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Minireview

COMPACT BONE STRUCTURE OF UNMODIFIED AND GENETICALLY MODIFIED RABBITS

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ABSTRACT

Bone, a relatively dense material in the sense of matrix, represents the main calcified tissue of the skeleton in mammals. Characteristic cells (osteoblasts, osteocytes, osteoclasts, bone lining cells) are embedded in the matrix. Bone exists in two usually fairly distinct forms: woven bone and lamellar bone (it is composed of compact bone and trabecular bone). Various observations and conclusions have been made concerning the development and histology of the compact bone tissue in mammals, including rabbits. Similar investigations were only rarely performed in transgenic animals. This review summarizes recent results based on the histological analyses of bone tissue in unmodified, as well as in genetically modified rabbits. We provide the information that juvenile genetically modified rabbits with the WAP-hFVIII gene construct dispose fibrolamellar bone tissue which is not observed in unmodified rabbits even in any ontogenetic stages. It is believed that morphological investigation together with molecular and physiological analyses, as well as the use of animal models can contribute to the more detailed information about processes in bone tissue structure with a potential application of the results in biology and human medicine.

Key words: bone tissue; transgenic rabbit; WAP-hFVIII gene; histology

INTRODUCTION

General characteristics of bone tissue in mammals

The skeleton provides structural support for the body, protecting internal organs and housing the bone marrow. It also functions as a reservoir of calcium and phosphate ions and plays a major role in the homeostasis of these minerals. Bone consists of an extracellular matrix, the organic phase of which is composed of the type I collagen, proteoglycans, and noncollagenous proteins including osteocalcin, bone sialoprotein, osteonectin, thrombospondin, and osteopontin. Bone matrix also contains growth factors and cytokines that have an important regulatory role in bone remodeling. The inorganic phase of bone matrix is composed mainly of calcium hydroxyapatite (Compston, 2001).

Bone is permeated and lined by various kinds of specialized cells. Osteoblasts, osteoclasts, and bone lining cells are present on bone surfaces, whereas osteocytes permeate the mineralized interior. Osteoblasts, osteocytes, and bone lining cells originate from local osteoprogenitor cells, whereas osteoclasts arise from the fusion of mononuclear precursors, which originate in the various hemopoietic tissues (Marks and Odgren, 2002).

About the level of the collagen fibers and its associated mineral, bone exists in two usually fairly distinct forms in mammals: woven bone and lamellar bone (Ricqles et al., 1991; Currey, 2002). Woven bone (immature bone, fibrous bone, primary bone tissue) is usually laid down very quickly, more than 4 μm a day and often much more, most characteristically in the fetus and in the callus that is produced during fracture repair.

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The collagen in this type of bone is variable, the fibers being 0.1-3 μm in diameter and they are oriented almost randomly, so it is difficult to make out any preferred direction over distances greater than about a millimeter (Boyde and Jones, 1998). Although woven bone is highly mineralized, it is often quite porous at the micron level. Woven bone texture has been noted in crocodilians, birds, frogs, salamanders and fish as well as in mammals. Lamellar bone (mature bone, secondary bone tissue) is more precisely arranged, and is laid down much more slowly than woven bone, less than 1 μm a day (Boyde, 1980). The collagen fibers and their associated mineral are arranged in sheets (lamellae), which often appear to alter in thickness. The final degree of mineralization of lamellar bone is less than that of woven bone (Currey, 2002). The collagen fibers in lamellar bone form branching bundles, 2-3 μm in diameter (Boyde, 1980), thicker than in most woven bone.

Macroscopically two types of lamellar bone can be distinguished in mammals: compact bone and trabecular bone (Wheater et al., 1987; Weiss, 1989; Creager, 1992; Stevens and Lowe, 1993; Schultz, 1997). Although both types of the bone are most easily distinguished by their degree of porosity or density (Gibson, 1985), true differentiation comes from histological evaluation of a tissue's microstructure. Compact bone (cortical bone) forms mainly the shafts of long bones (diaphyses), the surfaces of their extremities (epiphyses), short bones, and the outer and inner layer (*lamina externa et interna*) of the skull vault. Basic constituents of its structural organization are primary osteons and secondary osteons (Haversian systems). The main difference between both structures is that primary osteons are not surrounded by a reversal line. Lamellae around primary osteons merge smoothly with the surrounding bone. The secondary osteons consist of a central (Haversian) canal, which is surrounded by concentric rings (lamellae) of matrix. Between the rings of matrix, the bone cells (osteocytes) are located in spaces called lacunae. Small channels (canaliculi) radiate from the lacunae to the Haversian canal to provide passageways through the hard matrix. The Haversian canal is 30 to 70 μm in diameter and contains nutrient vessels, nerves, and connective tissue. The Haversian canals communicate with the periosteum, bone marrow, and each other through transverse or oblique channels called Volkmann's canals. Volkmann's canals can be differentiated from Haversian canals by their lack of concentric lamellae. The reversal (cement) line, ring of highly mineralized amorphous substances, separates the secondary osteon from its surroundings (Currey, 2002; Martiniaková et al., 2007). Several layers of lamellae can extend uninterrupted around the circumference of the shaft. They are located on the external surfaces of cortical bone immediately underneath the periosteum (outer circumferential lamellae), and on the internal surface adjacent to the endosteum (inner circumferential lamellae). Angular fragments of previous concentric and circumferential lamellae can fill the gaps between Haversian systems. They are called interstitial lamellae (Weiss, 1989). Trabecular bone (cancellous or spongy bone) constitutes the internal parts of the long bone extremities (epiphyses) and the middle layer (diploe) of the skull vault. The bone consists of irregular, sinuous convolutions of lamellae which create a system of trabeculae and griddles arranged in the direction of biomechanical stress (Rho et al., 1998; Urbanová and Novotný, 2005). The spaces between the trabecular meshwork are occupied by bone marrow (Stevens and Lowe, 1993). In contrast to compact bone, complete osteons usually are absent due to the thinness of the trabeculae. Spongy bone is more metabolically active than compact bone due to its much larger surface area for remodeling.

Classifications of bone tissue for research application

In general, the presence of vascular canals is clearly characteristic of bone tissue. Vascular bone tissues contain an intrinsic network of blood vessels. The nature, number, and orientation of vascular canals are used for classifications based on vascularization. One of the first classifications was made in 1913 by Foote, who described many sections of femoral diaphyses for all extant groups of tetrapods. Later Enlow and Brown (1956) classified bone tissue into three main categories including primary vascular, non-vascular, and Haversian bone tissue. Primary vascular bone tissue consists of primary osteons (simple or primary vascular canals). Various patterns of vascularization can occur in this bone category (e.g.: *longitudinal*, *radial*, *reticular*, *plexiform, laminar, lepidosteoid, acellularfibriform, protohaversian)*. In a bone tissue of the second category (non-vascular bone tissue) vascular canals (primary or secondary) are absent; the tissue is composed solely of cellular lamellae which surrounds a large central marrow cavity or covers some other tissue (e.g., in case of *necrosis*). Finally, Haversian bone tissue is composed of secondary osteons (Haversian systems). According to Enlow and Brown (1956) three subcategories - *irregular*, *endosteal*, *dense* – can be identified within the Haversian bone tissue.

In general, bone may be composed entirely of any one given tissue, but may also represent a combination of two or more tissue types. In an individual, one bone may be composed of one type and another bone within the same individual may be made of an entirely different type. Regions within the same bone may differ in structure. A basic bone type may become replaced or modified by a secondary tissue form.

According to Enlow and Brown (1956) and Ricqles (1975), taking into account not only the vascularization

pattern of the bone tissue, but also its fibrillar organization, Ricqles et al. (1991) distinguished four major categories of the bone vascularisation. In addition to non-vascular bone tissue and bone tissue with secondary vascular canals (secondary osteons) the authors also recognize bone tissue with primary canals oriented in one direction (*longitudinal canals*, *circular vascular canals, radial vascular canals*, *oblique vascular canals*) or more than one direction (*laminar vascularisation*, *plexiform vascularisation*, *reticular vascularisation*).

Both classifications of bone tissue mentioned above (Enlow and Brown, 1956; Ricqles et al., 1991) are internationally accepted and often used in various scientific publications for detailed description of bone tissue types in experimental animals (including rabbits).

Physiological variability in compact bone structure of rabbits

The rabbit is one of the most commonly used animals for medical research, being used in approximately 35% of musculoskeletal research studies (Neyt et al., 1998). This is in part due to ease of handling and the size. The rabbit is also convenient in that it reaches skeletal maturity shortly after sexual maturity at around 6 months of age (Gilsanz et al., 1988). Approximately 70-80% of rabbit's skeleton is composed of compact bone, which is found mainly in the shafts of long bones and surfaces of flat bones. Histological research of the compact bone microstructure can be carried out in two ways: qualitatively and quantitatively. The qualitative approach is used to identify the structural pattern of the compact bone tissue. The quantitative one is used for counts and measures of the basic structural characteristics (primary osteons, Haversian canals, secondary osteons).

According to Wang et al. (1998) rabbit long bones have a very different microstructure from humans. In comparison to the secondary bone structure of mature human bone, rabbits have a primary vascular longitudinal tissue structure, comprising vascular canals of primary osteons running parallel with the long axis of the bone, surrounding the medullary canal as well as the periosteal surface. The bone between these layers is comprised of dense Haversian bone tissue (Enlow and Brown, 1958; Martiniaková et al., 2003; 2005b). Also, primary vascular radial bone tissue can be found in rabbit's bones (Martiniaková et al., 2009). Using quantitative measurements Müller and Demarez (1934) found an average diameter of Haversian canals in rabbits of 12.6 μm; Martiniaková et al. (2003) noted a mean diameter of the canals in the *femur* diaphysis area of 17.49±7.52 μm According to Paaver (1973) the diameter of secondary osteons was 96±1.8 μm in rabbits. The maximum mean osteon diameter described by Martiniaková et al. (2005b) was 223.79+47.69 μm with a mean minimum diameter of 50.79+9.71μm. Whereas the basic structural pattern of bone tissue in a specific part of the skeleton seems to be inherited, some differences in quantitative values can also be affected by age, sex, mechanical strain or diet (Stoker and Epker, 1971; Martiniaková et al., 2005b; Copping, 2005; Martiniaková et al., 2009).

Stoker and Epker (1971) investigated age-related changes in endosteal bone remodeling and balance in the rabbit. Their results showed that there was a progressive, negative endosteal balance in rabbits analogically to that in humans. These findings had some value for the selection of animals for experimental studies on bone loss.

Age-related changes in histological structure of the *femur* between juvenile and adult rabbits were observed by Martiniaková et al. (2005b). These findings indicate that compact bone tissue displayed, in general, primary vascular longitudinal structure in both groups of rabbits. However, a density of secondary osteons was higher in adult individuals. The measured variables of the primary osteons' vascular canals, the Haversian canals and the secondary osteons were higher in juvenile rabbits ($P<0.001$), except for the area of Haversian canals. The study by Copping (2005) revealed also age-related changes in the microscopic structure of the paired frontal bone of the domestic rabbit. Qualitative changes included changes in bone tissue types and developmental processes, and presence or absence of primary and secondary osteons. Quantitative changes were measured by counting primary osteons in the outer table of each frontal bone half within 1.6 mm of the metopic suture. In this region, primary osteons were absent at birth, limited in number at 14 days and were numerous between one and three months of age. In animals older than three months, numbers of primary osteons generally decreased with increasing an age. No animals older than 16 months revealed primary osteons. Possible sources of variability in the relationship between observed primary osteon number and age included ability to recognize primary osteons, methods of sampling and processing of bone slices, sex related differences and diseases afflicting specimen.

Similarly to soft tissues a diet plays an important role also in bone development and its function. Rabbits can be successfully used as an animal model in the studies focused on the effects of various environmental risk factors on bone tissue structure. The effects of nickel and nickel-zinc combination on compact bone structure have been observed by Martiniaková et al. (2009) in rabbits. According to the authors, measured values for vascular canal parameters of primary osteons were significantly lower in rabbits fed the diet with higher Ni content as compared to the control group ($P<0.01$). Peroral administration of Ni-Zn combination to rabbits led to decrease in size of the secondary osteons ($P<0.05$). In

rabbits from the experimental groups, a smaller number of secondary osteons was identified when compared to the control group.

In relation to common laboratory methods applied in bone research, Andrade et al. (2008) recently found interesting effects of freezing on bone histological morphology in rabbits. Bone freezing increased cellular and nuclear areas at cancellous bone and diminished nuclear area at the cortical bone. Cortical bone collagen suffered denaturation proportionally to temperature decrease and to freezing duration. These alterations compromised the morphology of tissues after 90 or 120 days of freezing at the temperature of −70°C. Cells were died during freezing leading to reduce bone antigenicity.

Changes in bone structure of genetically modified rabbits

Transgenic animals are used as experimental models to perform phenotypic studies or for biomedical research (Sathasivam et al., 1999). Other applications include the production of human hormones and proteins such as insulin, anti-thrombin III (to treat intravascular coagulation), collagen (to treat burns and bone fractures), fibrinogen (used for burns and after surgery), human fertility hormones, human hemoglobin, human serum albumin (for surgery, trauma, and burns), lactoferrin (found in mother milk), tissue plasminogen activator, and particular monoclonal antibodies (including one that is effective against a particular colon cancer). Animals mostly used for this work are pigs, cows, sheep, goats, and rabbits.

The first transgenic rabbit was obtained two decades ago (Hammer et al., 1985; Brem et al., 1985) and some factors influencing the efficiency of rabbit transgenesis have been addressed (Chrenek et al., 1998; 2005; Murakami et al., 2002). The efficiency of transgenic rabbit production is low, ranging from 0.3 to 2.5%. In particular, problems such as low pregnancy rate, small litter size, cannibalism, mosaicism and low transgene transmission rates have been observed (Chrenek and Makarevich, 2008).

With respect to bone tissue structure, it was found that adenoviral-mediated transfer of human BMP-6 gene accelerates healing in a rabbit ulnar osteotomy model (Bertone et al., 2004). Therefore, BMP-6 gene could be potently osteoinductive *in vivo* resulting in acceleration of bone repair. In the study of Serhan et al. (2003) transgenic rabbits overexpressing 15- lipoxygenase and endogenous anti-inflammatory lipid mediators exhibited markedly reduced bone loss and local inflammation. The results suggested that lipoxins can be targets for novel approaches to bone diseases, e.g., periodontitis and arthritis, where inflammation and bone destruction are features. It was also published that IgH-transgenic rabbits carrying a productive VDJ-Cmu transgen were B cell-deficient, with a 50-100% reduction in serum IgM and IgG levels (Jasper et al., 2007). The bone marrow of newborn transgenic rabbits contained severely reduced levels of preB cells and almost no B cells. These IgH-transgenic rabbits provided a useful model for studies of B cell development both in bone marrow and in GALT.

In 2004 Chrenek and co-workers generated transgenic rabbits with human blood clotting factor VIII gene (hFVIII), which codes a protein with important therapeutic application for the treatment of Hemophilia A. Given gene construct (WAP-hFVIII), in case of integration, causes expression of recombinant protein in the mammary gland only. However, a slight hFVIII mRNA expression was also found in the spleen, lung and brain of transgenic rabbits (Chrenek et al. 2005), but it was not observed in the bone (Martiniaková et al., 2005a; 2006).

Despite of this fact, Martiniaková et al. (2005a; 2006; 2008a; 2008b) identified a new type of bone tissue - fibrolamellar tissue in juvenile transgenic rabbits with WAP-hFVIII gene construct. This tissue had not been observed in non-transgenic rabbits even at any ontogenetic stages. Fibrolamellar bone tissue was found particularly in large mammals whose bones have to grow in diameter rather quickly (Currey, 2002). It had been reported in *Canis* (dog), *Ovis* (sheep), *Sus* (pig), *Bison* (buffalo), and *Bos* (cattle), and also in fossil bones including *Pharahippus blackburgi* (primitive horse), *Kannemeyeria* (herbivorous mammal-like reptiles), and *Brachiosaurus* and *Plateosaurus* (extinct herbivorous dinosaurs) (Mori et al., 2005). The authors (Martiniaková et al., 2008a; 2008b) also observed non-mineralized parts of basic *substantia* in compact bone tissue of transgenic rabbits leading to a slightly reduced mineralization process in these animals. Histomorphometrical measurements had shown that measured variables of the primary osteons' vascular canals, the Haversian canals and the secondary osteons were higher in 1 month-old (Martiniaková et al., 2008a), 2 month-old (Martiniaková et al., 2008b) and 2.5 month-old (Martiniaková et al., 2006) transgenic rabbits in most cases. Therefore, these rabbits exhibited improvement in blood supplying.

The results observed by Martiniaková et al. (2005a; 2006; 2008a; 2008b) also indicated that changes in qualitative and quantitative histological characteristics of bone tissue in juvenile transgenic rabbits with WAP-hFVIII gene could be caused by genetic manipulations. In other words, fibrolamellar bone tissue had not been identified in rabbits at all. Moreover, it is known that microstructural changes in compact bone tissue between individuals of the same species had been widely conditional to different age (Martiniaková et al., 2005b), length of investigated bone (Jowsey, 1966) and

also to genetic factors (Beamer et al., 2001). However, investigated animals were examined at the same age and they were kept under standard conditions. Some changes in the bone length were observed between transgenic and non-transgenic individuals (Martiniaková et al., 2008a; 2008b). Nevertheless, changes in bone length identified between individuals of the same species had never led to the appearance of a new type of bone tissue in these animals. They were correlated mainly to different bone geometry and mechanical properties (Brianza et al., 2007). In addition, it is widely accepted that genetics plays a substantial role in variability of bone microstructure, with estimates of heritability ranging from 40-93 % (Kelly et al., 1991; Beamer et al., 2001).

CONCLUSION

The research of bone morphology and physiology in animals has not been realized as intensively as in humans in the past. However, studies of bones on cellular and molecular level have revealed a bone tissue as a dynamic, metabolically active and live-important part of the body. Animals can be successfully used as experimental models in many areas of biomedical research including bone biology. This review summarizes recent information about bone tissue structure of mammals with a focus on the compact bone structure variability of rabbits in physiological conditions as well as after genetic modifications. Moreover, we provide interesting findings that transgenesis of an individual by bone-unrelated gene construct can cause changes in compact bone (non-target) tissue structure. Anyway, morphological investigation together with molecular and physiological analyses, as well as the use of animal models can extend our knowledge about physiological processes in bone tissue including bone maintenance, development or remodeling. The results of this progressive research have a potential to be applied in human medicine to improve therapy of bone-related diseases and to design of new medicaments.

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