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Original article

Histological examination of frozen autograft treated by liquid nitrogen removed after implantation

Yoshikazu Tanzawa[1], Hiroyuki Tsuchiya[1], Toshiharu Shirai[1], Katsuhiro Hayashi[1], Zen Yo[2], and Katsuro Tomita[1]

[1] Department of Orthopaedic Surgery, Kanazawa University School of Medicine, 13-1 Takaramachi, Kanazawa 920-8641, Japan
[2] Department of Pathology, Kanazawa University Hospital, Kanazawa, Japan

Abstract
Background. Several oncological sterilization methods involving autoclaving, irradiation, or pasteurization have been developed for limb reconstruction of large bone defects following tumor excision. Studies involving histological examinations of these autografts have all found that osteogenesis occurs slowly. We have used frozen autografts treated by liquid nitrogen for limb reconstruction and have achieved excellent results for bone union. To determine if frozen autografts exhibit early bone remodeling, we investigated the repair processes of the frozen bones.
Methods. We analyzed frozen autografts treated by liquid nitrogen, retrieved at a mean of 19.1 months (2–75 months) after implantation because of complications or local tumor recurrence. The specimens were obtained from six patients with a mean age of 36.2 years (8–68 years). The six grafts comprised three osteoarticular grafts, two intercalary grafts, and one joint graft. We histologically reviewed the autograft-containing sections for tumor cell necrosis, evidence of cortical repair, the cortical junction, and joint cartilage.
Results. Tumor cells were completely eradicated from the frozen bone in all cases. In a specimen retrieved 5 months after implantation, a small area of the bone showed active osteocytes and osteoblasts. In three cases retrieved more than 1 year after implantation, osteocytes and osteoblasts were observed in broad portions of the frozen bones, indicating the onset of osteogenesis in the frozen bone at an early stage. The cortical host–graft junction showed incorporation along with continuity of bone trabeculae. In addition, we were able to find normal chondrocytes on the articular surface.
Conclusions. The frozen bone specimens in this study thus showed evidence of newly formed bone and earlier osteogenesis than has been previously reported. Our results suggest that frozen autografts may be considered one of the most useful recycled materials for biological reconstruction.

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Introduction

Because of recent advances in diagnostic imaging, neoadjuvant chemotherapy, and operative technique, limb salvage surgery is now a primary treatment option for malignant bone and soft tissue tumors of the extremities to improve quality of life. Various methods of reconstruction (e.g., massive prostheses, allografts, combinations of allograft and prosthesis, reconstruction with distraction osteogenesis using an external fixator[1]) are presently in use for limb reconstruction of large bone defects following tumor excision.

As an alternative to allografts, several oncological sterilization methods involving autoclaving,[2] irradiation,[3] and pasteurization[4] have been developed for reusing the resected bone as autografts during reconstruction. These methods, however, require special equipment or strict thermal control; especially in the case of autoclaving, anything less than these conditions may cause weakness of the treated bone and loss of bone inductivity.[5]

We have developed a new method of hypothermically sterilizing autografts with liquid nitrogen based on in vitro and in vivo experiments.[6] We have used frozen autografts treated by liquid nitrogen in patients since 1999. Advantages of biological reconstruction with frozen autografts include the following: simplicity, osteoinduction, osteoconduction, short treatment time, preservation of cartilage matrix, perfect fit, sufficient biomechanical strength, easy attachment of tendons and ligaments, desirable bone stock, and cryoimmunological activity.[7,8]

Studies involving histological examinations of oncologically sterilized autografts including autoclaved bone, pasteurized bone, and irradiated bone have all found that osteogenesis occurs slowly.[9–14] We previously published a case report describing the histological examination of a frozen autograft treated by liquid nitrogen that was removed 6 years after implantation. Its histology

Table 1. Details of the six patients

Case no.	Age/sex	Histology	Location	Duration in situ (months)	Reason for retrieval	Type of graft
1	68/F	Chondrosarcoma	Pelvis	2	Infection	Osteoarticular
2	56/M	Chondrosarcoma	Pelvis	5	Infection	Osteoarticular
3	40/M	MFH	Distal femur/proximal tibia	5	Infection	Joint
4	18/F	Osteosarcoma	Tibial shaft	22	Soft part recurrence	Intercalary
5	8/F	Osteosarcoma	Distal femur	17	Soft part recurrence	Intercalary
6	19/F	Osteosarcoma	Distal femur	75	Collapse of subchondral bone	Osteoarticular

MFH, malignant fibrous histiocytome

showed that bone remodeling and revascularization had occurred within the frozen bone.[15] However, to date, no study examining the regeneration of frozen bone has tracked the time course of histological changes within the frozen bone during the period of implantation.

In this study, we investigated the histological features of frozen autografts that had to be removed from six patients at different time points after implantation. We wanted to provide further insight into the bone incorporation and remodeling processes operating in the autografts. We also investigated whether chondrocytes can survive the freezing process used in the preparation of our autografts. We analyzed histologically the joint cartilage of these frozen autografts to elucidate the cartilage changes that liquid nitrogen treatment evokes.

Patients and methods

A total of 66 frozen autografts have been used in limb reconstructions at our facility from 1999 through 2007. Six patients subsequently had to have their autografts removed because of complications or local tumor recurrence (Table 1). Among these patients, the initial reconstruction surgery utilized plates and screws for the two patients with pelvic tumors and utilized intramedullary nails for the other four patients. In case 5, grafting was combined with bone transport using an Ilizarov external fixator to reconstruct a diaphyseal bone defect after implantation.[16,17] The transported segment was moved distally to the frozen bone autograft, with bone transport completed 5 months after implantation. The four nonchondrosarcoma patients received preoperative and/or postoperative chemotherapy.[18]

Two patients with chondrosarcoma of the pelvis (cases 1, 2) and one patient with malignant fibrous histiocytoma of the knee joint (case 3) developed deep infections, leading to retrieval of their autografts a short time (2–5 months) after initial surgery. Two patients with osteosarcoma (cases 4, 5) developed recurrences outside the area of the frozen bone, which were treated by tumor excision and amputation, respectively. In case 6, collapse of the implanted femur caused severe instability of the knee joint that was treated by excision and total knee replacement.

The mean age of the patients at the time of retrieval was 36.2 years (range 8–68 years). The mean duration in situ for all six specimens was 19.1 months (range 2–75 months). The six grafts comprised three osteoarticular grafts, two intercalary grafts, and one joint graft. Clinical details of the six patients are presented in Table 1.

The extirpated specimens were embedded in their entirety in paraffin and sectioned (5 μm thickness). The sections were stained with hematoxylin and eosin. We histologically reviewed the autograft-containing sections for evidence of cortical repair (vascular and bone regeneration) in all six cases. We also examined the cortical junction and joint cartilage in two and four cases, respectively.

Results

Tumor necrosis

No viable tumor cells were detected in any of the sections in any of the cases, and clusters of dead tumor cells were identified within the frozen bones. In one patient with chondrosarcoma of the pelvis (case 1), extensive regions of tumor necrosis containing cells that had lost their nuclei were observed (Fig. 1). These findings indicate that the freezing method can completely devitalize tumor cells.

Cortical repair

In cases 1 and 2, the grafted bone was composed of trabeculae of dead bone. Indicators of tumor necrosis included trabeculae containing empty lacunae and an absence of microvessels in the cortex. The area adjacent to articular cartilage revealed necrotic changes suggestive of abscess formation. Invasion of neutrophils and histiocytes and formation of fibrogranulation tissue were evident in the medullary area. No remodeling activity was observed after such a short duration (Fig. 2a).

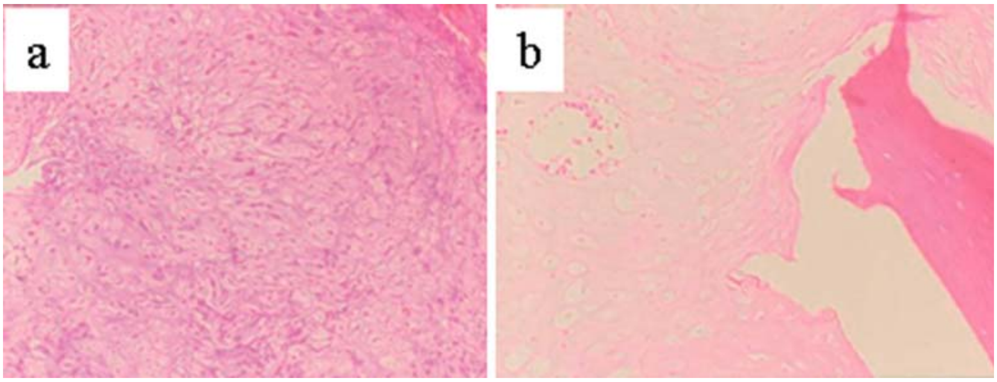

Fig. 1. Histology of tumor necrosis. **a** This specimen from case 1 shows a grade 1 chondrosarcoma before surgery. **b** All tumor cells have disappeared after liquid nitrogen treatment. **a**, **b** ×100

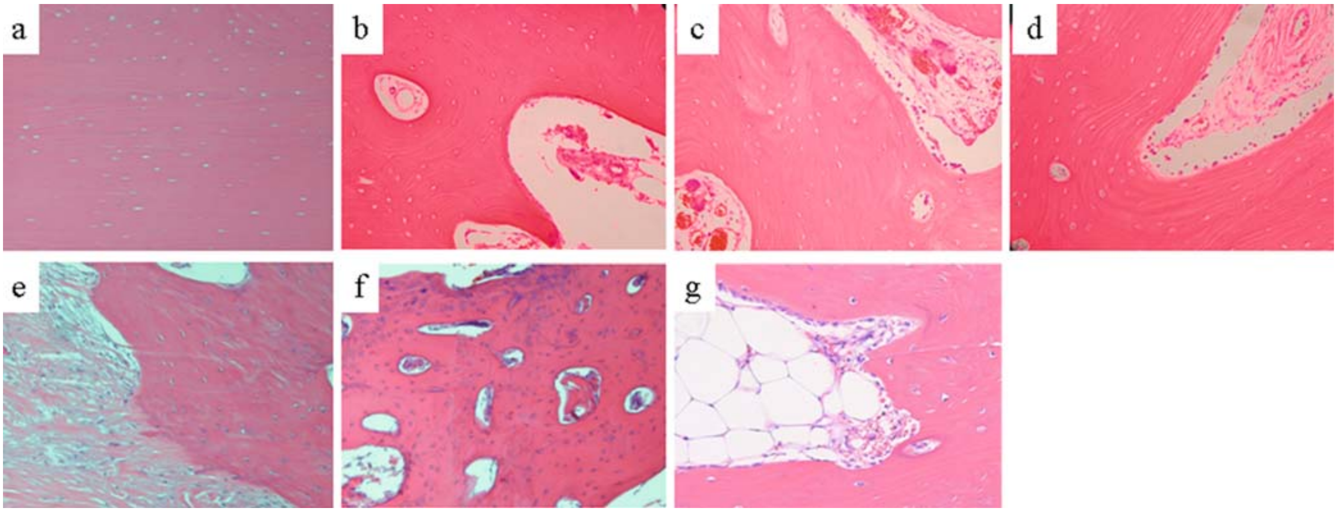

Fig. 2. Histology of cortical repair. **a** Case 2. **b** Case 3. **c, d** Case 5. **e, f** Case 6. **g** Case 6. **a–f** ×100; **g** ×200

In case 3, an autograft retrieved 5 months after implantation, a small area of the grafted bone that was in contact with the host bone showed vessels, active osteocytes and osteoblasts, along with some remodeling adjacent to the host–graft junction (Fig. 2b). However, the other portions of the graft demonstrated considerably lower tissue viability, ranging from totally nonviable to partially viable.

On the other hand, among the three cases retrieved more than 1 year postoperatively (cases 4–6), osteocytes and invasion of vessels were observed in broad portions of the grafted bones (Fig. 2c), and osteoblasts in the metaphysis were also present, which could represent evident osteogenesis (Fig. 2d). In case 6, most cortical areas in the diaphysis contained osteocytes and microvessels, and fibrovascular tissue was present between the cortex and medullary space. The fibrovascular tissue showed an abundance of vessels, and osteocytes appeared more numerous in the cortex next to this area (Fig. 2e). Surrounding osteocytes embedded in trabeculae of the subchondral bone in the epiphysis were also evident. In addition, many microvessels had invaded the bone, and the marrow was filled with fibrovascular tissue (Fig. 2f). A portion of the metaphysis was composed of areas of bone remodeling containing osteoclasts and osteoblasts (Fig. 2g).

Cortical junction

The cortical junction was examined in two specimens (cases 3, 5). In case 3, bone union on the distal side was confirmed 5 months after implantation by radiological findings showing that the radiolucent line had become unclear (Fig. 3a). Microscopically, the gap between the frozen bone and host bone contained a fibrous membrane with abundant, fibrovascular tissue (Fig. 3b). The cortical junction showed no continuity of the lamellar structure. In case 5, radiographs showed an evident cortical gap on the lateral side of the cortical junction, indicating that bone union has not yet occurred at the docking site 12 months after completion of bone transport (Fig. 3c). The retrieved femur was sliced sagittally, and whole sections were stained with hematoxylin and eosin for evaluation (Fig. 3d). Microscopy revealed

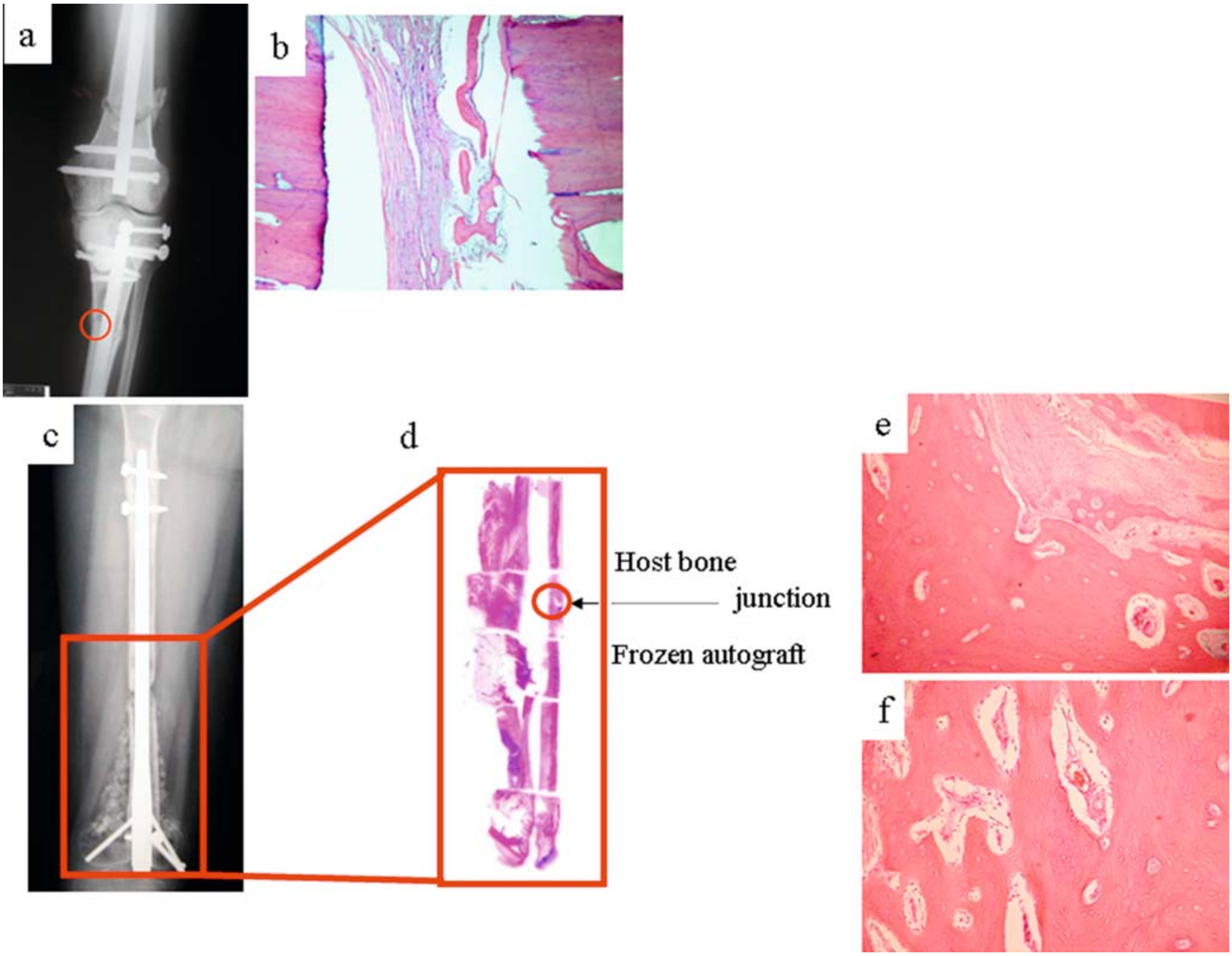

Fig. 3. Radiographs and histology of the host–frozen bone junction. Case 3: **a** Roentgenogram. **b** Microscopic examination of the region in exhibited in **a** (×40). Case 5: **c** Roentgenogram. **d** Retrieved sections. **e** Microscopic examination of the docking site (×40). **f** Medullary part of the cortical junction (×100)

the presence of a fibrous mem brane in the lateral part of the cortical junction (Fig. 3e). The medullary part of the cortical junction, however, showed incorporation, as evidenced by continuity of bone trabeculae and the presence of many osteocytes and vessels (Fig. 3f).

Joint cartilage

Four cartilage specimens were examined (cases 1, 2, 3, 6). The three specimens retrieved within 5 months after implantation (cases 1, 2, 3) showed fibrillation of the superficial surface and irregularities in the thickness of the frozen articular cartilage. The specimens were totally devoid of chondrocytes in the lacunae of the persisting articular cartilage (Fig. 4a). The fourth specimen (case 6) had severe degenerative changes, but some normal chondrocytes were observed in scattered areas of the articular surface (Fig. 4b). Nuclei of these chondrocytes showed hematoxylin staining and were arranged irregularly in the cartilage matrix. The joint surface exhibited irregularities, including fibrillation of the superficial surface.

Bone scintigraphy

In case 5, bone ^{99m}Tc-scintigraphy 5 months after implantation revealed a rim of increased uptake on the surface of the cortex (Fig. 5a) Uptake by the remainder of the frozen bone was comparable to host bone uptake, except for an area of low uptake on the medial part of the metaphysis (Fig. 5b).

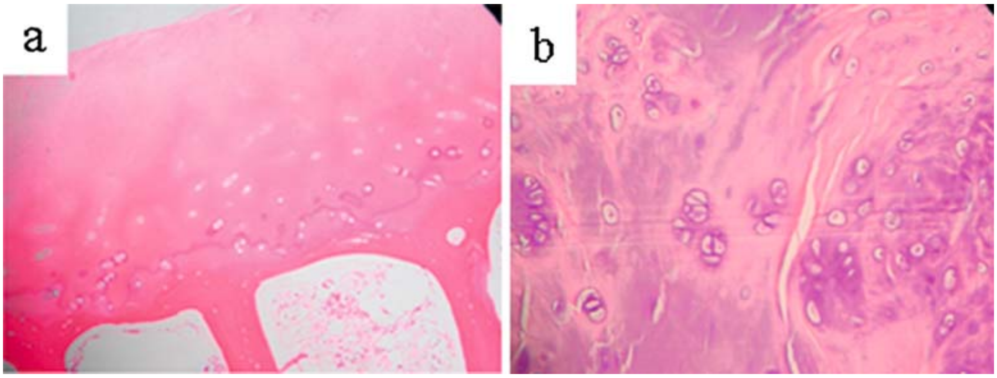

Fig. 4. Histology of frozen articular cartilage. **a** Case 3 (×100). **b** Case 6 (×200)

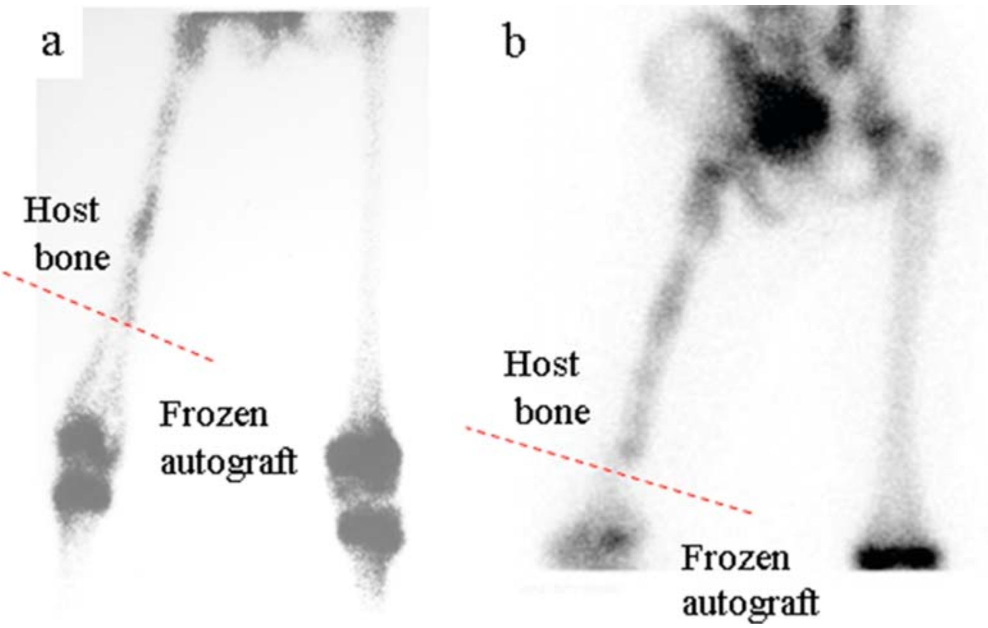

Fig. 5. Bone ^{99m}Tc-scintigraphy (case 5). **a** At 5 months after implantation. **b** At 15 months after implantation

Discussion

We previously have reported that reconstruction using tumor-bearing autografts treated by liquid nitrogen is an effective method for biological reconstruction.[7] We also published a case study of a frozen autograft removed 6 years after implantation that demonstrated that the frozen bone had been replaced with normal bone.[15] That case report, however, could not elaborate on the repair process of frozen bone, and to date no published study has provided such a description. In this study, we evaluated histological changes in frozen autografts at six time points following implantation.

Osteogenesis in the frozen bones of our patients developed earlier than has been reported in other studies involving nonfrozen bones[9–14] (Table 2). Griffiths et al.[19] reported that new bone formation in allografts begins at the junction between the host and graft bone and creeps toward the subchondral bone from the junction. One of our cases showed the presence of osteocytes and microvessels at the junction between host and autograft 5 months after implantation. These observations demonstrate that osteogenesis in the frozen bone begins at the junction, just as it does in allografts; and that osteogenesis within autografts begins within the first 5 months following implantation. Of all the published studies containing the histological analyses of retrieved human pasteurized bones, only one study has described early osteogenesis. In that study, Watanabe et al.[20] reported that histological examination of a pasteurized bone removed 9 months after implantation revealed evidence of osteocytes and microvascular migration into the pasteurized cortical bone. On the other hand, Sakayama et al.[21] published a study that described the pathological findings of pasteurized bone retrieved 5 months after implantation. In that study, the pasteurized bone was necrotic; vascularization and fibroblastic proliferation were observed in part of the marrow, but no new bone formation was seen. In contrast, we have observed that bone regeneration in

Table 2. Histological examination reports of each devitalized bone

Study subject and authors	Duration in situ	Histological features	
		Cortex	Cartilage
Allograft	5 years	Internal repair: 15%–20% of the graft was repaired	No survival of chondrocytes
Enneking[22]		Slow internal repair	
Pasteurized bone			
Watanabe[20]	9 months	Osteocytes and microvascular migration	
Kubo[14]	40 months	No signs of repair	
Irradiated bone			
Hatano[13]	13 months	No osteoblasts	
	34 months	Osteoblasts in the subchondral bone	Viable chondrocytes
Frozen bone	5 months	Osteoblasts in small portions	
Our institution	17 months	Osteoblasts in broad portions	
	75 months	Osteoblasts in entire portions	Normal chondrocytes

frozen autografts begins earlier than 5 months after implantation.

Ennecking and Campanacci[22] reported that the total extent of repair was approximately 30% in most of the allografts retrieved 2 years after implantation. Internal repair was confined to the ends and the periphery of the cortices; and it penetrated so slowly that only 15%–20% of the graft was repaired by 5 years, after which deeper repair seldom occurred. Thus, the incorporation process of allografts appeared to be rather slow among their study subjects. Hatano et al.[13] reported that a specimen of irradiated bone obtained 34 months after surgery showed surrounding osteoblasts and osteocytes embedded in trabeculae of the subchondral bone but that another specimen obtained 13 months after surgery did not show any osteoblasts.

In contrast, the specimen in our study removed 17 months after implantation showed osteoblasts in broad portions of the frozen bone, indicating that bone incorporation was proceeding at a rate faster than the reported rates for allografts and irradiated bones. No published report to date has proven histologically that bone regeneration occurs at such an early stage. The frozen bone specimen in our study removed 75 months after implantation displayed newly formed bone in entire portions, including the subchondral bone region most distant from the osteotomy site. Hatano et al.[13] reported that regeneration of the irradiated subchondral area occurs at the last stage of regeneration. Our results indicate that osteogenesis in frozen bone is completed within 6 years of implantation.

We observed that bone formation in frozen bone resulted from the migration of mesenchymal stem cells already present in the continuous normal medullary cavity, which may induce bone formation from the inside.[15] In case 5, incorporation at the junction was observed in the medullary part of the cortex, suggesting that bone formation might be promoted by mesenchymal stem cells from a normal medullary cavity.

Bone scintigraphy using a ^{99m}Tc-biphosphonate compound is a suitable method for assessing bone incorporation of bone grafts. We previously reported that the uptake on the inside of a frozen autograft was comparable to the uptake in host bone 6 years after operation and that the uptake in the graft gradually got closer to normal bone as bone incorporation progressed. In case 5, at 15 months after implantation most parts of the frozen bone showed uptake similar to that of the host bone, indicating substantial progress in bone incorporation of the frozen bone at that time.

Hatano et al.[13] examined osteochondral autografts following extracorporeal irradiation and detected viable chondrocytes in three of the five cartilage specimens. Their observation that chondrocytes can survive irradiation raises doubts as to the safety of irradiating cartilaginous tumor autografts. In our study, we were able to find normal chondrocytes on the articular surface, a puzzling observation because the freezing process used in the preparation of our autografts results in loss of chondrocyte viability. Although the mechanism of chondrocyte regeneration is unclear, a possible source of these new chondrocytes is synovium-derived stem cells, which are known to have chondrogenic differentiation potency, as they are capable of generating chondrocytes for cartilage repair.[23] However, we were able to confirm necrosis of tumor cells in the frozen autografts from our two chondrosarcoma patients. We have implanted frozen autografts into 15 chondrosarcoma patients, all of whom are recurrence-free after a mean of 78 months. The lack of viable chondrocytes predisposed some patients to joint degeneration. Cartilage frozen by liquid nitrogen develops osteoarthritic changes in time, as is seen in osteochondral allografts.[24] Salvage operations such as resurfacing total knee arthroplasty may be necessary for some patients in the future. Alternatively, ice-free cryopreservation through vitrification could be tried during the initial surgery to prevent chondrocytes from dying.[25]

Histological examination of the specimens in our study revealed clusters of dead tumor cells in the frozen bone, confirming that the tumor cells had been devitalized by our freezing method. Sakayama et al.[26] reported pathological findings for a frozen autograft in an osteosarcoma patient that was retrieved for histological examination because the patient died from lung metastases 2 years after implantation. An autopsy showed no histological evidence of local recurrence. Our two patients with osteosarcoma developed local recurrences arising from soft tissue outside the area of the frozen bone, but no tumor cells were identified inside the frozen bone area in either case. Also, among the 66 patients in whom we have implanted frozen autografts, to date we have observed no clinical evidence of tumor recurrence arising from the autografts.

In this study, our frozen bone specimens showed evidence of newly formed bone and earlier osteogenesis. Marciani et al.[27] reported on the good remodeling quality of liquid nitrogen-treated bone. In addition, Garusi et al.[28] found that bone graft frozen by liquid nitrogen acts the same as a normal graft in rats. Frozen autografts contain autogenous proteins, growth factors, and cytokines;[29] and they do not elicit an immune reaction. They have the advantages of early bone union, a low risk of bone resorption, and rapid progression toward incorporation. We believe that engraftment of frozen autografts engenders more vitalization, revascularization, and remodeling, leading to replacement by living bone. We conclude that frozen autografts may be considered one of the most useful recycled materials for biological reconstruction.

Each author certifies that his or her institution has approved the human protocol for this investigation and that all investigations were conducted in conformity with ethical principles of resecarch.

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