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Stress-Induced Activation of Apoptosis Signal-Regulating Kinase 1 Promotes Osteoarthritis

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Abstract

Apoptosis signal-regulated kinase 1 (ASK1) has been shown to affect a wide range of cellular processes including stress-related responses, cytokine and growth factor signaling, cell cycle and cell death. Recently, we reported that lack of ASK1 slowed chondrocyte hypertrophy, terminal differentiation and apoptosis resulting in an increase in trabecular bone formation. Herein, we investigated the role of ASK1 in the pathogenesis of osteoarthritis (OA). Immunohistochemistry performed on articular cartilage samples from patients with OA showed ASK1 expression increased with OA severity. In vitro analysis of chondrocyte hypertrophy, maturation and ASK1 signaling in embryonic fibroblasts from ASK1 knockout (KO) and wild type (WT) mice was examined. Western analysis demonstrated an increase in ASK1 signaling commensurate with chondrogenic maturation during differentiation or in response to stress by the cytokines, tumor necrosis factor alpha or interleukin 1 beta in WT, but not in ASK1 KO embryonic fibroblasts. Surgically induced moderate or severe OA or OA due to natural aging in WT and ASK1 KO mice was assessed by microCT of subchondral bone, immunohistochemistry, histology, and OARSI scoring. Immunohistochemistry, microCT and OARSI scoring all indicated that the lack of ASK1 protected against OA joint degeneration, both in surgically induced OA and in aging mice. We propose that the ASK1 MAP kinase signaling cascade is an important regulator of chondrocyte terminal differentiation and inhibitors of this pathway could be useful for slowing chondrocyte maturation and cell death observed with OA progression.

Osteoarthritis (OA) is a painful and debilitating disease associated with medical costs, totaling nearly \$185.5 billion per year (Lawrence et al., 2008; Kotlarz et al., 2009). It affects over 27 million people in the United States alone and can affect any joint, including the hand, foot, jaw, knee, and hip. As the result of normal joint wear and tear over time, almost everyone over the age of 70 will develop OA (Goekoop et al., 2011). Traumatic injury to the

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

joint early in life can accelerate the onset of disease development (Blagojevic et al., 2010; Neogi and Zhang, 2013; Svoboda, 2014), as articular cartilage injury initiates degenerative changes (Blagojevic et al., 2010; Gelber et al., 2000). Quite often, injury also involves synovial inflammation resulting in the production and release of reactive oxygen species (ROS), cytokines (e.g., tumor necrosis factor alpha; TNF α or interleukin-1 *beta*; IL-1 β) and growth factors (e.g., vascular endothelial growth factor; VEGF) into the joint space (Schiller et al., 2003). These factors exacerbate changes in the articular cartilage inducing clonal proliferation of surface chondrocytes and hypertrophy of deep zone chondrocytes; accompanied by apoptosis and protease degradation of the extracellular matrix (Regan et al., 2008; Scott et al., 2010; van der Kraan and van den Berg, 2012). It is through these activities that the mechanical integrity of the cartilage is compromised resulting in additional mechanical load on the subchondral bone plate which promotes remodeling and the formation of thicker, stiffer, sclerotic bone with reduced mineralization (Swagerty and Hellinger, 2001). Ultimately, all of these factors contribute to the complete erosion of the articular cartilage, leaving total joint replacement surgery as the only solution for most patients to restore joint function and relieve OA-induced pain and disability. A treatment to slow these processes could delay or prevent the need for joint replacement surgeries. Preventative measures that delay surgical intervention are especially important, as the lifetime of an implant is only 15 to 20 years, which means joint replacements in younger patients will typically require a second surgery.

We and others have reported that prolonged reactive oxygen species (ROS) production promotes chondrocyte proliferation, hypertrophy, and apoptosis in both the growth plate and articular cartilage in mice (Morita et al., 2007; Kishimoto et al., 2010; Eaton et al., 2014). Recently, we reported inhibition of Apoptosis Signal-Regulating Kinase-1 (ASK1) increased chondrocyte resistance to ROS-associated signaling, delayed chondrocyte apoptosis and increased the length of the hypertrophic zone in the growth plate (Eaton et al., 2014). ASK1 is a key enzyme in the transduction of ROS signaling and plays a role in LPS-induced arthritis, cardiomyocyte hypertrophy, neuronal degeneration and bacterial sepsis (Yamaguchi et al., 2003; Matsuzawa et al., 2005; Cho et al., 2009). As increased ROS production also plays a role in the promotion of articular cartilage degradation and OA progression after injury, we asked if ASK1 inhibition might prevent or slow the development of OA.

Injury-induced cytokines such as TNF α or IL-1 β are released, resulting in increased intracellular stress and activation of ASK1 in chondrocytes (Pickvance et al., 1993; Tsou et al., 2012; Bigoni et al., 2013). ASK1 then selectively phosphorylates c-Jun N-terminal kinase (JNK) and/or p38 MAPK to initiate signaling cascades that can lead to catabolic activity and cell death (Liu et al., 2012). Both JNK and p38 MAPK have been implicated as effectors of chondrocyte differentiation, hypertrophy and apoptosis (Takeda et al., 2000; Sayama et al., 2001; Van Laethem et al., 2006). Thus, therapeutic inhibition of ASK1 activation, delivered at the time of injury, could limit cartilage degradation and slow OA progression by reducing cell death and the catabolic effects of pro-inflammatory cytokines (Watanabe et al., 2005; Mnich et al., 2010). Normally, the adverse effects of ROS are balanced by production of antioxidants, and administration of antioxidant therapies has provided some relief of OA symptoms (McAlindon et al., 1996; Wang et al., 2007). However, anti-oxidant therapies tend to block all ROS initiated signaling; some of which are

required for normal cell function. In contrast, inhibition of ASK1 does not directly inhibit ROS or block all ROS-associated signaling, but only attenuates ROS-induced cytokine production, cell death and inflammatory cell infiltration (Harada et al., 2006). Additionally, ASK1 is constitutively expressed and only long term activation in the presence of inflammation and oxidative stress results in initiation of apoptotic pathways (Ichijo et al., 1997).

Herein, we show that ASK1 expression is increased with chondrocyte maturation and the highest concentration is present in pre and hypertrophic chondrocytes, in a pattern similar to that observed with apoptotic induction. In human articular cartilage tissue samples retrieved from joint replacement surgeries, increased ASK1 was observed with OA progression. In contrast, joint degeneration was attenuated in 2 year old ASK1 knockout (KO) mice, as evidenced by decreased cartilage and proteoglycan loss when compared to wild-type (WT) mice. Further, two mouse models of OA induction, one severe (partial meniscectomy) and one milder (joint destabilization/injury) confirmed inhibition of ASK1 retarded cartilage destruction as compared to WT. Additionally, when mouse embryonic fibroblasts from the ASK1 KO mouse were cultured in chondrogenic media in the presence of either TNF α or IL-1 β terminal differentiation was attenuated. Together these findings suggest inhibition of ASK1 slows chondrocyte hypertrophy and inhibits changes associated with osteoarthritic progression.

Materials and Methods

Femoral condyle cartilage sample collection

Femoral condyle samples were collected from female patients between 63–86 years of age who underwent total knee arthroplasty in compliance with Institutional Review board (IRB) at Thomas Jefferson University. Cartilage degeneration was mapped by gross observation of the femoral condyle topography (Fig. 1A). Condyles were cut into 15 $\times$ 15 mm subsections and categorized by morphology into the following groups: non-lesion area from the lateral condyle, a matching lesion area on the medial condyle and an adjacent peri-lesion area.

ASK1 KO and C57BL6 WT mice

Eight to ten week old male ASK1 deficient (ASK1 KO) mice and C57BL/6N wild-type (WT) mice were used in the present study. ASK1 KO mice were purchased from the Oriental Yeast Co., Tokyo, JP, with the permission of Dr. Hidenori Ichijo. ASK1 KO mice were then backcrossed onto the C57BL/6N (Charles River Laboratories) background for at least 10 generations to reduce genetic variation. NIH guidelines for the care and use of laboratory animals were observed and all animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Thomas Jefferson University. Experiments comparing all three genotypes used littermates and other experiments used mice from different litters matched by age.

Cell culture

We isolated primary mouse embryonic fibroblasts (MEFs) from each genotype at embryonic day E18.5 (E18.5) and performed both monolayer and micromass culture in chondrogenic

differentiation media DMEM with 1% Fetal Bovine Serum (FBS; Atlanta Biologicals, GA), ascorbic acid (Sigma–Aldrich, MO; 50 µg/ml), transforming growth factor beta (TGFβ; Gemini Bio Products, CA; 10ng/ml), Insulin-Transferrin-Selenium (ITS; Gibco, NY; 10µl/ml), and dexamethasone (Sigma; 10 nl/ml) for 2 wks. MEFs have been shown to perform similarly to bone marrow stem cells and can differentiate into chondrocytes, adipocytes and osteoblasts (Saeed et al., 2012). The following treatment concentrations were used: TNFα (PeproTech, Rocky Hill, NJ; 100 µg/L), ASK1 inhibitor (NQDI-1; Tocris, UK; 30 µM), and interleukin 1 beta (IL-1β; R&D Systems, MN; 2 µg/L).

OA induction by partial medial meniscectomy (PMM-ACL) surgery

Only male mice were used for this study. Mice were anesthetized at 8–10 weeks of age using isoflurane inhalation in the animal facility. Buprenex (Reckit Benckiser Pharmaceuticals, Richmond, VA; IP injection of 0.1~0.2 mg/kg) was given both before skin incision and an hour after surgery. PMM-anterior cruciate ligament (ACL) was induced in the right knee joint by transection of the anterior attachment of medial meniscotibial ligament (MMTL) and ACL described previously (Visco et al., 1996; Kamekura et al., 2005; Glasson et al., 2007). Briefly, the joint capsule was opened with an incision just medial to the patellar tendon and the MMTL and ACL were sectioned with micro-surgical scissors. The medial half portion of the meniscus was then removed. For control, surgery was performed on left knee joints also, but the ligaments were left intact and termed “sham joints.” All surgeries were weight-bearing following recovery from anesthesia. Mice were sacrificed 4 weeks after PMM-ACL surgery and subjected to (micro-CT) and histological analyses.

OA induction by destabilization of the medial meniscus (DMM) and full-thickness cartilage injury

Bilateral full-thickness articular cartilage defects were created in the knee joints of the right hind limbs of 8–10 week-old mice (n = 8) through microsurgery (Fitzgerald et al., 2008). Briefly, mice were given intraperitoneal injections of 0.1~0.2 mg/kg body weight of Buprenex (Hospira inc, IL) both before skin incision and an hour after surgery. Mice were anesthetized by isoflurane inhalation. A small (0.5 cm) medial parapatellar skin incision was made, the joint capsule was opened, the MMTL was transected, and the patella was luxated laterally to expose the trochlear groove articulating surface. A full-thickness punch was made in the articular cartilage by creating a circular defect in the cartilage with a sterile 27-gauge, 0.5-inch needle, using a circular motion until the subchondral bone was reached, confirmed by the appearance of a blood droplet following removal of the needle. Both the joint capsule and the skin were closed with 6–0 absorbable polypropylene sutures. The surgical areas were fully weight bearing within 2–3 h of surgery and the mice showed no signs of lameness or systemic effects for the duration of the experiment.

Micro-computed tomography (MicroCT) analysis

Each knee joint was harvested at the end of the experimental protocol, fixed in 4% paraformaldehyde, and subjected to microCT analysis (Scanco µCT 40; Basserdorf, Switzerland). The scans were performed in the long axis of the diaphysis, with an energy of 70 kVp, a current of 114 µA and a 200 ms integration time producing a resolution of 12 µm³ voxel size. Each scan comprised a minimum of 500 slices encompassing the knee joint,

femur, and tibia. For subchondral bone analysis, 50 slices of the both the medial and the lateral condyle were traced and evaluated. The 3D data sets were low-pass filtered using a Gaussian filter ($\sigma = 1.0$, support = 1) and segmented with a fixed threshold filter (212 mg HA/cm³) according to the current guidelines (Bouxsein et al., 2010). The morphometric parameter of the condyles' subchondral bone volume fraction (BV/TV) (i.e., the ratio of bone volume over total volume traced) was calculated.

Histology, imaging, OA grading, and image analysis

Mouse knee joints and human condyle samples were fixed in 4% paraformaldehyde, decalcified with 12.5% EDTA for two weeks— 1 month, and embedded in paraffin. Sections were cut at 6 μ m and mounted on slides (Superfrost/Plus; Thermo/Fisher Scientific Inc, CA). Standard histology including hematoxylin, eosin and alcian blue were performed. Stained sections (6 μ m) were imaged on an Optiphot microscope (Nikon, Melville, NY) with a Spot Color Camera (Diagnostic Imaging, Sterling, MI). Cartilage damage was scored by two observers (GE, QZ) blinded to sample identity and using a published OARSI scoring system by Glasson and colleagues (Glasson et al., 2007). Microscopic imaging of immunohistochemically stained slides was performed using a Nikon E800 microscope system (Nikon, Melville, NY) with a 12-bit cooled digital camera (Retiga EXi, QImaging, Burnaby, BC) with an LCD filter to acquire monochrome or color images. All images were photographed at the same setting, enhanced equally and intensity and localization analysis was performed using a custom written module to automate and standardize the procedure. All analysis was performed with Image Pro Plus 7.0 (MediaCybernetics, Silver Spring, MD).

Western analysis

Western blotting was performed as described previously.¹⁶ The following list of rabbit antibodies were used: collagen type X (COL-X; Abcam, Cambridge, MA), phosphorylated p38 (Cell Signaling, Danvers, MA), p38 (Invitrogen, Carlsbad, CA), phosphorylated and non-phosphorylated JNK (Millipore, Billerica, MA), phosphorylated and non-phosphorylated ASK (Cell Signaling, Danvers, MA), p65 nuclear factor kappa beta (NF κ B), matrix metalloproteinase 13 (MMP13), and vascular endothelial growth factor (VEGF), (Santa Cruz, Dallas, TX); as well as a mouse anti- β -actin (Santa Cruz).

Immunohistochemistry and markers of apoptosis

Immunohistochemistry was used to test for the prevalence of proteins of interest using detection with 3, 3'-diaminobenzidine (DAB) or immunofluorescence, as previously reported (Eaton et al., 2014). Briefly, tissue slides were deparaffinized, rehydrated, placed in either antigen unmasking solution (Vector; Burlingame, CA) or digested in 0.05 mg/ml bovine hyaluronidase (Calbiochem, Darmstadt, Germany) in PBS for 20 min at 37°C, then washed and permeabilized with 0.5% Triton. DAB slides were incubated with 3% H₂O₂ in methanol for 5 min then blocked in 4% Bovine Serum Albumin (BSA, Equitech-Bio, Kerrville, TX) with 0.1% Tween 20 in PBS. Primary antibodies were diluted with 1% BSA with 0.1% Tween 20 in PBS, placed on the slides and incubated overnight at 4°C. The following rabbit primary antibodies were used: anti-COL-X (1:200; Abcam, Cambridge, MA), ASK1 and cl-PARP (1:50; Cell Signaling, Danvers, MA), MMP13 and VEGF (1:100;

Santa Cruz, Dallas, TX), TNF α (1:200; Novus, Littleton, CO), gamma-H2AX (1:50; Bethyl, Montgomery, TX), and IL-1 α (1:100; Epitomics, Burlingame, CA). Secondary fluorescent antibodies, applied for 1 h were, Alexa Fluor 488 donkey or Alexa Fluor 594 goat anti-rabbit (1:200; Invitrogen, Eugene, OR), then washed in PBS and coverslipped using Vectashield Hard Set with 4',6-diamidino-2-phenylindole (DAPI, Vector, Burlingame, CA) For DAB, horse radish peroxidase-conjugated anti-rabbit (1:200; Vector, Burlingame, CA) was used followed by DAB peroxidase substrate kit (Vector, Burlingame, CA) incubated at 37°C for approx. 10 min. washed and counterstained with alcian blue and hematoxylin and mounted with PermOUNT (Thermo Fisher). A negative control sample was incubated with no primary antibody. All slides were imaged on an Eclipse E800 microscope (Nikon, Melville, NY) with an Evolution QEi Monochrome camera with LCD color filter (Q-imaging, Canada). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Abcam, Cambridge, MA) was performed as per instructions.

Statistical analysis

Statistical analysis between groups was performed using a student's *t*-test in normally distributed samples and the Mann–Whitney test for nonparametric data. A level of significance (α) or a p-value of less than 0.05, with a 95% confidence interval was determined and data are presented as the mean $\pm$ SEM. Western data comes from two individual samples from three independent analyses. The number of samples used for histology and microCT analysis varied from 3–8 mice per test and is noted for each experiment, which were performed on both littermates and mice from litters of the same age.

Results

Expression of ASK1 increases with human OA progression

Femoral condyle cartilage samples from lateral and medial sites were collected from 15 OA patients undergoing total knee arthroplasty (Fig. 1A). Histology showed the full thickness cartilage of the less affected lateral condyle could be divided into surface, mid, and deep zones, while the medial condyle perilesion sample, collected next to the cartilage denuded lesion, was thinner and had less alcian blue (proteoglycan) staining. Immunohistochemistry for ASK1 was performed and quantified for all 15 patient samples for all three zones of the lateral condyle and the medial perilesion sample (Fig. 1B and D). Expression of ASK1 was increased in the surface zone of the lateral condyle, where contact with the joint space and synovium would occur, but decreased with progression into the mid and deep zones (Fig. 1C). In the medial condyle perilesion sample, ASK1 expression was similar to that observed in the surface zone of the lateral condyle and significantly increased compared to the deep zone of the lateral condyle (Fig. 1E). A negative control for each region is shown in supplementary Figure S1 (surface—A, Mid—B, Deep—C zones). These findings confirm that increases in ASK1 expression are associated with areas where cell stress is known to be present in articular cartilage concurrent with the onset (lateral condyle surface zone) and progression (medial condyle) of OA.

ASK1 expression is increased in hypertrophic chondrocytes and lack of ASK1 decreases expression of hypertrophic markers

OA is associated with changes in chondrocyte maturation to more easily visualize how the expression of ASK1 during with chondrocyte maturation, growth plates from post-natal day (PD) 14 wildtype (WT) mice were subjected to immunofluorescent staining with an antibody to ASK1. Fluorescent intensity was increased with chondrocyte maturation culminating in the intense cytoplasmic staining of the hypertrophic chondrocytes (arrow; Fig. 2A). This region of the growth plate is characterized as the zone where terminal differentiation and chondrocyte death occurs, as evidenced by TUNEL, to indicate apoptosis. To determine how the lack of ASK1 affected chondrocyte maturation, WT and KO growth plates were also compared for expression of hypertrophic markers; VEGF, MMP13, and COL-X (Fig. 2B). There was a profound decrease of staining intensity of all three markers in the hypertrophic zone (HZ) of KO mice as compared to WT and negative control with no primary antibody (Suppl. Fig. 1D). Additionally, the HZ of the KO mouse was noticeably longer, indicating a delay in chondrocyte death, as we have previously reported (Eaton et al., 2014).

Stress cytokines activate ASK1 to increase chondrocyte maturation and hypertrophy

The inflammatory cytokines TNF α and/or IL-1 β are commonly present in the joint space during OA and contribute to accelerated articular chondrocyte maturation and cartilage degradation (Fernandez-Vojvodich et al., 2012). To investigate how the loss of ASK1 would affect chondrocyte maturation in response to these factors, mouse embryonic fibroblasts (MEFs) from WT and KO mice were cultured in chondrogenic media and treated with TNF α or IL-1 β for the last 24 h of the 13 day chondrogenic culture period. Western blot analysis comparing MEFs from both genotypes showed a similar phosphorylation of JNK and p38, but the addition of TNF α or IL-1 β to the media lead to increased phosphorylation of ASK1 and downstream kinases JNK and p38 in WT, but not in KO cells (Fig. 2C; n 4). Additionally, in the presence of TNF α or IL-1 β , NF κ B a well-known transcription factor regulating chondrocyte differentiation also exhibited decreased expression in the KO cells.

Interestingly, WT and KO MEFs cultured in chondrogenic media showed no appreciable difference in the expression of hypertrophic markers of chondrocyte differentiation (COL-X, MMP13, and VEGF). However, upon addition of TNF α or IL-1 β an increase in expression of all three proteins was observed in WT, but no response was observed in the KO cells (Fig. 2D). Taken together, these results suggest that in the presence of inflammatory cytokines, the absence or inhibition of ASK1 prevents MAPKinase signaling leading to the production of proteins associated with chondrocyte catabolism and hypertrophy.

Induction of OA after partial medial meniscectomy ACL surgery (PMM-ACL) is attenuated in ASK1 KO mice

To determine if the lack of ASK1 could also protect against OA-induced cartilage damage, the well-established partial medial meniscectomy (PMM-ACL) surgery was performed on ASK1 KO, heterozygous (Het) and WT littermates. This surgery, where the medial half of the hind right meniscus is resected, was performed on the right knee while a sham surgery was performed on the contralateral left limb. After 4 weeks, a comparison of the sham to

PMM-ACL knee joints showed the WT had severe degradation, cartilage loss, fibrosis, inflammatory infiltration and loss of proteoglycan staining (Fig. 3A). To determine the extent of OA pathology across all operated mice, scoring by two independent graders was performed using OARSI defined scoring system for mice (Glasson et al., 2007). The results are presented as a box and whisker plot with data points from each mouse superimposed (Fig. 3B). Using this scoring method, Het and KO mice showed less joint degradation when compared to WT. To determine if the progression of OA affected the subchondral bone, microCT analysis and 3D reconstruction was performed (Fig. 3C). The BV/TV fraction was significantly decreased for the femoral subchondral bone in the WT, as compared to the same regions in the Het and KO mice (Fig. 3D). This decrease in subchondral bone volume is characteristic of unbalanced remodeling associated with an inflammatory environment.

OA is attenuated in ASK1 KO mice after joint destabilizing (DMM) surgery

PMM-ACL surgery induced a very rapid deterioration of the joint cartilage. To more closely analyze a knee injury with gradual onset, less degenerate OA symptoms and joint inflammation, a second surgery was performed where the medial meniscus was destabilized and the articular cartilage of the trochlear groove injured by a 26 gauge needle passing through the cartilage into the subchondral bone (DMMi). Eight-weeks post-surgery WT and KO knees from the sham (Fig. 4A) were histologically compared to DMMi knees (Fig. 4B). Higher magnification comparing the WT and KO knees after surgery highlighted the greater degree of joint degradation in the WT (Fig. 4C). Boxes showing higher magnification images of specific cartilage regions highlight the increased cartilage hypertrophy, the loss of proteoglycan and areas completely devoid of cartilage in the WT, as compared to similar regions in the KO joints (Fig. 4C, boxes). Eight-weeks after DMMi surgery, OARSI grading of the KO mice generated a lower average OA score (2.16) when compared to the WT mice (4.42; $P=0.004$) (Fig. 4D). In addition, microCT evaluation of subchondral bone showed an increase in the WT BV/TV indicating increased load in response to articular cartilage degradation and loss, while the BV/TV of the KO mice showed no significant change (Fig. 4E).

ASK1 KO mice show decreased expression of markers of chondrocyte maturation and death

Immunohistochemical analysis was performed to determine the expression of chondrocyte maturation, inflammation, and DNA damage markers in the articular cartilage 8 weeks after DMMi and cartilage injury surgery. Articular cartilage from WT mice showed significant ASK1 staining while as expected the KO mice lacked ASK1 (Fig. 5A). The double-stranded DNA break marker, H2A histone family, member X (H2AX, Fig. 5B) and apoptosis marker cleaved poly (ADP-ribose) polymerase (cI-PARP, Fig. 5C) were also increased in WT, while in the articular chondrocytes of the ASK1 KO levels were lower. WT articular chondrocytes also displayed an increase in VEGF (Fig. 5D), MMP13 (Fig. 5E), and COL-X when compared to KO (Fig. 5F). Immunohistochemistry for ASK1 in a 2 month old mouse without OA shows the normal staining pattern (Suppl. Fig. E–F with brightfield) and the negative control with no primary antibody shows the specificity of the staining (Suppl Fig. G). These results suggest that the lack of ASK1 protects chondrocytes against inflammatory stress induced differentiation associated with OA pathology.

ASK1 KO mice show decreased wear of the articular cartilage during aging

To determine if the lack of ASK1 could also attenuate age-related articular cartilage degeneration, we examined mouse joints in aging mice for up to 2 years. No significant difference in cartilage thickness or joint degradation for WT or KO was observed up to 1 year of age (Fig. 6A). At 2 years, however, WT mice showed less proteoglycan, cartilage thinning, increased wear of the articular cartilage surface, chondrocyte hypertrophy, and calcification compared to age-matched ASK1 KO mice. OARSI grading of WT and KO mouse limbs at 2 years provided further evidence that KO mice showed a significant protection from developing OA (Fig. 6B). No difference in subchondral BV/TV was detected at 2 months, (Fig. 6C), but at later time points (6 months, 1 year, and 2 years) there was a significant decrease in the thickness of subchondral bone in the KO mice (Fig. 6D). Indeed, at 1 year WT mice showed a significant thickening of subchondral bone, consistent with OA progression as compared to the BV/TV of the KO mouse. Taken together, these results suggest that aged ASK1 KO animals show less severe histological and subchondral bone changes associated with chronic OA.

Discussion

The purpose of this study was to investigate the involvement of ASK1 MAP kinase signaling in articular chondrocyte catabolic activity during OA pathogenesis and progression. This study highlights the potential of inhibiting ASK1 activation to decrease degenerative effects leading to articular cartilage damage and the development of OA in humans and in three different mouse models. Specifically, ASK1 expression was upregulated in human OA cartilage and in the ASK1 KO mouse decreased cartilage damage was observed after surgically-induced arthritis and during aging. Immunohistochemistry performed on sections of injury-induced OA articular cartilage in ASK1 KO mice displayed a decrease in markers of chondrocyte terminal differentiation, DNA damage and cell death. These findings also correlated with decreased chondrocyte maturation and death observed in the hypertrophic zone of mouse growth plates in ASK1 KO mice. Similarly, MEFs from the ASK1 KO mice cultured in chondrogenic media and treated with inflammatory stress-inducing cytokines, TNF α or IL-1 β , known to activate ASK1, showed decreased chondrocyte maturation and death. Taken together the data reported herein suggests that inhibition of ASK1 may be a valuable strategy to protect cartilage after injury and slow osteoarthritic progression.

In a non-stressed environment ASK1 is virtually inactive, but oxidative or inflammatory stress leads to its phosphorylation and activation (Ichijo et al., 1997). Studies have shown that chondrocytes experience increases in oxidative metabolism and ROS signaling during differentiation, maturation and aging (Carlo and Loeser, 2003; Han et al., 2007; Pattappa et al., 2011) with high expression of ASK1 observed in hypertrophic chondrocytes undergoing terminal differentiation in both adult and embryonic mice (Tobiume et al., 1997, 2001). Furthermore, a number of studies have shown that increased levels of intracellular ROS induce degenerative changes in articular cartilage (Jallali et al., 2005; Yudoh et al., 2005; Loeser, 2010). In agreement with these studies, we found ASK1 expression increased in mouse and human OA articular cartilage, coordinate with areas of inflammatory stress and high ROS.

After injury to the joint, cytokine release and cell death create an inflammatory environment which promotes additional cell damage, inhibits tissue repair and regeneration which contributes to tissue destruction. Chondrocytes within the joint, like those of the growth plate, become stimulated by stress and inflammatory cytokines to reenter the cell cycle, undergo proliferation, pre-hypertrophy, hypertrophy, and finally apoptosis (Aigner and Gerwin, 2007). Based on our previous observation of mouse growth plates from ASK1 KO mice that displayed longer hypertrophic zones and decreased production of the maturation markers VEGF, MMP13, and COL-X (Eaton et al., 2014), we hypothesized that inhibition of ASK1 could decrease the damage due to OA initiating events. Results from both a severe and mild induction of OA using a mouse model, performed in this study, confirmed this hypothesis. Additionally, treatment of chondrogenic WT MEFs with inflammatory cytokines, TNF α or IL-1 β lead to increased ASK1, JNK, p38, and NF κ B signaling with coordinate increases in COL-X, MMP13, and VEGF, while the ASK1 KO MEFs had decreased expression of these signaling proteins and hypertrophic markers. Others have detailed the intracellular generation of ROS upon TNF treatment (Goossens et al., 1995; Lin et al., 2004) and described the ROS-dependent TNF-induced activation of ASK1 through dissociation of thioredoxin and the association of TRAF2 in the regulation of inflammation, proliferation and apoptosis (Saitoh et al., 1998; Wajant et al., 2003; Noguchi et al., 2005). These studies also corroborate the reduction in JNK and p38 activation and TNF-induced apoptosis in ASK1-deficient MEFs (Tobiume et al., 2001). Interestingly, it was also reported that sustained, but not transient JNK and p38 activation, was impaired in response to TNF, indicating the prolonged exposure or generation of ROS is specifically inhibited by the absence of ASK1.

In this study, the slowing of OA progression cannot be specifically attributed to chondrocytes without ASK1, as ASK1 is lost in immune, endothelial and bone cells as well, and this must be considered in a total KO mouse. For example, ASK1 signaling in immune cells is known to be required for activation and migration of macrophages and release of cytokines (Matsuzawa et al., 2005; Osaka et al., 2007). Additionally, in endothelial cells cytokine signaling is reduced when ASK1 is degraded through the ubiquitin-proteasome system (Zhao et al., 2007). Upon TNF α treatment, ASK1 dissociates from suppressor of cytokine signaling 1 (SOCS1) to become stabilized, leading to enhancement of TNF-induced MAPK activation (He et al., 2006). TNF α signaling in bone and osteoclasts can lead to increased bone remodeling; through increased osteoblast apoptosis and osteoclast activation. In our previous study, we reported enhanced bone formation in the ASK1 KO mouse both during development and in ectopic bone formation (Eaton et al., 2014). Together, our in vivo experiments indicate decreased ASK1-associated signaling probably in each of these cell types contribute to an overall quenching of inflammation and an attenuation of cytokine (TNF and IL-1) induced pathology after joint injury or during aging. However, the in vitro data supports a specific role for chondrocytes lacking ASK1, in that the expressions of terminal differentiation markers are reduced to specifically slow cartilage degeneration. Together this and other studies, highlight the potential of ASK1 inhibition as an effective strategy to reduce articular cartilage degeneration and OA progression. Optimally, a small molecule inhibitor of ASK1, given at the time of injury, could decrease early degenerative changes which are one of the highest risk factors for OA development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

ASK1 expression increases with osteoarthritis cartilage degeneration. (A) Femoral condyles retrieved from knee arthroplasty surgery indicating regions of tissue collection. H&E stained histology of articular cartilage tissue taken from the lateral (no lesion) and the medial (perilesion) regions. (B) Immunohistochemistry for ASK1 of the lateral condyle from the surface, mid and deep zones of the articular cartilage. (C) Quantification of the ASK1 staining from each zone of the lateral condyle for 15 patient samples. (D) Immunohistochemistry for ASK1 of the medial condyle of the articular cartilage. (E) Quantification of the ASK1 staining from the deep zone of the lateral condyle cartilage compared to the medial condyle perilesion cartilage, using 2 images from each of 15 patient samples. (* = $P < 0.05$; ** = $P < 0.01$).

Fig. 2.

Knocking out ASK1 decreases chondrocyte maturation and MAPK signaling in response to stress. (A) Fluorescence immunohistochemistry of the growth plate for ASK1 shows increased expression with terminal differentiation and the highest intensity in hypertrophic chondrocytes where cells also stain positive with TUNEL. (B) Immunohistochemistry performed on postnatal D14 mouse growth plates shows an increase in VEGF, MMP13, and COL-X in the WT during chondrocyte maturation, with decreased staining in the KO animals. Examination of all growth plates showed an increase over WT in the length of the hypertrophic zone (HZ-brackets) in the KO mice. (C) WT MEFs grown in chondrogenic media (13 days) treated with TNF α or IL-1 β (for 24 h) showed increased phosphorylation of JNK and p38, as well as increased expression of NF κ B, all of which were decreased in the KO cells. Graphs show densitometric analysis of the Westerns (percentage relative to untreated WT). (D) Similar increases in markers of chondrocyte maturation, COL-X, MMP13, and VEGF were observed in WT MEFs after treatment, but not in KO MEFs and graph indicates quantification. (n = 4; * = P 0.05; ** = P 0.01).

Fig. 3.

ASK1 KO mouse protected against OA changes after partial meniscectomy. (A) H&E and alcian blue (proteoglycan) stained sections of WT, Het, and KO mice after surgery show articular cartilage changes relative to sham limbs. Het and KO mice show less severe degradation, cartilage loss, and fibrosis than WT (compare areas marked with yellow arrows). (B) Box and whisker plot showing OARSI OA scores for representative WT, Het and KO mouse limbs. (C,D) MicroCT reconstruction of WT, Het, and KO knee joints after meniscectomy surgery showed a thinning in subchondral bone in WT, concurrent with early

stages of OA development, which is absent in Het and KO mouse knees (white arrows). (n = 7; * = $P \leq 0.05$; ** = $P \leq 0.01$; scale bars = 1mm).

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Fig. 4.

ASK1 KO mice are protected against OA 8 weeks after cartilage injury. (A,B) H&E and Alcian blue stained sections of WT and KO mice after surgery showed articular cartilage degradation compared to sham surgeries. Compared to WT, KO knees have thicker articular cartilage, smoother architecture, and less fibrosis, inflammatory infiltration and subchondral bone thickening (compare areas marked with yellow arrows). (C) Magnified views of WT and KO limbs showing areas of chondrocyte hypertrophy, osteophyte formation, and proteoglycan loss (insets). (D) Box and whisker plot showing OARSI OA scores for representative WT and KO mouse limbs. (E) MicroCT reconstruction of WT and KO limbs show an increase in subchondral bone thickness (BV/TV) in WT, but this effect is abrogated in KO. ($n = 3$; $* = P < 0.05$; $** = P < 0.01$; $\# = P < 0.05$ compared to unoperated; scale bars = $500 \mu\text{m}$).

Fig. 5.

ASK1 Promotes Chondrocyte Maturation and Hypertrophy in Articular Cartilage with OA.

(A) Immunohistochemistry performed on injury-induced OA mouse limbs clearly shows the presence of ASK1 in WT articular cartilage, but not in the KO mouse cartilage.

Accompanying the presence in ASK1 in WT articular chondrocytes was an increase in (B) H2AX, (C) cI-PARP (D) VEGF, (E) COL-X, and (F) MMP13, all of which were decreased in KO animals. (n = 2 mice-3 images each; scale bar = 100 μ m).

Fig. 6.

Characterization of WT and KO mice articular cartilage during aging. (A) H&E and Alcian blue (proteoglycan) stained sections of WT and KO mice showed comparatively similar articular cartilage of the knee joint at 2 months and 1 year, but 2 year old WT mice showed increased articular cartilage wear and less proteoglycan, compared to the cartilage thickness observed in the ASK1 KO mouse (compare areas marked with yellow arrows). (scale bar = 500 μ m) (B) Box plot showing OARSI OA scores for representative WT and KO mouse limbs. (C,D) MicroCT reconstruction of WT and KO hind limb joints at 2 months, 6 months, 1 year, and 2 years showed a decrease in subchondral bone thickness (BV/TV) compared to WT (arrows). (** = P 0.01 compared to WT; # P 0.05 compared to 2mo; ## P 0.01 compared to 2mo; n = 3/age; Scale bar = 1 mm).