Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes

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Abstract: The search for biocompatible materials that can support the growth and phenotypic expression of osteoblasts and chondrocytes is a major challenge in the application of tissue engineering techniques for the repair of bone and cartilage defects. Chitosan, a copolymer of glucosamine and *N*-acetylglucosamine, may provide an answer to this search. Chitosan is the deacetylated product of chitin, a ubiquitous biopolymer found in the exoskeleton of insects and marine invertebrates. Little is known about the utility of chitosan in propagating human osteoblasts and chondrocytes. In this study, we test the hypothesis that chitosan promotes the survival and function of osteoblasts and chondrocytes. Chitosan (4%, w/v in 2% HAc) was coated onto plastic coverslips that had been fitted into 24-well plates. Human osteoblasts and articular chondrocytes were seeded on either uncoated or chitosan-coated coverslips at 1×10^5/ cells per well. Cultures were incubated at 37°C, 5% CO_2 for a period of 7 days. Cell viability was assessed at that time using a fluorescent molecular probe. The phenotypic expression of osteoblasts and chondrocytes was analyzed by reverse transcriptase-polymerase chain reaction and immunocytochemistry. Osteoblasts and chondrocytes appeared spherical and refractile on chitosan-coated coverslips. In contrast, greater than 90% of cells on plastic coverslips were elongated and spindle shaped after 7 days of culture. Similar to cells propagated on uncoated control wells, greater than 90% of human osteoblasts and chondrocytes propagated on chitosan remained viable. Human osteoblasts propagated on chitosan films continued to express collagen type I whereas chondrocytes expressed collagen type II and aggrecan, as shown by reverse transcriptase-polymerase chain reaction analysis and immunostaining. The present *in vitro* work demonstrates the biocompatibility of chitosan as a substrate for the growth and continued function of human osteoblasts and chondrocytes. Chitosan may have potential use as a tissue engineering tool for the repair of osseous and chondral defects.© 2000 John Wiley & Sons, Inc. J Biomed Mater Res, 51, 586–595, 2000.

Key words: osteoblasts; chrondrocytes; chitosan; extracellular matrix.

INTRODUCTION

The need for more effective methods of repairing bone and cartilage defects has prompted investigation into the utility of tissue engineering approaches. One such approach is to generate histologically and functionally normal tissue by delivering healthy cells in a biocompatible scaffold. The cell-material complex can then be implanted to repair osseous and chondral defects. A number of natural and synthetic materials have been considered for their potential use as cell delivery vehicles. However, the identification of materials that can support osteoblast and chondrocyte survival and phenotypic expression remains a major challenge. One candidate material that may fulfill this need is chitosan.

Over the past 20 years, the biomedical applications of chitosan have been widely researched.[1] The material has been evaluated as a wound-healing agent, bandage material, skin grafting template, cholesterol-lowering agent, hemostatic agent, hemodialysis membrane, and drug-delivery vehicle.[2–10] Chitosan is the product of the partial deacetylation of the naturally occurring polysaccharide chitin, which is found in the exoskeletons of insects and marine invertebrates. It is a linear copolymer of *N*-acetyl-D-glucosamine and glucosamine, with the latter constituting greater than 80% of the molecule. Chitosan has been suggested to possess biological and material properties suitable for clinical applications; specifically, it has been reported to be nontoxic[6,11–16] and bioresorbable[17–20] when used in human and animal models.

Chitosan has been explored as a modulator of wound healing.[21–24] Studies of full-thickness dermal

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wounds in rabbits and rats have demonstrated that chitosan accelerates the healing response time.[6,25] Kim and Min[26] reported that a wound-covering material composed of polyelectrolyte complexes of chitosan and sulfonated chitosan hastened wound healing and yielded a "good-looking" skin surface. The effect of chitosan on wound healing in patients undergoing plastic surgery was noted by Muzzarelli et al.[27] The authors reported that surgical sites treated with chitosan exhibited better histo-architecture and vascularity compared with untreated controls. There were no inflammatory cells at the dermal layer of tissue treated with chitosan. *In vitro* studies have further clarified the contribution of chitosan to wound healing through its activation of fibrogenic mediators such as growth factors and cytokines.[28–34] This increased expression of growth factors enhances fibroblastic activity and promotes fibrous tissue synthesis.

The ability of chitosan to promote wound healing may also be attributed to its tendency to form polyelectrolyte complexes with the polyanion heparin,[35,36] which possesses anticoagulant as well as angiogenic properties.[37,38] This molecule enhances mitogenesis by the induction[39,40] and stabilization of fibroblast growth factor (FGF).[41,42] By forming a complex with heparin and acting to prolong the half-life of growth factors, chitosan promotes tissue growth and wound healing. These studies support the notion that chitosan promotes tissue growth and cellular differentiation. More recently chitosan has been proposed to serve as a nonprotein matrix for three-dimensional tissue growth. Chitosan could provide the biological primer for cell-tissue proliferation and reconstruction.[43]

Encouraged by the utility of chitosan as a wound-healing agent, investigators have explored the material's capacity to promote the growth of bone.[44–48] Using chitosan plugs, Muzzarelli et al.[44] succeeded in creating mineralized bone-like tissue in osseous defects in rats, sheep, and dogs. The authors noted that endosteal, periosteal, and bone marrow osteoblast-like precursors become entrapped in the chitosan-tissue mixture. Based on this observation, the researchers speculated that intramembranous bone formation may be promoted by simultaneous proliferative and angiogenic events. The same investigators confirmed the osteogenic potential of chitosan in patients undergoing apicectomy or wisdom tooth avulsion. The authors showed that patients receiving chitosan sponge implants readily developed newly formed bone in the defects. These implants resorbed readily and left no traces of chitosan 6 months after surgery.[49]

In the present study, we addressed the question of whether chitosan can serve as a supporting matrix for connective tissue growth. We tested the hypothesis that chitosan can support the normal function and expression of extracellular matrix components in human osteoblasts and chondrocytes.

MATERIALS AND METHODS

Preparation of chitosan

A 4% (w/v) solution of chitosan was prepared by dissolving chitosan powder (Seikagaku Corp., Tokyo, Japan) with a greater than 90% degree of deacetylation in 2% (v/v) acetic acid. The chitosan was allowed to fully dissolve over a 24-h period and then sterilized by autoclave. Twenty-four–well cell culture plates (Becton Dickinson, Lincoln Park, NJ) were fitted with 15-mm plastic coverslips (Nalge Nunc Intl., Naperville, IL) in order to facilitate the retrieval of cells from the wells. The chitosan solution was evenly coated onto the surface of the coverslips fitted in the 24-well plates. The solution was then allowed to dry over a period of 24 h to form a thin film. The acidity of the films was neutralized with 1 mL of a 0.5*M* solution of NaOH. Excess base was then removed through repeat washings with Hanks' balanced salt solution until film pH returned to a physiologic range (pH = 7.4). As controls, several coverslips were not coated with chitosan. Prepared plates were finally sterilized by exposure to 12,000 rads before use.

Preparation and propagation of cells

Normal human osteoblasts (Clonetics, Walkersville, MD) were maintained with osteoblast growth media (Clonetics) as monolayer cultures in 75-cm^2 flasks (Costar Corp., Cambridge, MA). Chondrocytes were obtained from the nonfibrillated articular cartilage of an osteoarthritic patient (71/M) during total knee arthroplasty. Cells were retrieved by 0.2% collagenase digestion and propagated in micro-carrier spinner cultures for 2 weeks.[50] Both osteoblasts and chondrocytes were cultured at 37°C and 5% CO_2 before seeding onto chitosan films. Osteoblasts from confluent flasks were trypsinized and centrifuged, then resuspended in serum-free media before their use. Chondrocytes in confluent spinner cultures were isolated from micro-carrier beads by digestion with 0.2% collagenase and centrifuged. The cells were resuspended in AIM-V serum-free media (GIBCO, Grand Island, NY). Osteoblasts and chondrocytes were seeded at 1×10^5/1 mL into wells containing uncoated and chitosan-coated plastic coverslips. All plates were subsequently incubated over a period of 7 days at 37°C and 5% CO_2.

Cell viability and morphologic analysis

Cultures were observed and photographed using phase contrast microscopy at day 2 and day 7 of incubation. Cell viability was assessed on day 7 using the Live/Dead Assay kit (Molecular Probes, Eugene, OR). Coverslips with attached cells were placed in a 1-mL solution of the vital dye calcein-AM (2 μM), and the nuclear stain ethidium homodimer (4 μM) in phosphate-buffered saline. The coverslips were incubated in the dark at room temperature with

out agitation for 40 min, then examined under fluorescent microscopy without subsequent washing.[51,52] Cell viability was indicated by bright green fluorescence in the cytoplasm of the cells. These cells also excluded trypan blue, confirming their viability. Dead cells were identified by the binding of ethidium homodimers to nucleic acids, giving off a bright red fluorescence. These results were also validated through fluorescent staining of ethanol fixed cells, which appeared bright red and stained with trypan blue.

Analysis of mRNA expression

RNA was isolated from trypsinized cells by the TRIzol Reagent method (Life Technologies, Rockville, MD). Isolated RNA (1μg) was used to create a total cDNA library with the Advantage RT-for-PCR Kit (Clontech Laboratories, Palo Alto, CA) using the oligo (dT_{18}) primer. Ten milliliters of the resulting reverse transcriptase product were expanded using the SuperTaq Plus (Ambion, Austin, TX) PCR Kit and primers specific to the sequences of interest (collagen type I, collagen type II, and aggrecan). The housekeeping gene GAPDH was analyzed using the G3PDH Control Amplimer Kit (Clontech Laboratories) to assure equal loading of the sample cDNA. The PCR products were analyzed by electrophoresis in a 1.5% agarose gel that contained ethidium bromide and were photographed with UV light excitation.

Immunocytochemical staining

Cells were retrieved from chitosan and control cultures by trypsinization and then fixed with cytospin collection fluid (Shandon, Pittsburgh, PA). Osteoblasts were incubated with polyclonal goat antibody to collagen type I (Fisher Scientific, Pittsburgh, PA), whereas chondrocytes were incubated with polyclonal goat antibody to collagen type I, collagen type II (Fisher Scientific), or monoclonal mouse antibody to keratan sulfate (ICN Biochemical, Cosa Mesa, CA). Staining for the collagens or keratan sulfate was visualized using the immunoperoxidase technique. Diaminobenzidine was used as the substrate, yielding a brownish color (Vector Laboratories Immunoassay Kit, Burlingame, CA). Cell preparations were counterstained with 1.0% toluidine blue. The specificity of the immunoperoxidase staining was verified through the omission of the primary antibody or use of unrelated primary antibodies. There was no staining observed under these conditions.

RESULTS

Viability and morphological analysis

By day 2 of culture, human osteoblasts seeded onto uncoated and chitosan-coated coverslips appeared refractile with well-defined morphology, with diameters ranging from 10 to 20 μm. Beyond 2 days of culture, greater than 90% of human osteoblasts on the surface of uncoated plastic coverslips assumed a spindle-shaped, fusiform appearance [Fig. 1(A)]. In contrast, fewer than 5% of osteoblasts on the surface of chitosan-coated coverslips assumed an elongated shape. Over 90% of these cells on the surface of chitosan maintained a plump, spherical to polygonal form [Fig. 1(B)]. These morphological differences were maintained through day 7 of culture. Osteoblasts on the surface of uncoated coverslips remained fibroblastic and fusiform in shape [Fig. 1(C)]. Those cells on chitosan-coated coverslips retained their round to oval shape [Fig. 1(D)]. Osteoblasts on uncoated and chitosan-coated coverslips were randomly distributed and did not overlap nor form clusters. Although an equal number of cells had been seeded into each well, osteoblasts on the surface of uncoated coverslips coverslips were observed at a lower density than on chitosan-coated coverslips. There were approximately 40 to 50 cells viewed per field at 100× magnification on the surface of uncoated coverslips. In contrast, 60 to 70 cells were counted per field at 100× magnification on chitosan-coated coverslips. The density of osteoblasts at day 7 ranged from 120 to 160 cells for uncoated coverslips and 120 to 130 for chitosan-coated coverslips per field at 100× magnification. Osteoblasts on the surface of the uncoated and chitosan-coated wells appeared to multiply by two-fold during the culture period.

Chondrocytes seeded onto uncoated and chitosan-coated coverslips appeared refractile with well-defined morphology by day 2 of culture. At day 2 of culture, chondrocytes propagated on uncoated coverslips showed similar morphology to osteoblasts, displaying a fibroblastic and flattened appearance [Fig. 2(A)]. Cells propagated on chitosan-coated coverslips appeared more round to oval in shape [Fig. 2(B)]. Human chondrocytes cultured on uncoated coverslips continued to display a fibroblastic, drawn-out morphology through day 7. In contrast, chondrocytes on chitosan-coated coverslips exhibited a more spherical appearance at day 7 of culture [Fig. 2(C) and (D)]. Chondrocytes seeded onto the surface of uncoated wells were observed at a density of 40 to 50 cells per field at 100× magnification. Sixty to 70 cells per field at 100× magnification were noted on the surface of uncoated coverslips. Chondrocyte density was also noted to be slightly less in uncoated wells with 90 to 110 cells counted per field, as compared with 110 to 130 cells per field at 100× magnification for cells on chitosan-coated wells. The uneven distribution of cells precluded statistical evaluation of cell number on the surface of uncoated and chitosan-coated coverslips.

At the end of the 7-day culture period, greater than 90% of human osteoblasts and chondrocytes stained a vivid green with the fluorescent vital dye, indicating

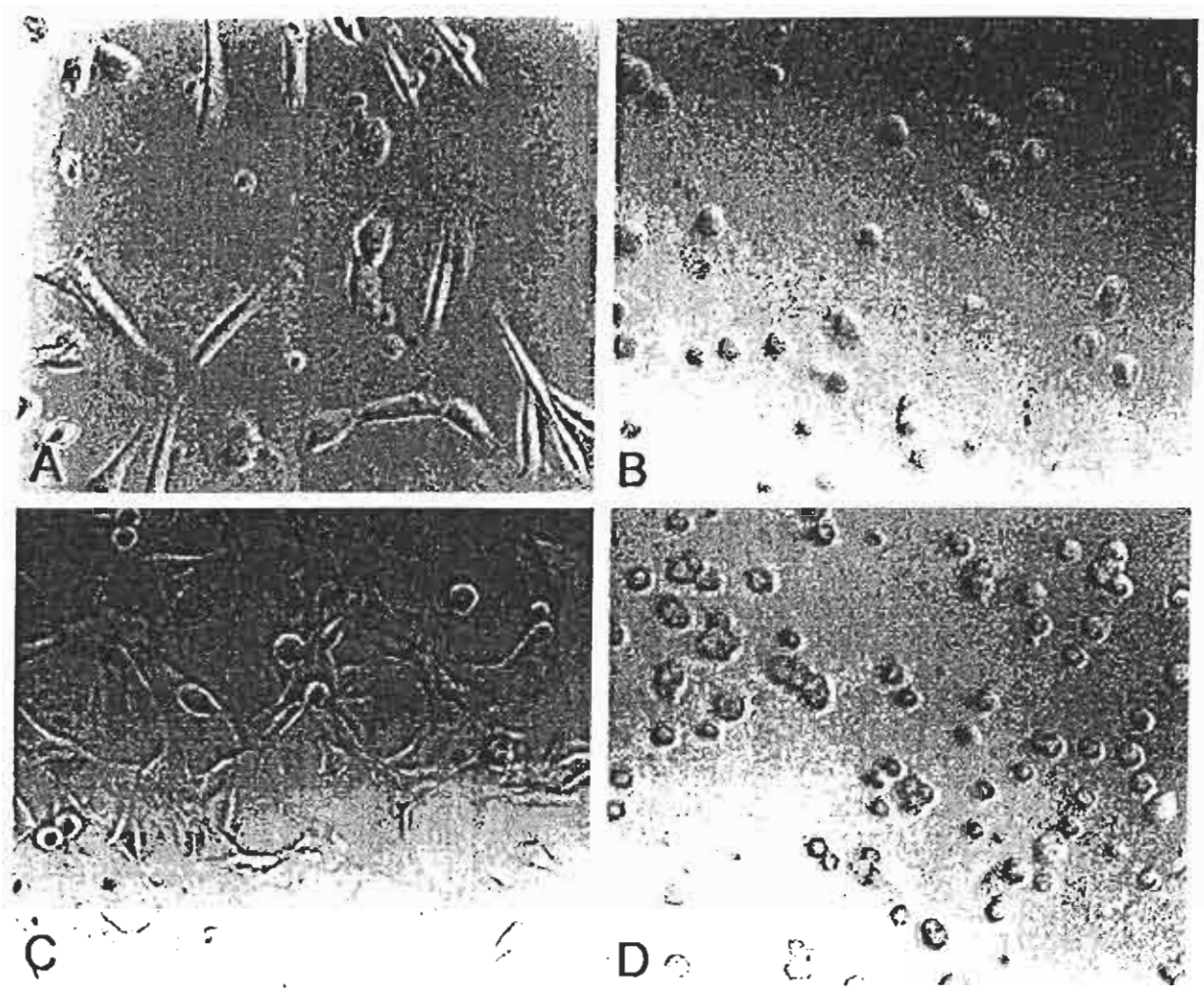

Figure 1. Phase photomicrographs of human osteoblasts (original magnification ×100). Cells analyzed at (A) day 2 on the surface of uncoated coverslips, (B) day 2 on the surface of chitosan-coated coverslips, (C) day 7 on the surface of uncoated coverslips, and (D) day 7 on the surface of chitosan coated coverslips. Note cells propagated on the surface of uncoated coverslips (A) and (C) are more fusiform and spindle shaped.

viability [Fig. 3(A–D)]. Fewer than 10% of cells were stained red, indicating minimal cell death after 7 days of culture on either uncoated or chytosan-coated coverslips.

Phenotype expression—mRNA analysis

Osteoblasts grown on either uncoated or chitosan-coated coverslips continued to transcribe mRNA for collagen type I [Fig. 4(A)]. Human chondrocytes maintained on plastic coverslips and chitosan films maintained their expression of matrix components collagen type II and aggrecan. There was no expression of collagen type I [Fig. 4(B)]. Both human osteoblasts and chondrocytes propagated on chitosan exhibited decreased intensity in the bands representing matrix component transcripts as compared with control cells. This decreased intensity may be due to inaccurate RNA determination, as chitosan interfered with the ultraviolet spectroscopic analysis of RNA. Thus, measured levels of RNA extracted from cells cultured on chitosan were inconsistent. This interference from chitosan contributed to subsequent unequal loading of RNA in reverse transcription and polymerase chain reactions, with fewer mRNA transcripts available for amplification.

Immunostaining patterns

Osteoblasts propagated on the surface of uncoated coverslips stained intensely for collagen type I. Cytoplasmic immunostaining of the osteoblasts was grana-

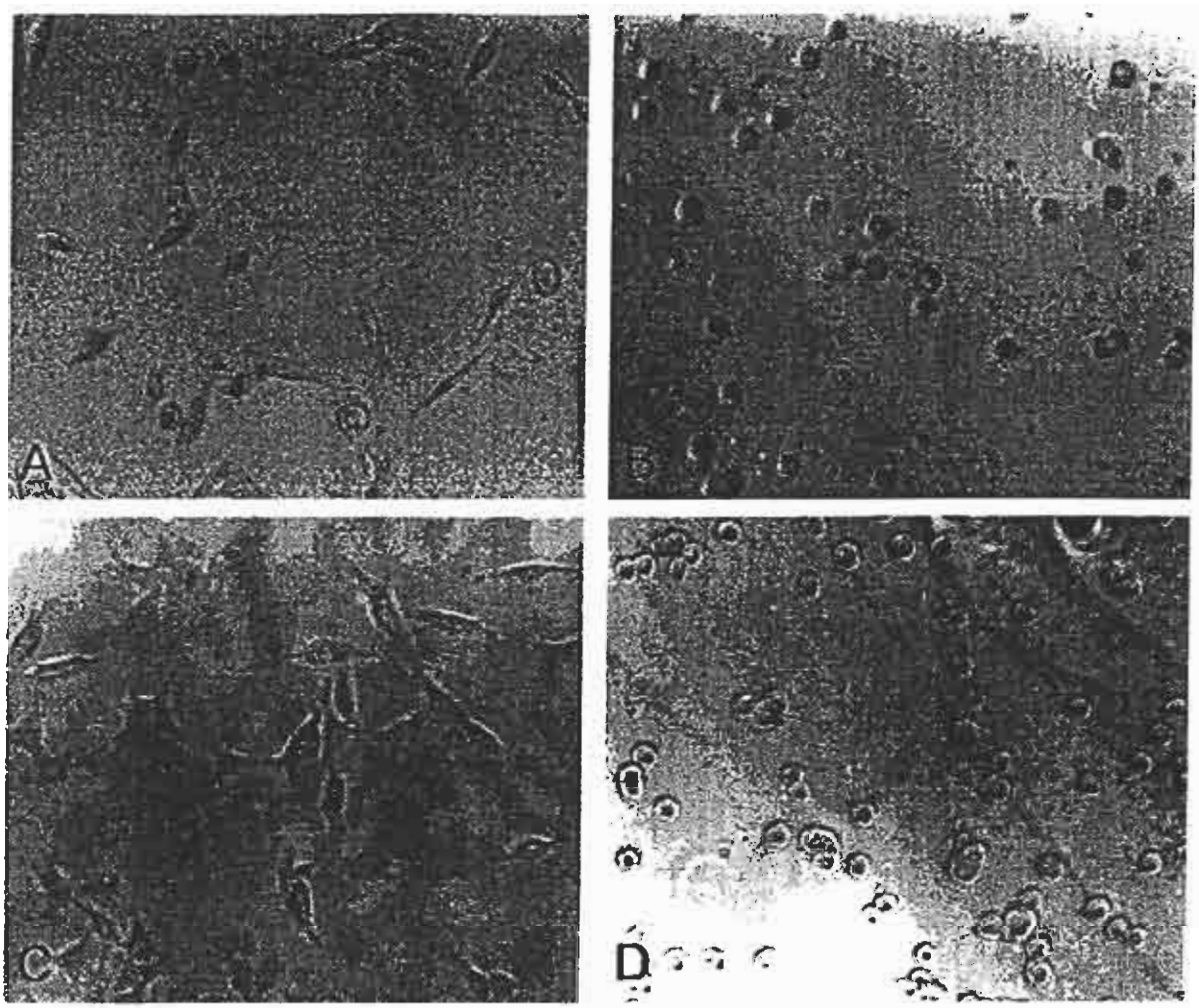

Figure 2. Phase photomicrographs of human chondrocytes (original magnification ×100). Cells analyzed at: (A) day 2 on the surface of uncoated coverslips, (B) day 2 on the surface of chitosan coated coverslips, (C) day 7 on the surface of uncoated coverslips, and (D) day 7 on the surface of chitosan-coated coverslips

lar, and the enzyme substrate yielded an even brown staining throughout the cell. The nuclei were counterstained with toluidine blue [Fig. 5(A)]. Osteoblasts from the surface of chitosan-coated coverslips also stained intensely for collagen type I [Fig. 5(B)]. Cytoplasmic immunostaining of these cells was granular, uneven, and blurred. Cell borders were indistinct, as clumping of cells obscured their margins. A halo of chitosan and stained material surrounded osteoblasts from chitosan-coated coverslips.

Chondrocytes cultured on uncoated and chitosan-coated coverslips had no detectable level of staining for collagen type I [Fig. 6(A)]. In contrast, these cells stained intensely for collagen type II and keratan sulfate [Fig. 6(B,C)]. Immunostaining of the human chondrocytes on both uncoated and chitosan-coated coverslips yielded an even brown staining throughout the cells. Chondrocytes from the surface of chitosan-coated coverslips had indistinct borders and clumped together. Again, cells propagated on the surface of chitosan were surrounded in a halo of chitosan and stained material. There was insignificant staining of osteoblasts and chondrocytes when the primary antibody was omitted or when an irrelevant anti-human immunoglobulin was used to replace the primary antibody.

DISCUSSION

In the present study we document for the first time the use of chitosan-coated surfaces in propagating human osteoblasts and chondrocytes. We demonstrate that cells cultured on chitosan coated surfaces remain viable and maintain a spherical morphology more similar to that displayed by osteoblasts and chondrocytes *in vivo*. Additionally, the higher density of os-

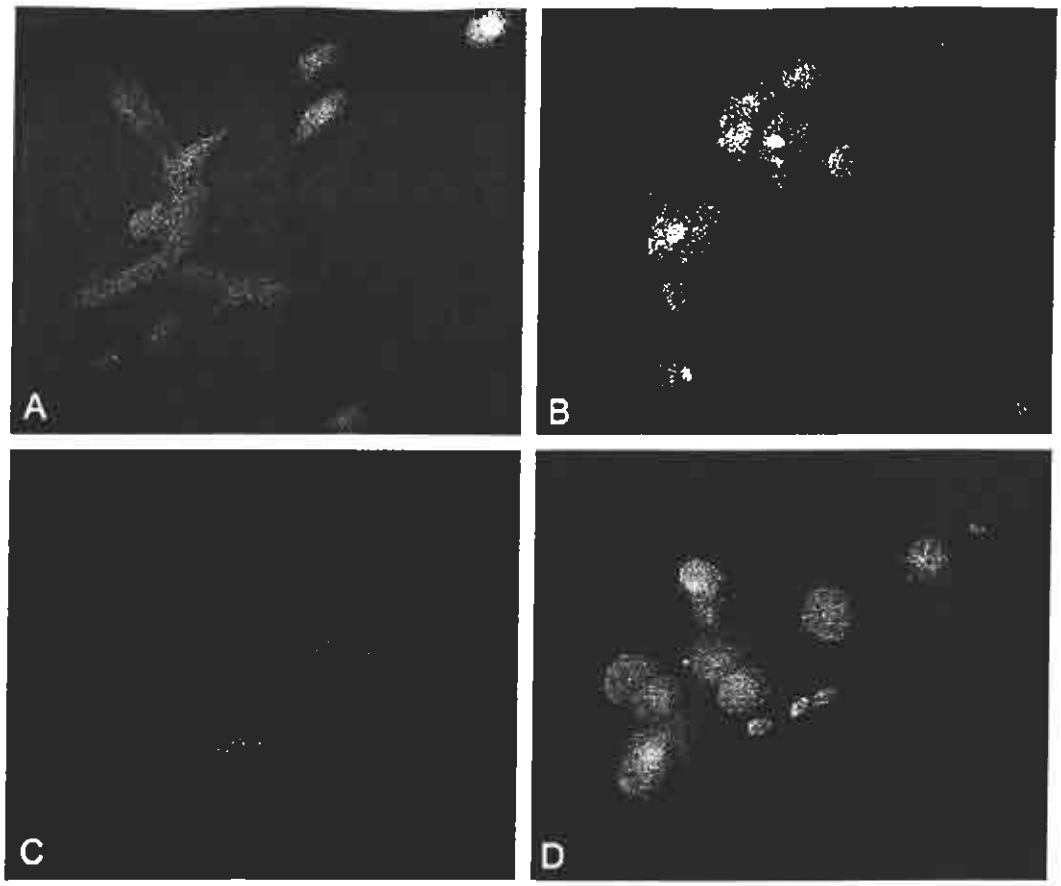

Figure 3. Fluorescent photomicrographs of human osteoblasts and chondrocytes labeled with Live-Dead Assay (original magnification ×250). Cells analyzed at day 7 of culture: (A) osteoblasts on the surface of uncoated coverslips, (B) osteoblasts on the surface of chitosan-coated coverslips, (C) chondrocytes on the surface of uncoated coverslips, and (D) chondrocytes on the surface of chitosan-coated coverslips

teoblasts and chondrocytes found on chitosan-coated coverslips, as compared with uncoated coverslips, suggests an increased affinity of these cells for chitosan-coated surfaces. However, because the cellular distribution was not uniform through the surface, statistical comparison was not feasible. Thus, the apparent difference in cellular density may not be statistically significant. The two-fold increase in cell density on both chitosan-coated and uncoated coverslips over the 7-day culture period also indicates that both human osteoblasts and chondrocytes continue to proliferate, while continuing to express tissue-specific extracellular matrix proteins. The observation that chitosan promotes human osteoblast and chondrocyte survival is not unexpected because it has been previously documented as a biocompatible substrate for the growth of various cell types.[53,54] Human epithelial cells, keratinocytes, and fibroblasts have all been demonstrated to attach and multiply on the surface of chitosan films. Moreover, these cells were found to have greater affinity for films containing higher concentrations of chitosan.[55]

We observed that human osteoblasts and chondrocytes, when propagated in monolayer culture, tend to exhibit a fibroblastic, flattened morphology. That these cells, when seeded onto a chitosan-coated surface, retain a rounded, oval shape and continue to express features of their original characteristics reinforce the relationship between morphology and cellular phenotype. This observation is reminiscent of other cellular systems in which morphology has been closely associated with cell-specific functions.[56–58] Alteration in shape appears to parallel changes in cell phenotype, in turn leading to a change in cell function. The ability of chitosan to help maintain cellular morphology and function was earlier noted by Yagi et al.[59] They demonstrated that rat hepatocytes propagated on the surface of chitosan-coated polystyrene plates maintained their spherical morphology in culture, while maintaining urea synthesis and drug catabolic activity. In contrast, hepatocytes propagated on collagen-coated plates underwent a dramatic morphological change, from spherical to flat, and lost their drug catabolic activity.

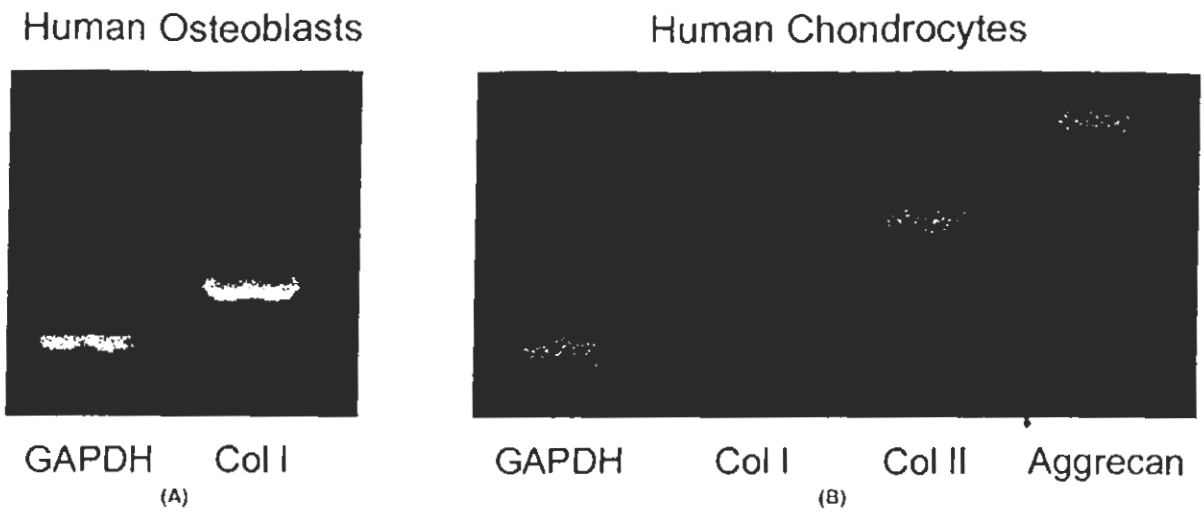

Figure 4. Osteoblast and chondrocyte mRNA expression by RT-PCR. (A) Osteoblast mRNA expression after 7 days of culture on chitosan-coated coverslips shows expression collagen type I. (B) Chondrocyte mRNA expression after 7 days of culture on chitosan-coated coverslips with no detectable level of collagen type I, and expression of collagen type II and aggrecan.

Recent studies of chondrocytes of animal origin further confirm the utility of chitosan for supporting cell growth and function. Denuziere et al.[25] have demonstrated that rabbit chondrocytes attach and proliferate on the surface of chitosan films. Sechriest et al.[60] also reported that bovine chondrocytes propagated on the surface of glycosaminoglycan (GAG)-augmented chitosan maintain their normal phenotypic characteristics, namely a spherical morphology, low mitotic rate, and synthesis of collagen type II and proteoglycan. Our investigation confirms the ability of both human osteoblasts and chondrocytes to attach, survive, and proliferate on the surface of chitosan. Similar to earlier studies, we demonstrate that these cells maintain a rounded appearance on a chitosan-coated surface and continue their production of cell-specific extracellular matrix proteins.

The ability of chitosan to support human cell attachment and survival could be attributed to its chemical properties. Chitosan is a binary polyheterosaccharide of *N*-acetylglucosamine and glucosamine in a β1→4 linkage. This polyheterosaccharide structurally resembles glycosaminoglycans, which are major components of the extracellular matrix of bone and cartilage. Glycosaminoglycans consist of long-chain, unbranched, repeating disaccharide units. Included among these disaccharide units are glucosamine residues, which constitute the basic structure of chitosan. Glucosamine residues are the framework of both keratan sulfate and hyaluronate, two predominant glycosaminoglycans found in articular cartilage. Glycosaminoglycans linked to a protein core are organized to form proteoglycans. These complex molecules are thought to play a key role in modulating cell morphology, differentiation, and function.[6,61,62] Previous studies have demonstrated the involvement of proteoglycans in cell–cell and cell–matrix interactions, as well as cell function.[63–65] The addition of proteoglycans to chondrocyte cultures has been shown to stimulate the synthesis of sulfated proteoglycans. Furthermore, removal of matrix proteoglycans results in decreased synthesis. Thus, the resemblance of chitosan to components of proteoglycans may be conducive to cell attachment and promoting function.

The success of propagating cells on the surface of chitosan films has prompted investigation into the potential of this natural polymer as a cellular scaffold. Elçin et al.[66] have reported that a porous three-dimensional chitosan matrix facilitated bovine adrenal chromaffin cell attachment and survival up to 14 days in culture. They showed that chromaffin cells seeded in chitosan scaffolds remained viable 2 weeks after implantation into the subarachnoid space of rats.

Our findings, as well as those of previous studies, suggest that chitosan is a biocompatible substrate for cell propagation. This polyheterosaccharide possesses the attributes of a practical scaffolding material and may serve as an effective template in the repair of osseous and chondral defects. The unique material properties and ability of chitosan to support viable and functioning human osteoblasts and chondrocytes make the material an attractive candidate for future use in bone and cartilage tissue engineering.

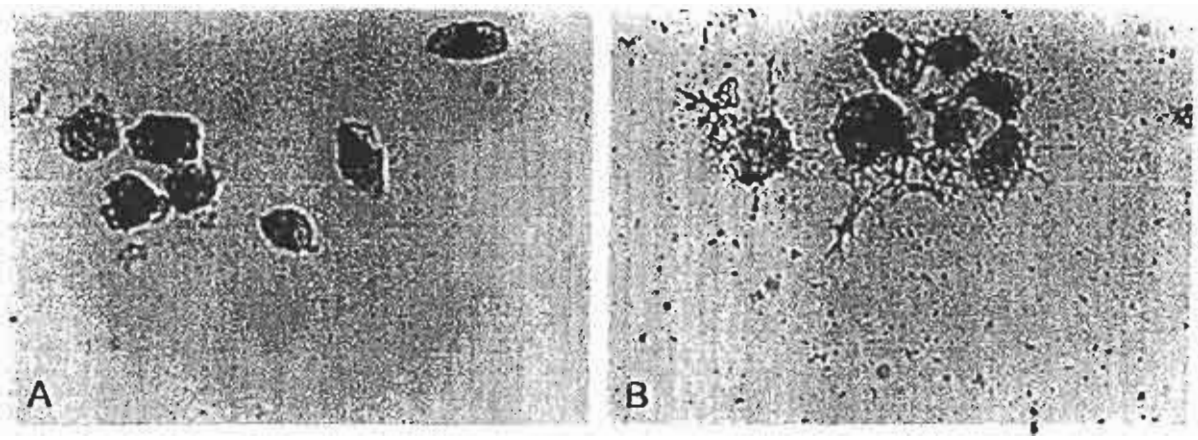

Figure 5. Immunostaining of osteoblasts (original magnification ×400). Cells retrieved on day 7 and stained for collagen type I from the surface of (A) uncoated and (B) chitosan-coated coverslips.

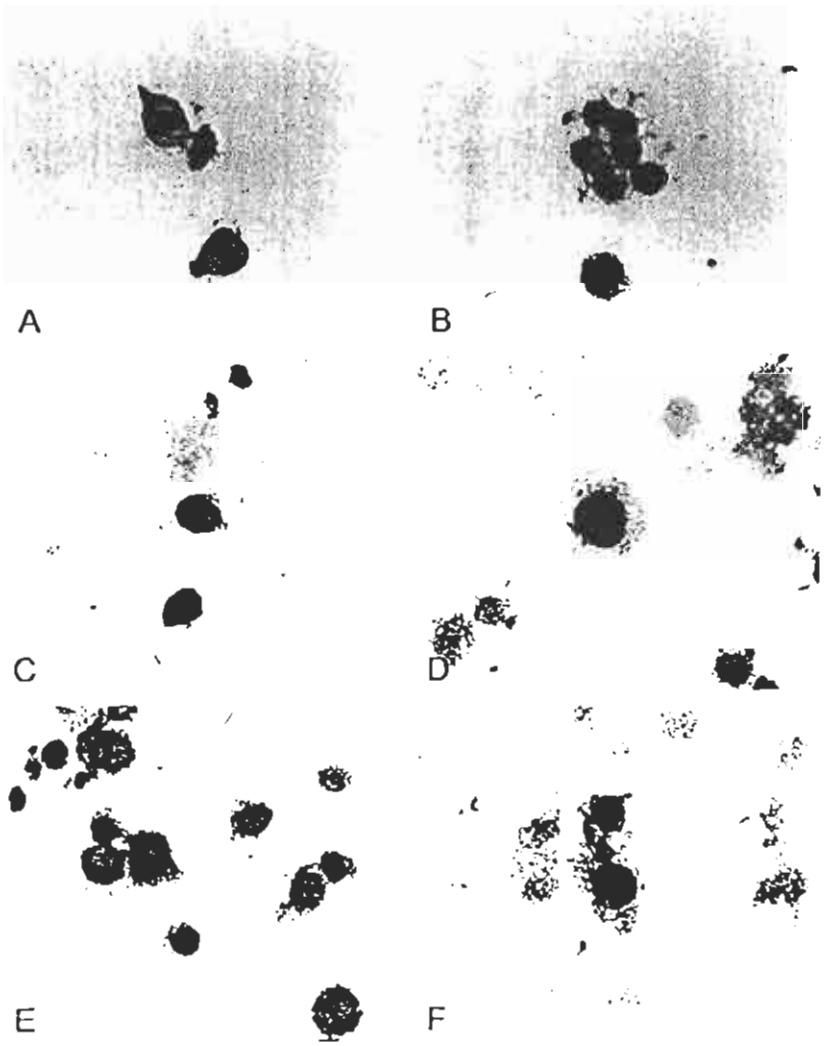

Figure 6. Immunostaining of chondrocytes (original magnification ×400). Chondrocytes from the surface of (A) uncoated or (B) chitosan-coated coverslips, did not stain for collagen type I. Chondrocytes from the surface of (C) uncoated and (D) chitosan-coated coverslips, stained for collagen type II. Chondrocytes from the surface of (E) uncoated and (F) chitosan-coated coverslips, stained for keratan sulfate.

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