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Ultrastructure of the periosteum from membrane bone

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INTRODUCTION

The use of free periosteal grafts has become increasingly popular as a potential source of osteogenesis in a variety of clinical situations (Skoog, 1965; Ritsila, Alhopuro, Gylling & Rintola, 1972; Mock, 1928; Satoh, Tsuchiya & Harii, 1983; Aitasalo & Lehtinen, 1985). The procedure is justified by evidence that periosteum is a source of osteoprogenitor cells, capable of proliferation and osteogenesis after injury (Tonna & Cronkite; 1961; Tonna, 1965; Baro & Latham, 1981; Tenenbaum, Palangio, Holmyard & Pritzker, 1986). The morphological basis of this functional capacity of periosteum is less clear; traditionally, the tissue is divided into an inner proliferative or cambial layer and an outer fibrous layer. The extent and composition of these layers has never been clearly defined, for there have been few ultrastructural studies (Tonna, 1975; Holtrop, 1975; Rhodin, 1974), although it has been claimed recently that there is a population of osteogenic cells in the cambial layer that is morphologically distinct from fibroblasts (Tang & Chai, 1986). All of the studies mentioned above describe the periosteum of a long bone, whereas the regions in which surgical manipulation of this tissue has proved most valuable consist of membrane bone (e.g. Skoog, 1965; Ritsila *et al.* 1972; Perko, 1979). Only one study has described periosteum in this situation (Habel & Maniscalco, 1981).

The present report describes a quantitative ultrastructural examination of periosteum from two areas of membrane bone, the palate and the skull, in young beagle dogs.

MATERIALS AND METHODS

Seven beagle dogs between 2 and 3 months old were anaesthetised intravenously with barbiturate and perfused via the ascending aorta with a solution of 0·9% sodium chloride at 37 °C for several minutes. Subsequently, a solution of 1·5% glutaraldehyde containing 2 mM calcium chloride in 0·08 M cacodylate buffer at pH 7·0 was perfused for 30–45 minutes or until the lips and pinnae of the ears showed evidence of fixation; this solution had an osmolality of 320–330 mOsmol.

Specimens of bone and overlying soft tissues (mucosa and skin) were removed from the hard palate and skull with a saw and immersed in 2·5% glutaraldehyde in cacodylate buffer, pH 7·0, for 12 hours at 4 °C. They were then rinsed in 0·1 M cacodylate buffer and decalcified in 0·1 M ethylenediamine tetra-acetic acid (EDTA; Warshawsky & Moore, 1965) for 2–4 weeks. After decalcification was complete, specimens were trimmed into blocks of 2–3 mm^3, washed in buffer and postfixed in 1·5% osmium tetroxide for 2–4 hours. They were dehydrated in ascending concentrations of ethanol, block stained in 2% uranyl acetate in 70% ethanol for 2

hours and embedded in Spurr's epoxy resin. Semithin (0·5–1·0μm) sections were prepared from each block and stained with toluidine blue for light microscopical examination; these included a full thickness of bone, periosteum and the overlying mucosa or dermis. Ultrathin sections were prepared on a Sorval MT-2B ultramicrotome, mounted on formvar-filmed single hole grids, stained with 0·5% aqueous uranyl acetate and lead citrate and examined in a Zeiss EM-10 transmission electron microscope.

Quantitation

Ten blocks of palatal tissue and six blocks of skull were randomly selected for quantitation. From each block, sections were cut and 7–10 electron micrographs taken at a magnification of $\times 1600$ to include the whole periosteum as well as adjacent bone and soft tissue. Positive film negatives were prepared and projected on a coherent lattice grid (Weibel, 1979) with 42 points at a total magnification of $\times 9000$. At this magnification, it is possible to identify clearly and distinguish cell and fibre types. Points were recorded as falling on cells, fibres, vessels or matrix. Cells were identified as fibroblasts, osteoblasts or other cells; fibres as collagen or elastin. Identification of cell types was based on the criteria for oral tissues illustrated by Schroeder & Munzel-Pedrazzoli (1973). In a separate count, the number of profiles of fibroblast-like cells that included all or part of the nucleus was recorded so as to permit determination of the relative numerical density and average volume of these cells.

Counts were entered directly on an IBM personal computer using a program written for this purpose. This converted point counts into volume density, as related to a reference volume of 1 cm^3 of tissue and numerical densities as number of cells per cm^3 of tissue. Numerical densities (N_v) were calculated using the formula $N_v = (K/\beta)(N_a^3/V_v)$ (Weibel & Gomez, 1962), where N_a is the number of cells in the test area, V_v the volume density, and K and β are constants depending on the shape of the cells. The shape of fibroblasts is highly variable, so a spherical shape was assumed and K and β were entered as 1 and 1·38 respectively (Muller, 1980). This assumption means that the values for numerical densities will be only approximate if cell shape departs radically from that of a sphere. A complete account of the quantitation procedures has already been published (Squier & Kremenak, 1982).

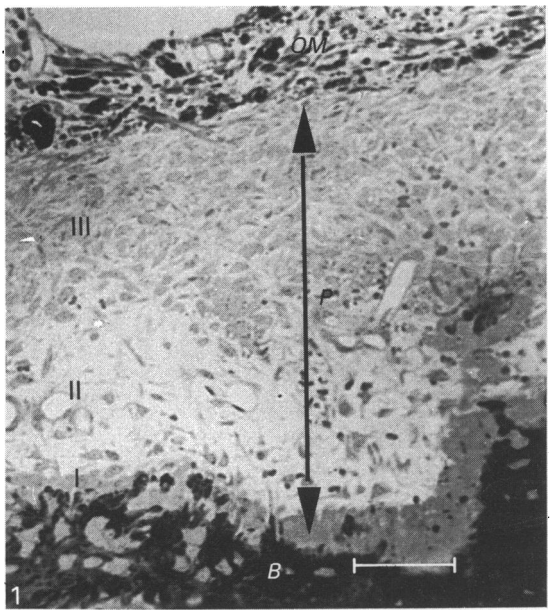
A mean and standard error were calculated for all values, and differences were examined using an analysis of variance (ANOVA). Differences significant at the 5% level were identified with Duncan's multiple range test.

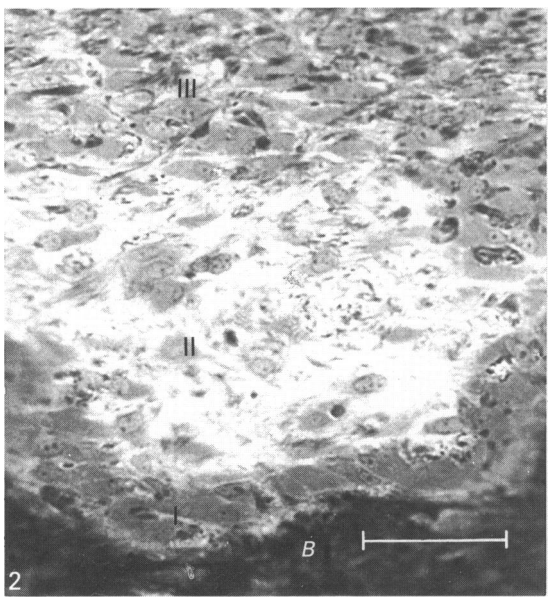
RESULTS

Microscopic examination of the periosteum from both palate and skull revealed a zonal organisation with a highly variable overall thickness (range 70–150μm). The light (Figs. 1, 2), and transmission electron micrographs (Fig. 3) illustrate these zones, which were identified as follows: Zone I, a layer of osteoblasts in contact with the bone surface and occasional smaller, compact cells, resembling fibroblasts; Zone II, a relatively translucent zone with capillaries; Zone III, a zone consisting of cells intermingled with collagen fibrils.

The thickness of these zones varied greatly in absolute terms, but Zone I was always relatively thin (10–20μm), whereas Zones II and III were always several times thicker (20–80 μm).

Although these zones are evident with both the light and electron microscope, the features on which a distinction can be made are revealed by quantitation (Table 1).





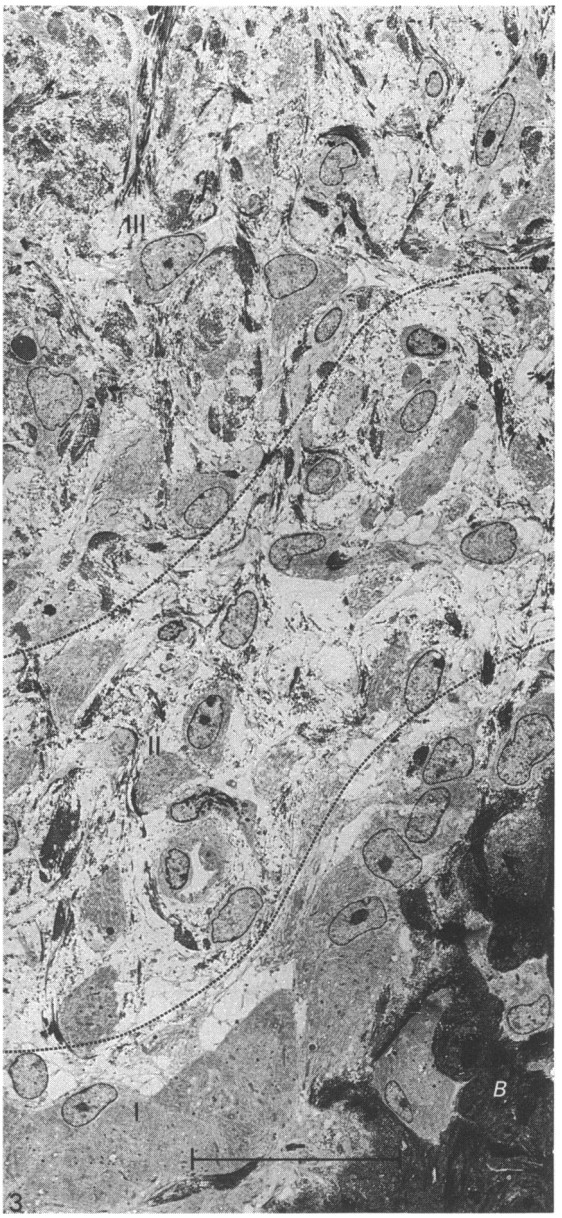


Fig. 1. Light micrograph of semithin epoxy-embedded section of periosteum (*P*) from palate showing three zones (I, II, III). *OM*, oral mucosa; *B*, mineralised surface of bone. Toluidine blue; bar, 25 μm.

Fig. 2. Light micrograph of semithin epoxy-embedded section of periosteum from skull showing three zones (I, II, III). *B*, mineralised surface of bone. Toluidine blue; bar, 25 μm.

Fig. 3. Transmission electron micrograph of periosteum from palate. Differences in the cell and fibre composition of the three layers I, II, III are evident. *B*, mineralised surface of bone. lead citrate and uranyl acetate; bar, 25 μm.

Zone I in both bony sites was predominantly occupied by osteoblasts, which represented 90% of the cells present. There was often a supra-osteoblast layer consisting of smaller, more compact cells with large quantities of endoplasmic reticulum (Fig. 3). The next major volumetric component was collagen fibrils, amounting to 15% of the volume. In Zone II, the largest volume was occupied by

Table 1. *Relative volume of tissue constituents* (*mean* ± S.E.M. mm^3/cm^3)

Tissue components	Palate			Skull		
	Zone 1	Zone 2	Zone 3	Zone 1	Zone 2	Zone 3
Fibroblasts	42±5	282±28	346±46	15±4	231±10	324±152
Osteoblasts	613±47	0	0	752±53	0	0
Other cells	4±1	9±3	3±2	0	5±1	2±1
Collagen	184±20	205±37	412±65	126±20	328±69	505±139
Elastin	0	0	1±1	0	0	0
Vessels	0	120±39	5±5	0	173±82	11±10
Nerves	0	0	0	0	4±4	0
Matrix	157±46	384±14	233±42	108±29	259±1	189±25

Table 2. *Fibroblast parameters in different zones of periosteum* (*mean values* ± S.E.M.)

Parameters	Palate			Skull		
	Zone 1	Zone 2	Zone 3	Zone 1	Zone 2	Zone 3
Numerical density cells/cm^3	$4{\cdot}7\times10^{7}$ $\pm1{\cdot}9\times10^{7}$	$2{\cdot}2\times10^{8}$ $\pm4{\cdot}0\times10^{7}$	$2{\cdot}1\times10^{8}$ $\pm1{\cdot}0\times10^{8}$	$3{\cdot}4\times10^{7}$ $\pm1{\cdot}6\times10^{7}$	$4{\cdot}5\times10^{8}$ $\pm2{\cdot}1\times10^{8}$	$6{\cdot}5\times10^{8}$ $\pm6{\cdot}1\times10^{7}$
Surface density (cm^2/cm^3)	154·7±29·9	671·7±171·2	942·0±430·4	113·9±55·4	1045·2±110·7	1805·0±492·3
Profile cross-sectional area (cm^2)	$3{\cdot}5\times10^{-7}$ $\pm1{\cdot}6\times10^{-7}$	$3{\cdot}89\times10^{-7}$ $\pm7{\cdot}0\times10^{-8}$	$4{\cdot}9\times10^{-7}$ $\pm1{\cdot}6\times10^{-7}$	$1{\cdot}9\times10^{-7}$ $\pm7{\cdot}4\times10^{-8}$	$2{\cdot}7\times10^{-7}$ $\pm1{\cdot}1\times10^{-7}$	$2{\cdot}8\times10^{-7}$ $\pm1{\cdot}1\times10^{-7}$
Cell volume (cm^3)	$3{\cdot}6\times10^{-10}$ $\pm2{\cdot}1\times10^{-10}$	$3{\cdot}8\times10^{-10}$ $\pm1{\cdot}0\times10^{-10}$	$5{\cdot}6\times10^{-10}$ $\pm2{\cdot}8\times10^{-10}$	$1{\cdot}6\times10^{-10}$ $\pm0{\cdot}6\times10^{-10}$	$2{\cdot}3\times10^{-10}$ $\pm1{\cdot}4\times10^{-10}$	$2{\cdot}4\times10^{-10}$ $\pm1{\cdot}4\times10^{-10}$
Cell surface/volume	42·9	176·8	168·2	71·2	454·4	752·1

matrix (32%), which probably accounts for its translucent appearance by microscopy. Fibroblasts and collagen were present in equal proportions (25%), and vascular elements, predominantly capillaries, occupied between 12% and 17% of the volume. The majority of cells were fibroblasts, with endothelium making up most of the remainder. Zone III had the highest relative volume of collagen fibrils (46%) and fibroblasts (34%) of the three layers, with matrix amounting to 18%. Fibroblasts made up 94% of all cell types present.

The values for the relative density of tissue components were compared between the three zones in each site and between the same zones in the two bony sites using a multivariate analysis of variance. Overall, for all components, there were significant ($P < 0{\cdot}05$) differences between the three zones in each site but not between the same zones in different sites. When individual components were examined, fibroblasts and ground substance were found to occupy a significantly ($P < 0{\cdot}05$) greater relative volume in the palate than in the cranium.

Fibroblasts showed a consistent difference between Zones I, II and III in both palate and skull (Table 2). The fibroblast-like cells adjacent to the osteoblasts in Zone I were different from fibroblasts in the other zones in that they had a significantly ($P < 0{\cdot}05$) lower numerical density and surface density; they also had a smaller cell volume and a lower cell surface-to-volume ratio than in other zones, but this was not statistically

significant. This means that they would tend to be more compact and isodiametric than normal fibroblasts. Such quantitative data is in accord with their appearance in electron micrographs as smaller and more regular cells than the fibroblasts evident in Zones II and III. There was a tendency for a gradation in the values of all the fibroblast parameters between Zones I and III, but the differences between Zones II and III were not significant.

DISCUSSION

In view of the important role that periosteum plays in healing following injury, and because of its use as a potential source of osteoprogenitor cells after grafting, it is surprising that there is so little precise information available on the organisation of this tissue. The original division of periosteum into two layers (Tonna, 1965) was used by Tang & Chai (1986), who demonstrated differences between the osteogenic cells of the cambial layer and fibroblasts of the fibrous layer. It seems clear that the almost continuous layer of cells on the bone surface in Zone I represent osteoblasts, since the surfaces we examined were in young dogs that were still growing. In resorbing surfaces, such as the nasal aspect of the growing palate, this zone contains large numbers of osteoclasts (unpublished observations). When a mucoperiosteal flap is raised on the palatal surface or when periosteum is dissected from calvaria (Tenenbaum *et al.* 1986) the differentiated osteoblasts are left on the bone surface. As this elevated tissue can form bone both *in vitro* (Tenenbaum *et al.* 1986) and when transplanted *in vivo* (Skoog, 1965; Ritsila *et al.* 1972), osteoprogenitor cells are presumably present in the tissue, possibly represented by the fibroblast-like cells that were observed adjacent to the osteoblasts in electron micrographs. The significant differences between such cells and those in Zones II and III tend to support the suggestion by Tang & Chai (1986) that there is a population of osteogenic cells morphologically distinct from fibroblasts.

The importance of adequate vascular perfusion of the periosteum in achieving osteogenesis has often been emphasised (Finley, Acland & Wood, 1978; Canalis & Burstein, 1985). In Zone II blood vessels occupy a greater relative volume than in either of the other zones. Simpson (1985), in a study of the blood supply to the periosteum of long bones, suggested that the intrinsic periosteal vessels lay between the cambial and the fibrous layers; our results indicate that the majority of vessels are within Zone II, with only half as many present, in volumetric terms, in the fibrous layer (Zone III).

Zone III is readily visible with the light microscope because of the affinity of collagen for many histological stains. Consequently, the term 'fibrous layer' is traditionally applied to this layer. However, these fibres are different from the large coarse bundles present in the submucosa or hypodermis and consist mainly of small bundles of fibrils. In quantitative terms, almost as much of this layer is occupied by fibroblasts as by collagen, but these cells are not prominent in histological preparations. Although cells are not quantitatively the largest component of Zone II, they are relatively conspicuous because of the small quantities of collagen and large amount of matrix (Figs. 1, 2). Thus, this zone has been described as the cambial layer in the literature.

The significant quantitative differences between the tissue components in the three zones of the periosteum at two different anatomical sites confirm the appearances seen in micrographs, and indicate that periosteum does have an organised microanatomy that is consistent in different regions of membrane bone.

SUMMARY

Specimens of skull and palate were taken from 7 beagle dogs after perfusion and the periosteum examined by light and electron microscopy. Three zones were evident in the periosteum, differing in terms of the proportion of cells, fibres and matrix. Zone I consisted of osteoblasts adjacent to the bone surface and a supraosteoblast layer of smaller, compact cells, Zone II was a relatively translucent zone with numerous capillaries and Zone III consisted of cells intermingled with collagen fibrils. Quantification of the relative volumes of tissue components using stereology indicated significant differences between the three zones in each bony site, but not between the two sites. Measurement of numerical density, surface density, profile cross-sectional area, cell volume and cell surface/volume ratio of fibroblast-like cells revealed marked differences between these cells in Zone I and the other two zones; it is possible that the fibroblast-like cells seen in Zone I represent osteoprogenitor cells. Zone II represents the classical cambial layer and contains the majority of the vascular elements present in the periosteum. Zone III contains large volumes of fibroblasts and collagen fibrils and corresponds to the classical fibrous layer. The similarity of this zonal organisation in different regions suggests that periosteum has a consistent structure in membrane bone.

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REFERENCES

AITASALO, K. & LEHTINEN, R. (1985). The influence of a free periosteal transplant on bone healing in the irradiated tibia. *Scandinavian Journal of Plastic and Reconstructive Surgery* **19**, 237–244.

BARO, W. B. & LATHAM, R. A. (1981). Palatal periosteal response to surgical trauma. *Plastic and Reconstructive Surgery* **67**, 6–14.

CANALIS, R. F. & BURSTEIN, F. D. (1985). Osteogenesis in vascularized periosteum. *Archives of Otolaryngology* **111**, 511–516.

FINLEY, J. M., ACLAND, R. D. & WOOD, M. B. (1978). Revascularized periosteal grafts: a new method to produce functional new bone without grafting. *Plastic and Reconstructive Surgery* **61**, 1–6.

HABEL, M. B. & MANISCALCO, J. E. (1981). Observations on the ultrastructure of the pericranium. *Annals of Plastic Surgery* **6**, 103–111.

HOLTROP, M. E. (1975). The ultrastructure of bone. *Annals of Clinical and Laboratory Science* **5**, 264–271.

MOCK, H. E. (1928). Periosteal transplants in the repair of delayed union, ununited fractures and loss of bone substance. *Surgery, Gynecology and Obstetrics* **46**, 641–657.

MULLER, W. (1980). Stereology of inflammatory connective tissue infiltrates in oral mucosa. *Pathology, Research and Practice* **166**, 271–248.

PERKO, M. A. (1979). Two stage closure of cleft palate. *Journal of Maxillofacial Surgery* **7**, 76–80.

RHODIN, J. A. G. (1974). *Histology. A Text and Atlas*, p. 196. New York: Oxford University Press.

RITSILA, V., ALHOPURO, S., GYLLING, U. & RINTOLA, A. (1972). The use of free periosteum for bone formation in congenital clefts of the maxilla. *Scandinavian Journal of Plastic and Reconstructive Surgery* **6**, 57–60.

SATOH, T., TSUCHIYA, M. & HARII, K. (1983). A vascularized iliac musculo-periosteal free flap transfer: a case report. *British Journal of Plastic Surgery* **36**, 109–112.

SCHROEDER, H. E. & MUNZEL-PEDRAZZOLI, S. (1973). Correlated morphometric and biochemical analysis on gingival tissues. Morphometric model tissue sampling and test of stereologic procedures. *Journal of Microscopy* **99**, 301–329.

SIMPSON, A. H. R. W. (1985). The blood supply of the periosteum. *Journal of Anatomy* **140**, 697–704.

SKOOG, T. (1965). The use of periosteal flaps in the repair of the primary palate. *Cleft Palate Journal* **2**, 332–339.

SQUIER, C. A. & KREMENAK, C. R. (1982). Quantitation of the healing palatal mucoperiosteal wound in the beagle dog. *British Journal of Experimental Pathology* **63**, 573–584.

TANG, X.-M. & CHAI, B.-F. (1986). Ultrastructural investigation of osteogenic cells. *Chinese Medical Journal* **99**, 950–956.

TENEBAUM, H. C., PALANGIO, K. G., HOLMYARD, D. P. & PRITZKER, K. P. H. (1986). An ultrastructural study of osteogenesis in chick periosteum *in vitro*. *Bone* **7**, 295–302.

TONNA, E. A. (1965). Response of the cellular phase of the skeleton to trauma. *Periodontics* **4**, 105–114.

TONNA, E. A. (1975). Electron microscopy of aging skeletal cells. III. The periosteum. *Laboratory Investigation* **61**, 609–632.

TONNA, E. A. & CRONKITE, E. P. (1961). Autoradiographic studies of cell proliferation in periosteum of intact and fractured femora of mice utilizing DNA labelling with H^3-thymidine. *Proceedings of the Society for Experimental Biology and Medicine* **107**, 719–730.

WARSHAWSKY, H. & MOORE, G. (1965). A method of fixation and decalcification of rat incisors for electron microscopy. *Anatomical Record* **151**, 431.

WEIBEL, E. R. (1979). *Stereological Methods*, Vol. 1. New York: Academic Press.

WEIBEL, E. R. & GOMEZ, D. M. (1962). A principle for counting tissue structures on random sections. *Journal of Applied Physiology* **17**, 343–348.