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Structural and Cellular Differences between Metaphyseal and Diaphyseal Periosteum in Different-aged Rats

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Abstract

In both physiological and pathological processes, periosteum plays a determinant role in bone formation and fracture healing. However, no specific report is available so far focusing on the detailed structural and major cellular differences between the periosteum covering different bone surface in relation to ageing. The aim of this study is to compare the structural and cellular differences in diaphyseal and metaphyseal periosteum in different-aged rats using histological and immunohistochemical methods.

Four female Lewis rats from each group of juvenile (7-week old), mature (7-month old) and aged groups (2-year old) were sacrificed and the right femur of each rat was retrieved, fixed, decalcified and embedded. 5 µm thick serial sagittal sections were cut and stained with Hematoxylin and Eosin, Stro-1 (stem cell marker), F4/80 (macrophage marker), TRAP (osteoclast marker) and vWF (endothelial cell marker). 1mm lengths of middle diaphyseal and metaphyseal periosteum were selected for observation. The thickness, total cell number and positive cell number for each antibody were measured and compared in each periosteal area and different-aged groups. The results were subjected to two-way ANOVA and SNK tests.

The results showed that the thickness and cell number in diaphyseal periosteum decreased with age (p<0.001). In comparison with diaphyseal area, the thickness and cell number in metaphyseal periosteum were much higher (p<0.001). There were no significant differences between the juvenile and aged groups in the thickness and cell number in the cambial layer of metaphyseal periosteum (p>0.05). However, the juvenile rats had more Stro1+, F4/80+ cells and blood vessels and fewer TRAP+ cells in different periosteal areas compared with other groups (p<0.001). The aged rats showed much fewer Stro1+ cells, but more F4/80+, TRAP+ cells and blood vessels in the cambial layer of metaphyseal periosteum (p<0.001).

In conclusion, structure and cell population of periosteum appear to be both age-related and site-specific. The metaphyseal periosteum of aged rats seems more destructive than diaphyseal part and other age groups. Macrophages in the periosteum may play a dual important role in osteogenesis and osteoclastogenesis.
Introduction

The periosteum is a type of connective tissue developed from mesodermal cells during the embryonic development. It is tightly attached to various bone surfaces through Sharpy’s fibres, forming a thin but very tough membrane. In both physiological and pathological processes, periosteum plays a determinant role in both bone formation and fracture healing in addition to the involvement of other important factors such as growth factors and mechanical loading [1-3]. The periosteum contains progenitor cells both osteogenic and chondrogenic in nature, as well as other related bioactive factors, and is highly vascularised [1, 4, 5]. Transplantation of autogenous and allogogenous periosteum have been applied successfully to repair various-sized bone or cartilage defects, in particular to large bone defects [6-10]. However, the availability of periosteum, morbidity associated with harvest and immunological concerns are still barriers against large scale in vivo application of periosteum transplantation. Interestingly, the progenitor cells in the bone marrow and periosteum have been successfully applied in bone tissue engineering with various scaffold materials [11-17]. Some researchers have attempted to make artificial periosteum by seeding progenitor cells onto a membrane and achieved positive results in repairing bone defects in vivo [18, 19]. However, current artificial periosteum, which is based on single type of cell structure, is still far from clinical application, primarily due to the questionable cell viability, stability and long-term function. One of the major challenges in tissue-engineered periosteum is to form the cellular structures similar to native periosteum, which can represent the stage of active bone formation. Therefore, it is imperative to understand the cellular structures of periosteum and their relationship during bone development and ageing. To date, however, no clear histological basis is available for the selection of donor-site or age-related periosteal grafts.

Previous studies have revealed that periosteum consists of two different layers [20]. The outer fibrous layer is composed of fibroblasts, collagen, elastin fibers, nerve, and microvascular network. The inner cambial layer is highly cellular containing mesenchymal stem cells, fibroblasts, osteogenic progenitors and osteoblasts. However, no study has documented the detailed structural and specific cellular differences between the periosteum on different bone surface, such as the difference between metaphyseal and diaphyseal periosteum.

Age is another important factor affecting the structure and function of periosteum. Some age-related changes in periosteum have been reported including decreased periosteal fibroblast number, fibrous layer thickness, osteoblast number, collagen formation, osteoid zones and vessel density throughout the periosteum [21-24]. Depending on the locations of bone formation or resorption periosteum shows corresponding structural changes with ageing [21, 25]. However, the detailed information about the age-related structural and cellular changes in different periosteal areas is still unclear. We hypothesize that age-related changes in periosteum are also site-specific. Therefore, the distribution of mesenchymal stem cells, macrophages, osteoclasts, blood vessels and structural changes in metaphyseal and
diaphyseal periostea from the femurs of different aged rats were investigated in this study.

Materials and Methods

Animal samples and slices

This study was carried out according to the guideline of the Animal Ethics Committee of the Queensland University of Technology. Three different aged groups of female Lewis rats were utilized with four rats in each group. The juvenile, mature, and aged groups were 7-week old, 7-month old, and two-year old rats respectively. The right femur of each rat was retrieved after the animals were sacrificed. The tissue samples were fixed with 4% paraformaldehyde for 12 hours at room temperature, then decalcified in 10% EDTA and embedded in paraffin. Serial sections of 5 µm thick sagittal slices were cut from the paraffin blocks using a microtome (Leica Microsystems GmbH Wetzlar, Germany). The slices near the central sagittal plane were used for subsequent experiments.

Definition and selection of observed tissue areas

The diaphyseal and metaphyseal periostea were selected for the observation. The diaphyseal periosteum was selected from 1 mm length of periosteum in the middle of diaphyseal area and the metaphyseal periosteum was selected from 1 mm length of periosteum from metaphyseal area starting from the mesial border of growth plate on upper metaphysis.

Structural observation

Four slices from each rat sample were stained with Hematoxylin and Eosin (HD Scientific supplies Pty Ltd, Kings Park, NSW, Australia). The images were captured under ×200 magnification. The thickness of fibrous and cambial layers on the middle line perpendicular to the periosteum in each microscopic field and cell number of each layer throughout each periosteal area were measured using Axion software (Carl Zeiss Microimaging GmbH, Göttingen, Germany) under a microscope (Carl Zeiss Microimaging GmbH, Göttingen, Germany). The data from the four slices were averaged and recorded for subsequent analysis.

Immunohistochemistry

Four specific cell markers, Stro-1 (mouse anti-human, Chemicom International Inc., Temecula, CA, USA), F4/80 (Rat anti-mouse, ABR-Affinity BioReagents Inc., Golden, CO, USA), TRAP (tartrate-resistant acid phosphatase)(mouse anti-human, Lab Vision Co., Fremont, CA, USA) and vWF (mouse anti-human, Chemicom International Inc., Temecula, CA, USA), were utilized to identify mesenchymal stromal cells, monocytes/macrophages, osteoclasts and blood vessels in each periosteal sample of different-aged groups. To validate the results, each experiment was repeated at least three times.
Prior to immunoperoxidase staining, endogenous peroxidase activity was quenched by incubating the tissue sections with 3% H$_2$O$_2$ for 20 minutes. All sections were blocked with 10% swine serum for 1h. The enzymatic treatment was used to expose epitopes by incubating the slices with proteinase K (ready-to-use, DakoCytomation, CA, USA,) for 10 minutes at room temperature. Sections were then incubated with optimal dilution of primary antibody Stro-1 (1:100), F4/80 (1:50), TRAP (1:20) and vWF (1:100) overnight at 4 °C. Sections were then incubated with a biotinylated swine-anti-mouse, rabbit, goat antibody (DAKO Multilink, CA, USA) for 15 minutes, and then incubated with horseradish peroxidase-conjugated avidin-biotin complex (ABC) for 15 minutes. Antibody complexes were visualized after the addition of a buffered diaminobenzidine (DAB) substrate for 4 minutes. The reaction was stopped by immersion and rinsing of sections in PBS. Sections were then lightly counterstained with Mayer’s haematoxylin and Scott’s Blue for 40 seconds each, in between 3 minute rinses with running tap water. Following this, the sections were dehydrated with ascending concentrations of ethanol solutions, cleared with xylene and mounted with a cover slip using DePeX mounting medium (BDH Laboratory Supplies, England).

Controls for the immunohistological staining procedures included conditions where the primary antibody was omitted. In addition, an irrelevant antibody (IgG), which was not present in the test sections, was used as a control.

Under ×400 magnification, the positive cell number from each cell population in each periosteal area was counted using a microscope (Carl Zeiss Microimaging GmbH, Göttingen, Germany) and AxioVision software (Carl Zeiss Microimaging GmbH, Göttingen, Germany). Each measurement included three different slices and the average was recorded for subsequent analysis. To eliminate the effect of the difference in total cell number and periosteum thickness in different groups on the positive cell number and blood vessel counting, the Strol-1$^+$, F4/80$^+$, and TRAP$^+$ cell numbers were normalized to the cell number per 100 total cells in each specific group. The blood vessel number was normalized to the vessel number in 0.03mm$^2$ periosteal area.

**Statistical analysis**

All the data were analyzed according to the age of rats and the periosteal sites by two-way ANOVA using the General Linear Model and post-hoc testing performed using Student-Neuman-Keuls comparison (SNK) for group homogeneity. The significance level was set at $p \leq 0.05$. Analysis was performed using SPSS software (SPSS Inc, Chicago Il).

**Results**

**Structural differences in different periosteal areas and aged groups**

The two-way analysis of variance model indicated that the age of rats and the
periosteal sites both influenced, independently and interactively, the thickness and cell number in the periosteum (p<0.001). In diaphyseal periosteum, both thickness and cell numbers decreased with age in both cambial and fibrous layers (Fig.1). However, no significant differences were found in the thickness of the fibrous layer between juvenile and mature groups (p=0.39). There was no difference in cell numbers in the fibrous layer between mature and aged rats (p= 0.07) (Fig.1). Both the thickness and cell numbers in the cambial layer decreased significantly with age (p<0.001) (Fig.1). Compared to the diaphyseal area, the thickness and cell number in fibrous and cambial layers were significantly higher in metaphyseal periosteum (p<0.001) (Fig.1).

In general, the metaphyseal periosteum of juvenile and aged animals was thicker and more cellular when compared with the mature group. Notably, there were no significant differences between the juvenile and aged groups in the thickness and cell numbers in the cambial layer of metaphyseal periosteum (p=0.07 for thickness and p=0.14 for cell number) (Fig.1). The mature rats had the thinnest and least cellular cambial layer in the metaphyseal area. The thickness of the fibrous layer in the metaphyseal periosteum from all three age groups was similar (p=0.09), while both mature and aged groups were much less cellular in this layer than the juvenile group (p<0.001) (Fig.1).

**Stro-1 expression in periosteum**

The normalized Stro-1+ cell number was used in this analysis. Site (p=0.04), age (p<0.001) and the interactions between site/age were also significant (p<0.001) in the model for Stro1 expression. In diaphyseal periosteum, Stro-1 was broadly expressed in the cambial and fibrous layers in juvenile rats (Fig. 2). Very few Stro-1 positive cells were found in either cambial or fibrous layers from mature and aged rats compared to the juvenile group (p<0.001) (Fig. 2).

In the metaphyseal area, the juvenile rats had significantly more Stro-1+ cells in both the cambial and fibrous layers compared with the mature and aged groups (p<0.001) (Fig. 2). In the fibrous layer of aged rats there were more Stro-1+ cells compared with the cambial layer and the fibrous layer of mature group (p<0.05). The mature and aged groups had similar number of Stro-1+ cells in the cambial layer. (Fig. 2).

**F4/80 expression in periosteum**

The normalized F4/80+ cell number was used for this analysis. Periosteal sites and age were both significant (p<0.001) factors in the model as well as their interactions. The juvenile rats had significantly more F4/80+ cells in both layers of the diaphyseal periosteum compared with the mature and aged groups (p<0.001) (Fig. 3). Nearly no F4/80+ cells were detected in the diaphyseal periosteum in the mature and aged groups (Fig. 3).
In the metaphyseal area, the F4/80$^+$ cells were found mostly in the cambial and fibrous layers of aged periosteum as well as in the cambial layer of juvenile periosteum. Significantly fewer positive cells were identified in the mature periosteum (p<0.001) (Fig. 3).

**TRAP expression in periosteum**

The normalized TRAP$^+$ cell number was used for this analysis. Site and age (both p<0.001) were significant factors in the model as well as the interaction between site/age (p<0.001). TRAP$^+$ cells were mostly detected in the both layers of the metaphyseal area in aged rats compared with the juvenile and mature rats (p<0.001) (Fig.4). Very few TRAP$^+$ cells were identified in diaphyseal periostum, although the cambial layer in the diaphyseal periosteum of aged rats had more positive cells than both the juvenile and mature aged groups (p=0.004) (Fig. 4). No difference was observed in the fibrous layer of diaphyseal periosteum in three age groups (p=0.147) (Fig. 4).

**vWF expression in periosteum**

The normalized vessel number was used in the analysis. Site and age (both p<0.001) were significant factors for vWF expression with the interaction of site/age also being significant (p<0.001). Blood vessels identified by the vWF staining in diaphyseal periosteum revealed that juvenile rats had a higher degree of vascularization in the cambial layer than the older age groups (p<0.001) with the exception of the fibrous layer (p=0.77)(Fig.5).

In metaphyseal areas the juvenile and aged rats had the higher degree of vascularization in the cambial layer compared to the mature group (p<0.001) (Fig.5). The degree of vascularization in the cambial layer of aged rats was also higher than the fibrous layer (p<0.001). The vessel number in the fibrous layer of mature rats was similar to that of juvenile rats (p=0.154), but greater than that of aged rats (p<0.05)(Fig.5).

**Discussion**

The indispensable role of periosteum in both bone formation and fracture healing is well documented. When the periosteum is stripped off, the healing of bone defects and surrounding soft tissues is seriously compromised [2, 26]. This phenomenon has inspired many studies to focus on the application of periosteal grafts in bone and cartilage regeneration. It is known that periosteum is a thin membrane-like connective tissue covering the external surfaces of most bones and several studies have revealed the general histological and ultrastructural structures of periosteum from various bones [22, 27]. However, the detailed cellular structure of periosteum and its site specificity in relation to ageing are not well understood. In this study, the age-related degeneration (shown as decrease in thickness and cell number) was observed in the diaphyseal periosteum, especially the decrease in cell numbers in both cambial and
fibrous layers in the aged group. However, in the metaphyseal areas, it is worth noting that the aged rats had thicker and more cellular cambial layer than the rats from the mature group although they were similar in the fibrous layer. There was also a noticeable change in cell populations within these areas, in particular an increased fractional number of osteoclasts. These observations would indicate aged-associated resorptive activity of cortical bone in metaphyseal areas, which in turn may have clinical relevance with more fractures reported in metaphyseal areas compared with diaphyseal areas in the elderly. In a recent study by Bliziotes M. et al.,[28] an increase in osteoclast numbers and eroded cortical bone surfaces was found more obvious at the femoral neck of nonhuman primates than at the shaft, especially in the castrated female animals. The nature of the regulation of periosteal bone turnover activity is not clear, but mechanical force and sex hormones are important modulators of periosteal activity. It has been found that low sex steroid levels in female animals are associated with an increase in periosteal resorptive activity [29] and the application of focal mechanical force results in an increase in local bone formation [30].

Stro-1 is thought to be a pluripotent cell marker found highly expressed on various stromal cells [31-33]. Stro-1 positive cells are capable of differentiating into multiple mesenchymal cell lineages including adipocytes, osteoblasts and chondrocytes, as well as hematopoiesis-supportive stromal cells, [32, 33]. In this experiment, a higher percentage of Stro-1 positive cells were found in diaphyseal and metaphyseal periosteum in juvenile rats indicating the high osteogenic/chondrogenic nature of periosteum in this stage. In mature and aged groups, Stro-1 positive cells were significantly decreased and the intensity of Stro-1 staining was weaker in all periosteal areas compared with the juvenile group. In juvenile rats, the expression of Stro-1 was found in periosteal cells, pre-osteoblasts, osteoblasts and early osteocytes embedded in osteoid. Only mature osteocytes were negative for Stro-1. The broad expression of Stro-1 in both layers of juvenile periosteum suggests that both cambial and fibrous layers may be involved in the new bone formation.

To our knowledge no other study has documented the age-related distribution of monocytes/macrophages or osteoclasts in periosteum even though periosteal bone turnover activity and osteoclast distribution have recently been documented in the femoral neck in a series of adult rhesus and Japanese macaques [28]. In our study periosteal monocytes/macrophages and osteoclasts were identified using F4/80 and TRAP as specific cell markers. F4/80 (or EMR1) is a specific monocyte/macrophage marker, while TRAP is mainly expressed by mature osteoclasts. Although both monocytes/macrophages and osteoclasts derive from the haemopoietic precursors and both can be multinucleate, there are still substantial differences existing between these two cell types [34]. The differentiation into the osteoclast lineage from haemopoietic precursors is found prior to macrophage commitment [35]. It has been reported that some growth factors produced by macrophages, such as BMP2 or TGF-β, could promote the osteogenesis and proliferation of osteoblasts and chondrocytes in vitro [36]. Macrophages can develop into osteoclasts only when certain stimuli exist, such as receptor activator of nuclear factor Kappa B ligand (RANKL),
Macrophage-Colony Stimulating Factor (M-CSF) or inflammation factors [37]. In this study numerous macrophages, but limited number of osteoclasts, were found in the diaphyseal and metaphyseal periosteum especially in the cambial layer of juvenile rats. In aged rats both macrophages and osteoclasts were increased in the metaphyseal area. Few macrophages and osteoclasts were found in mature rats and the diaphyseal periosteum of aged rats.

Blood vessels, identified by vWF staining, showed that both cambial and fibrous layers in different periosteal areas in juvenile rats were well vascularized while mature rats had blood vessels predominantly in the fibrous layer. In the juvenile rats more active periosteal osteogenic activity was found in both periosteal areas, which was demonstrated by increased cell numbers of mesenchymal stem cells and macrophages as well as increased thickness of the cambial layer. The high degree of vascularization in the periosteum of juvenile rats suggests a role in nutrient and osteoprogenitor cell supply. In diaphyseal periosteum, blood vessel numbers in the cambial layer decreased with age. However, in the metaphyseal region, the aged rats had more blood vessels in the cambial layer when compared to the juvenile and mature groups. The increase of periosteal vascularisation in aged rats could be related to increased bone resorptive activity. The increased number of blood vessels in the areas of osteoclastic bone resorption is also reported in bone metastasis [38] and ectopic bone resorption [39]. In bone development angiogenesis and bone resorption are closely associated with each other. Vascular endothelial growth factor (VEGF), the most critical growth factor for angiogenesis, has been found to stimulate osteoclast activity [40]. Therefore, osteoclast activity and angiogenesis can be regulated by the common mediators such as VEGF.

Based on the results obtained in this study, it could be concluded that the age-related periosteal structure and cell populations are site-specific. The diaphyseal periosteum showed age-related degeneration, whereas, the metaphyseal periosteum is more destructive in older age. Macrophages in the periosteum may play a dual, age dependent role in bone metabolism with osteogenesis in young rats and osteoclastogenesis in aged rats.
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Legends

Figure 1. Diagrams and pictures (×200) of H&E observation of diaphyseal and metaphyseal periosteum from different-aged groups. The thickness and cell number of the cambium layer in diaphyseal periosteum (Dia-c) decreased with age. No significant differences were found in the thickness of the diaphyseal fibrous layer (Dia-f) in juvenile and mature groups. There were no differences in cell numbers in the fibrous layer in mature and aged rats. There were no significant differences between the juvenile and aged groups in the thickness and cell number in cambial layer of metaphyseal periosteum (Meta-c). The thickness of metaphyseal fibrous layer (Meta-f) from three aged groups was similar to one another, while both mature and aged groups were much less cellular in this layer than in the juvenile group. *: p<0.05. J: Juvenile; M: Mature; A: Aged.

A: diaphyseal periosteum from the juvenile group; B: diaphyseal periosteum from the mature group; C: diaphyseal periosteum from the aged group; D: metaphyseal periosteum from the juvenile group; E: metaphyseal periosteum from the mature group; D: metaphyseal periosteum from the aged group; (In pictures, B: bone tissue; P: periosteum; C: cambial layer; F: fibrous layer)

Figure 2. Diagram and pictures (×400) of Stro-1+ cell distribution in different periosteal areas and age groups. Stro-1 was broadly expressed in both the cambial and fibrous layers of diaphyseal periosteum (Dia-c and Dia-f) from juvenile rats. Very few Stro-1+ cells were found in diaphyseal cambial and fibrous layers from mature and aged rats in comparison with the juvenile group. No significant difference was found in the Stro-1+ cell numbers in metaphyseal periosteum (Meta-c and Meta-f) of the juvenile and mature rats when compared with the diaphyseal area, while both mature and aged groups had much fewer positive cells than the juvenile group in this area. *: p<0.05. J: Juvenile; M: Mature; A: Aged.

A: diaphyseal periosteum from the juvenile group; B: diaphyseal periosteum from the mature group; C: diaphyseal periosteum from the aged group; D: metaphyseal periosteum from the juvenile group; E: metaphyseal periosteum from the mature group; D: metaphyseal periosteum from the aged group; (In pictures, B: bone tissue; P: periosteum)

Figure 3. Diagram and pictures (×400) of F4/80+ cell distribution in different periosteal areas and age groups. The juvenile rats had much more F4/80+ cells in cambial layer of diaphyseal periosteum (Dia-c) compared with fibrous layer (Dia-f) and the other two groups. In metaphyseal area, the F4/80+ cell number in both layers (Meta-c and Meta-f) from aged rats were more than other aged groups. *: p<0.05. J:
Fig. 4 Diagram and pictures (×400) of TRAP⁺ cell distribution in different periosteal areas and age groups. Few TRAP⁺ cells were found in the diaphyseal periostum (Dia-c and Dia-f) in any of the three groups. In the metaphyseal area, the aged rats had much more TRAP⁺ cells in the cambial layer (Meta-c) than the fibrous layer (Meta-f) and the other two groups. *: p<0.05. J: Juvenile; M: Mature; A: Aged.

Fig. 5 Diagram and pictures (×400) of vWF⁺ blood vessel distribution in different periosteal areas and age groups. Juvenile rats had higher degree of vascularization in the cambial layer of diaphyseal periostum (Dia-c) than the other two groups except in the fibrous layer (Dia-f). In metaphyseal area, the rats from aged group had highest degree of vascularization in cambial layer (Meta-c) among all three groups. The degree of vascularization in the cambial layer of aged rats was higher than the fibrous layer (Meta-f). The vessel number in the fibrous layer of mature rats was similar to that of juvenile rats, but more than that of aged rats. *: p<0.05. J: Juvenile; M: Mature; A: Aged.
References:


[38] Li M, Amizuka N. [Histopathological observations on osteolytic bone metastasis]. Clin Calcium 2006;16: 591-97.
