INTRODUCTION:
Cartilage is an avascular tissue and can act as a barrier against tumor invasion and some chronic inflammatory diseases. This avascularularity may be due to the local production of angiogenesis inhibitors. During our previous studies on expression of plasminogen in human articular cartilage, we isolated a unique cDNA fragment named plasminogen related gene-B (PRG-B) which predicts a 9kDa polypeptide designated as plasminogen-related protein-B (PRP-B). We reported that recombinant PRP-B (rPRP-B) inhibited tumor growth by blocking tumor angiogenesis in vivo and that this effect was at least partly mediated through the inhibition of basic fibroblast growth factor (bFGF) -induced tyrosine kinase signaling in endothelial cells (2, 3). However, the detailed antiangiogenic mechanisms of rPRP-B in other angiogenic diseases were not analyzed. In inflammatory diseases like RA and OA, activation of angiogenesis through the up-regulation of pro-angiogenic cytokines such as bFGF, tumor necrosis factor-a (TNF-a), interleukin-1ß (IL-1ß), vascular endothelial growth factor (VEGF) have been reported. Therefore, attempts have recently been made to employ angiogenesis-inhibitors for the treatment of arthritis. In this study, we investigated the effects of rPRP-B as an antiangiogenic protein for cytokine-stimulated human fibroblast-like synoviocytes (FLS).

MATERIALS AND METHODS:
Recombinant protein: The isolation and characterization of rPRP-B has been described previously (4).
Cell culture: FLSs were purchased from CELL APPLICATIONS, INC. FLSs were harvested in Synoviocyte Basal Medium (CELL APPLICATIONS, INC.) containing Synoviocyte Growth Supplement (CELL APPLICATIONS, INC.) at 37ºC in a humidified 5% CO₂. Cells between passage 3 and 5 were used for experiments.
Cell Proliferation assay: Recombinant human bFGF and IL-1ß were purchased from Oncogene Research Products and PeproTech EC, respectively. FLSs in Synoviocyte Basal Medium containing Synoviocyte Growth Supplement were split into 96-well plates (2000 cells/well) pre-coated with 1% gelatin and harvested for 24h. After washing with PBS, rPRP-B (5ng/ml) in Synoviocyte Basal Medium was added into the indicated wells for 1 hr. Cultures were then treated with bFGF or IL-1ß (5ng/ml) and the plates incubated for 48h. Following the incubation, viable cells were detected using a spectrophotometric CellTiter 96 Aqueous cell proliferation assay (Promega).
Enzyme-linked immunosorbent assay (ELISA): The levels of VEGF in cell culture supernatants were measured using the Quantikine immunoassay kit for human VEGF (R&D Systems). We plated FLSs at a density of 2000 cells/well in a 96-well culture plate. After serum starvation, cultures were treated with bFGF or IL-1ß with or without rPRP-B. Following the 48h incubation, 200µl of cell culture supernatants were collected from each well and ELISA was performed according to the manufacturer’s instructions.
Reverse transcription- polymerase chain reaction (RT-PCR): We investigated the effect of rPRP-B on expression of VEGF mRNA in FLSs by RT-PCR. FLSs were stimulated with bFGF and IL-1ß (5ng/ml) in Synoviocyte Basal Medium with or without rPRP-B (5ng/ml). After 48h incubation, the culture medium was removed and total RNA isolated using the RNAqueous-4PCR kit (Ambion) according to the manufacturer’s instructions. PCR was performed using specific primer pairs for VEGF or GAPDH, and the reaction products analyzed by agarose gel electrophoresis. The results were scanned and the images analyzed using image software to determine the pixel intensity value for each band. The values for VEGF mRNA in stimulated FLSs with or without rPRP-B were compared.
RESULTS:
Cell proliferation assay: rPRP-B could not inhibit bFGF or IL-1ß-induced proliferation of FLSs (data not shown).
ELISA: rPRP-B reduced the level of secreted VEGF in the culture supernatants of both bFGF and IL-1ß stimulated FLSs (Fig.1).

Discussion:
In this study, we analyzed the effect of rPRP-B on FLSs stimulated by the pro-angiogenic cytokines bFGF and IL-1ß. These results demonstrated that rPRP-B was capable of reducing VEGF expression at the mRNA and protein level in cytokine stimulated FLSs without affecting cell proliferation. VEGF is an important mediator of angiogenesis and is involved in the development of the vascular system. VEGF expression is up-regulated in macrophages and FLSs in RA synovial tissues. Moreover, VEGF expression level is correlated with disease severity. Therefore, our results suggest that rPRP-B can be a promising new anti-arthritic drug with a novel mechanism of action that is quite different from currently used anti-inflammatory drugs.

References:

Fig. 1: VEGF concentrations detected by ELISA in supernatants of bFGF or IL-1ß stimulated FLSs with or without rPRP-B.

Fig. 2: Expression level of VEGF mRNA isolated from bFGF or IL-1ß stimulated FLSs with or without rPRP-B.