Minced Cartilage without Cell Culture Serves as an Effective Intraoperative Cell Source for Cartilage Repair

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ABSTRACT: Traumatic articular cartilage injuries heal poorly and may predispose patients to the early onset of osteoarthritis. One current treatment relies on surgical delivery of autologous chondrocytes that are prepared, prior to implantation, through ex vivo cell expansion of cartilage biopsy cells. The requirement for cell expansion, however, is both complex and expensive and has proven to be a major hurdle in achieving a widespread adoption of the treatment. This study presents evidence that autologous chondrocyte implantation can be delivered without requiring ex vivo cell expansion. The proposed improvement relies on mechanical fragmentation of cartilage tissue sufficient to mobilize embedded chondrocytes via increased tissue surface area. Our outgrowth study, which was used to demonstrate chondrocyte migration and growth, indicated that fragmented cartilage tissue is a rich source for chondrocyte redistribution. The chondrocytes outgrown into 3-D scaffolds also formed cartilage-like tissue when implanted in SCID mice. Direct treatment of full-thickness chondral defects in goats using cartilage fragments on a resorbable scaffold produced hyaline-like repair tissue at 6 months. Thus, delivery of chondrocytes in the form of cartilage tissue fragments in conjunction with appropriate polymeric scaffolds provides a novel intraoperative approach for cell-based cartilage repair. © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:1261–1270, 2006

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INTRODUCTION

Tissue healing requires cell migration that redistributes cells from the surrounding tissues to the injury site. In cartilage tissue however, the ability of chondrocytes to migrate from their native lacunae site is very limited due to the supposed rigidity of the matrix.[1–4] While chondrocytes migrate and grow well in vitro, the limitation in chondrocyte migration in vivo is thought to be the major contributing factor to poor cartilage self-healing. To compensate for the migration deficiency, various surgical interventions for cartilage repair focus on delivering reparative cells or tissues.[5–8] Marrow stimulation attempts to tap marrow cells by breaching the subchondral bone, although the mechanical durability of resultant fibrocartilage is often unsatisfactory.[9] Osteochondral autograft transplantation transfers tissue plugs, consisting of the articular cartilage along with a portion of the subchondral bone, from less-weight bearing areas of the articulating surface to the defect area. However, donor tissue availability and morbidity, as well as poor interface integration in the chondral layer, continue to be issues that limit the application of this technique.[10,11]

Autologous chondrocyte implantation (ACI) directly establishes a chondrocyte presence in the treatment site through the delivery of culture-expanded chondrocytes.[12] Although it was originally believed that the active contribution of ACI was both from implanted chondrocytes and the periosteum sutured over the defect, it soon became clear that the periosteal flap could be replaced by other carrier materials such as collagen[13] and hyaluronan derivatives.[14] Long-term follow-up of ACI has suggested improvement in clinical symptoms and

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activity levels.[15–17] Despite variations among different surgeons,[18] the improved clinical results also correlate with the formation of hyaline-like cartilage in the repair tissue.[17] Recently, controlled and randomized clinical trials indicated that ACI is better than or comparable to other current cartilage therapies 1- or 2-years posttreatment.[10,19]

Despite clinical success, ACI is associated with technical hurdles that impede its broad adoption. Central to these is the cell culture preparation of sufficient numbers of chondrocytes for implantation. Consequently, ACI must be conducted as a two-stage surgical procedure: first cartilage tissue is harvested, from which chondrocytes are isolated as the starting cell source for expansion, and then cultured cells are implanted. In addition to the challenges of two-stage procedure, cell expansion itself is a technical and financial burden, given that autologous tissue must be processed individually. Because of these challenges, widespread adoption of ACI will likely continue to be limited even with continued refinements to the current practice.[20]

To overcome the challenges, we devised a non-culture-based approach to prepare a chondrocyte-loaded implant. Starting with the same amount of cartilage tissue as with ACI, a tissue fragmentation step is incorporated to mince the cartilage tissue mechanically into small tissue fragments before reimplantation. The purpose is to promote outgrowth of embedded chondrocytes through the increased tissue surface area. Using this methodology, we established that chondrocytes can effectively grow into adjacently placed scaffold materials and produce neo-cartilage in a surrogate mouse model. We also established, through a goat trochlear model, that cartilage fragments together with a resorbable scaffold and resorbable fixation staple could effectively treat chondral defects leading to a hyaline-like repair tissue in just 6 months.

METHODS

Approvals were obtained from the Institutional Review Board (IRB) of Rush University for the use of human tissue and from the Institutional Animal Care and Use Committee (IACUC) of Thomas D. Morris, Inc. for the use of animals in this study.

Cartilage tissue, harvested from the intercondylar notch of human subjects undergoing anterior cruciate ligament reconstruction and from the femoral condyles of adult bovine animals, was aseptically rinsed, weighed, and transferred (400 mg) into a petri dish. The tissue was minced into small fragments ($\sim$1 mm^3) with a surgical scalpel in the presence of PBS (Life Technologies, Bethesda, MD) and evenly loaded onto a scaffold made of polyglycolide/polylactide (PGA/PLA, Ethicon, Somerville, NJ) non-woven felt ($4 \times 5 \times 0.1$ cm) or polyglycolide/polycaprolactone (PGA/PCL, Ethicon) foam reinforced with polydiaxanone (PDS, Ethicon) mesh. The tissue fragments were retained on the scaffold with a coating (800 μl) of fibrin clot (Calbiochem, San Diego, CA) to form a construct for culture.

The prepared constructs or control scaffolds without cartilage fragments were placed in culture dishes (100 mm) in chondrocyte growth medium and cultured at 37°C in a humidified atmosphere containing 5% CO_2. The growth medium (all components from Life Technologies) contained DMEM-high glucose supplemented with 10% fetal calf serum (HyClone, Logan, UT) and HEPES (10 mM), nonessential amino acids (0.1 mM), L-proline (20 μg/ml), ascorbic acid (50 μg/ml), penicillin/streptomycin (100 u/ml each), and Amphotericin B (0.25 μg/ml). Punches (4 mm, $n = 3$) were made from the cultured constructs at 4 days, 3 weeks, and 6 weeks for histological analysis (MPI Research, Mattawan, MI).

Human and bovine chondrocytes served as positive controls in the SCID mouse experiments. The isolation and culture expansion of both human and bovine chondrocytes were performed as previously described.[21] Two days before implantation, chondrocytes were seeded onto scaffolds at 1 million cells per cm^2 of scaffold area. Bovine primary cells and human passage-3 cells were used for scaffold seeding.

Five-week old male Fox Chase SCID mice (Charles River Laboratories, Portage, MI) received four in vitro cultured punches each, surgically implanted in four subcutaneous pockets located in the lateral thoracic region.[22] Polyester 5-0 sutures (ETHIBOND, Ethicon) were used to secure the skin to the musculature around each implant to prevent subcutaneous migration. Four weeks postsurgery, the implants were harvested and fixed in 10% formalin. Each implant was bisected for hematoxylin/eosin (H&E) and Safranin-O (SO) histology and type II collagen immunohistochemistry (MPI Research).

Minced bovine cartilage was cultured as described above except without the supporting scaffold. The culture was pulse-labeled overnight with BrdU (10 μM, Calbiochem) before harvesting. Immunofluorescent detection of BrdU was performed as previously described.[23] Alexa Fluor® 488 conjugated BrdU antibody (1:20) and Ethidium homodimer-1 (1:2,000) (Molecular Probes, Eugene, OR) were used for staining and counterstaining, respectively. Gray-scale images were recorded originally, and individual image channels were then pseudo-colored to render red fluorescence for BrdU signal and green fluorescence for counterstaining.

Eight skeletally mature Spanish-Nubian goats (2 years old) with confirmed absence of radiographic evidence of stifle joint pathology were used in the study. The unilateral trochlear model consists of two, full-thickness, 7-mm-diameter chondral defects on each side of the trochlear ridge created through a mini-arthrotomy. Defect creation was controlled by utilizing manual curettes in a premarked area (via a skin biopsy punch)

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to prevent subchondral bone violation. The defects were either untreated (empty untreated) or treated with scaffolds loaded with cartilage fragments or with scaffold alone. The treatments were applied in random ($n = 5$ for each condition). The source for the cartilage fragments was autologous tissue collected during defect creation. After an implant was placed in the defect, a single PDS/PGA staple (DePuy Mitek, Norwood, MA) was centrally inserted through the subchondral bone to secure the implant. The operated limb was immobilized with a splint/cast for 2 weeks. Six months following implantation, the goats were euthanized. Comparisons between the surgical limb and the contralateral limb were made in each goat to document muscle wastage and range of

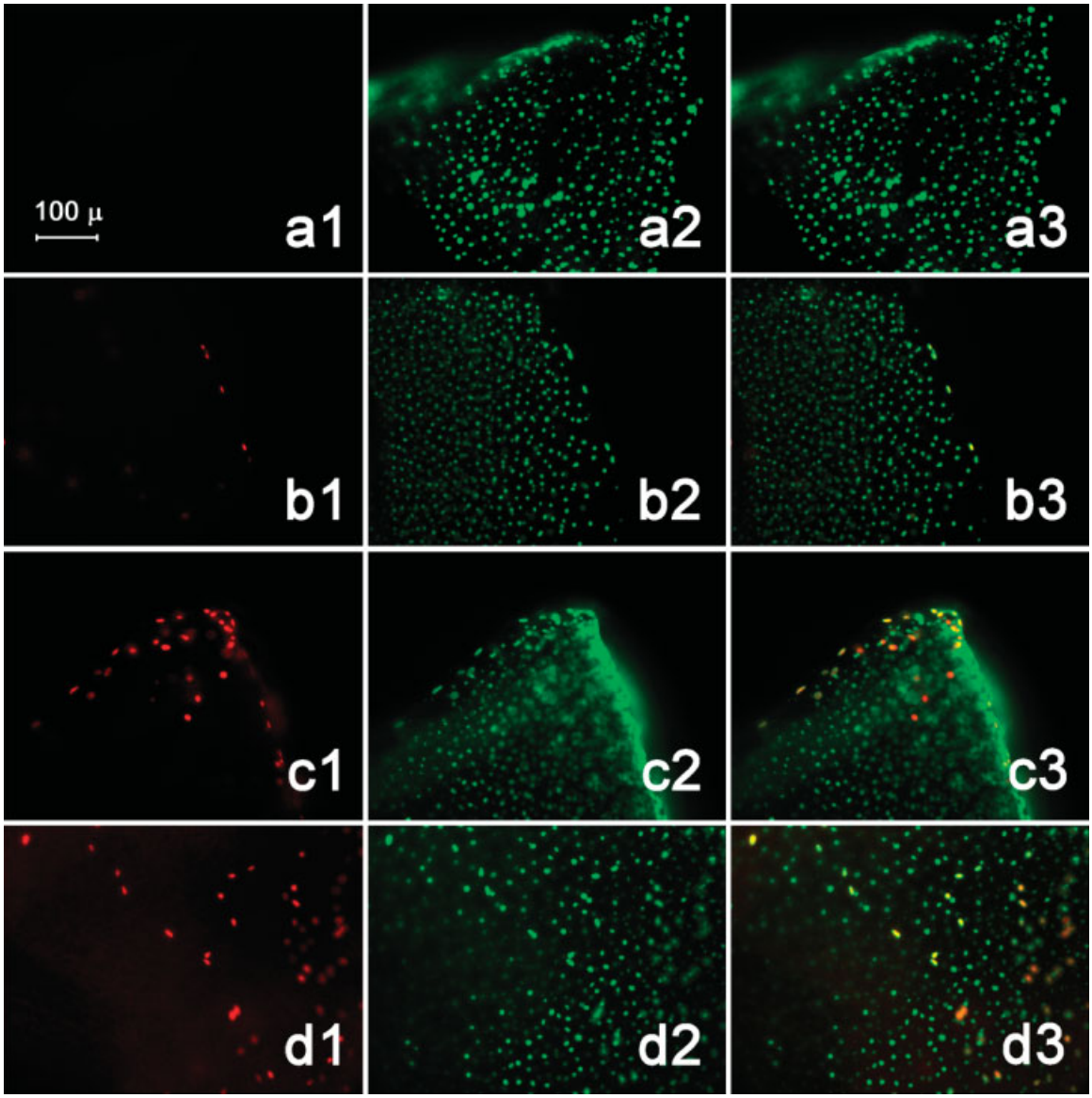

Figure 1. Bovine articular cartilage tissue was manually minced and cultured for various time points, and pulse labeled with BrdU overnight before harvesting. The harvested cartilage fragments from day-1 (**a1–a3**), day-2 (**b1–b3**), day-4 (**c1–c3**), or day-6 (**d1–d3**) cultures were then probed with a fluorophore conjugated BrdU antibody (**a1–d1**), and counterstained with ethidium homodimer-1 (**a2–d2**). Superimposed images of BrdU and the ethidium bromide are also presented (**a3–d3**). Note how chondrocytes became progressively activated with time, and appear preferentially on the cut surface of the cartilage fragments. Images were originally recorded in gray-scale under respective fluorescent filters, and individual image channels were then pseudo-colored to render red fluorescence for BrdU signal and green for the ethidium bromide counterstain.

motion. The surgical joints were then opened for gross evaluation.

Total joint health was assessed by evaluating the synovial fluid, synovial membrane, articular cartilage, meniscus, and bone. Reparative tissue was evaluated for evidence of unresorbed staple and scaffold, and overall tissue fill, uniformity, conformity, color, and integration. Osteochondral blocks encompassing the entire defect were dissected and fixed in 10% formalin. Histology using H&E and SO stains and immunohistochemistry for type I and type II collagens were performed (CTBR, Montreal, QC) on decalcified paraffin sections (5–6 μm). All slides were evaluated by an independent histopathologist in a blinded fashion and graded based on the modified O'Driscoll scale.[24]

RESULTS

Little or no BrdU incorporation was detected in cartilage fragments cultured for 1 day (Fig. 1a). However, as the culture progressed to 6 days, a progressive increase in the BrdU signal was detected in chondrocytes within the cultured cartilage fragments (Fig. 1b–d). Most importantly, the BrdU signal in chondrocytes seemed to localize along the tissue edge or on the surface, suggesting a correlation between tissue mincing and mitogenic activation of the chondrocytes (Fig. 1b1, c1).

Chondrocyte outgrowth into the scaffolds was limited at day 4 (Fig. 2a1), but increased by 3 weeks

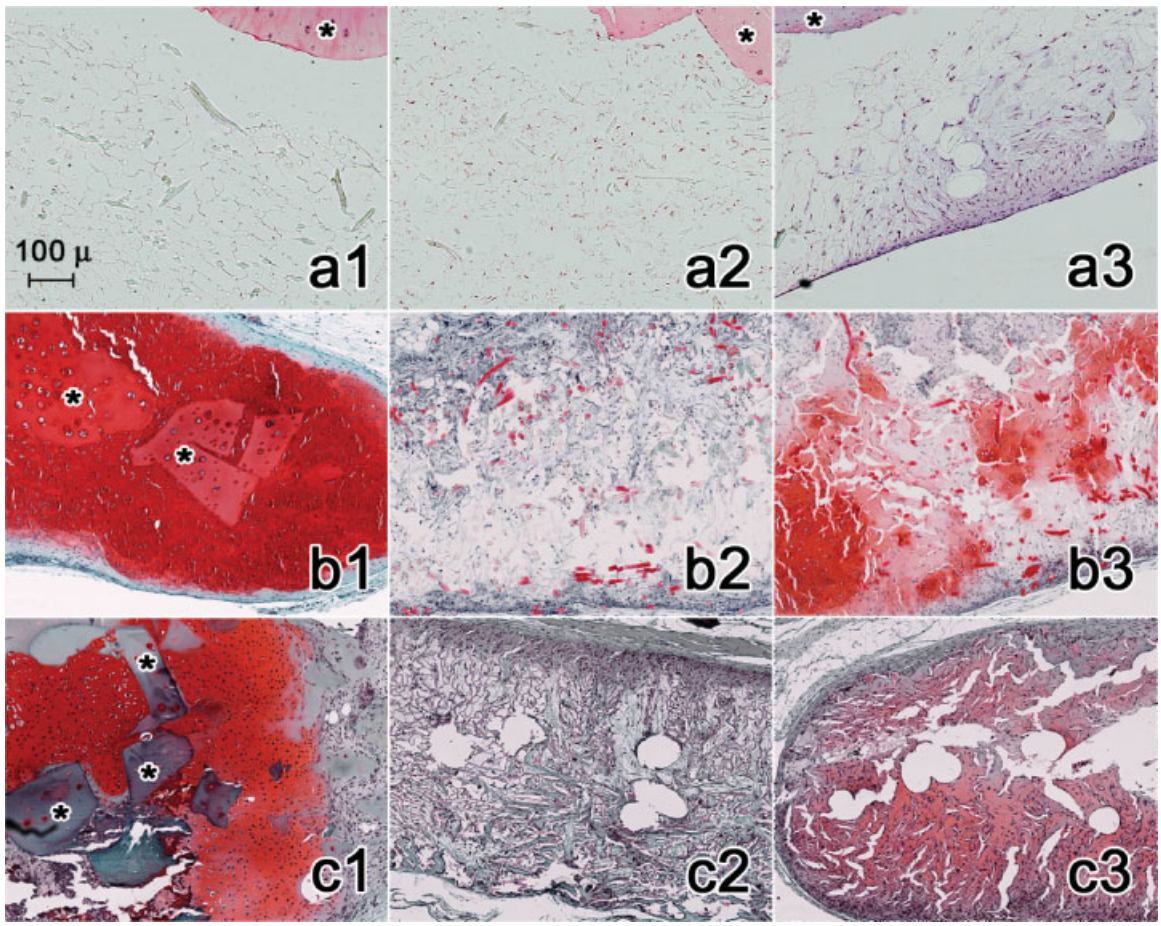

Figure 2. Manually minced articular cartilage tissue was cultured on top of a scaffold as described. Chondrocyte outgrowth from cartilage fragments was assessed by the evaluation of H&E cross-sections of cultured constructs of 4 days (**a1**), 3 weeks (**a2**), or 6 weeks (**a3**). Chondrogenic potential of the outgrown cells from the 6-week cultured constructs was further evaluated by implantation into the SCID mice. Four weeks postimplantation both bovine (**b1–b3**) and human (**c1–c3**) constructs from SCID mice were assessed by the evaluation of SO cross-sections for conditions of cartilage-loaded scaffold (**b1, c1**), scaffold alone (**b2, c2**) and scaffolds loaded with expanded chondrocytes (**b3, c3**). Note how cartilage fragments, similar to primary chondrocytes, elicit neocartilage formation in the surrounding environment. "*" indicates the presence of original cartilage tissue fragments.

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(Fig. 2a2) and became more uniform by 6 weeks (Fig. 2a3). Moreover, the outgrown cells were interconnected with newly deposited extracellular matrix (Fig. 2a3). In addition, an inverse relationship between tissue fragment size and the efficiency of the outgrowth (data not shown) was found when cartilage fragments of different sizes were analyzed.

Cartilage matrix from the SCID mice revealed by SO staining showed that newly established chondrocyte populations were able to produce and deposit cartilage matrix. Neo-cartilage formation was evident as discrete SO positive areas that had rounded chondrocyte-like cells and no vasculature (Fig. 2b1). Cartilage matrix specificity was further confirmed by collagen type II immunostaining (data not shown). Compared to constructs loaded with expanded primary chondrocytes from enzymatic digestion, the cartilage fragment-loaded constructs produced a more intense matrix, although the cell morphology and the cellularity of the neo-cartilage were similar for both conditions (Fig. 2, compare b1 and b3). Scaffold alone controls (Fig. 2b2) produced neither matrix deposition nor comparable cell morphology and cellularity. Similar results of neo-cartilage formation were obtained

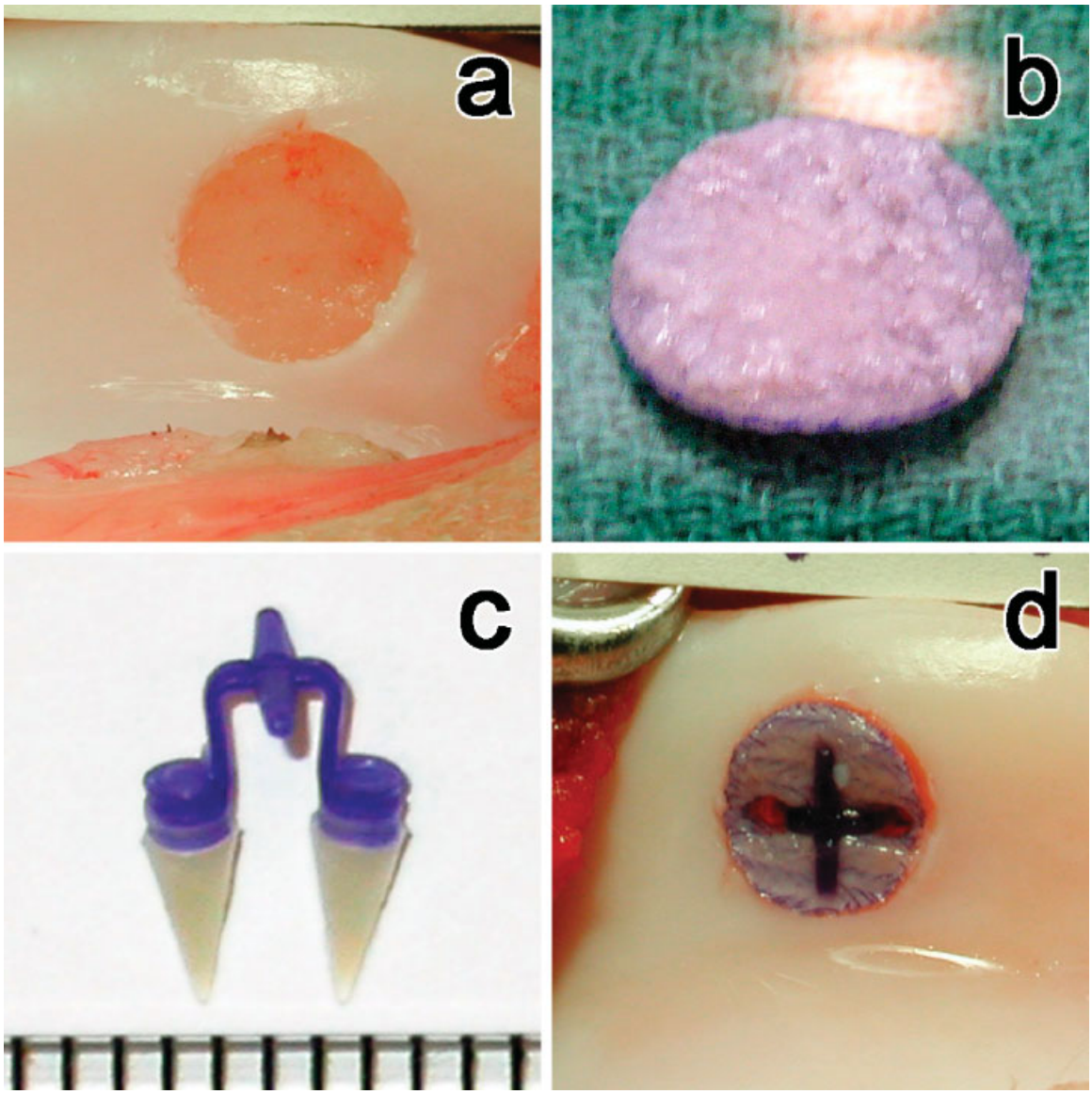

Figure 3. (**a**) A 7-mm full thickness chondral defect in trochlear groove. (**b**) A tissue-loaded implant coated with fibrin glue. (**c**) A resorbable PGA/PDS staple for implant fixation with a millimeter-scaled ruler at the bottom. (**d**) A defect treated with tissue-loaded implant.

with cartilage tissue fragments from five human donors (Fig. 2c). Both human cartilage fragments and dissociated human chondrocytes formed cartilage-like clusters (Fig. 2, compare c1 and c3), but the outgrown cells from cartilage fragments produced a more robust neo-cartilage.

No significant adverse events occurred during the goat study. Pictures of typical experimental setting of empty defect, cartilage fragment-loaded scaffold, a fixation staple, and implant-treated defect are shown in Figure 3. No signs of synovitis or other abnormalities were evident after 6 months implantation. Repair tissue from treatment with cartilage fragments generally resulted in whiter tissue and better surface congruency compared to other treatment groups, although all defects exhibited tissue fill. The best healing, as revealed by each of the replicates in all the treatment conditions in SO staining, occurred in defects treated with the cartilage fragment-loaded implants (compare Fig. 4a with b and c). In the cartilage fragment-loaded samples, hyaline-like cartilage usually filled the entire defect with complete integration to the normal adjacent cartilage and subchondral bone (Fig. 4d1, d4). SO staining of repair tissue was usually normal or near normal (Fig. 4a). The surface of the repair tissue was smooth and intact, although the repair cartilage was still

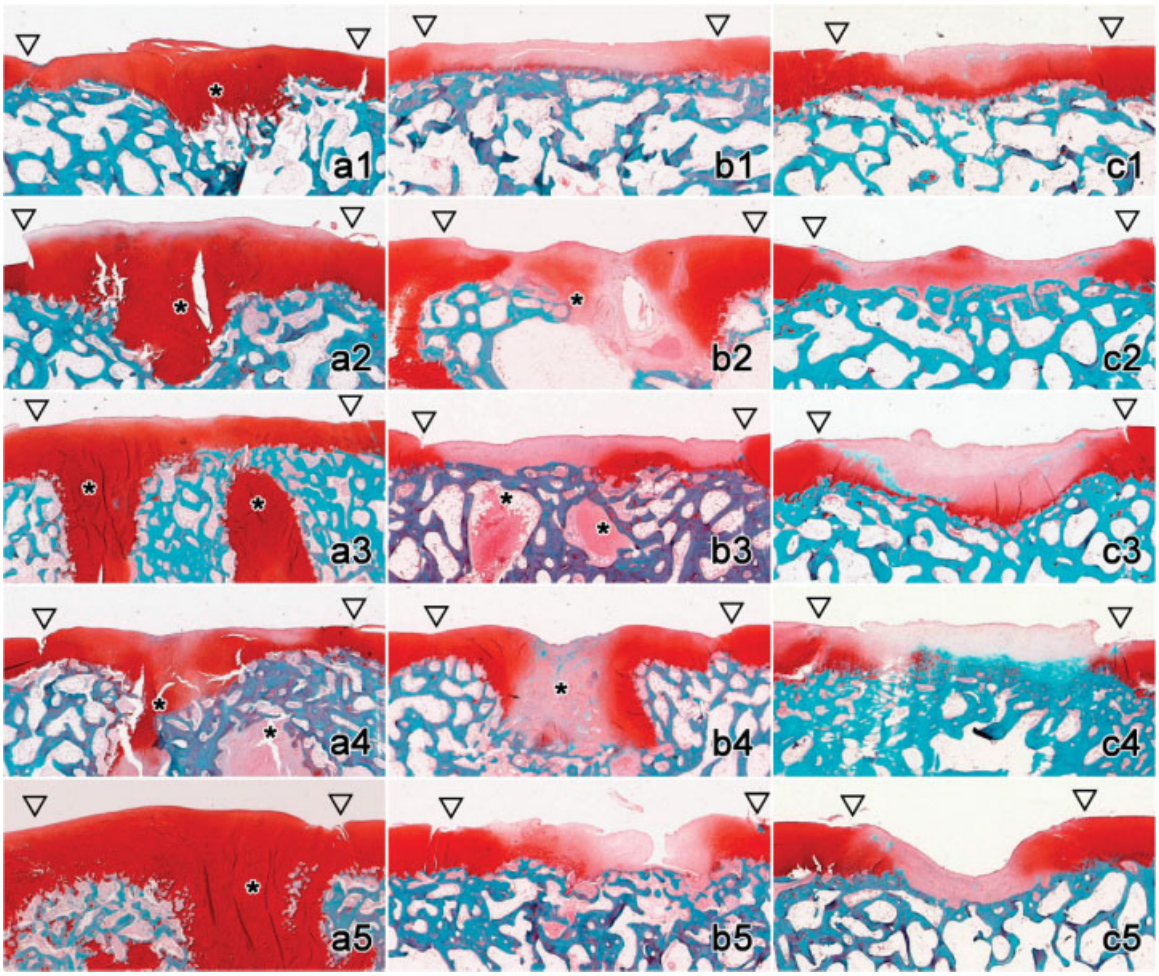

Figure 4. Full thickness 7-mm (diameter) chondral defects in the trochlear groove were treated with cartilage fragment-loaded scaffolds (**a, d, e**), scaffold alone (**b, f**), or left as empty untreated defects (**c**) for 6 months. Osteochondral blocks surrounding the defects from each of the treatment groups were evaluated through SO sections (**a–c**), H&E sections (**d**), and immunohistochemical sections (**e, f**). The high magnification H&E images (**d1–d4**) were derived from a single defect (**d**) and the white boxes represented the locations of the magnified areas. Immunohistochemistry evaluations for type I (**e1, f1**) and type II (**e2, f2**) collagens on paraffin sections were also performed. "*" (**e**) indicates a staple intrusion site, and "▽" indicates the margins of the defects.

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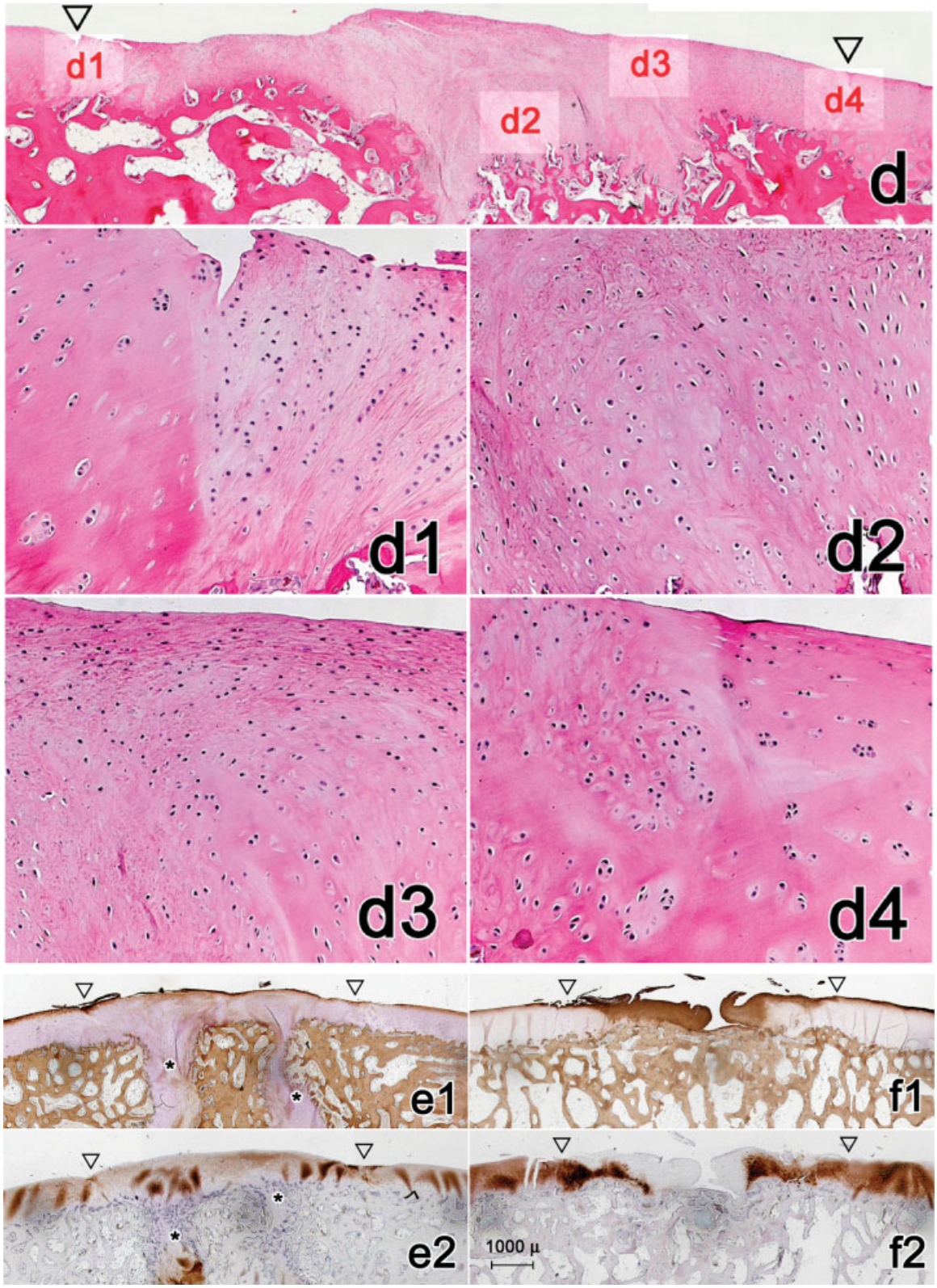

Figure 4. (*Continued*)

immature and lacked the normal zonal structure (Fig. 4d1–d4).

Active remodeling of subchondral bone was also apparent at the site of staple intrusion. Some staple sites were filled with cartilage-like tissue that extended down from the articular layer. However, neither the staple nor the scaffold material was observed microscopically in any of the specimens (Fig. 4), consistent with the resorption of the polymeric materials. Treatment with scaffold alone induced tissue repair with lesser quality (Fig. 4b1–b5). The tissue response usually consisted of fibrous tissue to fibrocartilage with severe disruption of the structural integrity of the articular surface (Fig. 4b2, b4, b5). Typically, the repair tissue was integrated with adjacent host cartilage but with less SO staining than that observed with the cartilage fragment-loaded implants. Subchondral bone repair was inconsistent (Fig. 4b2). Untreated defects were consistently filled with fibrocartilage (Fig. 4c1–c5) that integrated well into the adjacent cartilage, but lacked any structural organization. Immunostaining confirmed that defects treated with cartilage fragment-loaded constructs had the most consistent prevalence of type II collagen (compare Fig. e2 with f2) and lesser amounts of type I collagen compared with the scaffold alone treatment (compare Fig. 4e1 with f1). Histologic grading revealed a significantly better score with cartilage fragment-loaded implants (19.4 ± 2.1) versus empty untreated (16.4 ± 2.3; $p < 0.05$) and scaffold alone (14.4 ± 2.1; $p < 0.01$).

DISCUSSION

Therapeutic methods that promote a presence of chondrogenic cells in cartilage defects are much needed, as spontaneous migration of chondrocytes from the surrounding tissue is very limited. Clinical application of ACI is becoming an established technique for repairing cartilage lesions. To maintain the benefit of chondrocyte therapy while improving the preparation of the cell source, we proposed a novel approach where chondrocytes are delivered in the form of cartilage tissue fragments. This concept of cartilage fragments as the transplantable cell source is clearly distinct from a previously described clinical procedure in which autologous osteochondral morselized graft was used as the cell source.[25] Whereas morselized osteochondral graft consists of a small amount of cartilage tissue and a larger bone contribution, our approach relies strictly on the cartilage tissue. In addition, osteochondral graft tissue is typically implanted in a bleeding osteochondral defect to induce a cell contribution from the stimulated marrow. In contrast, since the cartilage fragments described in our approach are delivered in a non-bleeding chondral defect, the repair process is likely to be driven more by the chondrocytes than other cell types with osteogenic and chondrogenic potential, such as marrow-derived mesenchymal stem cells. Furthermore, we devised a scaffold-based system to provide an optimal three-dimensional structure for uniform tissue distribution in the defect.

BrdU incorporation and the outgrowth assessment in the current study demonstrated that chondrocyte migration and proliferation from the tissue fragments were effectively elicited via tissue morselization or fragmentation. We also established the reparative benefit of cartilage fragments in the treatment of critical-sized chondral defects in large weight-bearing animals. In just 6 months, the implantation of cartilage fragment-loaded scaffold resulted in repair tissue with hyaline-like features. The repair tissue was significantly better in the overall histological grading than any of the control treatment groups, including the scaffold alone and empty untreated groups. The consistent and significant healing benefit derived from the contribution of cartilage fragments demonstrates the feasibility of using this new concept to treat cartilage lesions. The repair potential of cartilage fragments was further demonstrated in the amount of tissue required for an effective healing. The neocartilage formation in SCID mice (Fig. 2b1, c1) as well as the chondral healing in goats (Fig. 4a1–a5) indicated that a small amount of cartilage tissue fragments is expected to be able to cover a large area for new tissue formation. In fact, less than 1/10 amount of the defect-filling tissue was used to treat an entire defect in the goat study. Based on this, a harvest of 200–300 mg of cartilage, similar to an ACI cartilage harvest, would allow treatment of a chondral lesion of at least 10 cm^2 in size.

Clinical application of the methodology described in this study could offer practical advantages compared to the current ACI technique. Because of the elimination of the culture step, the surgical procedure would be consolidated into a one-stage procedure. Cartilage biopsy harvesting and implantation could be accomplished intraoperatively, hence eliminating the burden of multiple surgeries. In addition, elimination of the need for periosteum and the application of staple-based fixation techniques would simplify the surgical practice and potentially reduce the associated complications.[26] Most importantly, the high cost associated with the

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technical and logistic complexities of the current culture-expansion processes would be avoided.

In the current study, we could not tease out the cellular contribution from the marrow or the synovial compartment to the repair process. Cells could potentially have migrated from small fissures in the underlying bone as a result of the staple intrusion or adhere to the scaffold from the synovial environment. Nevertheless, both these potentially confounding factors were present in our scaffold-alone control, so the addition of the cartilage fragments clearly plays a key role in the repair process and leads to a more mature, better integrated hyaline-like tissue. The cartilage regeneration potential of our approach is likely dependent on multiple parameters, such as the size and the loading density of the fragments, the chemical and structural aspects of the scaffold, and the method of harvest. These factors could influence specific sub-populations of cells, their state of activation, and their ability to migrate, populate, and differentiate in response to the local environment. These aspects will be the focus of future investigations that may better elucidate the underlying mechanisms that contribute to the successful repair observed with our approach. Careful clinical investigation, including randomized clinical studies, will be required before this approach becomes a therapeutic reality. We believe that the data presented here support a simple, cost-effective treatment for cartilage repair addressing a large clinical need that has been the focus of intense research for over 150 years.[27]

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