Increased Connective Tissue Extracellular Matrix in the *op/op* Model of Osteopetrosis

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Abstract

Mice that are homozygous for the recessive osteopetrosis spontaneous mutation (*op/op*) develop severe osteopetrosis due to a defect in the production of macrophage colony-stimulating factor (M-CSF) and a deficiency in monocyte-derived osteoclasts. Our study describes a novel soft tissue finding in an osteopetrosis (B6C3Fe *a/a-Csf1^{op}/J*) mouse model. Tissues were obtained from B6C3Fe *a/a-Csf1^{op}/J* mice and age-matched wild-type mice, processed for hematoxylin and eosin sections, and comprehensive light microscopic tissue evaluation was performed. Mutant mice had characteristic traits of *op/op* deficiency including missing incisors and domed skulls. Histologically, the bone marrow cavity was effaced by interweaving thick bony trabeculae consistent with osteopetrosis. An increase in a finely granular, basophilic interstitial extracellular matrix (ECM) was observed in the subcutaneous connective tissue of the *op/op* mice when compared with controls. Histochemically, the ECM was negative with periodic acid Schiff and stained dark blue with alcian blue at a pH of 2.5, indicating that it is composed primarily of nonsulfated glycosaminoglycans (GAGs). This work suggests an increased ECM that is composed mainly of GAGs

located in the subcutaneous tissue in *op/op* mice. This increase in ECM may be related to altered matrix production or turnover because of changes in M-CSF production.

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Introduction

Mice homozygous for the recessive osteopetrosis mutation (*op/op* mice) have osteopetrosis and a restricted capacity for bone remodeling and resorption due to osteoclast deficiency [1]. These mice typically have hypocalcemia, hypophosphatemia, generalized reduction in the number of macrophages as well as reduced size and number of osteoclasts, but no abnormalities in osteoblasts [2, 3]. On gross pathologic examination, *op/op* mice are toothless, have generalized skeletal sclerosis, domed skulls, short tails, lower body weight and growth rate, and lack incisors. On histologic examination of the bone, the proximal end of the tibia and the distal end of the femur are widened, the diaphysis does not have a well-defined cortex, the zone of hypertrophied chondrocytes occupies half the thickness of the epiphyseal plate and the maturation of the mandibular incisor extracellular matrix (ECM) does not take place [3, 4]. Partial or complete correction of these defects has been observed following treatment with colony-stimulating factor (CSF-1) [1, 5–7].

Fig. 1. Histopathology of a bone section from a control mouse. HE. $\times 10$.

Fig. 2. Histopathology of a bone section from an *op/op* mouse. Note that the bone marrow cavity is effaced by interweaving thick bony trabeculae consistent with osteopetrosis. HE. $\times 20$.

Connective tissue, one of the most abundant and widely distributed tissues in the body, is metabolically active and provides structural support. Connective tissues have 2 major components: cells and ECM. Fibers and ground substance, which exist outside the cells, are collectively called the ECM. Three major types of fibrous proteins appear in connective tissue: collagen, elastin and reticular fibers.

The ECM component of connective tissue is the dominant one and consists of organic material called ground substance within which fibers are embedded. In addition, there are structural glycoproteins of ECM. Ground substance is composed of glycosaminoglycans (GAGs) and proteoglycans. GAGs include hyaluronic acid, chondroitin sulfate, dermatan sulfate, heparin sulfate and keratin sulfate. GAGs are important components of the connective tissue that bind water and minerals. GAGs can normally be found in various tissues such as cartilage, heart valves and eye vitreous fluid.

Published reports on the histopathologic characterization of the *op/op* osteopetrosis mouse model have been limited to the skeletal system. Using histopathologic and histochemical evaluation, we evaluated various tissues and characterized changes observed in this osteopetrosis animal model. We report novel alterations in the soft connective tissues of the skin that were characterized by a generalized increase in connective tissue GAGs in the subcutaneous tissues. The relevance of this finding in this important model of human disease is that it fills a gap in the knowledge on abnormalities in ECM in osteope-

trolosis. In addition, it provides the foundation for potential future studies looking at mechanisms of this process and its relevance to human disease.

Materials and Methods

Study Animals

Four-week-old female B6C3Fe *a/a-Csf1^{op}/J* mice ($n = 8$) were used in the study. Mice exhibited the classic traits of *op/op* deficiency: missing incisors and domed skulls. Age-matched female mice ($n = 8$) were used as controls. The mice were obtained from Jackson Labs (Bar Harbor, Me., USA). All procedures were in compliance with the Pfizer Ann Arbor Laboratories Animal Care and Use Committee.

Study Tissue Samples and Histopathology

At the time of necropsy, the following tissues were collected: adrenal, bladder, bone, diaphragm, esophagus, eyes, heart, kidney, liver, lung, lymph node, muscle, ovaries, pancreas, sciatic nerve, spleen, skin, thymus, tongue, uterus and vagina. Tissues were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin wax. Paraffin sections, 3 μm thick, were prepared routinely and stained with hematoxylin and eosin and examined by light microscopy.

Histochemical Staining

To better characterize the increased matrix in the subcutaneous tissue, various carbohydrate stains (periodic acid Schiff, PAS, and alcian blue) were applied to skin sections using standard methods [8]. Three-micrometer sections were cut from formalin-fixed, paraffin-embedded blocks, mounted on positively charged glass slides and dried. For the alcian blue staining, different pHs were used (0.4, 1.0 and 2.5).

Color version available online

Fig. 3. Histopathology of the subcutaneous tissue from a control mouse. HE. $\times 10$.

Color version available online

Fig. 4. Histopathology of the subcutaneous tissue from an *op/op* mouse. Note the increase in a finely granular, basophilic interstitial ECM (arrows). HE. $\times 10$.

Color version available online

Fig. 5. Histochemical PAS and alcian blue (pH 2.5) staining of the subcutaneous tissue from a control mouse. Histochemical stain. $\times 10$.

Color version available online

Fig. 6. Histochemical staining of the ECM in an *op/op* mouse subcutaneous tissue. Note that the ECM is PAS negative and stained dark blue with alcian blue at a pH of 2.5 (arrows). Histochemical stain. $\times 10$.

Results

Histologically, the bone marrow cavity and hematopoietic elements of the *op/op* mice were effaced by interweaving thick bony trabeculae consistent with osteopetrosis (fig. 1, 2). No inflammatory, degenerative or vascular changes were noted. An increase in a finely granular,

basophilic interstitial ECM was observed in the subcutaneous connective tissue of the *op/op* mice when compared with controls (fig. 3, 4). No evidence of skeletal muscle degeneration or necrosis was noted.

Histochemically, the ECM was PAS negative and stained dark blue with alcian blue at a pH of 2.5, but not at pH 1 or 0.4 (fig. 5, 6). There were no other tissue differ-

ences between *op/op* and control animals, although 2 of the *op/op* mice had an incidental background finding of retinal photoreceptor degeneration.

Discussion

Monocyte/macrophage colony-stimulating factor (M-CSF) is a glycoprotein growth factor that stimulates the survival, proliferation and differentiation of mononuclear phagocytes (bone marrow precursor cell to monoblast to promonocyte to monocyte to macrophage) and osteoclasts [9, 10]. There are 3 isoforms of CSF-1: (1) secreted glycoprotein, (2) proteoglycan and (3) cell surface glycoprotein [10]. CSF-1 receptor is selectively expressed on mononuclear phagocytes [9]. About 94% of circulating CSF-1 is selectively cleared by macrophages by receptor-mediated endocytosis and intracellular destruction [9]. CSF-1 is found in serum or plasma and has a circulating half-life of 10 min [9]. Macrophage depletion increases the half-life of circulating CSF-1 [9].

Osteopetrosis is a heterogeneous group of heritable conditions in which there is a defect in bone resorption by osteoclasts. This condition is associated with an increased skeletal mass due to abnormally dense bone [11]. In humans, there are 2 forms of osteopetrosis: (1) infantile osteopetrosis, an inherited autosomal recessive defect, and (2) autosomal dominant osteopetrosis [11]. Children with infantile osteopetrosis lack teeth and have unerupted or decayed teeth [12].

The *op/op* mice have been found to have an insertion mutation in the coding region of the M-CSF gene, and are subsequently severely deficient in macrophages and osteoclasts [13]. Therefore, these mice develop osteopetrosis. Other murine models of osteopetrosis include the RANKL knockout, RANK knockout, TRAP knockout and osteoprotegerin-transgenic mice [11]. The *op/op* mice in this study had increased bone density consistent with osteopetrosis and exhibited the characteristic traits of osteopetrosis, including missing incisors and domed skulls.

Hyaluronan (hyaluronic acid, HYA, and hyaluronate) is found in high concentrations in the connective tissues of vertebrates [14]. HYA is the predominant GAG found in loose connective tissues. HYA has a tissue half-life of 12 h to 3 days and is catabolized by receptor-mediated endocytosis and lysosomal degradation locally in macrophages [14]. The lowest concentration is found in blood serum and urine [14]. Histochemically, HYA stains with alcian blue at pH 2.5. In our study, the increased matrix

appeared as a loosely arranged fibrillar or granular material that was alcian blue positive and PAS negative and was considered consistent with a mucinous material. The material separated and surrounded dense collagen bundles and caused slight expansion of the interstitium. The matrix stained dark blue with alcian blue at a pH of 2.5 only, but not at pH 1 or 0.4, indicating that it is composed primarily of nonsulfated GAGs. No abnormalities were evident in the collagenous matrix.

There is a significant reduction in the number of macrophages in various organs in the *op/op* mouse model [15]. In addition, there is the lower population and/or the activity of macrophages which results in defective mineralization in the bone [16]. The increased matrix in connective tissues may be related to a decreased rate of matrix internalization and clearance by macrophages due to decreased numbers of macrophages. In the *op/op* mouse model, ECM maturation does not take place, indicating a role for monocyte-derived macrophages in ECM clearance. This hypothesis is further supported by demonstrating that ECM biosynthesis and release is monocyte/macrophage density dependent in vitro studies [17]. Based on the histochemical findings in our study, the increase in matrix accumulation in connective tissues is probably related to an altered macrophage clearance mechanism of HYA. There are reports of HYA accumulation in cutaneous mucinosis, but this accumulation is not related to M-CSF inhibition. Impaired mucin degradation and enhanced mucin synthesis have been proposed as potential mechanisms of cutaneous mucinosis in humans, usually associated with thyroid disorders (hypothyroidism and hyperthyroidism). It is important to note that mucinous accumulation changes usually resolve following thyroid hormone supplementation [18]. Significant increases in concentrations of parafollicular cells in the thyroid gland are seen in osteopetrotic mice when compared with normal littermates during the first 3 months after birth. However, the parafollicular cell density 8 and 10 months after birth is not different and concentration of parafollicular cells returns to normal after bone matrix synthesis is reduced [19]. This is consistent with our study in which the mice used were of young age (4 weeks old).

Inherited retinal degeneration is widespread among laboratory mice and has been documented in various strains of mice around the world [20, 21]. Retinal degeneration characterized by loss of photoreceptors is reported as an autosomal recessive trait in Swiss-derived strains of mice, including SJL mice [22], and the allele responsible is designated as *rd* [23]. In this study, 2 mice from the *op/op* group had bilateral retinal degeneration character-

ized by loss of the outer photoreceptor layer. This finding is consistent with previously reported work that affected both pigmented and albino mice with complete loss of photoreceptors [22–25].

In conclusion, our work suggests that there is increased ECM that is composed mainly of GAGs located in the subcutaneous tissue in *op/op* mice. This increase in the

ECM may be related to altered matrix production or turnover because of changes in M-CSF production. Potential future studies may include: (1) effects of M-CSF injection or macrophage substitution on ECM accumulation and (2) quantification of subcutaneous macrophages to investigate macrophage HYA clearance.

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