.12 It inhibits in vitro as well in vivo the proliferation of normal hematopoietic stem cells and committed progenitors.12 AcSDKP possibly generated from thymosin-β413 is present in blood at nanomolar concentrations and is ubiquitously distributed in various tissues.14 In the circulation, AcSDKP is hydrolyzed by the NH2-terminal catalytic domain of angiotensin-converting enzyme (ACE)15 and its plasma level increases substantially during angiotensin-converting enzyme inhibitor (ACEI) administration.16 Thus, some of the effects of ACEI may be mediated by an increase in AcSDKP. Besides its role
in the regulation of hematopoiesis, AcSDKP plays another biological function as a promoter of angiogenesis.\textsuperscript{17} It acts as a stimulator of endothelial cells migration and differentiation into capillary-like structures. AcSDKP induces neovascularization in the chicken embryo chorioallantoic membrane and abdominal muscle in rat. Recent finding also reports the ability of AcSDKP to upregulate capillary density in rat hearts with myocardial infarction.\textsuperscript{18} Taken together, these data prompted us to investigate the contribution of this tetrapeptide to neovascularization in a model of unilateral hindlimb ischemia.

Materials and Methods

Mouse Ischemic Hindlimb Model

Experiments were conducted according to the French veterinary guidelines. All experiments were performed in 10 weeks C57Bl/6 mice or C57Bl/6 MCP-1 deficient animals. Mice underwent surgery to induce unilateral hindlimb ischemia, as previously described.\textsuperscript{3} Briefly, animals were anesthetized by isoflurane inhaling. The ligature was performed on the proximal origin of the right femoral artery, just above the origin of the circumflexa femoris lateralis. An osmotic minipump was then subcutaneously implanted on the back of the ischemic mice (Alzet, type 2001 and 2004) to deliver low (0.12 mg/kg per day) or high (1.2 mg/kg per day) doses of AcSDKP (IPSEN-Biotech, Paris, France) for 7 or 21 days. A group of mice was also treated with MCP-1 (1.5 μg/kg per day, osmotic minipump) for 21 days. The control animals received osmotic minipump delivering saline solution.

Cell Culture

Immortalized EA.hy926 endothelial cells were grown in Dulbecco Minimal Essential Medium (DMEM) supplemented with 10% fetal calf serum. For cell-based enzyme linked immunosorbent assay (ELISA) and reverse-transcriptase–polymerase chain reaction (PCR) studies, EA.hy926 cells were seeded into 25-cm² culture flask (Corning, VWR), grown to 90% confluence, and starved overnight in serum-free DMEM medium before stimulation. Cells were then incubated for 4 hours and 24 hours in serum-free DMEM in the absence or presence of AcSDKP at various concentrations. The cells and their supernatants were collected for determination of MCP-1 secreted protein and MCP-1 mRNA levels.

Cell Proliferation Assays

Human umbilical vein endothelial cells (Promocell, 5 × 10⁵ cells) were plated in gelatin-coated 96-well plates (Becton Dickinson) and allowed to adhere for 24 hours. The medium was removed, and the cells were washed twice with serum-free EGM2 medium. Serum-free medium containing 0.1% FBS, AcSDKP 10⁻⁷ M, AcSDKP 10⁻⁵ M, or 5% FBS was then added. After incubation at 37°C for 8 hours, 1 μCi/mL [3H]thymidine (Amersham) was added and cells were incubated for 30 minutes at 4°C. The number of CD45/CD11b-positive cells was counted and their supernatants were then isolated by density gradient centrifugation with Ficoll, and the number was expressed in cells by field, as previously described.\textsuperscript{3,19} Results were expressed as percentage of total number of cultured cells. In the second set of experiments, BM-MNCs isolated from the control mice were plated in the absence or presence of AcSDKP at various concentrations for 4 days and the number of EPCs was assessed, as described.

ELISA Quantification of MCP-1

Blood was collected and centrifugated at 1600g for 15 minutes at 4°C, plasma was then removed, aliquoted, and frozen at −20°C. MCP-1 concentration was determined using a mouse MCP-1 ELISA kit (R&D Systems). MCP-1 concentration in the supernatants of EA.hy926 endothelial cells was evaluated using the human MCP-1 ELISA kit (R&D Systems).

Determination of Plasma AcSDKP Levels

Blood was collected into pre-chilled heparinized tubes. Lisinopril (Sigma) was immediately added to the blood samples (10⁻⁴ M final concentration) to prevent AcSDKP degradation. Samples were centrifugated at 1600g for 15 minutes at 4°C and the plasma stored at −20°C until use. AcSDKP concentration was determined using a highly specific competitive enzyme immunoassay with acetylcholinesterase conjugate as a tracer (SPIbio) as previously described.\textsuperscript{17}

Quantification of Neovascularization

Vessel density was evaluated by 4 different methods, as previously described:\textsuperscript{2} (1) high-definition microangiography using barium sulfate (1 g/mL) injected in the abdominal aorta, followed by image acquisition with a digital X-ray transducer and computerized quantification of vessel density expressed as a percentage of pixels per image occupied by vessels in the quantification area; (2) assessment of capillary density by immunostaining with a rabbit polyclonal antibody directed against total fibronectin (dilution 1/50, Chemicon International); (3) evaluation of arteriole density by immunostaining using a mouse monoclonal antibody directed against human smooth muscle actin α-1 (dilution 1/50; Dako Cytomation); and (4) laser Doppler perfusion imaging to assess in vivo tissue foot perfusion.

Flow Cytometry Analysis of Sca1/c-kit–Positive Cells in Blood

Seven days after the onset of ischemia, mononuclear cells were isolated from peripheral blood (300 μL) of control and AcSDKP-treated mice. Mononuclear cells were then incubated with fluorescein isothiocyanate (FITC) conjugated monoclonal antibody against Sca-1 (D7) and phycoerythrin conjugated monoclonal antibody against c-kit (2B8) (all purchased from BD Pharmingen).

Isolation of Bone Marrow Mononuclear Cells and Endothelial Progenitor Cells Differentiation Assay

Bone marrow cells were obtained by flushing the tibias and femurs of control and AcSDKP-treated mice for 7 days. Low-density bone marrow mononuclear cells (BM-MNCs) were then isolated by density gradient centrifugation with Ficoll, as previously described.\textsuperscript{2} Dual positive staining for both AcLDL-Dil and B-1 lectin characterized endothelial progenitor cells (EPCs). EPCs were counted and the number was expressed in cells by field, as previously described.\textsuperscript{2,19} Results were expressed as percentage of total number of cultured cells. In the second set of experiments, BM-MNCs isolated from control mice were plated in the absence or presence of AcSDKP at various concentrations for 4 days and the number of EPCs was assessed, as described.

Flow Cytometry Analysis of Circulating CD45/CD11b–Positive Cells

Blood was collected from control and AcSDKP-treated mice. Seven days after the onset of ischemia, low-density mononuclear cells were then isolated by density gradient centrifugation with Ficoll and incubated with antibody against CD45 (1:100; Pharmingen) and with antibody against CD11b (0.25 μg/million cells; BD Pharmingen) for 30 minutes at 4°C. The number of CD45/CD11b–positive cells was then analyzed by flow cytometry.

Real-Time Quantitative Reverse-Transcription PCR

Real-time quantitative reverse-transcription PCR was performed on a Light Cycler (Roche Diagnostics). Quantification was performed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. The sequence of primers used were, for MCP1: forward 5’-CTTGAGCATCCACGTGTGG-3’; reverse 5’-CCCCCTCCTTCTTGGGTC-3’; and for GAPDH: forward 5’-GACTGGTGATGGCCCCCTCTG-3’; reverse 5’-GAGTTCCACACCCTCTGGTC-3’. The detection of amplification products was carried out using SYBR Green (Roche Applied Science). To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and subsequent agarose gel electrophoresis. For data analysis, Light Cycler 3.5 Software was used.
Immunohistochemistry

Seven-micrometer frozen tissue sections from the mouse gastrocnemius muscle were used for immunohistochemistry. Macrophages were identified with a rat monoclonal antibody directed against Mac-3 (1:50, BD Pharmingen). Tissues sections were then analyzed using avidin-biotin horseradish peroxidase visualization systems (Vectastain ABC kit elite; Vector Laboratories, Biovalley) and counterstained with hematoxylin. Mac-3–positive cells were counted in randomly fields in the ischemic area of gastrocnemius muscle using Histolab software. MCP-1 was detected in randomly fields of tissues sections with a goat polyclonal antibody against mouse JE/MCP-1 (1:20; R&D System) and with a fluorescent anti-goat antibody (1:50; Dako Cytomation).

Statistical Analysis

Results were expressed as mean±SEM. One-way analysis of variance ANOVA was used to compare each parameter. Post-hoc Bonferroni t test comparisons were then performed to identify which group differences account for the significant overall ANOVA. P<0.05 was considered significant.

Results

Ischemia and Endogenous Synthesis of AcSDKP

We first analyzed the effect of ischemia on AcSDKP levels. We showed that ischemia enhanced AcSDKP plasma levels by 2.3-fold when compared with sham-operated animals (P<0.05; Figure Ia, available online at http://atvb.ahajournals.org). AcSDKP-positive staining was mainly observed around vascular structures. The number of capillary positive for AcSDKP tended to be higher in ischemic compared with nonischemic tissue but this difference did not reach statistical significance (Figure Ib).

AcSDKP Induces Neovascularization in Mouse Ischemic Hindlimb

To assess AcSDKP proangiogenic potential, postischemic neovascularization was analyzed in mice treated with low-dose (0.12 mg/kg per day) or high-dose (1.2 mg/kg per day) of AcSDKP for 7 or 21 days. AcSDKP treatment increased AcSDKP plasma levels by 3.7-fold (7.36±0.89 nM versus 1.99±0.42 nM in AcSDKP-treated mice versus control animals, respectively; P<0.05).

At day 7, angiographic score, capillary density and foot perfusion were not significantly affected in mice receiving 0.12 mg/kg per day or 1.2 mg/kg per day of AcSDKP. In contrast, at day 21, angiography scores, arteriole density, capillary number, and foot perfusion were increased in high-dose AcSDKP-treated mice by 1.9-, 1.8-, 1.3-, and 1.6-fold, respectively, compared with control animals (P<0.05, P<0.01, P<0.01, respectively) (Figures 1 and 2).

AcSDKP Does Not Affect Endothelial Cell Proliferation

The observed in vivo efficacy of AcSDKP to improve vessel growth in the ischemic hindlimb prompted us to determine the molecular and cellular mechanisms involved in this process. An increase in capillary number following AcSDKP administration suggests that this tetrapeptide may affect endothelial cells proliferation. To address this hypothesis, we analyzed the effect of AcSDKP on EA.hy926 endothelial cells and human umbilical vein endothelial cells proliferation. However, proliferation of endothelial cells was not modulated by AcSDKP treatment in our experimental conditions, whatever the type of endothelial cells (Figure Ila, Iib, and Iic, available online at http://atvb.ahajournals.org).

AcSDKP Does Not Affect BM-MNC Differentiation Into EPC

Neovascularization does not rely exclusively on sprouting of pre-existing vessels, it also involves postnatal vasculogenesis. In response to ischemia, a subset of EPC was shown as being mobilized from bone marrow, they incorporate the ischemic area and participate to vessel growth. Furthermore, AcSDKP was previously reported to regulate the proliferation of bone marrow-derived hematopoietic CD34+ stem cells. We therefore analyzed the effect of AcSDKP on the mobilization of BM-MNC and their differentiation into EPC.

BM-MNC Mobilization

EPC are thought to derive from Sca-1/c-kit–positive mononuclear cells. Nevertheless, the percentage of Sca-1/c-kit–positive mononuclear cells in the peripheral blood remained unchanged in AcSDKP-treated mice compared with untreated animals (Figure IId).
BM-MNC Differentiation Into EPC

BM-MNC were isolated from control and AcSDKP-treated mice and then cultured for 4 additional days on gelatin/vitronectin. AcSDKP administration had no effect on the number of EPC derived from BM-MNC (Figure IIe). Furthermore, BM-MNC differentiation into EPC was unaffected when BM-MNC were isolated from untreated mice and next cultured for 4 days in presence of 10^{-7} M AcSDKP (data not shown). Taken together, these results suggest that AcSDKP promoted postischemic neovascularization without affecting postnatal vasculogenesis.

AcSDKP Activates Inflammatory Reaction

The lack of effect of AcSDKP on endothelial cell proliferation and BM-MNC led us to hypothesize that AcSDKP may enhance vessel growth by activating the inflammatory reaction.

AcSDKP Increases the Number of CD45/CD11b-Positive Cells in Blood

We analyzed the role of AcSDKP on the monocytes/macrophages mobilization. The number of circulating CD45/CD11b-positive cells in blood was determined by flow cytometry. Treatment with AcSDKP (1.2 mg/kg per day) for 7 days increased the number of circulating CD45/CD11b-positive cells by 1.4-fold compared with controls (18.0±1.1% versus 13.1±1.3% for AcSDKP-treated mice versus untreated mice, respectively; P<0.05).

AcSDKP Increases Macrophages Infiltration in Ischemic Area

Similarly, the number of infiltrated macrophages in ischemic area of AcSDKP-treated mice was increased by 2.3-fold compared with control mice, 7 days after the onset of ischemia (9.67±0.2 cells/mm^2 versus 4.2±0.6 cells/mm^2 for AcSDKP-treated mice versus untreated mice, respectively; P<0.001). Hence, AcSDKP was shown to enhance inflammatory reaction in ischemic tissue.

AcSDKP Activates MCP-1 Signaling

We next sought to define the molecular pathway involved in AcSDKP-induced activation of inflammation. It is well-known that accumulation of leukocytes at inflammatory sites is regulated by chemokines. Among them, MCP-1 represents a major critical rate-limiting step in monocytes/macrophages infiltration in ischemic area.5,9

AcSDKP Induces MCP-1 Secretion by Endothelial Cells In Vitro

We first investigated the effect of AcSDKP on MCP-1 levels in endothelial cells. AcSDKP (10^{-7} M) was shown to increase MCP-1 mRNA levels by 1.5-fold compared with untreated endothelial cells (P<0.05; Figure 3a). AcSDKP also upregulated MCP-1 protein content in the supernatant of treated endothelial cells (1811±360 pg/mL versus 1465±313 pg/mL, respectively; P<0.001; Figure 3b).

AcSDKP Increases MCP-1 mRNA and Protein Levels In Vivo

We next determined the effect of AcSDKP on MCP-1 levels in vivo in the ischemic hindlimb model. Ischemia enhances MCP-1 mRNA level by 12-fold compared with nonischemic tissue (P<0.05; Figure 3c). Furthermore, AcSDKP (1.2 mg/kg per day) increases MCP-1 mRNA content by 90-fold compared with control ischemic mice (P<0.01; Figure 3c). Administration of AcSDKP also raised MCP-1 plasma levels by 3-fold compared with control ischemic mice (P<0.001) (Figure 3d). Finally, MCP-1–positive staining was mainly localized around capillaries of ischemic tissue and was markedly improved by AcSDKP treatment (Figure 3e).

AcSDKP Proangiogenic Effect Required MCP-1–Related Pathway

We next analyzed the requirement of MCP-1 in AcSDKP-induced neovascularization using mice deficient for MCP-1.
Postischemic Neovascularization

AcSDKP treatment increased angiography scores, capillary numbers and foot perfusion by 1.7-, 1.4-, and 1.6-fold in wild-type mice compared with untreated animals \((P < 0.05)\) versus untreated wild-type mice. This effect was fully blunted in MCP-1–deficient mice. In addition, MCP-1 treatment enhanced revascularization in wild-type animals supporting the hypothesis that MCP-1 plays a pivotal role in vessel growth in this setting (Figure 4a, 4b, and 4c).

Inflammatory Reaction

Changes in neovascularization were associated with modulation of the inflammatory reaction. At day 21 after the onset of ischemia, few macrophages were seen in the interstitial space of control mice. The number of tissue infiltrated macrophages markedly increased in wild-type mice treated with 1.2 mg/kg per day of AcSDKP compared with untreated mice \((P < 0.001)\). In contrast, AcSDKP-induced macrophages infiltration was abrogated in MCP-1–deficient mice (Figure 4d).

Discussion

The main results of this study reveal the efficacy of AcSDKP to improve postischemic neovascularization in the context of hindlimb ischemia. A detailed analysis of the molecular and cellular mechanisms involved in this effect suggests an activation of the proinflammatory MCP-1–related pathway.

AcSDKP, an endogenous tetrapeptide produced constitutively by the endothelium, is a potent angiogenic factor both in vitro and in vivo.\(^{23}\) We could extend those observations showing that AcSDKP also promotes postischemic neovascularization. The proangiogenic effect of AcSDKP is not mediated by activation of endothelial cells proliferation. In addition, although AcSDKP modulates primitive hematopoietic stem cells proliferation and probably their homing, we do not observe any effect of AcSDKP on BM-MNC differentiation into EPC. Alternatively, our results suggest that AcSDKP enhances endothelial cells ability to produce MCP-1. MCP-1 may activate specific pathways involved in the angiogenic phenotype. MCP-1 upregulates hypoxia-inducible factor 1 alpha gene expression and subsequently vascular endothelial growth factor-A (165) expression.\(^{24}\) In addition, MCP-1 induces chemotaxis of human endothelial cells at nanomolar concentrations in the absence of leukocytic infiltrates.\(^{10}\) MCP-1 also represents a major critical rate-limiting step in monocytes/macrophages infiltration in ischemic area.
Exogenous administration of MCP-1 or a deficiency in its receptor, CCR2, regulate monocyte recruitment in the adventitia of growing arteries after vessel occlusion. Monocytes accumulation and activation play a pivotal role in collateral growth and angiogenesis by secretion of key angiogenic factor such as tumor necrosis factor-α, bFGF, or vascular endothelial growth factor. In addition, we evidenced that AcSDKP-induced monocytes/macrophages infiltration is fully blunted in MCP-1–deficient mice suggesting that MCP-1 is an upstream regulator of inflammation in AcSDKP-related pathway. Taken together, the present findings suggest that AcSDKP enhances MCP-1 production by endothelial cells and thereby attracts monocytes/macrophages in ischemic area to promote neovascularization.

AcSDKP was shown to enhance the conversion of the prometalloproteinase-1 into its active form, suggesting that this tetrapeptide is able to stimulate the acute phases of neovascularization characterized by matrix degradation and inflammatory cells infiltration. Surprisingly, AcSDKP partially prevented and reversed the inflammatory reaction within the heart. The reason of this discrepancy is unclear but, in this latter study, AcSDKP was administered up to 4 months after the onset of ischemia. In the ischemic hindlimb model, inflammation plays a major role during the acute phase of neovascularization. Therefore, long-term administration as well as the remodeling process occurring in the treated heart might explain the difference in AcSDKP-related effects on inflammation between ischemic heart and hindlimb.

Our study highlights the concept that AcSDKP may be involved in angiogenesis-related diseases. In support of this view, AcSDKP serum levels are increased in adult patients with hematologic malignancies. Upexpression of thymosin-β4, the putative precursor of AcSDKP, also occurs in neoplastic tissues suggesting that AcSDKP may be implicated in different pathological states characterized by activation of angiogenesis. A growing body of evidences suggest that ACEI promotes postischemic neovascularization. Treatment with ACEI also increases AcSDKP concentration in plasma highlighting the hypothesis that AcSDKP may partially mediate ACEI proangiogenic potential.

In conclusion, this study provides the first evidence to our knowledge that AcSDKP improves MCP-1–dependent postischemic neovascularization and supports the concept that AcSDKP administration might constitute a potential therapeutic...
strategy in the treatment of ischemic diseases. However, MCP-1 and inflammation have been shown to promote atherosclerotic plaque growth, suggesting putative side effects of AcSDKP-induced MCP-1 upregulation. Further studies should determine whether therapeutic angiogenic strategy based on systemic and/or local administration of AcSDKP may promote side effects especially in the setting of atherosclerosis.

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Figure I online
Figure II online
**FIGURE LEGENDS ONLINE**

**Figure I:** A) Quantitative evaluation of AcSDKP plasma levels in sham-operated animals (Sham) and control mice with hindlimb ischemia (Isch), 7 days after the onset of ischemia. B) Representative photomicrographs and quantitative evaluation of AcSDKP-positive vessels (positive staining appears in brown, arrows indicating representative examples of AcSDKP-positive capillaries) in sections of ischemic and non ischemic gastrocnemius muscle from control animals. n= 4, *P <0.05 versus sham-operated animals.

**Figure II:** A) Quantitative evaluation of EA.hy926 endothelial cells proliferation, 24 h after treatment with $10^{-11}$M, $10^{-9}$M, $10^{-7}$M of AcSDKP. B) Potential positive effect of AcSDKP on HUVEC proliferation. Cont indicates untreated HUVEC; Cont+FBS, HUVEC treated with 10 % foetal bovin serum (FBS); AcSDKP $10^{-7}$M, HUVEC treated with AcSDKP $10^{-7}$M in absence of FBS; AcSDKP $10^{-9}$M, HUVEC treated with AcSDKP $10^{-9}$M in absence of FBS, Values are mean ± SEM, n= 3. ***P<0.001 versus untreated HUVEC. C) Potential negative effect of AcSDKP on HUVEC proliferation. Cont indicates untreated HUVEC; Cont+FBS, HUVEC treated with 10 % foetal bovin serum; AcSDKP $10^{-7}$M + FBS, HUVEC treated with AcSDKP $10^{-7}$M in presence of FBS; AcSDKP $10^{-9}$M, HUVEC treated with AcSDKP $10^{-9}$M in presence of FBS, Values are mean ± SEM, n= 3. ***P<0.001 versus HUVEC treated with 10% foetal bovin serum. D) Quantitative evaluation of the percentage of Sca-1/c-kit positive cells in the blood of mice treated with or without 0.12 mg/kg/day (AcSDKP 0.12) or 1.20 mg/kg/day (AcSDKP 1.20) of AcSDKP, 7 days after the onset of ischemia. E) Quantitative evaluation of the percentage of cells stained with both AcLDL-Dil and BS-1 lectin. BM-MNC were isolated from mice treated with or without 0.12 mg/kg/day (AcSDKP 0.12) or 1.20 mg/kg/day (AcSDKP 1.20) of AcSDKP, 7 days after the onset of
ischemia. BM-MNC were then cultured for 4 days. EPC were characterized as adherent cells with double positive staining for AcLDL-Dil and BS-1 lectin.