INTRODUCTION

Bone morphogenetic proteins are members of the TGF-β superfamily of growth and differentiation factors. Members of the BMP family were originally isolated from bone and defined by their ability to induce de novo ectopic bone formation. Human BMP-9 shares 50-55% homology with BMPs 2, 4, 5, 6, 7, and 8 at the amino acid level [1]. Implantation of recombinant human (rh) BMP-9 induces ectopic bone formation, but the relatively large quantity of implanted rhBMP-9 required to induce cartilage and bone formation in vivo led the discoverers of this molecule to suggest that its primary function may be as a growth or differentiation factor at nonskeletal sites [1]. Consistent with this assertion, others have suggested functions for BMP-9 outside of the skeletal system. BMP-9 is expressed in the fetal and adult liver and stimulates proliferation of the HepG2 hepatocyte cell line and primary rat hepatocytes; induces and maintains the neuronal cholinergic phenotype in the developing central nervous system; and modulates hemopoietic progenitor cell generation and colony formation in vitro.

Though BMP-9 is not predominantly expressed during bone formation and rhBMP-9 is not as potent as other BMPs in inducing bone formation in vivo, injection of adenoviral vectors coding for BMP-9 does induce ectopic bone formation and produces potentially therapeutic spinal arthrodesis [2]. To explore the effects of BMP-9 on chondrocytes in a well-defined, three-dimensional, in vitro model of chondrogenesis, we investigated the effects of BMP-9 on the growth and biochemical composition of tissue-engineered cartilage. This paper is, to the best of our knowledge, the first to explore the effects of BMP-9 on chondrocytes in a well-defined in vitro system and reports the effects of BMP-9 on engineered cartilage growth, ECM deposition, and mineralization.

METHODOLOGY

Isolation of chondrocytes. Primary chondrocytes were isolated from full-thickness bovine calf articular cartilage by digestion with type II collagenase and resuspended in culture medium (DMEM) containing 4.5 g/l glucose, 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 10 mM HEPES, 0.1 mM nonessential amino acids, 0.4 mM proline, and 50 µg/ml ascorbic acid as previously described. Chondrocytes were harvested from a total of 3 knee joints.

Preparation of scaffolds, cell seeding and culturing. In brief, PGA was extruded into 13 µm diameter fibers, processed to form a 97% porous non-woven mesh, die punched into discs 5 mm in diameter by 2 mm thick, and sterilized with ethylene oxide. Five million freshly isolated chondrocytes were seeded per scaffold. After seeding, cell-polymer constructs were transferred into 6-well plates (one construct and 6 ml of medium per well), and cultured on an orbital shaker at 50 rpm. Medium supplemented with the appropriate concentration of supplemented BMP-9 was completely exchanged three times per week for 4 weeks. Recombinant human BMP-9 was a generous gift from Wyeth (Cambridge, MA).

Biochemical analysis of engineered tissues. Engineered tissues (n = 3 for each treatment) were weighed, frozen, lyophilized, and digested with proteinase K at 60°C for 16 hours (1 mg/ml proteinase K in buffered solution). Sulfated GAG content was determined spectrophotometrically at 525 nm after reaction with dimethylmethylene blue dye, using bovine chondroitin sulfate as standard. Hydroxyproline content was determined spectrophotometrically after acid hydrolysis and reaction with p-dimethylaminobenzaldehyde and chloramine-T, and the amount of total collagen was calculated using a 1:10 ratio of hydroxyproline to collagen. The number of cells per cell-polymer construct was assessed from the DNA content using a spectrofluorometer and a conversion factor of 7.7 pg DNA per chondrocyte. Undegraded polymer does not account for a significant fraction of the wet weight of a 4-week construct (approximately 1 to 2%).
Histological analysis of engineered cartilage. Samples for histological analyses were fixed in 2% glutaraldehyde in PBS for 15 minutes, then in 10% neutral formalin, and then embedded in paraffin. Sections (5 µm thick) were cut through the center of the construct and placed on glass slides. Deparaffinized sections were stained with hematoxylin and eosin to visualize morphology, alizarin red to visualize mineralization, or immunostained for type-I collagen.

RESULTS
Macroscopic and Histological Analysis. Over 4 weeks of in vitro cultivation, the cell-polymer constructs developed into cartilaginous tissues that macroscopically resembled native cartilage while roughly retaining the cylindrical shape of the original polymeric scaffold. Only constructs cultured for 4 wks in the presence of 10, 50, or 100 ng/ml BMP-9 contained hypertrophic chondrocytes. Adjacent to some, but not all, of these hypertrophic chondrocytes, H&E-staining revealed mineralization (data not shown). Alizarin red staining co-localized with regions of mineralization seen in H&E-stained sections (data not shown). Mineralization was located ~100 to ~300 µm from the surface of the construct but not all areas in this region were mineralized.

Biochemical Analysis. In the absence of supplemented BMP-9 (i.e., control medium), the wet weight of the cell-polymer construct increased with time, a trend that was potentiated by all concentrations of BMP-9 tested (Fig. 1). After 4 weeks of cultivation, all concentrations of BMP-9 significantly increased the wet weight (52-95% increase) compared to control constructs. Concurrent with the increase in total wet weight of the constructs, relative to unsupplemented controls, BMP-9 increased the amount of the major structural constituents of the constructs, GAG (58-111% increase) and collagen (23-53% increase) and also increased the number of cells (71-107% increase) (Fig. 2).

DISCUSSION
In contrast to other BMPs (BMP-2, -12 and -13 [3] or growth factors (IGF-1, TGF-β, bFGF, IL-4, PDGF [4]) previously tested in this in vitro tissue-engineered cartilage system, BMP-9 is the only factor to induce mineralization. The effects of BMP-9 on ECM deposition are qualitatively similar to what we previously reported with BMP-2, -12, and -13 in the same in vitro tissue-engineered cartilage system [4] with all four BMPs increasing the wet weight of the constructs, increasing the GAG percent, and decreasing the collagen percent. The notable difference between BMP-9 and BMP-2, -12, and -13, however, is the dose required to elicit these effects; BMP-9 is active at much smaller concentrations than the other BMPs. The higher potency of BMP-9 would not be readily predicted from previous in vivo studies that have shown that much more rhBMP-9 than rhBMP-2 is required to induce ectopic bone formation. Taken together, these data suggest that BMP-9 is a potent modulator of mature chondrocyte activity.

REFERENCES