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Fluorescent Viability Stains Overestimate Chondrocyte Viability in Osteoarticular Allografts

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Background: Allografts from many tissue banks are carefully processed and stored with the goal of preserving chondrocyte viability. However, the importance of living chondrocytes for graft stability is unclear, in part because actual viabilities of individual allografts at the time of placement are seldom known.

Hypotheses: Cell yields from allograft and fresh cartilage differ significantly if chondrocyte viability in allografts is lower than indicated by fluorescence staining with conventional viability probes. In addition, transmission electron microscopy will show significant differences in the percentage of morphologically abnormal chondrocytes in allograft and fresh cartilage.

Study Design: Controlled laboratory study.

Methods: Fluorescence viability staining, chondrocyte yield, and chondrocyte characteristics were studied in 8 commercial osteochondral allografts (7 hemicondyles, 1 talus) and 4 freshly harvested cartilage samples from an adult distal femur (age, 46 years), from an adult talus (age, 51 years), and from an adult tibial plateau (age, 29 years) and from a juvenile distal tibia (age, 9 years). Selected fresh and allograft specimens were repeatedly frozen and thawed to deliberately kill chondrocytes by membrane disruption. The findings were analyzed to determine if allograft and fresh cartilage were significantly different with respect to each of the 3 different outcome measures.

Results: Although fluorescent staining indicated that approximately 75% of chondrocytes were viable (calcein AM–labeled) in allograft cartilage, counterstaining with 4,'6-diamidino-2-phenylindole showed that fewer than 30% contained identifiable nuclei. In contrast, 100% of cells labeled as viable contained nuclei in fresh cartilage. Killing chondrocytes by freeze-thawing before staining did not diminish calcein AM staining in allograft cartilage but caused a significant reduction in fresh cartilage. The average yield of chondrocytes from allograft cartilage was less than 200 000/100 mg tissue, significantly lower than in fresh cartilage, which averaged more than 1.5 million/100 mg tissue. The yield from freeze-thawed controls was less than 24 000/100 mg. Cell numbers increased after 7 days of culture in all cases except for chondrocytes from freeze-thawed cartilage, an indication that the isolated cells were viable. Morphologic analysis by transmission electron microscopy revealed significant increases in the numbers of chondrocytes with pyknotic or absent nuclei or with disintegrated plasma membranes in allograft versus fresh cartilage.

Conclusion: Conventional fluorescence probes are unreliable for analyzing chondrocyte viability in osteoarticular allografts. Alternative methods for assessment of viability, such as cell culture and ultrastructural imaging, may provide more accurate assessment of viability in allografts.

Clinical Relevance: Conventional staining methods that overestimate chondrocyte viability in osteoarticular allografts may mislead investigators attempting to assess the effects of chondrocyte viability on graft stability following implantation. A more reliable means to measure chondrocyte viability will be required to accurately assess these effects.

Keywords: allograft; cartilage; chondrocyte; viability

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Articular injuries leading to focal cartilage defects are common, occurring in isolation or with other knee injuries. It has been estimated that articular cartilage injuries occur in as many as 5% to 10% of all acute knee injuries presenting with knee hemarthrosis.[19] Without intervention, the notoriously limited capacity of cartilage for self-repair[3,11,22,25] dooms many patients with joint injuries to progressive degenerative disease. The main goal of intervention is to restore a smooth, structurally intact articular surface. Methods of treatment

include abrasion chondroplasty, autologous chondrocyte implantation, microfracture, mosaicplasty, osteochondral autograft transfer, and osteochondral allograft transplantation.[1,5,10,22,25] Typically, these procedures result in the production of fibrocartilage or a mixture of fibrocartilage and hyaline cartilage.

Osteochondral allografts have primarily been used to treat osteochondral and chondral defects in the femoral condyle and tibial plateau.[1,11,25] This method was popularized by Gross,[9] and these grafts reportedly have a clinical success rate of 75% to 95% at 5 years' follow-up and 64% to 85% at 10 years' follow-up.[2,4,8,9,15,24] Unlike cell-based alternatives, osteochondral allografts immediately restore a smooth, mechanically functional joint surface. Moreover, initial studies have added substantially to the practical appeal of the approach by indicating that allografts could be stored for up to 40 days without significant loss of mechanical properties or chondrocyte viability.[1,3,4]

The long-term durability of osteoarticular grafts is thought to depend on de novo cartilage extracellular matrix synthesis by autologous chondrocytes that survive the transplantation process.[1,3,23,25] Storage conditions are considered critical for maintaining chondrocyte viability. Freezing cartilage sharply decreases chondrocyte viability and may disrupt the extracellular matrix.[1,17,21,22] In contrast, storage at 4°C does no significant harm to the matrix and is thought to better preserve chondrocyte viability. Results from several studies indicate that the majority of chondrocytes remain viable for 14 to 60 days in cartilage stored in serum-free culture medium at 4°C.[1,20,22,25]

A variety of methods have been used to determine chondrocyte viability in cartilage, including lactate dehydrogenase assays, MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5 dephenyltetrazolium bromide; Thiazolyl blue) assays, and $^{35}SO_4$ metabolic radiolabeling.[12] However, despite questions regarding the reliability of calcein AM and ethidium homodimer (EtHd) for viability assessment in cartilage, they remain the most commonly used reagents for analysis of allograft viability.[12] Calcein AM passes through cell membranes and fluoresces only after cleavage by cellular esterases.[6] The cleaved fluorescent molecule cannot diffuse out through intact cell membranes. Thus, the stain accumulates as a bright green signal in the cytoplasm of living cells under epifluorescent illumination (488 nm excitation). In contrast, EtHd, which is often used in conjunction with calcein AM, is unable to pass through intact cell membranes but can enter the nuclei of cells with compromised membrane integrity, where it binds by intercalation with DNA. Ethidium homodimer fluoresces red-orange on 488 nm excitation without a requirement for enzymatic conversion. Sections of cartilage labeled with calcein AM and EtHd may be counterstained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) to label all cell nuclei present in the section.[13] The blue fluorescent DAPI signal labels all cells that contain nuclei whether the cells were alive or dead at the time of fixation. In principle, the number of cells stained with calcein AM and EtHd should equal the number stained with DAPI. However, the number of DAPI-stained nuclei actually counted frequently exceeds the number of calcein AM + EtHd counted by 10% to 20%. This result is likely due to the higher signal to noise ratio of the DAPI fluorescence signal, which leads to more efficient counting.

The yield of viable chondrocytes isolated by enzymatic digestion cartilage has been used to assess the effects of storage conditions on chondrocyte viability.[16] This study showed declines in the numbers of chondrocytes isolated from fresh cartilage (94% viable) versus chondrocytes isolated from cartilage stored for 72 hours at 4°C in serum-containing medium (47% viable) or in serum-free culture medium (84% viable).

Dead or dying chondrocytes in cartilage display characteristic morphologic abnormalities when examined by transmission electron microscopy. Chen et al,[7] reporting on the effects of mechanical loading of cartilage, detected increased numbers of chondrocytes lacking intact plasma membranes, an unequivocal indication of death. Many other chondrocytes in loaded cartilage showed irregular nuclear membranes or condensed chromatin, characteristic of cells undergoing apoptosis. These results were concordant with calcein AM/EtHd staining, which showed extensive loss of chondrocyte viability–loaded explants.

Osteochondral allografts are prepared by commercial suppliers using rigorously standardized procedures designed to preserve living chondrocytes. However, conditions during shipping, and during handling in the operating room, are less well controlled and may have detrimental effects on chondrocyte viability. Thus, viability at the time of implantation might vary substantially. Initial studies in our laboratory sought to measure such variations using the conventional calcein AM/EtHd fluorescence stains. Unfortunately, staining patterns in many allograft samples could not be reconciled with counterstains that indicated nuclei were often absent in lacunae counted as viable.[14] This suggested that the viability of chondrocytes in allografts is overestimated by calcein AM/EtHd–based assays. On the basis of these findings, we hypothesized that if viability was actually equivalent in allograft and fresh cartilage as indicated by calcein AM/EtHd, the yield of viable chondrocytes isolated from the 2 groups would be similar. Moreover, we hypothesized that transmission electron microscopy would show that the percentage of morphologically intact chondrocytes would be similar in allograft and fresh cartilage.

METHODS

Cartilage Harvest

Osteochondral allografts (7 hemicondyles, 1 talus) used for implantation were retrieved from the operating room after graft placement. All allografts tested were prepared by a commercial tissue bank and had been preserved in serum-free culture medium at 4°C. Based on the supplier's product description, the average storage time was 14 days. However, exact storage times for each sample were not known. The age of the donors was not given, but exclusion criteria described by the tissue bank indicated that donors were older than 18 years and younger than 50 years. Allografts were kept submerged in culture medium and

processed immediately after retrieval. Three or more 4-mm-diameter cartilage disks were cut from 1 to 2 cm around the graft excision site. Care was taken to keep the sample moist during excision.

All fresh cartilage samples were harvested as surgical discard material from amputated lower limbs with Institutional Review Board approval. One sample was obtained from an adult (age, 46 years) distal femur (DF), a second was obtained from an adult (age, 51 years) talus (Tal), and a third from an adult (age, 29 years) tibial plateau (TP). A fourth sample was harvested from the distal tibial surface (DT) of a 9-year-old child. None of these specimens showed any histological abnormalities suggestive of arthritis, such as fibrillation or proteoglycan loss, when evaluated by safranin-O staining. Cartilage was excised from joints, submerged in sterile saline or culture medium (DMEM with 10% fetal bovine serum; Invitrogen, Grand Island, NY), and delivered to the laboratory within 4 hours of harvest.

Freeze-Thaw Controls

Selected fresh and allograft specimens were repeatedly frozen and thawed to deliberately kill chondrocytes by membrane disruption. This procedure was performed by first placing explants on dry ice until they were solidly frozen (3 minutes), then transferring the frozen explants to culture medium warmed to 37°C until they were completely thawed (5 minutes). Freeze-thawing was repeated 3 times to ensure that most cells were killed.

Viability Staining

Full-thickness fresh and allograft cartilage specimens were cut into 4-mm disks and were incubated overnight in calcein AM and EtHd to detect live and dead cells, respectively. For viability staining, cartilage disks were immediately distributed in 48-well culture plates in 1.0 mL culture medium and incubated overnight in calcein AM (5 μM) and EtHd (0.5 μM) (Molecular Probes, Carlbad, Calif). Stained cartilage disks were cryoembedded, sectioned, and mounted on Superfrost Plus Slides (Fisher Scientific, Pittsburg, Pa) using Vectashield DAPI H-1200 Mounting Medium (Vector Laboratories, Burlingame, Calif). Cartilage cryosections were imaged on an Olympus BX60 microscope (Olympus, Tokyo, Japan) using ultraviolet illumination for DAPI detection and 488-nm illumination for the calcein AM and EtHd detection.

Quantitative Measurement of Viability Stains

A quantitative histologic approach was developed to count cells in cartilage stained with viability probes. Seven allografts and 4 fresh cartilage specimens were processed. A computer-controlled stepper motor-driven microscope stage (Prior Scientific, Rockland, Md) and digital camera (QImage, Sykesville, Md) were used to scan entire 4-mm-wide cartilage cryosections with a 10× objective. Commercial software (Image-ProPlus, Media Cybernetics Inc, Bethesda, Md) was used to assemble multiple individual microscope fields (0.4 mm × 0.6 mm) into large (10-20 mm^2), high-resolution (2 pixels per micrometer) composite images. Cell counts were performed within each of the individual fields (n = approximately 30) making up the composite image using a custom-designed MATLAB-based digital image analysis program (Mathworks, Natick, Mass) on a personal computer. The program automatically identifies cells based on size and shape, automatically thresholds, and then performs a red/green color separation to split orange-red–stained (EtHd) cells from green-stained (calcein AM) cells. The color-separated populations were counted to obtain total live (green) and total dead (orange-red) data. Percentage of live cells was calculated from these counts [green cells/(green cells + red cells) × 100]. To obtain total nuclei present in the section, a similar image analysis routine counted DAPI-stained (blue) cells in corresponding composite images scanned with 390-nm light. At least 2 sections per cartilage disk were scanned and analyzed. This represented a minimum of several hundred cells counted per specimen.

Chondrocyte Yield

Four allograft and 4 fresh cartilage specimens were used in culture experiments. Chondrocyte cultures were established by digestion of the cartilage extracellular matrix (ECM) using collagenase and pronase as described.[18] Full-thickness cartilage specimens (100-500 mg) were first weighed and then digested overnight in a culture incubator in culture medium containing enzymes. Chondrocytes collected from the digests were suspended in culture medium without enzymes and were manually counted on a hemocytometer. The cell suspensions were plated in 100-mm dishes and incubated for 7 days with periodic medium exchange (every 3-4 days). After 7 days of incubation, the monolayer cultures were trypsinized and counted again to determine if the populations grew over time.

Ultrastructural Analysis: Electron Microscopy

Cartilage samples from 3 fresh and 4 allograft samples were cut into small blocks (<1 mm thick) and were fixed for 10 to 14 days in 2% glutaraldehyde. The blocks were rinsed in cacodylate buffer, postfixed in 1% osmium, then dehydrated and embedded in Spurr's resin (Electron Microscopy Sciences, Hatfield, Pa). Thin sections were applied to copper grids and stained with uranyl acetate and lead nitrate. The sections were imaged by transmission electron microscopy (TEM) on a JEOL JEM-1230 microscope (JEOL, Tokyo, Japan) at a magnification of 1000 to 4000×. Chondrocytes were scored for nuclear abnormalities (absent or pyknotic nuclei) and cytoplasmic abnormalities (blebbing, shrinkage, or retraction). Chondrocytes displaying any of these features were scored as "abnormal." At least 2 blocks per specimen were examined, and at least 12 microscope fields were imaged from each block. A total of 136 allograft sections and a total of 84 fresh cartilage sections were imaged and counted.

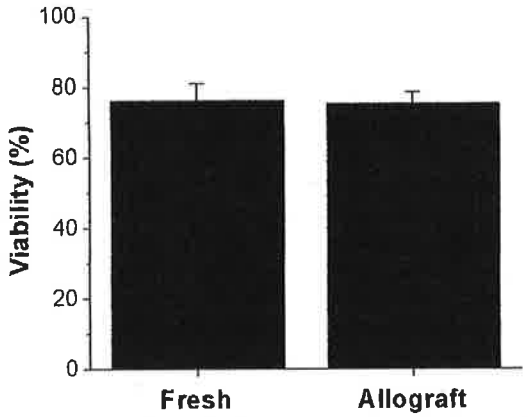

Figure 1. Chondrocyte viability in fresh and allograft cartilage by calcein AM/EtHd staining. The average percentages of viable chondrocytes in fresh cartilage (n = 4) and in allograft cartilage (n = 8) were measured using calcein AM and EtHd staining. The bars represent standard errors.

Statistics

The Student *t* test was used to evaluate the significance of differences in morphologic characteristics and viability between allograft and fresh cartilage.

RESULTS

Fluorescent Staining for Viability

Average percentage viability as calculated from calcein AM/EtHd staining was 75% ± 3.2% for allografts and 76% ± 4.8% for fresh cartilage (Figure 1). Calcein AM, EtHd, and DAPI staining patterns differed for fresh, allograft, and freeze-thawed control cartilage (Figure 2). Quantitative image analysis showed that the number of calcein AM–stained chondrocytes was 53.1 ± 7.5 cells/field in fresh cartilage and 34.5 ± 2.5 cells/field in allograft cartilage, a difference that was significant ($P = .003$) (Figure 3). The number of EtHd-stained cells in fresh and allograft cartilage was 10.8 ± 1.6 cells/field and 11.7 cells/field, respectively, and was infrequent (Figure 3). Fresh cartilage had significantly more abundant DAPI-stained cells (89.7 ± 8.6 cells/field) versus allograft cartilage (12.9 ± 2.2 cells/field) ($P < .001$).

Repeated freeze-thawing was used to deliberately kill chondrocytes in allograft and fresh cartilage before viability staining. Freeze-thawing of fresh cartilage dramatically altered the pattern of staining in a predictable manner: few cells were positive for calcein AM (3.2 ± 0.8 cells/field), and many stained positively with EtHd (49.1 ± 5.8 cells/field). Many cells were also stained with DAPI (74.3 ± 8.2 cells/field). The absence of calcein AM and presence of EtHd staining confirmed that freeze-thawing killed chondrocytes in fresh cartilage. In striking contrast, freeze-thawing did not alter the staining pattern in allograft cartilage, which retained large numbers of calcein AM–stained lacunae (32.4 ± 3.9 cells/field) and remained largely negative for EtHd (0.3 ± 0.3 cells/field) and DAPI

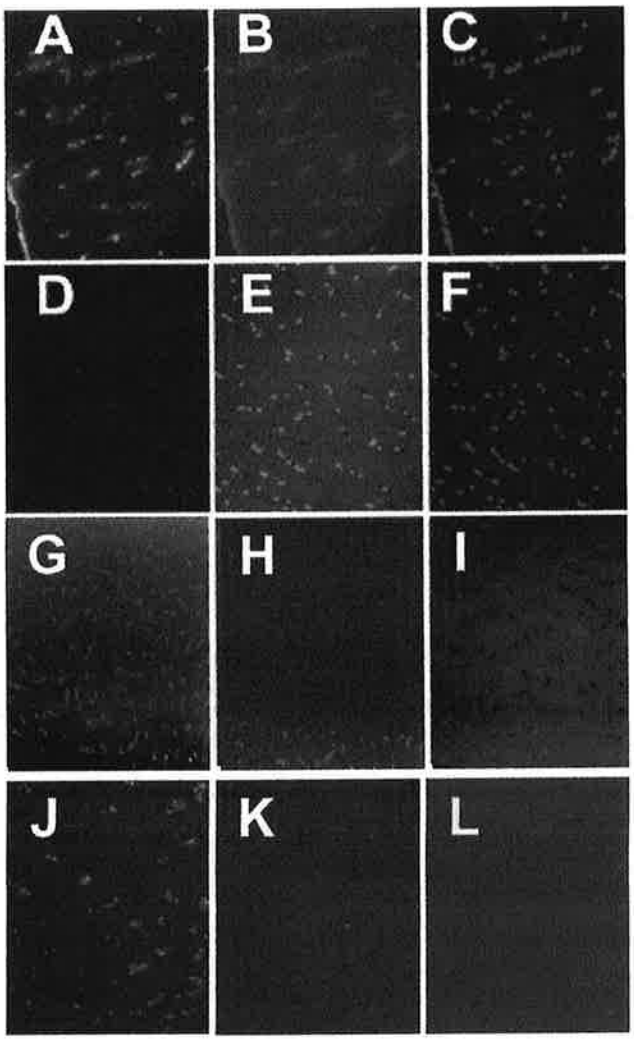

Figure 2. Pattern of calcein AM, EtHd, and DAPI staining in cartilage. Fresh cartilage (A, B, C), freeze-thawed fresh cartilage (D, E, F), allograft cartilage (G, H, I), and freeze-thawed allograft cartilage (J, K, L) were stained with calcein AM (A, D, G, J), with EtHd (B, E, H, K), or with DAPI (C, F, I, L).

(13.2 ± 1.9 cells/field). This indicated that few calcein AM–stained lacunae actually contained DNA detectable either by DAPI or EtHd.

Chondrocyte Yields and Growth in Culture

Allograft and fresh cartilage samples were digested to liberate chondrocytes from the matrix for cell culture. The yield of cells from each specimen was recorded, and the cells were incubated for 1 week to assess population growth (Figure 4). Initial chondrocyte yields for allograft cartilage ranged from 41 000/100 mg cartilage to 311 000/100 mg cartilage. Initial yields for fresh cartilage ranged from 500 000/100 mg to 1.4×10^6/100 mg. A negative control consisting of fresh cartilage, which was frozen and thawed repeatedly to deliberately kill chondrocytes, yielded 22 000 chondrocytes/100 mg cartilage

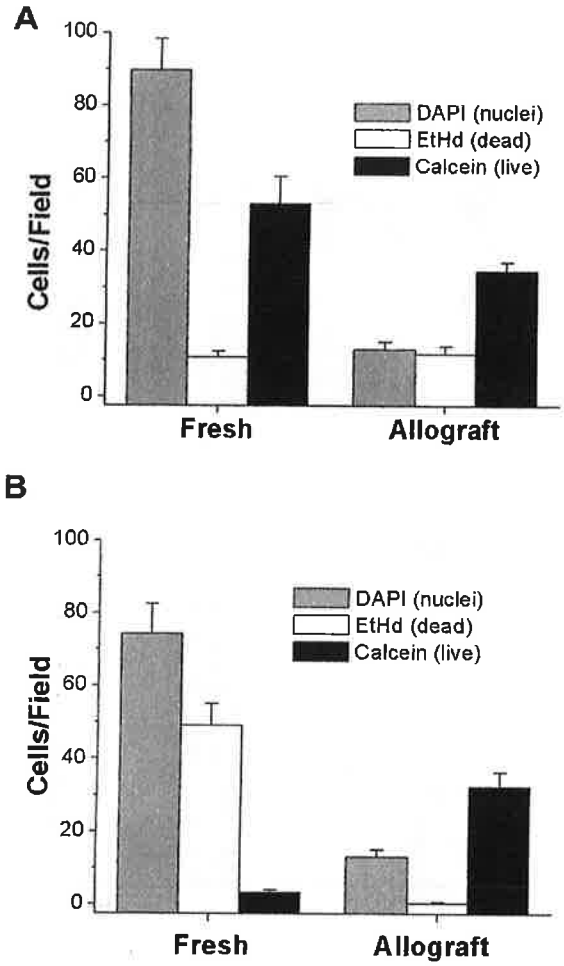

Figure 3. A, numbers of chondrocytes in fresh and allograft cartilage stained with calcein AM, EtHd, or DAPI. B, numbers of chondrocytes in fresh and allograft cartilage stained with calcein AM, EtHd, or DAPI after repeated freeze-thawing. Means and standard errors for 7 allografts and 4 fresh samples are shown.

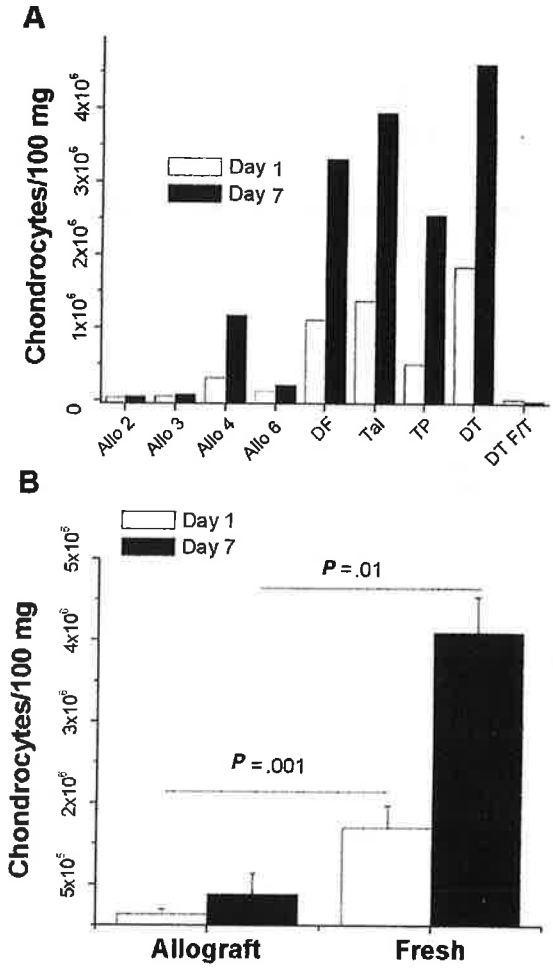

Figure 4. A, chondrocytes isolated from 4 fresh (DF, Tal, TP, DT) and 4 allograft cartilage samples (Allo 2, 3, 4, 6) were counted immediately after enzymatic isolation (day 1) and after 7 days of in vitro population growth (day 7). Fresh DT cartilage was freeze-thawed before digestion (DT F/T). Cell numbers were normalized to tissue wet weight (chondrocytes per 100 mg tissue). B, means and standard errors for the allograft and fresh cartilage samples shown in A. As indicated by the *t* test results ($P = .01$ or $P = .001$), fresh samples yielded significantly greater numbers of chondrocytes at both time points.

initially (Figure 4A). The average yield of viable chondrocytes was significantly higher in fresh cartilage than in allograft cartilage immediately after isolation ($P = .001$) and after 7 days in culture ($P = .010$) (Figure 4B) .The growth of the cultures, measured in terms of population doublings (PD), ranged from 0.5 to 1.9 PD for chondrocytes from allografts and from 1.3 to 2.3 PD for chondrocytes from fresh cartilage. Chondrocytes isolated from negative controls were the only culture in which the population declined during 1 week in culture (–2.24 PD). Although the average PD for allografts (0.92 PD) was lower than the average for fresh cartilage (1.7 PD), the difference was not significant ($P = .10$).

Ultrastructural Analysis of Chondrocytes

Transmission electron microscopy was used to assess the ultrastructural characteristics of chondrocytes in cartilage samples. Based on calcein AM/EtHd results, the authors predicted that equal numbers of cells in allograft and fresh cartilage would show characteristic signs of degeneration or death. Chondrocytes were scored for nuclear abnormalities (absent or pyknotic nuclei) and cytoplasmic membrane abnormalities (blebbing, shrinkage, or retraction) (Figure 5). Chondrocytes in allograft cartilage displayed significantly more of these abnormalities than chondrocytes in fresh cartilage: morphologic abnormalities among chondrocytes in allografts ranged from 40% to 90%, while the range for fresh cartilage was 1% to 15% abnormal (Figure 6A). The number of normal chondrocytes in fresh cartilage averaged 2.6 ± 0.16/field, significantly greater than in allograft cartilage, which averaged 1.1 ± 0.09/field

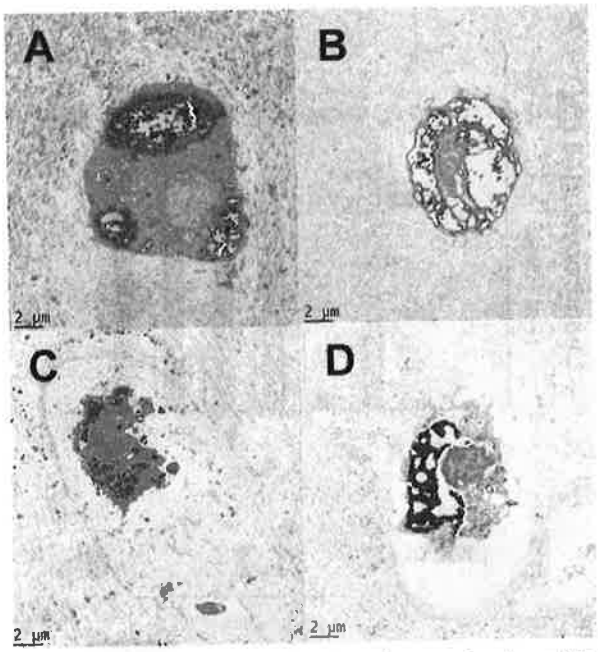

Figure 5. Images of chondrocytes from a fresh cartilage source (A) and from allograft specimens (B, C, D). The chondrocyte in A fully occupies the lacuna and shows an organized cytoplasm with secretory vessels. The nucleus is also clearly defined and shows a regular border. Abnormal cells in B, C, and D show retraction or collapse of the cytoplasm with irregular pyknotic nuclei (B, D) or no apparent nucleus (C).

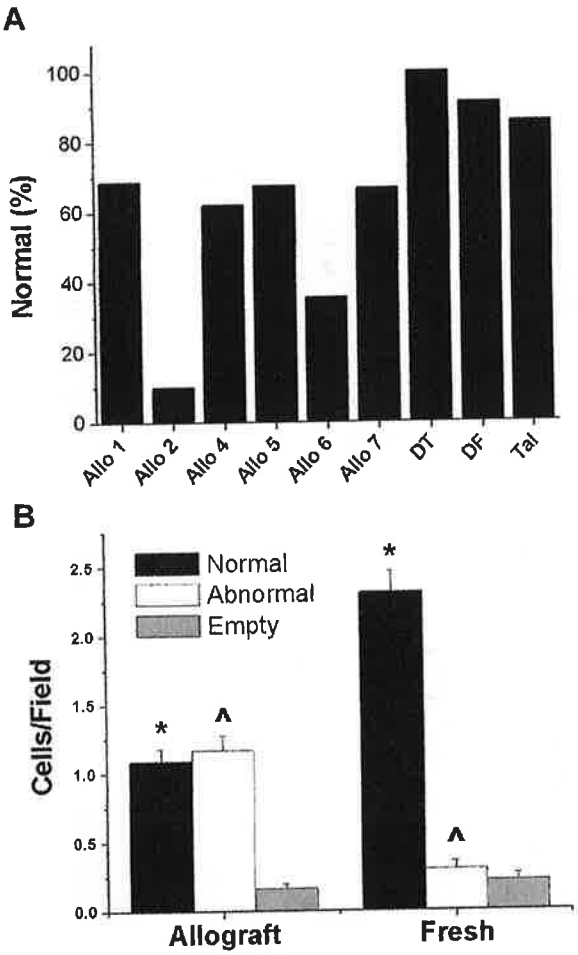

Figure 6. Normal and abnormal chondrocytes and empty lacunae were counted in 6 different allografts (Allo 1, 2, 4, 5, 6, 7) and 3 different fresh cartilage specimens (DT, DF, Tal). A, percentage of normal chondrocytes for each cartilage specimen. B, normal and abnormal chondrocytes, and empty lacunae per microscope field. Columns show means and standard deviations for all fresh and allograft specimens. The number of normal cells was significantly greater in fresh cartilage specimens (*), and the number of abnormal chondrocytes was significantly greater in allograft specimens (^) ($P < .05$).

(Figure 6B). Conversely, the average number of abnormal chondrocytes in allograft (1.2 ± 0.11/field) was significantly greater than in fresh cartilage (0.3 ± 0.06/field).

Although both cell yield and TEM data indicated that there were significantly fewer viable cells in allograft cartilage than calcein AM/EtHd staining indicated, the 2 alternative measures did not correlate well with one another: linear regression analysis of the 2 data sets showed a coefficient (R) of 0.20 and a P value of .60.

DISCUSSION

If calcein AM/EtHd–staining results indicating similar viabilities in allograft and fresh cartilage were accurate, cartilage digests should have yielded similar numbers of chondrocytes per volume of tissue. However, we found that the mean cell yields for allografts (132 000 cells/100 mg) were 10-fold lower than for fresh cartilage (1.2×10^6/100 mg). After 1 week of incubation, the numbers of chondrocytes in cultures from fresh cartilage remained higher than in cultures from allograft cartilage. However, the rate of population growth was similar in cultures from both types of tissue, an indication that the cells counted in the initial isolates were viable in both allograft and fresh cartilage. Thus, our findings suggest that, relative to fresh cartilage, the viable cell density in allograft cartilage was much lower than estimated by calcein AM/EtHd staining, which indicated that viability in allografts was nearly 100% that of fresh cartilage.

We predicted that, if viabilities were similar in allograft and fresh cartilage, equal numbers of cells would show characteristic signs of degeneration or death by TEM. Chondrocytes with abnormal nuclei or compromised plasma membranes were observed in both allograft and fresh cartilage. However, quantitative analysis revealed that the density of abnormal cells in allografts was 4-fold greater than in fresh cartilage. The average for allografts was 55% abnormal, similar to the level previously reported in a TEM study of allograft cartilage after 37 days in storage at 4°C.[22] Thus, as for chondrocyte yields, TEM analysis revealed significant

differences in cellularity between allograft and fresh cartilage that were not apparent by calcein AM/EtHd staining.

Cell yields and TEM, the alternative measures of viability we used, did not correlate well with one another. The reason for this discrepancy is unknown. However, bulk processing for cell isolation is expected to reflect sample-wide viability, whereas TEM examines only a small fraction of the sample and may be more subject to sampling error. At present, it appears that a number of independent tests should be performed to accurately determine viability in cartilage.

As expected, repeated freeze-thawing of fresh cartilage significantly depleted calcein AM staining and increased EtHd staining in fresh cartilage. In contrast, calcein AM staining persisted, and there was no significant increase in EtHd staining in allograft cartilage after freeze-thawing. This lack of EtHd staining indicated that DNA was absent or reduced in allograft cartilage, a finding that was consistent with the observation that DAPI staining was reduced in allograft cartilage. These results supported the conclusion that calcein AM/EtHd staining discriminated between living and dead chondrocytes in fresh cartilage but not in allograft cartilage.

The original purpose of this study was to evaluate the viability of typical, commercially supplied allografts used by orthopaedic surgeons in the operating room. Thus, we studied allografts as received from a commercial tissue bank without controlling for donor age or storage times. Much of the variability we observed among the 8 different allografts samples was likely due to the effects of these factors.

Our data indicate that alternative measures of viability should be used to evaluate chondrocytes in allograft cartilage. These may include the measures we employed in this study and other methods, including $^{35}SO_4$ incorporation, that are not subject to the high background of false-positives given by the calcein AM/EtHd–staining system. The cause of the calcein AM background problem we observed in allografts is unknown. However, our results suggest that an enzymatic activity that activates calcein AM fluorescence is preserved for some time after chondrocyte death in allografts stored at 4°C. Future work will determine if other viability probes that depend on intracellular enzymatic activity such as nitro blue tetrazolium or MTT are subject to the same problems when applied to allografts.

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