Acute Injury induced Fibrosis Is Medated By Lysophosphatidic Acid

Ling Wu, PhD, Frank Petrigliano1, MD, Siyoung Lee1, BSc, David McAllister, MD, Denis Evseenko, PhD.
University of California, Los Angeles, Los Angeles, CA, USA.

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Introduction: Articular cartilage is a highly specialized tissue which forms the surfaces in synovial joints. Pathological and disorganized regeneration of cartilage tissue after injury often results in deposition of excessive inert fibrotic tissue with inferior biomechanical properties. This process includes replacement of highly specialized hyaline cartilage by biomechanically-inferior, disorganized fibrotic tissue enriched in collagen type I (COL I). Lysophosphatidic acid (LPA) is recently identified as a novel potential regulator of COL I biosynthesis that may play a mechanistic role in fibrocartilage formation. LPA is a small lipid produced by an ectoenzyme autotaxin (ATX), also known as phospholipase D, concentrated on the outward face of the plasma membrane of articular chondrocytes and BMSCs. The goal of this study is to investigate the molecular mechanisms underlying fibrosis after joint injury.

Methods: Full thickness cartilage defects were generated in the femoral intra-condylar region of a knee joint in rats. At weeks 1, 2 and 4, rats were sacrificed and dissected for histological examination. PLGA (polylactic-co-glycolic acid) microspheres were fabricated to release BrP-LPA. Tissue samples were fixed, dehydrated, and embedded in paraffin using routine procedures. Immunohistochemistry was performed to examine the expression of collagens.

Results: Using immunohistochemistry, we examined the expressions of the two receptors in articular chondrocytes and synovial tissue of adult human donor and found that these two major receptors for LPA were abundantly present on articular chondrocytes and synovium (Fig. 1A and 1B). In contrast, ATX was minimally expressed by chondrocytes at all stages, although other cell types including vascular cells evidenced abundant expression (Fig. 1A). The ATX protein is highly expressed in osteo-chondral defects in the rat knee. Rat knee joint injury model was employed to study the levels of ATX expression after full-thickness cartilage injury. In this system, cells from the bone marrow migrate into the site of injury and form a scar that repairs the defect but has poor mechanical properties. Immunohistochemical staining confirmed little ATX expression by rat articular chondrocytes in uninjured joints (Fig. 2A); however, ATX was expressed at high levels by stromal cells filling the defect at Day 7 following injury (Fig. 2B). The expression of ATX at Day 14 and 28 after injury was significantly lower than at Day 7 (Fig. 2C). Significant COL I deposition at the site of cartilage injury was clearly present at Day 7 and further increased by Day 14 (Fig. 2B and 2C). LPA stimulates COL I deposition by human chondrocytes and BMSCs. Chondrocyte pellets were cultured either in chondrogenic medium (control), in chondrogenic medium containing LPA (LPA) or chondrogenic medium containing both LPA and chemical inhibitor BrP-LPA (LPA+BrP-LPA). Results of histological examination indicated that both control and LPA-treated samples deposited cartilaginous matrix strongly stained with alcian blue. However, LPA-treated pellets produced high levels of COL I in addition to COL II, while control pellets primarily expressed COL II. After normalization to DNA, LPA-treated pellets showed almost 3-fold higher levels of COL I deposition compared to control, while inhibition of the ATX/LPA axis abolished this increase. qPCR was performed
next to examine the ratio of COL II and COL I gene expression in cultured pellets. After treatment with LPA, the COL II/COL I ratio in chondrocyte pellets decreased in a dose-dependent manner. We next studied the effects of LPA on COL I deposition by BMSCs. Since BMSCs have considerable amount of endogenous ATX activity, the effects of LPA were studied in the presence of the ATX inhibitor S32826 (1 μM) added to all tested groups, which selectively inhibits the activity of ATX but not the LPA receptors. LPA treatment resulted in almost 3-fold increase in COL I deposition by BMSCs and increase in pellet size, which was completely reversible by inhibiting the LPA receptors via BrP-LPA. Specific inhibitors for G-protein interaction with LPA G-protein coupled receptors (Pertussis Toxin), PI3 kinase (PI 828), MEK1/MEK2 (U0126) and p38 MAP kinase (SB230580) completely abolished the increase of COL I production induced by LPA, while the selective inhibitors for Phospholipase C (U73122) and Rho kinase (Y27623) did not block the effects of LPA. Next, we focused on PI3 kinase (PI3K) and p38 MAP kinase (p38 MAPK) pathways, as they demonstrated the most prominent inhibition of COL I deposition. qPCR confirmed that inhibition of PI3K, MEK1/MEK2 and p38 MAPK abolished the stimulatory effects of LPA on COL I gene expression. Using the same methods, we also tested the effects of the inhibitors alone on COL I deposition. Our data showed that blocking of PI3 kinase significantly reduced the COL I expression at both mRNA and protein level. Next, we studied phosphorylation of PI3K and p38 MAPK in chondrocytes in response to LPA treatment at different time points by immunofluorescent staining using antibodies against phospho-PI3K and phospho-p38 MAPK. Phosphorylation of both kinases peaked at 1 hour after LPA treatment and remained high for p38 MAPK for up to 24 hours, while phosphorylation levels of PI3K steadily decreased after 2 hours.

We studied the effects of ATX and LPA inhibition on the COL I deposition at the site of cartilage injury in vivo. BrP-LPA was administered via local intra-articular application using PLGA microspheres loaded with BrP-LPA in combination with systemic delivery of the drug via IP injection. Control animals were injected with PLGA microspheres without the drug and also given IP injections of physiological saline. Three weeks post-surgery, COL I deposition in the defects was markedly inhibited by BrP-LPA (Fig. 3A). Notably, control defects were filled with fibrotic tissue rich in COL I fibers with a minimal cellular component, while BrP-LPA treated animals showed significantly higher numbers of cells expressing COL II but minimal levels of COL I in the area of the defect (Fig. 3A and B). These experiments revealed significant improvement of chondrogenesis though the increased COL II deposition and tissue remodeling as well as reduction of the excessive COL I fibers in the site of injury in the presence of BrP-LPA.

**Discussion:** Our study demonstrates that the ATX/LPA axis is activated after acute full-thickness cartilage injury resulting in deposition of fibrotic tissue at the site of injury. Inhibition of the ATX/LPA axis in injured rats significantly improved chondrogenesis at the site of injury, reducing COL I deposition and fibrotic tissue formation.

**Significance:** The current study demonstrates that LPA signaling plays a major role in the regulation of COL I biosynthesis by articular chondrocytes and BMSC both in vivo and in vitro. Our experimental data suggest that controlled inhibition of the ATX/LPA axis may significantly improve the quality of regenerated cartilage tissue via reduced COL I production and fibrosis in the site of injury and thus improve the existing clinical approaches for cartilage restoration.
Figure 1. ATX expression is limited to bone marrow stromal cells in normal human joints. Human adult articular chondrocytes express minimal levels of ATX, while the LPA receptors LPAR 1 and 2 are highly expressed by chondrocytes and synovial cells.

Figure 2. Fibrocartilage formation during the healing of full-thickness cartilage defects in rat knee joints. (A) Control rat knee joints only evidence ATX expression in bone marrow stromal cells, while articular chondrocytes express collagen II (COL II) and low levels of collagen I (COL I). (B) Seven days after the creation of full-thickness defects, ATX and COL I are abundantly expressed in the fibrotic tissue. By day 14 after injury (C), ATX expression had decreased in the injured area, while a rich fibrocartilaginous matrix highly positive for COL I was deposited. Arrows indicate higher magnification images of the boxed area. Scale bars = 50μm.
Figure 3. Pharmacological inhibition of the ATX/LPA axis reduces COL I deposition in the site of joint injury. (A) Three weeks following full-thickness osteochondral defects, rat knee joints treated with BrlP-LPA showed less COL I accumulation and fiber formation while evidencing increased COL II deposition. Dashed line shows the initial site of injury. Arrows indicate enlarged images of the box area. Scale bar = 50 μm. (B-C) Quantitative analysis documented the concomitant decrease in COL I (B) and increase in COL II (C) following BrlP-LPA treatment.