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● *Original Contribution*

LOW-INTENSITY PULSED ULTRASOUND PROMOTES CHONDROGENIC PROGENITOR CELL MIGRATION *VIA* FOCAL ADHESION KINASE PATHWAY

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Abstract—Low-intensity pulsed ultrasound (LIPUS) has been studied frequently for its beneficial effects on the repair of injured articular cartilage. We hypothesized that these effects are due to stimulation of chondrogenic progenitor cell (CPC) migration toward injured areas of cartilage through focal adhesion kinase (FAK) activation. CPC chemotaxis in bluntly injured osteochondral explants was examined by confocal microscopy, and migratory activity of cultured CPCs was measured in transwell and monolayer scratch assays. FAK activation by LIPUS was analyzed in cultured CPCs by Western blot. LIPUS effects were compared with the effects of two known chemotactic factors: *N*-formyl-methionyl-leucyl-phenylalanine (fMLF) and high-mobility group box 1 (HMGB1) protein. LIPUS significantly enhanced CPC migration on explants and in cell culture assays. Phosphorylation of FAK at the kinase domain (Tyr 576/577) was maximized by 5 min of exposure to LIPUS at a dose of 27.5 mW/cm^2 and frequency of 3.5 MHz. Treatment with fMLF, but not HMBG1, enhanced FAK activation to a degree similar to that of LIPUS, but neither fMLF nor HMGB1 enhanced the LIPUS effect. LIPUS-induced CPC migration was blocked by suppressing FAK phosphorylation with a Src family kinase inhibitor that blocks FAK phosphorylation. Our results imply that LIPUS might be used to promote cartilage healing by inducing the migration of CPCs to injured sites, which could delay or prevent the onset of post-traumatic osteoarthritis. (E-mail: james-martin@uiowa.edu) **© 2014 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Low-intensity pulsed ultrasound, Articular cartilage, Post-traumatic osteoarthritis, Focal adhesion kinase, Cell migration.**

INTRODUCTION

Osteoarthritis (OA), a joint disorder characterized by progressive loss of cartilage, results in the clinical symptoms of joint pain, stiffness and restricted motion. Although the etiology of OA is unclear, risk factors include aging, obesity and history of joint overuse (Buckwalter et al. 2005; Martin and Buckwalter 2002, 2003). Joint trauma is also strongly associated with OA, particularly when it leads to extensive cartilage damage and chondrocyte death (Buckwalter and Brown 2004; Buckwalter and Martin 2004; Martin et al. 2009). Diverse approaches to the repair of intra-articular cartilage injury have been implemented, including surgical procedures, cell transplantation and scaffold injection; however, these methods have met with limited success (Buckwalter and Mankin 1998; Buckwalter and Martin 2004).

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There have been frequent attempts to use low-intensity pulsed ultrasound (LIPUS) to stimulate cartilage repair. These studies have found that LIPUS stimulates cartilage anabolism by enhancing the production of matrix molecules, including proteoglycan and collagen (Khanna et al. 2009; Korstjens et al. 2008; Naito et al. 2010; Parvizi et al. 1999; Zhang et al. 2002, 2003). In addition, LIPUS has been proven to attenuate the progression of cartilage degradation *in vivo* and has been proposed as a tool to induce mesenchymal stem cells (MSCs) to differentiate into articular chondrocytes (Cui et al. 2007; Ebisawa et al. 2004; Gurkan et al. 2010; Lai et al. 2010; Park et al. 2007; Schumann et al. 2006). However, progress in understanding the mechanism of these benefits has been slow.

Dynamic regulation of cell-extracellular matrix adhesion is required for cell migration, as well as for cell proliferation, differentiation and survival (Lauffenburger and Horwitz 1996; Parsons et al. 2000). The formation and turnover of integrin-associated focal adhesion complexes are regulated not only by cytoskeleton-linked proteins

such as talin, vinculin, α-actinin and paxilin, but also by intracellular signaling proteins such as focal adhesion kinase (FAK), c-Src, protein kinase C, phosphatidylinositol 3-kinase and Rho kinase (Amano et al. 1997; Choi et al. 2008; Critchley and Gingras 2008; Humphries et al. 2007; Huveneers and Danen 2009; Lim et al. 2003; Merlot and Firtel 2003; Mofrad et al. 2004; Schaller 2010). Integrins trigger signal transduction via tyrosine phosphorylation (Clark and Brugge 1995; Giancotti and Ruoslahti 1999; Howe et al. 1998), and there is substantial evidence indicating that FAK is a major player in relaying signals from integrins to downstream factors, which, in turn, causes cell motility (Cary et al. 1996; Hanks et al. 1992; Horwitz and Parsons 1999; Ilic et al. 1995; Mitra et al. 2005; Parsons 2003; Petit and Thiery 2000; Sieg et al. 1999, 2000; Zhao and Guan 2011).

Focal adhesion-associated protein tyrosine kinases such as FAK and Src family kinases (SFKs) play significant role in cell motility (Cabrita et al. 2011; Sanchez-Bailon et al. 2012). Current understanding suggests that inhibited recruitment of SFKs leads to blockage of FAK phosphorylation at Tyr 576/577, which triggers the signaling pathway for cell migration (Calalb et al. 1996; Chaturvedi et al. 2008; Ciccimaro et al. 2009; Green et al. 2009; Hauck et al. 2001). Directional cell migration is critical to living organisms to maintain homeostasis or immune response. *N*-Formyl-methionyl-leucyl-phenylalanine (fMLF) and high-mobility group box protein B (HMGB1) are well-known chemo-attractants for directional cell migration toward target locations (Carrigan et al. 2007; Degryse et al. 2001; Filaferro et al. 2013; Palumbo et al. 2009).

Previously, we reported the discovery and characteristics of a chondrogenic progenitor cell (CPC) response to cartilage injury. We found that mechanically induced chondrocyte death caused CPCs to migrate from nearby healthy cartilage toward injured cartilage, resulting in repopulation of the matrix within 7–14 d (Seol et al. 2012). Those CPCs exhibited significantly higher expression of genes involved in cell migration, and their migratory activity in response to chemo-attractants was remarkably higher than that of normal chondrocytes. Thus, CPCs could accelerate the repair of injured cartilage by replenishing extracellular matrix macromolecules such as proteoglycans and collagen fibers. Therefore, we hypothesized that LIPUS stimulation promotes the migratory activity of CPCs toward injured sites in articular cartilage and this action is mediated by FAK activation.

METHODS

Osteochondral explants and CPC isolation

Mature bovine stifle joints were obtained from a local abattoir (Bud's Custom Meats, Riverside, IA, USA), and approximately 2.5 $\times$ 2.5 cm^2 of osteochondral explants, including the central loaded area from the tibial plateau, was prepared by manually sawing. The explants were gently rinsed in Hanks' balanced salt solution (HBSS) (Invitrogen Life Technologies, Carlsbad, CA, USA) and cultured for 2 d in 45% Dulbecco's modified Eagle medium and 45% Ham's F-12 supplemented with 10% fetal bovine serum (Invitrogen Life Technologies), 100 U/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B at 37°C, 5% CO_2 and 5% O_2. To make a traumatized cartilage injury model, explants were rigidly fixed before a single blunt impact with 14 J/cm^2 using a customized drop-tower device and then returned to culture. Five to seven days post-impact, the explants were gently rinsed with HBSS , and the cartilage part was incubated in 0.25% trypsin-EDTA (Invitrogen Life Technologies) for 10 min under the same culture conditions. The CPCs from typsin-EDTA-treated cartilage were then isolated as previously described (Goodwin et al. 2010; Martin et al. 2009; Sauter et al. 2012; Seol et al. 2012).

LIPUS apparatus

A manually controlled square-wave pulser-receiver (Panametrics-NDT, Waltham, MA, USA) was used to generate multiple LIPUS doses by adjusting pulse voltage and pulse repetition frequency. A 1-, 3.5- or 5-MHz plane water-immersible transducer (NDT Systems, Huntington Beach, CA, USA) with an effective area of 28 mm^2 and outer diameter of 25 mm were used in our experiments. During LIPUS stimulation, the surface of the transducer was directly immersed facing the cartilage side of osteochondral explants and cultured CPCs in monolayers containing culture medium at a distance of approximately 1 cm. LIPUS output power levels were evaluated with a radiation force balance, and the calculated intensity was regarded as spatially averaged and temporally averaged because it measures overall acoustic power without providing spatial and temporal pressure levels (Preston 1986; Zeqiri and Bickley 2000).

CPC migration in injured cartilage by confocal microscopy

Either partial- or full-thickness cartilage defects were created to examine the effect of LIPUS stimulation on CPC migration. Full-thickness cartilage defects were aseptically created in an osteochondral explant using a 2-mm biopsy punch (Miltex, York, PA, USA), and TISSEEL fibrin hydrogel (Baxter Healthcare, Westlake Village, CA, USA) was injected into the defects. Twenty-four hours after their creation, defects were stimulated for 7 consecutive days with LIPUS (1 MHz, 36.7 mW/cm^2, 20 min/d) (Fig. 1b). Partial-thickness cartilage defects were approximately