

U0126, an Inhibitor of MEK1/2, Increases Tumor Necrosis Factor- α -Induced Apoptosis, but not Interleukin-6 Induced Apoptosis in C-28/I2 Human Chondrocytes

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Abstract

Background: Activation of the SAPK/MAPK signaling pathway by pro-inflammatory cytokines is known to induce apoptosis in cultured articular chondrocytes. C-28/I2, an immortalized human juvenile chondrocyte cell line was employed to determine the extent to which recombinant human (rh) forms of the pro-inflammatory cytokines, tumor necrosis factor- α (rhTNF- α), interleukin-6 (rhIL-6) and oncostatin M (rhOSM) induced apoptosis.

Methods: The induction of apoptosis in the presence or absence of these cytokines was measured by the DAPI/TUNEL assay, by whether or not pro-caspase-3 was activated and by the extent to which poly-ADP-ribose polymerase (PARP) was degraded.

Findings: Only rhTNF- α , and rhIL-6 significantly increased apoptosis in C-28/I2 chondrocytes, although rhOSM exhibited a strong trend ($p=0.067$) towards increasing the frequency of apoptotic chondrocytes. The number of apoptotic C28/I2 chondrocytes was significantly increased ($p=1.3 \times 10^{-5}$) by the combination of rhTNF- α and U0126 (10 μ M) compared to rhTNF- α alone. However apoptosis was not further increased by combining rhIL-6 with U0126. The LI-COR[®] western blot system showed that U0126 (10 μ M) inhibited the phosphorylation of extracellular signal-regulated kinase-2 (p-ERK2) by phorbol myristate acetate-treated immortalized myometrial cells, U0126 (10 μ M) did not alter total U-ERK2. Western blot analysis also revealed that the increased frequency of apoptotic C-28/I2 chondrocytes induced by rhTNF- α and rhOSM, but not rhIL-6, was associated with PARP degradation. However, none of the cytokines resulted in pro-caspase-3 activation.

Conclusion: These results showed that rhTNF- α and rhIL-6 were strong inducers of apoptosis in the immortalized C-28/I2 human chondrocyte cell line. They also suggested that inhibiting ERK2 phosphorylation via U0126-mediated inhibition of MEK1/2 activity, increased rhTNF- α -induced C-28/I2 chondrocyte apoptosis.

Keywords: Apoptosis, Arthritis, Chondrocytes, Cytokines, Extracellular signal-regulated kinase, Inflammation, Mitogen-activated protein kinase

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Introduction

Over the last 30 years or so, *in vitro* studies of human chondrocytes isolated from non-arthritic and/or osteoarthritic (OA) human cartilage furthered the understanding of how articular cartilage was likely to respond to the elevated levels of pro-inflammatory

cytokines present in the milieu of inflamed rheumatoid arthritis (RA) or OA synovial joints [1-5]. However, the growth and phenotypic stability of cultured human chondrocytes obtained by enzymatic dissociation of RA or OA cartilage specimens may be seriously compromised by the effects of antecedent drug therapies *in vivo* as well as chondrocyte expansion *in vitro*. This

would especially be the case where the “resident” cartilage was sampled from the knee or hip of RA and OA patients. Furthermore, the interpretation of results from cultured human chondrocyte studies may also be affected by any number of several structural changes in the cartilages from which these chondrocytes are obtained. For example, altered structures could include the thicker cartilage from a patient with early RA or OA, versus the thinner, residual cartilage from patients with end-stage RA or OA. To avoid having to cope with these and other anatomically-related issues as well as potential tissue sampling problems, such as needing to avoid problems inherent in sampling cartilage from osteophytic spurs of OA cartilage specimens, investigators have more recently opted to employ immortalized human chondrocyte cell lines for many of these *in vitro* studies. This paradigm shift in the use of these human chondrocyte lines has ensured a plentiful source of phenotypically-stable human chondrocytes in order to measure their overall responsiveness to pro-inflammatory cytokines [6-8].

C-28/I2 is an immortalized juvenile human chondrocyte cell line which has been employed to study human chondrocyte gene expression, as well as hormonal, growth factor, and cytokine responsiveness [9-15]. Thus, the results of several systematic analyses published since 1994 have shown that these chondrocyte cell lines, which have been rigorously examined, defined those phenotypic characteristics that enable the C-28/I2 chondrocyte line, among several established human chondrocyte cell lines, to serve as a “model” cell culture system for fine-tuning various aspects of human chondrocyte metabolism.

One of the phenotypic characteristics of the C-28/I2 chondrocyte cell line crucial to its use as a model system for human chondrocyte metabolism is its strong responsiveness to pro-inflammatory cytokines such as interleukin-1 β [9]. Specifically, IL-1 β was shown to decrease the expression of the aggrecan (ARGN) gene and the Type II collagen (COL2A1) gene while increasing expression of the matrix metalloproteinase (MMP) genes, MMP-1, MMP-3, and MMP-13 [9,15], in such a manner that it was considered to be similar to the response of primary cultures of human chondrocytes to IL-1 β . Importantly, the response of C-28/I2 chondrocytes to IL-1 β was also accompanied by changes in the ‘profile’ of several actively transcribed chondrocyte genes. This ‘profile’ resembled an “inflammation phenotype” which was consistent with several aspects of synovial joint pathology associated with the inflammation of arthritis, including increased cyclooxygenase-2 (COX-2), Type I collagen (COL1A1), and secretory phospholipase-A2 (PLA2G2) gene expression [6].

The results of a previous study also defined the growth requirements for establishing a stable phenotypic expression by C-28/I2 chondrocytes. These results stressed that high-density culture conditions were essential for maintaining a stable chondrogenic phenotype [8]. Thus, high cell density was considered to be a stringent regulatory requirement for C-28/I2 chondrocytes to faithfully express human articular cartilage-related genes, most critically, the cartilage-specific transcription factor, SOX9 [13]. Therefore, in the present study, C-28/I2 chondrocytes were routinely initiated at high cell density (10^5 /

ml) and, as such, chondrocyte apoptosis was analyzed when chondrocytes reached a maximally confluent state as determined by microscopic inspection.

The primary objective of this study was to determine the apoptotic response of C-28/I2 chondrocytes to 3 recombinant human (rh) pro-inflammatory cytokines, namely, rhTNF- α , rhIL-6 and rhOSM, all of which were known to be significantly elevated in the sera and synovial fluid of patients with RA and OA [16]. We then sought to determine the extent to which apoptosis was altered by rhTNF- α or rhIL-6 in the presence or absence of the IL-6 receptor neutralizing monoclonal antibody, tocilizumab or by U0126, an inhibitor of the upstream kinase, MEK1/2 whose activity is required to regulate the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2). With regard to the latter, U0126 was employed at a concentration of 10 μ M which was used to probe the potential effect of inhibiting p-ERK1 (p44) and p-ERK2 (p42) via MEK1/2 in an immortalized cell line of human myometrial cells, which was known to phosphorylate ERKp42 after incubation with phorbol myristate acetate (PMA). Thus, MEK1/2 activates the phosphorylation of p44 and p42 via their activation loop residues located at Thr202/Tyr204 and Thr185/Tyr185, respectively [17].

The phosphorylation of ERK1/2 is associated with both cell survival and/or apoptosis via the regulation of several critical downstream targets of p-ERK1/2 that include transcription factors c-myc/N-myc, Ets-1/Ets-2, c-fos, ELK-1, and STAT-1. These are all known to be involved in the regulation of cell proliferation and/or induction of apoptosis [17-23], thus making the regulation of ERK1/2 phosphorylation relevant to pro-inflammatory cytokine-induced chondrocyte apoptosis.

Methods

The immortalized juvenile human chondrocyte cell line, C-28/I2, was kindly provided by Drs. Mary B. Goldring and Miguel Otero (The Hospital for Special Surgery, Weill Medical College of Cornell University, New York, N.Y.). Dulbecco’s Modified Eagle’s Medium/Ham’s F-12 medium (1:1) and fetal bovine serum were purchased from Mediatech, Inc. The recombinant human cytokines, rhTNF- α , rhIL-6, and rhOSM, were obtained from R&D Systems and Prospec Bio, respectively.

Antibodies reactive with total ERK1/2 (U-ERK1/2) (Immunogen-Purified mouse ERK1/2; Clone-L34F12; Isotype-mouse monoclonal IgG1) and p-ERK1/2 polyclonal antibody (Immunogen-Purified rabbit p-ERK1/2; phosphorylation sites, ERK1, Thr202/Tyr204, ERK2, Thr185/Tyr187) were purchased from Cell Signaling Technology. Purified mouse-anti-human caspase-3 antibody (Immunogen-Human CPP32; amino acids, 1-219; Clone-Human CPP32; amino acids, 1-219; Isotype-Mouse IgG2a) was purchased from BD Transduction Laboratories. Purified mouse anti-human poly-ADP ribose polymerase (PARP) antibody (Immunogen-Purified human PARP; Clone-7D3-6; Isotype-Mouse IgG1) was purchased from BD Pharmingen. The GAPDH-reactive antibody was a mouse monoclonal antibody (Immunogen-rabbit muscle GAPDH; Clone-6C5; Isotype-rabbit IgG1) which was purchased from Santa Cruz Biotechnology. According to the manufacturer the anti-caspase-3 antibody will identify the 32kDa form of pro-

caspase-3, as well as p20, p17, and p12 subunits of activated caspase-3, on western blots, whereas the anti-PARP antibody was shown to react with intact PARP as well as several forms of degraded PARP [Data Sheet, BD Pharmingen].

U0126 (1, 4 diamino-2, 3-dicyano-1, 4, bis [2-aminophenylthio] butadiene) was purchased from Cell Signaling Technology. The monoclonal antibody, tocilizumab, [IgG subclass, IgG1κ], that neutralizes the interaction between IL-6 and IL6Rα, was provided through a contract between Genentech/Roche group and Case Western Reserve University. DAPI was obtained from Sigma-Aldrich. The TUNEL assay kit used for determining the frequency of apoptotic cells was purchased from Roche.

Analysis of C-28/I2 chondrocyte apoptosis

For the analysis of apoptosis, a stock of C-28/I2 chondrocytes was thawed from storage in a liquid nitrogen tank. The cells were re-suspended and plated into 12-well plates (growth area=22.1 mm²) at 10⁵ cells/ml in 1 ml DMEM/F12 (1:1) supplemented with 10% FBS. The chondrocytes were grown to a maximally confluent state which was generally reached after 5-7 days, and then the various assays (see below) conducted at that time.

The number of apoptotic chondrocytes was determined by the 4', 6-DiAmidino-2-Phenyl/Indole/Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (DAPI/TUNEL) assay as previously described [24]. C-28/I2 chondrocytes were incubated with either DMEM/F12 (1:1) plus 0.5% FBS, DMEM/F12 (1:1) plus 10% FBS, DMEM/F12 (1:1) plus DMSO and 0.5% FBS, or rhTNF-α (20 ng/ml), rhIL-6 (50 ng/ml), or rhOSM (50 ng/ml) or various combinations of rhTNF-α (20 ng/ml) and U0126 (10 μM), rhIL-6 (50 ng/ml) and U0126 (10 μM), rhIL-6 plus tocilizumab (200 ng/ml) or rhOSM (50 ng/ml) which were all maintained in DMEM/F12 (1:1) plus 0.5% FBS for 24 hr.

U0126, was first dissolved in DMSO and then diluted with DMEM/F12 (1:1). Thus, media used for determining the effects of U0126 on C-28/I2 chondrocyte apoptosis contained the identical concentration of DMSO present in the culture medium of the "no additions" (N/A) control group.

A morphological assessment of C-28/I2 chondrocyte size, shape and distribution in the control group as well as after treatment with rhTNF-α (20 ng/ml) or rhIL-6 (50 ng/ml) was performed in situ by examining the cells with phase contrast microscopy as well as after staining of the chondrocytes with hematoxylin-eosin (H&E), the stained cells being visualized by light-microscopy.

Preparation of C-28/I2 chondrocyte protein for SDS-PAGE

The culture dishes were kept on wet-ice throughout the entire cell collection procedure. C-28/I2 chondrocytes were collected with a rubber scraper in ice-cold phosphate buffered-saline (PBS). The pelleted chondrocytes were subjected to sonication. The protein-containing supernatant was centrifuged for 3 min at 5 × 10³ g to remove particulate material. An aliquot of the pelleted chondrocytes was re-suspended and used to determine cell protein using the bicinchoninic acid assay. Then the protein concentration in the supernatant was diluted to 1 mg/ml, and

the resultant crude protein-containing lysate stored at -20°C until used for the various assays as indicated below, including SDS-PAGE.

Experimental conditions for western blot analysis of pro-caspase-3 activation and PARP degradation

In a companion analysis to the DAPI/TUNEL assay, C-28/I2 chondrocytes were maintained in DMEM/F12 (1:1) supplemented with 0.5% FBS, with either, "no additions" (N/A; control) or DMSO (DMSO - control), or treated with rhTNF-α (20 ng/ml), rhIL-6 (50 ng/ml), rhOSM (50 ng/ml) or with combinations of rhTNF-α or rhIL-6 and U0126 (10 μM) for 24 hrs. In some experiments, C-28/I2 chondrocytes were incubated with rhTNF-α or rhIL-6 with or without tocilizumab (200 ng/ml) to determine the extent to which tocilizumab neutralizing the interaction between IL-6 and IL-6Rα resulted in the inhibition of pro-caspase-3 activation and/or PARP degradation.

A standard western blot analysis was employed to determine whether C-28/I2 chondrocyte apoptosis was associated with pro-caspase-3 activation and/or PARP degradation. Cell lysates were diluted in gel loading buffer consisting of 40% glycerol, 1 mM Tris-HCl, 2.5% β-mercaptoethanol, 8% sodium dodecyl sulfate, and 0.01% bromophenol blue and then heated for 5 min at 95°C. Heated cell lysates were then subjected to denaturing conditions in 4-20% SDS-PAGE using the Novex Tris-glycine system (Life Technologies). Protein (2μg/lane) was loaded on to the gel and after SDS-PAGE the separated proteins were electrotransferred to polyvinyl difluoride membranes (Bio-Rad Laboratories). For western blotting, the membranes were blocked with 5% nonfat milk in 20 mM Tris-HCl, 500 mM NaCl (pH 7.5) containing 0.05% Tween 20 (TTBS) for 1 h at room temperature. Afterward membranes were incubated with primary anti-caspase-3 antibody, anti-PARP antibody and anti-GAPDH overnight at 40°C. Western blots were washed 3 times with TTBS and incubated at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology). The blots were then treated with HyGLO™ Chemiluminescent HRP Antibody Detection Reagent (Denville). The western blot image was captured using the ImageQuant LAS 4000 system (GE Healthcare).

The Odyssey Two-Color and IN-Gel (LI-COR®) Western Blot System

We had previously shown that C-28/I2 chondrocytes primarily produced ERK2 [25]. To accomplish this, the LI-COR® western blot system was used to detect an infrared fluorescence antibody conjugate to capture U-ERK1/2 and p-ERK1/2 produced by the PMA-responsive hTERT-HMA/B immortalized cell line of human myometrial cells [26,27]. Thus, this human myometrial cell line was used to confirm that U0126, employed at 10μM in the C-28/I2 chondrocyte apoptosis assay, was capable of inhibiting the phosphorylation of ERK1/2 without altering total U-ERK1/2.

Statistical analysis

Data from the DAPI/TUNEL assay was first subjected to a normalcy test. Then a one-way ANOVA for multiple group comparisons

of unequal size was employed to compare the means of DAPI/TUNEL-positive chondrocytes in control and the various treatment groups. A p-value of <0.05 was considered significant. The Honest Significant Difference (HSD) value was calculated by Tukey's post hoc analysis to determine the p-value when multiple treatment groups were compared.

Results

C-28/I2 chondrocyte apoptosis measured by DAPI/TUNEL assay

The number of apoptotic C-28/I2 chondrocytes was enumerated with the DAPI/TUNEL assay. The results showed that there was no significant difference in the number of DAPI/TUNEL-positive C-28/I2 chondrocytes maintained in DMEM/F12 (1:1) supplemented with either 0.5% FBS (i.e., apoptosis analysis conditions) or with 10% FBS (i.e., chondrocyte growth conditions) (Figure 1). We also determined the extent to which rhTNF- α , rhIL-6, and rhOSM, induced apoptosis in C-28/I2 chondrocytes. In that regard, Figure 1 showed that rhTNF- α or rhIL-6 significantly increased the number of DAPI/TUNEL-positive chondrocytes when compared to the 0.5% FBS medium-containing control group. Of note, rhTNF- α significantly increased the number of apoptotic chondrocytes compared to either rhIL-6 or rhOSM (Tukey's HSD=7.81; treatment groups=3; degrees of freedom=9; $p < 0.05$). Although treatment of C-28/I2 chondrocytes with rhOSM also increased the number of DAPI/TUNEL-positive chondrocytes from $0.85 \pm 0.18\%$ in the 0.5% FBS-containing control group to $2.2 \pm 1.2\%$ (mean \pm SD, $n=3$) this difference produced a p-value of 0.067, which was not statistically significant. Nevertheless, the calculated $\Delta\%$ between control and rhOSM-treated C-28/I2 chondrocytes was 58.9% indicating a strong positive apoptotic response of these chondrocytes to rhOSM.

The number of DAPI/TUNEL-positive C28/I2 chondrocytes was significantly increased in medium containing DMSO/0.5% FBS compared to 0.5% FBS-containing medium. This was a critical result because it provided the appropriate control group for comparing the differences in apoptotic responsiveness in the various treatment groups, when U0126 was combined with various treatments (see below). This was due to the fact that the DMSO/0.5% FBS-treated group was used as the control group for C-28/I2 chondrocytes that were incubated with the various cytokines and U0126. In that regard, treating C-28/I2 chondrocytes with rhTNF- α and U0126 significantly increased the number of DAPI/TUNEL-positive chondrocytes compared with the DMSO/0.5% FBS medium-containing control group or when the TNF- α plus U0126 group was compared to the rhTNF- α group ($p < 6 \times 10^{-3}$) (Figure 1). By contrast, the combination of rhIL-6 and U0126 did not alter the number of DAPI/TUNEL-positive C-28/I2 chondrocytes compared to rhIL-6 (Figure 1).

Microscopic Evaluation

A companion microscopic analysis revealed that there were fewer C-28/I2 chondrocytes when these cells were maintained in DMEM/F12 medium (1:1) plus 0.5% FBS (Figure 2: Panels A and B) compared to DMEM/F12 (1:1) medium plus 10% FBS (Figure

2; Panels C and D), although no statistically significant difference in DAPI/TUNEL-positivity was detected among these two control groups (Figure 1). However, a few morphological abnormalities in C-28/I2 chondrocytes were noted in the rhTNF- α and rhIL-6-treated groups (Figure 2). The most prominent of these morphological changes was the presence of fewer chondrocytes as visualized by phase microscopy (Figure 2, Panels E and G). However, smaller chondrocytes with condensed nuclei were seen in all of the groups, including the control group, but there appeared to be more stellate-shaped chondrocytes, many of which showed condensed nuclei in the rhTNF- α and rhIL-6-treated groups (Figure 2; Panels E, F, G and H) when compared to the shape and size of the chondrocytes in Figure 2, Panel A. Of note, there were many fewer cells attached to the plastic culture dish substratum in the rhTNF- α - or rhIL-6-treated chondrocyte groups that were employed for staining with H&E compared to the living chondrocytes visualized by phase microscopy which most likely resulted from the loss of many of the loosely attached apoptotic chondrocytes during the formalin fixation and post-formaldehyde fixation washing preceding H&E staining.

Pro-Caspase-3 Activation and PARP Degradation

Although the DAPI/TUNEL assay by itself ostensibly provided a valid molecular-based assessment of the frequency of apoptotic C-28/I2 chondrocytes, we also employed conventional western blotting to determine the extent to which the various treatment groups activated pro-caspase-3 and/or induced PARP degradation.

PARP

PARP cleavage is considered a valid surrogate measurement of apoptosis. For example, during apoptosis PARP is cleaved by either activated caspase-3 or caspase-7 or other enzymes from the "intact" form of PARP into 2 major fragments of 89 kDa and 24 kDa [28]. However, other additional PARP cleavage products which migrate on polyacrylamide gels between 116 kDa and 24 kDa PARP may also be produced by the action of caspase-3/caspase-7 or by the action other enzymes, including calpain-1, cathepsins-B, -D and -G, granzyme-A, granzyme-B or MMP-2 [28]. Importantly, the molecular sizes of PARP degradation fragments often differ according to which enzyme class has mediated PARP degradation.

The results of the western blot analysis (Figure 3) showed that the "intact" form of PARP migrated to a position on the gel consistent with 124 kDa. This is the molecular size of "intact" PARP reported by other investigators [28]. In addition, various smaller PARP entities in this case, assumed to PARP fragments were detected in both of the control groups (Table 1) where, in fact a measurable number of DAPI/TUNEL-positive chondrocytes were also found (Figure 1). However, the PARP fragment pattern produced by rhTNF- α -treated C-28/I2 chondrocytes was vastly different from the PARP fragment pattern in both control groups. Thus, no "intact" PARP was detected in C-28/I2 chondrocytes incubated with rhTNF- α . Moreover, 2 PARP fragments migrating to 96kDa and 77 kDa which were also seen in the rhTNF- α -treatment group was not detected in the control groups. (Table 1). In addition, the

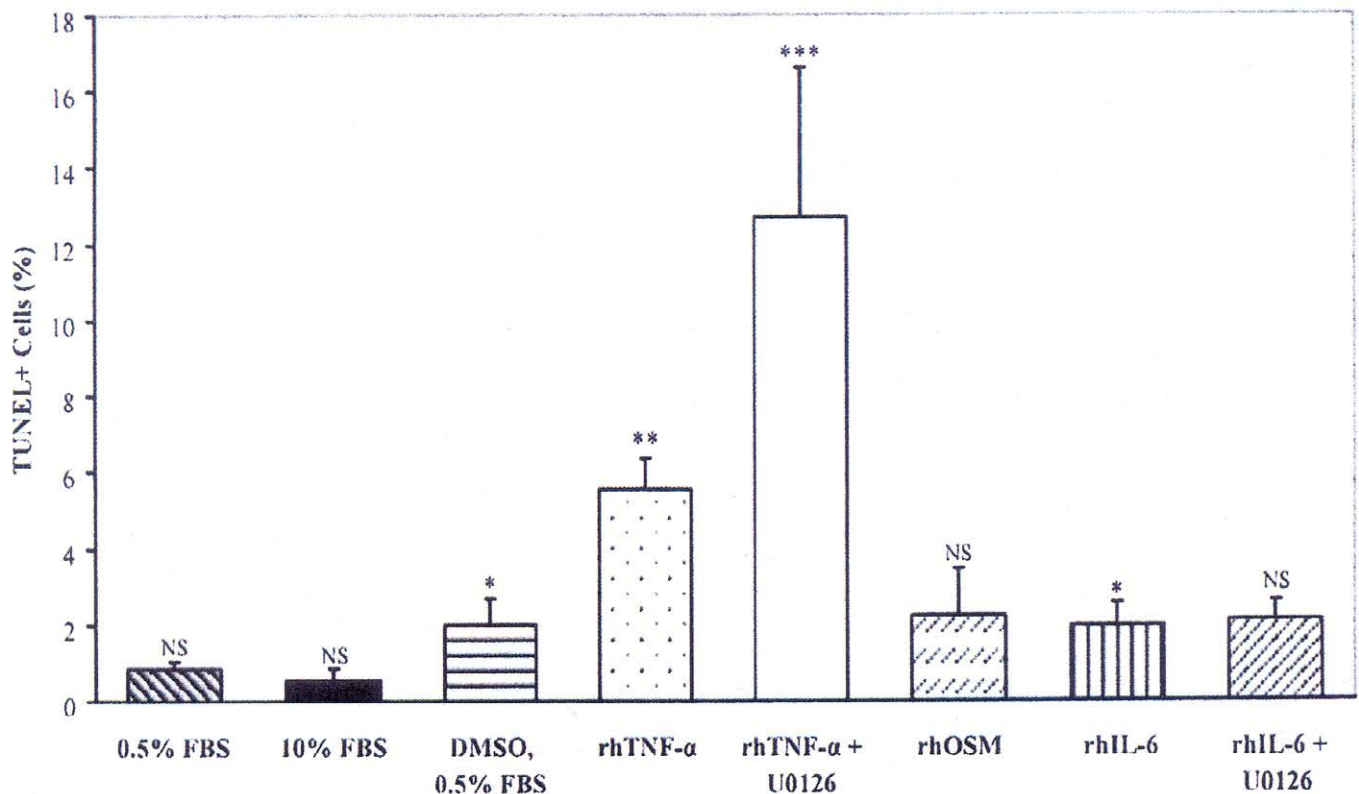


Figure 1 C-28/I2 Chondrocyte Apoptosis Induced by rhTNF- α , rhIL-6, rhOSM, and by rhTNF- α or rhIL-6 in Combination with U0126. C-28/I2 chondrocytes were grown to a confluent state in DMEM/F12 (1:1) supplemented with 10% FBS. After washing the cells in warmed PBS at room temperature, chondrocytes were incubated for 24 hrs in DMEM/F12 (1:1) containing either 0.5% FBS (n=4), DMEM/F12 (1:1) containing 10% FBS (n=5), DMEM/F12 (1:1) containing DMSO/0.5% FBS (n=5), or DMEM/F12 (1:1) containing 0.5% FBS plus rhTNF- α (20 ng/ml; n=4), rhIL-6 (50 ng/ml; n=4), rhOSM (50 ng/ml; n=4), DMEM/F12 (1:1) containing DMSO/0.5% FBS, rhTNF- α and U0126 (10 μ M; n=7), or rhIL-6 and U0126 (n=4). Cell nuclei and apoptotic cells were enumerated by the DAPI/TUNEL assay as previously described [24]. The mean \pm SD is shown as well as the comparison groups which were analyzed for the level of significance.

These were:

0.5% FBS versus 10% FBS; 0.5% FBS versus rhOSM; DMSO, 0.5% FBS versus rhIL-6 + U0126,

NS=Not Significant, $p > 0.05$;

0.5% FBS versus DMSO, 0.5% FBS; 0.5% FBS versus rhIL-6, * $p = 0.01$;

0.5% FBS versus rhTNF- α , ** $p = 2.8 \times 10^{-5}$;

DMSO, 0.5% FBS versus rhTNF- α + U0126, *** $p = 1.3 \times 10^{-5}$.

124 kDa PARP form was not detected in C-28/I2 chondrocytes incubated with rhTNF- α plus U0126 or after incubation with rhOSM (**Table 1**). Interestingly, a similar pattern of intact PARP as well as 3 smaller PARP forms (i.e., 95 kDa, 73 kDa, 58 kDa) were detected when C-28/I2 chondrocytes were incubated either with tocilizumab (TOC) alone or with rhIL-6 plus TOC (**Figure 3; Table 1**). These particular PARP fragment profiles was identical to the pattern of PARP fragments detected in both control groups as well as after chondrocytes were incubated rhIL-6 alone (**Table 1**).

Caspase-3

The western blot analysis of caspase-3 (CPP32) revealed a similar pattern of anti-caspase-3 antibody-positive bands in the 2 control groups as well as in the various treatment groups (**Figure 3**). Based on the results of this western blot, the purified mouse anti-human caspase-3 antibody detected a form of caspase-3 which migrated

between 33 kDa and 29 kDa and assumed to be pro-caspase-3. However, although it appeared that the active “intermediate and mature” forms of caspase-3 which according to previously reported analyses would be comprised of 19 kDa/20 kDa, 17 kDa and 12 kDa subunits [23,29,30], were not detected in any of the control or treatment groups. Thus, in the majority of the incubation conditions, including the 2 control groups, there were 2 caspase-3 antibody-positive bands, the aforementioned bands migrating between 29 kDa and 33 kDa which were prominent as well, as a less prominent anti-caspase-3 antibody band which migrated between 30kDa and 29kDa. This caspase-3 pattern was most clearly evident in C-28/I2 chondrocytes incubated with rhIL-6 (**Figure 3**). Of note, the anti-caspase-3 antibody-positive band was undetectable in C-28/I2 chondrocytes incubated with rhIL-6 plus U0126. Two additional findings also merit comment; 1) the single caspase-3-positive band in the rhOSM-treated

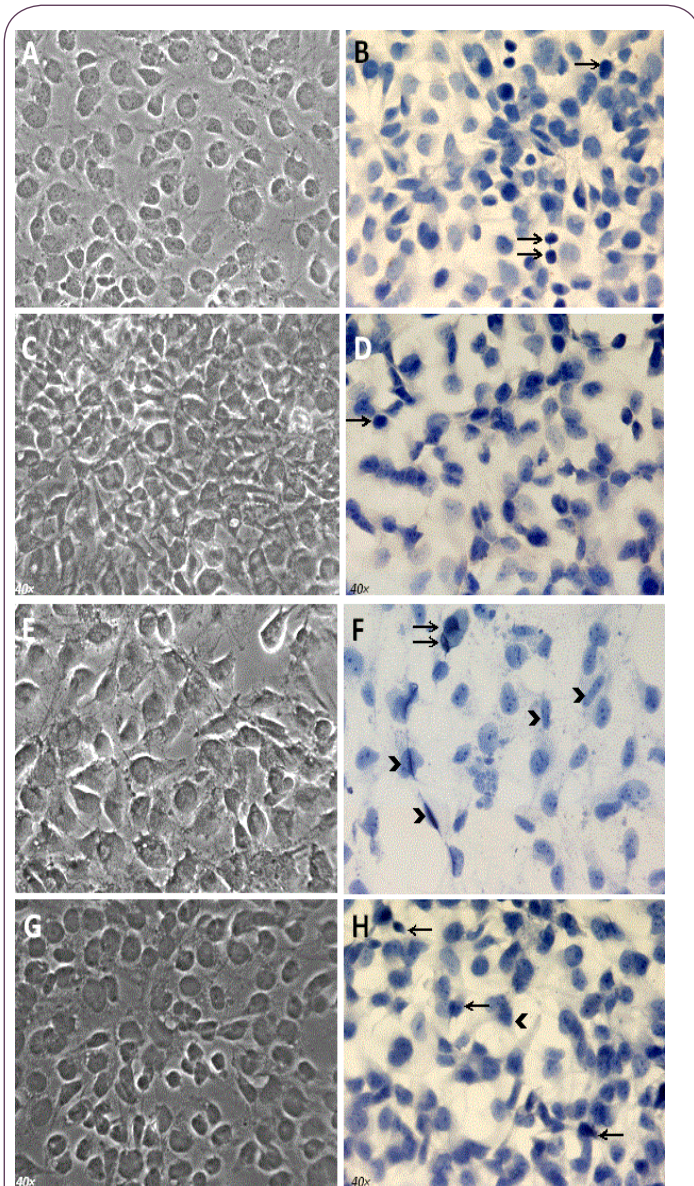


Figure 2 Morphological Changes in C-28/I2 Chondrocytes after Incubation with rhTNF- α or rhIL-6. Panel A, DMEM/F12 + 0.5% FBS (phase microscopy); Panel B, DMEM/F12 + 0.5% FBS (H&E); Panel C, DMEM/F12 + 10% FBS (phase microscopy); Panel D, DMEM/F12 + 10% FBS (H&E); Panel E, rhTNF- α (20 ng/ml) (phase microscopy); Panel F, rhTNF- α (20 ng/ml) (H&E); Panel G, rhIL-6 (50 ng/ml) (phase microscopy); Panel H, rhIL-6 (50 ng/ml) (H&E). There are fewer attached C-28/I2 chondrocytes in Panels E-H. There are more stellate-shaped chondrocytes in Panels E-H (arrowheads) with additional evidence of condensed nuclei (arrows). Chondrocytes with condensed nuclei were also seen in the control groups, Panels A and C (phase microscopy), Panels B and D (H&E).

group migrated to 32 kDa (Tables 1 and 2) and 2 incubation with either rhTNF- α plus U0126 or rhIL-6 plus U0126 did not alter the caspase-3 migration pattern compared to rhTNF- α or rhIL-6 alone.

GAPDH

Only 1 anti-GAPDH antibody reactive band was detected with the anti-GAPDH antibody on this western blot (Figure 3). This form of GAPDH migrated to the expected molecular size of GAPDH (i.e., 37kDa/38kDa) which was detected at that migration position in the control and all of the treatment groups (Figure 3; Table 1). However, there was an indication that the content of GAPDH was also influenced by a few of the incubation conditions most notably, by rhOSM, rhIL-6 and by TOC alone (Figure 3).

Activation of p-ERK2 by PMA in hTERT-HMA/B cells and Inhibition of p-ERK2 by U0126

We employed the LI-COR[®] western blotting system, which showed that the anti-ERK1/2 antibody reactive band produced by the immortalized human myometrial cell line, hTERT-HMA/B, was predominantly total U-ERK2 (Figure 4). Moreover, treatment of hTERT-HMA/B with PMA resulted in phosphorylation of U-ERK2 as previously reported [26,27]. The LI-COR[®] western blot analysis also showed that U0126 employed at 10 μ M essentially eliminated p-ERK2 without altering total U-ERK2 (Figure 4) indicating that 10 μ M was an effective U0126 concentration for inhibiting upstream MEK1/2 whose kinase activity is essential for the phosphorylation of ERK1/2. This result was important because 10 μ M was the concentration of U0126 employed in the analysis of C-28/I2 chondrocyte apoptotic responsiveness to rhTNF- α and rhIL-6.

Discussion

Our laboratory had previously shown that rhTNF- α was a potent pro-inflammatory cytokine inducer of apoptosis in human chondrocytes which had been enzymatically dissociated from OA knee cartilage [24]. This was indicated by the increased number of DAPI/TUNEL-positive chondrocytes in the rhTNF- α -treated group compared to a control group. The results of the DAPI/TUNEL assay demonstrating that apoptosis had been induced by rhTNF- α was further validated by showing the accumulation of ethidium bromide-positively stained intranucleosomal DNA fragments in the rhTNF- α -treated group [24], the migration pattern of which on 1.2% agarose gels was consistent with that of DNA fragments produced by apoptotic cells.

The results of the current study extended those previous findings to now show that rhTNF- α increased the number of apoptotic cells in the immortalized human C-28/I2 chondrocyte cell line. In addition we demonstrated, for the first time, that rhIL-6 was also a pro-apoptotic pro-inflammatory cytokine for C-28/I2 chondrocytes, although rhIL-6 was not nearly as effective an inducer of apoptosis in C-28/I2 chondrocytes as was rhTNF- α .

The concentration of rhTNF- α (20 ng/ml) used in this study was principally based on the concentration range of rhTNF- α (10-20 ng/ml) we employed in a previously published study of rhTNF- α -induced apoptosis in chondrocytes derived from human osteoarthritic cartilage [24], whereas the concentrations of rhIL-6 and rhOSM (50 ng/ml) were chosen on the basis of presumptive elevated levels of these pro-inflammatory cytokines in RA patients, both of which are known to be involved in the clinical progression of RA. Thus, one limitation on the interpretation of the DAPI/TUNEL analysis may reflect, in part, the concentration of these pro-inflammatory cytokines early on in RA pathogenesis, but may

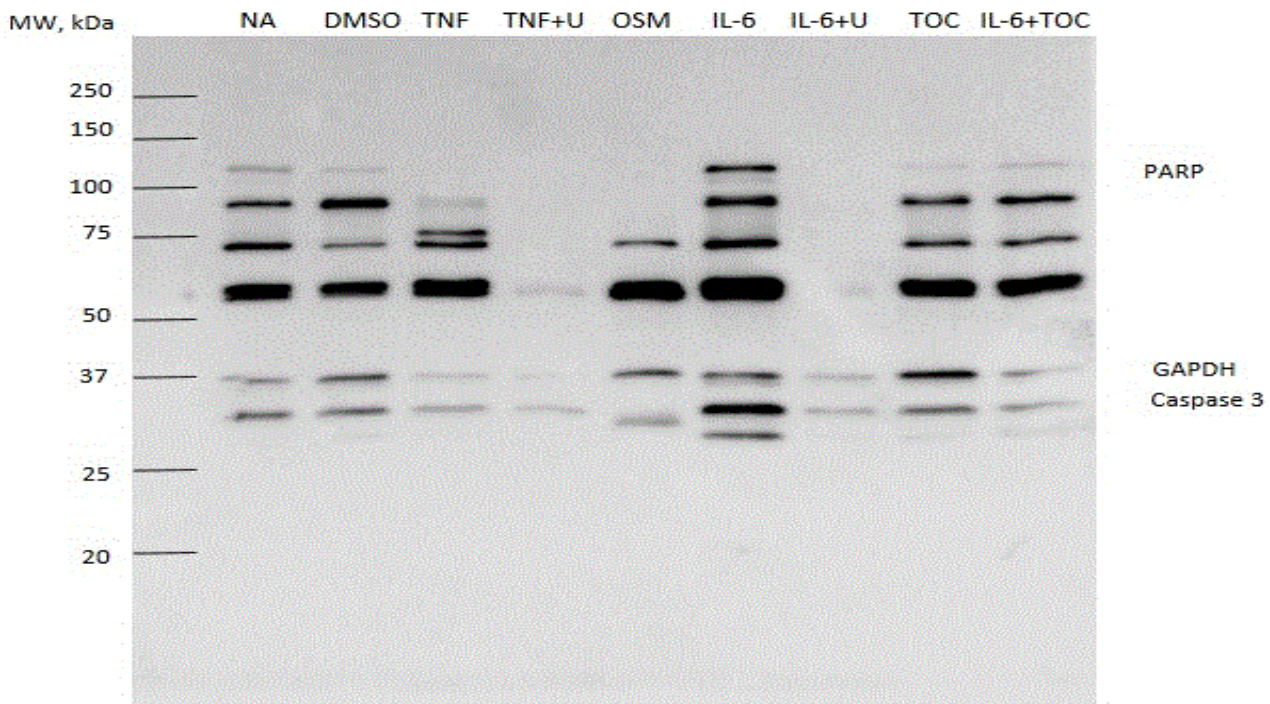


Figure 3 Western Blot Analysis of Pro-Caspase-3, Activated Caspase-3 and PARP Degradation.

C-28/I2 were grown to a confluent state in DMEM/F12 (1:1) supplemented with 10% FBS. After washing the cells in warmed PBS at room temperature, chondrocytes were incubated in DMEM/F12 (1:1) containing either 0.5% FBS (NA) or 0.5% FBS plus DMSO (DMSO), rhTNF- α (20 ng/ml), rhTNF- α plus U0126 (10 μ M), rhOSM (50 ng/ml), rhIL-6 (50 ng/ml), rhIL-6 (50 ng/ml), rhIL-6 plus U0126, tocilizumab (200 ng/ml) or rhIL-6 plus tocilizumab (200 ng/ml), for 24 hrs. Western blots were probed for reactivity with caspase-3 using a mouse anti-human caspase-3 antibody. The caspase-3 antibody is expected to detect both pro-caspase-3 (~32 kDa) and activated caspase-3 (20 kDa/21 kDa). The anti-PARP antibody is expected to detect undegraded PARP (~124 kDa) as well as various PARP degradation products. The anti-GAPDH antibody was employed to determine if any of the treatment groups altered the content of total GAPDH.

Western blot-Left to right; NA, DMEM/F12 plus 0.5% FBS; DMSO, DMEM/F12 plus 0.5% FBS + DMSO; TNF, rhTNF- α (20 ng/ml); TNF+U, rhTNF- α plus U0126 (10 μ M); OSM, rhOSM (50 ng/ml); IL-6, rhIL-6 (50 ng/ml) IL-6+U, rhIL-6 + U0126; TOC, tocilizumab (200ng/ml); IL-6 + TOC, rhIL-6 + TOC.

Table 1: Molecular size calculations for PARP, Caspase-3 and GAPDH

*Intact PARP (Molecular Sizes of PARP fragments)

** Inactive caspase-3 (CPP32)

*** Uncharacterized anti-caspase-3 antibody reactive band

Treatment Group	PARP (kDa)	Caspase-3 (kDa)	GAPDH (kDa)
NA	124* [92, 72, 56]	33**, 29***	37
DMSO	123* [92, 71, 58]	33**, 30***	38
rhTNF- α	[96, 77, 72, 58]	34**	38
rhTNF- α + U0126	[58]	33**	38
rhOSM	[72, 57]	32**	38
rhIL-6	125*, 93, 72, 57	34**, 30***	38
rhIL-6 + U0126	NOT DETECTABLE	33**	38
TOC	125* [95, 73, 58]	33**, 30***	38
rhIL-6 + TOC	125* [95, 73, 58]	34**, 30***	38

not be an accurate indicator of the level of these cytokines after medical therapy of RA is initiated by either by TNF- α blockade or neutralization of the IL-6/IL-6R interaction. The concentration of tocilizumab [200 ng/ml] employed in this study to determine whether tocilizumab altered the pro-caspase-3 activation and

PARP degradation after treating C-28/I2 chondrocytes with rhIL-6 (**Table 1**) was based on the results of our prior study [31] which showed that this concentration of tocilizumab [i.e., 200 ng/ml] inhibited rhIL-6-induced MMP-9 production.

An examination of C-28/I2 chondrocytes by phase microscopy,

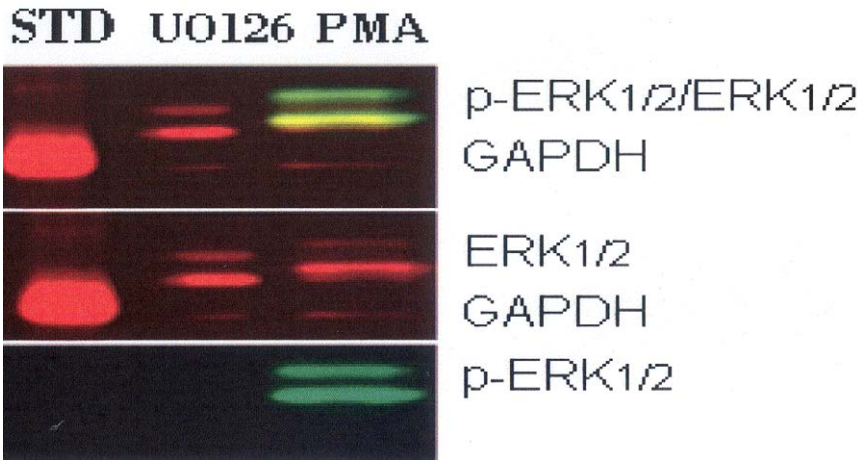


Figure 4 Effect of U0126 on Total U-ERK1/2 and p-ERK1/2 in hTERT-HM^{A/B} Cells.

The immortalized human myometrial cell line, hTERT-HM^{A/B} was pre-incubated with U0126 (10 μ M) for 10 min followed by incubation with PMA (100 nM) for 10 min or with PMA alone for 10 min. The Odyssey Two-Color and IN-Gel (LI-COR[®]) western blot system was used to detect an infrared fluorescence antibody conjugate which captures total U-ERK1/2 and p-ERK1/2 with an anti-U-ERK1/2 or anti-p-ERK1/2 antibody (Cell Signaling Technology). Lanes as shown are: STD, gel loading control; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; U0126, hTERT-HM^{A/B} cells pre-incubated with U0126 (10 μ M) followed by incubation with PMA (100 nM) for 10 min; hTERT-HM^{A/B} cells treated with PMA (100 nM) for 10 min. Total U-ERK1/2 is depicted in red, p-ERK1/2 is depicted in green and the total U-ERK1/2/p-ERK1/2 merge is depicted in yellow.

as well as after staining formalin-fixed cells with H&E confirmed that the increase in the frequency of apoptotic chondrocytes in the rhTNF- α , and rhIL-6-treated groups was associated with morphological changes in the shape, the number and overall distribution of the chondrocytes on the coverslip.

Both PARP degradation and caspase-3 activation are considered to be 2 surrogate biomarkers of apoptosis. Thus, the western blot analysis, which probed for evidence of PARP degradation confirmed that incubation of C-28/I2 chondrocytes with rhTNF- α or rhIL-6 did indeed result in PARP degradation. We had previously shown that hydrostatic pressure applied to human OA chondrocytes *in vitro* at a pressure level which was consistent with elevated biomechanical stress on synovial joints (i.e., 5 megapascals; 5mPA) also induced chondrocyte apoptosis [23]. In that study, 5 mPA-induced human OA chondrocyte apoptosis was associated with an increase in TNF- α , c-myc, p53, bax- α and iNOS gene expression, suppression of bcl-2 gene expression as well as pro-caspase-3 activation and PARP degradation. Taken together, the present western blot results indicated that intact PARP was undetectable in response to rhTNF- α or rhOSM in C-28/I2 chondrocytes but not in response to rhIL-6. Thus after incubation with rhTNF- α and rhOSM only faster migrating PARP forms were detected whereas with rhIL-6 the "intact" form of PARP as well as other forms of degraded PARP was detected. In addition, the PARP fragments found in the rhIL-6 group were identical in their migration pattern to those forms of PARP that were produced by C-28/I2 chondrocytes in the control groups, an indicator that rhIL-6-induced C-28/I2 chondrocyte apoptosis was not associated with degradation of "intact" PARP.

In contrast to PARP degradation produced by rhTNF- α and rhOSM, we could find no evidence for the activated 20kDa/21kDa forms of caspase-3 despite the assertion by the manufacturer that

the purified mouse anti-human caspase-3 antibody used in this western blot analysis purportedly recognizes both pro-caspase-3 (CPP32) as well as activated caspase-3 [BD Technical Data Sheet]. Thus, this finding strongly suggested that although PARP degradation was most often considered to be caspase-dependent [29] and caspase-3-dependent, in particular [23,32], the pattern of PARP degradation produced by C-28/I2 chondrocyte undergoing apoptosis in response to rhTNF- α , rhIL-6, and rhOSM appeared to be caspase-3-independent, thus potentially implicating other enzymes in the degradation of PARP in C-28/I2 chondrocytes [28]. Although the effect of tocilizumab on rhIL-6-induced C-28/I2 chondrocyte apoptosis was not assessed in the DAPI/TUNEL assay, the results of the PARP degradation analysis suggested that tocilizumab itself, as well as the combination of rhIL-6 and tocilizumab, had no effect either on caspase-3 activation or on PARP degradation as did rhIL-6.

Recently, Wylie et al., [25] showed that the ratio of total U-ERK2 (p42) to total U-ERK1 (p44) in C-28/I2 chondrocytes was 2.33 ± 0.05 (mean \pm SEM, n=3), an indicator that C-28/I2 chondrocytes predominately synthesized U-ERK2. The results of that study also demonstrated that rhIL-6 or rhOSM increased the ratio of p-ERK2 to β -actin in C-28/I2 chondrocytes without altering the ratio of total U-ERK2 to β -actin. Furthermore, the combination of rhIL-6 and U0126 reduced the ratio of p-ERK2 to β -actin.

Several previously published studies had commented on the biological significance of varying ratios of ERK1 to ERK2 in cultured cells. Thus, in most mammalian tissues and cell lines studied to date ERK2 appeared to be the predominating ERK isoform, whereas ERK1 was quantitatively much less abundant. In principle this evidence should adequately explain the dramatic biological effects observed when ERK2 is either ablated in mice or knocked down in cells [33-37]. Thus, the results reported in

the original study from Pouyssegur's group on the significance of total ERK1 versus total ERK2 [37] was noteworthy with respect to the findings with C-28/I2 chondrocytes because the most significant biochemical feature of ERK1-deficient cells was the strong hyperphosphorylation of the ERK2 isoform.

Importantly, U0126, (an upstream inhibitor of MEK1/MEK2, the activation of which is required for the phosphorylation of ERK1/2) [17,38], in combination with rhTNF- α , not only increased the number of DAPI/TUNEL-positive C-28/I2 chondrocytes, but also produced the most marked effect on PARP degradation where, for example, only a single 58 kDa PARP fragment was detected. However, the effect of rhTNF- α and U0126 on C-28/I2 chondrocyte apoptosis could not be mimicked by rhIL-6 in combination with U0126. This result suggested that although activation of ERK2 was a highly plausible mechanism accounting for rhTNF- α -mediated apoptosis of C-28/I2 chondrocytes, inhibition of p-ERK2 by U0126 in rhTNF- α -treated C-28/I2 chondrocytes may have had the additional effect of shifting MAPK signaling from the ERK pathway to the p38 kinase and/or JNK1/2 pathways. Thus, "cross-talk" between signal transduction pathways [24] constitutes one plausible mechanism responsible for increasing C-28/I2 chondrocyte apoptosis after treatment with rhTNF- α and U0126. Indeed, Loesser et al., [39] in reviewing MAPK signaling in normal chondrocytes, similarly concluded that TNF- α activated p38 MAPK and JNK1/2, which we also confirmed to be the case with human OA chondrocytes as well [24]. Furthermore, this conclusion was borne out by the results from an analysis on the effects of rhIL-6 and U0126 in restoring the level of p-JNK46, the latter having been inhibited by rhIL-6 alone (data not shown). That rhIL-6 plus U0126 also was found to reduce the ratio of p-p38 MAPK to β -actin [25] tends to support the conclusion that "cross-talk" between MAPK signaling pathways could be responsible for regulating the induction of C-28/I2 chondrocyte apoptosis by rhIL-6.

Pelletier et al. [40] had previously shown that PD98059, another MEK1 small molecule inhibitor, decreased, rather than increased, canine OA chondrocyte cell death. The mechanism attributed to the PD98059 effect on canine OA chondrocyte apoptosis was not only its capacity to inhibit MEK1 but also its effect on reducing COX-2, inducible nitric oxide (iNOS) and p-p38 MAPK, the latter having been previously shown to be involved in increasing OA chondrocyte apoptosis [19]. In contrast, Schmidt et al., [41] showed that iNOS mRNA expression was reduced in C-28/I2 chondrocytes in association with the inhibition of p38 MAPK, NF- κ B, and JAK2/STAT1A activation. Thus, various interpretations of the results in the present study must include the possibility going forward that 1) inhibition of MEK1/2 by U0126 and PD98059 produce contrasting results insofar as chondrocyte apoptosis is concerned; 2) that C-28/I2 chondrocytes behave differently than canine OA chondrocytes as a response to inhibiting MEK1/2; and 3) that induction of C-28/I2 chondrocyte apoptosis in response to rhTNF- α , also involves activation of p38 MAPK and/or JNK1/2 MAPK, as was previously shown to be the case with cultured human OA chondrocytes [24].

Wylie et al., [25] also showed that rhOSM strongly activated C-28/I2 chondrocyte ERK2. Thus, combined with the present results

which showed that rhOSM caused a robust positive trend towards increasing C-28/I2 chondrocyte DAPI/TUNEL-positivity as well as causing PARP degradation suggested that ERK2 activation was also likely to be a primary mechanism involved in the increased level of C-28/I2 chondrocyte apoptosis in response to rhOSM, and even more so, because the addition of U0126 did not alter this result.

These results became even more pertinent to the role of ERK2 as a regulator of apoptosis in view of the data published by Seidel and Graves [42], who showed that Ets-1, one of several transcription factors involved in regulating apoptosis possessed a "Pointed" domain and an ERK2 docking site. Based on the results of their experiments, Seidel and Graves [42], proposed that this structurally defined ERK2 docking site could represent the component which explains the common mechanism by which activated ERK2 regulates apoptosis. Thus it can be proposed that activated ERK2 in C-28/I2 chondrocytes in response to rhTNF- α , rhIL-6 or rhOSM, eventually interacts with various cytoplasmic and nuclear substrates, including Ets-1, as well as promoting the interaction of p-ERK2 with other protein scaffolds and anchoring proteins which further regulates gene transcription associated with apoptosis [43].

By further assessing the effect of rhTNF- α , rhIL-6, or rhOSM on C-28/I2 chondrocyte apoptosis we should also be able to shed additional light on alternative signal transduction mechanism(s) responsible for the regulation of pro-apoptosis proteins which result in the loss of chondrocyte viability in RA or OA articular cartilage [19,44-47]. In that regard, the results of this study with respect to the effect of combining rhIL-6 with tocilizumab on caspase-3 activation and PARP degradation in C-28/I2 chondrocytes was quite unexpected considering our current understanding of the mechanism by which tocilizumab neutralizes the interaction of IL-6 with the IL-6 receptor mechanism resulting in the inhibition of JAK/STAT signaling [45]. Thus, neither rhIL-6, tocilizumab, nor rhIL-6 plus tocilizumab had any effect on the degradation of PARP. However, the extent to which tocilizumab would have inhibited rhIL-6-induced C-28/I2 chondrocyte apoptosis warrants further study.

Of note, the chronic inflammatory state of RA synovial joints resulting from autoimmune activation of T-cells, B-cells, macrophages, and other accessory cells (i.e., dendritic cells) of the innate and adaptive immune system [47-49], often results in both "apoptosis-resistance" in RA synovial tissue [50-52] and a reduction in chondrocyte vitality, the latter event generally attributed to the capacity of pro-inflammatory cytokines to induce apoptosis [24]. In addition, the profound loss of chondrocyte viability as OA progresses from an indolent state to an inflammatory disease believed to be generally accompanied by a process resulting from chondrocyte apoptosis is generally considered to be a hallmark of this disease process. Thus, chondrocyte apoptosis is thought to be at an elevated level as a result of articular cartilage responding to the elevated levels of synovial fluid pro-inflammatory cytokines, such as TNF- α , IL-6, and OSM, among other pro-inflammatory cytokines [53].

In conclusion, human chondrocyte model *in vitro* culture systems such as the one employed in this study with C-28/I2 chondrocytes

could produce the pre-clinical data moving forward which would determine the clinical efficacy of various agents with the potential to inhibit chondrocyte apoptosis in well-validated animal models of RA or OA. The results from these studies could then provide the impetus for developing drugs which would be specifically designed to inhibit pro-inflammatory cytokine-induced chondrocyte apoptosis in various forms of arthritis which we [5,54,55] have previously proposed to be the case.

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