General and Comparative Endocrinology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

High metabolic activity of tissue-nonspecific alkaline phosphatase not only in young but also in adult bone as demonstrated using a new histochemical detection protocol

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ARTICLE INFO

Article history:
Received 22 March 2017
Revised 5 May 2017
Accepted 10 May 2017
Available online xxxx

Keywords:
TNAP
Zinc
Metabolism
Growth
Development
Calcification
Mineralisation
Cartilage
Bone
Glyoxal fixation
Ageing
Animal model
Horse
Equine

ABSTRACT

Tissue-nonspecific alkaline phosphatase (TNAP) is playing a key role in bone calcification, as has been demonstrated in different mammalian species including human and rodents. However, to investigate age-related changes during life history, histochemical demonstration of TNAP is severely hampered, particularly in the elderly, by technical difficulties associated with sectioning calcified tissue. Sufficient fixation must precede decalcification since poorly fixed bone tissue is exposed to the deleterious effects of decalcification reagents. In order to find a method that would allow cryosectioning of bone without loss of TNAP activity, we assessed the efficacy of different fixation reagents regarding the effects on structural integrity and TNAP activity using liver and osseous tissue from younger and older horses. The results of this study reveal that glyoxal-based fixatives sufficiently preserved bone tissue for successful cryosectioning without compromising TNAP activity. The method described combines the demonstration of TNAP activity with optimal preservation of tissue morphology in osseous tissue of younger and even of older mammals. As a model species, we selected horse bones in light of potentially higher similarities to ageing history and lifelong locomotion in humans as compared to other, mostly smaller, experimental model species with a much shorter life span and artificial locomotive activity when kept in cages. This may serve as a basis for future studies addressing the impact of different life traits in iconic, domestic and companion animals, which are often patients in veterinary medicine, as well as for basic research on human physiology and pathologies of the musculoskeletal system.

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1. Introduction

In the complex process of skeletal growth, alkaline phosphatases (APs, E.C.3.1.3.1) are key players in the initiation of bone mineralisation (Millán, 2013). The APs are dimeric enzymes, present in most organisms, where they catalyse at alkaline pH the hydrolysis of phosphor-monoesters to release and deposit inorganic phosphate (McComb et al., 1979) in the extracellular matrix, particularly along collagen fibrils and within the lumen of chondrocyte- and osteoblast-derived matrix vesicles (Anderson, 1969; Ali et al., 1970; Glimcher, 2006; Millán, 2013; Buchet et al., 2013). In humans, there exist four isozymes, whereby three are tissue-specific: the intestinal, placental, and germ cell APs. A fourth isozyme, termed tissue-nonspecific AP (TNAP), is expressed at high levels in bone, liver and kidney (Harris, 1990; Millán, 2013) exhibiting the same amino acid sequence, but differing by post-translational glycosylation (Nosjean et al., 1997; Linder et al., 2009). Humans with mutations in the TNAP isozyme have defective bone mineralisation and – depending on the nature of the mutation – phenotypes can vary between severe hypo-mineralisation in mid-life to markedly impaired mineralisation in stillborn foetuses (Whyte, 2010). The importance of TNAP for normal mineralisation was strengthened by the development of knockout mice, which had normal bones at birth, but developed skeletal hypo-mineralisation similar to that seen in patients with hypo-phosphatasia (Waymire et al., 1995; Narisawa et al., 1997; Fedde et al., 1999; Narisawa, 2015). According to Narisawa et al. (1997), this indicates that in the mouse, TNAP appears not to be essential for the initial events of bone mineral deposition but to play a role in the maintenance of this process after birth. The distribution of TNAP during bone growth has been extensively investigated in rodents, and pronounced TNAP expression was reported in hypertrophic chondrocytes of the growth plate as well as in osteoblasts (Roach, 1999; Fedde et al., 1999).

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http://dx.doi.org/10.1016/j.ygcen.2017.05.008
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Please cite this article in press as: Maly, I.P., et al. High metabolic activity of tissue-nonspecific alkaline phosphatase not only in young but also in adult bone as demonstrated using a new histochemical detection protocol. Gen. Comp. Endocrinol. (2017), http://dx.doi.org/10.1016/j.ygcen.2017.05.008

However, less is known about the activity of TNAP in calcified adult bone tissue, which remains a challenge because both, fixation and thorough decalcification of bone are required. It is known that tissue TNAP activity is very sensitive to fixation processes (Doty and Schofield, 1984): according to Erben (1997) and Roach (1999), formaldehyde fixation greatly reduced TNAP activity, and Miao and Scutt (2002) reported destroyed TNAP activity upon fixation of samples with neutral phosphate-buffered formalin (NBF). For this reason, for histochemical demonstration of TNAP activity, Miao and Scutt (2002) recommended fixation of bone samples by periodate-lysine-paraformaldehyde (PLP) according to McLean and Nakane (1974).

In our preliminary experiments we used a fixation protocol as described by Miao and Scutt (2002), but could not detect any TNAP activity. Thus, to determine the influence of every single step of the procedure on TNAP activity, we tested different fixation conditions on unfixed cryosections of horse liver tissue firstly. The respective findings were then adopted to osseous tissue of horses at different ages. Horse bones were selected as a model in light of potentially higher similarities to ageing history and life traits in humans as compared to other, mostly smaller, experimental model species with a much shorter life span and potentially different locomotive activity when kept in cages. This study may serve as a basis for future studies on the function of TNAP not only in developing and growing bones, but can also help to better understand the role of TNAP in physiology and pathologies of the skeletal system in human and other mammals.

2. Materials and methods

2.1. Source of specimens

Fresh bone specimens of equine femoral condyles and livers from 3 horses of different age, 8 months (young animal), 3 years (adolescent), and 8 years (2 years after growth plate closure, compare Henson et al., 1995), respectively, were obtained from the commercial slaughterhouse in Basel, Switzerland and immediately stored on ice after harvest.

2.2. Tissue processing and fixation procedures on liver specimens

Liver tissue samples were cut into small blocks with a razor blade, frozen in isopentane cooled with liquid nitrogen to −50 °C and stored in air-tight tubes at −80 °C until further processing. Ten micrometer thick liver sections were obtained at a cryostat temperature of −20 °C and placed on Super Frost Plus slides (Menzel-Glaeser, Braunschweig, Germany), dried over night at room temperature and fixed at 4 °C in five different fixatives for 24 or 48 h, respectively: (a) 100% Ethanol; (b) 10% neutral buffered formalin (NBF); (c) Periodate-lysine-paraformaldehyde fixative (PLP, McLean and Nakane 1974); (d) Glyo-Fixx™ (purchased from Thermo Shandon, Pittsburgh, PA, USA), and (e) Glyoxal fixative (GF). Proprietary glyoxal fixatives are reportedly useful for histology and immunohistochemistry (Dapson, 2007), but to the best of our knowledge, there are no published recipes for fixatives of proven efficacy that contain glyoxal as principal ingredient. Thus, we created our own GF from the published components, which contains 8% glyoxal, 20% ethanol, 0.1 mM $ZnCl_2$ and 50 mM glycolic acid, adjusted to pH 4.4 with 1 N NaOH, which served as a compromise of both, good histological morphology and TNAP activity.

2.3. Impact of EDTA treatment on TNAP activity in liver sections

Liver sections were incubated in EDTA solution consisting of 10% EDTA and 50 mM Tris, adjusted to pH 7.8 with 1 N NaOH at 4 °C for one day.

2.4. Reactivation of TNAP in liver sections after EDTA treatment

In a first experiment, the chelated liver sections were exposed to 50 mM $MgCl_2$ or to 5 mM $ZnCl_2$ solution for 1 h before staining. In a second experiment, liver sections were exposed to 50 mM $MgCl_2$ solution for 2 h before staining. In a third experiment, sections were exposed to 5 mM $ZnCl_2$ solution for 1 h and subsequently to 50 mM $MgCl_2$ solution for another hour prior to staining.

2.5. Final protocol for the demonstration of TNAP activity in bone tissue

2.5.1. Tissue processing, fixation, degreasing, decalcification and infiltration

Bone specimens were washed in PBS and cut into 4 mm thick slices using a low speed diamond saw (Isomet, Buehler, Switzerland). The slices were fixed in Glyo-Fixx™ or GF for 48 h at 4 °C, rinsed in cold PBS for 2 h and subsequently degreased by incubation at 4 °C for 6 h in 70% ethanol followed by extraction overnight at 4 °C in 100% ethanol. Thereafter, bone segments were transferred into 70% cold ethanol and stored at 4 °C. Degreased bone specimens were decalcified in EDTA solution (consisting of 10% EDTA and 50 mM Tris adjusted to pH 7.8 with 1 N NaOH) at 4 °C. The EDTA solution was continuously stirred and changed every other day. Using this protocol, the specimens were completely decalcified within a time frame of 4–6 days. To remove EDTA from the decalcified tissues, the specimens were rinsed using running tap water for 2 h and placed into infiltration solution (12% PVA (MW 13,000–23,000, 87–89% hydrolysed, Sigma-Aldrich St. Louis, MO, USA), 3% propylene glycol, 0.5% BSA, 20 mM Tris-HCl buffer pH 7.6) for 48 h at 4 °C as previously described (Maly et al., 2013). Bone samples were then removed from the infiltration solution and plunged into isopentane cooled to −65 °C in liquid nitrogen. After freezing, samples were stored at −20 °C until cryo-sectioning was performed.

2.5.2. Cryomicrotomy

Liver and bone samples were mounted to the cryotome stage using the above infiltration solution. Cryostat sections (6 μm thick) were cut using a CryoStar NX70 cryostat (Thermo Scientific, Switzerland) at −23 °C, mounted on SuperFrost microscope slides and air dried.

2.5.3. Reactivation of TNAP in bone sections

Before staining, bone sections were exposed to 5 mM $ZnCl_2$ solution for 1 h and subsequently to 50 mM $MgCl_2$ solution for another hour prior to staining.

2.6. Histochemical demonstration of TNAP activity

TNAP histochemistry was performed as previously described (Maly et al., 2013; Eppler et al., 2016). Briefly, TNAP activity was visualized with a BCIP (5-bromo-4-chloro-3′-indolyphosphate)/NBT (nitro-blue tetrazolium chloride) alkaline phosphatase substrate system (0.57 mM BCIP, 0.30 mM NBT in AMPD buffer (50 mM 2-Amino-2-methyl-1,3-propanediol, 5 mM $MgCl_2$, 100 mM NaCl, adjusted to pH 9.5 with 1 M HCl) at room temperature for 20 min. To test for false non-enzymatic deposition of the reaction product, control tissue slices were incubated in the same medium supplemented with mM tetramisole hydrochloride as a specific inhibitor of alkaline phosphatase activity (Van Belle, 1972; Henson et al., 1995). After washing in 0.5% acetic acid for 10 min, glass coverslips were mounted using solution containing 10% Moviol 4–88 (Sigma, Switzerland), 25% glycerol and 0.2% acetic acid.

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