Regulation of inflammation signalling by resveratrol in human chondrocytes *in vitro*☆

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1. Introduction

Pro-inflammatory cytokines like interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) are well known as mediators of degradation and apoptosis in chondrocytes during degenerative joint diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA) in humans as well as in animals [1–4]. It is known, that IL-1β and TNF-α up-regulate matrix degrading enzymes such as matrix metalloproteinases (MMPs) and pro-inflammatory mediators such as cycloxygenase-2 (COX-2) leading to cartilage matrix destruction and joint inflammation. IL-1β and TNF-α are also known to activate the ubiquitous transcription factor NF-κB [5], which in chondrocytes leads to further pro-inflammatory cytokine

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production and thus to further cartilage degradation [6]. However, the mechanism of chondrocyte apoptosis during joint disease has not been fully elucidated. Since several arthritis associated catabolic signalling pathways converge towards the induction of apoptosis, the inhibition of these signalling pathways in the apoptotically activated chondrocytes seems to be a promising approach in OA therapy.

A great variety of dietary phytochemicals and herbal remedies have been proposed for OA therapy including stinging nettle extract, catechines such as epigallocatechin, trypterygium wilfordii hook F extract, resveratrol and curcumin [6–14]. Some of these are already known to inhibit NF-κB in chondrocytes and other cell types [7,13,15–17]. The clinical efficacy for some of the discussed phytopharmaceuticals such as curcumin and Tripterygium wilfordi hook F has been documented. Curcumin has been in use in Ayurveda Indian medicine as an anti-inflammatory agent for a long time and its potential against arthritis was first reported in 1980 [18,19]. In a 20-week placebo-controlled study of ethanolic extract of Tripterygium wilfordi hook F (TWHF), a dose-dependent effect was seen in the American College of Rheumatology 20 (ACR20) response in patients with RA [20]. The *in vivo* efficacy of resveratrol against OA has been reported from animal studies with rabbits [21,22]. Here, resveratrol significantly reduced cartilage degeneration in the joints. However, clinical efficacy of resveratrol against OA has still to be evaluated in well controlled clinical trials.

The natural polyphenolic compound resveratrol (*trans*-3,4′-trihydroxystilbene) occurs in the skin of red grapes, vines, various other fruits, peanuts and root extracts of the weed *Polygonum cuspidatum* [23]. In plants, resveratrol functions microbiologically as a phytoalexin that protects against fungal infections and exhibits antiprotozoal activity [24]. Additionally, it demonstrates anti-inflammatory, anti-tumor, immunomodulatory, cardioprotective, anti-oxidative and chemopreventive capabilities [25–28]. Resveratrol has also been found to modulate the expression of a series of intracellular signalling proteins, cellular proliferation, inflammation and apoptosis and to decrease COX-2 and NOS-2-expression in different cell lines [24,26,28,29]. It was reported to down-regulate cell cycle regulatory molecules, inhibitors of apoptosis and induce activation of pro-apoptotic molecules in UVB-stimulated mouse skin [27,30,31]). Recently, we showed that resveratrol has an anti-apoptotic effect on chondrocytes by inhibiting the IL-1β-induced stimulation of caspase-3 and the cleavage of the DNA repair enzyme poly(ADP-ribose)polymerase (PARP) in human articular chondrocytes *in vitro* [14]. Contrary to this, resveratrol leads to apoptotic cell death in several tumour cell lines, through the induction of activated caspase-3, accumulation of p53 and p21 and cleavage of PARP [32].

p53 is a tumor suppression protein and can induce growth arrest, apoptosis and can thus prevent accumulation of DNA-damaged cells and cancer cells [33–37]. Degradation of p53 is mediated by ubiquitin-dependent and ubiquitin-independent pathways and this is regulated by NAD(P)H:quinon oxidoreductase 1 (NQO1) [38–41]. NQO1 binds to p53 and stabilizes it, whereas dicoumarol, an inhibitor of NQO1 activity, disrupts NQO1–p53 binding and thus induces ubiquitin-independent proteosomal degradation of p53 [38–42]. Tsvetkov et al. showed that curcumin, a natural phenolic compound, inhibits NQO1 activity *in vitro* and *in vivo* through disrupting the binding of NQO1 to p53, inducing ubiquitination-independent degradation of p53 and inhibiting thus p53-mediated apoptosis [43].

Reactive oxygen species (ROS), which are involved in the signal transduction pathways, induce apoptosis in different cell types [44–48] and play critical roles in cell regulation, proliferation and even as second messengers [49,50]. It has been reported that mitochondria are the major source of ROS that mediate cytokine-induced signalling [51,52]. For instance, the cytokine TNF-α changes the membrane permeability of mitochondria leading to cytochrome c release and subsequent caspase activation, leading to apoptosis [53–57]. Furthermore, reports show that *N*-acetylcysteine acting as an antioxidant [58] blocks the production of ROS in cells.

The present study was undertaken to characterize the inflammation signalling effect of resveratrol, which is known to exert potential chemopreventive effects, in pro-inflammatory cytokine stimulated chondrocytes.

2. Material and methods

2.1. Antibodies

Monoclonal anti-β-actin (A4700) and anti-human IL-1β (I3642) antibodies were purchased from Sigma (Munich, Germany). R&D System, Inc. (Heidelberg, Germany) provided the polyclonal anti-active caspase-3 (AF835) antibody. Monoclonal anti-PARP [poly(ADP-ribose)polymerase] (7D3-6) antibodies were purchased from Becton Dickinson (Heidelberg, Germany). Monoclonal anti-p53 antibodies (sc-56186) were obtained from Santa Cruz Biotechnology. The chondrocyte growth medium and supplements (Ham's F-12/Dulbecco's modified Eagle's medium (50/50) containing 10% fetal calf serum, 25 μg/ml ascorbic acid, 50 IU/ml streptomycin, 50 IU/ml penicillin, 2.5 μg/ml amphotericin B, essential amino acids and L-glutamine) were obtained from Seromed (Munich, Germany). Resveratrol was purchased from Sigma (Munich, Germany). Resveratrol was prepared as a 100 mg/ml solution in ethanol and then further diluted in cell culture medium. IL-1β was obtained from Strathman Biotech GmbH (Hannover, Germany). Caspase specific inhibitor Z-DEVD-FMK was obtained from Calbiochem (La Jolla, CA). Dicoumarol was dissolved in 0.13 M NaOH, *N*-acetylcysteine as anti-oxidant and MTT were purchased from Sigma (Munich, Germany). Alkaline phosphatase linked sheep anti-mouse (AP326A), and sheep anti-rabbit (AP304A) secondary antibodies for western blotting were obtained from Chemicon International (Temecula, CA, USA).

2.2. Chondrocyte isolation and culture

Cartilage explants from healthy femoral head articular cartilage obtained during joint replacement surgery for femoral neck fractures were used to isolate primary human articular chondrocytes [59]. Cartilage slices were digested primarily with 1% pronase for 2 h at 37 °C and subsequently with 0.2% (v/v) collagenase for 4 h at 37 °C. Primary chondrocytes were cultured at 0.1×10^6 cells/ml in Petri dishes in monolayer culture for a

period of 24 h at 37 °C with 5% CO_2. Cartilage samples were derived from human patients with full informed consent and local ethics committee approval.

2.3. Experimental design

Chondrocyte monolayer cultures were washed three times with serum-starved medium and incubated for 1 h with serum-starved medium (0.5% FCS). Serum-starved human articular chondrocytes were either pre-stimulated with 10 ng/ml IL-1β alone for 1 or 24 h before being co-treated with IL-1β and with various concentrations (1–200 μM) of resveratrol for 1, 12, 24, 36 and 48 h or were exposed to 10 ng/ml IL-1β alone for 0, 5, 10, and 15 min before being co-treated with 50 μM resveratrol and 10 ng/ml IL-1β for 15 and 30 min. The experiments described in the present study were specifically designed to mimic the cellular events that occur in the clinical condition of OA: chondrocytes were pre-exposed to pro-inflammatory cytokines (IL-1β) before each treatment with resveratrol was initiated in the presence of the inflammatory agents IL-1β/TNF-α.

2.4. Cell proliferation assay

For the cell proliferation assay, chondrocytes were cultured for 24 h and then treated with 10 ng/ml IL-1β, 50 μM resveratrol, co-treated with 10 ng/ml IL-1β and 50 μM resveratrol or left untreated and evaluated after 1, 12, 24, 36 and 48 h. The viability and proliferation of cells was determined by the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromid)) dye uptake method. The MTT assay is an established method for the spectrophotometric measurement of cell proliferation as a function of mitochondrial activity in cells. It is therefore an indirect proof for cell proliferation and useful for testing in multi-well format. This assay is based on the hydrolysis of a tetrazolium ring by mitochondrial dehydrogenase enzymes to form an insoluble blue reaction product (formazan). The chondrocytes (2500 well^{-1}) were seeded in triplicate in a 96-well plate for 24 h and then treated as described above. Final samples were adjusted in a final volume of 0.1 ml and incubated for 24 h at 37 °C, then examined for cell number. One hundred microlitre of medium and 10 μl of MTT solution (5 mg/ml PBS, sterile) were added to each well and incubated for 4 h at 37 °C/5% CO_2. Subsequently, 100 μl of 10% SDS (w/v) in 0.01 M HCl was added and the samples were further incubated for 18 h. The transmission signal was determined at 570/630 nm using a micro plate reader (Bio-Rad, Munich, Germany). A sample without cell loading was used as a baseline value. The assay was performed in triplicate and the results are provided as mean values with standard deviations from three independent experiments.

2.5. Apoptosis assay

For the apoptosis assay, chondrocytes were induced to undergo apoptosis by incubation with 10 ng/ml IL-1β followed by a co-treatment with the above indicated concentrations of resveratrol or dicoumarol and evaluated after 1, 12, 24, 36 and 48 h. For statistical analysis, ultrathin sections of the samples were prepared and evaluated with an electron microscope (TEM 10, Zeiss, Jena, Germany).

2.6. Transmission electron microscopy (TEM)

Chondrocytes were fixed 1 h with Karnovsky-fixative and post-fixed in 1% OsO_4 solution (0.1 M phosphate buffer). Monolayer cell pellets were rinsed and dehydrated in an ascending alcohol series before being embedded in Epon and cut on a Reichert-Jung Ultracut E (Germany). Ultrathin sections were contrasted with 2% uranyl acetate/lead citrate. A transmission electron microscope (TEM 10, Zeiss, Jena, Germany) was used to examine the sections. The number of cells with morphological features of apoptotic cell death was determined by scoring 200 cells from 30 different microscopic fields. Isolated cell membrane fraction was centrifuged (10,000 g/5 min), resuspended in PBS containing 2 mM $MgCl_2$, and centrifuged again. The pellet was prepared for EM as described above as control.

2.7. Plasma membrane isolation

For isolation and preparation of the chondrocytes plasma membrane fraction the interface between 1.4 and 0.8 M sucrose of the gradient was carefully removed and diluted with 4 volumes of STM 0.25 (50 mM Tris/HCl, pH 7.4, 0.25 M sucrose, 5 mM $MgSO_4$, 2 mM dithioerythritol, 10 μg/ml leupeptin, 1 mM PMSF). The chondrocytes cell membranes were sedimented at $5000 \times g$ for 20 min, and the pellets were resuspended in STM 0.25. This method yielded a cell membrane fraction with a specific activity of 5′-nucleotidase 2.5 times higher than in the homogenate as previously described [60].

2.8. Western blot analysis

For western blot analysis chondrocyte proteins were extracted with lysis buffer (50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% (v/v) aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, PMSF) on ice for 30 min as previously described [59,61]. Protein determination was performed with the bicinchinonic acid system (Uptima, France) using bovine serum albumin (BSA) as a standard. The total protein concentrations of samples were adjusted to the same level before proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Separated proteins were transferred onto nitrocellulose membranes and membranes were blocked with 5% (w/v) skimmed milk powder in phosphate buffered saline (PBS)/0.1% Tween 20 for 1 h at ambient temperature (AT). Following blocking, membranes were incubated with the primary antibodies overnight at 4 °C. After thorough washing with the blocking buffer, the samples were incubated with alkaline phosphatase conjugated secondary antibodies for 1 h. Membranes were then washed in 0.1 M Tris, pH 9.5, containing 0.05 M $MgCl_2$ and 0.1 M NaCl. Specific antigen–antibody complexes were revealed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (*p*-toluidine salt; Pierce, Rockford, IL) as substrates for alkaline phosphatase. Immunoblots were semi-quantitatively analysed using the computer program ''Quantity one'' (Bio-Rad, Munich, Germany).

2.9. Measurement of reactive oxygen species (ROS)

Serum-starved chondrocytes were incubated with IL-1β 10 ng/ml alone or being co-treated with 10 ng/ml IL-1β and resveratrol (50 μM) or 10 ng/ml IL-1β and *N*-acetylcysteine (NAC) (500 μM) for the indicated time periods, followed by washing in PBS and incubation with dihydro-rhodamine 123 (DHR-123: 5 mM stock solution in DMSO) at a final concentration of 1 μM for 1 h. The fluorescence intensity resulting from DHR 123 oxidation was measured by a FACS flow cytometer analysis with excitation at 488 nm and was detected between 515 and 550 nm.

2.10. Statistical analysis

The results are expressed as the means ± S.D. of a representative experiment performed in triplicate. Data shown are representative of three independent experiments. The means were compared using Student's *t* test assuming equal variances. Statistical significance was at $p < 0.05$.

3. Results

3.1. Resveratrol blocks IL-1β-reduced chondrocyte proliferation

Because IL-1β inhibits the proliferation and induces cell death, we began studying the effects of resveratrol on the proliferation of the adhered chondrocytes treated with or without IL-1β. Human chondrocytes were cultured in a 96-well plate and treated with IL-1β, resveratrol, with either IL-1β and resveratrol or left untreated for the indicated times. The viability and proliferation of the chondrocytes cultivated only in the presence of IL-1β were significantly lower compared to those of chondrocytes treated with resveratrol, resveratrol and IL-1β or left untreated (Fig. 1).

3.2. Resveratrol inhibits IL-1β protein synthesis

In light of the above results, we hypothesized that resveratrol inhibits the mechanism of action of IL-1β. To test this idea, we incubated chondrocytes first with IL-1β for 15 min and then co-treated them with resveratrol for 30 min. Using western blot analysis to measure active, mature (cleaved) IL-1β in chondrocytes, we found that resveratrol significantly inhibited the expression of mature IL-1β (Fig. 2I). Mature IL-1β is released by cleavage of the 31 kDa inactive cytoplasmic form by the IL-1β-converting enzyme after binding with the cytoplasma membrane during secretion [62]. To test the effect of resveratrol on membrane-bound IL-1β, we isolated the cell membrane fraction and performed western blot analysis. As shown in Fig. 2II, resveratrol inhibited significantly the amount of membrane-bound IL-1β in chondrocytes. This result confirms clearly the above result, which showed that resveratrol reduces the production of the mature IL-1β.

3.3. Resveratrol inhibits IL-1β-induced degenerative features and apoptosis in chondrocytes

Untreated monolayer chondrocytes showed a typical rounded or flattened shape with small cytoplasmic processes, a large mostly euchromatic nucleus with nucleoli and a well structured cytoplasm (Fig. 3A). Treatment of chondrocyte monolayer cultures with 10 ng/ml IL-1β for 1, 12, 24, 48 h led to degenerative changes such as multiple vacuoles, swelling of rough ER and clustering of swollen mitochondria (Inset D, Fig. 3) and degeneration of other cell organelles. After longer incubation periods (48 h) more severe features of cellular degeneration were seen in response to IL-1β treatment. These include areas of condensed heterochromatin in the cell nuclei and multiple and autophagic cytoplasmic vacuoles. The flattened monolayer chondrocytes became more and more rounded, lost their microvilli-like processes and became apoptotic (Fig. 3B–E). Chondrocytes that were pre-treated with IL-1β (1 h) and then co-treated with IL-1β and 50 μM

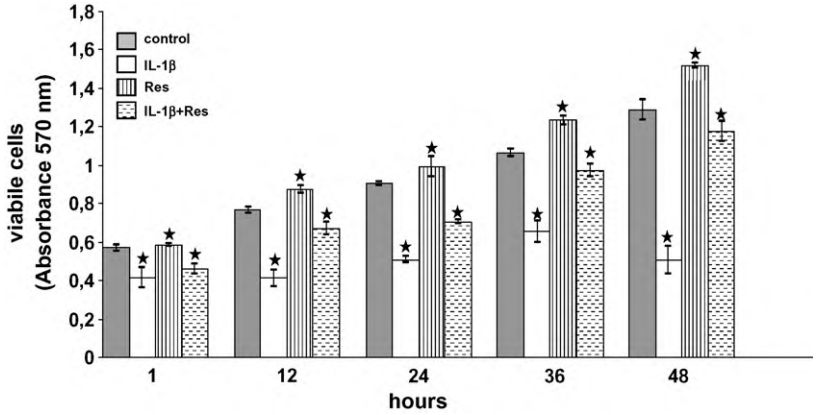

Fig. 1 – Effect of resveratrol and IL-1β on the proliferation of chondrocytes *in vitro*. Serum-starved human articular chondrocytes (0.1×10^6 cells/ml) were exposed to 10 ng/ml IL-1β, 50 μM resveratrol, co-treated with 10 ng/ml IL-1β and 50 μM resveratrol or left untreated for 1, 12, 24, 36 and 48 h. Cell viability was examined by MTT assay. The MTT assay is a spectrophotometric measurement of the cell viability as a function of the mitochondrial activity. This assay was performed in triplicate and the results are provided as mean values with standard deviations from three independent experiments. Values were compared to the control and statistically significant values with $p < 0.05$ were designated by an asterisk (*).

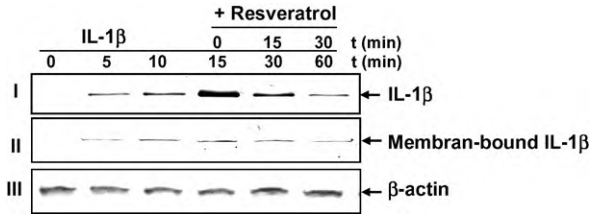

Fig. 2 – Effect of resveratrol on IL-1β protein synthesis. (I) Serum-starved human articular chondrocytes were exposed to 10 ng/ml IL-1β alone for 0, 5, 10 and 15 min before being co-treated with 50 μM resveratrol and 10 ng/ml IL-1β for 15 and 30 min. The amount of mature IL-1β protein produced by the chondrocytes was determined by western blotting analysis. The arrow points to the 17.5 kDa mature IL-1β protein. (II) Effect of resveratrol on cell membrane-bound IL-1β. After incubation of chondrocytes with or without resveratrol, as described above, the cell membranes of the chondrocytes were isolated (see Section 2) and the amount of membrane-bound IL-1β was determined by western blotting analysis. The arrow points to the 31 kDa inactive membrane-bound IL-1β. Expression of the house-keeping protein β-actin (III) was not affected.

resveratrol for 1, 12, 24 and 48 h showed less severe cellular degeneration on the ultrastructural level as early as 1 h after co-treatment (Fig. 3F–I). Even further, in the presence of resveratrol (for 12–48 h) the morphological degenerative features and mitochondrial swelling (Inset H, Fig. 3) of the chondrocytes nearly disappeared (Fig. 3F–I). The chondrocytes regained a flattened shape and numerous microvilli-like cytoplasmic processes. To test, whether this process is a caspase-dependent pathway, serum-starved chondrocytes were pre-stimulated with 10 ng/ml IL-1β before being co-treated with 10 ng/ml IL-1β and 50 μM caspase inhibitor Z-DEVD-FMK for the same time periods. The results clearly showed a time-dependent decrease in the morphological degenerative features and mitochondrial swelling (Inset L, Fig. 3) of the chondrocytes (Fig. 3J–M). Statistical evaluation of the data clearly highlighted changes in the number of cells with mitochondrial changes before and after IL-1β-treatment. Co-treatment with resveratrol or the caspase inhibitor Z-DEVD-FMK clearly decreased the number of cells with mitochondrial changes (Fig. 4).

3.4. *Resveratrol inhibits IL-1β-induced activation of caspase-3 and cleavage of PARP*

Since electron microscopy investigation demonstrated that IL-1β-induced apoptosis (Fig. 3B), and caspase-3 appears to be involved in the induction of apoptosis in chondrocytes, we wanted to determine whether resveratrol also suppresses caspase-3 activation and PARP cleavage in with IL-1β-treated chondrocytes. To determine this, chondrocytes were pre-incubated with IL-1β (10 ng/ml) for 24 h and then co-treated with different concentrations of resveratrol (0, 0.1, 1, 10 and 100 μM) for 24 h. As shown in Fig. 5, co-treatment with resveratrol after incubation with IL-1β down-regulated the level of biologically active caspase-3 and inhibited the cleavage of the DNA-repair enzyme PARP, thereby interrupting the apoptotic cascade.

3.5. *Resveratrol inhibits IL-1β-induced release of reactive oxygen species in chondrocytes in a time-dependent manner*

The chondrocytes were treated with IL-1β (10 ng/ml) alone or being co-treated with 10 ng/ml IL-1β and resveratrol (50 μM) or 10 ng/ml IL-1β and NAC (500 μM) for the indicated time. Release of reactive oxygen species (ROS) in IL-1β-treated chondrocytes was measured by the flow cytometry method. Chondrocytes produced reactive oxygen species in a time-dependent manner (Fig. 6).

3.6. *Resveratrol induces ubiquitin-independent degradation of p53 and inhibits p53-dependent apoptosis in chondrocytes in vitro*

Following these results we wanted to determine whether resveratrol may either promote or inhibits IL-1β-induced p53 accumulation in chondrocytes. Therefore, we examined the effect of resveratrol on the accumulation of p53 and the induction of apoptosis in with IL-1β-treated chondrocytes. As shown in Fig. 7A, resveratrol inhibits p53 accumulation in a dose-dependent manner with the strongest effect at 200 μM. The degree of inhibition of p53 accumulation with 200 μM resveratrol was similar to that with 300 μM dicoumarol alone or with 100 μM dicoumarol and 100 μM resveratrol (Fig. 7B). Chondrocytes undergo apoptosis when cultured with IL-1β after 48 h (Fig. 3E). Western blot analysis of the p53 level in chondrocytes cultured with IL-1β, with resveratrol and dicoumarol for 48 h showed that both resveratrol and dicoumarol decreased the level of p53 in a dose-dependent manner. Furthermore, we observed resveratrol (100 μM) and dicoumarol (100 μM) together had a synergistic effect on the decrease of the p53 level (Fig. 7B). Interestingly, these results indicate the ability of the natural phenolic compound, resveratrol to induce p53 degradation and thus inhibit p53-induced apoptosis in a dose-dependent manner in chondrocytes *in vitro* (Fig. 8).

4. Discussion

The present study leads to the following findings: (1) IL-1β-induced suppression of chondrocytes proliferation is relieved by resveratrol co-treatment. (2) Resveratrol inhibits membrane-bound IL-1β and mature IL-1β protein production in chondrocytes. (3) Incubation of chondrocytes with IL-1β results in morphological alterations, the main findings were swollen mitochondria and widened endoplasmic reticulum and apoptosis, these effects are abolished through co-treatment with resveratrol or caspase inhibitor Z-DEVD-FMK. (4) Furthermore, co-treatment of the IL-1β-stimulated cells with resveratrol blocks activation of caspase-3 and PARP cleavage. (5) IL-1β induces production of ROS in chondrocytes and this was significantly suppressed by resveratrol or NAC. (6) Finally, IL-1β treatment in chondrocytes results in apoptosis and this was accompanied by accumulation of tumor

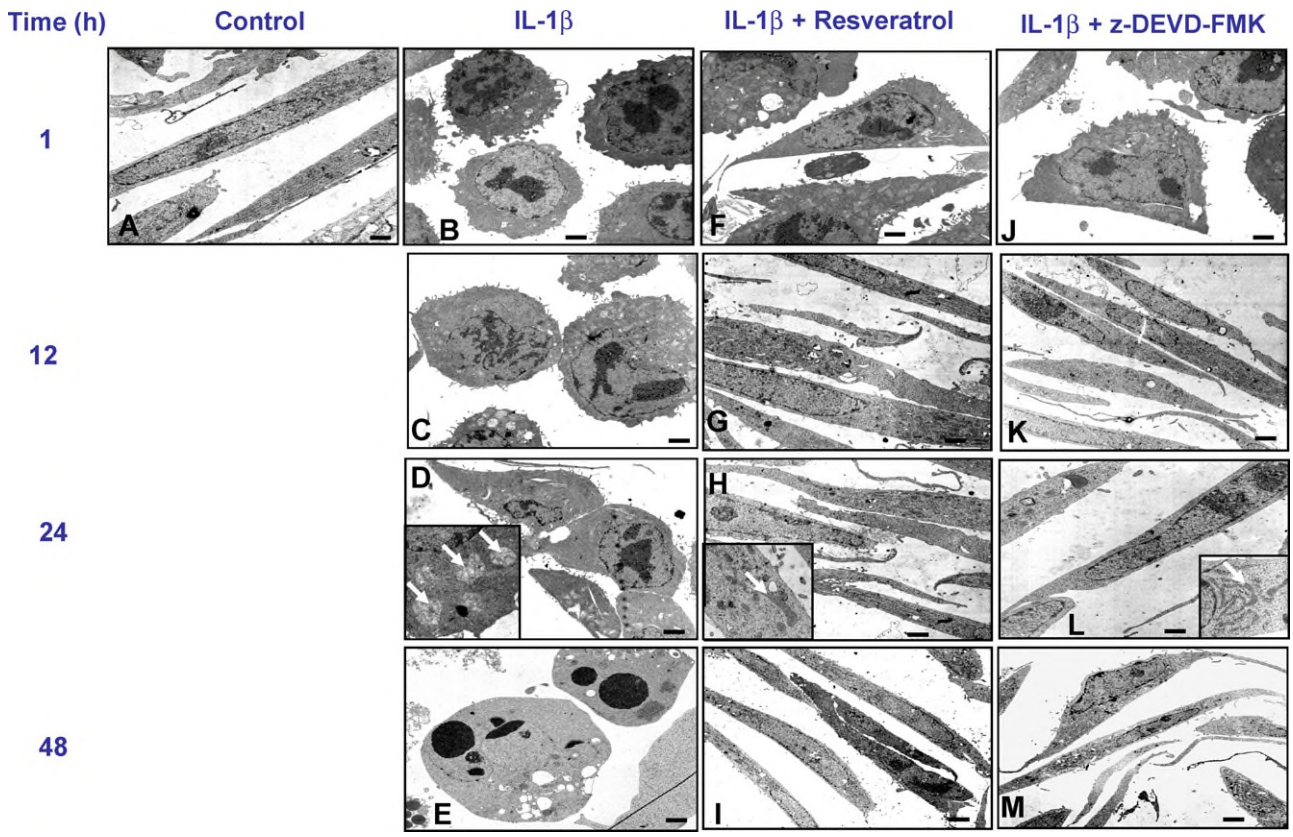

Fig. 3 – Effect of resveratrol and the caspase inhibitor Z-DEVD-FMK on IL-1β-induced mitochondrial changes and apoptosis in chondrocytes *in vitro*. Serum-starved human articular chondrocytes were either left untreated or exposed to 10 ng/ml IL-1β alone for 1, 12, 24 and 48 h (B–E) or pre-stimulated for 1 h with 10 ng/ml IL-1β before being co-treated with IL-1β and 50 μM of resveratrol (F–I) or 50 μM of caspase inhibitor Z-DEVD-FMK (J–M) for 12, 24, 48 h and evaluated with TEM. Chondrocytes, treated with 10 ng/ml IL-1β exhibited characteristic features of degeneration: annular chromatin condensation at the nuclear envelope of chondrocytes and swelling of mitochondria and rough ER in a time-dependent manner (B–E). Chondrocytes that were pre-treated with IL-1β and then co-treated with IL-1β and 50 μM resveratrol (F–I) or the caspase inhibitor Z-DEVD-FMK (J–M) showed less severe cell degenerations at the ultrastructural level. In control cultures no ultrastructural changes were observed (A). A–M: ×5000; Bar = 1 μM.

suppressor gene protein p53. (7) Resveratrol inhibits p53 accumulation and induces ubiquitin-independent degradation of p53. (8) These molecular events may be accounted for by inhibition of IL-1β-induced p53- and ROS-dependent apoptosis, a process we believe to be mediated by resveratrol.

Resveratrol belongs to the group of compounds called phytoalexin that are naturally produced in plants in the skin of fruits like red grapes and cranberries during environmental stress such as microbial infection (fungal, protozoa and other pathogens) or UV irradiation [24,63]. Resveratrol was first isolated from the root extract of the orient medicinal plant *Polygonum cuspidatum* and its potent anti-inflammatory properties have made it a favourite ingredient in asian herbal medicine [64]. Resveratrol has been identified in almost 70 species of plants, including red grapes, peanuts and mulberry. It possesses anti-inflammatory, immunomodulatory, antioxidant, cardioprotective and chemopreventive effects [25,26,28]. Further, Resveratrol is an anti-inflammatory dietary phytochemical that has previously been shown to antagonize some catabolic effects of TNF-α and IL-1β via inhibition of NF-κB [28,32,64].

Pro-inflammatory cytokines (TNF-α and IL-1β) have been shown to initiate chondrocyte degradation and apoptosis *in vitro* [3,65]. IL-1β protein has been implicated in articular cartilage in early events of degenerative joint diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA). These catabolic cytokines have the additional effect of aggravating local tissue inflammation by stimulating chondrocytes and synoviocytes to produce more pro-inflammatory cytokines. These in turn, activate expression of enzymes such as matrix metalloproteinases (MMPs) in chondrocytes and synoviocytes, leading to cartilage matrix breakdown [66]. Indeed, IL-1β and TNF-α also induce chondrocyte cell programmed death [1] which occurs in degenerative joint diseases of humans as well as animals [2–4].

Recently, we showed in chondrocytes that IL-1β-induced suppression of the collagen type II and β1-integrin signal receptor synthesis and activation of caspase-3 and PARP cleavage were blocked by resveratrol [14]. However, the mechanism of these effects of resveratrol in chondrocytes has not been fully understood.

Reversal of IL-1β-induced suppression of proliferation and viability in chondrocytes by resveratrol is an important sign

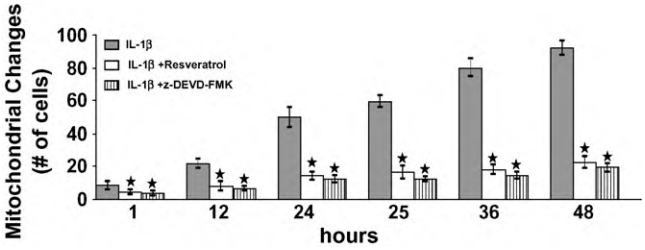

Fig. 4 – Effect of resveratrol and the caspase inhibitor Z-DEVD-FMK on IL-1β-induced mitochondrial changes in chondrocytes *in vitro*. Serum-starved human articular chondrocytes were either exposed to 10 ng/ml IL-1β for 1 h, co-treated with 10 ng/ml IL-1β and 50 μM resveratrol, treated with the caspase inhibitor Z-DEVD-FMK or left untreated for 1, 12, 24, 25, 36 and 48 h. The number of cells with mitochondrial changes was determined by scoring 200 cells from 30 different microscopic fields in ultrathin sections evaluated with an electron microscope. This assay was performed in triplicate and the results are provided as mean values with standard deviations from three independent experiments. Values were compared to the control and statistically significant values with $p < 0.05$ were designated by an asterisk (*).

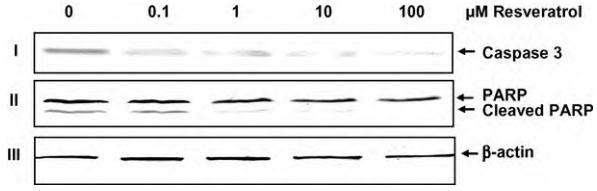

Fig. 5 – Effects of resveratrol on IL-1β-induced activation of caspase-3 and of PARP cleavage in chondrocytes. Chondrocytes were pre-incubated with IL-1β (10 ng/ml) for 24 h and then co-treated with different concentrations of resveratrol (0, 0.1, 1, 10 and 100 μM) for 24 h. Western blot analysis confirmed the activation of caspase-3 (I) and the cleavage of PARP (II) in response to IL-1β-stimulation (10 ng/ml for 24 h). Co-treatment with resveratrol after incubation with IL-1β down-regulated the level of biologically active caspase-3 and inhibited the cleavage of the DNA-repair enzyme PARP, thereby interrupting the apoptotic cascade (I and II). Expression of the house-keeping protein β-actin (III) was not affected by co-treatment with resveratrol and IL-1β.

for its positive effect on chondrocytes viability, differentiation and function. Furthermore, we observed that the IL-1β-stimulated cell membrane binding of inactive IL-1β and thus the production of mature IL-1β protein was blocked by resveratrol in a time-dependent manner. Indeed, Estrov et al. [32] already reported that resveratrol inhibits significantly the production of IL-1β protein in OCIM2 cells. These results indicate that inhibition of IL-1β protein production is part of the stimulatory mechanism of resveratrol on chondrocyte proliferation and viability.

Because most cell types require stimulation of the caspase pathway for apoptosis to occur, we asked whether resveratrol might block this pathway in chondrocytes treated with IL-1β. We treated the chondrocytes with IL-1β and caused morphological alterations with degenerative processes in cell organells, swelling of the mitochondria and rough endoplasmic reticulum and apoptosis. Co-treatment with resveratrol or the caspase inhibitor Z-DEVD-FMK partially reversed the apoptotic effect of IL-1β, suggesting that resveratrol inhibits the caspase pathway. Furthermore, we investigated the effect of resveratrol on caspase-3, because caspase-3 is a key executioner of apoptosis [67,68]. Moreover, the activation of caspase-3 results in cleavage of the DNA-repair enzyme PARP. As we expected, we found that resveratrol inhibited IL-1β-activation of caspase-3 and consequently blocked cleavage of PARP in a dose-dependent manner. These results highlight that this pathway is a caspase-dependent pathway. Interestingly, it appears that activation of caspase-3 does not represent an irreversible commitment to apoptosis in chondrocytes. In fact, our electron microscopy studies on IL-1β-stimulated human chondrocytes treated with resveratrol and the caspase inhibitor Z-DEVD-FMK show that there are no differences between these two agents in terms of patterns of intracellular proteins activated and ultrastructural changes.

Discrepancies between our findings and the results from other authors such as Estrov et al. [32], that resveratrol treatment inhibited the nuclear transcription factor NF-κB and activated caspase-3, thus inducing apoptosis in acute myeloid leukemia cells (AML cells), may be due to several reasons.

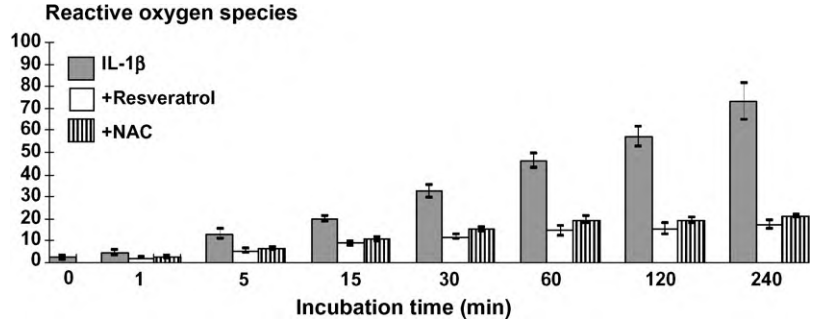

Fig. 6 – Resveratrol inhibits IL-1β-induced reactive oxygen species in a time-dependent manner in chondrocytes *in vitro*. Serum-starved human articular chondrocytes were exposed to 10 ng/ml IL-1β or co-treated with 10 ng/ml IL-1β and resveratrol (50 μM) or with NAC (500 μM) for the indicated time. Release of reactive oxygen species (ROS) was measured for time intervals as indicated by the flow cytometry method.

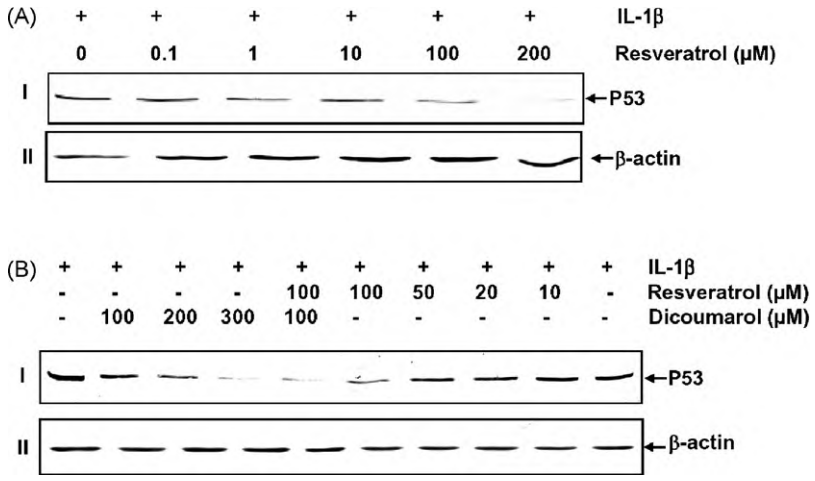

Fig. 7 – Resveratrol induces ubiquitin-independent degradation of p53 and inhibits p53-dependent apoptosis in chondrocytes *in vitro*. (A) Serum-starved human articular chondrocytes were exposed to 10 ng/ml IL-1β for 24 h (AI, first line) before being co-treated with 10 ng/ml IL-1β and resveratrol for the indicated concentrations (AI). (B) Serum-starved human articular chondrocytes were exposed to 10 ng/ml IL-1β for 24 h (BI, first line) before being co-treated with 10 ng/ml IL-1β and the indicated concentrations of resveratrol or dicoumarol. p53 was determined by western blotting. Expression of the house-keeping protein β-actin (AII, BII) was not affected by co-treatment with resveratrol, dicoumarol and IL-1β.

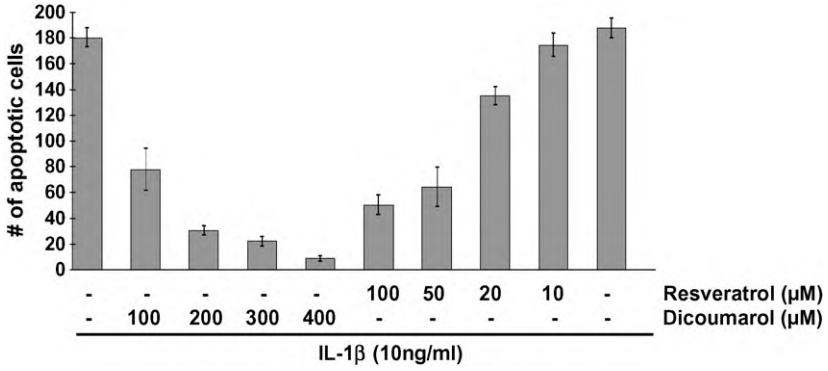

Fig. 8 – Resveratrol inhibits p53-dependent apoptosis in IL-1β-treated chondrocytes. Serum-starved human articular chondrocytes were exposed to 10 ng/ml IL-1β for 24 h (first column) before being co-treated with 10 ng/ml IL-1β and the indicated concentrations of resveratrol or dicoumarol for 24 h. The number of cells with morphological features of apoptotic cell death was determined by scoring 200 cells from 30 different microscopic fields in ultrathin sections evaluated with an electron microscope. This assay was performed in triplicate and the results are provided as mean values with standard deviations from three independent experiments.

Indeed, different cell types used (in this paper we used healthy human chondrocytes), cells from different species used and the knowledge that phytopharmaceuticals can act differently in different cell types may be the reason for the differing influence of resveratrol in chondrocytes compared to AML cells. Further, varying between species, cell types and stimulants, transcription factors such as NF-κB can have different impacts such as anti-apoptosis and apoptosis, proliferation, anti-inflammation and inflammation, stress responses and many others.

Reactive oxygen species (ROS) activate cell damage and it has been reported that this process is involved in diseases such as osteoarthritis [69,70]. Recently, it has been reported that ROS production during chondrocytes hypertrophy plays an important role and it has been shown that ROS levels were up-regulated during this process and is accompanied by inhibition of chondrocyte proliferation [71]. It has been shown that resveratrol has an antioxidant activity [72,73]. Indeed, the present study shows that resveratrol and NAC, a specific inhibitor of ROS, is a potent inhibitor of ROS production in chondrocytes *in vitro*. Results from this study, not only show the possible use of resveratrol in the prevention of osteoarthritis, but also provide interesting results which are involved in its function as an antioxidant.

Here, we report that resveratrol induces p53 degradation and thus, inhibits p53-dependent apoptosis. Resveratrol induced degradation of p53 by ubiquitin-independent pathway and so inhibited p53-mediated apoptosis, like several NQO1 inhibitors including dicoumarol [39,41,43]. The present study has shown that resveratrol like dicoumarol disrupts p53

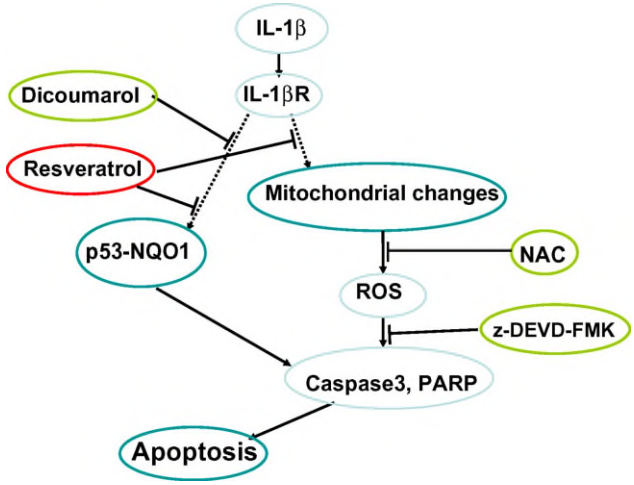

Fig. 9 – Working model for the inhibition effect of resveratrol on IL-1β-induced apoptosis in chondrocytes *in vitro*. Binding of IL-1β to its receptor (IL-1βR) alters mitochondrial membrane permeability and thus generation of ROS. It is known, that the mitochondria are the major source of cellular ROS involved in cytokine-induced apoptosis. ROS induces, through unknown mechanisms, activation of caspase-3 and finally apoptosis. In addition, IL-1β induces accumulation of p53. Accumulation of p53 activates caspase-3 and leads to apoptosis. These apoptosis pathways can be inhibited by the extracellular addition of known agents (Dicoumarol, NAC, Z-DEVD-FMK) as well as by resveratrol. Therefore, we propose that resveratrol inhibits cytokine-induced apoptosis through (a) inhibiting accumulation of p53 and inducing p53 ubiquitin-independent degradation and (b) inhibiting ROS production and release.

by inhibiting NQO1 activity and its complexes. The exact mechanism by which resveratrol acts to degrade p53 has not been totally elucidated but this event might be caused through sterical changes in the p53 molecule.

Thus, we propose a cartoon as shown in Fig. 9 to explain the mechanism of resveratrol and its potent action in preventing chondrocyte degradation and apoptosis. Namely, extracellularly added resveratrol is incorporated into intracellular compartments. There, it reduces IL-1β-induced mitochondrial ROS release and prevents IL-1β-induced p53 accumulation.

Taken together, our data, in combination with our previous studies suggest that resveratrol, through suppression of (1) the IL-1β- (2) the ROS- and (3) the tumor suppressor protein p53-production, may have a potential anti-inflammatory and anti-apoptotic effect in chondrocytes during degenerative processes in joint cartilage and may therefore become a potential agent in the treatment of osteoarthritis.

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