Retention of Allogeneic Calvarial Periosteum as Soft Tissue around Allogeneic Transplanted Teeth in Alveolar Bone

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Purpose: Periodontal ligament and cementum are important parts of the tooth-supporting apparatus. However, the mechanism behind the ability of the periodontal ligament to produce cementum and to maintain a non-calcified fibrous tissue between the root of a tooth and the alveolar bone is not fully understood. A part of the calvarial periosteum seems to share developmental similarities with the periodontal ligament with regard to cell types and morphology. The aim of this study was to compare the ability of two types of allogeneic periosteum to form a periodontal ligament-like structure around allogeneic transplanted teeth in alveolar bone.

Materials and Methods: Teeth for transplantation were extracted from adult beagle dogs. The crown, cementum and periodontal ligament of the extracted teeth were removed. Periosteum was obtained from the calvaria and tibia in three-month-old beagle dogs. The roots were wrapped with the calvarial periosteum (CP group), tibia periosteum (TP group), or surgical collagen sheets (SC group) and were transplanted into surgically created bone cavities in edentulous sites. Roots only were transplanted as a control group. The transplanted teeth were completely covered with a submerged barrier membrane.

Results: Histomorphometrical analysis was performed at 24 weeks after surgery. New cementum formation was not observed in any of the groups. More substantial connective tissue adhesion occurred around the transplanted roots of the CP group as compared with the TP, SC, and control groups.

Conclusions: The calvarial periosteum seems to possess the same competency as periodontal ligament tissue with regard to the maintenance of non-calcified tissue between tooth and alveolar bone. However, cementum formation may be a unique ability of the periodontal ligament.

Key words: periosteum, periodontal ligament, cell differentiation, cement formation, neural-crest derived mesenchyme, non-calcified tissue

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INTRODUCTION

One of the goals of periodontal therapy is the regeneration of the destroyed attachment between the tooth and alveolar bone. The attachment consists of cementum with inserted functionally oriented fibres. To date, several regenerative periodontal therapies have been investigated but the ultimate goal of reforming the complete attachment apparatus has not been achieved (Nyman et al, 1982; Hancock, 1989; Heijl, 1997). The complete regeneration of the attachment apparatus includes the maintenance of a fibrous tissue between the root of the tooth and the alveolar bone, and the insertion of functionally oriented fibres in the cementum. Cementum is classified according to its position and time of formation (primary, secondary) and its content of cells and fibres (acellular afibrillar cementum, acellular extrinsic fibre cementum, cellular mixed stratified cementum, cellular intrinsic fibre cementum) (Listgarten and Kamin, 1969; Schroeder, 1992). The formation of cementum is regulated by cementoblasts of the periodontal ligament. Osteoblasts forming bone are also present in the periodontal ligament; however, the difference between osteoblasts and cementoblasts is not clear (Ten Cate, 1998). There is no distinct evidence that cementoblasts are derived

from the same stem cells or the same lineage as osteoblasts. Furthermore, despite the fact that it contains many osteoprogenitor cells (Isaka et al, 2001), it is still not clear what mechanism is responsible for the maintenance of the periodontal ligament (a very thin fibrous tissue between the calcified tissues), the cementum and the alveolar bone (Lekic and McCulloch, 1996). The lack of information with regard to the characteristics of the periodontal ligament is one of the reasons that a definitive periodontal regeneration therapy has not been established.

Amongst the tissues that abut the bone, the periodontal ligament is considered to be the tissue that is most similar to the periosteum. The periosteum is also a very thin fibrous tissue that contains many osteoprogenitor cells (Tonna, 1979; Wlodarski, 1989; Gallay et al, 1994). The outer surface of the alveolar bone is covered by periosteum while the periodontal ligament covers the internal tooth socket of the alveolar bone. Moreover, the periosteum is continuous with the periodontal ligament. It has also been reported that the same biological substances (e.g. growth factors, hormones, etc.) influence the cementum adjacent to the periodontal ligament and the bone lying beneath the periosteum (Iwasaki et al, 1995; Ballock et al, 1997; Chien et al, 1999; Chai et al, 2000). The periosteum of the cranial frontal bone and the periodontal ligament are thought to share developmental similarities (Gilbert, 1997; Ten Cate, 1998). Comparative studies of the periosteum and periodontal ligament may provide clues for clarifying the characteristics of the periodontal ligament cells, cementoblasts and osteoblasts.

The aim of this study was to clarify the healing pattern of transplanted teeth in alveolar bone wrapped in allogeneic calvarial periosteum through an examination of the behaviour of the periosteum around transplanted teeth in alveolar bone.

MATERIALS AND METHODS

Animals

This animal study was performed in accordance with guidelines approved by the Council of the American Physiological Society for the use of animal experiments. A total of 8 (3-year-old) male young adult beagle dogs weighing between 10 and 15 kg and 3 (3-month-old) male puppy beagle dogs were used. The animals were deemed clinically healthy as determined by physical examinations, and all were found to have a clinically healthy periodontium without tooth loss. All dogs were inbred.

Surgical Procedures

For all surgical procedures, the animals were sedated by an intramuscular injection of ketamine hydrochloride (10 mg/kg body weight) (Meiji, Japan) and an intravenous injection of sodium pentobarbital (0.6 mg/kg body weight) (Abbott Laboratories, USA). The mandibular incisors and premolars on both sides of the dental arches were extracted to provide recipient beds for transplantation of the roots. Three months later, after complete healing of the bone socket, a mucoperiosteal flap was elevated in the area from the mandibu-

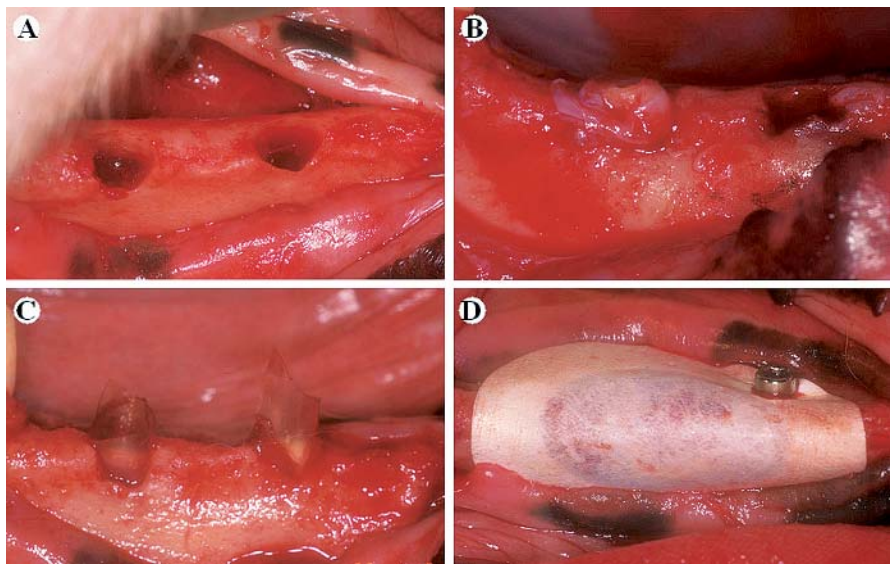

Fig. 1 Surgical procedures in this study. **A**. Two bone cavities were made at the recipient area. **B**. The periosteum wrapped roots were transplanted into the bone cavities. **C**. The collagen sheet wrapped roots were transplanted into the bone cavities in the same manner as periosteum wrapped roots. **D**. The bone cavities were then completely covered with ePTFE membrane, and fixed with a screw.

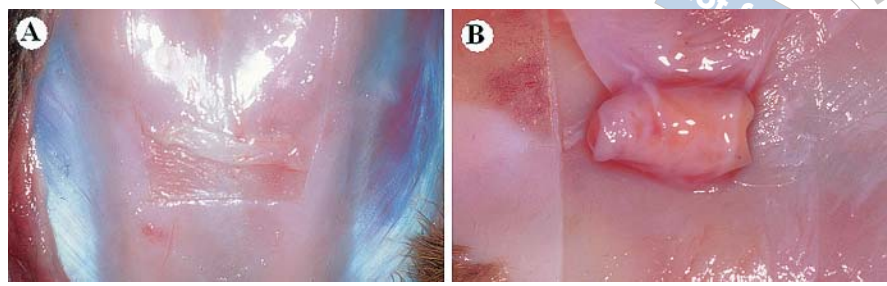

Fig. 2 The view of calvarial periosteum. **A.** The calvarial periosteum was obtained from calvaria of dogs. **B**. The extracted root was wrapped by the calvarial periosteum.

lar canine to the first molar including the recipient area on each side. Two bone cavities for transplantation of roots were then made at the recipient area on each side (Fig. 1). Each bone cavity was drilled at a low speed using a round surgical bur under saline irrigation. The cavity was made to match the shape of the root to be transplanted in an apical-coronal direction, with its long axis parallel to the buccal and lingual bony plates. It was first undersized and thereafter adjusted to the root size to ensure adaptation of the root to its cavity. The mandibular premolars and incisors for transplantation were extracted at the same time from other young male adult beagle dogs. The crowns of these teeth were cut off at the level of the cementum-enamel junction with a diamond disk, under a saline spray. The cementum and periodontal ligament of the extracted teeth were removed with burs and curettes under irrigation with sterile saline. The pulp tissue was then extirpated under aseptic conditions and the root canal was filled with calcium hydroxide cement (Neo Dental Chemical Products, Japan). At the same time, the skin of the skull and legs of male puppy beagle dogs were incised with a surgical scalpel with the periosteum then being obtained from the tibia and nasal side of the frontal bone (Fig. 2). The periosteum was removed carefully in order to protect against contamination by the fat and muscle tissue. The root was wrapped by the calvarial periosteum (CP group) or tibia periosteum (TP group) with the bone side of the periosteum on the root surface (Fig. 2). In a third group, the same procedure was followed but instead of periosteum we used a surgical collagen sheet (Koken, Japan) (SC group). A small part of the periosteum was used for histological assessments.

The periosteum wrapped roots were then transplanted into the recipient bone cavities on one side (Fig. 1B), while the roots wrapped with the collagen sheet or the unwrapped roots were transplanted into the bone cavities on the contralateral side (Fig. 1C). The transplanted roots on each side were thereafter completely covered with an expanded polytetrafluoroethylene (ePTFE) membrane (W.L. Gore and Associates, USA), which was fixed with screws (Institut Straumann AG, Switzerland) (Fig. 1D). Wound closure was initiated by fenestration of the periosteum at the base of the buccal and lingual gingival flaps in an effort to increase flap relaxation. The buccal and lingual gingival flaps were replaced and sutured with 4-0 silk sutures to ensure complete coverage of the ePTFE membranes. Penicillin G (100,000 U/ml/10 kg) was administered subcutaneously on the day of surgery. The dogs were fed soft food to minimize trauma to the flaps during the postsurgical phase. The sutures were removed at 10 days after surgery.

Biopsies and Histological Assessments

Six dogs were euthanized 24 weeks after surgery and two dogs were euthanized at 6 weeks with an overdose of sodium pentobarbital (65 mg/kg, i.v.) (Abbott Laboratories, USA). Block biopsies of the transplanted areas, including the transplanted roots and the surrounding tissues were obtained and were fixed in 10% neutral buffered formaldehyde, decalcified in 10% formic acid, and embedded in paraffin after dehydration in a graded alcohol series. The biopsies from 7 of the dogs were serially sectioned at 6 μm in the bucco-lingual plane. Sagittal sections were made in the biopsies obtained from 1 dog. All sections were stained with hematoxylin and eosin (H&E) and were examined under a light microscope. New cementum, connective tissue adhesion and bone ankylosis were analyzed around the transplanted root and were compared between each group. In order to confirm the presence of new cementum or bone ankylosis, a cementum-like deposit on the root was confirmed if the deposit made contact with the bone in all the sections. Eight specimens from the biopsy of the mid-portion of the root were chosen at 60 μm intervals for histomorphometric analysis in both the mesial and distal direction for specimens cut in the bucco-lingual plane, and the lin-

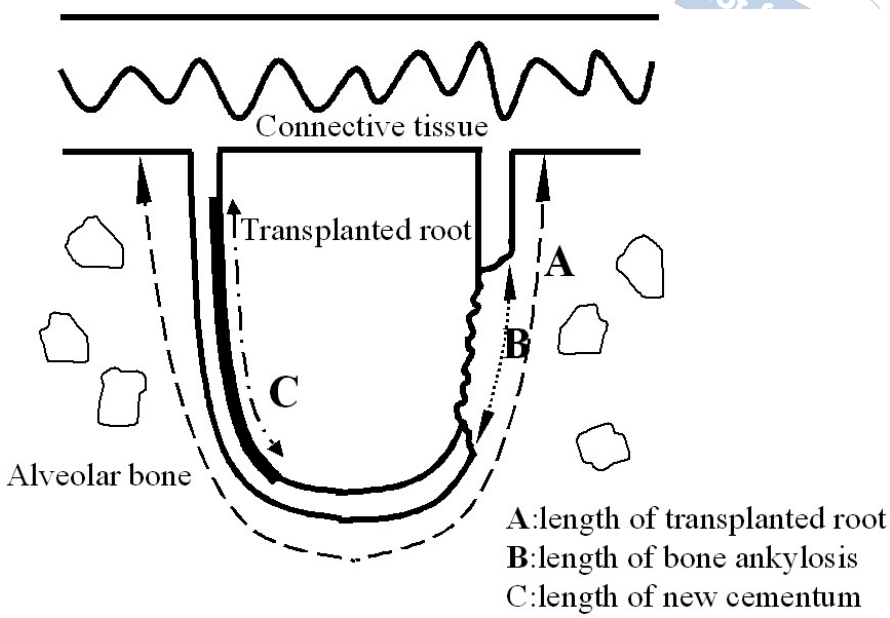

Fig. 3 Schematic drawing showing parameters of the histomorphometric analysis. **A**. Length of entire surface of transplanted root. **B**. Length of bone ankylosis. **C**. Length of new cementum.

gual and buccal direction for specimens cut in the sagittal plane. Histomorphometric analysis was performed using a microscope with the aid of a computer program for assisted image analysis (Nikon, Japan) to determine the extent of new cementum, connective tissue adhesion and bone ankylosis.

The percentage of bone ankylosis (Fig. 3) was defined as the mean value of the ratio of the length of the bone ankylosis to the surface length of the transplanted root. The sum of the length of the bone ankylosis was divided by the surface length of the transplanted root, with the resulting fraction multiplied by 100 to yield the percentage bone ankylosis. The percentage of connective tissue adhesion and new cementum was measured and calculated in the same manner as the bone ankylosis determination.

Statistical Analysis

Means and S.D. were calculated for the CP, TP, SC and control groups. The statistical significance of differences between each group was analyzed using a Student's *t*-test.

RESULTS

Clinical Observations

The gingiva over the submerged transplanted roots covered with ePTFE membrane demonstrated slight inflammatory reactions throughout the postsurgical phase in all groups. Clinical healing progressed uneventfully in general except for ePTFE membrane exposure. At the time of euthanasia, the covering gingiva exhibited very mild signs of inflammation. ePTFE membrane exposure was observed in 1 of the 8 dogs during the third week after surgery in the CP group. However, none of the ePTFE membranes had been displaced or perforated at the time of euthanasia of the animals.

Histological Observations and Assessments

All specimens in each of the CP, TC, SC and control groups were used for histological analysis. In all of the 4 groups, inversion of the epithelium into the bone defect was completely prevented by the ePTFE membrane. Infiltration of inflammatory cells could not be detected under the membrane in any group. The histological observations of small parts of the calvarial and tibia periosteum revealed that they consisted of fibrous tissue with several cell layers and there was no contamination by fat and muscle tissue (Fig. 4).

The CP, TP and SC groups were euthanized 24 weeks after surgery. There was no new cementum found in any of the sections from all groups. Connective tissue adhesion and bone ankylosis were assessed in the tissue surrounding the transplanted roots and were compared between each group. In the CP group, connective tissue adhesion was observed surrounding most of the root in all of the specimens (Fig. 5). Histomorphometric analysis showed that the percentage of

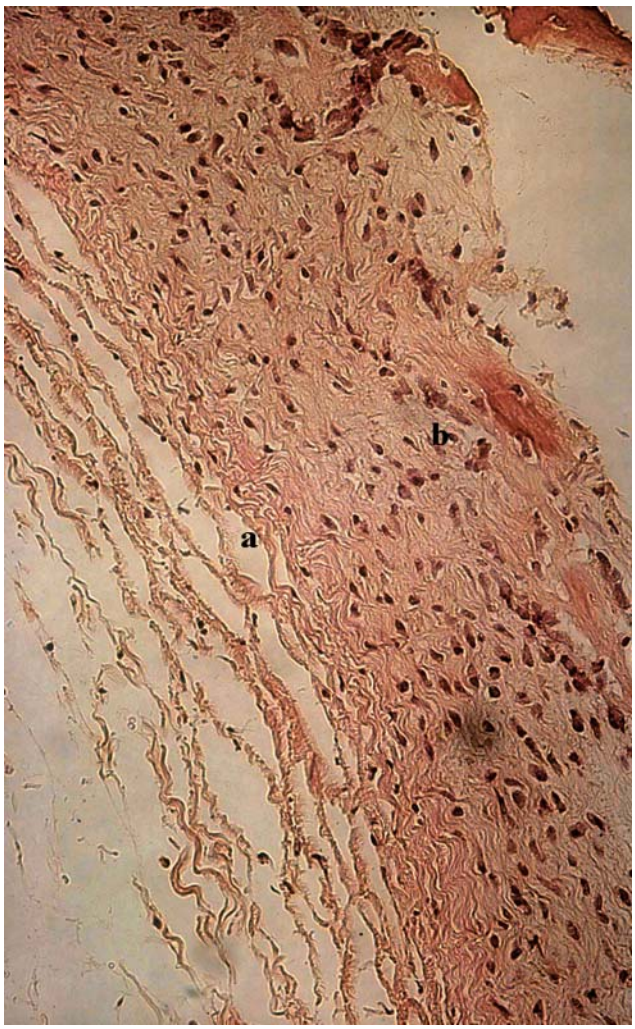

Fig. 4 Histological section of pieces of periosteum from calvaria (×200, H&E stain). (a) Inside layer. (b) Outside layer.

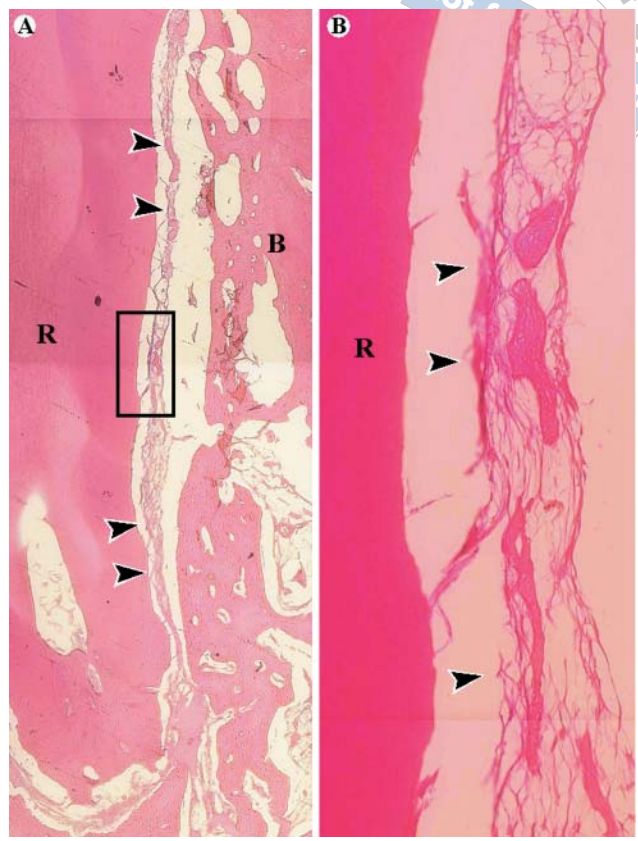

Fig. 5 Histological section of CP group at 24 weeks after surgery. **A**. Connective tissue adhesion occurred all over the root surface. Arrow heads mark the soft tissue between alveolar bone (B) and transplanted root (R). (×10, H&E stain). **B**. Higher magnification of the connective tissue adhesion. R=transplanted root (×50, H&E stain).

connective tissue adhesion ranged from 60 to 90% in the CP group. Bone ankylosis was observed in the remaining areas in the CP group. Most of the connective tissue adhesions were composed of loose and disorganized connective tissue; however, dense connective tissue was observed in a few areas in the CP group. The connective tissue adhesion resembled intact periodontal ligament width-wise. However, the orientation of the fibres of the connective tissue adhesion was parallel to the root surface. Exposure of the ePTFE membrane had no effect on the amount of connective tissue adhesion and bone ankylosis. Inversely, connective tissue adhesion was observed in a few parts of the surrounding root in the TP and SC groups whereas there was substantial bone ankylosis in all other parts that surrounded the root in both of these groups (Figs. 6, 7). Histomorphometric analysis showed that the percentage range for connective tissue adhesion in the TP and SC groups was 20 to 45% and 2 to 10%, respectively. There was no difference between the bucco-lingual and sagittal specimens in any of the groups. Sagittal sections also demonstrated substantial connective tissue adhesion in the CP group whereas there were only small amounts in the TP and SC groups.

The control group was euthanized 6 weeks after surgery. In all of the transplanted roots of the control group, bone ankylosis occurred in all areas (Fig. 8). Histomorphometric analysis showed that the percentage of connective tissue adhesion ranged from 5 to 10% in the control group.

There was no significant difference between the CP and TP group in terms of percentage of connective tissue adhesion and bone ankylosis, whereas a significant difference was observed in both parameters be-

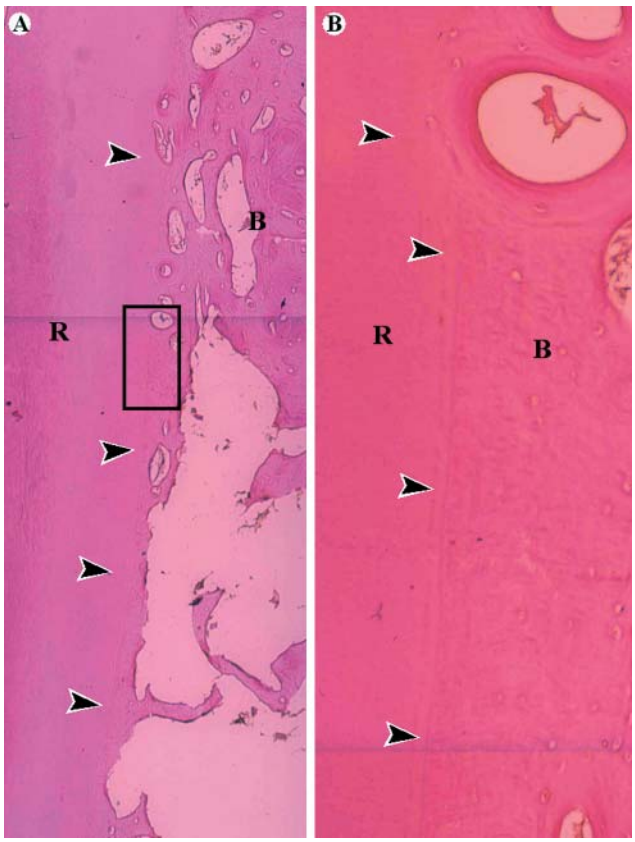

Fig. 6 Histological section of TP group at 24 weeks after surgery. **A**. Bone ankylosis was observed all over the root surface. Arrow heads mark the bone ankylosis between alveolar bone (B) and transplanted root (R). (×10, H&E stain). **B**. Higher magnification of the bone ankylosis. R=transplanted root (×50, H&E stain).

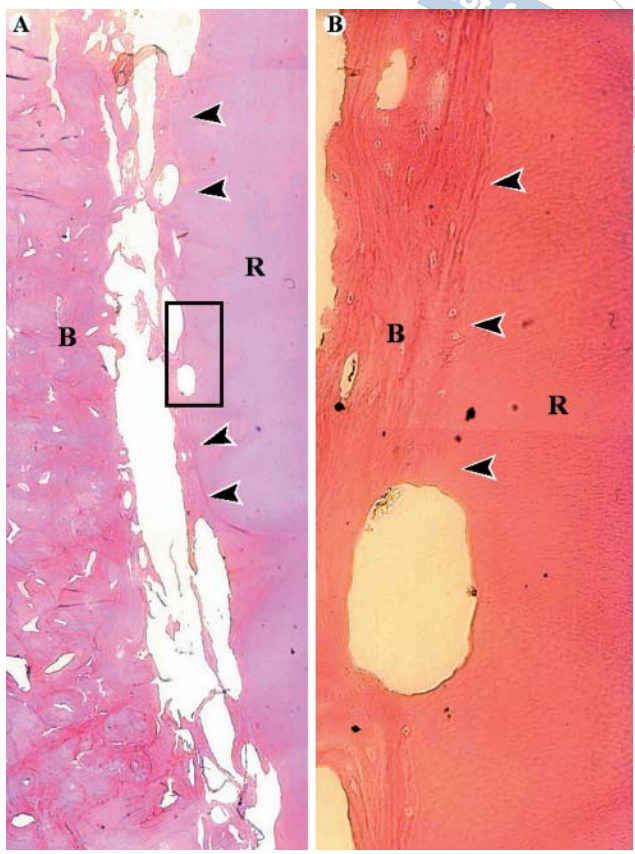

Fig. 7 Histological section of SC group at 24 weeks after surgery. **A**. Bone ankylosis was observed all over the root surface. Arrow heads mark the bone ankylosis between alveolar bone (B) and transplanted root (R). (×10, H&E stain). **B**. Higher magnification of the bone ankylosis. R=transplanted root (×50, H&E stain).

tween the CP, the SC, and the control groups (Table 1). There was no significant difference between the TP and SC groups in terms of the percentage of connective tissue adhesion and bone ankylosis, whereas a significant difference was observed in both parameters between the control group and the TP group (Table 1).

DISCUSSION

The periodontal ligament is an important tooth-supporting tissue. However, its detailed characteristics have not been clarified. There is especially no data as to whether the periodontal ligament is a discrete tissue or if it is similar to other tissues (e.g. articular ligament, periosteum, etc.), since periodontal ligament specific proteins and molecules have not been identified. In order to clarify these questions, we postulated that the biology of calvarial periosteum is similar to that of the periodontal ligament based on developmental similarities and we performed a comparison of the periodontal ligament with periosteum. Presently it remains unclear which type of cell plays a key role in the periodontal ligament, although periodontal ligament consists of many types of cells (e.g. fibroblasts, cementoblasts, osteoblasts, etc.). Since our approach was to

Table 1 The mean ratio of the connective tissue adhesion in transplanted roots

CP	TP	SC	Control
78.9±8.1	30.8±10.1	22.5±6.5	5.0±3.6
	*		
		*	
			*

Values are the percentage of the connective tissue adhesion to all around roots (n=8)

* P<0.05 CP: calvaria periosteum, TP: tibia periosteurm, SC: surgical collagen sheet

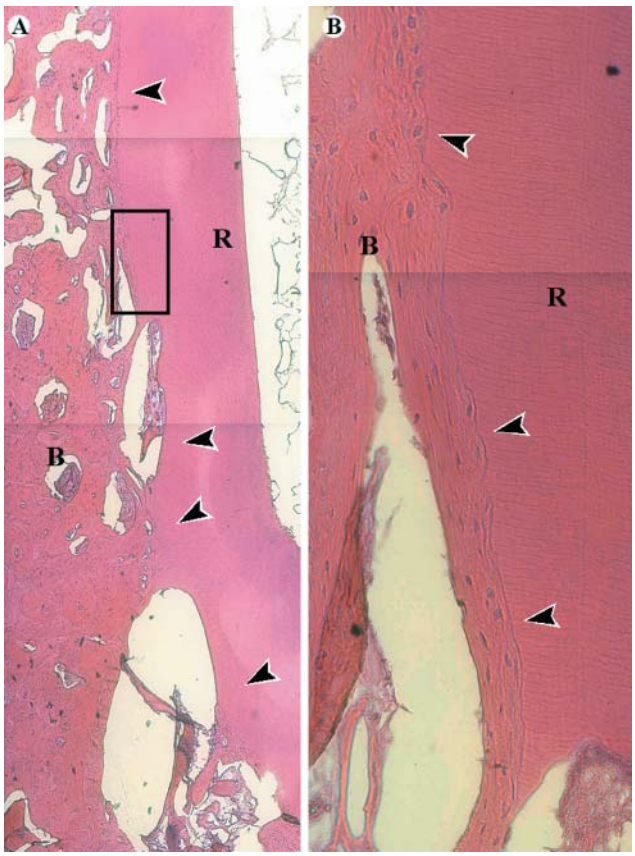

Fig. 8 Histological section of control group at 6 weeks after surgery. **A**. Bone ankylosis was observed all over the root surface. Arrow heads mark the bone ankylosis between alveolar bone (B) and transplanted root (R). (original magnification ×10, H&E stain). **B**. Higher magnification of the bone ankylosis. R=transplanted root (×50, H&E stain).

compare the two tissues *in vivo*, we considered it necessary to put each of the tissues into the same environment. The tooth transplantation model is suitable for placing periosteum into a similar environment as periodontal ligament, as grafting of the periosteum has already been established clinically (Peltomaki and Ronning, 1997; Santiago et al, 1998). Tooth transplantation has also been established as both a clinical technique (Andreasen et al, 1981; Nethander et al, 2003) and as an experimental method (Runyon et al, 1986; Garcia and Saffar, 1990; Isaka et al, 2001). Furthermore, submersion of the transplanted tooth provides a stable environment for the grafted tissue, since occlusal force and bacterial infection are excluded. Thus, by combining these techniques, the grafted periosteum should be intact and be able to exhibit biological activity soon after the grafting procedure in the submerged tooth transplantation model. In fact, the amount of non-calcified tissue around the transplanted teeth was different between the CP, TP and SC groups, revealing that grafted periosteum retained its function. Thus, this model seems suitable for investigating the behaviour of periosteum around the tooth in alveolar bone.

We chose the allogeneic tooth and periosteum transplantation model in this study because it is known that there are two ways to obtain periosteum: from the bone of infantile dogs, or from the bone of adult dogs during initial healing after fractures. However, it is difficult to ensure that dogs survive after calvaria bone fractures. Moreover, it is easier if periosteum is obtained from infantile dogs, since the periosteum of an infantile dog is thicker than that seen in adults. Therefore, we chose the periosteum obtained from infantile dogs for allogeneic transplantation studies. Unfortunately, infantile dogs are not suitable for tooth implantation, since permanent tooth germs exist in the mandibular bone. Furthermore, it is important that an adequate spaced recipient bed, away from the residual tooth, should be considered in order to avoid the periodontal ligament of residual tooth coming into contact with the transplanted periosteum. Therefore, allogeneic tooth implantation (from one adult dog to another adult dog) has also been used, as there are no major rejection problems found in allografting between blood relatives of beagle dogs (Bijnen et al, 1979; Sveen et al, 1980; Finco et al, 1985; Liu et al, 2001; Schwartz and Andreasen, 2002). All dogs used in this study were blood relatives, because all the dogs were inbred.

It is known that periosteum contains many osteoprogenitor cells, and participates in bone remodelling and fracture repair (Heppenstall, 1980; Wlodarski, 1989; Gallay et al, 1994). In the present study, it was confirmed that osteoprogenitor cells were present (high ALPase activity, PTH-responded cAMP, etc.) in both periosteum cells derived from calvaria and tibia, indicating an osteoblast phenotype (data not shown). However, connective tissue adhesion was observed in the CP and TP group whereas substantial bone ankylosis was observed in the control group. Furthermore, the SC group, which was wrapped in a collagen sheet, also showed substantial bone ankylosis. These results suggest that tissue composed of only fibrous elements is not sufficient for survival as soft tissue in alveolar bone, and that periosteum can maintain soft tissue between calcified tissue, teeth and alveolar bone. Surprisingly, the amount of connective tissue adhesion in the CP group was significantly greater than that of the TP group. Cranial periosteum used in this study, was taken from the nasal side of the frontal bone. The nasal side

of frontal bone and face components are derived from the neural crest (ectoderm), whereas the tibia periosteum is derived from the mesoderm (Gilbert, 1997; Ten Cate, 1998; Moore and Persaund, 1999). It has been shown that the ossification system of the skull is different from that of the tibia. This suggests that the characteristics of calvarial periosteum cells are different from tibia periosteum cells and that the maintenance of soft tissue between the tooth and alveolar bone may depend on the developmental origin of the cells. The periodontal ligament, like the calvarial periosteum, is also derived from the neural crest (Ten Cate, 1998). The neural crest derived cells may play a role in the maintenance of the soft tissue space between the tooth and alveolar bone.

Cementum formation was not found in any of the groups. This indicates that cementum formation is not a co-function to the maintenance of non-calcified tissue and that both functions are regulated separately. The fact that periosteum failed to form cementum indicates two possibilities. First, cementum formation is one of the unique abilities of the periodontal ligament, and there may be no stem cells for cementoblasts in other tissues, even in the developing periosteum. Therefore the cementum formation may be regulated only by cells in the periodontal ligament. A second possibility is that a specific molecule (e.g. growth factor or hormone, etc.) may be needed for differentiation of stem cells into cementoblasts even though stem cells in other tissues may have the potential to differentiate into cementoblasts. Along this line, the presence of epithelial cell rests of Malassez in the periodontal ligament is one of the differences between the periosteum and periodontal ligament (Hamamoto et al, 1989; Lambrichts et al, 1993; Ten Cate, 1998). It is therefore of interest that such epithelial cells are known to derive from epithelium involved in enamel formation during development (Ten Cate, 1998).

Recently, enamel matrix derivative (EMD) has been introduced as a new modality in regenerative periodontal treatment (Gestrelius et al, 1997a). Histological studies have shown that treatment with EMD in periodontal defects results in regeneration similar to the original structure, which has been defined as a formation of a new layer of acellular cementum with inserted collagen fibres (Heiji et al, 1997; Heiji, 1997). Enamel-associated molecules like EMD have been reported to promote proliferation, migration, adhesion and differentiation of cells associated with periodontal tissue healing (Gastrelius et al, 1997b). It could be argued that epithelial cell rests of Malassez play a role in the differentiation of stem cells into cementoblasts. However, further studies are required for the understanding of the differentiation process of cementoblasts.

Periosteum, which covers the alveolar bone, is contiguous with the periodontal ligament in intact situations. As periodontal ligament covers the bone surface of the tooth socket like periosteum it may be involved in bone remodelling. It is known that the same biological substances (e.g. growth factor, hormones, etc.) influence the cementum, which periodontal ligament abuts, and the bone that the periosteum abuts (Iwasaki et al, 1995; Ballock et al, 1997; Chien et al, 1999; Chai et al, 2000). In the healing process after long bone fractures, periosteum covers the fracture portion in order to isolate the space for bone formation from invasion by other tissues and allows for ossification to be initiated under reconstructed periosteum (Heppenstall, 1980). After tooth extraction, the extraction socket is also covered by periosteum and bone fills the space beneath the periosteum (Lin et al, 1994). Furthermore, bone formation in a tooth socket with retained periodontal ligament occurs faster than that of the bone socket without the periodontal ligament (Anderson, 1981; Garcia and Saffar, 1990). It is possible that the periodontal ligament differentiates into periosteum to cover the extraction socket and isolates the area of bone formation and/or provides osteoprogenitor cells in the area after tooth extraction. Periodontal ligament may be a tooth dependent periosteum-like tissue. Recently *Periostin* was identified as a periosteum marker gene, which is also expressed in the periodontal ligament (Horiuchi et al, 1999; Oshima et al, 2002). These data may support our hypothesis.

Dense connective tissue was observed in few areas surrounding the root in the CP group. However, the orientation of the fibres of the connective tissue adhesion was parallel to the root surface as occlusal force is needed for the formation of functional oriented fibres of the periodontal ligament (Cohn, 1965, 1966; Levy and Mailland, 1980; Tran Van and Mailland, 1981). Thus, it is possible that the parallel fibres change into functional oriented fibres if the transplanted root becomes functional.

In the future, use of the periosteum may be beneficial for periodontal tissue regeneration through a combination of tissue engineering and/or genetic engineering, including the use of master genes for cementogenesis.

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