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Ionizing Radiation Causes Active Degradation and Reduces Matrix Synthesis in Articular Cartilage

J.S. Willey^{1,2}, D.L. Long², K.S. Vanderman^{2,3}, and R.F. Loeser²

¹Department of Radiation Oncology, Wake Forest School of Medicine Comprehensive Cancer Center, Winston-Salem, NC

²Department of Internal Medicine, Section of Molecular Medicine, Wake Forest School of Medicine, Winston-Salem, NC

³Department of Orthopaedic Surgery, Wake Forest School of Medicine, Winston-Salem, NC

Abstract

Purpose—Little is known regarding radiation effects on adult articular (joint) cartilage, though joint damage has been reported following cancer treatment or occupational exposures. The goal of this study was to determine if radiation can reduce cartilage matrix production; induce cartilage degradation; or interfere with the anabolic effects of IGF-1.

Materials and Methods—Isolated chondrocytes cultured in monolayers and whole explants harvested from ankles of human donors and knees of pigs were irradiated with 2 or 10 Gy γ -rays, with or without IGF-1 stimulation. Proteoglycan synthesis and IGF-1 signaling were examined at Day 1; cartilage degradation throughout the first 96 hours.

Results—Human and pig cartilage responded similarly to radiation. Cell viability was unchanged. Basal and IGF-1 stimulated proteoglycan synthesis was reduced following exposure, particularly following 10 Gy. Both doses decreased IGF-induced Akt activation and IGF-1 receptor phosphorylation. Matrix metalloproteinases (ADAMTS5, MMP-1, and MMP-13) and proteoglycans were released into media after 2 and 10 Gy.

Conclusions—Radiation induced an active degradation of cartilage, reduced proteoglycan synthesis, and impaired IGF-1 signaling in human and pig chondrocytes. Lowered Akt activation could account for decreased matrix synthesis. Radiation may cause a functional decline of cartilage health in joints after exposure, contributing to arthropathy.

Keywords

Radiation; chondrocyte; cartilage; IGF-1; arthritis

Introduction

Ionizing radiation can induce a severe acute and persistent reduction in the structural integrity of exposed skeletal tissue (Baxter et al. 2005; Willey et al. 2010). During the course of radiation therapy (RT) for tumor treatment, the incidental irradiation of non-cancerous (normal) skeletal tissue in joints can be fairly substantial (Konski & Sowers 1996; Luxton et al. 2004). While various reports demonstrate the extent and nature of bone and

Corresponding Author: Jeffrey S. Willey, Wake Forest School of Medicine, Radiation Biology Section, 4th Floor NRC Building, Box 571059, Medical Center Blvd, Winston-Salem, NC 27106, Phone: 864-506-0672, jwilley@wakehealth.edu.

Declaration of Interest Statement

The authors report no declarations of interest.

growth plate cartilage damage following exposure (Damron et al. 2008; Kwon et al. 2008; Pritchard et al. 2010; Willey et al. 2010; Jia et al. 2011; Alwood et al. 2012), radiation effects on articular cartilage within the joint structure are undefined and relatively unstudied. Progressive degeneration and arthritis have been reported in various joints exposed to radiation (Kolar et al. 1967). Specifically, cartilage thinning, pain, swelling, and ultimately erosion of subchondral bone have been described from hips following pelvic RT and from joints within the hand following occupational exposures (Kolar et al. 1967). The effects of radiation on cartilage may be masked by both the considerable delay existing between RT and joint symptoms, and the increased incidence of hip fractures following RT with subsequent alteration of joint loading (Kolar et al. 1967). Thus little research has investigated the contribution of radiation effects on articular cartilage to arthropathy.

Studies of radiation effects on articular, embryonic, or physal cartilage have focused on radiation-induced changes in functional matrix production and metabolism (Hugenberg et al. 1989; Jikko et al. 1996). Mature articular cartilage is generally considered radiation resistant in terms of cell viability, especially relative to other tissues with higher proliferative capacity (Cornelissen et al. 1996; Jikko et al. 1996) or when comparing articular versus physal cartilage within a single irradiated rodent model (Baserga et al. 1961). Direct radiation effects on matrix production or breakdown are therefore of primary interest in studies that use articular cartilage to model the functional and cellular consequences of direct articular cartilage irradiation for therapeutic purposes (e.g., radiation synovectomy; (Hugenberg et al. 1989)). Identifying radiation effects on matrix metabolism also provides insight into the mechanisms behind bone growth deficits observed in pediatric patients who receive RT (Cornelissen et al. 1996; Hiranuma et al. 1996; Margulies et al. 2006; Damron et al. 2008; Damron et al. 2009). While published results on cartilage matrix response to irradiation are inconsistent when using tissues or cells from embryonic or rapidly growing animals (Matsumoto et al. 1994; Jikko et al. 1996), limited data suggests radiation can negatively impact matrix metabolism in adult animal models (Hugenberg et al. 1989). Overall, radiation effects on matrix metabolism remain unclear.

A clear understanding of radiation effects on articular cartilage is necessary in order to determine how radiation can contribute to arthropathy in humans following exposure. Cartilage matrix consists primarily of proteoglycan (PG) polymers (particularly aggrecan) and type-II collagen (Leong et al. 2010), which determine the mechanical properties of the tissue. The fixed negative charges of the highly sulfated glycosaminoglycans (GAG) covalently linked to the core protein of the PG imparts compressive stiffness by attracting water (Torzilli et al. 1997; Yoo et al. 2011). Reducing PG content in cartilage matrix can thus lower compressive stiffness. Progressive destruction and loss of matrix contributes to arthropathies such as osteoarthritis (Goldring & Marcu 2009). Osteoarthritic or aged chondrocytes can exhibit both an increased production of matrix metalloproteinases (MMP) capable of eroding matrix and resistance to insulin-like growth factor (IGF)-1 signaling, which is critical for new matrix production via activation of the PI-3Kinase / Akt pathway (Goldring & Marcu 2009; Loeser 2009; Yin et al. 2009). While few studies have investigated radiation as a cause for arthropathy, direct measurement of articular cartilage mechanical properties following exposure are absent from the literature. However, recent preclinical evidence published in preliminary form has shown that radiation can induce an acute reduction in the surface mechanical properties of mouse and pig articular cartilage, specifically lowering compressive stiffness (Lindburg 2011). A weakening of cartilage at articular surfaces in response to irradiation, specifically by altering matrix metabolism, could contribute to overall joint erosion. Clinical evidence may support this notion: though not directly linked with radiation exposure, the incidence of total joint replacement is substantially increased in adults who were treated for cancer as children (Oeffinger et al. 2006).

Our goal was to identify if irradiating mature articular cartilage and chondrocytes harvested from adult human donors could both cause degradation of matrix as well as reduce matrix formation. We examined GAG release from irradiated explants, as well as new PG synthesis from cultured monolayers of differentiated adult articular chondrocytes. As IGF-1 responsiveness is critical for cartilage PG synthesis, we also investigated the response of irradiated cells to this growth factor. Finally, as primary human cultures and tissues are often unfeasible for use in normal tissue injury studies, we document the response of large animal (porcine) cartilage to irradiation as a model for human tissue.

Methods

Reagents and Antibodies

Collagenase-P was purchased from Roche Diagnostics (Manheim, Germany). Pronase was from Calbiochem (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1), antibiotics, and fetal bovine serum were from Invitrogen (Grand Island, NY, USA). IGF-I was from Austral Biologicals (San Ramon, CA, USA). [35S]sulfate was from Amersham Biosciences (Piscataway NJ, USA), and PicoGreen double-stranded DNA assay reagent was from Invitrogen. Antibodies and their sources were as follows: Akt, Akt phospho (p)-Akt (S473), p-Akt (T308), IGF-1 receptor (R) beta, p-IGF-1R (Y1135/1136), from Cell Signaling Technology (Beverly, MA, USA); MMP-1 and MMP-13, and α -actin from Abcam (Cambridge, MA, USA); and ADAMTS 5 from Chemicon (Billerica, MA, USA).

Chondrocyte and Cartilage Explant Preparation and Culture

Normal, non-osteoarthritic human ankle cartilage was obtained from tissue donors within 48 hours of death, through the National Disease Research Interchange (Philadelphia, PA, USA) and Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL, USA). Cartilage was aseptically removed from the talotibial articular surface of the ankle. Articular cartilage was also harvested from the medial and lateral femoral condyles of 3 month old pigs, donated by the Departments of Cardiothoracic Surgery, and Plastic and Reconstructive Surgery at Wake Forest School of Medicine. For cell monolayer studies, cartilage was digested with 0.2% Pronase (Calbiochem, San Diego, CA) for 1 h and then with 0.025% Collagenase-P (Roche Diagnostics, Manheim, Germany) overnight. Monolayer cultures were then established by plating cells in 6-well plates at a density of 1×10^6 cells/ml (2 ml/well) in DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum. Cells were cultured for 6 days to ensure confluence prior to irradiation. For explant studies, full-thickness cartilage discs were harvested using a 4-mm biopsy punch. Prior to irradiation, disks were cultured for 3 days in serum-free DMEM/Ham's F-12 supplemented with 1% mini-ITS plus ascorbate (5 nM insulin, 2 g/ml transferrin, 2 ng/ml selenous acid, 25 g/ml L-ascorbic acid phosphate magnesium salt n-hydrate (Wako, Richmond, VA, USA), 420 g/ml bovine serum albumin, and 2.1g/ml linoleic acid) for recovery before experimental treatments. Media was changed the day of exposure.

Stimulation and Irradiation Procedure

Chondrocytes were switched to serum-free DMEM/Ham's F-12 medium overnight before being irradiated. Serum-free conditions are well tolerated by chondrocytes and allow for the evaluation of IGF-1 signaling and proteoglycan synthesis without the effects of serum (Starkman et al. 2005; Yin et al. 2009). Cell monolayers and disks were transferred to a ^{137}Cs irradiator, and exposed to a 2 Gy or a 10 Gy dose at 364 rad/min. One hour after exposure, select groups of cells were stimulated with IGF-1 (100ng/ml) for 24 hours until lysates were prepared or [35S] sulfate was added to measure proteoglycan synthesis (details below).

Chondrocyte Viability and Signaling

The effects of radiation and IGF-1 stimulation on chondrocyte survival were assessed using the LIVE/DEAD cell assay (Molecular Probes, Eugene, OR, USA), as described previously (Del Carlo & Loeser 2002). To serve as a positive control for cell death, 70% methanol was added to select control and irradiated cells for a half hour prior to performing the LIVE/DEAD assay. To assess radiation's effect on IGF-1 signaling, immunoblotting was performed, as previously described (Yin et al. 2009). Cellular protein was prepared using cell lysis buffer (Cell Signaling Technology; Beverly, MA, USA) supplemented with Phosphatase Inhibitor Mixture 2 (Sigma-Aldrich; St. Louis, MO, USA) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich). Lysates were then centrifuged to remove insoluble fractions, and the soluble protein concentration was quantified with BCA reagent (Pierce Biotechnology; Rockford, IL, USA). Loaded samples containing equal amounts of total protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane (Bio-Rad; Hercules, CA, USA). The ECL detection kit (Amersham Biosciences; Piscataway NJ, USA) was used for blot visualization. Band densitometry analysis was performed using Eastman Kodak Co. 1D 3.6 image analysis software.

Glycosaminoglycan Release Assay

The release of sulfated GAGs from explants into conditioned media was assessed using the dimethylmethylene blue (DMB) assay (Blyscan Analysis; Bicolor Ltd; Newtownabbey, Ireland) per the manufacturers protocol, but using 100 μ l of media for human tissues and 20 μ l from pig tissues. Bovine nasal cartilage (Sigma-Aldrich; St. Louis, MO, USA) was used to generate a standard curve.

Examination of Secreted MMPs and ADAMTS5

Immunoblotting was performed on conditioned media samples harvested from monolayer studies to assess the presence of MMP-1, MMP-13 (as indicated by the secreted proenzyme), and ADAMTS 5. 50 μ l of media collected 24 hours after irradiation was loaded from each condition and immunoblotted as described above. The concentration of MMP-13 was also measured via ELISA (R&D Systems; Minneapolis, MN, USA) from the conditioned media collected from human chondrocytes).

Proteoglycan Synthesis Assay

The [³⁵S] sulfate incorporation assay was performed to measure synthesis of newly formed GAGS within the matrix. Chondrocytes were cultured in serum-free medium in monolayer with or without overnight stimulation using 100 ng/ml IGF-I. The following day, media was replaced with fresh serum-free media for 1 hour, followed by a 4 hour incubation with [³⁵S] sulfate. Media was collected from wells. Following the addition of 1 ml serum-free media, wells were scraped and suspensions were spun at 10,000G for 10 minutes to isolate cell pellets. Pellets were digested overnight at 56°C with 500 μ g/mL of papain (Sigma-Aldrich; St. Louis, MO, USA) digestion. Incorporation of radiolabeled GAGs into newly formed PG was quantified from both media and digested pellet (cell and cell-associated PG) using the Alcian blue precipitation method (Starkman et al. 2005) and then normalized to DNA content of the samples. DNA was quantified by the PicoGreen double-stranded DNA assay (Invitrogen; Grand Island, NY, USA), per the manufacturer's protocol.

Statistics

Data were analyzed using SigmaPlot version 12.0 (Systat Software Inc., Richmond, CA, USA). All statistical comparisons utilized a two-way ANOVA with a Tukey's post-hoc test to account for treatment effects and variance attributed to cells and tissues harvested from

different donors. For all tests, $P < 0.05$. Statistics were performed using raw data, and distinguished between the variance associated with treatment and donor effects in order to correctly assess if radiation treatment was altering cartilage metabolism or cell signaling. Graphs are presented as percentage of control. A significant donor effect was identified for all tests, indicating that baseline levels for tested parameters differed between individuals (data not shown). Likewise, a significant interaction effect (treatment X donor) was identified for most tests, indicating that high variability between donors affected the magnitude (but not direction) of the response to treatment.

Results

Cell Viability and DNA content

The chondrocytes were plated at high density and experiments performed at confluency using unpassaged cultures in order to ensure cells were differentiated and non-proliferative and thus characteristic of mature chondrocytes within adult articular cartilage. Cell viability at 1 day after exposure was unchanged regardless of treatments (2 Gy or 10 Gy of radiation with and without IGF-1) for both isolated monolayer human chondrocytes (Figure 1A and B) and for isolated monolayer porcine chondrocytes (Figure 1C). In an additional experiment, we cultured chondrocytes out to 21 days and observed no change in viability after a 10 Gy dose (data not shown).

DNA content of the cell lysates was quantified for use as a referent for PG synthesis (at Day 1) and GAG release, and as an indirect measure of viability. At Day 1, DNA content (ng/ml) from cells following 2 Gy (2.90 ± 0.8) and 10 Gy (2.63 ± 0.4) exposure without IGF-1 stimulation, and from 2 Gy (2.92 ± 0.2) and 10 Gy (2.64 ± 1.4) groups with IGF-1 treatment, was not different from non-irradiated controls without IGF-1 stimulation (2.99 ± 0.6) and with IGF-1 stimulation (2.40 ± 0.5). As treatments did not alter these values, DNA content of cell monolayers or digested explants were used as a referent for data obtained from other assays for the purpose of normalization to cell number.

Cartilage Breakdown

As high individual variation for GAG release and PG synthesis values between individuals was observed, we present the average raw values from all individuals within each group (Table 1 and 2, respectively) and the average fold change relative to control for each measure (Figure 2 and 4, respectively), plotting the fold change for each individual and determining the average with error: statistics were performed on raw data. While the magnitude of the GAG release scores varied, the response within and between individuals was similar: GAG content increased in media following irradiation. The released GAG content was determined from media collected 48 hours after exposure, and then again at 96 hours from cultures with fresh media applied at 48 hours in order to examine for delayed GAG release. Thus the data indicate GAG release during Day 1 and 2 after exposure (48 hours), and Days 3 and 4 after irradiation (96 hours). The GAG released into the media (normalized to DNA content of corresponding culture) from human explants was significantly greater following 10 Gy exposure (main dose effect), regardless of when assays were performed ($P < 0.05$; Table 1). GAG release following 2 Gy exposure regardless of when media was collected (main dose effect) was marginally greater ($P = 0.053$). Overall, at 48 hours following irradiation, GAG presence in the media was elevated by 68% at 2 Gy, and 48% at 10 Gy (Figure 2A). From media collected at 96 hours, GAG content was 34% and 50% greater after 2 Gy and 10 Gy, respectively. From pigs, radiation resulted in a 65% increase in GAG content released into media at 48 hours after 2 Gy, and a significant 119% increase in GAGs present after 10 Gy ($P < 0.05$; Figure 2B). GAG content in media harvested at 96 hours (final 48 hours of culture) was unchanged between groups.

MMP-1, MMP-13, and ADAMTS 5 were all increased in conditioned media from isolated human chondrocytes following exposure to both 2 Gy and 10 Gy (Figure 3A). ADAMTS 5 is also produced by pig chondrocytes following exposure (Figure 3C). IGF-1 stimulation did not prevent any increase in ADAMTS 5 production. As radiation appeared to alter the presence of several standard loading controls as determined from immunoblotting media (data not shown), we performed ELISA of the conditioned media from human sample examining MMP-13 levels at 1 Day. ELISA confirms MMP-13 is elevated after exposure, statistically greater at 10 Gy with ~135% increase ($P < 0.05$). The 80% increase at 2 Gy did not reach statistical significance (Figure 3B).

Matrix Synthesis

The presence of newly formed proteoglycans at 1 day following irradiation was quantified with and without overnight IGF-1 stimulation. In general, from humans and from pigs, radiation decreased PG synthesis (Figure 4). Similar to GAG release, the individual variation for PG synthesis was substantial enough to necessitate presenting both raw values (Table 2) and the average fold change relative to control within groups (Figure 4). Again, the high variation did not affect the response within and between individuals: PG synthesis was lowered with radiation. From humans, counts relative to DNA content were significantly lower from combined media and pellet samples (and from both individually, data not shown) relative to control at 2 Gy (−13%; $P < 0.01$) and 10 Gy (−16%) (Figure 4A; $P < 0.05$). IGF-1 stimulation caused a marked increase in PG synthesis of approximately 123% relative to control ($P < 0.001$). The IGF-1 mediated increase in new PG synthesis was impaired with radiation, with counts significantly lower than non-irradiated + IGF-1 stimulation at both 2 Gy and 10 Gy ($P < 0.001$). Relative to non-irradiated controls without IGF-1 stimulation, IGF-1 stimulation only increased PG synthesis by 78% at 2 Gy ($P < 0.001$), and 35% at 10 Gy ($P < 0.01$).

A very similar pattern was seen from pig chondrocytes following exposure as for human cells: radiation significantly impaired PG synthesis at 10 Gy (−48%, Figure 4B; $P < 0.001$). IGF-1 stimulation increased PG production following exposure (+92% at 2 Gy, $P < 0.001$; and +87% at 10 Gy, $P < 0.05$) over non-irradiated control without IGF-1 stimulation. However, radiation impaired this response, with a significant reduction in counts relative to non-irradiated controls with IGF-1 stimulation at 2 Gy and 10 Gy ($P < 0.001$).

IGF-1 Signaling

Since Akt activation promotes PG and collagen synthesis, and as OA chondrocytes exhibit reduced sensitivity to IGF-1 mediated Akt activity, we examined the effect of radiation on IGF-1 signaling. Phosphorylation of Akt at both the serine (473) and threonine (308) activation sites was observed 1 day after irradiation in human (Figure 5A) and pig (Figure 5C) cells stimulated with IGF-1. However, from both human and pig cells, Akt phosphorylation with IGF-1 stimulation was impaired at both 2 and 10 Gy (Figure 5A and C). While radiation induced a marked reduction in IGF-1 mediated Akt phosphorylation, a modest reduction following irradiation was observed in both phosphorylation of IGF-1R with IGF-1 stimulation from human cells (Figure 5A) and basal IGF-1R phosphorylation without IGF-1 stimulation (Figure 5B). Additionally, a slight overall reduction in total IGF-1R both with and without IGF-1 stimulation was observed (Figure 5A).

Discussion

The radiation dose provided to synovial joints during the course of RT is minimized in an attempt to reduce the incidence of bone damage and growth abnormalities. Little consideration is given to radiation effects on articular cartilage, as radiation-induced bone

damage, including spontaneous hip fracture, is the most obvious (with growth deficiency) and potentially catastrophic skeletal disorder following irradiation (Baxter et al. 2005). Joint deterioration in the irradiated hip is generally attributed to the altered loading associated with hip fracture. However, degenerative arthropathy and arthritis have been described in irradiated hips in the absence of fracture, or in joints of the hand following occupational exposures (Kolar et al. 1967). The incidence of arthropathy can be fairly substantial: in the most extensive study to date, which was reported 40 years ago, ~23% of those who received occupational exposure to hands developed arthropathy over the course of a 10 year follow up (Kolar et al. 1967). The overall incidence of arthropathy of the hip was ~8% at 10 years. No definitive or well supported mechanism has been established as to how radiation can contribute to arthropathy (Cornelissen et al. 1996).

In this study, exposure of primary human and porcine chondrocytes and explants to ionizing radiation lowered PG synthesis, increased matrix degradation, and was associated with elevated MMP and ADAMTS 5 production, and impaired IGF-1 signaling. Though these changes were observed early after exposure (within the first week) from cultured explants and cells, they could negatively affect the functional and mechanical properties of the tissue if the findings are translated *in vivo*. Cell viability was not affected by radiation exposure from these confluent, non-proliferating cell cultures, in agreement with other studies (Hong et al. 2010). As these tissues were removed from the joint prior to exposure, radiation likely had a direct effect on cartilage and chondrocyte metabolism. The lowered sensitivity to IGF-1 signaling (with lowered Akt activation when stimulated with IGF-1) could account for a reduced PG production. The rapidity over which these changes occurred largely support a published abstract describing the quick reduction in surface compressive modulus from animal models of irradiated articular cartilage, including from pigs (Lindburg 2011). Thus from human and porcine tissue, radiation can induce early deficits in cartilage, though our observations are from early time points following exposure and changes in cartilage following irradiation can be time dependent (Mosier et al. 1983).

The reduction in PG synthesis following irradiation from our pig and human tissue largely agrees with the dose-dependent reduction in PG synthesis observed following irradiation of articular cartilage in an adult canine model (Hugenberg et al. 1989). The majority of the other published accounts have reported radiation effects on cartilage matrix synthesis from embryonic, rapidly growing, or physal tissue, most commonly from rabbits, in order to identify mechanisms for bone growth deficiency following irradiation within pediatric RT patients. These studies yielded conflicting results. In irradiated monolayers or 3-D cultures generated from digested articular cartilage of 21 day old rabbits (Matsumoto et al. 1994), no reduction in PG synthesis was observed at doses of 10 Gy in cells that had been plated 5 days before exposure. However, from another study of 28 day old rabbits, cells exposed to 10 Gy did exhibit reduced PG production, despite being cultured in similar conditions (Jikko et al. 1996). Likewise, studies examining irradiated embryonic cartilage reported a dose dependent decline in matrix production following doses greater than 20 Gy (Cornelissen et al. 1990), and substantially reduced following doses > 100 Gy (de Ridder et al. 1988; Cornelissen et al. 1990). As the results between our adult human and large animal model compare favorably with the adult canine model and some inconsistency exists among studies using rapidly growing and immature animal models, the larger and more mature animal models may be more appropriate to model the effects of radiation on articular cartilage health than tissue from growing or smaller animals.

Degradation of the type II collagen and proteoglycans present within the matrix of articular cartilage involves several enzymes (Leong et al. 2010). In this study, we document an increase in ADAMTS 5 and the collagenases MMP-1 and MMP-13 in conditioned media from human and pig cells following 2 Gy and 10 Gy exposure. Of particular note, MMP-13

plays a major role in cleavage of type II collagen during osteoarthritis (Mitchell et al. 1996; Long & Loeser 2010). Additionally ADAMTS 5 (aggrecanase-2) is a key proteolytic enzyme for aggrecans in the cartilage matrix and is a primary enzyme leading to matrix dysfunction during OA (Verma & Dalal 2011). The expression of matrix metalloproteinases and aggrecanases in human chondrocytes has been linked with activation of mitogen activating protein (MAP) kinases (Beier & Loeser 2010). Though causality cannot be determined, radiation has previously been shown to increase the activation of the MAP kinases ERK, JNK, and p38 from rabbit chondrocytes within the first day of exposure to 10 Gy (Hong et al. 2010).

The increased presence of ADAMTS 5 and matrix metalloproteinases in the conditioned media of irradiated human and pig cells was associated with a release of GAG from explants. This degradation of cartilage matrix is in agreement with observed GAG release from cartilage explants harvested from the knees of adult dogs (Hugenberg et al. 1989), though concentration or activity of MMPs were not measured. In contrast, MMP activity (MMP-1, 2, 3, and 9) in conditioned media of articular and growth plate chondrocytes cultured from 28 day old rabbits was unchanged after a 10 Gy dose (Jikko et al. 1996). Moreover, unlike the present study, irradiation of cartilage explants did not affect PG degradation, indicated by GAG release into the media (Jikko et al. 1996). Similar to the PG synthesis response, degradation of cartilage following exposure may be dependent on the radiation model (*in vitro* vs. *in vivo* vs. *ex vivo*), age of animal at exposure, developmental maturity of the model, and dose (Hugenberg et al. 1989; Jikko et al. 1996). These data also suggest that articular chondrocytes from pigs could prove to be a useful model for radiation-induced arthropathy in humans. While we may assume the GAG release was mediated by the MMP and ADAMTS 5 concentration in the cultured media following exposure, we did not test blocking function using an inhibitor of these proteolytic enzymes following exposure (Lin & Liu 2010).

The nature of both the impaired proteoglycan production and increase in PG degradation from irradiated human and pig chondrocytes following irradiation is unclear. Other reports identifying reduction of proteoglycan synthesis from embryonic cartilage note evidence of apoptosis coincident with reduced matrix production following doses greater than 100 Gy (de Ridder et al. 1988; Cornelissen & de Ridder 1990). We observed no differences in cell death using the LIVE-DEAD assay, and no drop in DNA content. Likewise, at doses of 10 Gy or less, others have observed no differences in chondrocyte viability (Jikko et al. 1996; Hong et al. 2010). One potential explanation for our observed reduction in PG synthesis is a potential imbalance between IGF-1 activation of the PI-3-Kinase / Akt pathway and MAP kinase activation following exposure. IGF-1 signaling and Akt activation are necessary for proteoglycan synthesis in cultured human chondrocytes (Starkman et al. 2005; Yin et al. 2009). Specifically, we identify reduced Akt phosphorylation (S473 and T308) following irradiation at both 2 and 10 Gy in humans when stimulated with IGF-1, with a marginal reduction in total and phosphorylated IGF-1R. And as noted, activation of MAP kinases can both cause cartilage destruction through increased MMP and aggrecanase production, and impaired PG synthesis (Yin et al. 2009). An imbalance between PI-3-Kinase / Akt activation and MAP kinase activity after irradiation could contribute to cartilage deterioration. As endogenous Akt phosphorylation without IGF-1 stimulation is difficult to visualize via immunoblotting, we cannot draw robust conclusions regarding radiation effects on Akt activation in the absence of IGF-1 stimulation. However, we did note that activation of the IGF-1 receptor was lower after irradiation with overexposed blots (Fig. 4B), and the downstream effects could decrease Akt phosphorylation, resulting in the lowered PG synthesis observed following 2 Gy and 10 Gy without IGF-1.

Acquisition of a senescence-associated secretory phenotype following irradiation could be one possible explanation for the observed alteration in matrix metabolism. This phenotype is characterized by an increase in MMP, cytokine, and chemokine production (Loeser 2009). Increased intracellular concentration of reactive oxygen species (ROS) may contribute to this phenotype and to impaired IGF-1 signaling (Yin et al. 2009). Radiation has recently been shown to induce senescence among young rabbit articular chondrocytes early after exposure (Hong et al. 2010). Activation of MAP Kinases contributed to this phenotype, specifically p38, and was mediated by elevated oxidative stress (Hong et al. 2010). Thus it is possible that a radiation-induced a senescent phenotype contributed to the observed functional deficits.

In summary, exposure to 2 Gy and 10 Gy doses of ionizing radiation lowered proteoglycan synthesis, induced active degradation of matrix, and impaired IGF-1 signaling in human and pig cartilage and chondrocytes. IGF-1 mediated phosphorylation of Akt at multiple activation sites was lowered following irradiation, and could account for the lowered PG production. An imbalance may exist between the PI-3 Kinase / Akt pathway and MAP Kinase pathways following irradiation of chondrocytes, resulting in both active matrix degradation and lowered formation, though our observations remain speculative. A weakening of articular cartilage through persistent radiation-induced changes in matrix metabolism or permanent deterioration of mechanical properties could contribute to arthropathy following direct irradiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Cell viability of human and pig chondrocytes following irradiation with 2 or 10 Gy γ -rays. “NR” represents “non-irradiated”. A). Live (green) versus dead (red) human chondrocytes at 1 day following exposure to 2 and 10 Gy of radiation using a LIVE/DEAD assay, with and without IGF-1 stimulation (100X magnification). 70% MeOH was used as a control for dead cell imaging. B) and C). Counts are from n = 2 independent studies of irradiated human (B) and pig (C) chondrocytes.

Figure 2.

Glycosaminoglycans (GAG) released into the media from explants of human (A) and pig (B) cartilage. GAG content was quantified after exposing explants to 2 and 10 Gy of radiation. Media was harvested at 48 hours after exposure or at 96 hours (48 hours after an exchange of fresh media). Plots represent fold change versus control (0 Gy). Data are $n = 3$ independent experiments from both humans and pigs, each using data from two explants per treatment. Error bars indicate SEM. The asterisk (*) indicates a significant main radiation effect of 10 Gy regardless of time relative to 0 Gy control following a two-way ANOVA of raw data and a Tukey's Post Hoc Test ($\alpha = 0.05$). The (#) indicates a significant difference relative to control at 48 hours after exposure.

Figure 3.

Effect of radiation on MMP production in human and pig chondrocytes from media collected at one day after irradiation with 2 Gy or 10 Gy γ -Rays. “NR” represents “non-irradiated”. A). Representative blots from human chondrocytes for ADAMTS 5 (aggrecanase-2), MMP-1, and MMP-13. Results are representative of n = 3 independent experiments. B). Results from an ELISA for MMP-13 concentration in conditioned media of human chondrocytes one day after irradiation (n = 2 independent experiments). Error bars indicate SEM. Statistics were performed on raw data. The asterisk (*) indicates significant difference from 0 Gy following a two-way ANOVA (main effects were individual and dose) and a Tukey’s Post Hoc Test indicating difference between groups ($p = 0.05$). C). ADAMTS 5 concentration from the conditioned media of pig explants irradiated with either 2 or 10 Gy doses. Data represent n = 3 studies.

Figure 4.

Proteoglycan synthesis from human (A) and pig (B) chondrocytes at one day after irradiation with 2 or 10 Gy and IGF-1 stimulation. “NR” represents “non-irradiated”. Data are representative of $n = 2$ (human) and $n = 3$ (pig) independent studies per treatment condition. Error bars indicate SEM. These data are determined from $[^{35}\text{S}]$ incorporation into newly formed proteoglycans, and calculated as counts per minute relative to DNA content of the cells. Data are plotted as the average fold change for individuals relative to NR, No IGF. Statistics were performed on raw data. The symbols above horizontal lines indicates significant difference between groups following a two-way ANOVA (main effects were individual and dose) and a Tukey’s Post Hoc Test indicating difference between groups ($\alpha = 0.05$). The topmost lines indicate differences between IGF-1 and non-IGF-1 treated groups. (* $P < 0.01$; # $P < 0.05$).

Figure 5.

Effects of radiation on IGF-1 activation of Akt and IGF-1R in human (A and B) and pig (C) chondrocytes. “NR” represents “non-irradiated”. One hour after irradiation using 2 or 10 Gy γ -Rays, groups of cells were treated with IGF-1 overnight (100ng/ml). Immunoblots are representative results from a minimum of $n = 3$ independent experiments. Error bars indicate SEM. Lysates were immunoblotted for: A) p-Akt (S473 and T308), total Akt, p-IGF-1R, total IGF-1R, and β actin. Densitometry for each protein is identified by the figures on the right. For p-Akt blots, total Akt was used as the referent. For IGF-1R blots, β actin density was used as the referent. B) Immunoblot for pIGF-1R were overexposed to identify differences in the 2 Gy and 10 Gy lanes versus non-irradiated (NR) lanes. Densitometry is

not presented for overexposed blots. C). P-Akt (S473) and total Akt blots from pig lysates. Densitometry of the blot is identified by a graph on the right.

Table 1

Proteoglycan release from human and pig cartilage as determined from sulfated GAG content in media surrounding explants at 48 and 96 hours after irradiation.

	Non-Irradiated	2 Gy	10 Gy
<i>Human</i>			
48 Hours $\mu\text{g GAG} / (\text{ng DNA} / \text{ml})$	3.38 ± 0.71	5.52 ± 0.8	5.04 ± 1.35^a
96 Hours $\mu\text{g GAG} / (\text{ng DNA} / \text{ml})$	3.53 ± 0.98	4.62 ± 1.55	5.19 ± 1.01^a
<i>Pig</i>			
48 Hours $\mu\text{g GAG} / (\text{ng DNA} / \text{ml})$	0.71 ± 0.12	1.20 ± 0.54	1.90 ± 0.49^b
96 Hours $\mu\text{g GAG} / (\text{ng DNA} / \text{ml})$	0.77 ± 0.21	0.77 ± 0.31	0.89 ± 0.22

Note. All values shown as mean $\pm$ SD for $n = 3$ independent experiments and using a Two-Way ANOVA accounting for individual variation and dose (main effects) within each time point. For humans, 10 Gy resulted in a significantly greater release of GAGs regardless of time point ($^aP = 0.05$), with 2 Gy at both time points being marginally greater than control ($P = 0.053$). A 10 Gy dose increased GAG release after 48 hours in pigs ($^bP = 0.05$).

Table 2

Proteoglycan synthesis values from human and pig chondrocytes at one day following irradiation.

	Non-Irradiated		2 Gy		10 Gy	
	.	IGF-1	.	IGF-1	.	IGF-1
Human Chondrocytes CPM / (ng/ml DNA)	1382 ± 85	3480 ± 71 ^a	1081 ± 68 ^b	2771 ± 86 ^a	1113 ± 175 ^b	1722 ± 65 ^a
Pig Chondrocytes CPM / (ng/ml DNA)	297 ± 70	549 ± 5 ^a	204 ± 48	417 ± 9 ^b	74 ± 10 ^a	119 ± 9

Note. CPM represents “counts per minute”. All values are shown as mean ± SD for n = 2 (human) and 3 (pig) independent experiments.

^a $P < 0.01$ and

^b $P < 0.05$ versus non-irradiated control without IGF-1 stimulation, following Two-Way ANOVA (main effects are Treatment and Individual) with Tukey's Post-Hoc test.