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N-acetyl-seryl-aspartyl-lysyl-proline stimulates angiogenesis in vitro and in vivo

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Abstract

N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), a natural inhibitor of pluripotent hematopoietic stem cell proliferation, has been suggested as capable of promoting an angiogenic response. We studied whether Ac-SDKP stimulates endothelial cell proliferation, migration, and tube formation; enhances angiogenic response in the rat cornea after implantation of a tumor spheroid; and increases capillary density in rat hearts with myocardial infarction (MI). In vitro, an immortal BALB/c mouse aortic endothelial 22106 cell line was used to determine the effects of Ac-SDKP on endothelial cell proliferation and migration and tube formation. In vivo, a 9L-gliosarcoma cell spheroid (250–300 μm in diameter) was implanted in the rat cornea and vehicle or Ac-SDKP (800 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ip) infused via osmotic minipump. Myocardial capillary density was studied in rats with MI given either vehicle or Ac-SDKP. We found that Ac-SDKP 1) stimulated endothelial cell proliferation and migration and tube formation in a dose-dependent manner, 2) enhanced corneal neovascularization, and 3) increased myocardial capillary density. Endothelial cell proliferation and angiogenesis stimulated by Ac-SDKP could be beneficial in cardiovascular diseases such as hypertension and MI. Furthermore, because Ac-SDKP is mainly cleaved by ACE, it may partially mediate the cardioprotective effect of ACE inhibitors.

Keywords

endothelial cell; capillaries; myocardial infarction

N-ACETYL-SERYL-ASPARTYL-LYSYL-PROLINE (Ac-SDKP), an endogenous inhibitor of pluripotent hematopoietic stem cell proliferation (5, 17), is ubiquitously distributed in various tissues and biological fluids such as plasma, urine, and circulating mononuclear cells of humans and animals (29, 30). Ac-SDKP is cleaved from its precursor thymosin- β 4, most likely by prolyl oligopeptidase (7), and is hydrolyzed almost exclusively by angiotensin-converting enzyme (ACE) at the Asp-Lys peptide bond (12). After treatment with ACE inhibitors (ACEi), Ac-SDKP concentrations increased 4- to 6-fold in plasma and 34- to 42-fold in urine (1, 2, 14). Thus some of the protective effects of ACEi may be mediated by an

increase in endogenous Ac-SDKP. In addition to the antiproliferative effect of Ac-SDKP on the hematopoietic system, when administered after two-thirds hepatectomy in rats it reportedly reduced hepatocyte proliferation by up to 50% (20). We recently showed that Ac-SDKP is also able to inhibit cardiac fibroblast proliferation and collagen synthesis in vitro and left ventricular interstitial fibrosis in vivo in rats with renovascular or aldosterone-salt hypertension without affecting left ventricular hypertrophy or systemic blood pressure (25, 32, 33). In rats with heart failure after myocardial infarction (MI), Ac-SDKP prevented and reversed cardiac interstitial fibrosis and perivascular collagen deposition (42). However, although Ac-SDKP acts as a negative regulator of hematopoietic stem cells and fibroblasts, it was found to promote vascular smooth muscle cell and endothelial cell proliferation in vitro and vascularization in vivo (18, 28), suggesting that it may be a potential stimulus for angiogenesis.

Angiogenesis is defined as formation of new blood vessels, characterized by dissolution of the bond between the endothelium and the underlying basement membrane, cell migration and proliferation and tube formation, culminating in the development of a new capillary (34). Regulation of angiogenesis is dependent on a biological balance between factors promoting vascular growth, such as VEGF and FGF, and angiostatic substances such as angiostatin and endostatin that inhibit new vessel formation (6). Although both Ac-SDKP and its precursor thymosin- β 4 are capable of stimulating angiogenesis (16, 21) as well as inhibiting hematopoietic stem cell proliferation (4, 23), it is not known whether Ac-SDKP is able to stimulate neovascularization in the heart following myocardial ischemia via an angiogenic response. Thus, in the present study, we investigated 1) the role of Ac-SDKP in endothelial cell proliferation, migration, and tube formation in vitro and the effects of Ac-SDKP on 2) angiogenesis stimulated by a tumor spheroid in the rat cornea and 3) myocardial capillary density in rats with MI in vivo.

METHODS

Cell Culture

BALB/c mouse aortic endothelial 22106 immortal cells were generously donated by Dr. Lei Wang (Department of Neurology, Henry Ford Hospital, Detroit, MI). The cells were grown in F-12K medium (American Type Culture Collection) supplemented with 10% FBS and 1% antibiotic and antimycotic solution (Invitrogen) and cultured in a 75-cm² flask (Corning) at 37°C and 5% CO₂. Medium was routinely replaced and cells passed by trypsinization when they reached 70% confluence.

[³H]-thymidine Incorporation

[³H]-thymidine incorporation was performed as described previously (32). Briefly, cells were seeded onto 6-well plates containing F-12K supplemented with 10% FBS at a density of 0.5×10^5 cells per well and allowed to grow until subconfluent, occupying 60% to 70% of the surface of the plate. They were cultured in serum-free DMEM for 24 h and then treated with 2.5% FBS either alone or combined with Ac-SDKP (Bachem) (0 to 10 nM) for 24 h in DMEM containing [³H]-thymidine (1 μ Ci/ml) and 10^{-6} mol/l captopril to prevent degradation of Ac-SDKP. Each well was washed once with 1 ml of ice-cold PBS, and then 1

ml of ice-cold 10% TCA was added. The plates were scraped and cell lysates transferred to Eppendorf vials and then vacuum-filtered through Whatman filters and washed three times with 5 ml of ethanol-TCA (70:5%). Filters were counted for 1 min in vials containing 4-ml scintillation cocktail. The results are the mean of three independent experiments.

Cell Migration

Endothelial cell migration was evaluated using standard 48-well chemotaxis chambers (Neuro Probe) as described by Wang et al. (39). Briefly, cells were resuspended in migration medium (Iscove's modified Dulbecco's medium + 0.1% FBS) at 5×10^5 cells/ml. Then, 25 μ l solutions containing various concentrations of Ac-SDKP (0 to 10 nM) and 10^{-6} mol/l captopril in triplicate were placed in the lower wells of the chemotaxis chambers, and 50- μ l aliquots of cells (2.5×10^4) were added to the upper wells. The contents of the upper and lower wells were separated by a polycarbonate membrane (12 μ m pore size, Neuro Probe) coated with 0.1% gelatin. Cells were allowed to migrate for 5 h at 37°C, after which time the filters were removed and the nonmigrating cells on the top of the membrane were removed with cotton swabs. Migrating cells became attached to the bottom of the membrane, where they were fixed with methanol and stained with Diff-Quick (Dade Behring; Düringen, Switzerland). Migration was quantified by counting cells in 3 random fields/well at $\times 40$ magnification using an inverted phase-contrast microscope (Nikon Labophot). Assays were performed in triplicate and repeated five times. Results are expressed as the fold increase over control values.

Tube Formation

The effect of Ac-SDKP on endothelial cell tube formation was examined by capillary-like tube formation assay as described by Chen et al. (9). Briefly, 96-well plates (Corning) were coated with 100 μ l Matrigel (13 mg/ml, BD Biosciences) that was allowed to solidify for 30 min at 37°C. Cells (2×10^4 cells/150 μ l/well) were plated onto the surface of the Matrigel in serum-free F-12K medium containing Ac-SDKP at different concentrations (0–10 nM) and 10^{-6} mol/l captopril and cultured at 37°C in a 5% CO₂ humidified atmosphere for 4 h. Matrigel wells were digitized under a $\times 2.5$ objective (Olympus BX40) for measurement of total tube length using a video camera (Sony DXC-970 MD) interfaced with an MCID image analysis system (Imaging Research). Tracks of endothelial cells organized into networks of cellular cords (tubes) were counted and averaged in three randomly selected fields. Each assay was performed in triplicate, and the results are the mean of four independent experiments.

Corneal Micropocket Assay and Imaging Analysis of Neovascularization

Preparation of tumor spheroid.—9L-gliosarcoma cells were cultured in a T25 flask and detached at 80% confluence. They were transferred to a T75 flask coated with agar, where spheroids formed within 3–5 days. Spheroids were selected under a microscope and transferred to 24-well plates just before implantation.

Spheroid implantation.—Male Sprague-Dawley rats weighing 100 – 300 g (Charles River Laboratories) were anesthetized, and one eye was secured in place with a hemostat. An intrastromal linear keratotomy (1 to 2 mm long and half the thickness of the cornea) was

made at the center of the cornea with a no. 11 blade, and a lamellar micropocket was created. A spheroid 250–300 μm in diameter was implanted into the base of the micropocket, and the eye was gently flushed with saline. After the same procedure was applied to the other eye, the rat was placed on a heating pad until it woke up. Infusion of vehicle ($n = 13$) or Ac-SDKP ($800 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ip via osmotic minipump, $n = 11$) was started on the day of operation and continued for 10 days.

Imaging analysis.—Rats were anesthetized, and black ink (no. 44031, Sanford) injected into the left ventricle until the corneal vessels turned black. Both eyes were removed, and the cornea was photographed with a microcomputer imaging system (Nikon Eclipse E800, Mager Scientific; Dexter, MI). Corneal areas exhibiting neovascularization were analyzed quantitatively using the grid-guided histomorphometric point counting method (31).

Myocardial capillary density.—To induce MI, male Lewis inbred rats weighing 255–275 g (Charles River Laboratories) were anesthetized, and the left anterior descending coronary artery was ligated as described previously (43). Saline (vehicle; $n = 10$) or Ac-SDKP ($800 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ip; $n = 9$) was infused via osmotic minipump 7 days before MI and continued for 4 mo. At the end of the experiment, the rats were euthanized and the hearts excised. Sections of the left ventricle were pretreated with 3.3 U/ml neuroaminidase type V (Sigma; St. Louis, MO) and then stained with rhodamine-labeled *Griffonia simplicifolia* lectin I to show the capillaries (43). From each slice, three radially oriented fields from the noninfarcted area were selected at random and photographed at a magnification of $\times 100$. Capillary density was expressed as the number of capillaries per mm^2 of myocardium.

Data Analysis

Data are expressed as means $\pm$ SE. The dose-response test is based on a linear regression, with the response being the dependent variable and the $\log_{10}$ of the dose being the independent variable. Tests for each dose versus control and tests for adjacent levels were performed using Fisher's protected least-significant difference test. This is a two-stage test in which the overall significance of the model is first assessed by F -test; then, if this test is found to be significant at the 0.05 level, each pairwise evaluation is carried out by t -test using information obtained at all doses to estimate mean square error. We also performed Dunnett's test, which is appropriate for testing all groups versus a standard (control) group; this is a slightly more conservative approach that controls for the type I experiment-wise error rate. In almost every case, there was no difference in statistical significance between these two methods. To maintain consistency between sections of tests, Fisher's least-significant difference test was chosen for all results. All tests were two sided. For capillary density and corneal neovascularization, an unpaired Student's t -test was used. Significance was assessed at the 0.05 level.

RESULTS

Effect of Ac-SDKP on DNA Synthesis

Endothelial cell proliferation was assessed by [^3H]-thymidine incorporation, a measure of DNA synthesis. Ac-SDKP (0.01 to 10 nM) increased [^3H]-thymidine incorporation in a dose-dependent manner (Fig. 1), although only the highest dose (10 nM) produced a significant effect ($P < 0.05$).

Effect of Ac-SDKP on Endothelial Cell Migration

Cell migration was studied using a chemotaxis chamber. Migrating cells became attached to the bottom of the chamber (Fig. 2A). Ac-SDKP at 10 nM significantly increased the number of migrating cells compared with control. Ac-SDKP at 0.01 and 0.1 nM had no effect on endothelial cell migration; however, higher doses (1 and 10 nM) significantly stimulated migration compared with control (1.52- and 1.51-fold over control, respectively; $P < 0.05$) (Fig. 2B).

Effect of Ac-SDKP on Endothelial Cell Tube Formation

The angiogenic effect of Ac-SDKP in vitro was examined by Matrigel tube formation. No tubes were seen when cells were incubated with control medium (Fig. 3A, *left*), whereas in the presence of Ac-SDKP newly formed capillary-like tubes became obvious (Fig. 3A, *right*). Quantitatively, Ac-SDKP increased tube length from 3.21 ± 0.39 mm/mm² (control) to 3.55 ± 0.50 , 4.58 ± 0.44 , 4.82 ± 0.72 , and 5.33 ± 0.61 mm/mm² at 0.01, 0.1, 1, and 10 nM, respectively, although the increase was only significant at 1 and 10 nM (Fig. 3B).

Effect of Ac-SDKP on Corneal Neovascularization

Corneal micropocket assay is a suitable method to evaluate the effect of Ac-SDKP on angiogenesis in vivo. In normal eyes, the cornea is clear, transparent, and free of vessels (Fig. 4A, *left*). Implantation of a tumor spheroid stimulated vessel formation (2.2 ± 0.6 mm²/cornea) (Fig. 4A, *middle*), which was markedly enhanced by systemic administration of Ac-SDKP (5.8 ± 1.4 mm²/cornea; $P < 0.05$ vs. vehicle) (Fig. 4A, *right*).

Effect of Ac-SDKP on Myocardial Capillary Density Post-MI

Rats with MI exhibited a significant decrease in capillary density compared with sham-operated rats. Ac-SDKP significantly increased the number of capillaries compared with vehicle (Fig. 5A). Quantitative analysis showed that capillary density in the sham group was $2,450 \pm 96$ number/mm²; MI reduced capillary number to $1,414 \pm 72$ number/mm², whereas Ac-SDKP increased it to $1,842 \pm 83$ number/mm² ($P < 0.05$ vs. MI-vehicle) (Fig. 5B).

DISCUSSION

In the present study, we found that Ac-SDKP is capable of inducing angiogenesis both in vitro and in vivo because 1) it stimulated endothelial cell proliferation and migration as well as tube formation, 2) it increased myocardial capillary density in rats with MI, and 3) it promoted neovascularization in the rat cornea when stimulated by implantation of a tumor cell spheroid.

Ac-SDKP was originally described as a natural inhibitor of pluripotent hematopoietic stem cell proliferation. It prevents cells from entering into the S phase of the cell cycle and maintains them in the G0/G1 phase, thus reducing the cell damage caused by chemotherapy or ionizing radiations (3, 17, 40). Moreover, Ac-SDKP has been shown to inhibit fibroblast proliferation, thereby reducing cardiac and renal fibrosis (26, 33, 42). Interestingly, unlike its inhibitory effect on fibroblast proliferation, Ac-SDKP has been shown to enhance endothelial cell as well as smooth muscle cell proliferation (18, 28). For example, Liu et al. (18) reported that Ac-SDKP at nanomolar concentrations stimulated endothelial cell migration and differentiation, two essential components of new vessel formation. They also showed that in vivo, Ac-SDKP promoted angiogenesis in the chicken embryo chorioallantoic membrane and in rat abdominal muscle (18). In the present study, we confirmed that Ac-SDKP is able to stimulate endothelial cell proliferation and migration as well as tube formation in vitro and further showed that Ac-SDKP administered chronically enhanced corneal neovascularization and increased myocardial capillary density post-MI in vivo. Taken together, these findings suggest that Ac-SDKP might be a potent mediator of angiogenesis.

The tetrapeptide Ac-SDKP is endogenously released from its precursor thymosin- β_4 (11, 41). It has been shown that thymosin- β_4 shares similar biological properties with Ac-SDKP, such as inhibiting hematopoietic stem cell proliferation and stimulating angiogenesis (8, 21). However, the mechanism by which Ac-SDKP and thymosin- β_4 mediate endothelial cell proliferation and angiogenic response is not understood. Liu et al. (18) showed that in cultured endothelial cells, Ac-SDKP enhanced the release of matrix metalloproteinase (MMP)-1, a family of highly homologous Zn^{2+} -endopeptidases. Using a hindlimb ischemic model in mice, Johnson et al. (13) found that the postischemic angiogenic response was significantly diminished in MMP-9-null mice, this suggests that MMPs secreted by endothelial cells play a crucial role in the angiogenic response by degrading the basement membrane of existing blood vessels and allowing endothelial cells to migrate and proliferate, forming solid endothelial cell sprouts and eventually capillary tubes and vascular loops (24, 27).

Ac-SDKP is released from thymosin- β_4 by prolyl oligopeptidase (7) and degraded exclusively by ACE (12, 18). Inhibition of ACE resulted in a 4- to 6-fold increase in Ac-SDKP in plasma and a 34- to 42-fold increase in urine (1, 2, 14). Thus increased Ac-SDKP may contribute to some of the cardioprotective effects of ACE inhibitors, such as reducing interstitial fibrosis. ACEi also reportedly increased capillary density in an animal model of hindlimb ischemia and capillary length in the hypertensive rat heart (10, 36). We previously found that ACEi increased cardiac capillary density in rats with MI (19), although conflicting results have been reported by other investigators. Volpert et al. (37) and Wang and Prewitt (38) showed that the ACEi captopril inhibited tumor-induced angiogenesis and reduced capillary density in the cremaster muscle of hypertensive rats. Although we do not have a good explanation for this discrepancy, it may be due to the different experimental models used and the different tissues evaluated. In the present study, we further showed that chronic infusion of Ac-SDKP increased the number of capillaries in the rat heart post-MI, similar to the effect observed with ACEi in rat models of MI, indicating that Ac-SDKP could mediate the angiogenic effect of ACEi. Angiogenesis, the formation of new blood vessels, is

not only critically involved in a number of normal physiological processes, such as embryonic development, ovulation, and wound healing, but is also a critical step in many pathological conditions, including MI. Angiogenic response and neovascularization in the heart is an important physiological process that frequently occurs in myocardial ischemia and infarction. It provides blood flow and oxygen to sustain metabolism, thus reducing infarct size and improving cardiac reserve and prognosis post-MI (34, 35). Thus maintenance and formation of microvessels may be important targets for treatment of myocardial ischemia and infarction. We believe our study clearly demonstrates for the first time that Ac-SDKP increases myocardial capillary density and promotes angiogenesis in rat hearts after MI. The antifibrotic and angiogenic properties of Ac-SDKP may provide a new strategy for treatment of heart failure due to MI.

Limitations

There are several limitations of this study. First, we could not explain why Ac-SDKP has such diverse effects on cell growth, inhibiting proliferation of hematopoietic stem cells, hepatocytes, and fibroblasts, while at the same time stimulating proliferation of endothelial cells and vascular smooth muscle cells (28). Second, although we have convincingly demonstrated that Ac-SDKP stimulates angiogenesis in vitro and in vivo, the underlying mechanisms were not explored in the present study. Besides activating MMPs as suggested by Liu et al. (18), we need to know whether Ac-SDKP is able to stimulate release of growth factors known to regulate angiogenesis and neovascularization. Third, because angiogenesis has been implicated in tumor invasion and metastasis, we need to clarify whether Ac-SDKP also stimulates tumor growth and metastasis due to its angiogenic action. However, this is unlikely, because both Ac-SDKP and ACEi have been proven to reduce hematotoxicity during chemotherapy and radiation-induced normal tissue injury during tumor treatment (15, 22).

In conclusion, the present study shows that Ac-SDKP, a natural inhibitor of pluripotent hematopoietic stem cell proliferation, is able to stimulate endothelial cell proliferation and migration and tube formation in vitro and increase neovascularization in the rat cornea and capillary density in the rat heart after MI in vivo. The pathophysiological significance and clinical relevance of these effects, as well as the mechanisms responsible, need to be clarified in future studies.

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Fig. 1.

Effect of *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) on endothelial cell proliferation expressed as [³H]-thymidine incorporation, a measure of DNA synthesis. Ac-SDKP increased [³H]-thymidine incorporation in a dose-dependent manner. * $P < 0.05$ vs. control (C).

Fig. 2.

Effect of Ac-SDKP on endothelial cell migration evaluated with a chemotaxis chamber. *A*: representative images showing that Ac-SDKP at 10 nM significantly increased the number of migrating cells in the bottom of the chamber compared with control. *B*: effect of various doses of Ac-SDKP on cell migration. Ac-SDKP at 0.01 and 0.1 nM had no effect on cell migration; however, higher doses (1 and 10 nM) significantly stimulated migration compared with control (1.52- and 1.51-fold over control, respectively; * $P < 0.05$ vs. control).

Fig. 3.

Effect of Ac-SDKP on tube formation using Matrigel assay. Representative image (*A, left*) shows that no tubes were seen when cells were incubated with control medium, whereas in the presence of Ac-SDKP newly formed capillary-like tubes became obvious (*A, right*, a representative image best showing the tube formation-stimulating effect of Ac-SDKP). The effect of Ac-SDKP tended to be dose dependent, although only higher doses reached statistical significance (*B*). * $P < 0.05$ vs. control.

Fig. 4.

Effect of Ac-SDKP on rat corneal neovascularization using corneal pocket assay. *A*: representative images show that in the normal eye, the cornea is transparent and free of vessels (*left*). Implantation of a tumor spheroid-stimulated neovascularization (*middle*), which was markedly enhanced by systemic administration of Ac-SDKP (*right*). *B*: quantitative results are shown in the bar graph. # $P < 0.01$ vs. sham; * $P < 0.05$ vs. vehicle..

Fig. 5.

Effect of Ac-SDKP on myocardial capillary density in rats with myocardial infarction (MI).

A: representative images showing the number of capillaries (bright red staining) in sham infarction (Sham-MI), MI treated with vehicle and MI treated with Ac-SDKP (MI + Ac-SDKP). Myocardial capillary density in the vehicle group was markedly decreased compared with sham, and Ac-SDKP significantly increased the number of capillaries (*A* and *B*). [#] $P < 0.01$ vs. sham; ^{*} $P < 0.05$ vs. MI + vehicle.