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VITAMIN D DEFICIENCY LEADS TO SENSORY AND SYMPATHETIC DENERVATION OF THE RAT SYNOVIUM

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Abstract—Vitamin D deficiency is associated with increased susceptibility to inflammatory arthritis. Sensory and sympathetic synovial nerves are critical to the development of inflammatory arthritis and spontaneously degenerate in the early phases of disease. These nerves contain vitamin D receptors and vitamin D influences nerve growth and neurotrophin expression. We therefore examined the density of synovial nerves and neurotrophin-containing cells in vitamin D-deficient rats. Seven-week-old Sprague–Dawley rats were fed either control or vitamin D-deficient diets for 4 weeks. Knee synovium sections extending from the patella to the meniscus were immunostained for total nerves, myelinated and unmyelinated nerves, sympathetic nerves, peptidergic and non-peptidergic sensory nerves, and neurotrophins and immune cell markers. In control rats, intimal innervation by unmyelinated sensory fibers was denser than subintimal innervation. In contrast, sympathetic innervation was confined to the subintima. Many sensory axons contained markers for both peptidergic and non-peptidergic nerves. Nerve growth factor (NGF) was primarily expressed by intimal CD163-negative type B synoviocytes, while neurturin, a ligand selective for non-peptidergic sensory neurons, was expressed by synovial mast cells. In vitamin D-deficient rats, there were significant reductions in sensory nerves in the intima and sympathetic nerves in the subintima. While there was no significant change in NGF-immunoreactivity, the number of neurturin-expressing mast cells was significantly reduced in the intima, suggesting that intimal reductions in sensory nerves may be related to reductions in neurturin. Vitamin D deficiency therefore may increase susceptibility to inflammatory arthritis by depleting sensory and sympathetic synovial nerves as a result of reduced synovial neurotrophin content.

Key words: vitamin D, synovium, sensory, sympathetic, arthritis, nerves.

INTRODUCTION

Rheumatoid arthritis (RA) is a debilitating inflammatory disease affecting 1.3 million Americans (Helmick et al., 2008). While genetic risk factors for RA have been identified, environmental factors are also implicated (Arend and Firestein, 2012). For example, vitamin D deficiency has been strongly associated with RA development in humans and inflammatory arthritis in rodent models. An inverse relationship between vitamin D intake and RA risk has been established (Song et al., 2012). In addition, patients with active RA had significantly lower serum vitamin D levels than patients with silent RA (Moghimi et al., 2012). Vitamin D levels in RA patients were also inversely correlated with disease activity scores, pain, and disability (Kroger et al., 1993; Cutolo et al., 2006; Patel et al., 2007; Haque and Bartlett, 2010; Rossini et al., 2010; Turhanoglu et al., 2011; Attar, 2012), and vitamin D-deficient RA patients had an increased number of tender joints (Kerr et al., 2011). A positive association has been observed between vitamin D deficiency and undifferentiated inflammatory arthritis (Heidari et al., 2012), a condition which frequently progresses to active RA, which suggests that vitamin D deficiency may play a role in initiation or early stage of the disease. In rodents, vitamin D deficiency or vitamin D receptor deletion increased disease severity and delayed resolution of inflammatory arthritis (Zwerina et al., 2011; Moghaddami et al., 2012). Conversely, supplementation with the active hormone, 1,25-dihydroxyvitamin D3 or a vitamin D analog prevented development or halted progression of collagen-induced arthritis, and reduced the severity of Lyme-induced arthritis (Cantorna et al., 1998; Larsson et al., 1998). Collectively, evidence supports the conclusion that vitamin D deficiency increases the susceptibility to and severity of inflammatory arthritis.

The exact mechanisms of how vitamin D deficiency contributes to RA severity remain poorly understood. It has been speculated that normal vitamin D levels may be protective by attenuating inflammatory cell activation (Wen and Baker, 2011). Alternatively, vitamin D may

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Abbreviations: αSMA, alpha smooth muscle actin; CGRP, calcitonin gene-related peptide; CD163, cluster of differentiation 163; DAPI, 40,6-diamidino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid; GFRα2, glial-derived neurotrophic factor family receptor alpha 2; MCT, mast cell tryptase; NFH, neurofilament H; NGF, nerve growth factor; PBS, phosphate buffered saline; PGP9.5, protein gene product 9.5; RA, rheumatoid arthritis; SP, substance P; TH, tyrosine hydroxylase.

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influence joint innervation, as abundant evidence has shown that joint innervation plays an important role in RA development. In humans, hemiplegia protects against RA development in the paralyzed limbs (Jacqueline, 1953; Thompson and Bywaters, 1962), and unilateral sciatic nerve transection delayed onset and severity of adjuvant arthritis in denervated limbs of rats (Courtright and Kuzell, 1965). Sensory C fibers appear to be particularly important, as systemic reductions in peptidergic sensory axons induced by capsaicin administration in rats diminished the severity of adjuvant-arthritis (Colpaert et al., 1983; Levine et al., 1986). Sympathetic nerves are also important as guanethidine-induced sympathectomy, catecholamine depletion by reserpine, and administration of β-blockers propranalol, butoxamine or ICI 118,5510 all decreased severity and increased latency to onset of adjuvant arthritis (Levine et al., 1986, 1988). Interestingly, both sympathetic and sensory synovial axons have been reported to undergo partial spontaneous degeneration in RA patients and in the early phases of many rodent inflammatory arthritis models (Konttinen et al., 1990; Mapp et al., 1990, 1994; Imai et al., 1997; Miller et al., 2000; Hukkanen et al., 2002; Weidler et al., 2005; Dirmeier et al., 2008) and synovial axon loss has been suggested to be an early indicator of RA (Levine et al., 1984; Takeba et al., 1999). Therefore, while the relationship between synovial sensory and sympathetic innervation and RA remains to be fully elucidated, these nerves appear to be integrally involved in the development and progression of RA.

We and others have shown previously that peripheral innervation can be regulated by vitamin D levels. Both sensory and sympathetic neurons possess vitamin D receptors, and therefore have the potential to respond directly to vitamin D (Tague et al., 2011; Tague and Smith, 2011). *In vitro*, the extent of neurite outgrowth from cultured primary sensory or central neurons was influenced by the addition of varying levels of 1,25-dihydroxyvitamin D3 (Brown et al., 2003; Tague et al., 2011). Likewise, vitamin D deficiency, *in vivo*, promoted a marked increase in deep tissue sensitivity, and altered the density of skeletal muscle (but not cutaneous) nociceptor innervation (Tague et al., 2011). Vitamin D can also regulate neurotrophin expression in target cells (Kalueff et al., 2004), suggesting that it may also indirectly influence peripheral innervation. Accordingly, this study was conducted to establish whether vitamin D deficiency influences synovial innervation, which might influence the onset and modulation of RA.

EXPERIMENTAL PROCEDURES

Animals and diets

Animal protocols and procedures were in accordance with NIH guidelines for the care and use of laboratory animals and approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. As described previously (Tague et al., 2011), weaned female Sprague–Dawley rats (Harlan Laboratories Inc., Madison, WI, USA) were fed *ad libitum* normal chow until the introduction of experimental diet as outlined below. At 31 days of age, rats were ovariectomized via bilateral hindflank incisions and administered ketoprofen 5-mg/kg s.c. (Ketofen; Fort Dodge Animal Health, Fort Dodge, IA, USA) as a postoperative analgesic. Ovariectomized rats were used to eliminate estrous cycle-driven variations in neuronal VDR expression (Tague and Smith, 2011) and because this model may incorporate risk factors associated with human populations with musculoskeletal pain, RA, and vitamin D deficiency (post-menopausal/estrogen-suppressed females) (Gaugris et al., 2005; Alexander et al., 2007; Khan et al., 2010; Myasoedova et al., 2010). At 48 days of age, rats were randomly assigned to treatment groups and fed one of two diets; control (n = 5): 2.2 IU/g vitamin D (cholecalciferol), 0.47% Ca, 0.3% P (TD.07370; Harlan TekladMadison, WI, USA), or VD- (n = 4): vitamin D-depleted, 2.5% Ca, 1.5% P (TD.07541; Harlan Teklad,). The VD- diet was based on previous studies that reported that increasing dietary calcium from 0.47% to 2.5% normalized serum calcium in prolonged vitamin D deficiency (1.5% P is needed as a counterbalance) (Weishaar and Simpson, 1987) and rats fed this diet exhibited rapid increases in musculoskeletal sensitivity (Tague et al., 2011). After 4 weeks on the diet, serum 25(OH)D concentrations in VD- rats were reduced below 10 nmol/L (compared 54–82 nmol/L in controls) and we found no differences in serum calcium or phosphorous (Tague et al., 2011). At which time subjects were deeply anesthetized and perfused with 50 ml of cold 0.9% saline containing 10 units/ml heparin (APP Pharmaceuticals, Lake Zurich, IL, USA) at a rate of 40 ml/min, followed by 150–200 ml of 4% formaldehyde, prepared in phosphate buffered saline (PBS) from paraformaldehyde (Sigma–Aldrich, St. Louis, MO, USA).

Tissue processing

The bones from the left hind leg were cut out at mid-thigh and ankle leaving the knee joint intact. Knee samples were post-fixed in Zamboni's fixative overnight at 4 °C, washed in PBS changed daily for three days at 4 °C, decalcified in PBS containing 10% EDTA (Sigma) (pH 7.4) for two weeks at 4 °C, and cryoprotected overnight at 4 °C in 30% sucrose. The knee was cut in half along the transverse plane, embedded in tissue freezing media (Electron Microscopy Sciences, Hatfield, PA, USA) frozen on dry ice, and cryosectioned at 20 μm. Each slide contained two sections approximately 400 μm apart. Slides from each animal were stained with hematoxylin and eosin or Giemsa to examine overt morphological changes.

Immunostaining

General. Each staining was completed and analyzed in a batch with a single slide from each animal containing the same section numbers. Thawed sections were pre-incubated in 1.5% donkey serum (Jackson ImmunoResearch, West Grove, PA, USA), 0.5% gelatin (Sigma–Aldrich), and 0.5% Triton X-100 (Sigma–Aldrich) prepared in Superblock (Thermo Scientific, Waltham, MA, USA) for 1 h, incubated overnight with primary antibodies, followed by a 2-h incubation with secondary