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Temporal expression of proteoglycans in the rat limb during bone healing

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Abstract

Proteoglycans found in the bone extracellular matrix and on the cell surface can complex with HBGFs such as the FGFs, TGFs and BMPs which are known to play key roles in regulating fracture healing. Here we have studied the expression of key PGs during the bone repair process in order to determine the relationship between PG expression and healing status. We created non-critical sized trephine defects just proximal to the distal end of the tibial crest of adult male Wistar rats and examined the healing process histologically as well as by monitoring the temporal expression of mRNA transcripts for ALP, OP and OC, together with HSPG, CSPG and FGF-FGF receptor expression. Following surgery, animals were allowed to recover, and then euthanized after 7, 14, 21 and 28 days post-surgery, at which time tissue was harvested for histological examination and total RNA extracted and the mRNA transcripts examined by quantitative real-time PCR. HS and CSPG expression was generally observed to increase in the days immediately following injury, reaching peak expression two weeks post-surgery. This was followed by a gradual return to basal levels by day 28. The expression patterns of PGs were broadly similar with those of ALP, OP and FGFRs. The increase of mRNA expression for many key PGs detected during bone healing coincided with the elevation of bone markers and FGFRs, and provides further evidence that PGs involved in bone repair act in part through susceptible growth factors, including the FGF/FGFR system. The data presented here indicates that increased proteoglycan expression is involved in the early stages of bone healing at a time when previous studies have shown that the levels of HBGFs are maximal. Hence there exists a rationale for an exploration of the use of exogenous PGs as an adjunct therapy to potentiate the powerful effects of these factors and to augment the natural healing response.

Keywords: Growth factor; Osteoblast; Fracture repair; Heparan sulfate

1. Introduction

Bone healing is a complex morphogenetic process involving the coordinated participation of many types of cartilage-forming, bone-forming, and bone-resorbing cells. (Yamazaki et al., 1999). Cellular activity is regulated by local growth factors, especially HBGFs such as the FGFs, TGFs and BMPs (Barnes et al., 1999; Bolander, 1992) that have also been shown to stimulate bone healing in vivo (Cowan et al., 2005; Kawaguchi et al., 2001). The effect of these GFs is regulated through their binding to cognate, high-affinity receptors, a process that is controlled by a complex interaction with extracellular PGs (Selleck, 2000).

PGs are a ubiquitous family of macromolecules, consisting of a core protein and one or more covalently attached GAG chains, that are found in the ECM, particularly in basement membranes, as well as on or adjacent to cell surfaces (Selleck, 2000). Current data suggests that every tissue has a unique PG profile, which in turn helps to dictate its regenerative capacity. The first PG to be characterised in bone was found to have CS GAG chains, and was thus a CSPG. Fisher et al. (1983) analysed developing subperiosteal bone from fetal calves, young rats and human foetuses and demonstrated the presence of two structurally, chemically,

Abbreviations: HBGFs, heparan-binding growth factors; PG, proteoglycans; FGFs, fibroblast growth factors; FGFR, FGF receptor; TGFs, transforming growth factors; BMPs, bone morphogenetic proteins; ALP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; GF, growth factors; GAG, glycosaminoglycan; ECM, extracellular matrix; CSPG, chondroitin sulfate proteoglycan; HPRT, hypoxanthine guanine phosphoribosyl transferase.

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and immunologically distinct CSPGs. The "small" fraction, with one or two CS chains, consisted in fact of two different CSPGs: decorin (PG-II), with one CS chain, and biglycan (PG-I) with two CS chains (Fisher et al., 1989). Both are strongly expressed in normal bone ECM. While the core proteins of decorin and biglycan are very similar, so conferring broadly similar properties, they are also quite distinct with respect to their pattern of expression. Decorin tends to be uniform throughout osteogenic and non-osteogenic layers of periosteum, while biglycan is expressed in pre-osteogenic cells, both in the periosteum as well as in undifferentiated mesenchymal cells in the bone vascular canals. These latter are thought to be pre-osteogenic cells available for recruitment for the formation of the secondary centre of ossification (Bianco et al., 1990). Subsequent analysis of developing human fetal vertebrae has shown that decorin is maximally expressed at sites of appositional bone growth (under the periosteum), whereas biglycan predominates at growth plates (Bianco et al., 1990).

Decorin has also been shown to have an important interaction with members of the TGF-β family. These are predominantly expressed by platelets into cartilage and bone ECM, where they stimulate osteoprogenitor cell proliferation via activation of their cognate serine/threonine membrane receptors (Alliston and Derynck, 2000). The most intense immunostaining for TGF-β ligands is seen during endochondral ossification (Lieberman et al., 2002). Yamaguchi et al. (1990) showed that TGF-βs bind reversibly to decorin via its core protein, which, through its close association with type I collagen, creates a latent reservoir of TGF-β in the ECM. During periods of bone formation and remodeling, decorin enhances the binding of TGF-β1 to its receptors (Takeuchi et al., 1994), resulting in the recruitment, proliferation, and differentiation of osteoprogenitor cells (Alliston and Derynck, 2000). One of the downstream actions of TGF-β1 on osteoblasts is to stimulate the synthesis and secretion of matrix components such as type I collagen, fibronectin, as well as decorin itself (Takeuchi et al., 1993). Biglycan also interacts with TGF-βs (Hildebrand et al., 1994), but the significance of this for osteogenesis and wound healing is yet to be elucidated. Versican, a large CSPG, is expressed in the developing rat mandible, especially in areas of woven bone (Nakamura et al., 2005) and in the limb bud during prechondrogenic condensation (Shibata et al., 2003). It is thought to function as an anti-adhesion molecule during the initiation of matrix assembly (Knudson and Knudson, 2001). However, its temporal expression during bone healing is unknown.

Relatively poorly characterised, but known to be crucial in bone is another group of PGs which contain HSGAG chains, the HSPGs. HSPGs are exported to the bone progenitor cell surface in three major forms: the cell membrane-intercalated syndecans, the membrane-associated glypicans and the secreted, ECM-resident perlecans. Importantly, perlecan is the most abundant PG present during the early stages of skeletogenesis (Arikawa-Hirasawa et al., 1999). These PGs are also ubiquitously expressed throughout the body, and play particularly important roles during embryogenesis, where they help control morphogenesis (David, 1992). HSPGs have many core protein isoforms, again reflecting the locations and functions of the molecules (Ruoslahti, 1989). Employing osteoblastic MC3T3-E1 cells derived from new-born mouse calvaria, Takeuchi et al. (1990) showed that HSPGs are profusely expressed in vitro during the process of mineralization. It has further been shown with osteosarcoma cells that HSPGs are inserted into the plasma membrane either via plasma membrane-spanning hydrophobic domains in their core proteins, or via covalent linkage to plasma membrane glycosylphosphatidylinositol structures (McQuillan et al., 1992). Using immunohistochemical techniques, Nakamura and Ozawa (1994) were the first to demonstrate the presence of HSPGs throughout in vivo bone. HSPGs were also localised to the osteoblastic Golgi apparatus, demonstrating that these cells were the primary site of synthesis.

When HBGFs are bound to PG/GAG chains, they are protected from extracellular protease degradation (Ruoslahti and Yamaguchi, 1991), a process that has important sequelae for wound healing. Endogenous HBGF-PG complexes act as a large capacity reservoir of GFs that can be rapidly dissociated and mobilised following tissue damage. Such influxes of bioactivity can be prolonged, as the PGs sustain their mitogenic ligands for longer periods on their signaling receptors (Ornitz, 2000), thus potentiating their activity. As such, PGs are potential candidates for clinical application. Despite this potential, relatively little is known about the expression pattern and effects of HSPG and CSPGs in healing tissues, particularly during bone healing.

Bone healing has been well characterised histologically. The process of bone healing in non-critical size defects can be divided into several stages; initial phases, with cellular invasion into the defect, followed by phases of formation of callus tissue, composed of woven bone with varying amounts of cartilage. The callus is then modeled over time through the actions of osteoclasts and osteoblasts, to restore the lamellae and thus normal bone architecture (Campbell et al., 2003; Hiltunen et al., 1993). However, critical bone defects do not heal spontaneously (Schmitz and Hollinger, 1986).

In this study, using non-critical trephine defects in the tibiae, we have examined the bone-healing response of male adult Wistar rats over a 28-day period. We have characterised the temporal expression of PGs thought be critical to bone repair and correlated their expression with the various stages of bone healing. We hypothesized that the healing response would follow a pattern involving the early response of versican, biglycan and decorin CSPGs, thought to be involved in the TGF-β-mediated recruitment, proliferation and differentiation of osteogenic progenitors (Alliston and Derynck, 2000; Takeuchi et al., 1993), followed by the expression of HSPGs that would mediate tissue mineralization (Takeuchi et al., 1990). This was indeed the case. The results suggest that augmented PG exposure may be a valid therapeutic procedure for improved bone healing.

2. Materials and methods

2.1. Animals and surgery

Twenty 300 g, 70-day-old male Wistar rats were used in this study, and all work was conducted after approval from the Animal Ethics Committee of the Institute of Molecular and Cell Biology, Singapore. Surgery was performed on sixteen animals,