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Activated protein C stimulates osteoblast proliferation *via* endothelial protein C receptor

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ABSTRACT

Introduction: Bone is continually remodeled by the action of osteoblasts, osteocytes, and osteoclasts. Resting osteoblasts are able to proliferate and differentiate into mature osteoblasts when physiologically required, as after tissue injury. Activated protein C (APC) is a serine protease that functions in anticoagulation, anti-inflammation, anti-apoptosis, cell proliferation, and wound repair. In this study, we examined the effect of APC on osteoblast proliferation and differentiation.

Materials and Methods: We examined the presence of protein C in human fracture hematoma by immunohistochemical staining. We then evaluated the effect of APC, diisopropyl fluorophosphate-inactivated APC (DIP-APC) or protein C zymogen on normal human osteoblast (NHOst) proliferation using tetrazolium salt assay in the presence or absence of aprotinin, hirudin, protein C, antibody against protein C, endothelial protein C receptor (EPCR) or protease-activated receptor (PAR)-1. Finally, activation of p44/42 MAP kinase was evaluated by Western blot analysis.

Results: Both APC and DIP-APC increased osteoblast proliferation in a dose-dependent manner, while protein C did not. The APC-induced increased proliferation of osteoblast was not affected by aprotinin, hirudin, and anti-protein C antibody which inhibits the protease activity of APC. Treatment with protein C or anti-EPCR antibody which inhibits APC binding to EPCR inhibited APC-mediated osteoblast proliferation, while treatment with anti-PAR-1 antibody did not. APC promoted the phosphorylation of p44/42 MAP kinase within osteoblasts; this effect was inhibited by the anti-EPCR antibody.

Conclusions: APC stimulates osteoblast proliferation by activating p44/42 MAP kinase through a mechanism that requires EPCR but not PAR-1 or the proteolytic activity of APC. APC generated at fracture sites may contribute to fracture healing by promoting osteoblast proliferation.

1. Introduction

Activated protein C (APC) is an anticoagulant serine protease that physiologically controls blood coagulation by inactivating factors Va and VIIIa in the presence of plasma protein S [1,2]. Protein C zymogen is activated by the thrombin-thrombomodulin complex in the presence of the endothelial protein C receptor (EPCR) [3]. APC also regulates cell inflammation and apoptosis [4], stimulates cell proliferation [5], and promotes wound repair [5–7] in the presence of EPCR. These activities of APC with EPCR can also be mediated by the activation of protease-activated receptor-1 (PAR-1) [4,7,8], a member of the seven transmembrane domain G protein-coupled receptor (GPCR) family [9,10]. Thrombin-catalyzed PAR-1 activation is important in a wide variety of pathological processes, including inflammation and wound healing [11–13].

Bone is continually remodeled by multiple cell types, primarily osteoblasts, osteocytes and osteoclasts [14,15]. A large proportion of osteoblasts, the bone-forming cells that are derived from mesenchymal stem cells, progressively differentiate into osteocytes when they are embedded in bone matrix at local sites. However, a small population of these cells becomes bone-lining cells, thought to be resting osteoblasts [15]. Osteoblasts express PAR-1, which mediates thrombin-induced proliferation of these cells [16–19].

Because there are many physiologically active substances including coagulation factors in fracture hematomas, APC may be present at fracture sites. In addition, previous studies have shown that PAR-1 is expressed by osteoblasts [16–19] and that APC activates PAR-1 in the presence of EPCR to induce anti-inflammatory, anti-apoptosis, and wound healing effects [4,5,7]. Therefore, we hypothesized that APC generated by thrombin at fracture sites may have pathophysiological effects on osteoblasts. In the present study, we demonstrated that APC stimulates osteoblast proliferation by activating p44/42 mitogen activated protein kinase (p44/42MAPK, also known as ERK1/2) through an EPCR-dependent but PAR-1-independent mechanism.

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2. Materials and Methods

2.1. Protein C, APC and Antibodies

Protein C was purified from human plasma according to the method of Suzuki et al. [2] and APC was prepared from protein C by treatment with thrombin [2]. Diisopropyl fluorophosphate-inactivated APC (DIP-APC) was prepared according to the method of Grey et al. [20]. The protease activity of DIP-APC was confirmed to be less than 1% using Boc-Leu-Ser-Thr-Arg-MCA (Peptide Research Institute, Osaka, Japan), fluorogenic substrate specific for APC.

Rabbit anti-human protein C antibody was obtained from Sigma (St. Louis, MO). Anti-human EPCR monoclonal antibodies, RCR-252 and RCR-92, the former inhibits the binding of APC to EPCR and the latter does not, were provided by Dr Kenji Fukudome, Saga University School of Medicine. Anti-human PAR-1 antibodies, ATAP-2 and WEDE-15, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and BECKMAN COULTER (Fullerton, CA), respectively. Peroxidase-labeled goat anti–rabbit IgG antibody (Histofine Simplestain Max PO) and negative control IgG for immunostaining were from Nichirei (Tokyo, Japan), and DAKO (Carpenteria, CA), respectively. Alexa Fluor® 488 goat anti-mouse IgG was from Invitrogen (Carlsbad, CA). Anti-p44/42 MAPK antibody and anti-phosphorylated p44/42 MAPK antibody were from Cell Signaling (Beverly, MA).

2.2. Cell Culture

Normal human osteoblasts, purchased from Bio Whittaker (Walkersville, MD), were alkaline phosphatase-positive and able to form von Kossa-positive nodules. The cells were maintained at 37 °C in osteoblast growth medium (Bio Whittaker) supplemented with 10% fetal calf serum (FCS), 50 μg/mL ascorbic acid, and 0.1% gentamicin/amphotericin B solution in a 5% CO_2/95% air atmosphere on plastic dishes.

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (Walkersville, MD), and cultured in endothelial cell growth medium-2 (EGM-2) (Clonetics) containing 10% heat-inactivated FCS.

2.3. Immunohistochemical Staining

Tissue specimens were obtained from patients aged 26 to 83 years who were undergoing open reduction and internal fixation for isolated closed fractures four or five days after injury. Patients who had open fractures, multiple traumas, a previous malignancy or connective tissue disorder, osteoarthritis, rheumatoid arthritis, inflammatory bowel disease, metabolic disease, or were using certain medications, including steroids, anticoagulants, or antiplatelet agents, were excluded. Written informed consent was obtained from all patients, and the protocol was approved by the Human Studies Subcommittee of Mie University Graduate School of Medicine (Permission No. 605). Fracture hematomas were obtained at the time of surgery. The hematoma was manually removed before any manipulation or irrigation to avoid blood contamination in the operating field and placed in sterile polypropylene containers. Specimens were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), sectioned on a cryostat to 6 μm, mounted on glass slides (Matsunami, Osaka, Japan), and stored at -80 °C. Specimens were thawed and fixed in freshly prepared 4% neutralized formaldehyde in phosphate-buffered saline (PBS). After quenching with 3% H_2O_2 in methanol and equilibrating in PBS, samples were incubated with either a rabbit anti-human protein C antibody or a negative control IgG at room temperature overnight. Sections were incubated with peroxidase-labeled goat anti–rabbit IgG antibody at room temperature for 30 min. Peroxidase activity was detected with diaminobenzidine (DAB) (Sigma). Sections were then counterstained with hematoxylin and dehydrated.

To estimate the amount of protein C in fracture hematoma tissues, small fragments of the fracture hematomas were vigorously vortexed in sterile polypropylene tubes for 30 min and then centrifuged at 15,000 g at 4 °C for 15 min. The protein C concentration in the supernatant was determined by an enzyme-linked immunosorbent assay (ELISA) using mAbs for protein C as described previously [21].

The EPCR protein in osteoblasts was also detected using immunohistochemical analysis. On day 8, the osteoblasts were washed with PBS and fixed in 4% formaldehyde for 20 min and permeabilized for 20 min in PBS containing 0.05% Tween 20. The cells were incubated with 50 μg/ml of anti-EPCR antibody (RCR-252) for 3 h at room temperature, followed by incubation for 30 min with Alexa Fluor® 488 goat anti-mouse IgG (1:200) and DAPI (1:500, DOJINDO, Kumamoto, Japan), and the cells were observed using a conventional fluorescence microscope.

2.4. Proliferation Assay

We evaluated the effect of APC, protein C and DIP-APC on osteoblast mitosis using a cell counting kit (DOJINDO) and a BrdU labeling and detection kit (Roche, Indianapolis, IN). The cells were grown in 96-well plates. After washing twice with PBS, subconfluent osteoblasts were serum-starved for 24 h. Various concentrations of APC, protein C, or DIP-APC were subsequently added to the culture medium. After a 24-h incubation, the assays were performed as follows.

DNA synthesis was assessed by BrdU incorporation. After adding the BrdU labeling solution to each well, cells were incubated for an additional 4 h before fixation. Incorporated BrdU was detected by the direct immunoperoxidase method according to the manufacturer's instructions. The absorbance was read at 405 nm using a microplate reader (Bio-Rad, Hercules, CA) with a reference wavelength of 490 nm. Viable cell numbers were assessed by the tetrazolium salt assay using a commercially available cell counting kit. This kit utilizes the principle that the tetrazolium salt WST-1 is cleaved to formazan by cellular mitochondrial dehydrogenases. Larger numbers of viable cells result in more increased overall mitochondrial dehydrogenase activity. After a 24-h culture with APC-, protein C-, or DIP-APC-containing medium, WST-1 labeling solution was added to the culture medium. The total number of viable cells was subsequently evaluated by measuring the absorbance at 450 nm.

We then assessed the effect of protein C zymogen, aprotinin (Wako, Osaka, Japan) and an anti-protein C antibody (HC-4), which recognizes an epitope on the light chain of protein C and inhibits APC activity, on APC-induced proliferation of osteoblasts. Protein C, aprotinin or the anti-protein C antibody was added to cells 30 min before APC treatment. After a 24-h incubation, viable cell numbers were assessed by the tetrazolium salt assay as described above.

2.5. Effect of Anti-EPCR and Anti-PAR-1 Antibodies on APC-induced Osteoblast Proliferation

To examine the roles of EPCR and PAR-1 in APC-induced osteoblast proliferation, osteoblasts were pre-incubated with RCR-252 (20 μg/ml), RCR-92 (20 μg/ml), or ATAP-2 (20 μg/ml) at 37 °C in 96-well plates for 30 min. After adding APC (50 nM) or PBS to the culture medium, osteoblasts were incubated for an additional 24 h. Osteoblast proliferation was then evaluated by the tetrazolium salt assay.

2.6. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed as described with the following minor modifications [22]. Briefly, total RNA was extracted from osteoblasts and HUVECs (control cells) by the acidic phenol-guanidine isothiocyanate method using RNAzol reagent (Biotex Laboratories, Houston, TX). First-strand cDNA was synthesized using total cellular RNA (4 μg)