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Biological Effects of the Plant-derived Polyphenol Resveratrol in Human Articular Cartilage and Chondrosarcoma Cells

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Abstract

The natural phytoestrogen resveratrol (RSV) may have therapeutic potential for arthritic conditions. RSV is chondroprotective for articular cartilage in rabbit models for arthritis, but its biological effects on human articular cartilage and chondrosarcoma cells are unknown. Effects of RSV on human articular cartilage homeostasis were studied by assessing production of matrix-degrading enzymes (MMP-13, ADAMTS-4, and ADAMTS-5), as well as proteoglycan production and synthesis. The counteractions of RSV against catabolic factors (e.g., FGF-2 or IL-1 β) were examined by *in vitro* and *ex vivo* using monolayer, three-dimensional alginate beads and cartilage explants cultures, respectively. RSV improves cell viability of articular chondrocytes and effectively antagonizes cartilage-degrading protease production that was initiated by catabolic and/or anti-anabolic cytokines in human articular chondrocytes. RSV significantly also enhances BMP7-promoted proteoglycan synthesis as assessed by ³⁵S-sulfate incorporation. Protein-DNA interaction arrays suggest that RSV inhibits the activation of transcription factors involved in inflammation and cartilage catabolic signaling pathways, including direct downstream regulators of MAPK (e.g., AP-1, PEA3) and NF κ B. RSV selectively compromises survival of human chondrosarcoma cells, but not primary articular chondrocytes, revealing cell-specific activity of RSV on non-tumorigenic versus tumor-derived cells. We propose that RSV exerts its chondroprotective functions, in part, by deactivating p53-induced apoptosis in human primary chondrocytes, but not human chondrosarcoma. Our findings suggest that RSV has potential as a unique biologic treatment for both prevention and treatment of cartilage degenerative diseases.

Keywords

articular cartilage; cartilage degeneration; regeneration; osteoarthritis; chondrosarcoma; resveratrol; matrix metalloprotease; MMP13; proteoglycan; cell survival; cancer

Introduction

Osteoarthritis (OA) is a progressive and functionally debilitating disease characterized by erosion of articular cartilage. Clinical management of OA involves symptomatic treatment

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aimed at minimizing joint pain and inflammation, but most current treatment strategies fail to address or interfere with early biochemical and pathophysiologic processes underlying the disease process itself. As a result, recent research efforts have focused on the development of novel biologic therapies aimed at slowing and/or reversing cartilage degradation at a molecular level.

Under normal conditions, articular chondrocytes maintain a dynamic equilibrium between synthesis and degradation of extracellular matrix (ECM) components, including type II collagen fibrils surrounding and restraining large, hydrated aggregates of proteoglycans, allowing normal cartilage to function as a shock absorber and withstand compressive loads (Nakata et al., 1993). In OA, perturbations in the normal metabolic properties of chondrocytes result in the destruction of the ECM *via* release of destructive enzymes, including matrix metalloproteinases (e.g., MMP-13) and aggrecanases (e.g., ADAMTS4 and ADAMTS5). Stimulation of these proteases by chondrocytes themselves, growth factors, and inflammatory cytokines further perpetuates ECM destruction and disease propagation (Im et al., 2007a; Im et al., 2007b; Martel-Pelletier et al., 2001; Muddasani et al., 2007a). Several groups have demonstrated that the signaling cascades generated by inflammatory cytokines (e.g., IL-1) or fibroblast growth factor-2 (FGF-2 or basic FGF) favor catabolism by stimulating protease production and inhibiting proteoglycan deposition in human adult articular cartilage or intervertebral disc tissue *via* ERK/MAPK activation (Im et al., 2007b; Im et al., 2003; Le Maitre et al., 2004; Le Maitre CL, 2007 Aug 9; Shinmei et al., 1988). FGF-2 also mediates striking antagonistic effects on cartilage anabolic activity in conjunction with IGF-1 and BMP7, and both FGF-2 and IL-1 modify chondrocyte gene expression when stimulated by mechanical injury (Cravero et al., 2009; Ellman et al., 2008; Im et al., 2008).

Resveratrol (RSV, trans-3,4',5-trihydroxystilbene) is a natural polyphenol compound found in various plants including grapes and red wines. Its powerful and diverse biological effects have been well documented in the literature. RSV is considered to be anti-oxidative, anti-inflammatory, anti-aging, anti-cancer (due to anti-proliferative, anti-angiogenic and/or anti-heparinase activity), and anti-viral (Bertelli et al., 1999; Bhat et al., 2001; Fremont, 2000; Haider et al., 2002; Huang et al., 2001; Ignatowicz and Baer-Dubowska, 2001; Jang et al., 1997; Leiro et al., 2004; Martinez and Moreno, 2000). Recently, Elmali and colleagues reported a significant protective effect of RSV injections on articular cartilage degradation in rabbit models for OA and RA *via* histological analysis *in vivo* (Elmali et al., 2007; Elmali et al., 2005). In human articular chondrocytes, Shakibaei (Shakibaei et al., 2007) and Czaki (Csaki et al., 2008) elucidated anti-apoptotic and anti-inflammatory regulatory mechanisms mediated by RSV. Previously, we have demonstrated the potent anabolic and anti-catabolic potential of RSV in bovine intervertebral disc cartilage (Li et al., 2008b).

The aim of the present study is to determine the potential benefits of RSV to impede the progression of adult human articular cartilage degeneration by assessing its biological effects on both normal and arthritic human articular chondrocytes. The impact of RSV on proteoglycan accumulation and catabolic factor-mediated matrix-degrading enzyme expression (MMPs and aggrecanases) is elucidated *in vitro* and *ex vivo*, in addition to evaluation of downstream mediators regulated by RSV. Further, to assess the specific chondroprotective effects of RSV on knee joint chondrocytes, we compared RSV-mediated effects in non-tumor human articular chondrocytes and synoviocytes to RSV-mediated effects in chondrosarcoma cells.

Materials & Methods

Chondrocyte isolation and culture

Normal human knee joints were obtained within 24 hours of death from donors (age ranging from 40 to 65) through the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL) for articular chondrocytes and synoviocytes with approval by the local ethics committee and consent from families. Prior to dissection, each specimen was graded for overall degenerative changes based on the modified 5-point scale of Collins (Muehleman et al., 1997). Surgically removed cartilage samples from OA patients (age ranging from 40 to 65) were obtained from the Orthopedic Tissue and Implant Repository Study with consent from the patients. Human tissues were handled according to the guidelines of the Human Investigation Committee of Rush University Medical Center. Chondrocytes and synoviocytes were isolated by enzymatic digestion of cartilage using pronase for 1 hour, followed by overnight digestion with collagenase-P as described previously (Im et al., 2003; Loeser et al., 2005a). Alginate beads and monolayers were prepared for long-term and short-term cell culture analyses, respectively.

For alginate bead cultures, isolated chondrocytes were resuspended in 1.2% alginate (2×10^6 cells/mL) and beads were formed by drop-wise addition into a CaCl_2 solution as previously described (Hauselmann et al., 1992). Briefly, beads were cultured at 8 beads/well in 24-well plates in 1 mL/well of DMEM/Ham's F-12 medium (1:1) supplemented with 1% mini-ITS+ (insulin-transferrin-selenium) and 0.1% ascorbic acid (Gruber et al., 2006). Cells were treated with RSV at 10, 25, 50, or 100 μM (Calbiochem, San Diego, CA), FGF-2 at 50 ng/mL (NCI, Bethesda, MD), or BMP7 at 100 ng/mL (Stryker Biotech, Hopkinton, MA). Triplicate wells were used for each condition. The medium in all cases was changed every other day over a 21-day period before DMMB analysis.

For monolayer cultures, isolated cells were washed and suspended in culture media at 3×10^6 cells/mL, and the cells were then seeded onto 12-well plates using 1 mL media/well. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 (1:1) containing 10 % fetal bovine serum and antibiotics (complete media). The cells were treated with RSV (1–200 μM), FGF-2 (100 ng/mL), and IL-1 β (1 ng/mL) (Amgen, Thousand Oaks, CA). The supernatant was removed 24 hours after the initiation of each treatment and subjected to immunoblotting, as described below. For nuclear extraction, experiments were terminated 45 minutes after treatment was initiated.

Tumor cells lines such as human chondrosarcoma (105KC) and lung cancer cells (A549) were cultured as previously described (Loeser et al., 2005b). Transient transfection was performed using previously published protocols (Im et al., 2003). Human lung cancer cells and human chondrosarcoma cells were cultured as described (Liu et al.).

Adult human articular cartilage explant culture (ex vivo organ culture)

Previously, we noted that cellular response to FGF-2 is greater in damaged cartilage and/or osteoarthritic chondrocytes compared to normal cartilage (grade 0 or 1). Based on our observations, for explant cultures, early-stage OA femoral cartilage (grade 2, average age 50-year-old) was used for the study. Full-thickness explants of 4-mm diameter were excised from cartilage using a biopsy puncher. Explants then recovered in complete media for 2 days before switching to mini-ITS+-supplemented media (1%). After another 2 days, explants were treated with agents of interest for 7 days.

Histological assessment

Human articular cartilage explants were fixed in 4% paraformaldehyde and embedded in paraffin (n=4). Serial sections of 10 μ m thickness were prepared and placed onto slides. Paraffin was removed from sections with xylene, followed by stepwise rehydration of sections in ethanol and distilled water. For Safranin-O Fast Green staining, sections were immersed in 0.1% Fast Green for 3 min, followed by 0.1% Safranin-O for 15 min. An unblinded investigator grouped the slides and randomly numbered them. These groups were then graded by two different blinded investigators (H-JJ and XL). A relative grade was assigned from 0–4, where 0 means no staining (PG loss) and 4 means the most intense stain (normal disc) based on Safranin-O. Two independent examinations were performed, and the repeatability of grading on the two occasions was determined using Cohen kappa statistics.

Dimethylethylene blue (DMMB) assay for proteoglycan accumulation and DNA assay for cell numbers

The alginate culture system offers the possibility to quantify the accumulation of the cell-associated matrix (CM). At the end of the 21-day alginate culture period, the medium was removed and the alginate beads were collected and processed for proteoglycan assays using the DMMB binding method, as previously described (Gruber et al., 2006; Li et al., 2008a; Li et al., 2008b; Loeser et al., 2005a). The CM was separated from the remainder of the matrix by centrifugation and proteoglycan accumulation per cell in the CM was quantified (Altman et al., 1986). Live and Dead assessments of cells in alginate were performed by PicoGreen Calcein AM to stain live cells, and ethidium bromide homodimer 1 to stain dead cells following the manufacturer's protocol (Molecular Probes, Eugene, OR). Survival was measured weekly throughout the entire culture period for 21 days. At least 100 cells were counted in triplicate for each data point, similar to previous experiments (Loeser et al., 2003).

³⁵S-Sulfate incorporation into newly synthesized proteoglycans

The same labeling protocol was used for all cultures. On day 7 of culture in alginate, the medium was removed and replaced by fresh medium. One hour later, this medium was replaced with fresh medium containing [³⁵S]-sulfate at 20 μ Ci/mL (Amersham Corp., Arlington Heights, IL). After incubation for 4 hours, the labeling medium was removed and beads were rinsed twice in cold 1.5 mmol/L SO₄ wash medium. Beads were dissolved and digested with 400 μ L papain (20 μ g/mL in 0.1M sodium acetate, 0.05 MEDTA, pH 5.53) at 60°C for 16 hours. Sulfate incorporation was measured using the Alcian blue precipitation method, as previously described (Loeser et al., 2000). All samples were analyzed in duplicate and normalized for DNA content using Hoechst 33258 as previously described (Loeser et al., 2000).

Protein-DNA interaction arrays using nuclear extracts

Nuclear extracts were prepared from cells cultured in 6-well monolayer plates using the Nuclear Extraction Kit (Panomics, Inc., Fremont, CA) according to the manufacturer's protocol. Protein concentrations were determined using the Micro bicinchoninic acid assay (Pierce, Rockford, IL). Biotin-labeled DNA binding oligonucleotides (TranSignal Probe Mix) were pre-incubated with nuclear extracts with different treatments to allow for the formation of protein/DNA complexes. The probes in these complexes were then extracted and hybridized to the TranSignal protein/DNA array membranes following the procedure outlined in the user manual of the Panomics TranSignal spin protein/DNA array kit. Membrane detection was conducted following the procedure in the Panomics manual.

Transient transfection of MMP13 luciferase promoter construct

Nucleofection was optimized for human articular chondrocytes based on the manual of the Nucleofector™ kit (Lonza, Walkersville, MD), as described previously (Loeser et al., 2005b; Pulai et al., 2005). The MMP-13 luciferase promoter plasmid construct (Im et al., 2003) was used for transfection at a concentration of 1 µg/reaction. After 24 hours, cells were co-treated with RSV (100 µM) + FGF-2 (100 ng/mL) or RSV + IL-1β (1 ng/mL), and incubated for another 24 hours. *Renilla* vector (pRL-TK, 100 ng/reaction) was co-transfected as an internal control, as we described previously (Yan et al.). Both *Renilla* and firefly luciferase activity were measured simultaneously using a dual-luciferase reporter assay system (Promega, Madison, WI) and a luminometer (Berthold, Huntsville, AL).

Cell Survival/Toxicity Assays

After two days of RSV treatment, cell viability was measured using the Cell Titer 96 Aqueous One Solution Cell Proliferation and Toxicity Assay. The MTS tetrazolium compound is reduced in metabolically active cells, which are quantified by measuring the optical density at 490nm.

Cell Stimulation and Immunoblotting

Experiments were terminated with removal of medium and/or cell lysate preparations as described above. The conditioned media or cell lysates were collected and the total protein concentrations were determined by a bicinchoninic acid protein assay (Pierce, Rockford, IL). In each case, equal amounts of protein were resolved by 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane for immunoblot analyses as previously described (Li et al., 2008b). Antibodies were purchased from either R&D System (Minneapolis, MN) or Abcam (Cambridge, MA).

Active MMP-13 Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of active MMP-13 was quantified in the conditioned media using an InviLISA® Human Act MMP-13 Assay Kit (Protealmmun GmbH, Berlin, Germany). Briefly, conditioned medium was added into wells of a microtiter plate coated with MMP-13 specific antibodies. After 2-hour incubation at room temperature, biotinylated antibody was added to each well to detect the bound active-MMP-13. Then, with the substrate incubation, color development from MMP-13 activity was measured using the plate reader, with the wavelength of 450 nm. A highly specific monoclonal antibody for the activated form of human MMP-13 permits specific detection of the active form of MMP-13 at a sensitivity of 7 pg/mL.

Reverse transcription and quantitative real-time polymerase chain reaction

Total cellular RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) following the instructions provided by the manufacturer. Reverse transcription (RT) was carried out with 1 µg total cellular RNA using ThermoScript RT-PCR system (Invitrogen) for first strand cDNA synthesis in 20 µL of reaction volume. For real-time qPCR, cDNA was amplified using the Bio-Rad MyiQ Real-Time PCR Detection System. RT product was subjected to real-time PCR in a 20-µL total reaction mixture containing 10 µL Bio-Rad iQ SYBR Green supermix (Bio-Rad, Hercules, CA). A threshold cycle (CT value) was obtained from each amplification curve using the iQ5 Optical System Software (Bio-Rad) provided by the manufacturer. Relative mRNA expression was determined using the µCT method, as detailed by manufacturer guidelines (Bio-Rad). The housekeeping gene s18RNA was used as internal control in the reaction for normalization. The primer sequences and the conditions for their use are summarized in Table 1.

Data analyses

Statistical significance was determined by Student's t-test, or one-way repeated measures ANOVA followed by Sidak *post-hoc* test using the SPSS 17 program. All data were expressed as the mean $\pm$ standard error. Statistical analysis was performed using the Statview (Version 5.0, SPSS, Chicago, IL) program package. The Cohen kappa value was calculated using the internet-based program (http://department.obg.cuhk.edu.hk/researchsupport/Cohen_Kappa_data.asp). $P < 0.05$ was defined as significant for all tests.

Results

RSV has net anabolic effects on primary human articular cartilage

To investigate whether RSV inhibits matrix degradation *ex vivo*, we incubated full-thickness femoral cartilage explants (grade 2) with individual catabolic agents (FGF-2, 100 ng/mL; IL-1 β , 10 ng/mL; LPS, 1 μ g/mL), in the presence or absence of RSV (200 μ M, 500 μ M). After a 9-day treatment period, explants were fixed, paraffin-embedded, and stained with Safranin-O Fast Green to assess gross PG contents. As shown in Figure 1, all catabolic agents induce marked PG loss, with the severity ascending in the order of FGF-2, LPS, and IL-1 β (Figure 1 *b, h, e*). RSV effectively antagonizes these catabolic actions in a dose-dependent fashion, and at its higher dose (500 μ M), nearly abolishes PG loss (Figure 1 *d, g, j*). Our findings suggest that RSV counteracts potent inflammatory catabolic factors in cartilage, thus conferring chondroprotection.

In long-term *in vitro* cultures, human knee articular chondrocytes cultured in alginate beads for 21 days in the presence of RSV were analyzed using DMMB assay for accumulation of PG in the CM. Incubation of cells in alginate beads with RSV significantly increased PG accumulation per cell in a dose-dependent manner (Fig 2A). At concentrations of 50 μ M and 100 μ M RSV, PG accumulation increases by 50% and 100% ($p < 0.05$), respectively, compared to control. Treatment with BMP7 (100 ng/mL) was included as a positive control. The results also demonstrate that RSV rescues PG losses induced by FGF-2. When given alone, FGF-2 (50 ng/mL) decreases PG accumulation by ~50% compared to control (untreated). However, pre-treatment with RSV (100 μ M) reverses FGF-2 (50 ng/mL)-mediated reduction of PG deposition (Fig. 2A) without altering cellular proliferation as assessed by DNA assays (Fig. 2B). Of note, treatment of human articular chondrocytes with RSV (regardless of with or without FGF-2) slightly, but significantly ($*p < 0.05$) improves cell viability, similar to viability observed in the presence of BMP7 (data not shown), as revealed by the Live and Dead cell assay (Calcin AM). Cell viability is maintained above 95% throughout the long-term culture period of 21 days compared to control viability of 86% (Fig. 2C).

Next, we examined if the increase in PG accumulation is mediated by a RSV-induced synthesis of PG in human articular chondrocytes by quantifying the incorporation of 35 S-sulfate. When cells were incubated with RSV (100 μ M), no significant increase was observed in PG synthesis after normalization with DNA ($p < 0.1$). These results suggest that PG synthesis may not be the major reason for the stimulation of PG accumulation in the presence of RSV. However, when cells were co-cultured with RSV and BMP7, PG synthesis is significantly ($p < 0.01$) enhanced compared to either factor alone, revealing biological synergism between the two compounds (Fig. 2D).

RSV counteracts degradative effects of catabolic mediators in primary human articular chondrocytes

We next investigated the mechanism by which RSV promotes PG accumulation in human articular chondrocytes. Using monolayer cell cultures, we monitored mRNA levels of matrix-degrading enzymes, including MMP-13, ADAMTS4 and ADAMTS5, in response to treatment with RSV (100 μ M). To understand the efficacy of RSV to negate cartilage-catabolic responses, chondrocytes were treated in the absence or presence of the catabolic mediators FGF-2 (100 ng/mL) or IL-1 β (1 ng/mL). Under basal conditions, RSV reduces the mRNA level of MMP-13 by ~3 fold, but has limited effects on ADAMTS4 and ADAMTS5 (Fig. 3A). In contrast, FGF-2 stimulates expression of all three ECM proteases by up to 2.5-fold, while IL-1 β stimulates primarily MMP-13 and ADAMTS4, and to a lesser extent, ADAMTS5. Strikingly, co-treatment of either mediator with RSV results in potent attenuation of catabolic factor-stimulated cartilage-degrading enzyme expression to values comparable to basal levels (Fig. 3A).

Given that MMP-13 is the most potent collagen type II-degrading enzyme (Martel-Pelletier et al., 2001), and MMP-13 mRNA levels exhibit the most robust increase in expression after stimulation with catabolic factors (Fig 3A), we assessed MMP-13 protein levels to validate that modulations in mRNA levels translate into changes in protein levels. Immunoblotting results for MMP-13 show that protein levels are highly increased by both FGF-2 (100 ng/mL) and IL-1 β (1 ng/mL). RSV significantly silences MMP-13 production not only the basal conditions ($p < 0.05$) but also in the presence of FGF-2 ($p < 0.01$) and IL-1 β ($p < 0.01$) (Fig. 3B; upper panel). Immunoblotting results were quantified by densitometric analyses (Fig. 3B; lower panel).

Transient transfection experiments with a MMP-13 promoter (–1600 bp)/Luciferase reporter plasmid construct using human primary chondrocytes further support our gene expression results. Treatment of cells with RSV inhibits basal MMP-13 promoter activity (Fig. 3C; $p < 0.05$) as well as FGF-2 or IL-1 β –enhanced transcription of MMP-13 (Fig. 3C; $p < 0.01$, respectively). Hence, the inhibitory effect of RSV on basal and cytokine-induced MMP-13 mRNA levels is, at least in part, controlled by transcriptional mechanisms. Next, MMP-13 enzyme activity was assessed by ELISA to specifically detect the activity of MMP-13 (Fig. 3D). In this ELISA kit, a highly specific monoclonal antibody for the activated form of human MMP-13 permits the detection of an active form of MMP-13 at a sensitivity of 7 pg/mL. Our results demonstrate that treatment with RSV indeed attenuates MMP-13 enzyme activity that was stimulated by either FGF-2 or IL-1 β (Fig. 3D).

RSV inhibits cytokine-dependent activation of ERK/MAPK but not PI3K/AKT signaling pathway in primary articular chondrocytes

Previously, we demonstrated the critical catabolic role of the ERK/MAPK cascades in the upregulation of MMP-13 after stimulation with IL-1 β and FGF-2 in adult human articular chondrocytes and chondrocyte-like disc cells (Ellman et al., 2008; Im et al., 2007b; Muddasani et al., 2007b). FGF-2 rapidly activates ERK/MAPK as well as PI3K/Akt signaling pathways within 5 minutes as represented by phosphorylation of ERK1/2 (Fig. 4A) and Akt (Ser473) (Fig. 4B). Stimulation with IL-1 β activates only ERK/MAPK but shows no influence on the PI3K/Akt pathways in human articular chondrocytes (data not shown). Thus, we hypothesized that RSV may impinge on the upregulation of MMP-13 by regulating the ERK/MAPK signaling pathway. Results demonstrate that treatment with RSV markedly attenuates the phosphorylation of ERK/MAPK (Fig. 4A) upon stimulation of cells with FGF-2 or IL-1 β within 5 minutes, and these activation patterns are strongly correlated with the pattern of MMP-13 production (lower panel). In contrast, treatment with RSV has

only minimal or no effect on the activation of the PI3K/Akt pathway, and has no influence on FGF-2-mediated activation of Akt signaling (Fig. 4B).

RSV deactivates multiple downstream transcription factors that are activated by IL-1 β

We further sought to understand the molecular mechanisms by which RSV attenuates cartilage degradation in the presence of inflammatory factors. Using nuclear extracts from human articular chondrocytes, we examined changes in transcription factor protein-DNA binding activity using multiplex protein-DNA interaction arrays. The array panel contains a selected set of downstream transcription factors that are involved in inflammatory pathways and articular cartilage homeostasis. After stimulation of human articular chondrocytes with IL-1 β for 45 minutes, multiple transcription factors are activated compared to baseline levels (Fig. 5A *versus* 5B). The pro-inflammatory cytokine IL-1 β induces apoptosis in chondrocytes during degenerative joint diseases such as OA as well as rheumatoid arthritis (RA) (Blanco et al., 1999; Csaki et al., 2008; Hashimoto et al., 1998; Heraud et al., 2000). Consistent with previous reports, DNA-protein interaction array results demonstrate that p53 DNA binding activity is significantly stimulated by IL-1 β (10 ng/mL) along with AP-1, AP-2, Ets1/PEA3, NF κ B, p53, Sp1, and multiple STATs that are critical for cytokine signals. In the presence of RSV, the IL-1 β -induced activation of transcription factors is significantly suppressed, including p53 DNA binding activity (Fig. 5C). These data demonstrate that RSV elicits selective changes in transcription factor protein-DNA binding activities to execute its biological activities such as anti-inflammatory and chondroprotective functions in adult human articular cartilage.

RSV selectively compromises survival of human chondrosarcoma cells, but not primary articular chondrocytes and synoviocytes

Our protein-DNA interaction results further confirm that the chondroprotective effect of RSV may be, at least in part, *via* the RSV-mediated suppression of p53 activity, as previously suggested (Csaki et al., 2008). However, RSV has also been shown to induce cell death in cancer cells (Zhou et al.), and these paradoxical results led us to examine the selective biological function of RSV on tumor-derived (human chondrosarcoma) *versus* non-tumor derived cells (human primary articular chondrocytes from knee and ankle as well as synoviocytes). A human lung cancer cell line was included as a positive control (Liu et al.).

To understand the effects of RSV on cell survival, we treated a panel of primary human chondrocytes, synoviocytes and human cancer cell lines with varied doses of RSV (0, 10, 25 and 50 μ M) and monitored cell viability using MTS assays, which measure metabolically active live cells (Fig. 6A–E). The data reveal that RSV does not appreciably alter the viability of non-tumorigenic cells isolated from unaffected ankle tissue (Fig. 6A) or knee joints (Fig. 6B), nor does RSV affect survival of knee joint synovial cells (Fig. 6C) at the range of concentrations tested (between 10 to 50 μ M). In contrast, RSV treatment of cancerous cells results in a major decrease in the viability of human chondrosarcoma cells (Fig. 6D; $p < 0.05$) and human lung cancer cells (Fig. 6E; $p < 0.01$) at concentrations of 25 μ M or higher. Thus, RSV does not affect survival of non-cancerous cell types, but is cytotoxic for chondrosarcoma cells and other tumor-derived cell types at the concentrations tested, suggesting that the biological role of RSV in human tissues is highly cell type specific.

Discussion

Starting in the early 1990s, a unique French paradox first led researchers to the discovery of potential health benefits of RSV in humans. Much like the American diet, the French diet

consisted of a high fat content, yet their citizens had a lower incidence of heart disease than Americans. As the French were avid wine drinkers, researchers began to focus on possible beneficial effects of wine on heart disease. Subsequent studies led to the discovery of RSV, a key ingredient in red wines and grapes, as a potential health-promoting compound. Over the past decade, several profound biological effects of RSV in a variety of tissues have been documented in the literature. The vast therapeutic potential of RSV is reflected by its antioxidant and anti-aging effects, as well as its neuroprotective, cardioprotective, and anti-inflammatory roles (Bertelli et al., 1999; Bhat et al., 2001; Fremont, 2000; Haider et al., 2002; Huang et al., 2001; Ignatowicz and Baer-Dubowska, 2001; Jang et al., 1997; Leiro et al., 2004; Martinez and Moreno, 2000). In the past year alone, dozens of studies recognizing the potential of RSV have been published, uncovering many of the underlying mechanisms by which it exerts its effects. These studies potentially pave the way for use of RSV as a treatment for a wide range of health problems in the future. More specifically, the role of RSV in cartilage homeostasis has only recently been investigated, and early results indicate that RSV may have therapeutic potential for arthritic conditions.

Elmali and colleagues were the first to demonstrate the potential for RSV to slow cartilage degradation in experimental OA (Elmali et al., 2005) and RA (Elmali et al., 2007) models in rabbits. In the OA model, rabbits underwent unilateral knee ligament transection to induce degeneration and were given intra-articular injections of RSV (Elmali et al., 2005). Histological evaluation after 5 weeks revealed that rats treated with RSV plus dimethylsulphoxide (DMSO) exhibit significantly reduced destruction of cartilage tissue and decreased PG loss compared to those treated with DMSO alone (control). Similar results were found in the RA model (induced by intra-articular injection with lipopolysaccharide) (Elmali et al., 2007). Findings from both studies suggest that intra-articular injections of RSV may protect cartilage against the development of experimentally-induced inflammatory and degenerative arthritis. Our findings support and expand those of Elmali and colleagues, as we clearly demonstrate that RSV has potent anti-catabolic and anabolic capabilities at a molecular level in human articular cartilage.

Overproduction of matrix-degrading enzymes by chondrocytes plays a central role in matrix degeneration in arthritic cartilage (Inada et al., 2004; Lindy et al., 1997; Raggatt et al., 2006; Vincenti and Brinckerhoff, 2002; Yammani et al., 2006), because catabolic mediators such as IL-1 and FGF-2 upregulate these enzymes in cartilage (Im et al., 2003; Muddasani et al., 2007a). This study shows that RSV antagonizes the catabolic factor-mediated upregulation of multiple matrix-degrading enzymes, providing evidence that RSV is indeed capable of slowing the catabolic processes involved in cartilage degeneration. Further, we illustrate the potent long-term anabolic effects of RSV *via* increased PG accumulation and biological synergism with the well-known anabolic growth factor BMP7, suggesting that RSV could be a potent therapeutic compound for articular cartilage regeneration.

Another unique finding in this study is the cell-specific nature by which RSV exerts its effects in normal versus tumor cells. The ability of RSV to delineate between normal chondrocytes and malignant cells surmounts a tremendous hurdle in chemotherapy treatment. Chondrosarcomas are malignant cartilage tumors that are resistant to conventional cancer treatments (e.g., chemotherapy and radiotherapy). At least a subset of the signaling pathways and metabolic processes involved in tumor progression are analogous to those perturbed in OA. For example, modifications in pathways mediating insulin-like growth factor (IGF) signaling and expression of MMPs likely contribute to both diseases – cancer and arthritis (Beech et al., 1998; Sugita et al., 2004). Therefore, compounds that target either OA or chondrosarcoma may be beneficial for both, and provide the impetus for studies on the identification of new treatment strategies for both sarcomas and arthritic conditions.

Our findings suggest that RSV imparts little or no cytotoxic effects on normal or arthritic articular chondrocytes. In contrast, RSV does exert cytotoxic effects in tumor-derived cells, possibly via the induction of apoptosis. More specifically, cell viability is preserved in primary chondrocytes from normal chondrocytes, OA chondrocytes, and primary synovial cells treated with RSV. However, treatment with RSV is cytotoxic to both chondrosarcoma cells and lung cancer cells. The preservation of cell viability in OA chondrocytes is consistent with our previous demonstration that RSV decreases the IL-1 β -mediated activation of p53 in bovine disc cells (Li et al., 2008b). Similarly, Csaki et al demonstrated that IL-1 β -induced apoptosis is accompanied by accumulation of p53 (Csaki et al., 2008). Given that RSV inhibits p53 activation and accumulation in bovine disc cells, we postulate that these same molecular events may explain chondroprotection *via* anti-apoptotic activity in the presence of RSV in articular cartilage. Others have found similar results in osteoblasts and osteosarcoma cells, where RSV selectively compromises cell viability of osteosarcoma cells but not osteoblasts (Li et al., 2009). Because RSV does not affect normal chondrocytes nor osteoblasts, its cytotoxicity appears to be restricted to cancer cells. Tumor-derived cells typically have mutations in the p53 pathway.

Interestingly, RSV may also exert cellular environment-specific effects, for example, in healthy *versus* degenerative articular cartilage. DMMB studies demonstrate that the RSV-mediated increase in PG accumulation is possibly grade-dependent in human knee articular chondrocytes (data not shown). Studies with age-matched chondrocytes from knee cartilage (grades 0, 1 or 3) that were cultured in alginate beads, reveal that PG accumulation per cell is greater after 21 days in grade 3 knee cartilage compared to grade 0 or 1 knee cartilage. Thus, the biological activity and anabolic impact elicited by RSV on cartilage may be linked to the severity of degeneration.

As an anti-inflammatory dietary phytochemical, it has previously been shown that RSV antagonizes catabolic effects of TNF- α and IL-1 β by inhibiting the NF κ B pathway in a variety of tissues (Estrov et al., 2003; Gehm et al., 1997; Kundu et al., 2006). In adult human articular cartilage, our results indicate that the biological anti-catabolic effects of RSV occur *via* inhibition of the ERK/MAPK pathway, and they do not mechanistically interfere with FGF-2-mediated activation of the PI3K/AKT pathway. In bovine disc cells, RSV inhibits the FGF-2 or IL-1 β -dependent activation of the ERK/MAPK pathway in coordination with NF κ B cascades (Li et al., 2008b). Consistent with findings in disc cells, transcription factor profiling by *in vitro* protein-DNA interaction arrays suggests that RSV inhibits the induction of NF κ B activity that may lead to significant modulations in the levels of several downstream transcription factors (e.g., AP-1, AP-2, Ets1/PEA3, and multiple STATs).

Although RSV has exciting potential therapeutic benefits for OA, it is necessary to recognize several limitations of this study before translation to a clinical setting. First, it would be necessary to define the mechanistic basis for the dual effects mediated by RSV. For example, it would be informative to define what molecules cause for the differences by which RSV affects tumor-related cells but not non-tumorigenic cells. In addition, our *in vitro* findings will need to be followed up with *in vivo* studies to determine if our results are corroborated by clinically relevant structural components of arthritic joints clinically. For example, given the limited vascularity of articular cartilage *in vivo*, increased oral intake of RSV from red wine or grape consumption would likely not be a feasible option for treatment of articular cartilage degeneration. Rather, direct injections of RSV into the joint may serve as a more feasible therapeutic option to slow the progression of arthritis. Additional studies are warranted to determine the proper dosing, concentration, and potential use of scaffolds for injection therapy. RSV therapy may only be beneficial when cellular components are still viable (e.g., the early- and intermediate-degenerative stages of disease progress). Despite increased anabolic activity of RSV in grade 3 chondrocytes, grade 4 (or end-stage)

arthritic chondrocytes were not tested in this study. At end-stages of degeneration where a lack of cellular components exists, RSV therapy may be limited and may need combination with cell-based therapy or other tissue-engineering techniques. Finally, development of RSV derivatives to increase its pharmacological bioavailability in the body may dramatically increase the potency of RSV as a future drug.

In summary, we have shown that RSV exerts anabolic, anti-catabolic, anti-inflammatory and chondroprotective effects in adult human articular cartilage *in vitro* as well as *ex vivo*. Via cell-specific effects, RSV may selectively target arthritic cells but not normal cells, and exert cytotoxic effects on sarcoma cells but not arthritic cells. Taken together, our results indicate that RSV has considerable promise for treatment of joint degenerative diseases in the future.

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Figure 1. RSV counteracts catabolic factor-mediated PG depletion in adult human articular cartilage *ex vivo*

Full-thickness femoral cartilage explants cultured in mini-ITS+-supplemented media were treated with catabolic agents with or without RSV in two different doses (200 and 500 μ M). After 7-day culture, explants were assessed for PG contents by Safranin-O Fast Green staining. (a) Control; (b) FGF-2 (100 ng/mL); (c) FGF-2 + RSV 200; (d) FGF-2 + RSV 500; (e) IL-1 β (10 ng/mL); (f) FGF-2 (100 ng/mL) + RSV 200; (g) IL-1 β (10 ng/mL) + RSV 200; (h) LPS (1 μ g/mL) (i) LPS (1 μ g/mL) + RSV 200; (j) LPS (1 μ g/mL) + RSV 500. Each slide represents results from at least 4 donor samples.

Figure 2. Anabolic effects of RSV on PG in adult human articular chondrocytes

Cells isolated from human knee joint articular cartilage were cultured for 21 days in 1.2% alginate beads in serum-free medium with mini-ITS+ (insulin-transferrin-selenium; control) or the control medium plus RSV 10 to 100 μM in the presence or absence of FGF-2 (50 ng/mL). Control medium plus BMP7 100 ng/mL was used as a positive control. At the end of the culture period, the beads were dissolved in sodium citrate and cell pellets were separated by centrifugation. The amount of cell-associate PG was quantified by DMMB assay (**A**), and after normalization to cell numbers using DNA measurement (**B**). Samples were measured in triplicate and expressed as a percentage of the day 21 control cultures (mean and SEM; $n=4$). Weekly cell viability was assessed throughout the long-term culture period of 21 days using PicoGreen Calcein AM (Live and Dead assay) to stain live cells, and ethidium bromide homodimer 1 to stain dead cells. At least 100 cells were counted in triplicate for each data point (**C**). On day 7 of culture in alginate, the medium was removed and replaced by fresh medium. One hour later, this medium was replaced with fresh medium containing [^{35}S]-sulfate at 20 $\mu\text{Ci}/\text{mL}$. After incubation for 4 hours, sulfate incorporation was measured using the Alcian blue precipitation method. All samples were analyzed in triplicate ($n=3$) and normalized for DNA content using Hoechst 33258 (**D**).

Figure 3. Inhibition of MMP-13, ADAMTS4 and ADAMTS5 by RSV after stimulation with catabolic cytokines

Human articular chondrocytes isolated from knee joints (n=6) were cultured in monolayer in 12-well plates (3×10^6), and were serum starved by changing the media to serum-free DMEM/F-12 with antibiotics for 24 hours before treatment. Cells were then treated with FGF-2 (100 ng/mL), IL-1 β (1 ng/mL), and/or RSV (100 μ M). After 24 hours, cells were collected for total RNA extraction to perform real-time qPCR (A), whereas the conditioned media was subjected to immunoblotting analyses using anti-MMP13 antibody (B, upper panel) followed by quantification by densitometric analyses (lower panel). Transient transfection of MMP-13 luciferase promoter construct (1 μ g/reaction) was performed using Nucleofection and human primary articular chondrocytes in duplicates (n=3). After 24 hours, cells were co-treated with RSV (100 μ M) + FGF-2 (100 ng/mL) or RSV + IL-1 β (1 ng/mL), and then incubated another 24 hours. *Renilla* vector (pRL-TK, 100 ng/reaction) was co-transfected as an internal control. Both *Renilla* and firefly luciferase activity were measured simultaneously using a dual-luciferase reporter assay system and a luminometer (C). Enzyme activity of MMP-13 in the conditioned medium was assessed in triplicates by Active MMP-13 ELISA kit in which a highly specific monoclonal antibody for the activated form of human MMP-13 permits to detect specifically active form of MMP-13 at the sensitivity of 7 pg/mL (D) (n=3).

Figure 4. RSV-antagonized activation of ERK/MAPK, but not PI3K/AKT, is correlated with MMP-13 production after challenge with cytokines

Cells in monolayer were stimulated with FGF-2 (100 ng/mL) or IL-1 β (10 ng/mL) in the presence or absence of RSV (100 μ M) for 5 minutes, then the cell lysates were collected followed by immunoblotting analyses using anti-phosphospecific ERK1/2 (**A**, *upper panel*). Total ERK was blotted for normalization (*middle panel*). In another set of experiments, cells were incubated as above for 24 hours, and the conditioned medium was collected to analyze for the MMP-13 production (**A**, *lower panel*) (n=7). Cells in monolayer were incubated with FGF-2 in the presence or absence of RSV in a time course (0, 5, 15 and 60 minutes), and the cell lysates were analyzed by immunoblotting using anti-phosphospecific Akt(Ser473) antibody. Total Akt was used for normalization (**B**) (n=3).

Figure 5. RSV inhibits multiple downstream regulatory molecules that are activated by IL-1 β

Human primary articular chondrocytes isolated from knee joints were cultured in 6-well plates (3×10^6) and serum starved for 24 hours. For treatment, cells were incubated with IL-1 β 10 ng/mL and/or RSV 100 μ M for 45 minutes. Cells were harvested and nuclear extracts were prepared from the cells. Biotin-labeled DNA binding oligonucleotides (TranSignal Probe Mix) were pre-incubated with nuclear extract samples of each treatment to allow the formation of transcription factor protein-DNA complexes. The probes in the complexes were then extracted and hybridized to the TranSignal Protein-DNA array membranes that contain 54 transcription factor DNA-binding sites. The response elements on the array are spotted in duplicate: the first row is DNA spotted normally, the second row is DNA diluted 1:10 ($n=3$).

Figure 6. RSV selectively compromises survival of human chondrosarcoma cells, but not primary articular chondrocytes and synoviocytes

A panel of primary human chondrocytes, synoviocytes and human cancer cell lines were treated with different doses of RSV (0, 10, 25 and 50 μ M). After two consecutive days of treatments, cell viability was measured using the Cell Titer 96 Aqueous One Solution Cell Proliferation and Toxicity Assay, which measures metabolically active live cells. The MTS tetrazolium compound is reduced in metabolically active cells which are quantified by measuring the optical density at 490nm. The results represent duplicates with three independent experiments ($n=3$; * $p<0.05$; ** $p<0.01$).

Figure 7. Summary of RSV-mediated effects in articular cartilage

RSV exerts anti-catabolic, anti-inflammatory, and chondroprotective effects in articular chondrocytes by inhibition of key chondrocyte catabolic signaling pathways to mediate upregulation of PG and suppression of MMP-13 expression.

Table 1

The primer sequences and the conditions

Gene	Primer sequence	Annealing Tm.	NCBI Access No.
18S RNA	ACCAGAGCGAAAGCATTGCCAAG TCGGCATCGTTTATGGTCGGAAC	55	X03205.1
MMP13	ACCCTGGAGCACTCATGTTTCCTA TGGCATCAAGGGATAAGGAAGGGT	55	NM_002427
ADAMTS4	ACTGGGCTACTACTATGTGCTGGA CTTCTTCTTGGAGCCAATGATGCG	55	NM_005099
ADAMTS5	CTGTGACGGCATCATTGGCTCAAA TTCAGGAATCCTCACCACGTCAGT	55	NM_007038