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Scanning electron microscopic and light microscopic observations on morphological changes of freeze-dried bone implantation in rats: Comparison with fresh autogenous bone transplantation

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Summary. Bone remodelling after the implantation of freeze-dried autogenous bone in rat parietal bone was compared with fresh autogenous bone transplantation, using a scanning electron and light microscope revealed the time intervals after transplantation/implantation. The light microscope revealed the time delay of the bone remodelling in the implantation, compared with the transplantations. The scanning electron microscope showed that the differences between the two groups were in the states of bone union and bone resorption. In the fresh bone group, the newly-formed bone filled the spaces between host and the transplanted bones at 2 to 3 weeks after the transplantation: the newly-formed bone fused and melted into the transplanted bone. New bone formation was more dominant on the bone surface in *the dura mater* side than in the skin side. The union was almost completed at 5 weeks. In freeze-dried bone implantation, the bone union in the contact space was very poor and the implanted bone was mainly covered by the new bone, which developed from the host bone surface in *the dura mater* side at 2 to 3 weeks after the implantation. What is noteworthy is that bone resorbed areas showing numerous Howship's lacunae were mainly observed on the host bone surface in the vicinity of newly-formed bone. However in freeze-dried bone implantation, the bone resorption was greater on the host and implanted bone surface than that of fresh bone transplantation: the resorption of host bone was considerably larger at certain periods after freeze-dried bone implantation. The present results show that the healing process of freeze-dried bone implantation, even though autogenous bone was used, differed from that of fresh autogenous bone transplantation, and the differences are concerned not only with time sequences but also with qualitative changes. This suggests that the host would have some different responses to the freeze-dried autogenous bone from fresh materials.

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Key words: Bone implantation, Fresh bone tranplantation, Freeze-dried bone, Scanning electron microscope, Rat

Introduction

In the reconstruction of bone defects, fresh autogenous bone has long been used as a transplanted material. However, autogenous bone transplantation gives some problems to the patients; the limitation of sources of large segments of the materials, additional surgical incisions, increased postoperative morbidity, and weakened donor bone sites (see Burchardt for a review, 1983). Hence allogeneic bone implantation has been developed as an alternative for autogenous bone transplantation. It has been recommended that the allogenic bone should be freeze-dried, becuase the process of freezing and drying may diminish the bone-antigenicity which can cuase retardations of both re-vascularization and new bone formation (Burchardt et al., 1977, 1978; Friedlaender et al., 1976, 1978).

In experimental studies, the freeze-dried allogeneic bone was incorporated in the same manner as autogenous bone, although the process was slightly delayed (Kreuz et al., 1951), and the freeze-dried allografts were considered to have a similar sufficiency to autografts (Jonck et al., 1981). Clinically successful results have been reported in small bone cyst (Spence et al., 1969). However, long term clinical and experimental evaluations have revealed some complications (e.g. fatigue fractures, non-unions, complete resorption of the implant materials) (Burchardt et al., 1977, 1978). The morphological experimental studies on vascular and cellular invasion, osteogenesis, and remodelling of transplanted/implanted bone were too few to finally evaluate the application of freeze-dried bone implantation (Burwell, 1965, 1976; Nogami and Urist, 1974; Urist et al., 1974; Thorogood and Gray, 1975). Most morphological studies have been made by the observations on the sections of materials,

and in these studies it has been very difficult to imagine the holistic dynamic changes of the transplanted/implanted bone: the conclusions which were made have been controversial. It is also unclear whether the complications of freeze-dried bone implantation described above have been caused by the remaining antigenicity.

It has been shown that the scanning electron microscope is ideally suited for the direct examination of bone surface and to easily understand the whole three-dimensional images (Boyde and Hobdell, 1969). Scanning electron microscopic studies have been made to clarify the morphological characteristics of bone surface under physiological condition in adult (resting, forming, and resorbing surfaces) (Boyde, 1972). Until now, however courses of fresh bone transplantation or of freeze-dried bone implantation have not been observed under a scanning electron microscope.

It is the aim of the present study to reveal the differences of three-dimensional whole image of bone formation between fresh bone transplantation and freeze-dried bone implantation. To this end, scanning electron microscopy as well as conventional light microscopy were used. Because any antigenicity of grafts can affect bone-formation, we used autogenous bone, which was freshly transplanted or implanted after freezing and drying, and their healing processes were compared. The results from the present study might provide some criticism of application of freeze-dried bone implantation.

Materials and methods

Animals

Fifity-six male Wistar rats (aged 8 weeks, weighing about 200 grams) were used in this study. They were fed conventional commercial food pellets (CE-2, Clea Japan, INC., Tokyo, Japan) and kept under optimum conditions (rom temperature 22° C; humidity 55%; lighting 300 - 500 lux; bad smell less than 20 ppm).

The rats were anesthetized by an intraperitoneal injection with sodium pentobarbital (40 mg/kg), and their parietal bones were exposed by ablation of the periosteum through a dermal incision. The parietal bone, measuring approximately 2 mm x 4 mm, was ressected by a dental bur for the bone tranplant/implant with special care to avoid perforating the dura mater.

Fresh autogenous bone transplantation (Control)

Twenty-eight rats were used for the fresh autogenous bone transplantation. The resected bones were rinsed in a sterile normal saline solution for 10 minutes to remove the blood. Then they were re-transplanted in the same place of each calvaria as fresh autogenous bone transplantation, and each transplanted bone was fixed by skin sutures.

Freeze-dried autogenous bone implantation

Another twenty-eight rats were used for the freeze-dried autogenous bone implantation. The resected bone were deep-frozen to –80° C for 6 hours and freeze-dried to less than 5% of their original moisture content for 2 days (Freeze Dryer FL-60, Japan Freezer Co., LTD, Tokyo, Japan). The freeze-dried bones were reconstituted in a sterile normal saline solution including antibiotics (0.5% Ampicillin, Fuji Pharmaceutical Co., LTD, Tokyo, Japan) for 20 minutes at room temperature. Then they were re-implanted in the same site as each parietal bone as an autograft.

Tissue preparation

In both fresh bone transplantation and freeze-dried bone implantation, four groups of 7 rats (five rats for scanning electron microscopic observation and two rats for light microscopy) were formed. At 1, 2, 3, and 5 weeks after transplantation/implantation, the animals of each group were anesthetized by intraperitoneal injection with sodium pentobarbital, and then sacrificed by transcardial perfusion with a fixative containing 1.25% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/l cacodylate buffer (pH 7.4) for 20 minutes at room temperature. The transplanted/implanted bones with peripheral host bone and soft tissue were removed.

For scanning electron microscopy, organic substances of the specimens were dissolved by 5% sodium hypochloride for 20 minutes at room temperature. They were rinsed in 0.1 mol/l cacodylate buffer (pH 7.4) 3 times. And then they were postfixed in a 1% osmium tetroxide solution in 0.1 mol/l cacodylate buffer (pH 7.4) for 90 minutes and dehydrated by a graded series of ethanol. After immersion in isoamyl acetate, specimens were critical-point dried with liquid carbon dioxide, mounted on stabs, coated by gold in a vacuum device, and examined with a scanning electron microscope (JSM-25, JEOL, Tokyo, Japan).

For light microscopy, the specimens were rinsed in 0.05 mol/l cacodylate buffer (pH 7.4) 3 times after the fixation. They were decalcified in 5% ethylendiamine tetraacetic acid at room temperature for 7 days, and embedded in a water-soluble plastic media (JB-4, Polysciences INC., Warrington, USA), and 2 µm thick serial sections were made. The sections were stained with hematoxylin and eosin, and observed with a light microscope.

Results

Fresh autogenous bone transplantation (Control)

1 week after transplantation

Under the scanning electron microscope, newly formed bone, which showed a spongy-like appeareance, grew from host bone surface of both skin and *dura mater* sides,

and islands of newly-formed bone were sometimes observed on the transplanted bone. A small amount of newly-formed bone on cut surfaces of host bone was sometimes observed at corners of transplant beds. The development of newly-formed bone was more prominent on *the dura mater* side (Figs. 1a, b). The newly-formed bone from host side united to a part of the transplanted bone, and in one of the specimens covered a great part of the transplanted bone surface. The newly-formed bone was constructed by thin trabeculae twined around each other, and the trabeculae were constituted by aggregation of many small spherical mineral clusters (about 1 μm in diameter) (designated by Boyde and Hobdell, 1969) (Fig. 2). Many vascular tunnels were observed among the trabeculae. The bone resorption, which was characterized by the depressed areas (Howship's lacunae), was observed around the pre-existing vascular spaces on the host and the transplanted bone surface (Fig. 3a); just like the «resorbing surface» in physiological bone, described by Boyde (1972). Howship's lacunae showed a circle-like shape surrounded by sharp edges, and the bottom of the lacuna showed a fibrous structure (Fig. 3b).

Light microscopically, new bone formation was observed on the host bone surface in *the dura mater* side only (Fig. 4). The new bone was thin and trabecular-like and it was enclosed by a layer of osteoblasts. Spindle- or round-shaped cells assembled in the connective tissues near the new bone. Multinucleated large cells (osteoclast) were observed near the cut surface of host and grafted bones. Many, but not all, of the bone lacunae in the grafts contained osteocytes, while in the host bone, the osteocytes existed in all lacunae. No conspicuous invasion of blood vessels nor bone marrow tissue in transplanted bone were observed.

2 weeks after transplantation

Scanning electron microscopy revealed that the newly-formed bone filled in most of the spaces between host and transplanted bone in both skin and *dura mater* sides; the newly-formed bone fused the transplanted bone and the union of newly-formed bone was more weighty in *the dura mater* side, though there was still an area of non-union (Figs. 5a, b). On the transplanted bone surface, the insular spongy-like bone was sometimes observed in the skin side, while, in the dura mater side, the newly formed bone was covering most of the area (Fig. 5b). In addition to the spongy-like bone with many vascular spaces, smooth-surfaced newly-formed bone was also recognized: it was built by fibrous bundles with a regular arrangement, and the thin fibres which branched off the bundles had small spherical mineral clusters (Fig. 6). On the spongy-like bone surface, the spherical mineral clusters on the trabeculae increased, and additionally some lacunae in which the osteoblast existed (osteoblastic lacuna; Boyde, 1972) were occasionally observed. The lacunae were about 15 μm long and 10 μm wide, and the back wall of the osteoblastic lacuna was also constructed by small nodules, and there were some openings of canaliculi which contained the processes of osteoblasts. The bone resorbed areas were observed not only around the pre-existing vascular spaces on host and transplanted bone surfaces, but also on the cut surfaces of host bone.

Light microscopically, the newly-formed bone was thicker and denser than that of 1 week after transplantation and was united to the transplanted bone (Fig. 7a). The blood vessels invaded the transplanted bone, and the osteoclasts surrounded the vessels (Fig. 7b). At the centre of the transplanted bone, bone marrow tissue containing blood vessels, a layer of osteoblasts lining the inner surface, and small round blood cells were often seen. The osteocytes were seen in all lacunae of the transplanted bone.

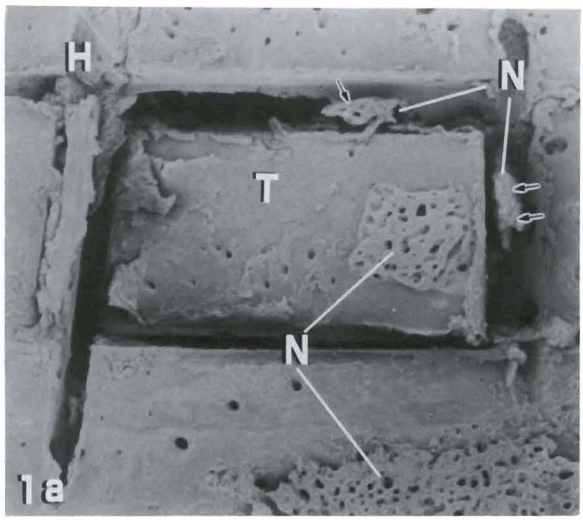
3 weeks after transplantation

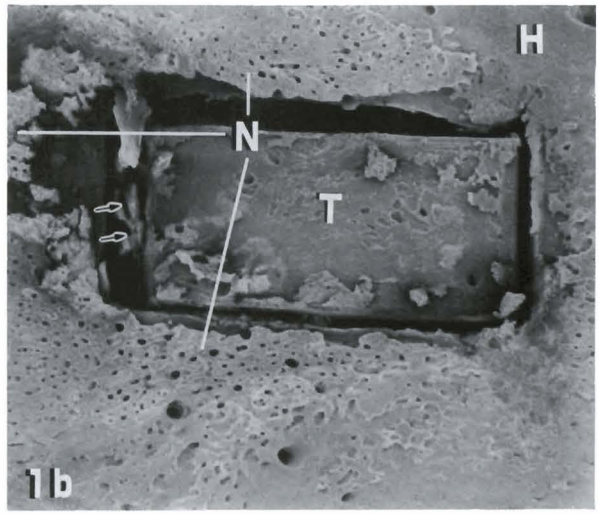
Scanning electron microscopy showed that the bone unions were seen in the all the spaces between host and transplanted bones in skin and *dura mater* sides (Figs. 8a, b). In the skin side, the bone union principally showed a spongy-like appearance, while in *the dura mater* side, smooth-surfaced bone formed bone union. Smooth-surfaced newly-formed bone was rarely observed. The smooth-surfaced newly-formed bone fused and melted into transplanted bone: the boundary between smooth-surfaced bone and the transplanted bone was not distinct. On higher magnification, the smooth-surfaced bone was characterized by numerous osteoblastic lacunae, the concentration of small spherical mineral clusters and small nodules like rice grains showing a regular arrangement (Fig. 9). These were close to, but not identical, to «forming surface» (Boyde, 1972). Some of the smooth-surfaced bone, especially in *the dura mater*» side, showed not only the atypical «forming surface» but also the «resorbing surface, characterized by shallow Howship's lacunae of various size. The resorbed area was also observed on both the pre-existing host and transplanted bones adjacent to the newly-formed spongy-like bone of the united area in the skin side.

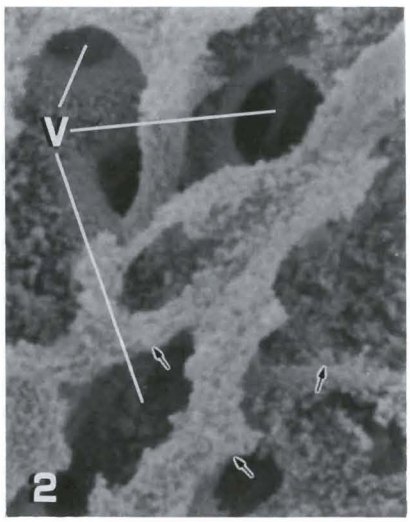
In light microscopic observation, the host bone and the grafted bone was perfectly united by newly-formed bone (Fig. 10). The newly-formed bone was observed around the bone marrow and the blood vessels in the transplanted bone.

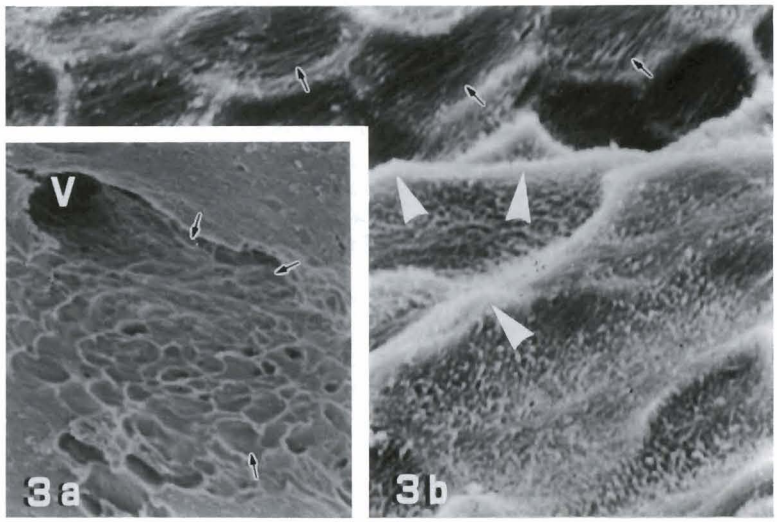
5 weeks after transplantation

Under the scanning electron microscope, it was impossible to distinguish newly formed bone from pre-exisisting transplanted bone or host bone (Figs. 11a, b). In *dura mater* side, the bone union expanded and the transplanted bone was perfectly covered by newly-formed bone (Fig. 11b). However, in skin side, the non-united part still existed. On higher magnification, the bone surface showed the typical «forming surface», characterized by many osteoblastic lacunae and a more regularly arranged nodular pattern (Fig. 12), and the









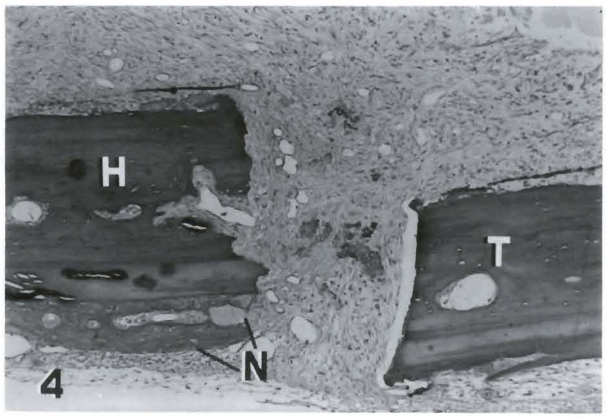


Fig. 1. Scanning electron micrograph (SEM) at 1 week after transplantation. Bone union (arrows) is seen in a part of the spaces between host and transplanted bone. H = host bone. T = transplanted bone. N = newly formed bone. a) Skin side. x 18. b) Dura mater side. The spongy-like newly formed bone exists on a great part of host bone and on a part of transplanted bone. x 18

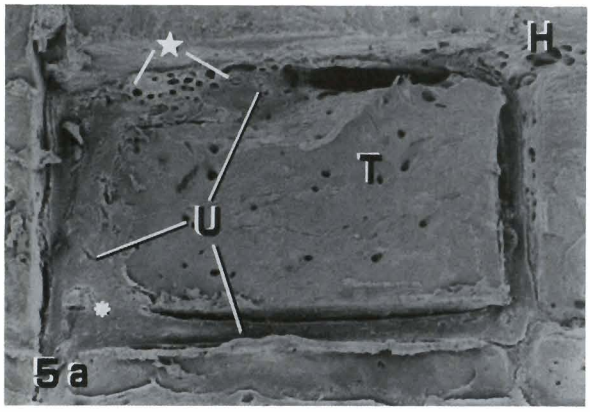
Fig. 2. High magnification of the newly formed bone at the dura mater side in Fig. 1b. Many vascular spaces (V) exist among thin trabecular-like bone with many small spherical mineral clusters (arrows). x 1,200

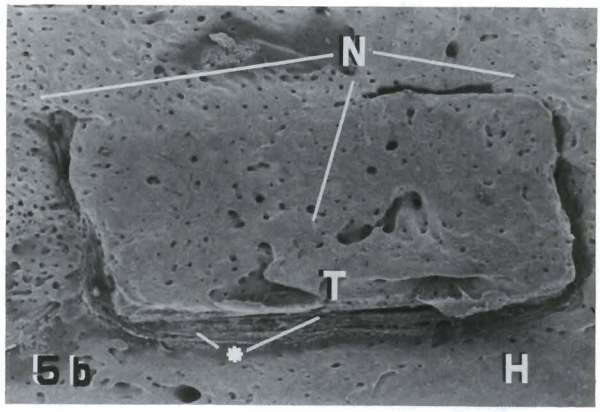
Fig. 3. SEM of one resorbed area surrounding vascular spaces of the host bone surface. a) Continuous lacunae (arrows) can be seen in the resorbed area around the vascular space (V). x 120. b) Higher magnification of the lacunae. A lacuna is enclosed by sharp edges (arrow heads) and the bottom of the lacuna shows fibrous structures (arrows). x 1,700

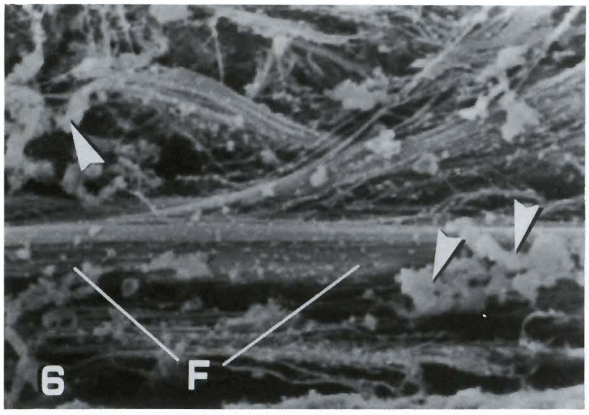
Fig. 4. Light micrograph (LM) 1 week after transplantation. upper part of the figure shows the skin side. Newly formed bone (N) is on host bone surface in dura mater side. No bone unions are in this section. H = host bone. T = transplanted bone. x 80

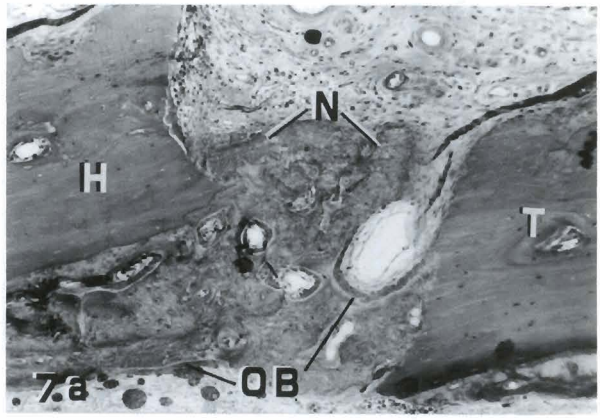
«resorbing surface». The «forming surface» and the «resorbing surface» were always in close proximity to each other.

Light microscopically, the host bone and the grafted bone were perfectly united, and the boundary of newly-formed bone and pre-existing bone was unclear.









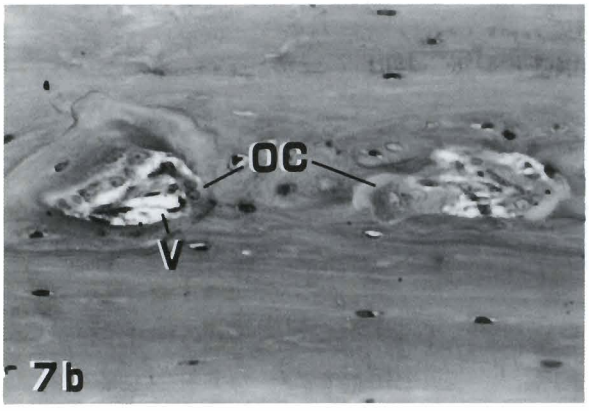


Fig. 5. SEM 2 weeks after transplantation. Bone union (U) exists between host and grafted bone. H = host bone. T = transplanted bone. a) Skin side. Newly formed bone of united area is distinguished in two parts showing spongy-like shape (white star) and smooth surface (asterisk). x 18. b) Dura mater side. Newly formed bone (N) covers transplanted bone. Smooth surface (asterisk) of newly formed bone is seen in united area. x 18

Fig. 6. Higher magnification of smooth surface in Fig. 5a. Thick fibrous bundles (F) with regular arrangement and thin fibres with spherical mineral clusters (arrow heads) branching off the bundles are seen. x 1,200

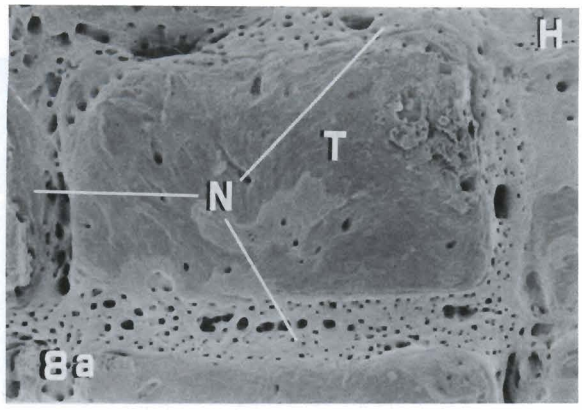
Fig. 7. LM 2 weeks after transplantation. Upper part of the figure shows skin side, and lower is dura mater side. a) Newly formed bone (N) is formed at dura mater side and bone union is seen between host (H) and transplanted bone (T). OB = Osteoblast. x 80. b) High magnification of transplanted bone. Invasion of blood vessels (V) and osteoclasts (OC) is seen around the vessels. x 250

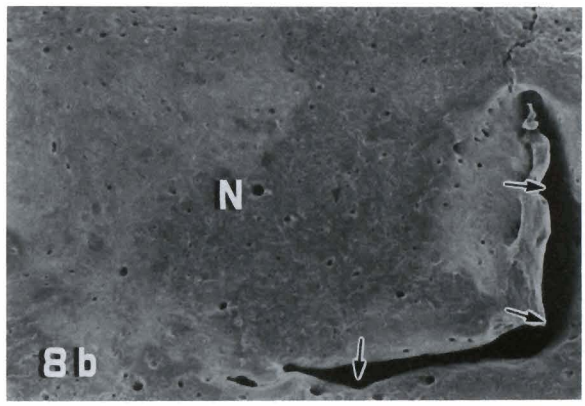
Freeze-dried autogenous bone implantation

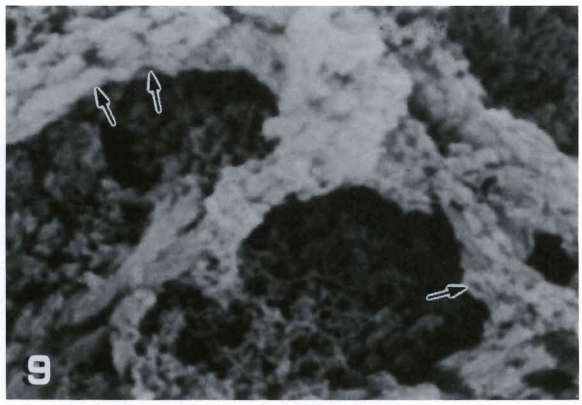
1 week after implantation

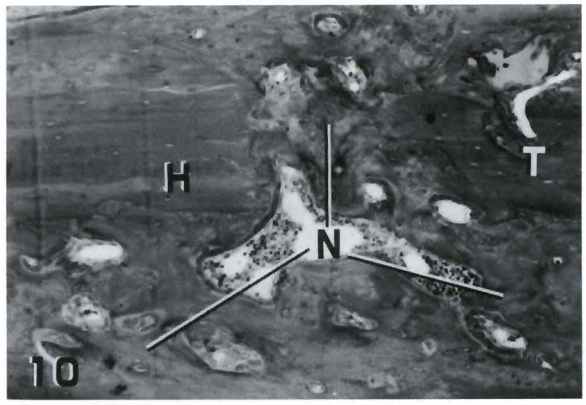
There was no calcified union between the implanted bone and the host bone; after anorganic treatment, the implanted bone was detached from the implant bed of the host bone (Fig. 13a). New bone formation was not observed in the skin side. In *the dura mater* side, however, the new bone was formed on the host bone surface and protruded towards the implant bed (Fig. 13b). The newly-formed bone had a spongy-like appearance with a large number of vascular spaces. On cut surfaces of host bone, there was no new bone formation, but the bone resorbed area, showing many Howship's lacunae, was observed near the newly formed bone (Fig. 14).

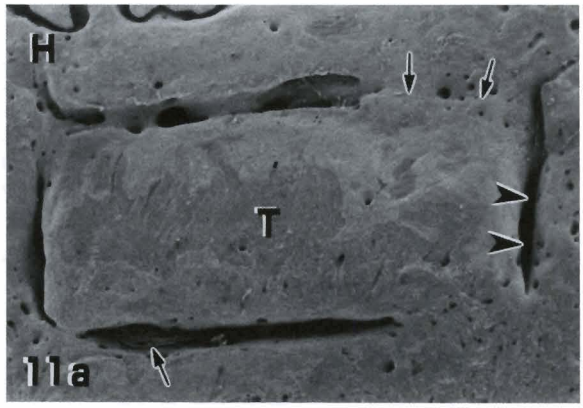
Light microscopically, the newly-formed bone was observed on the host bone surface under the periosteum only in *the dura mater* side. Newly formed bone was constructed by fine trabecular bone with a layer of osteoblasts, the same as in fresh bone transplantation. The space between the host bone and the implanted bone was filled with dense fibrous connective tissue with blood vessels. The osteoclasts in Howship's lacunae were

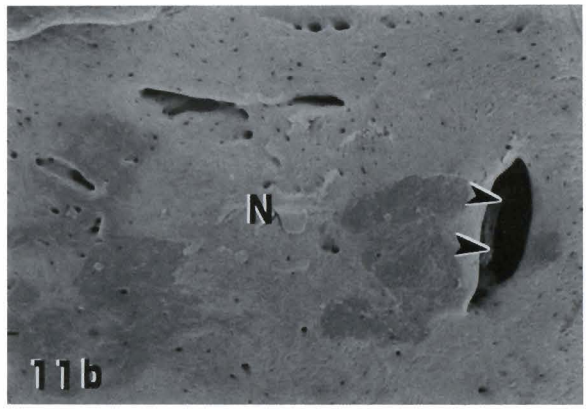












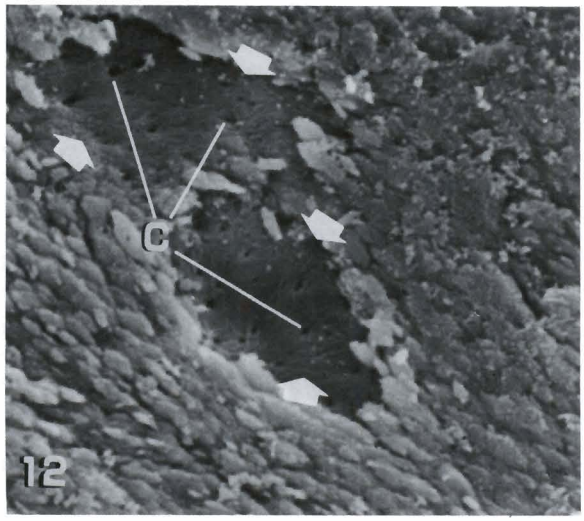


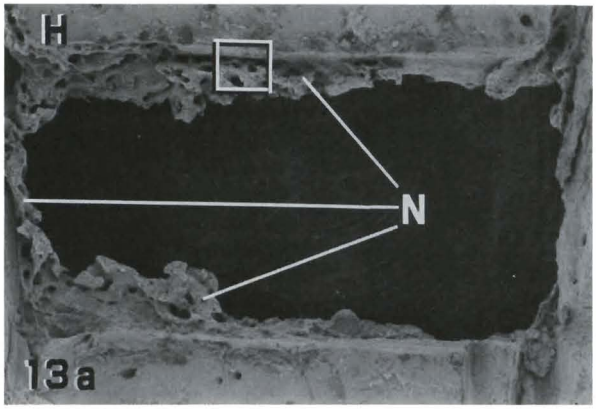
Fig. 8. SEM 3 weeks after transplantation. H = host bone. T = transplanted bone. N = newly formed bone. a) Skin side. Bone union by spongy-like newly formed bone exists in almost all areas between host and transplanted bone. x 18 b) Dura mater side. Bone surface of united area cannot be distinguished from host and transplanted bone surface, except for non-union (arrows). x 18

Fig. 9. Higher magnification of the newly formed bone in Fig. 8b. Calcified nodules like rice grains (arrows) are seen in a concentration of small spherical mineral clusters. x 2,500

Fig. 10. LM 3 weeks after transplantation. Upper part of the figure shows skin side at the lower part is dura mater side. Host (H) and transplanted bones (T) are perfectly united by newly formed bone (N) from dura mater side of host bone. x 85

Fig. 11. SEM 5 weeks after transplantation. H = host bone. T = transplanted bone. Arrow heads = non union. a) Skin side. Bone surface of united area (arrows) is similar to that of host and transplanted bone. x 18. b) Dura mater side. Bone union exists in most parts between host and transplanted bone and morphology of all bone surfaces is similar. N = newly formed bone. x 18

Fig. 12. High magnification of the newly formed bone in Fig. 11b. Calcified nodules like rice grains with regular arrangement can be seen on whole bone surface. arrows = osteoblastic lacunae. C = canalicle of osteoblastic lacuna. x 1,800



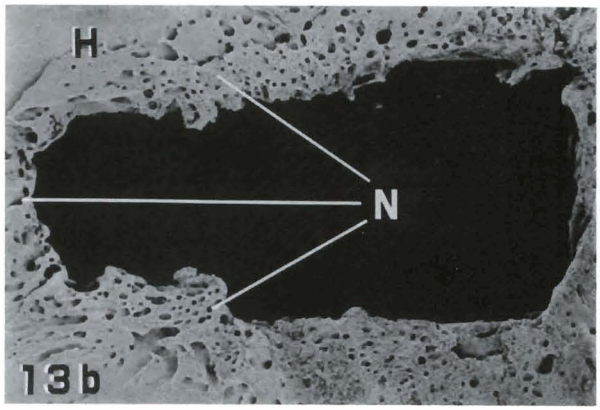


Fig. 13. SEM 1 week after freeze-dried bone implantation. No implanted bone can be seen in the implant bed. Newly formed bone (N) can be seen on dura mater side on host bone (H), but not skin side. a) Skin side. x 18. b) Dura mater side. x 18

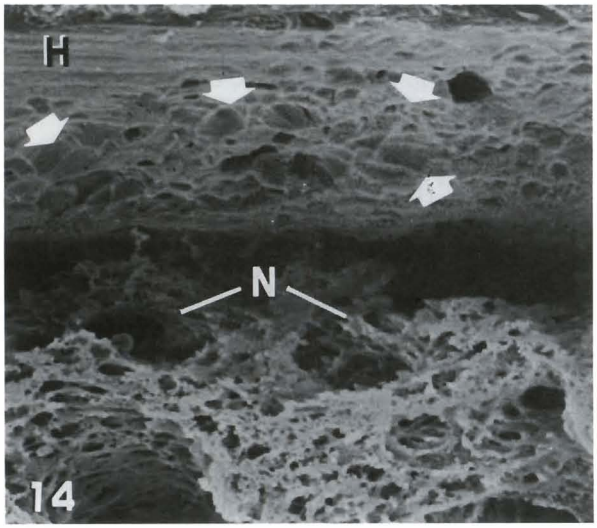


Fig. 14. Higher magnifications of outlined area in Fig. 13a. Continuous Howship's lacunae (arrows) can be seen at the cut surface of the host bone near the newly formed bone (N). x 350

seen on the cut surface of the host bone near the newly-formed bone. All osteocytic lacunae of the implanted bone were empty. No remodelling or invasion of blood vessels was observed.

2 weeks after implantation

There was no bone union in the skin side (Fig. 15a). The implanted bone was connected to the host bone by newly-formed bone protruding from the host bone surface in *the dura mater* side, and most of the implanted bone in *the dura mater* side was covered by the newly-formed bone (Fig. 15b). However, some of the characteristic features which were frequently observed in the fresh bone transplantation were not observed: no islands of newly-formed bone on the implanted bone; no newly-formed bone filling the spaces between host and implanted bone; no smooth surfaced newly-formed bone. A part of the surface of implanted bone was resorbed and had a rougher appearance than that of transplanted fresh bone.

Light microscopically, there were no remarkable changes, compared with the specimens of 1 week after implantation, though the newly-formed bone in *the dura mater* side was more developed toward the implanted bone (Fig. 16). In the implanted bone, the osteocytic lacunae were still empty and there was no bone marrow containing cells and blood vessels.

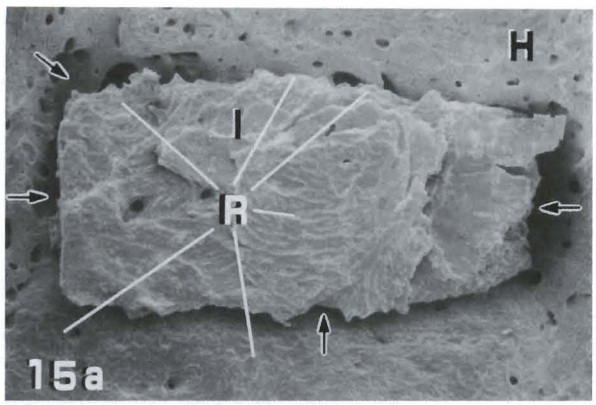
3 weeks after implantation

Scanning electron microscopy revealed that the bone union was formed in the skin side at last (Fig. 17a), although the unions by newly formed bone existed mainly at the corner of the implanted bed. Both host and implanted bone surfaces near the newly formed bone were greatly resorbed. In *the dura mater* side, the implanted bone was partly covered by the newly formed bone, but the boundary was clear: the melting of newly-formed bone into implanted bone was rare (Fig. 17b). Some specimens showed the regression of host bone caused by prominent resorption (Fig. 18). Newly formed bone surface showed nodular structures with a somewhat irregular arrangement, which was similar to that of 3-week-old specimens of the fresh bone transplantation.

Light microscopically, the newly formed bone in *the dura mater* side connected more widely to the implanted bone, though the fibrous tissue containing many fusiformed cells intercalated between the host and implanted bone (Fig. 19). In the implanted bone, fibrous tissues and blood vessels were observed in pre-existing cavities, but the osteocytic lacunae were still empy. No replacement of the implanted bone with newly formed live bone was observed.

5 weeks after implantation

Scanning electron microscopy showed that the newly formed bone covering the implanted bone increased greatly, but that not all the space was filled by newly formed bone neither in the skin side nor in *the dura mater* side (Figs. 20a,b). The surface of the bone union



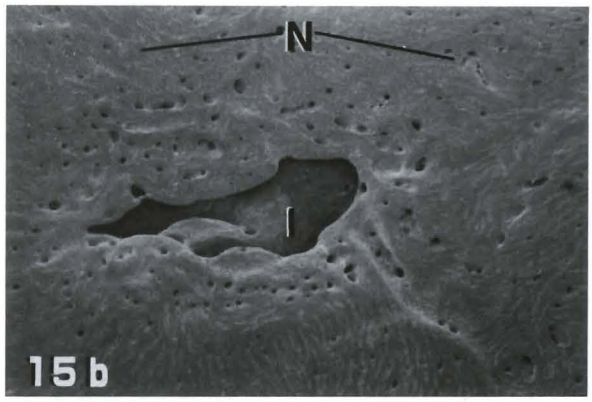


Fig. 15. SEM 2 weeks after implantation. H = host bone. I = implanted bone. a) Skin side. Bone union in the space (arrows) between host and implanted bone is not formed. R = Bone resorbed area. x 18. b) Dura mater side. Implanted bone (I) is covered by newly formed bone (N) from host side. x 18

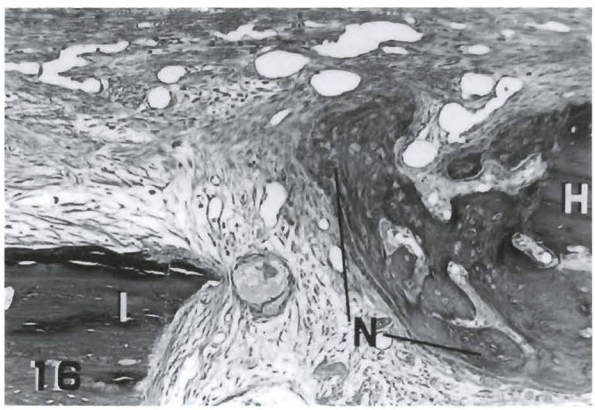


Fig. 16. LM 2 weeks after implantation. Upper part of the photograph shows skin side and the lower part is dura mater side. Newly formed bone (N) is seen on host bone (H) surface in dura mater side. No bone union is seen in this section. I = implanted bone. x 80

was rougher with variously-shaped vascular spaces, when compared with that of fresh bone transplantation, though the newly formed bone on implantation also showed the «forming surface» and «resorbing surface», like in fresh bone transplantation.

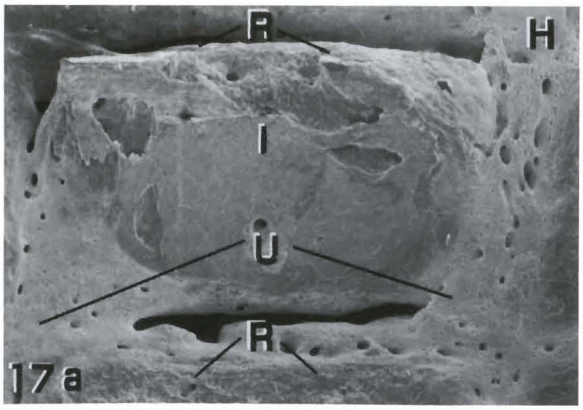
Light microscopy revealed that the replacement of implanted bone by the newly formed bone appeared (Fig. 21). This replacement was carried out in two ways. First, the surface of implanted bone, especially in *the dura mater* side, was replaced by newly formed bone. Secondly, the new bone was also formed around the blood vessels which invaded the implanted bone. This live bone tissue had irregular lamellar structures showing a mosaic pattern. The non-replaced implanted bone tissue showed a typical lamellar pattern, but no osteocytes were observed.

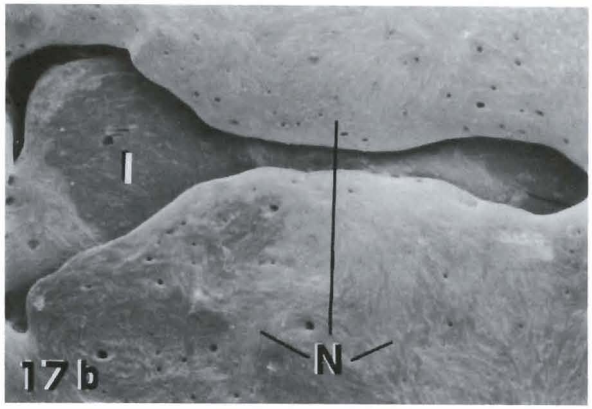
Discussion

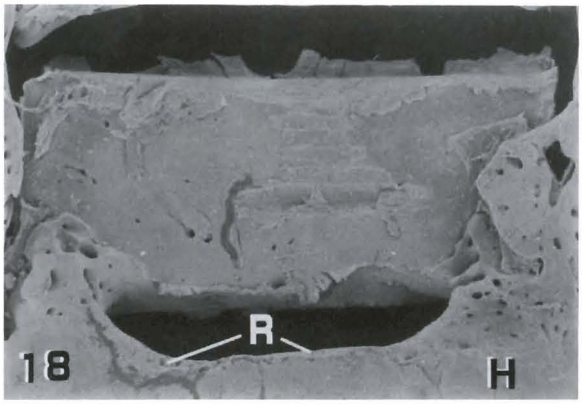
Previous light microscopic observations suggested that, in fresh autogenous bone transplantation, the necrotic grafted bone was gradually resorbed by the osteoclast along pre-exisisting Harversian canals, and then when the appropriate cavity size was obtained, the resorption ceased, osteoblasts appeared and the new bone formation was begun (Burchardt, 1983 for a review). Also in the freeze-dried bone implantation, the invasion of blood vessels into the implanted bone (i.e. re-vascularization) has been consiered to be an essential mechanism for its incorporation (Kreuz et al., 1951; Heiple et al., 1963). They suggested that the healing process of allogenic freeze-dried bone implantation was similar to that of fresh autogenous bone transplantation. However, the incorporation of freeze-dried bone was slower than that of fresh autogenous bone, and there were some complications (e.g. fatigue fractures, non-union, complete graft resorption) (Burchardt et al., 1977, 1978). These have been considered the results of remaining antigenicity of allogeneic freeze-dried bone.

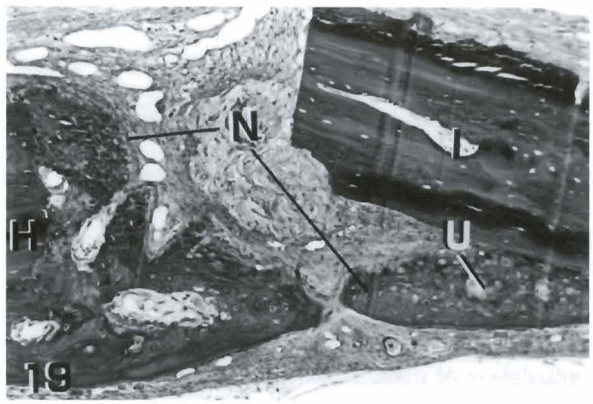
In the present study using autogenous bone, light microscopic results could not show significant differences between the healing process of fresh bone transplantation and that of freeze-dried implantation. However, the healing process in the implantation was slower than in transplantation of fresh materials. This may indicate that the delay of the healing process in freeze-dried bone implantation is not due to the antigenicity of the implanted bone.

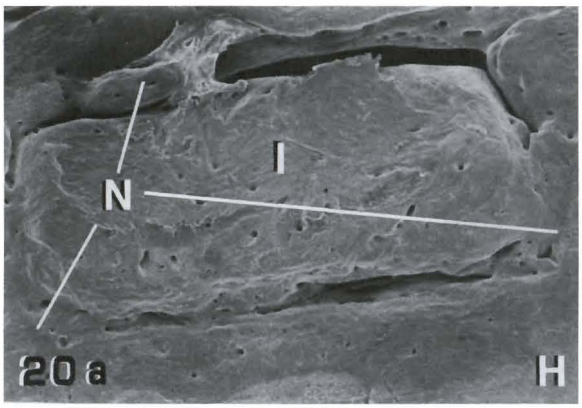
Furthermore, scanning electron microscopy in the present study revealed that the differences between the healing process in fresh bone transplantation and that in freeze-dried bone implantation concerned not only time sequences but also quantitative changes. In fresh bone transplantation, the union was made by newly formed bone which filled in the contact spaces between the host and the transplanted bone in the early stage of the transplantation: the newly formed bone fused and melted into the transplanted bone. On the contrary, in freeze-dried bone implantation, the bone union was made by the covering of the new bone, which protruded from the host bone surface, especially in *the dura mater* side: the fusion and melting of newly formed bone into the graft were not salient.

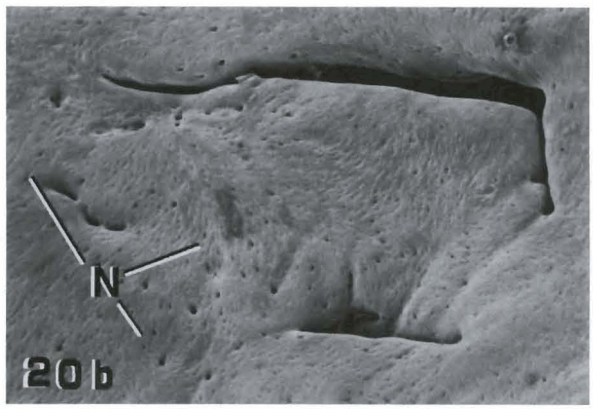












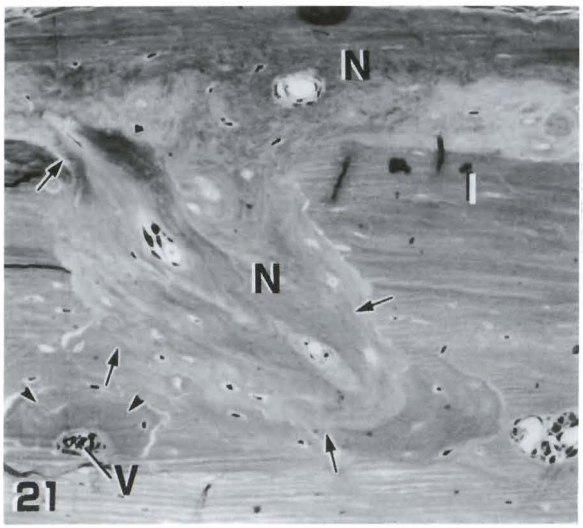


Fig. 17. SEM 3 weeks after implantation. H = host bone. I = implanted bone. a) skin side. Bone union (U) between host and implanted bone can be seen at the corner of implant bed. R = resorbed area. x 18. b) Dura mater side. Implanted bone is covered by newly formed bone (N) from host side. x 18

Fig. 18. SEM of another 3-week-old specimen. Note the extensive resorbed area (R) of the cut surface of host bone (H).

Fig. 19. LM 3 weeks after implantation. Bone union (U) between host (H) and implanted bone (I) can be seen in dura mater side. N = newly formed bone. x 80

Fig. 20. SEM 5 weeks after implantation. H = host bone. N = newly formed bone. a) Skin side. Newly formed bone from host bone connects to implanted bone (I) extensively. x 18. b) Dura mater side. Implanted bone is completely covered by newly formed bone from host bone. x 18

Fig. 21. LM of the newly formed bone (N) in the implanted bone (I) at 5 weeks after implantation. In implanted bone, invasion of newly formed bone (arrows) and new bone formation (arrow heads) around blood vessels (V) are seen. x 200

Also on the bone resorption, there is a difference between the fresh bone transplantation and the freeze-dried bone implantation. In the transplantation, the characteristic lacunae which had indicated the osteoclastic bone resorption (Jones and Boyde, 1970) were seen around the pre-existing vascular spaces on the host and transplanted bone surfaces, while in freeze-dried bone implantation, the bone resorption was greater on the host and implanted bone surfaces than that of the transplantation. It was noteworthy that the resorption of host bone was considerably large at certain periods after the implantation. This reaction should not be an immune response to «not-self», because of using autogenous bone in the present study. One may argue that the resorption of host bone might be as a consequence of bone-necrosis caused by disturbance of blood supply in the implantation procedures. Should this mechanism indeed be operating, we suspect that the implanted bone as a non-viable biologic material (Burchardt, 1987) might be more extensively resorbed than viable host bone. However, this was not the case. It is therefore concluded that the host reaction caused by non-viable biologic material like a freeze-dried bone, even though it is a «self», is considerably different from that after transplantation of fresh materials.

As described above, there are considerable differences in the incorporation patterns between fresh bone transplantation and freeze-dried bone implantation. However, both in transplantation and in implantation, newly formed bone in the early stage showed a spongy-like appearance. Spongy-like bone with many vascular spaces surrounded by the great number of spherical mineral clusters has been reported in the developmental stage of the calvaria and the mandibles of fetal or newborn mammals including man (Boyde and Hobdell, 1969). The newly formed bone in 3 and 5 weeks after the transplantation/implantation came near to mature bone in physiological conditions; characterized by the forming, resting and resorbing surfaces (Boyde and Hobdell, 1969; Jonse and Boyde, 1970), though the resting surface was not salient in the present study. The changes of newly formed bone in the transplantation/implantation can be analogous to ontogenetic change of bone.

It should be pointed out that the bone resorption was always observed in close proximity to the newly formed bone throughout the present examination, though the bone resorption in the implantation was greater than in the transplantation. Some *in vitro* studies suggest that bone resorption by osteoclasts is coupled with bone formation by osteoblasts (Rodan and Martin, 1981; McSheehy and Chambers, 1986; Thomson et al., 1986). The present study confirms that the coupling phenomenon between bone formation and bone resorption also occurs *in vivo*. The close relation between the bone resorbed area and the newly formed bone indicates that the coupling phenomenon can play an important role in bone resorption after bone transplantation/implantation.

It has been reported that increased blood supply plays a significant role in bone formation (Goldberg and Lance, 1972; Ray, 1972). In the present study, bone formation from host bone was more dominant on the bone surface in *the dura mater* side than that of the skin side, in the transplantation as well as the implantation. The connective tissue, including the periosteum in the skin side, was severely injured by implant procedures. On the other hand, the periosteum in *the dura mater* side was not so damaged. It is likely that blood supply in the skin side was less than in *the dura mater* side after the transplantation/implantation, and therefore periosteal reaction for the bone formation may have been retarded in the skin side. New bone formation after transplantation/implantation can be influenced by the degree of recovery of peripheral soft tissues.

From the present results, it is quite clear that the failure of freeze-dried bone implantation was not caused by the inflammation or the immune response alone. It would appear to us that the host might have some other recognitions to the bone treated by freezing and drying, in spite of being autogenous bone. How and what kind of cells in the host recognize the nonviable biological material like freeze-dried bone and how the cells respond to the material are still unknown. More detailed experimental studies about the host cell response to the nonviable biological materials will be needed for sucess in clinical use.

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