ACTIVATED PROTEIN C SUPPRESSES OSTEOCLAST DIFFERENTIATION

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INTRODUCTION:
Activated protein C (APC), an anticoagulant serine protease, physiologically inactivates blood coagulation cofactors, factor Va and factor VIIIa. Besides its function in the blood coagulation system, APC has been recently demonstrated to play an important role in the regulation of inflammation, cell proliferation and apoptosis via endothelial protein C receptor (EPCR) and/or proteinase-activated receptor-1 (PAR-1). Bone remodeling depends on a delicate balance between formation and resorption of bone tissues, in which bone-forming osteoblasts and bone-resorbing osteoclasts play central roles. During physical trauma such as fractures, hypercoagulability may occur at the sites of fracture. But the role of the blood coagulation system in bone remodeling is still unclear. In the present study, to elucidate the relationship between the blood coagulation system and bone remodeling we examined the effect of APC on RANKL-induced osteoclast differentiation, and found that APC suppresses human osteoclast differentiation through inactivation of NF-κB via EPCR.

MATERIAL AND METHODS:

Cell Culture
Normal human osteoclast precursor cells and culture medium were purchased from Lonza Walkersville, Walkersville, Maryland. Normal human osteoclast precursor cells were cultured using osteoclast precursor growth medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml) in the presence of soluble RANKL (100 ng/ml) and M-CSF (50 ng/ml) at 37°C in 5% CO2 atmosphere.

Osteoclast Differentiation Assay
The effect of APC on osteoclast differentiation was evaluated using TRAP staining kit (Cell Garage, Tokyo, Japan). Normal human osteoclast precursor cells were seeded at 1x10⁴ cells/well in a 96-well plate and cultured in osteoclast precursor growth medium. On days 4 and 7, the culture medium was replaced by fresh medium containing soluble RANKL and M-CSF. On day 8, osteoclasts were detected as TRAP-positive cells using a TRAP staining kit. TRAP-positive multinucleated cells (3 nuclei) were counted manually using a light microscope.

Effect of Anti-EPCR and Anti-PAR-1 Antibodies on RANKL-induced Osteoclast Differentiation
Two anti-human EPCR antibodies (RCR-252 and RCR-92) and anti-human PAR-1 antibody (ATAP-2) were used for studying the role of EPCR and PAR-1 on RANKL-induced osteoclast differentiation by APC. After treatment with APC and each antibody, osteoclast differentiation was assessed by TRAP staining.

Evaluation of NF-κB Activation
Normal human osteoclast precursor cells (2x10⁵ cells/well) were cultured in a 96-well plate in the presence of soluble RANKL (100 ng/ml) and M-CSF (50 ng/ml). Medium was changed on days 4 and 7, and then they were treated with different concentrations of APC. On day 8, the level of NF-κB activation in osteoclast precursor cells was determined using ELISA specific for NF-κB following the manufacturer’s instruction.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)
Total RNA was extracted from osteoclasts by the acidic phenol-guanidine isothiocyanate method using RNAzol reagent. First-strand cDNA was synthesized using the SuperScript First Strand Synthesis System kit (Invitrogen) and the total RNA extracted from osteoclasts as a template following the manufacturer’s instructions. PCR reaction was performed by standard protocol using a pair of primers specific for EPCR and PAR-1. PCR products were run on 2% agarose gel and the bands were visualized by ethidium bromide staining and ultraviolet transillumination.

Immunofluorescence staining
On day 8, the cells 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 anti-EPCR (50 µg/ml) or anti-PAR-1 antibodies (4 µg/ml) at 3 h at room temperature, followed by incubation for 30 min with Alexa Fluor® 488 goat anti-mouse IgG (1:200, Invitrogen), rhodamine phalloidin (1:100, Invitrogen) and DAPI (1:500, DOJINDO), and the cells were observed using a conventional fluorescence microscope.

RESULTS:

Effect of APC on RANKL-induced Osteoclast Differentiation
APC showed significant inhibition of TRAP-positive multinucleated cells formation in normal human osteoclast precursor cells in a dose dependent manner.

Effect of Anti-EPCR and Anti-PAR-1 Antibodies on RANKL-induced Osteoclast Differentiation
To elucidate the role of EPCR and PAR-1 in APC-mediated inhibition of RANKL-induced osteoclast differentiation, the effect of two anti-EPCR antibodies were examined. RCR252, which blocks APC-binding to EPCR, inhibited osteoclast differentiation; however, RCR92, which does not block APC-binding to EPCR, showed no effect. On the other hand, ATAP-2 did not inhibit the effect of APC on osteoclast differentiation.

Effect of APC on RANKL-induced activation of NF-κB
Among various transcription factors that have been shown to play critical roles in the expression of genes required for osteoclastogenesis, we first examined the effect of APC on RANKL-induced NF-κB activation in human osteoclast precursor cells. APC dose-dependently reduced RANKL-induced NF-κB activation.

Expression of EPCR and PAR-1 in Osteoclasts
RT-PCR analysis showed that both EPCR and PAR-1 mRNA expressed by osteoclasts. Immunofluorescence analysis also confirmed that osteoclasts express both EPCR and PAR-1 on their cell surfaces.

DISCUSSION:

The anticoagulant serine protease, APC was initially found to be a physiological regulator of the blood coagulation system. To date, it has been demonstrated that APC also regulates inflammation, cell proliferation and wound repair. Recently, we have shown that APC stimulates osteoblast proliferation via EPCR (manuscript submitted), however, there is little information on the effect of APC in musculoskeletal cells such as osteoblasts and osteoclasts. In the present study, to elucidate the relationship between the blood coagulation and bone remodeling we evaluated the effect of APC on osteoclast differentiation. Firstly, we showed that APC inhibits RANKL-induced osteoclast differentiation. This finding suggests that APC may regulate both osteoblasts and osteoclasts. It is known that APC affects cellular function exerts via EPCR, the physiological receptor of APC, and/or PAR-1. We confirmed both EPCR and PAR-1 are expressed by osteoclasts as measured by RT-PCR and immunohistochemical analysis. Our data are consistent with previous reports showing that PAR-1 is expressed by musculoskeletal cells such as osteoblasts and osteoclasts. The inhibitory effect of APC on osteoclast differentiation was reduced by anti-EPCR antibody that inhibits APC-binding to EPCR, but not by anti-PAR-1 antibody. These data suggest that the effect of APC on osteoclast differentiation is mediated by EPCR. Furthermore, data on NF-κB measured using specific ELISA suggest that APC inhibits RANKL-induced NF-κB activation in osteoclasts. The data are also consistent with previous data showing that APC inhibits NF-κB nuclear translocation in a monocytic cell line.

In conclusion, we provided evidence that osteoclasts express both EPCR and PAR-1, and that APC suppresses human osteoclast differentiation through inactivation of NF-κB via EPCR. This is the first report showing that APC affects osteoclast differentiation.

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