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The Changing Sensory and Sympathetic Innervation of the Young, Adult and Aging Mouse Femur

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Abstract—Although bone is continually being remodeled and ultimately declines with aging, little is known whether similar changes occur in the sensory and sympathetic nerve fibers that innervate bone. Here, immunohistochemistry and confocal microscopy were used to examine changes in the sensory and sympathetic nerve fibers that innervate the young (10 days post-partum), adult (3 months) and aging (24 months) C57Bl/6 mouse femur. In all three ages examined, the periosteum was the most densely innervated bone compartment. With aging, the total number of sensory and sympathetic nerve fibers clearly declines as the cambium layer of the periosteum dramatically thins. Yet even in the aging femur, there remains a dense sensory and sympathetic innervation of the periosteum. In cortical bone, sensory and sympathetic nerve fibers are largely confined to vascularized Haversian canals and while there is no significant decline in the density of sensory fibers, there was a 75% reduction in sympathetic nerve fibers in the aging vs. adult cortical bone. In contrast, in the bone marrow the overall density/unit area of both sensory and sympathetic nerve fibers appeared to remain largely unchanged across the lifespan. The preferential preservation of sensory nerve fibers suggests that even as bone itself undergoes a marked decline with age, the nociceptors that detect injury and signal skeletal pain remain relatively intact.

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Key words: skeletal, nociceptors, pediatric, genetic disorders, geriatric.

INTRODUCTION

The study of chronic skeletal pain most commonly focuses on diseases such as osteoarthritis, low back pain and fragility fractures which are due in large part to the age-related decline in the mass, quality and strength of the skeleton (Heaney et al., 2000; Melton et al., 2004; Mantyh, 2014). However, there are over 500 human genetic disorders of bone and cartilage. In many cases, the first symptom that prompts diagnosis of these disorders is chronic skeletal pain in the young neonatal and pediatric patient (McCarthy, 2011; Boyce, 2017). Genetic disorders of bone and joint which are accompanied by significant skeletal pain include; osteogenesis imperfecta (Rauch et al., 2002; Semler et al., 2012; Hoyer-Kuhn et al., 2014, Ward et al., 2016, Boyce, 2017), giant cells tumor (Chawla et al., 2013; Karras et al., 2013, Martin-Broto et al., 2014, Gossai et al., 2015), aneurysmal bone cyst (Lange et al., 2013; Pelle et al., 2014), fibrous dysplasia (Boyce et al., 2012; Naidu et al., 2014), Paget's disease (Grasemann et al., 2013) and juvenile arthritis (Clinch and Eccleston, 2009, Boyce, 2017).

Given that much of the young skeleton is cartilaginous and the peripheral nervous system is still developing (Brandi and Collin-Osdoby, 2006; Sacchetti et al., 2007), a major question is where in the young skeleton are the nociceptors that drive this pain and do nerves in the young bone have the same organization, distribution and density as sensory and sympathetic nerve fibers in adult bone? Similarly, an interesting but largely unanswered question is whether as the mass, quality and strength of bone and cartilage decline with age (Exton-Smith et al., 1969; Woolf and Pfleger, 2003), do the nerves that innervate the skeleton also undergo a marked decline?

One issue that has greatly hindered attempts to directly compare the innervation of young, adult and aging skeletons is that whereas young bone generally requires no decalcification prior to tissue sectioning (as it is mostly cartilaginous) both the adult and aging bone are highly calcified and require significant decalcification before tissue sectioning (Hukkanen et al., 1992, Mantyh et al., 2010; Jimenez-Andrade and Mantyh, 2012,

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Abbreviations: CGRP, calcitonin gene-related peptide; DAPI, 4′,6-diamidino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid; NGF, nerve growth factor; TH, tyrosine hydroxylase; TrkA, tropomyosin-related kinase receptor A.

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Chartier et al., 2014). Previous studies have suggested that many antibodies that work well in the non-calcified tissues frequently showed marked loss of immunostaining when subjected to the decalcification process (Arnold, 1988; Shi et al., 1991; Schulze et al., 1997; Hayat, 2002; Mach et al., 2002, Mantyh et al., 2010, Chartier et al., 2014).

To address this problem, in the present study the young mouse femur was used as a positive control, as this skeletal tissue can be processed for immunohistochemistry with or without decalcification and then stained with antibodies raised to a variety of antigens. While many antibodies showed significant loss of signal with decalcification, three antigens that showed virtually no loss of immunostaining between the non-decalcified and decalcified young femur and which worked very well in the young, adult and aging femur were; calcitonin gene-related peptide (CGRP) which labels thinly or unmyelinated peptidergic sensory nerve fibers (Kruger et al., 1989, Hukkanen et al., 1993, Clinch and Eccleston, 2009), tyrosine hydroxylase (TH) which is expressed by post-ganglionic adrenergic sympathetic nerve fibers (Parfitt, 2006; Manolagas and Parfitt, 2010), and platelet endothelial cell adhesion molecule (PECAM-1 and also known as CD31) which labels the endothelial cells of blood vessels (Chartier et al., 2014).

In light of the above observations, in the present study antibodies to CGRP, TH and CD31, age-related changes in sensory nerves, sympathetic nerves and blood vessels were explored in the mouse femur. The femur was chosen as it is the largest load bearing bone in the body. It clearly shows an age-related decline in mass and strength, and fracture of this bone in humans and mice is accompanied by significant pain (Jimenez-Andrade et al., 2007, 2009; Koewler et al., 2007; Freeman et al., 2008, Chartier et al., 2014). The mouse was chosen as it is the most commonly used experimental species in modeling human genetic diseases of bone and cartilage (McCarthy, 2011) as well as in studies of the injured, diseased and aging skeleton (Mantyh, 2014).

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed with young (n = 30), adult (n = 30) and aging (n = 30) male C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) that were 10 days post-partem, 3 months and 24 months of age, respectively. For all qualitative assessments, the number of animals was equal to or greater than 10 and for all quantitative data the "n" was $\geq$5. The mice were housed in accordance with the National Institutes of Health guidelines under specific pathogen-free conditions in autoclaved cages maintained at 22 °C with a 12-h alternating light/dark cycle and access to food and water ad libitum. All procedures adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Institutional Animal Care and Use Committee at the University of Arizona (Tucson, AZ, USA).

Preparation of tissue for immunohistochemistry and histology

Tissue from young (10 days) mice and adult (3 months) and aging (24 months) mice were euthanized and processed according to previously published protocols (Thompson et al., 2016). Young mice (10 days) were deeply anesthetized with CO2 delivered from a compressed gas cylinder then decapitated. Young hindlimbs were then excised and placed in 4% formaldehyde/12.5% picric acid solution in 0.1 M PBS (pH 6.9 at 4 °C) overnight. Following fixation, young tissue was either placed in PBS (pH 7.4) for 48 h and then cryoprotected in 30% sucrose at 4 °C or the femurs were then placed in decalcified solution for 2 weeks in 0.5 M ethylenediaminetetraacetic acid (EDTA) (PBS, pH 8.0 at 4 °C) and then cryoprotected in 30% sucrose at 4 °C for at least 48 h before sectioning. Thus, half of the young femurs were placed in the same decalcification solution as required for the mineralized adult and aging femurs while the other young femurs were cryo-sectioned without going through the decalcification process (i.e., the control limbs). These controls were performed in order to assess the potential effects that the decalcification process had on immunohistochemical staining of a variety of antibodies.

Adult and aging mice (3 and 24 months, respectively) were deeply anesthetized with ketamine/xylazine (0.01 ml/g, 100 mg/10 kg, s.c.) and perfused intracardially as previously described (Chartier, 2014). Similar to young tissue that was placed in the decalcification solution, adult and aging tissue was placed in 4% formaldehyde/12.5% picric acid solution in 0.1 M PBS (pH 6.9 at 4 °C) overnight. Following fixation, the femurs were decalcified for approximately 2 weeks in 0.5 M EDTA (PBS, pH 8.0 at 4 °C). In all cases, the EDTA solution was changed every day and in the adult and aging animals the decalcification was monitored radiographically with a Faxitron MX-20 digital cabinet X-ray system (Faxitron/Bioptics, Tucson, AZ, USA). Following total decalcification, each femur was cryoprotected in 30% sucrose at 4 °C for at least 48 h before being sectioned.

Tissue sections of young, adult and aging femurs were cut at either 20 or 60 μm serially and thaw mounted with two sections of bone per gelatin-coated slide. Sections at 20-μm thickness were stained with Safranin O and 60-μm-thick sections were used for immunofluorescence staining.

Immunohistochemistry and histology

In the present study, we focused on the distal end of the femur although a similar organization and age-related changes reported here were observed in other parts of the femur. A full protocol of the histology and immunohistochemical techniques used here can be found in a previous publication from our lab (Chartier et al., 2014). Briefly, following sectioning slides were dried at room temperature (RT) for 30 min and then washed in PBS for 3 × 10 min. Next, the slides were blocked with 3% normal donkey serum (Jackson ImmunoResearch, Cat# 017-11-121; West Grove, PA, USA) in PBS with