Analysis of Ancient Bones

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Contributor: Ksenija Šerstņova , Edgars Edelmers , Maksims Zolovs , Māra Pilmane

The analysis of bone microstructure and histological examination currently provides valuable insights into various facets of bone biology, ancient human existence, and bone-related diseases.

bone microstructure micro-computed tomography immunohistochemistry tissue factors

1. Introduction

The analysis of ancient bone remains initiates with a macroscopic evaluation, which is subsequently followed by the identification of their histomorphological structure and comprehension of the bone formation and resorption processes. Bone, categorized as a type of connective tissue, comprises various cell types and an expansive extracellular matrix encompassing the bone cells [1]. Osteocytes are cells that are necessary for maintaining bone homeostasis in the human skeleton, are the most numerous (90–95%), and develop through the osteoblast differentiation process, which occurs together with cell morphological and ultrastructural changes [2][3]. Mature osteocytes are located in lacunae and are interconnected with adjacent osteocytes and other bone cells by their dendritic processes extended in canaliculi, as a result forming a canalicular system (LCS) for cell communication. This system provides osteocytes with oxygen and nutrients, coming from closely located blood vessels, as well as enables intercellular transport of small signaling molecules between the bone cells [4][5].

2. Bone Tissue Factors and Proteins

As active and multifunctional bone cells, osteocytes are involved in numerous physiological processes induced by different factor/protein expressions in and outside bone tissue. First, osteocytes take part in several processes related to bone formation and participate in the synthesis and mineralization of osteoid matrix by producing proteins such as osteocalcin (OCN) and alkaline phosphatase (ALP). In places of bone tissue damage, they are known to secrete osteopontin (OPN) for the induction of new bone formation. By releasing signaling molecules through LCS, osteocytes are capable of inhibiting or stimulating osteoblasts and, thus, of regulating bone formation. Secondly, osteocytes take part in osteoclastogenesis by secreting RANKL, which is essential for normal bone remodeling, and osteoprotegerin (OPG), which protects the skeleton from extensive osteoclastic bone resorption [2][6]. Thirdly, bone tissue is an important reservoir of calcium, and under the induction of parathyroid hormone (PTH), osteocytes cause local demineralization and the further release of calcium in the bloodstream. Together with other functions, the osteocyte is considered to be a mechanosensory and mechanotransducer cell, as it is capable of responding to mechanical stimuli and loads. Moreover, osteocytes tend to act as endocrine cells

that secrete sclerostin protein, which promotes bone resorption, and fibroblast growth factor-23 (FGF-23), the phosphate regulator, in response to increased levels of phosphate in serum [5][7].

Among all previously mentioned factors and proteins that give osteocytes the ability to regulate the activity of osteoblast, osteoclasts, as well as bone remodulation processes, and some other factors can be detected in osteocytes. The process of osteoblasts differentiation into osteocytes is under the control of several transcription factors, such as runt-related transcription factor 2 (Runx2), which can further be expressed by osteocytes [8]. Transforming growth factor β (TGF-β) is predominantly found in osteoblasts but is also detected in osteocytes of human and animal bone tissue. Moreover, TGF-β2 and TGF-β3 isoforms play a role in endochondral and intramembranous bone formation, as they signal to promote osteoprogenitor cell proliferation as well as differentiation into the osteoblasts [9][10]. Heino et al., 2002 [11], found that TGF-β secreted by mouse osteocytes inhibits osteoclastogenesis and osteoclastic bone resorption without affecting the number of mature osteoclasts. Bosetti et al., 2007 [12], proved that cooperation between TGF-β and basic fibroblast growth factor (FGF/FGF-2) also induces osteoblast proliferation as well as inhibits ALP activity with osteoblast mineralization. Expressed by periosteal cells, osteoblasts, and osteocytes, and stored in ECM, FGF-2 is important for bone development, maintenance, and fracture healing, as disruption of the FGF-2 gene in mice causes a decrease in bone formation [13][14]. Bone morphogenetic proteins (e.g., BMP2, BMP4) are members of the TGF-β superfamily that among many functions also play an important role in bone formation. Runx2 is essential for BMP-2 to promote osteoblast differentiation and maturation [9][10]. As for matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases-2 (TIMP-2), they need to cooperate in a balanced manner for normal bone development. MMP-2 is an enzyme responsible for ECM degrading, while TIMP-2 can inhibit the catalytic activity of MMP-2 at its high concentrations and activate pro-MMP-2 at low concentrations, as dysregulation of MMP-2/TIMP-2 expression may lead to pathological conditions [15].

Under bone infection and inflammatory conditions, both innate and adaptive immune cells can regulate bone resorption by secreting different inflammatory (IL-1β, TNF-α, IL-6, IL-17) and anti-inflammatory factors (IL-4, IL-10, and gamma interferon). Inflammatory cytokines stimulate osteoclast formation through the induction of RANKL, while anti-inflammatory cytokines inhibit osteoclastogenesis and prevent bone loss [16].

Although the impact of those numerous factors/proteins/cytokines on bone cells and ECM properties provide researchers with relevant information about the process of bone formation and resorption during embryogenesis, in post-natal life, and within the chronological-related changes, there is a lack of data on their presence in samples of archeological human remains. One of the topics of fossil bone studies is related to the analysis of proteins (collagen, osteocalcin), as they can survive for a long period of time and provide researchers with information about the ancient human paleo diet and bioarchaeology. Schmidt-Schultz and Schultz [17][18], in a series of research on human archaeological bone, separated ECM proteins (collagen, osteonectin, osteopontin, and alkaline phosphatase) via electrophoresis and noticed that their quality is the same as that of recent bones. Schmidt-Schultz and Schultz [19] extracted growth factors (insulin growth factor II (IGF-II), BMP-2, and TGF-b) from ancient compact human bone and tooth dentin and suggested that macroscopic and microscopic analysis in combination with biochemical techniques could allow researchers to obtain more information about the history and evolution of

diseases. Smith et al. [20] in their study used ELISA to determine the relative amount of OCN in the animal bone and noticed that its amount decreases with the increase in diagenetic bone changes. Scott et al. [21] analyzed human femur cortical bone samples using ELISA and did not find any correlation between osteocalcin concentrations and diagenesis, suggesting that the protein can survive across a range of time periods and variable burial environments. It is noticeable that preservation conditions, such as age, humidity, temperature, pH, microorganism invasion, and burial context, may also affect the quality of bone tissue [22].

3. Bone Microstructure

As for the bone microstructure of ancient human bone samples, it is widely studied by osteoarcheologists and biological anthropologist investigators to answer questions about people from the past, their habitat, socioeconomic status, life quality, and diseases in comparison with modern conditions [23][24]. The most common techniques for such studies are computed tomography and/or X-ray radiography, as well as the usage of high-powered microscopes to examine the remains. All these techniques allow researchers to obtain detailed information and visual pictures of bones, without affecting or breaking them [25].

The understanding and studies of bone microstructural change, e.g., osteon population density (OPD), osteon area (On.Ar), bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular spacing/pore size (Tb.Sp), provides researchers with interesting data about the health status of populations and pathological and living conditions of the past [26]. Ancient human skeletons from archaeological sites are a rich source of information on the history of numerous bone diseases, e.g., skeletal dysplasia [27], Paget's disease [28] [29], and osteoporosis. Osteoporosis is one of the most widely examined pathologies of skeletal system change in the study of both the past and the present [30][31].

Not only diet but also lifestyle activities can affect the skeletal system, as exercise is known to increase bone density and improve overall bone health. Chirchir et al. [32] results showed that among chimpanzees, Australopithecus africanus, Paranthropus robustus/early Homo, Homo neanderthalensis, and early Homo sapiens, only recent modern humans have low trabecular density throughout the limb joints, potentially resulting from increased sedentism and reliance on technological and cultural innovations. Later, Chirchir and his colleagues [33] subsequently found that the decrease in physical activities, which came about due to the shift towards farming and industrial practices, played a significant role in the diminished bone density observed in contemporary humans compared to their Holocene predecessors. Gosman and Ketcham [34] conducted a research study on skeletal samples of subadult and young adult individuals from the Late Prehistoric Ohio Valley. Their investigation revealed distinct and interconnected patterns of growth, development, general functional activities, trabecular distribution, and the architecture of the bones. Pitfield et al. [35] also noticed differences in skeletal samples of children from medieval England, as after 7 years of age low-status children from York or Newcastle had more habitual loading on their arm bones than the high-status children in Canterbury, resulting from physical activities as the former entered the workforce.

The combination of Micro-CT imaging technique and histological analysis offers a powerful approach to scientific investigations, particularly when using the Zeiss Xradia Versa 3D X-ray microscopes (XRM)—it is highly versatile and capable of producing superior 3D image quality for a broad array of materials under different conditions. Importantly, this allows for the preservation and continued use of valuable samples, enabling studies that require four-dimensional visualization and in situ analysis. When Micro-CT imaging is combined with histological analysis, it allows for a complementary study of both the macroscopic and microscopic structures of the sample. Histological analysis can provide detailed insights into the cellular level structure and organization, while Micro-CT gives an overview of the overall structural organization in three dimensions. This combination can provide a more holistic and in-depth understanding of the sample, offering superior insights that might not be obtainable when using each technique in isolation [36].

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