Ankle Bipolar Fresh Osteochondral Allograft Survivorship and Integration: Transplanted Tissue Genetic Typing and Phenotypic Characteristics

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Background: Fresh osteochondral allografts represent a treatment option for early ankle posttraumatic arthritis. Transplanted cartilage survivorship, integration, and colonization by recipient cells have not been fully investigated. The aim of this study was to evaluate the ability of recipient cells to colonize the allograft cartilage and to assess allograft cell phenotype.

Methods: Seventeen ankle allograft samples were studied. Retrieved allograft cartilage DNA from fifteen cases was compared with recipient and donor constitutional DNA by genotyping. In addition, gene expression was evaluated on six allograft cartilage samples by means of real-time reverse transcription-polymerase chain reaction. Histology and immunohistochemistry were performed to support molecular observations.

Results: Of fifteen genotyped allografts, ten completely matched to the host, three matched to the donor, and two showed a mixed profile. Gene expression analysis showed that grafted cartilage expressed cartilage-specific markers.

Conclusions: The rare persistence of donor cells and the prevailing presence of host DNA in retrieved ankle allografts suggest the ingrowth of recipient cells into the allograft cartilage, presumably migrating from the subchondral bone, in accordance with morphological findings. The expression of chondrogenic markers in some of the samples argues for the acquisition of a chondrocyte-like phenotype by these cells.

Clinical Relevance: To our knowledge, this is the first report describing the colonization of ankle allograft cartilage by host cells showing the acquisition of a chondrocyte-like phenotype.

Severe posttraumatic ankle arthritis is a debilitating condition posing a reconstructive challenge for young active patients in whom implants and arthrodesis are not desirable. Fresh osteochondral allograft transplantation has been established as an option to replace the damaged area in ankle osteochondral defects, with surgical indications expanded to include total joint substitutions and reconstructive solutions[1,2]. The rationale for fresh osteochondral allograft is to obtain osseous healing with the host bone incorporating and replacing the donor bone while maintaining the hyaline cartilage architecture[3-5]. Long-term follow-up studies have demonstrated high rates of clinical success[6-8].

Despite the widespread use of fresh osteochondral allograft, little is known about allograft survivorship and integration following transplantation. Several studies have supported long-term in vivo survival of allograft cells, frequently with a high viability rate, but without determination of allograft cell origin[3-5,9,10]. This issue has been addressed by few studies, mostly case reports of knee allograft retrievals, in which it is generally believed that host cells cannot colonize the graft, except for the formation of host fibrocartilage[10]. Knee allograft osteochondral plugs after three years contained only viable donor cells[11] and a mixed cell population composed of both donor and recipient cells in a femoral condyle fresh

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osteochondral allograft was demonstrated after twenty-nine years[12].

Conversely, meniscal, tendon, and ligament allografts show a progressive replacement of donor cells by host ones. In the goat, patellar tendon and anterior cruciate ligament fresh allografts quickly lose donor cells, replaced in a few weeks by host cells[13]. Similar findings were obtained in human meniscal fresh allografts after three months[14].

In this study, we analyzed ankle bipolar fresh osteochondral allografts, in which host articular cartilage was totally replaced during surgery, therefore ensuring that grafted cartilage only belongs to the donor. The main goal of this work was to explore the potential of recipient cells to integrate into allograft cartilage and to identify the cell population mostly involved. The secondary aim was to investigate the influence of allograft repopulation on clinical outcome over time. To this end, after eighteen months, cartilage graft retrievals were genotyped and several markers were evaluated in a subgroup of the same samples. Allograft cartilage colonization by recipient cells with the synthesis of specific cartilage markers by allograft cells was observed, suggesting their differentiation toward a chondrocyte-like phenotype.

Materials and Methods

Case Series

Seventeen patients (thirteen men and four women) with a mean age (and standard deviation) of 35 ± 8.2 years (range, eighteen to forty-five years), affected by posttraumatic Grade-3 ankle arthritis[15], underwent bipolar fresh osteochondral allograft (seven right ankles and ten left ankles). The mean time (and standard deviation) between diagnosis and surgery was 13 ± 7 months. Inclusion criteria were a patient age of fifty-five years or less and unilateral end-stage ankle arthritis. Exclusion criteria were ankle anatomy disruption, osteopenia, osteonecrosis, rheumatoid arthritis, infections, and vascular and neurologic diseases[16]. The donors, identified through the bone bank program for tissue donation after families' donor consent, were eight males and four females, five of which donated both ankles and who had a mean age (and standard deviation) of 31 ± 8.8 years (range, nineteen to forty-four years). Donor tibia and talus size were measured with use of computed tomography (CT) scans and appropriate candidates were selected on the basis of ankle size. No tissue or blood-type matching was performed[17]. Donor ankle harvesting, performed within six hours from asystole, involved the excision of the entire joint with the intact capsule and synovial membrane, which was placed in 1000-mL Dulbecco modified Eagle medium (DMEM) with L-glutamine, $NaHCO_3$, and antibiotics, and then was stored at 4°C. To maintain chondrocyte viability, transplantation was performed within fifteen days at a mean time (and standard deviation) of 14.3 ± 1.6 days[3,18]. In all cases, the fresh osteochondral allograft quality before implant was excellent, showing intact articular surfaces (see Appendix) and viable cells according to the International Cartilage Repair Society (ICRS) cell viability subscore (see Appendix).

All patients gave informed consent and the study was approved by the institutional ethics committee. Patients were evaluated preoperatively, at eighteen months, and at the time of the latest follow-up at a mean time (and standard deviation) of 46 ± 9.6 months by means of radiographs and the American Orthopaedic Foot & Ankle Society (AOFAS) score[19].

Surgical Technique

Surgical treatment consisted of graft preparation and surgery in the recipient. The recovered ankle had all soft tissues and fibula removed. The medial malleolus internal surface was prepared with a probe-jig and a standard pneumatic saw. To obtain a correct fitting between the graft and the recipient, the same jig was used on the medial side of both the graft (during its preparation) and the recipient. With use of this jig, we obtained the appropriate match between the graft and the prepared osseous tibial surface of the recipient. With use of two Kirschner wires as a guide, 1-cm-thick osteochondral surfaces were obtained. The patient, under general or spinal anesthesia, was placed in a supine position with a tourniquet on the proximal thigh. An anterior incision was used and the internal surface of the medial malleolus was prepared with a probe-jig and a standard pneumatic saw. Furthermore, under fluoroscopic control, two Kirschner wires were positioned to help the articular surface cut, performed with a standard pneumatic saw removing both damaged surfaces. Allograft surfaces were positioned in the host ankle and were fixed with twist-off screws (see Appendix). Postoperative radiographs were made.

Sample Collection

Patient peripheral blood and donor residual osteochondral tissue were recovered at surgery. Biopsies of the grafted areas of fifteen cases were obtained during implant removal at a mean time (and standard deviation) of 19 ± 6 months by harvesting osteochondral cylinders perpendicularly from the anterior region of the talus, using an 8-gauge-diameter corer, in a weight-bearing area. The biopsies of two cases (Cases 7 and 10) were obtained at revision surgery for failure at a mean time (and standard deviation) of 24 ± 6 months, and all of the available cartilage tissue was recovered. Cartilage samples were used for genotyping and mRNA expression analyses, and osteochondral samples were used for histology and immunohistochemistry. Because of the limited tissue amount, it was not possible to perform all analyses in all specimens.

Genomic DNA Isolation

Recipient constitutional DNA was obtained from patient peripheral blood, and donor constitutional DNA was obtained from residual donor osteochondral tissue. Allograft DNA was obtained from cylindrical biopsies washed in phosphate buffered saline (PBS) after removal of any subchondral bone by a scalpel. Frozen tissue samples were pulverized with the grinding mill Mikro-Dismembrator S (Sartorius, Firenze, Italy). Total DNA was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Allograft Genotyping

To assess if allograft cartilage contained recipient or donor cells, genotyping by microsatellite analysis (see Appendix) was performed on total DNA from allograft cartilage samples compared with recipient and donor DNA. Allele patterns of fifteen cases were obtained by polymerase chain reaction (PCR) of CD4, von Willebrand factor type A (VWA), feline sarcoma (FES) oncogene, thyroid peroxidase (TPOX), and p53 short tandem repeats, as described[20]. PCR products were electrophoresed and were stained with SYBR Green dye (Roche, Indianapolis, Indiana). Gel images were acquired with Kodak Image Station 4000MM (Kodak, Rochester, New York). When donor material was absent, retrieved allograft matching was attributed by calculating the likelihood ratio[21] (see Appendix).

Allograft Gene Expression Analysis

Six retrieved allograft cartilage samples (30 to 80 mg) obtained from cylindrical biopsies after bone removal were analyzed (samples 3, 8, 10, 15, 16, and 17) and were compared with a series of controls (C) including healthy cartilage (C1 and C2), freshly isolated chondrocytes from healthy cartilage (C3), cultured chondrocytes at the culture passage P1 (C4) and P10 (C5), cultured synovial fibroblasts at the culture passage P3 (C6), freshly isolated osteoblasts (C7), and bone marrow-derived mesenchymal stem cells (C8). Chondrocyte, synovial fibroblast, osteoblast, and bone marrow-derived mesenchymal stem cell isolation and culture were as described[22-24].

Total RNA was extracted with RNA Pure reagent (EuroClone, Milan, Italy) from pulverized cartilage samples and pelletted cells (C3 to C8). After reverse transcription by random priming with use of the SuperScript-VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, California), expression of specific

TABLE I Summary of Allograft Colonization, Histological Appearance, and Clinical Outcome for the Seventeen Analyzed Cases*

	Demographic Characteristics										
	Recipient		Donor			ICRS I Score† *(points)*			AOFAS Score† *(points)*		
Cases	Age	Sex§	Age	Sex§	Genotyping	Recipient	Allograft	Donor	Preoperatively	At the 18-Month Follow-up	At the Latest Follow-up
1	24	M	19	M	A = R	6	8	17	22	90	68
2	31	M	42	M	A = R	—	—	—	31	74	82
3	43	F	26	F	A = R	6	8	—	30	65	79
4	39	M	24	F	A = R	—	9	—	33	90	92
5	24	M	19	F	A = R	6	11	16.5	18	90	90
6	40	M	29	M	A = R	5	14	16.5	36	69	72
7	32	M	40	M	A = R	—	9	—	22	58	Failure
8	45	F	33	F	A = R	3	9	16	36	71	75
9	43	M	40	M	A = R	—	14	—	22	69	48
10	34	M	40	M	A = R	—	5	16	17	70	Failure
11	36	M	44	M	A = D	—	15	—	38	71	76
12	37	M	21	M	A = D	8	8	15	22	65	80
13	38	M	19	F	A = D	5	14	16.5	38	71	74
14	44	M	40	M	A = mixed	—	15	16	23	74	77
15	38	M	29	M	A = mixed	6	16	16.5	28	74	85
16	17	F	33	F	—	6	8	16	22	64	45
17	24	M	29	M	—	—	7	—	28	86	90

*Allograft colonization was evaluated by genotyping comparing retrieved allograft (A) allele patterns to recipient (R) and donor (D) allele patterns. †Histological appearance was evaluated by the ICRS I score on recipient and donor osteocartilaginous tissues at time of surgery and on retrieved allograft at time of implant removal (eighteen months) or at revision for failure (Cases 7 and 10). ‡Clinical outcome was evaluated by the AOFAS score preoperatively, at the eighteen-month follow-up, and at the time of the latest follow-up (forty-six months). §M = male and F = female.

markers was evaluated by real-time PCR (see Appendix) in a LightCycler Instrument (Roche, Mannheim, Germany) with SYBR Premix Ex-Taq (Takara, Osaka, Japan). The amplification protocol was 95°C at ten seconds and forty-five cycles (95°C at five seconds and 60°C at twenty seconds). Cycle threshold (C_t) values were determined for each sample and mRNA levels were quantified compared with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene following the formula $(1 + E)^{\Delta C_t}$, where E is the reaction efficiency (approximated to 1 because for each transcript E was >90%) and ΔC_t is the difference between the GAPDH and the specific C_t.

Histology and Immunohistochemistry

Recipient, retrieved allograft, and donor specimens were fixed in 10% buffered formalin, were washed and were decalcified in 4% hydrochloric acid and 5% formic acid at room temperature, were dehydrated in alcohol, and were embedded in paraffin. Serial sections were stained with hematoxylin and eosin and 0.1% Safranin-O/0.02% Fast Green (Sigma-Aldrich, Seelze, Germany). ICRS I score[25] was applied to quantitatively evaluate cartilage repair.

Collagen type-II, CD73, CD90, matrix metallopeptidase 13 (MMP-13), caspase-3, and tartrate-resistant acid phosphatase (TRAP) markers were evaluated by immunohistochemistry. Specific unmasking was carried out depending on the antigen[17,26]. Samples were incubated with monoclonal antibodies against human collagen type II (2.5 μg/mL, Chemicon, Temecula, California), CD73 (5 μg/mL, Serotec, Oxford, United Kingdom), CD90 (5 μg/mL, Serotec), MMP-13 (5 μg/mL, R&D Systems, Minneapolis, Minnesota), caspase-3 (5 μg/mL, R&D Systems), and TRAP (5 μg/mL, Novocastra, Newcastle, United Kingdom). Sections were then treated with biotinylated secondary antibodies and Alkaline Phosphatase-Labeled Streptavidin (BioGenex, Fremont, California). Reactions were developed with use of Fast Red substrate (BioGenex). Slides were counterstained with hematoxylin (Sigma-Aldrich). Positive and negative controls were run together with test samples. Images were captured with an Eclipse 90i microscope (Nikon, Melville, New York) and were examined by two independent observers. Semiquantitative analysis evaluated the percentage of positive cells (CD73, CD90, MMP-13, caspase-3) or positive area (collagen type II) and the number of multinucleated cells on whole sections.

Statistical Analysis

Immunohistochemical quantification, AOFAS scores, and ICRS I scores were expressed as the mean and the standard deviation. Spearman rank correlation examined the relationship among ICRS I score, AOFAS score, and age. The Mann-Whitney test evaluated by exact methods for small samples was used to analyze the influence of donor or recipient sex on AOFAS and ICRS I scores. The Kruskal-Wallis test for multiple comparisons with the post hoc Dunn test was used for immunohistochemical data. The non-parametric Jonckheere-Terpstra test evaluated the relationship between scores and genotyping.

For all tests, $p < 0.05$ was considered significant.

Relative mRNA expression was expressed as the mean and the 95% confidence intervals calculated with the resampling bootstrap method for small samples.

The hierarchical cluster analysis of gene expression profiles was performed with use of cosine similarity as the matrix of distances (see Appendix).

Source of Funding

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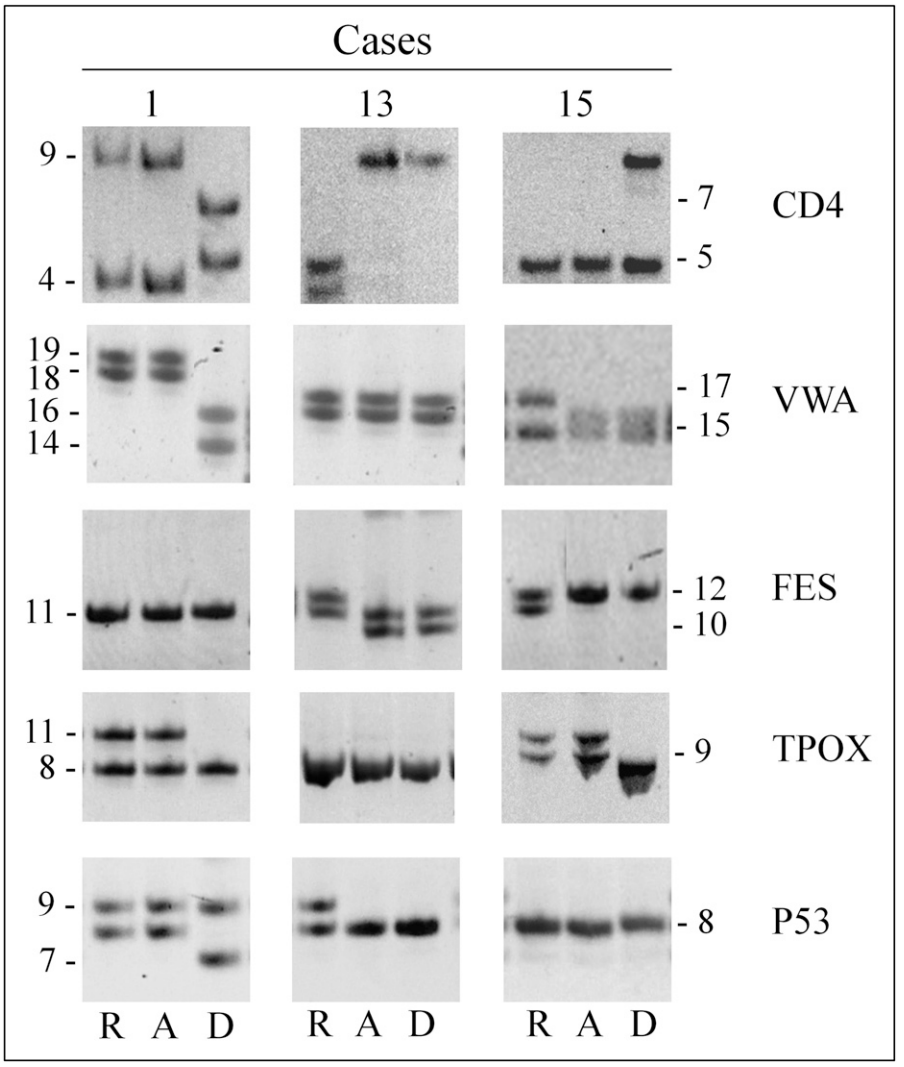

Fig. 1

Gel photographs showing genotyping of three retrieved allograft representative cases (for overall results, see Appendix). Total DNA from retrieved allograft cartilage (A) was compared with recipient (R) and donor (D) constitutional DNA by PCR analysis at five short tandem repeat sequences (CD4, VWA, FES, TPOX, and P53). PCR products were run on acrylamide gels. Case 1: complete matching between allograft and recipient DNA (A = R); Case 13: complete matching between allograft and donor DNA (A = D); and Case 15: allograft partial matching with both recipient and donor DNA, indicating a mixed population of recipient and donor cells in the allograft (A = mixed). Allele length was estimated by a side-by-side comparison with allelic ladders made up from a mixture of known alleles and is expressed as the number of repeats[27] as indicated beside gel images.

Results

Clinical Outcome

No intraoperative complications were observed. Postoperatively, one infection occurred, requiring a surgical irrigation at nine months, but was completely healed at the twelve-month follow-up. Two failures (Cases 7 and 10) occurred: Case 7 at twenty months and Case 10 at twenty-eight months; the failure of Case 7 was due to fibular nonunion and allograft collapse, but no clear reason for the failure of Case 10 was evident. The AOFAS score improved from 27.4 ± 7.0 preoperatively to 73.6 ± 9.7 at eighteen months (seventeen cases; $p < 0.05$). At the time of the latest follow-up (46 ± 9.6 months; fifteen cases), the AOFAS score was 75.5 ± 13.7 points ($p < 0.05$). No relationship among the AOFAS score, patient or donor age, and sex was found.

Allograft Genotyping

Allele patterns at five short tandem repeats[27] for the fifteen retrieved allograft cartilage samples (A) genotyped, compared with constitutional DNA allele patterns from recipient (R) and donor (D) samples, are reported in the Appendix. In Figure 1, an example of each possible matching (A = R, A = D, A = mixed) is shown.

Ten retrieved allografts (Cases 1 to 10), including the two failures, showed an allograft-recipient matched profile (A = R), indicating the exclusive presence of recipient cells in allograft cartilage. In Cases 2 and 4, missing donor DNA, the identity between allograft and recipient was supported by the perfect matching at all short tandem repeats, with a likelihood ratio (hypothesis of identity versus no identity) of 8.8×10^5 for Case 2 and 3.9×10^7 for Case 4, which means that it is 8.8×10^5 and 3.9×10^7 more likely that DNA belongs to the recipient than to some unknown person from the population.

Only three cases (Cases 11, 12, and 13) showed an allograft-donor matched profile (A = D), indicating the exclusive presence of donor cells in allograft cartilage. Case 11 was typed even without donor material, because the non-matching of recipient and allograft DNA at all short tandem repeats excludes their identity, confirming allograft-donor matching.

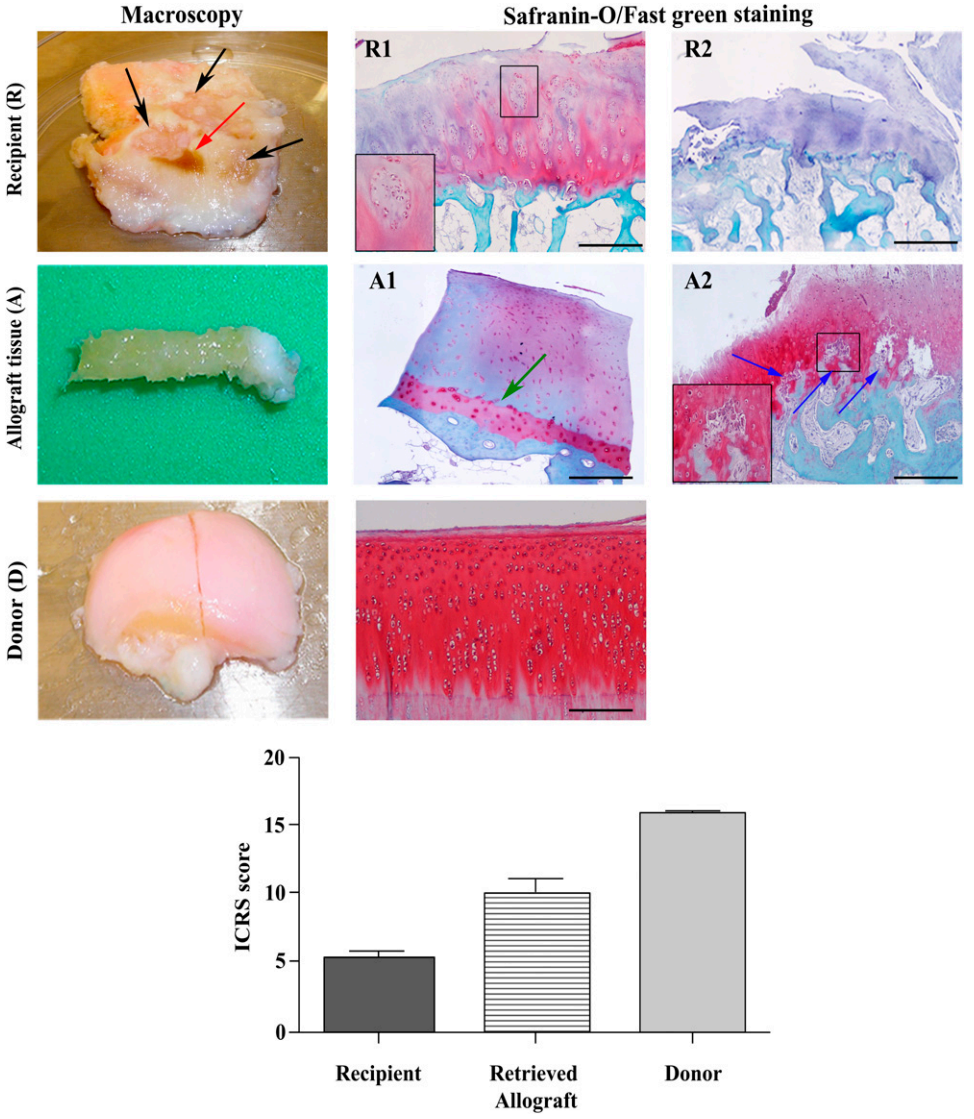

Fig. 2

Macroscopic photographs and histological images showing analysis of representative recipient (R), retrieved allograft (A), and donor (D) samples (500 μm; 50-μm insets). The top row shows that, for the recipient, fissuring (black arrows) and eburnation (red arrow) processes were noticed in macroscopic analysis. Several histological aspects in cartilage tissue, extending from the presence of cell clusters (white arrows) (R1) to cartilage delamination and depletion of proteoglycans (R2), were noticed. The middle row shows that, for the retrieved allograft, a good cell distribution with tidemark duplication crossed by mononucleated (green arrow) (A1) and multinucleated (blue arrows) cells (A2) were observed. The bottom row shows that, for the donor, a glossy cartilage surface was noticed in donor biopsies by macroscopic analysis. A good cartilaginous tissue with high proteoglycan component was observed by histology. A bar graph showing the ICRS I scores for the analyzed samples, which are represented as the mean and the standard deviation (the minimum of 0 points indicated poor cartilage repair and the maximum of 18 points indicated very good repair).

Two cases (Cases 14 and 15) showed a mixed profile, with both donor and recipient DNA present in the allograft cartilage (A = mixed). In Case 14, the allograft showed a tri-allelic profile at CD4 and VWA loci, indicating the presence of more than one individual genome in the cartilage sample, namely, recipient and donor DNA; in Case 15, two short tandem repeats matched with recipient DNA (CD4-TPOX) and two (VWA-FES) matched with donor DNA, indicating once again the presence of both donor and recipient DNA.

No correlation was found between genotyping and patient or donor age or the AOFAS score.

Allograft Cartilage Cell Characterization

Allograft cartilage RNA from six samples (Cases 3, 8, 10, 15, 16, and 17) and eight controls (C1 to C8) was analyzed for the expression of cartilage and bone markers. C1, C2, and C3 were chosen to compare allograft cells with differentiated chondrocytes, and C4 and C5 were chosen to compare allograft cells with dedifferentiated chondrocytes, as the monolayer culture induces a loss of chondrocyte phenotype[28,29]. C6, C7, and C8 were also used for comparison.

Results are expressed as RNA copy number/100,000 GAPDH copies. Transcript levels were extremely heterogeneous among samples, suggesting a different allograft behavior in the different cases (see Appendix). In particular, collagen type-II expression was detected in four samples (Cases 8, 15, 16, and 17), two of which (Cases 15 and 16) showed high mRNA levels, comparable with differentiated chondrocytes (C1 to C3). None of the other controls expressed detectable levels of collagen type II. Collagen type-IX expression was similar to C3, while aggrecan and sex-determining region Y-box 9 (SOX9) appeared expressed, even if at lower levels compared with cartilage controls. Allograft samples strongly expressed collagen type I, suggesting some level of fibrosis; they expressed cathepsin B as cartilage controls and MMP-13, indicating some tissue remodeling.

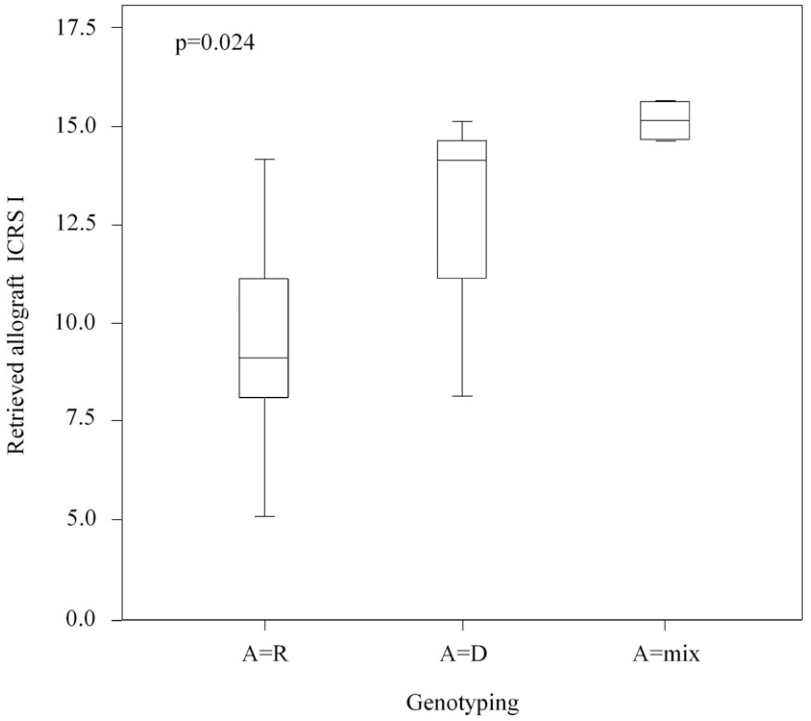

Fig. 3

Box plot representation of retrieved allograft ICRS I scores related to genotyping (A = R, A = D, or A = mixed). The black horizontal lines represent the medians and the box plots represent the 25th to 75th percentile range of the data. The minimum and maximum values are also indicated. A positive trend was observed with use of the Jonckheere-Terpstra test (p = 0.024), with the samples with a mixed genotype showing the highest score (corresponding to the best cartilage repair).

Osteoblast markers were expressed with some variability as in controls.

A hierarchical cluster analysis on the six samples combined with the controls was performed (the thirteen analyzed genes being the clustering parameters). Three main clusters (A, B, C) were evidenced according to how closely correlated samples and controls were (see Appendix). In cluster A, four (samples 3, 8, 16, and 17) of six allografts are grouped with mesenchymal stem cells (C8), while the remaining allografts (Cases 10 and 15) fall into cluster C, together with differentiated chondrocytes (C1 to C3). The results of cluster analysis agree with the migration of host precursor cells from the

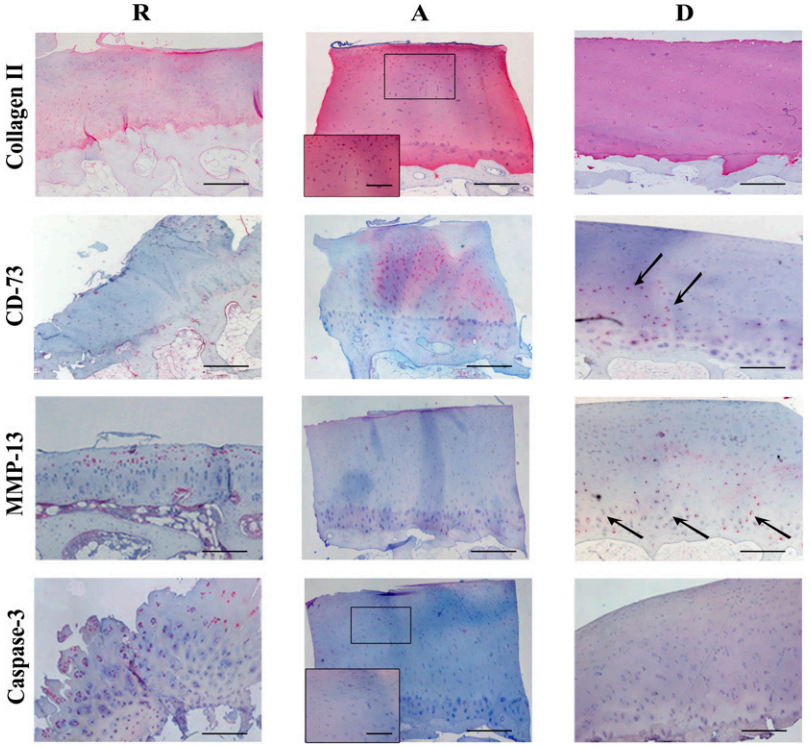

Fig. 4

Photographic panel showing immunohistochemical analyses for collagen type-II, CD73, MMP-13, and caspase-3 markers from representative recipient (R), retrieved allograft (A), and donor (D) specimens (500 μm; 50-μm insets). Black arrows in the CD73 and MMP-13 indicate positive cells within the cartilaginous extracellular matrix.

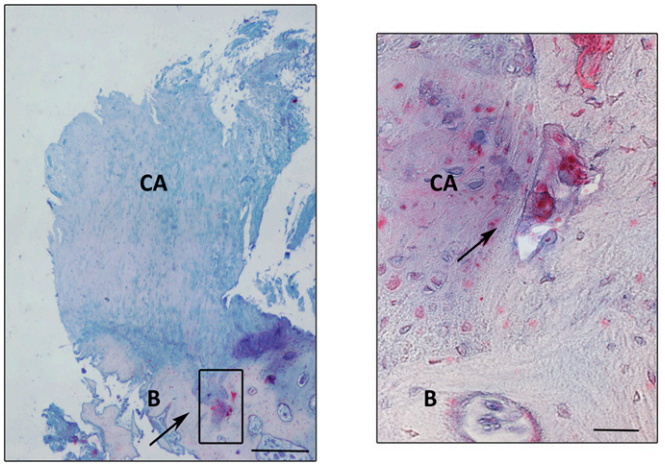

Fig. 5

TRAP immunohistochemical analysis of a representative retrieved allograft specimen. A positive staining was observed in the multinucleated osteoclast cells (black arrows) from subchondral bone (B), which are invading cartilage tissue (CA). The box identifies the area magnified (500 μm; 50-μm insets) on the right, the area located between cartilage (CA) and bone (B) compartments, containing two multinucleated cells positive to TRAP.

subchondral bone and their partial or progressive differentiation in chondrocyte-like cells. In cluster B, dedifferentiated chondrocytes (C4 and C5), osteoblasts (C7), and synovial fibroblasts (C6) are grouped.

Histology and Immunohistochemistry

Recipient samples displayed macroscopically several fibrillation areas with fissures and eburnations. Histology showed several levels of severity, from a cartilaginous matrix with abundant cell clusters to a complete matrix loss, with a low ICRS I score of 6 ± 1.3 points (Fig. 2 and Appendix).

Different degrees of tissue repair were observed in retrieved allograft biopsies after eighteen months: macroscopically, a cartilage surface with fibrillation areas was reported, while histology showed cartilage tissue with good cellular viability and arrangement, moderate proteoglycan content, and various non-continuous areas in the tidemark crossed by mononucleated and/or multinucleated cells from subchondral bone in all of the specimens, reporting a score of 11 ± 3 points, with an area infiltrated by multinucleated cells of 15% ± 7% (Fig. 2). The two failures (Cases 7 and 10) showed different degenerative aspects including loss of cartilage matrix, cell apoptosis, low proteoglycan content, and altered cartilage mineralization, recording a low score of 8.5 ± 0.5 points (data not shown). The retrieved allograft ICRS I score was higher in cases with the mixed matched genotype (15.5 ± 0.7 points) compared with those with the donor-matched genotype (12.3 ± 3.8 points) and the recipient-matched genotype (9.7 ± 2.9 points) ($p = 0.024$) (Fig. 3).

Donor specimens showed a glossy cartilage surface. Histology showed a well-organized cartilaginous tissue with a smooth surface, good proteoglycan content, and regular cellular arrangement (Fig. 2). Cell viability, as assessed by the ICRS I viability subscore, displayed the maximum value (see Appendix). A positive correlation was found between the donor ICRS I score (15 ± 5.2 points) and the eighteen-month AOFAS score (73.6 ± 9.7 points), suggesting that donor cartilage quality influences the outcome (rho = 0.636, $p = 0.048$).

In Table I, genotyping data, donor and recipient age and sex, and ICRS I and AOFAS scores are summarized.

High extracellular collagen type-II expression was noticed in retrieved allograft (90% ± 4%) and donor (91% ± 3%) specimens compared with recipients (30% ± 5%) ($p < 0.001$ for both comparisons). A cellular positivity for the CD73 marker was detected in all specimens (35% ± 2%). Similar findings were observed for CD90 (data not shown). A moderate cellular positivity for MMP-13, involved in cartilage destruction, was detected in recipients (60% ± 3%) with respect to retrieved allograft (25% ± 3%; $p < 0.01$) and donor (15% ± 4%; $p < 0.001$) specimens (retrieved allograft versus donor: $p < 0.05$). Apoptosis-related caspase-3 cellular expression was different in recipient (63% ± 4%), retrieved allograft (17% ± 3%), and donor (5% ± 2%) samples ($p < 0.01$ for all comparisons) (Fig. 4).

TRAP staining on retrieved allograft samples showed a marked positivity (98% ± 2%) only in multinucleated cells invading the cartilage matrix near the tidemark (Fig. 5).

Discussion

Fresh osteochondral allograft is a biological option for ankle osteochondral defect treatment, but it is a technically demanding procedure with reported complications and failures[30,31]. Fresh osteochondral allograft biology and integration are therefore critical topics to better understand allograft behavior. In particular, chondrocyte survivorship seems a key factor in allograft maintenance[3-5,9,10,32]. Another important question poorly investigated[11-14] is whether original donor chondrocytes survive or are replaced by host cells.

We analyzed seventeen ankle bipolar fresh osteochondral allografts after eighteen months, describing the origin of allograft cells and their phenotype. Out of fifteen allografts, ten completely matched to the host, three matched to the donor, and two showed a mixed profile. The prevalent host matching indicates that ankle fresh osteochondral allografts frequently lose donor cells, partially replaced in a short time by host cells. This last finding would suggest a role of the graft as a scaffold for recipient cell colonization, raising the question whether viability at the time of transplantation is really necessary. Data on large frozen allografts suggest an incomplete integration, even if no data were provided for grafts similar to those described here. We believe that donor cell viability has an important role both because all viable cells can contribute to allograft matrix maintenance and because host cells can initially colonize only the deeper cartilage zone. In agreement with this, allografts with a mixed genotype showed the best ICRS I score (Fig. 3). However, genotype was not correlated with the AOFAS score; therefore, genotyping at eighteen months does not have a predictive value on clinical outcome. Clinical outcome was good to excellent in fifteen of seventeen patients as indicated by the AOFAS scores. Histological-immunohistochemical evaluations gave evidence of a well-organized cartilage tissue with high collagen

type-II content and various processes of tissue remodeling, as indicated by MMP-13 and TRAP expression, in accordance with matrix remodeling required for recipient cell invasion of the graft.

Some allograft samples expressed collagen type-II mRNA as normal cartilage. This seems particularly relevant as this marker is peculiar to differentiated chondrocytes[28,29]. We genotyped only two collagen type-II positive samples, because of the low amount of cartilage tissue, finding recipient DNA in one specimen and a mixed donor and recipient population in the other. As the surgical procedure implies a total replacement of host cartilage by donor cartilage, in the sample matching with host DNA, collagen type-II expression may only arise from host cells migrated to the allograft cartilage that probably acquired a partial chondrocyte-like phenotype. Notably, all collagen type-II positive allografts had a good clinical outcome.

Cluster analysis, further characterizing allograft cell phenotype, indicated that allograft samples show expression profiles similar to mesenchymal stem cells or to chondrocytes, supporting allograft colonization by precursor cells from subchondral bone, as also validated by the presence of multinucleated TRAP-positive osteoclasts at the cartilage-bone boundary in all of the specimens, creating interruptions in the tidemark that allow cell migration toward the cartilage (Fig. 5). No tissue remodeling was observed at the superficial cartilage layer; thus, it would seem unlikely that recipient colonizing cells can come from synovium, which have been described as a possible source of mesenchymal stem cells[33,34]. Cells colonizing the allograft are probably stem cells, in accordance with allograft positivity for mesenchymal markers (Fig. 4).

Moreover, the observation that some allografts share the same cluster with chondrocytes confirms that invading cells, once embedded in a cartilage matrix, start to express chondrocyte-specific genes. This tissue remodeling is probably progressive and first limited to the deeper cartilage zone, in accordance with the finding of some samples retaining donor DNA profile or a mixed profile. The acquisition of a chondrocyte-like phenotype probably occurs only in some cells and at a different level in the different samples, as indicated by the heterogeneous expression profiles observed. Accordingly, the possibility that invading recipient mesenchymal stem cells can form fibrocartilage should not be excluded.

Further studies at longer follow-up could also clarify if samples retaining donor cells would progressively acquire a host-matched genotype, as observed in meniscal allografts[14]. This would explain how a non-host-matched genotype does not imply the worst clinical outcome. Actually, the time of remodeling is presumably variable among individuals. The two failures cannot be attributed to allograft repopulation as they showed the same host-matched genotype as the majority of the samples.

In conclusion, in our case series, ankle fresh osteochondral allografts appear to behave more similarly to meniscal, ligament, and tendon allografts, where donor cells are rapidly replaced by host cells[13,14,35], than to knee fresh osteochondral allografts[11,12], where host cells seem unable to colonize donor cartilage.

To our knowledge, this is the first study on the genetic characterization of ankle bipolar fresh osteochondral allografts highlighting allograft cartilage colonization by recipient cells through progressive remodeling at the cartilage-bone border, where mesenchymal stem cells integrate within the proximal part of the cartilage and start to acquire a chondrocyte-like phenotype.

Appendix

eA Figures showing the surgical field of representative cases and a dendrogram obtained by hierarchical clustering of six allograft samples and eight defined control cell types, tables showing the summary of primer sequences and Amplicon size for real-time reverse transcription PCR (RT-PCR) analysis, allograft genotyping, relative mRNA expression of different markers in cartilage allograft samples and in controls, and ICRS-I subscores for the seventeen analyzed cases, and text explaining the surgical technique, genotyping, likelihood ratio, and cluster analysis used in this study are available with the online version of this article as a data supplement at jbjs.org. ■

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