INTRODUCTION
Mesenchymal stem cells derived from the bone marrow (BM-MSCs) can be acquired without permanently damaging tissues, can be efficiently expanded in monolayers, and most importantly can differentiate into chondrogenic cells for the potential treatment of injured articular cartilage [1,2]. In order to use BM-MSCs in vitro for the repair of injured articular cartilage, a biomaterial is required to serve as both a delivery vehicle and a three dimensional structure for developing tissue. This biomaterial must be biocompatible, mechanically stable, and capable of not only promoting chondrogenic expression of cells, but also allowing deposition of extracellular matrix. Many synthetic and natural polymers have been tested as scaffolds for tissue engineering, including fibrin gels. Fibrin gels consist of fibrinogen and thrombin solutions, which generate a crosslinked fibrin clot in a process that mimics the last stage of physiologic coagulation system. It has also been shown that fibrin gels are capable of supporting chondrogenic potential that can induce a cartilage-specific extracellular matrix [3]. However, fibrin is also intrinsically unstable due to fibrinolysis. Stabilization can be achieved with a variety of fibrinolytic inhibitors that have been tested for in-vivo safety and efficacy [4]. Since previous studies have demonstrated that treatment of transforming growth factor-β (TGF-β) can induce chondrogenesis of BM-MSCs [2,5], we hypothesize that the three dimensional fibrin gel cultures can provide a supportive environment for chondrogenesis of BM-MSC. Therefore, the objective of this study was to not only examine the chondrogenesis of rabbit BM-MSCs cultured in fibrin gels with the treatment of TGF-β1 but also to examine the effect of various fibrinolytic inhibitors on chondrogenesis.

MATERIALS AND METHODS
Isolation of Rabbit BM-MSCs
Rabbit BM-MSCs were obtained from the tibias of New Zealand White rabbits (3 months old) and expanded in monolayer cultures based on a previously reported protocol [6]. The chondrogenic potential of BM-MSCs was examined using pellet culture.

Preparation of Cell-Fibrin Constructs
After the cells were trypsinized and counted, 2x10^5 cells were pelleted by centrifugation at 500 rpm for 10min. The cells were resuspended in 100µl of fibrinogen solution, which consisted of equal parts of 80 µg/ml fibrinogen and DMEM and then mixed with 100µl of 5 IU/ml thrombin solution. The entire mixture was then added to one well of a 48 well plate and incubated at 37°C in 5% CO_2 for 30 min.

Chondrogenesis of BM-MSCs and Fibrinolysis Inhibition
After cell-fibrin construct preparation, specimens were cultured in a defined serum-free medium consisting of high-glucose DMEM, 1% ITS (BD Biosciences, Bedford MA), 50µg/ml ascorbic acid, .01M HEPES buffer and 10^3 M dexamethasone (Sigma, St. Louis MO). Then the samples were divided into four groups: Aprotinin control, Aprotinin and TGF-β1, Aminohexanoic acid control, Aminohexanoic acid and TGF-β1. The specimens of the TGF-β1 group and control group were cultured in serum-free medium with and without the supplement of 10 ng/ml TGF-β1, respectively. In addition, the aprotinins were supplemented with 0.35 TIU aprotinin and the aminohexanoic acid were supplemented with 2 mg/ml of aminohexanoic acid. All cell-fibrin constructs were cultured in a humidified incubator maintained at 37°C in 5% CO_2, for 21 days. The culture medium was changed every 2-3 days. The expressions of chondrogenic genes were analyzed for each group using reverse transcription-polymerase chain reaction (RT-PCR) analysis after 7, 14, and 21 days. The RNA was extracted from half of the cell-fibrin construct using the reagent Trizol (GibcoBRL) according to the manufacturer’s instructions. The RT-PCR was performed using the GeneAmp PCR system (Applied Biosystems, Foster City, CA) and the ThermoScript RT-PCR system (GibcoBRL). The gene expressions of the specimens were normalized with the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene. In addition, deposition of extracellular matrix proteins was examined by histological and immunohistochemical evaluations. The remaining half of the cell-fibrin construct was fixed in 10% buffered formalin and paraformaldehyde. The specimens were then dehydrated with ethanol, cleaned with xylene, embedded in paraffin, and cut into 5 µm sections. Proteoglycans were detected by staining sections with toluidine blue.

RESULTS
The chondrogenic gene expressions of the four groups at two time periods are shown in Figure 1. After 14 days, the aprotinin and TGF-β1 group exhibited a stronger gene expression of aggrecan than the aprotinin control group and both aminohexanoic acid groups. Also at this time period, the control groups for both inhibitors show a weaker expression for collagen II than their TGF-β1 treated groups. After 21 days, the TGF-β1 treated groups for both aprotinin and aminohexanoic acid showed a stronger gene expression for both chondrogenic genes than their respective control groups. In addition, the specimens of the TGF-β1 groups exhibited stronger toluidine blue staining as compared with the specimens of the control groups for both inhibitors. The aprotinin and TGF-β1 group also exhibited a much stronger staining than the aminohexanoic and TGF-β1 group, suggesting that aprotinin is more conducive to deposition of extracellular matrix.

DISCUSSION
The analysis of the chondrogenic gene expression verifies our hypothesis that fibrin gels are capable of supporting the chondrogenesis of rabbit BM-MSCs. In addition, the fibrinolytic inhibitors needed to stabilize the fibrin gels have an affect on the chondrogenesis of the BM-MSCs. The toluidine staining shows that the aprotinin promotes extracellular matrix deposition when compared to aminohexanoic acid.

REFERENCE

Figure 1. Typical chondrogenic gene expression of fibrin gels of the four groups at 14 days (Apro = Aprotinin and Amino = Aminohexanoic acid)

Figure 2. Toluidine blue staining of (a)(b) aminohexanoic acid and (c)(d) aprotinin treated specimens (a)(c) without and (b)(d) with the treatment of TGF-β1 after 21 days.