Lohmann, CH, Schwartz, Z, Niederauer, GG, Carnes, DL Jr, Dean, DD and Boyan, BD. Pretreatment with platelet derived growth factor-BB modulates the ability of costochondral resting...

ARTICLE *in* BIOMATERIALS · FEBRUARY 2000

Impact Factor: 8.56 · DOI: 10.1016/S0142-9612(99)00132-5 · Source: PubMed

ELSEVIER

Biomaterials 21 (2000) 49–61

Biomaterials

Pretreatment with platelet derived growth factor-BB modulates the ability of costochondral resting zone chondrocytes incorporated into PLA/PGA scaffolds to form new cartilage in vivo

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Received 2 April 1999; accepted 16 July 1999

Abstract

Optimal repair of chondral defects is likely to require both a suitable population of chondrogenic cells and a biodegradable matrix to provide a space-filling structural support during the early stages of cartilage formation. This study examined the ability of chondrocytes to support cartilage formation when incorporated into biodegradable scaffolds constructed from copolymers (PLG) of polylactic acid (PLA) and polyglycolic acid (PGA) and implanted in the calf muscle of nude mice. Scaffolds were fàbricated to be more hydrophilic (PLG-H) or were reinforced with 10% PGA fibers (PLG-FR), increasing the stiffness of the implant by 20-fold. Confluent primary cultures of rat costochondral resting zone chondrocytes (RC) were loaded into PLG-H foams and implanted intramuscularly. To determine if growth factor pretreatment could modulate the ability of the cells to form new cartilage, RC cells were pretreated with recombinant human platelet derived growth factor-BB (PDGF-BB) for 4 or 24 h prior to implantation. To assess whether scaffold material properties could affect the ability of chondrogenic cells to form cartilage, RC cells were also loaded into PLG-FR scaffolds. To determine if the scaffolds or treatment with PDGF-BB affected the rate of chondrogenesis, tissue at the implant site was harvested at four and eight weeks post-operatively, fixed, decalcified and embedded in paraffin. Sections were obtained along the transverse plane of the lower leg, stained with haematoxylin and eosin, and then assessed by morphometric analysis for area of cartilage, area of residual implant, and area of fibrous connective tissue formation (fibrosis). Whether or not the cartilage contained hypertrophic cells was also assessed. The amount of residual implant did not change with time in any of the implanted tissues. The area occupied by PLG-FR implants was greater than that occupied by PLG-H implants at both time points. All implants were surrounded by fibrous connective tissue, whether they were seeded with RC cells or not. The amount of fibrosis was reduced at eight weeks for both implant types. When RC cells were present, the amount of fibrosis was less than seen in cell-free scaffolds. Pretreatment with PDGF-BB caused a slightly greater degree of fibrosis at four weeks than was seen if untreated cells were used in the implants. However, at eight weeks, if the cells had been exposed to PDGF-BB for 24 h, fibrosis was comparable to that seen associated with cell-free scaffolds. The cells supported an equivalent area of cartilage formation in both scaffolds. PDGF-BB caused a time-dependent decrease in cartilage formation at four weeks, but at eight weeks, there was a marked increase in cartilage formation in PDGF-BB-treated cells that was greatest in cells exposed for 4 h compared to those exposed for 24 h. Moreover, PDGF-BB decreased the formation of hypertrophic cells. The results indicate that in this model, RC cells produce cartilage; pretreatment of the RC cells with PDGF-BB promotes retention of a hyaline-like chondrogenic phenotype; and the material properties of the implant do not negatively impact on the ability of the cells to support chondrogenesis.

Keywords: Chondrocytes; Resting zone cells; Tissue engineering scaffolds; PDGF-BB; Polylactic acid; Polyglycolic acid

1. Introduction

In traumatic joint lesions and degenerative joint diseases, partial and full thickness chondral defects are common features. Current surgical therapy of these cartilage

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0142-9612/00/$ - see front matter
PII: S0142-9612(99)00132-5

defects is based on abrasion of the damaged cartilage and drilling of the subchondral bone to permit invasion of pluripotent progenitor cells into the defects [1,2]. Other strategies include osteochondral allografting or transplantation [3,4] as well as periosteal [5] and perichondrial [6,7] tissue grafting. While there is evidence that the resurfaced cartilage may convert from fibrous to hyaline cartilage, the mechanical properties are inferior to normal cartilage [8], leading to eventual failure [9].

Recently, several encouraging attempts to resurface joints have been made using cultured chondrogenic cells [10–15]. However, clinical outcome is still unpredictable. Although hyaline cartilage has been found in some healed defects, the mechanical mismatch between donor cartilage and subchondral bone still may lead to implant failure [16]. Moreover, cartilage cell therapy includes another critical procedure, the expansion of the articular cartilage cells for about four to six weeks in culture to achieve an appropriate number of cells to be implanted into the defect. After serial subpassages the articular cartilage cells tend to lose their phenotype in terms of aggrecan and collagen production [17], and may even begin to mineralize their matrix [18].

Despite these potential problems, it is becoming increasingly clear that optimal cartilage repair may require the addition of chondrogenic cells. In order to fill the defect site with cartilage, it is important that the chondrocytes be capable of both proliferation and differentiation to some extent [19]. This is particularly important at the interface of the cartilage and the subchondral bone to prevent delamination of the repair cartilage. However, chondrocyte hypertrophy and terminal differentiation, evidenced by matrix mineralization, should be minimized in a chondrogenic implant for a joint defect since this can lead to osteophyte formation.

To resolve this problem, cells at various stages of maturation along the chondrocyte lineage pathway have been used. Pluripotent perichondrial cells have been shown to have potential for cartilage defect repair when implanted into rabbit chondral defects [20]. Mesenchymal stem cells developed hypertrophic cartilage and fibrous tissue when implanted into goat knees [21]. In both instances, the interface between the pre-existing cartilage and the neocartilage tended to be fibrotic. Articular chondrocytes have been used by a number of investigators since they are presumed to be at an appropriate maturation state because they are derived from the articular cartilage itself. It is necessary to expand these cells in culture to obtain sufficient numbers for adequate seeding of the defect site and this can result in expression of a fibrous phenotype [22]. Alternatively, articular chondrocytes may enter the endochondral lineage with subsequent endochondral differentiation [18].

Recently we investigated the response of costochondral chondrocytes to a number of regulatory factors to better understand the transition of resting zone cartilage (RC) cells from production of a hyaline-like matrix, similar to that seen in articular cartilage, to production of a mineralizable matrix like that seen in the hypertrophic zone of the growth plate. These studies demonstrated that platelet-derived growth factor-BB (PDGF-BB) [23], like bone morphogenetic protein-2 (BMP-2) [24], increased [^{3}H]-thymidine incorporation. However, unlike BMP-2, which elicited no response at the concentrations used, PDGF-BB increased sulfation of the extracellular matrix. In addition, PDGF-BB prevented the RC cells from acquiring a more mature growth zone chondrocyte phenotype, whereas BMP-2 caused a shift in phenotypic expression [25,26]. The fact that the RC cells retained their chondrocyte phenotype following treatment with PDGF-BB is particularly important since Hunziker and Rosenberg [27] treated partial-thickness defects in articular cartilage with mitogenic growth factors (basic fibroblast growth factor [FGF-2], TGF-β1, epidermal growth factor, and insulin-like growth factor-1), but the cavities of the defects were filled with fibrous connective tissue instead of cartilage.

The nature of the cell delivery system may also play a role in determining the in vivo effectiveness of a particular cell type, whether or not a pretreatment is used. Different scaffolds have been investigated to provide a good wound healing environment in cartilage defects with variable outcomes. While our studies have used poly(D,L-lactide-co-glycolide) (PLG) scaffolds, most studies using scaffolds for cartilage repair have used polylactic acid (PLA) foams or polyglycolic acid (PGA) felts [11,28–31]. Porous PLA foams have been used for the delivery of perichondrial cells into full thickness defects in the rabbit femoral condyle [15]. Whereas Chu et al. [20] reported that hyaline cartilage formation occurred in the rabbit model, others have reported that polymer implants could only support fibrocartilaginous repair [32]. Scaffolds produced from PGA have been shown to support chondrogenesis in osteochondral defects in rabbits [11]. Teflon and polyester felts caused the development of intraarticular synovitis and failed to support normal cartilage formation [33]. Recently, PGA implants were shown to enhance proteoglycan synthesis and production of a mature cartilage matrix by chondrocytes [34] and PGA felts have been used successfully to grow cartilage ex vivo, indicating that alpha-hydroxypolyester scaffolds are not toxic to cartilage cells, particularly under conditions of fluid flow [35].

In this study, we tested the hypothesis that RC chondrocytes can form cartilage when implanted heterotopically in biodegradable scaffolds and that the material properties of the scaffold will influence the formation of neocartilage in a non-weight-bearing site. We also tested the hypothesis that pretreatment of the cells with PDGF-BB prevents chondrocyte hypertrophy and further endochondral maturation, as indicated by our

in vitro experiments, and that this effect can be achieved with short exposures to the growth factor.

2. Materials and methods

2.1. Implants

Implants for this study were manufactured and provided by OsteoBiologics, Inc. (San Antonio, TX). Two types of implants were prepared, each with a volume porosity of approximately 70%. PLG-H implants were constructed from PLG, of which 80% had a 75 : 25 lactide : glycolide ratio and an inherent viscosity of 0.76 dl/g, and 20% was poly(D,L-lactide-co-glycolide)-H with a 75 : 25 lactide : glycolide ratio, an inherent viscosity of 0.2 dl/g and a carboxyl end group. The carboxyl group was created as described by Tracy et al. [36], using water to terminate the polymerization rather than alcohol. Both polymers were procured from Boehringer Ingelheim (Ingelheim, Germany). The modified polymer was selected for its hydrophilicity in order to make the implant more wettable, thus potentially providing a better surface for cell attachment. The implants were fabricated by dissolving the polymer (80 wt% 75 : 25 PLG + 20 wt% 75 : 25 PLG-H) in acetone and precipitating the dissolved polymer in ethanol. The precipitate was kneaded repeatedly to form a uniform consistency and was then placed in a perforated mold under vacuum at 55°C for 4 d. This procedure is similar to previously published reports [37,38].

Fiber-reinforced implants (PLG-FR) consisted of a 90 wt% matrix (75 : 25 PLG) with 10 wt% reinforcing fibers (PGA). The chopped PGA fibers had a diameter of about 15 μm and an average length of approximately 2.6 mm (Albany International, Albany, NY). Similar to the procedure described above, the matrix polymer was dissolved in acetone, the fibers dispersed in ethanol, and the two combined to precipitate the matrix. After kneading to disperse and orient the fibers, the polymer was placed in a mold under vacuum at 65°C for 4 d. To characterize the mechanical properties of this scaffold, compression testing was performed in the axial direction and parallel to the predominant orientation of the fibers. Slivka et al. [39] have shown that the addition of such short fibers to a porous scaffold results in a predominant orientation of the fibers and an increase in the compressive modulus and yield strength.

After manufacture, residual amounts of acetone and ethanol were measured using gas chromatography. Bulk porosity of the scaffolds was calculated to be around 70% as determined from the scaffold dimensions, mass, and density of raw materials. Routine quality control tests were run on both raw materials and manufactured implants. The molecular weight of the PLG was determined with high-performance liquid chromatography using a gel permeation method. Based on polystyrene standards, the weight average molecular weight for the PLG-H implants was 50 800, and the polydisperity index of the implants was 2.5. Because the fibers, being composed of 100% PGA, do not dissolve in the chromatography solvent, their molecular weight was not assessed. The weight average molecular weight for the PLG used in preparing the PLG-FR implants was 94 500, and the polydispersity index for the PLG-FR implants was 2.0.

The glass transition temperature (T_g) of the polymer was determined using a differential scanning calorimeter. Two consecutive runs were performed, and the initiation of the endothermic shift on the thermogram of the second run (T_g onset, Run 2) was reported. The T_g (onset, Run 2) of the PLG-H implants was measured to be 46.6°C, whereas the T_g (onset, Run 2) of the PLG-FR was measured to be 50.2°C.

For mechanical testing, 6 mm diameter and 3 mm high cylindrical samples were precisely cut from porous blocks of the same implant type. This specimen size was selected for testing to minimize error resulting from limited sensitivity of the testing apparatus load cell. Since the determination of the mechanical properties takes into account the height and cross-sectional area of the test specimen, the resulting stiffness/yield stress is applicable for the polymer, regardless of the small changes in the specimen size used for implantation as described below.

For each implant type, five specimens were tested. The scaffolds were conditioned for 1 h in deionized water and then tested in a 37°C water bath. Unconfined, parallel plate compression testing was performed at a strain rate of 10% per min with a calibrated Instron Testing Apparatus (Model 5542, Charlotte, NC). Prior to testing, the thickness and diameter of each specimen were measured. Load deformation data were collected and analyzed according to ASTM D1621-94, 'Standard Test Method for Compressive Properties of Rigid Cellular Plastics'. This ASTM test method provides several advantages. Because the implants are porous, parallel plate compression testing is preferable over indentation testing since a flat-ended tip of the order of 1–2 mm could easily produce erroneous results when placed on an irregular surface with pores in the range of 50–300 μm. Numerous researchers have used compression testing for both cartilaginous [40] and implant [41–44] materials. From the load deformation data and post-processing analysis, the Young's modulus for PLG-H and PLG-FR was found to be 1.65 ± 0.14 MPa and 31.7 ± 7.4 MPa, respectively, and the yield stress for PLG-H and PLG-FR were 0.155 ± 0.014 MPa and 2.07 ± 0.49 MPa, respectively.

2.2. Cell culture

The culture system for RC cells has been described in detail previously [45]. Rib cages were removed from 18 male 125 g Sprague-Dawley rats and placed in

Dulbecco's modified Eagle medium (DMEM). The resting zone cartilage was separated, sliced and incubated overnight in DMEM containing 10% FBS, 50 μg/ml sodium ascorbate and 3% penicillin–streptomycin–fungizone in 5% CO_2 at 37°C and 100% humidity. DMEM was then replaced by two 20 min washes with Hank's balanced salt solution (HBSS), followed by sequential incubations in 1% trypsin for 1 h and 0.02% collagenase for 3 h. All enzymes were prepared in HBSS. Cells were isolated from the digested extracellular matrix by filtration, collected by centrifugation at 500 *g* for 10 min, and resuspended in DMEM. Cells were then plated at a density of 10 000 cells/cm^2. Media were exchanged at 24 h and then at 72-h intervals. Confluent first passage cells were used for the experiments.

2.3. Pretreatment of cells

RC chondrocytes were divided into three different groups. Group I cells were cultured, loaded into the scaffolds, and implanted. The other two groups were pretreated as described below. Confluent cultures of RC cells were incubated in media containing 37.5 ng/ml rhPDGF-BB (R & D Systems, Minneapolis, MN) for 4 h (Group II) or 24 h (Group III). Previous studies demonstrated that this concentration of growth factor stimulated RC proliferation and sulfate incorporation, but had no effect on alkaline phosphatase specific activity or collagen synthesis [23]. Higher concentrations of PDGF-BB did not elicit any greater response, and lower concentrations did not stimulate proliferation and sulfate incorporation to the same degree. Moreover, even when the RC cells were treated with PDGF-BB for up to 120 h, they failed to exhibit a 1,25-$(OH)_2D_3$-dependent stimulation of alkaline phosphatase specific activity typical of hypertrophic chondrocytes. In contrast, when RC cells were treated for 36 h with 24,25-$(OH)_2D_3$ [46] or for 72 h with TGF-β1 [47] or BMP-2 [25,26], the cells exhibited a 1,25-$(OH)_2D_3$-dependent increase in this enzyme activity, indicating a shift to a more mature phenotype.

2.4. Incorporation of the cells into the implant

Preliminary studies were performed to optimize the incorporation of the chondrogenic cells into the implants and to ensure cellular viability at the time of the implantation. The effects of prewetting the implants, pretreatment time, swirling speed (rpm), and loading the cells with or without vacuum were examined. Confocal and light microscopy were previously performed by Richard Coutts, M.D. and David Amiel, Ph.D. (University of California, San Diego, CA), to demonstrate loading of the cells into the implants. All subsequent studies were done with the optimal protocol outlined below.

Confluent cartilage cells were released from the culture flasks by trypsinization, serum was added to inhibit the trypsin, and the cells collected by centrifugation. Viability of the cells was assessed by trypan blue dye exclusion and found to be >95%. Cells were resuspended in 500 μl of DMEM at a concentration of 1.5×10^6 cells/ml and transferred to a 24-well tissue culture plate. Eight implants 3 mm in diameter and 1 mm in height were prewetted in 400 μl of DMEM in a 3 ml syringe sealed with a Luer-lok by pulling a vacuum on the syringe several times. This implant size was selected because of the relatively small size of the mouse calf muscle. Each group of eight prewetted implants was then placed in one well of a 24-well plate containing RC cells and gently swirled at 100 rpm for 2 h and 25 min at room temperature. The implant was then removed from the cell suspension and implanted as described below. As a control, implants were also prepared without cells. The media left in the cell culture wells were examined for cells, the cells counted, and then assessed for viability by trypan blue exclusion. Cell viability ranged from 75.4 to 92.2%. Of the total number of cells in suspension, 45.3–85.3% were incorporated into the scaffolds. Approximately 50% of the cells were loaded onto the four-week scaffolds, and approximately 80% of the cells were loaded onto the eight-week scaffolds. However, the range of loading efficiency was such that these differences were not statistically significant.

2.5. Implantation protocol

Forty-eight inbred male Nu/Nu mice (Harlan, Indianapolis, IN) with reduced immune systems (nude mice) were used in this study. Scaffolds were prepared under aseptic conditions in a class 100 clean-room. Four mice were used for each treatment group such that two identical implants were implanted intramuscularly into each animal with one implant in each calf muscle. This resulted in an $N = 8$ for each variable. In previous studies using mouse calf muscle implantation to test bone induction ability of various materials [48–50], we verified that there were no differences in outcome if each implant was an independent *N* or if each animal was an independent *N*. We opted for the former statistical design because it gave us an opportunity to consider the outcome of each cell/scaffold construct and to reduce the number of animals required for the study.

Nude mice were anesthetized by intramuscular injection of ketamine : xylazine : acepromazine (35 : 1 : 1). Both legs of each mouse were disinfected using povidone-iodine. Skin incisions of 0.5 cm were made in each leg and a pouch prepared by blunt dissection of the calf muscle. The implant was inserted into the pouch, and the incision was closed with resorbable cat gut suture. To assess the ability of the cells to support cartilage formation and whether this ability was modulated by growth factor pretreatment, PLG-H scaffolds were used. Controls included scaffolds with no cells and scaffolds

Table 1
Number of implants in each experimental group at the time of implantation and at harvest

	Number of implants	
	4 weeks[a]	8 weeks[a]
PLG-H scaffolds		
No cells	8(4)	8(8)
Cells + vehicle	8(7)	8(8)
Cells + PDGF-BB/4 h	8(8)	8(8)
Cells + PDGF-BB/24 h	8(8)	8(5)
PLG-FR scaffolds		
Cells + vehicle	8(8)	8(7)

[a]The first number in each column is the number of implants placed in each group, while the second number is the actual number of implants harvested and available for analysis.

containing cells pretreated with vehicle only. To determine if the ability of cells to form cartilage was scaffold-dependent, PLG-FR scaffolds loaded with resting zone cells, but without any pretreatments, were used. PLG-FR scaffolds with cells-only were compared directly with PLG-H scaffolds with cells-only, since the aim of the study was to see if the type of scaffold altered chondrocyte response and not to determine the host response to PLG-FR scaffolds alone. The various treatment groups are shown in Table 1. All aspects of animal care and use were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Health Science Center at San Antonio.

2.6. Histological evaluation

Tissue at the implant sites was harvested at four weeks and eight weeks post-operatively. At harvest, the animals were killed by an overdose injection of pentobarbital. Tissue was recovered from the implant sites and then fixed in 10% neutral formalin. Following fixation, the tissue was decalcified, embedded in paraffin, and sectioned. Sections (5–8 μm thick) were cut perpendicular to the long axis of the tibia and fibula through the entire implant and stained with haematoxylin/eosin or Safranin-O/Fast Green. The numbers of sections containing the implant varied among the specimens. Moreover, the plane of the section through the implant could not be assured because the implants tended to migrate to a variable extent from their original orientation at the time of placement. Therefore, we used the following approach to minimize experimental bias [48–50]. One section per implant, containing the largest implant area, was evaluated by two examiners for area of newly formed cartilage, implant size, area of fibrous connective tissue, and the presence of hypertrophic cartilage. Specimens lacking the implant were not included in any further analysis, since such a condition could indicate that the implant did not stay at the site. The number of implants remaining at the implant site in each group is indicated in Table 1.

Histomorphometric analysis was performed on the same histological sections. The entire section was examined using an Olympus microscope equipped with 4, 10 and 20× objectives, the image captured by a video camera, and the data stored and analyzed with NIH Image software. Calibration was performed according to the instructions accompanying the software. The area of new cartilage formed, area of residual implant, and area of fibrous connective tissue in mm^2 were measured. In measuring the implant area, it was assumed that all empty regions were originally occupied by implant material, since the solvents used to process the tissue dissolve PLG, leaving voids. Although the PLG was dissolved during processing, the tissue retained the conformation of the implant, so its area could be inferred by measuring the voids.

We attempted to measure the area of hypertrophic cartilage as well, since our review of the tissue histology had indicated that hypertrophic cells were present. Because the neocartilage was relatively immature, especially at four weeks, it was difficult to discriminate between large synthetic chondrocytes, prehypertrophic cells, and hypertrophic cells. We felt it was scientifically more accurate to note the presence of hypertrophic cells as a plus or minus rather than to arbitrarily set a perimeter around the cells. Hypertrophic chondrocytes were defined as being swollen and exhibiting a loss of both extracellular and intracellular staining in the haematoxylin and eosin sections.

2.7. Statistical analysis

The results of the morphometric analyses are shown as the mean ± SEM of the specimens examined. Since the number of sections for each implant available for histologic analysis varied, we selected the section with the largest implant diameter for analysis. Thus, each implant was an N of 1 for statistical purposes. Mortality affected the final number of samples available for analysis from each group. Two of the four mice implanted with PLG-H scaffolds alone died, reducing the N for that group to 4. One PLG-H implant containing cells that had not received pretreatment from the four-week group was not found at the time of harvest, reducing the N to 7. In the PLG-H group pretreated with PDGF-BB for 24 h and implanted for eight weeks, one mouse died and one additional implant could not be recovered, reducing the N for that group to 5. Finally, in the eight-week PLG-FR group, one implant could not be recovered, reducing the N for that group to 7. For experiments assessing the effects of PDGF-BB on cartilage formation and chondrocyte hypertrophy, treatment groups were compared

with scaffolds containing untreated cells. To assess whether fibrosis was due to the scaffold or the cells on the scaffold, PLG-H scaffolds alone were compared with PLG-H scaffolds containing cells. To determine whether the type of scaffold affected cartilage formation, PLG-H scaffolds containing untreated cells were compared with PLG-FR scaffolds containing untreated cells. To assess if there were time-dependent effects, each four-week treatment group was compared with the corresponding eight-week treatment group. Significant differences between groups were determined by the use of Bonferroni's modification of Student's *t*-test. P values $\leqslant 0.05$ were considered significant.

3. Results

3.1. Gross morphology

The shape of the PLG-H and PLG-FR implants at the time of implantation was identical. PLG-H implants at the time of harvest had an oval or oblong configuration. This was more evident at four weeks (Fig. 1A). By eight weeks, fibrous connective tissue was found throughout the implant mass (Fig. 1B). PLG-FR implants retained the contours of the original implant throughout the eight-week implantation period (data not shown).

3.2. General histology

All of the implants were encapsulated by fibrous connective tissue. The greatest amount of fibrous encapsulation was seen when no cells were incorporated into the implants (Fig. 1). PLG-H implants containing RC cells exhibited a reduction in fibrosis between four and eight weeks post-implantation. Those implants that contained untreated RC cells (Fig. 2C and D) had less fibrous connective tissue after eight weeks than did the implants containing PDGF-BB-treated cells (Fig. 3C and D).

Cartilage was present in all scaffolds containing RC cells after both four and eight weeks of implantation. Depending on the plane of section, cartilage was observed to form along the edge of the scaffold or as islands within the scaffold.

Regardless of whether chondrocytes were pretreated with PDGF-BB, the PLG-H and PLG-FR implants contained different stages of cartilage maturation with different degrees of hypertrophy. If hypertrophy was seen, it was always found in the center of the neocartilage. Formation of hypertrophic cartilage was time-dependent. At four weeks, some of the cells from untreated RC cultures were increased in size (Fig. 2B), and at eight weeks, typical hypertrophic chondrocytes were evident (Fig. 2D). However, newly formed cartilage in the implants that had been loaded with cells pretreated for 4 h with PDGF-BB contained little or no hypertrophic cartilage (Fig. 3).

New cartilage formation with PLG-FR implants was similar to that formed with the PLG-H implants (Fig. 4). As noted above for the PLG-H implants, chondrocytes in the center of the neocartilage were increased in size at four weeks (Fig. 4A), and some had a typical hypertrophic appearance after eight weeks (Fig. 4B). There were differences, however. The PLG-H implants appeared to have coalesced such that the cartilage formed adjacent to the main body of residual implant material. In contrast, the PLG-FR scaffolds retained their internal structure, and as a result, the cartilage islands were separated by the polymer architecture.

The presence of cartilage in the haematoxylin and eosin sections was confirmed by staining parallel sections with Safranin-O (Fig. 4). Safranin-O-positive staining was present both inside and outside the implant, coincident with the area deduced to be cartilage by haematoxylin and eosin staining.

3.3. Histomorphometric analysis

3.3.1. Cartilage area

The area of neocartilage formed varied significantly with the pretreatment received by the RC cells as well as with the time of implantation (Fig. 5). When untreated RC cells were loaded into the implants there was no difference in the amount of newly formed cartilage between four and eight weeks of implantation. Resting zone cells pretreated with PDGF-BB for 4 or 24 h produced less cartilage than untreated cells at four weeks; however, by eight weeks, the amount of cartilage was significantly increased 3.4-fold (4 h pretreatment) and 1.7-fold (24 h pretreatment) in comparison to the untreated cells. The effects of pretreatment were time-dependent; there was a 6.1-fold (4 h pretreatment) and 5.5-fold (24 h pretreatment) increase in neocartilage formation at eight weeks compared to the amount of cartilage at four weeks. RC cells pretreated with PDGF-BB for 24 h before implantation produced 42% less cartilage at eight weeks than cells pretreated for 4 h.

The type of scaffold implanted did not have a significant effect on the amount of neocartilage formed (Table 2). RC cells formed new cartilage at both four and eight weeks when loaded into PLG-FR scaffolds. The amount of cartilage increased with time, but did not differ significantly from the amount of cartilage seen in PLG-H scaffolds at the same time points.

3.3.2. Residual implant area

There was no significant difference in the amount of residual PLG-H implant area among all groups tested, regardless of pretreatment or length of implantation time (Fig. 6A). The amount of residual PLG-FR scaffold was

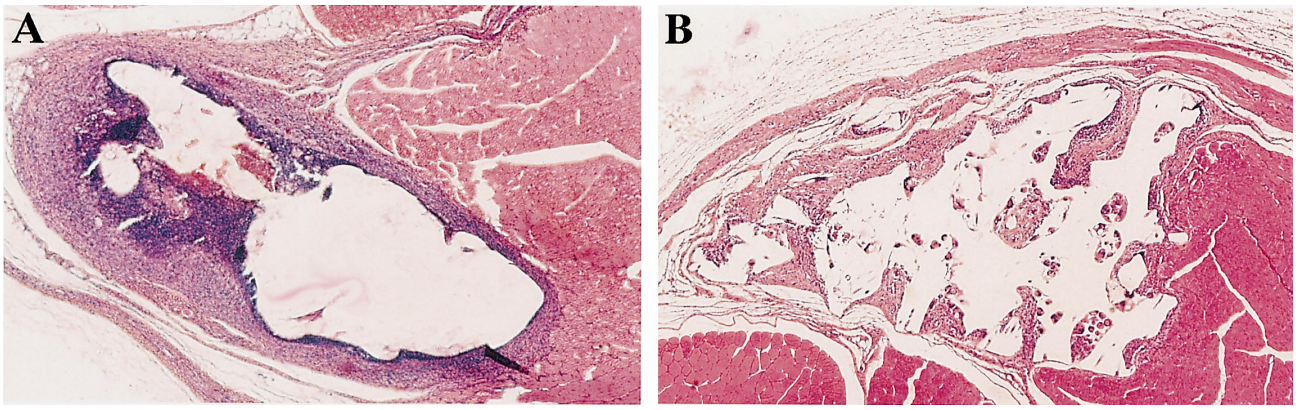

Fig. 1. Photomicrograph of PLG-H implants retrieved after intramuscular implantation in nude mice for four weeks (panel A) or eight weeks (panel B). Implants were not loaded with cells. At four weeks, the implant is surrounded with a fibrous envelope and inflammatory infiltrate. At eight weeks of implantation, the fibrous tissue is reduced and inflammation resolved. Sections were stained with hematoxylin and eosin; original magnifications were 4×.

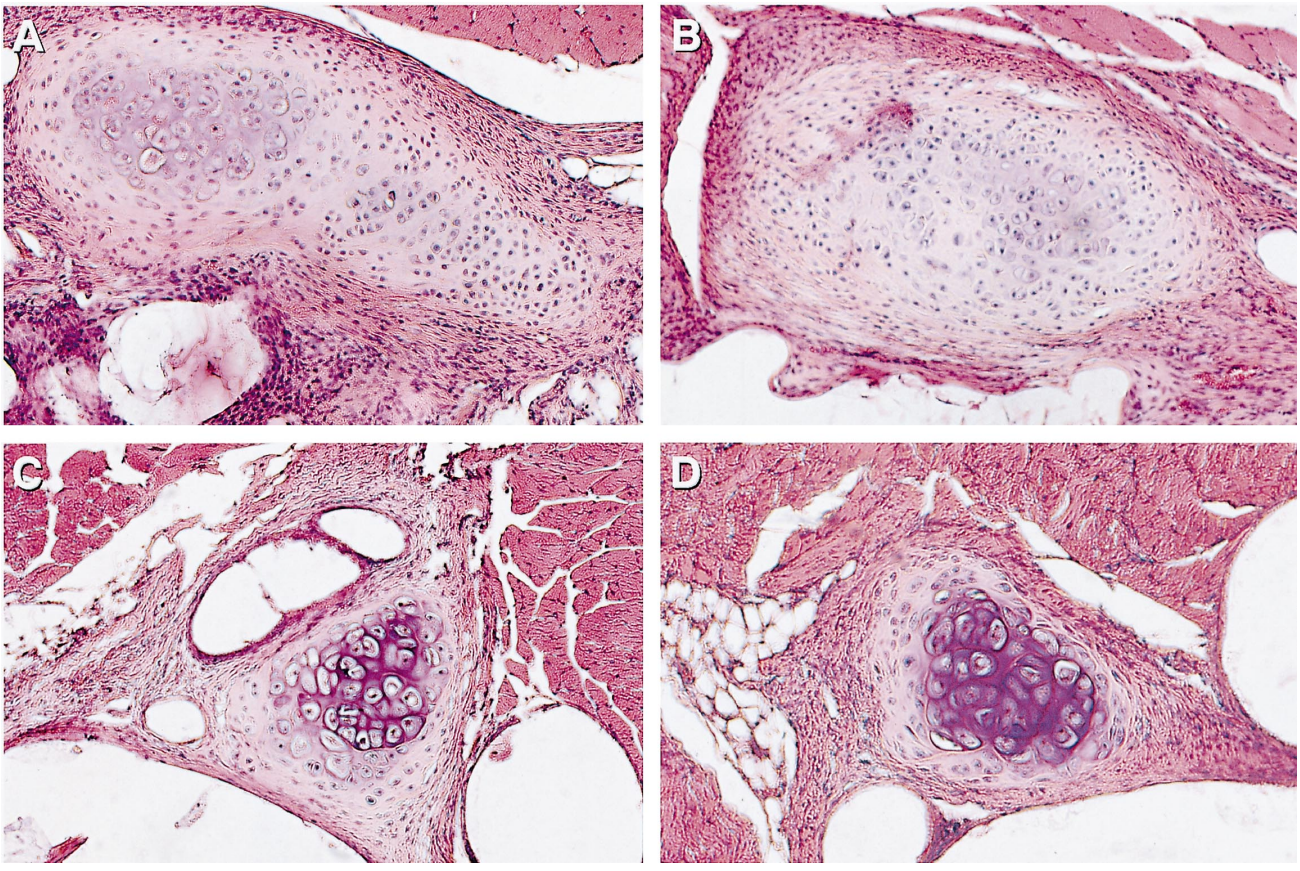

Fig. 2. Photomicrograph of neocartilage formed after intramuscular implantation of PLG-H scaffolds containing untreated resting zone cells in nude mice for four weeks (panels A and B) or eight weeks (panels C and D). PLG-H implants were loaded with untreated resting zone cells. Cartilage formation was observed to occur as islands or along the edges of the implants. Sections were stained with hematoxylin and eosin; original magnification for all panels was 10×.

also not significantly different between the four and eight-week groups (Table 2). However, the implant area of the PLG-FR was generally greater than that of the PLG-H implants.

3.3.3. *Area of fibrous connective tissue*

All PLG-H implants contained fibrous connective tissue, whether cells were present or not (Fig. 6B). Incorporation of RC cells into the PLG-H scaffolds resulted in

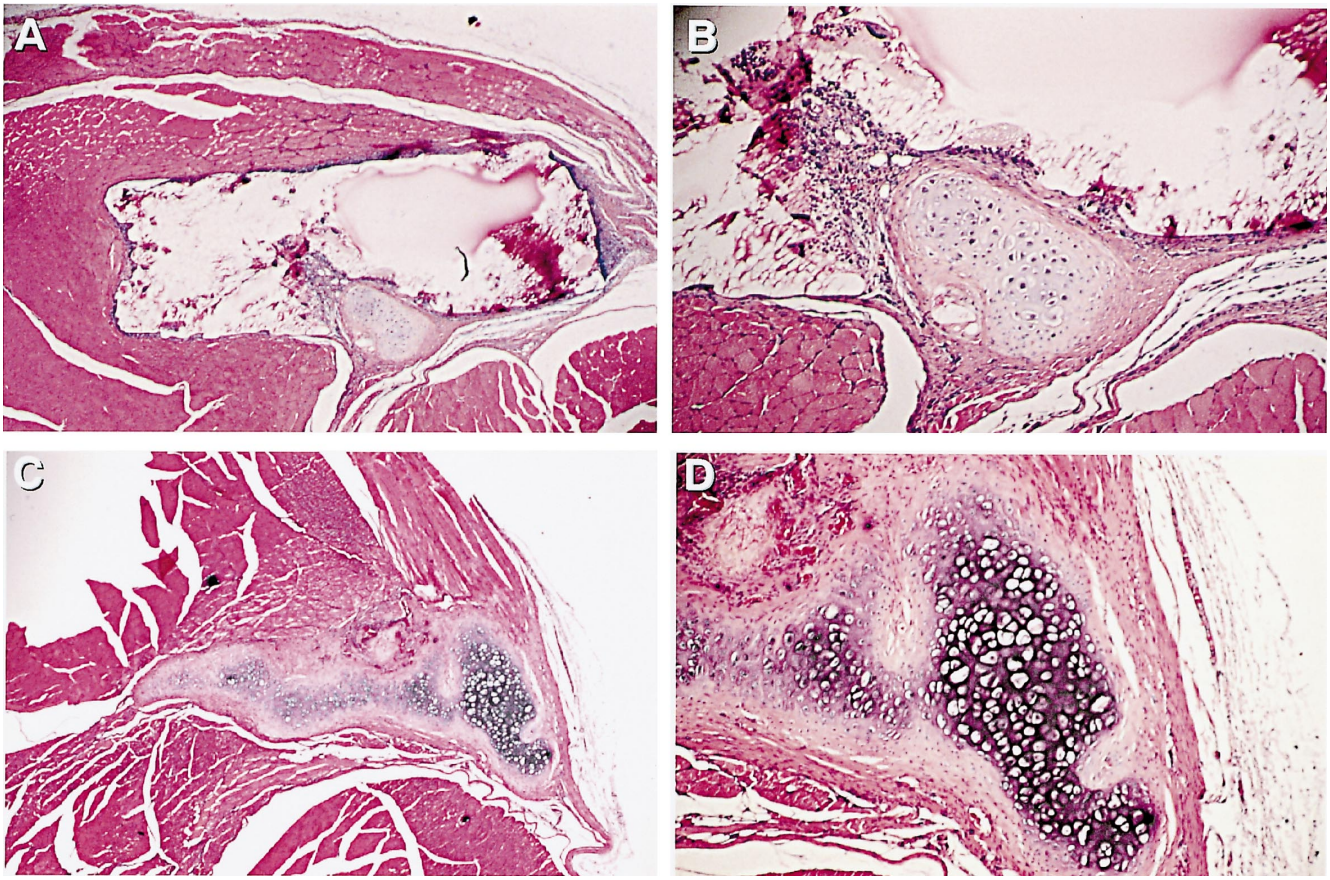

Fig. 3. Photomicrograph of neocartilage formed after intramuscular implantation of PLG-H scaffolds containing resting zone chondrocytes pretreated with PDGF-BB in nude mice for four weeks (panels A and B) or eight weeks (panels C and D). PLG-H implants were loaded with resting zone cells that had been pretreated with PDGF-BB for 4 h prior to implantation. Sections were stained with hematoxylin and eosin; original magnifications were 4× (panels A and C) or 10× (panels B and D).

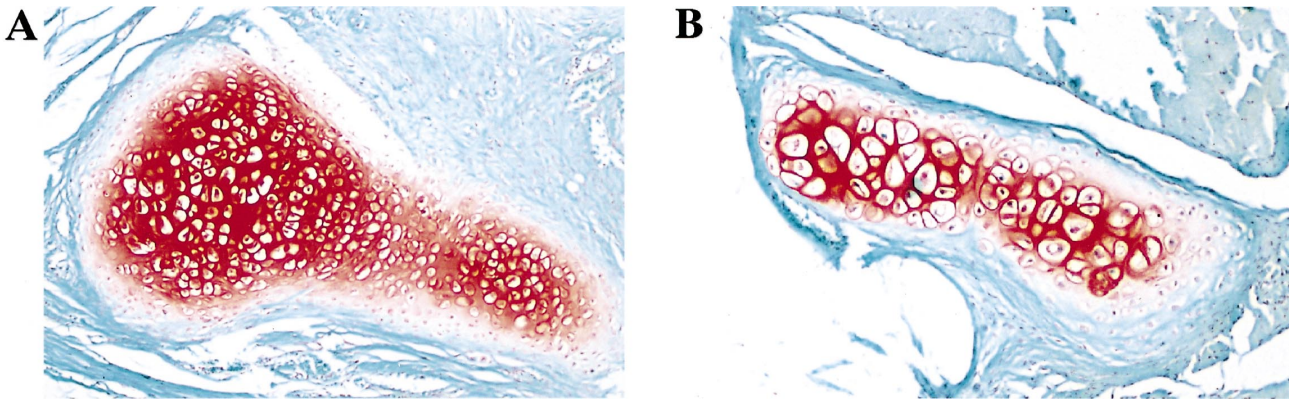

Fig. 4. Photomicrograph of neocartilage formed after intramuscular implantation of scaffolds containing untreated resting zone chondrocytes in nude mice for four weeks (panel A) or eight weeks (panel B). PLG-FR implants (panel A) or PLG-H implants (panel B) were loaded with untreated resting zone cells. Sections were stained with Safranin-O; original magnifications were 10× (panel A) and 20× (panel B).

a reduction in fibrous connective tissue formation at eight weeks. In contrast, pretreatment of the RC cells with PDGF-BB resulted in fibrous connective tissue formation comparable to that seen in the cell-free implants (Fig. 6B). When RC cells were implanted in PLG-FR scaffolds, the amount of fibrous tissue was similar to that seen with PLG-H scaffolds implanted for the same period of time (Table 2). There was slightly more fibrous

connective tissue measured around the PLG-FR implants, but this was not statistically significant. The area of fibrosis was reduced by almost 50% between four and eight weeks for both scaffold formulations, although this was only statistically significant in the PLG-H group.

3.3.4. Hypertrophic cartilage

Cartilage maturation was also affected by length of growth factor pretreatment and time of implantation (Fig. 6C). After four weeks, hypertrophic cartilage was found in three of seven implants containing RC cells that were not treated with PDGF-BB, while four of eight implants contained hypertrophic cartilage at eight weeks. In contrast, when RC cells were pretreated for 4 h with PDGF-BB, only one of eight implants at four weeks and zero implants at eight weeks contained hypertrophic cartilage. Pretreatment of RC cells for 24 h prior to implantation did not result in the formation of hypertrophic cartilage at four weeks, whereas three of eight implants exhibited evidence of hypertrophic cartilage at eight weeks. When RC cells were implanted in PLG-FR scaffolds, hypertrophic cartilage was noted in two of eight implants at four weeks and in four of seven implants at eight weeks (Table 2).

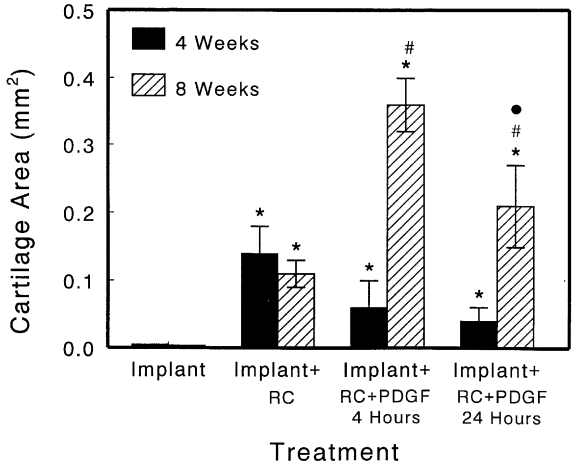

Fig. 5. The effect of PDGF-BB pretreatment of resting zone cells prior to implantation (PLG-H scaffolds) on their ability to induce new cartilage formation in nude mouse calf muscle. The morphometric analysis shows the area of new cartilage. * $P < 0.05$, all groups versus implant alone; # $P < 0.05$, four weeks versus eight weeks; • $P < 0.05$, PDGF-BB treatment for 24 h versus 4 h.

4. Discussion

The results of this study indicate that cells derived from the resting zone of costochondral growth plate cartilage are not only incorporated into porous PLG scaffolds, but can also develop new cartilage when implanted at a heterotopic site. The nature of the scaffold was not a factor in this non-weight-bearing model based on the failure to detect statistically significant differences in the parameters measured, even though the scaffolds themselves behaved very differently in terms of structural integrity. The shape of the PLG-H implants changed, whereas the shape of the PLG-FR implants did not, because of the difference in the chemical and mechanical properties of the two implants. The PLG-H scaffolds, having a much lower stiffness, a lower T_g, and faster degradation rate, were not able to maintain their structural integrity as well as the PLG-FR scaffolds did. Implant stiffness can affect the structural integrity of the scaffold as degradation starts occurring. Even though the muscle site has minimal load-bearing, some forces are placed on the implant as the muscles contract and relax.

Pretreatment of the cells prior to loading them onto the scaffolds had a significant effect on chondrogenesis. PDGF-BB caused a time-dependent increase in the amount of new cartilage formation and prevented the cells from hypertrophy and further progression in the endochondral maturation pathway. This confirmed previous in vitro experiments [23] indicating that

Table 2
Effect of scaffold design on cartilage and connective tissue formation by resting zone chondrocytes

Parameter	Implantation duration			
	4 weeks		8 weeks	
	PLG-H	PLG-FR	PLG-H	PLG-FR
Area of cartilage (mm^2)	0.14 ± 0.04	0.13 ± 0.05	0.11 ± 0.02	0.95 ± 0.41
Area of residual implant (mm^2)	1.38 ± 0.2	2.06 ± 0.25	1.27 ± 0.26	1.75 ± 0.19
Area of fibrosis (mm^2)	0.70 ± 0.2	0.92 ± 0.16	0.36 ± 0.13*	0.56 ± 0.12
No of implants with hypertrophic cartilage	3/7	2/8	4/8	4/7

Confluent, first passage resting zone cells were loaded into PLG-H and PLG-FR scaffolds without pretreatment and then implanted intramuscularly for four or eight weeks in nude mice. Area of new cartilage formation, residual implant area, and area of fibrosis was assessed by histomorphometry. Values are the mean $\pm$ SEM for the total number of implants analyzed ($n = 7$ or 8).

*$P < 0.05$, four weeks versus eight weeks.

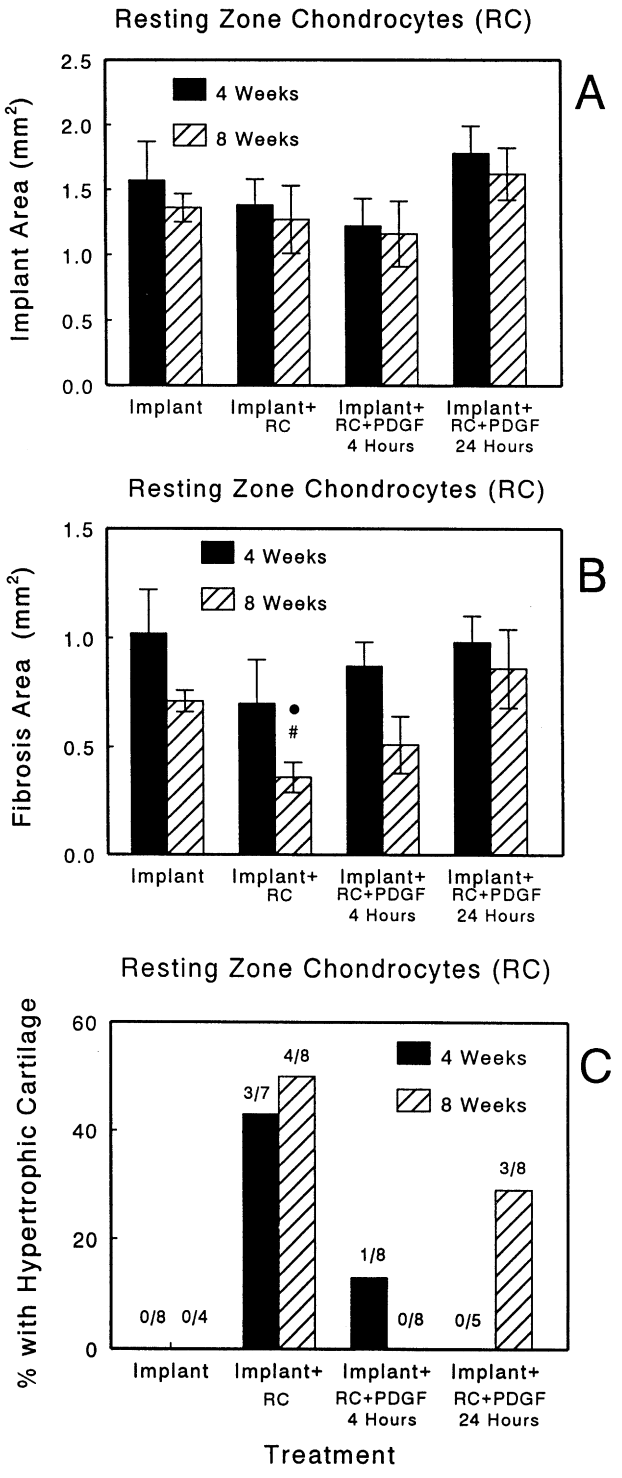

Fig. 6. The effect of pretreating resting zone chondrocytes with PDGF-BB prior to implantation on their ability to form neocartilage in the calf muscle of nude mice. PLG-H scaffolds were implanted alone (cell-free) or with resting zone cells that were either untreated or pretreated with PDGF-BB for 4 or 24 h prior to loading into the scaffolds and implantation. Panel A: residual implant area (mm^2) was determined by morphometric analysis. Panel B: area of fibrosis (mm^2) was determined by morphometric analysis. $^{\#}P < 0.05$, four weeks versus eight weeks; $^{\bullet}P < 0.05$, implant containing untreated cells versus implant alone or implants containing cells treated for 24 h with PDGF-BB and implanted for eight weeks. Panel C: percent of implants containing hypertrophic cartilage for each treatment group. The absolute number of implants containing hypertrophic cartilage is shown above each bar.

PDGF-BB prevents resting zone cells from acquiring a hypertrophic chondrocyte phenotype, while retaining their chondrogenic expression.

Our results show that RC cells can undergo further maturation along the endochondral lineage when implanted heterotopically in a non-weight-bearing site, supporting our in vitro studies showing that these cells form multilamellar nodules containing hypertrophic cells in long-term culture [51]. Treatment of resting zone cells in vitro with a number of factors, including TGF-β1 [47], BMP-2 [24–26,52], and 24,25-$(OH)_2D_3$ [46,53], for extended periods of time can induce the acquisition of a hypertrophic chondrocyte phenotype, as evidenced by 1,25-$(OH)_2D_3$-dependent increases in alkaline phosphatase activity. However, treatment with PDGF-BB does not cause this to occur [23]. The present study confirms the validity of our in vitro cell culture model, since pretreatment of the resting zone cells with PDGF-BB prior to implantation appeared to arrest the cells in a pre-endochondral maturation state. This effect was seen with relatively short 4-h exposures to PDGF-BB and was not appreciably different when the exposure was increased to 24 h.

The in vitro studies showed that PDGF caused increased proliferation and extracellular matrix sulfation without causing an increase in alkaline phosphatase, indicating that the cells remained in a relatively less differentiated state [23]. This effect may play a role in the reduced levels of cartilage observed at four weeks and the higher levels of fibrous connective tissue in the implant sites. By eight weeks, chondrogenesis was increased, potentially due to the enhanced pool of chondrocytes with a resting zone cell phenotype. Others have noted that mesenchymal cell proliferation is stimulated by PDGF [54] and have investigated the potential of this factor as a mitogen for enhancing hard and soft tissue wound repair [55,56]. These observations suggest that PDGF-BB treatment of RC cells prior to implantation might not only inhibit the hypertrophy of the cells, but also might provide a better outcome of the cartilage in longer time courses.

Pretreatment of the cells with bioactive factors like PDGF-BB also carries caveats as well. Although the amount of fibrous connective tissue around the scaffold containing untreated resting zone chondrocytes was reduced at eight weeks, there was no decrease noted in sites implanted with PDGF-BB-treated cells. Thus, while PDGF-BB may prevent the cells from hypertrophy [55], this factor may also stimulate connective tissue to develop [57], possibly via the upregulation of TGF-β [58]. It is possible that proliferating tissue in the scaffolds containing RC cells caused a general inflammatory response in the muscle pouch, resulting in fibrous tissue around the implants.

This study also demonstrates that the PLG scaffolds, in two different chemical compositions and with different mechanical properties, do not exhibit any toxic effects on

the chondrocytes or the surrounding muscle tissue up to eight weeks in vivo. Implantation in nude mice reduces the severity of an immune response [59], but it does not eliminate the toxic effects of an implanted material. Chondrocytes in the scaffolds exhibited the morphological features of normal cartilage cells. While fibrous tissue was present, particularly at four weeks, its incidence was reduced at eight weeks when cell/scaffold constructs were used. It is likely that this fibrotic response would have resolved itself as the scaffolds degraded. This was already evident in the cell-free scaffolds, although not yet statistically significant. The degradation characteristics of the scaffolds are currently being evaluated. Preliminary data indicate that PLG-H implants completely degrade in three to four months, and PLG-FR implants completely degrade in four to six months (unpublished observations).

Others have shown that the mechanical environment of cells has a significant effect on their metabolism and expression of extracellular matrix [60]. However, we did not find significant differences in neocartilage or fibrous tissue formation when RC cells were loaded on the two different scaffolds, even though PLG-FR implants were 20 times stiffer than the PLG-H implants. This suggests that RC chondrocytes are not significantly affected by the differences in the mechanical and chemical properties of the implants used in our non-weight-bearing model. The fact that cartilage and fibrous tissue formation in the two implants was comparable under non-weight-bearing conditions suggests that site-specific requirements for mechanical stability in weight-bearing articular cartilage defects can be met without compromising the amount of cartilage that can form. We have recently presented evidence supporting this hypothesis in an in vivo osteochondral defect model using these scaffolds and similarly prepared costochondral chondrocytes [61].

Acknowledgements

The authors greatly appreciate the assistance of Ms. Sandra Messier in the preparation of the manuscript and the technical assistance of Roland Campos in the performance of the experiments. In addition, we would like to thank OsteoBiologics, Inc., San Antonio, TX, for providing the scaffolds used in the study and to particularly acknowledge the assistance of Michael Slivka, Larry Cullen, Jeff Garza, and Neil Leatherbury. We also thank Dr. Kristine Kieswetter (OsteoBiologics, Inc.) for her help in preparing the histologic sections and Drs. Richard Coutts and David Amiel at the University of California at San Diego for performing the confocal and light microscopy of the implants. This work was supported by OsteoBiologics, Inc., the Center for the Enhancement of the Biology/Biomaterials Interface at the University of Texas Health Science Center at San Antonio, and the B. Braun Foundation (Melsungen, Germany).

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