MOBILIZATION OF ENDOTHELIAL PROGENITOR CELLS
IN FRACTURE HEALING AND DISTRACTION OSTEOGENESIS
*Lee, DY; *Kim, JA; +*Cho, T-J; *Lee, KS; *Yoo, WJ; * Choi, IH
++ Seoul National University Children’s Hospital, Seoul, Korea
*tjcho@snu.ac.kr

INTRODUCTION:
Vascular proliferation and invasion play an important role in new bone formation. Endothelial regeneration had been considered exclusively attributable to the sprouting of pre-existing vessels (angiogenesis). After Asahara et al. (1) first introduced the concept of endothelial progenitor cells (EPCs), multiple studies have confirmed that EPCs derived from the bone marrow contributed to new vessel formation in the injured sites to some extent (vasculogenesis). Mobilization of EPCs was reported in various clinical situations such as acute myocardial infarction, exercise-induced ischemia, surgical injuries, and vascular trauma (2,3). It is highly likely that a fracture would provoke EPC mobilization to let it contribute to fracture healing (FH). Distraction osteogenesis (DO) is a special form of bone healing process, in which exuberant new vessel formation and increase in blood supply to the operated limb has been well described (4,5). The purposes of current study were to delineate the temporal pattern of EPC mobilization in fracture healing and distraction osteogenesis; and to identify the signals from the injured site, which mobilize EPCs and induce their homing to the bone healing site.

METHODS
All animal protocol received prior approval by the institutional animal care and use committee.

Mouse Fracture Model
Closed fractures at mid-diaphysis level were induced manually in both tibiae. Five animals were harvested at each time points until post-fracture 14 days. Total mononuclear cells (MNCs) were isolated from peripheral blood by density gradient centrifugation. MNCs were plated on fibronectin-coated chamber slides at 1 X 10^6 per well, and were grown in endothelial cell growth medium. In 48 hours, adherent cells were incubated with DiI-acetylated-LDL for 1 hour and then counterstained with FITC-labeled lectin from Ulex Europaeus. Two independent investigators counted and averaged the number of EPCs per well by counting 10 randomly-selected high-powered fields on fluorescence microscopy. Another set of MNCs from each time points was co-stained with PE-conjugated anti-mouse VEGFR2 rat antibody and FITC-conjugated anti-mouse CD133 rat antibody. They were analyzed using FACStar flow cytometer. The number of positive cells was compared with IgG isotype control.

Total RNA was extracted from the callus tissue at each time points. RNase protection assay was performed to assay mRNA expression of IL-3, IL-11, IL-7, GM-CSF, M-CSF, G-CSF, LIF, IL-6, Stem cell factor (SCF), and housekeeping genes. RT-PCR was performed to assay mRNA expression of VEGF, MCP-1, SDF-1, VCAM-1, ICAM-1, Plasma VEGF concentration was assayed at each time points by ELISA technique using Quantikine VEGF ELISA kit (R&D systems).

Rat Distraction Osteogenesis Model
Bilateral monofixators were applied to both tibiae and corticotomy was made at the mid-diaphysis. After 7 days of latency period, the fragments were distracted 0.25 mm twice a day for 7 days, and then the regenerate bone was consolidated. Circulating EPCs in the peripheral blood were counted at each time points, and compared with no-distraction group.

RESULTS
EPC Mobilization in Fracture Healing
Fracture transiently increased number of EPCs in the peripheral blood. The number of adherent EPCs which incorporated both DiI-acetylated-LDL and FITC-lectin increased rapidly after fracture, peaked at 3 days and decreased slowly over a subsequent 10-day period (Fig. 1). On FACS analysis, VEGF-R2 positive fraction in the mononuclear cells peaked at 3 days, and VEGFR2+CD133+ dual positive fraction representing more immature precursor cells peaked at 1 day after fracture.

Quantitation of mRNA expressions using RNase protection analysis showed that different expression patterns between cytokines during fracture healing (Fig. 2). Among the cytokines known to mobilize EPCs, SCF was up-regulated throughout the fracture healing process but G-CSF, GM-CSF were not. Inflammatory cytokines such IL-6, IL-11 were up-regulated in early healing process and expression of M-CSF was increased steadily during healing process. RT-PCR suggested that mRNA expression of VEGF, MCP-1, VCAM-1 might be up-regulated in fracture callus than in control tissue (Fig. 3). The plasma level of VEGF increased immediately post-fracture, peaking at 12 hours, and then gradually decreased to a basal level around 14 days after fracture.

EPC Mobilization in Distraction Osteogenesis
During DO, the proportion of circulating EPCs peaked on POD 5, and then decreased gradually, which increased again during distraction period through consolidation period (Fig. 4).

DISCUSSION:
Adherent Dil-LDL positive cells and VEGF+CD133+ cells are considered to represent circulating EPCs derived from the bone marrow. We confirmed in this study that EPCs actually were mobilized into peripheral circulation by signals from fracture and distraction osteogenesis. VEGF, MCP-1, SCF induced during fracture healing seem to play roles in EPC mobilization. Tension stress at the regenerate tissue during DO may induce EPC mobilization from the bone marrow. Role of the mobilized EPC in bone healing needs further investigation.

REFERENCES:

ACKNOWLEDGEMENT
This study was supported by Korea Research Foundation Grant (KRF - 2004-041-E00208).