**The Influence of Resveratrol on the Synovial Expression of Matrix Metalloproteinases and Receptor Activator of NF-κB Ligand in Rheumatoid Arthritis Fibroblast-Like Synoviocytes**

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Z. Naturforsch. 68 c, 336 – 342 (2013); received July 30, 2012/May 21, 2013

Medication of rheumatoid arthritis (RA) remains challenging and often controversial concerning side effects or long-term complications. We investigated the effect of resveratrol, a phytoalexin discussed for its chondro-protective and anti-inflammatory qualities, on the synovial expression of matrix-degrading enzymes like matrix metalloproteinases (MMPs) and bone-remodelling proteins in RA fibroblast-like synoviocytes (FLS). Interleukin-1β-stimulated RA-FLS were treated with 100 μM resveratrol for 24 h. To evaluate the effect of resveratrol on the amount of bound/combined MMPs, a Luminex® xMAP multiplexing technology was used. The alteration in expression of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegrin (OPG) was measured by quantitative real-time polymerase chain reaction (qRT-PCR). Resveratrol reduced the expression of MMP-1 (p = 0.022), MMP-3 (p = 0.021), and MMP-9 (p = 0.047). qRT-PCR showed a significant reduction in the relative abundance of the transcripts of OPG (p = 0.012) and RANKL (p = 0.018). Our in vitro findings indicate that resveratrol could be a new target for further pharmacological studies in the field of RA. In the future it could play a role as a possible substitute or supplement to currently used drugs against RA to prevent cartilage matrix degradation and pathological bone resorption due to inhibition of MMPs and RANKL.

**Key words:** Resveratrol, Rheumatoid Arthritis, Matrix Metalloproteinases

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**Introduction**

The polyphenolic phytoalexin resveratrol (3,4',5-trihydroxystilbene) is abundant in grape skins (with higher concentration in red as compared to white wine), peanuts, the roots of the weed Polygonum cuspidatum as well as in other fruits and vegetables (Cardile et al., 2007; Csaki et al., 2008). It is produced by plants in response to stress and has a wide range of pharmacological activities. Resveratrol possesses several pharmacological activities, such as anti-inflammatory (Csaki et al., 2008; Bereswill et al., 2010), antiproliferative (Cardile et al., 2007; Gao et al., 2001, Glehr et al. 2013), pro-apoptotic (Byun et al., 2008; Glehr et al., 2013), antioxidant (Bhat et al., 2001), and cardioprotective (Lakota et al., 2009), with no obvious toxicity (Jang et al., 1997; Bhat et al., 2001; Baur and Sinclair, 2006).

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by synovial proliferation leading to cartilage and bone destruction (Lee et al., 2006). Contributing to the local production of cytokines, fibroblast-like synoviocytes (FLS) play a pivotal role in the pathogenesis of RA (Sipe et al., 1994; Burger et al., 2006). Cytokines such as interleukin-1β (IL-
1β) up-regulate matrix-degrading enzymes like matrix metalloproteinases (MMPs) leading to cartilage matrix destruction and joint inflammation (Burrage et al., 2006). RA-FLS also release large amounts of receptor activator of nuclear factor-κB ligand (RANKL), which mediates the differentiation of bone-resorbing osteoclasts from their macrophage precursors (Susa et al., 2004). Osteoclasts are highly specialized cells particularly involved in cartilage degradation and bone remodelling throughout life (Teitelbaum, 2000). Osteoprotegrin (OPG) functions as a decoy receptor by blocking the interaction between RANKL and its cognate receptor RANK. The RANK/RANKL system is necessary for osteoclast development and function (Yasuda et al., 1998).

It has been demonstrated that intra-articular injection of resveratrol reduces the severity of cartilage lesions and synovial inflammation in an experimental inflammatory arthritis rabbit model (Elmali et al., 2005). Wang et al. (2012) furthermore observed the effect of resveratrol on cartilage and chondrocytes apoptosis in experimental osteoarthritis (OA) in rabbits. Histological evaluation of cartilage tissue revealed significantly reduced cartilage destruction. Resveratrol reduced the apoptosis rate of chondrocytes and the production of NO. The protective effects of resveratrol were enhanced as a function of its concentration up to 50 μmol/kg (Wang et al., 2012).

The present study was designed to investigate the effect of resveratrol on the expression of MMPs, OPG, and RANKL in RA-FLS.

**Material and Methods**

*Harvest of the synovial tissue and expansion of RA-FLS*

RA-FLS were isolated from synovium of ten RA patients (average age, 64.5 years; range, 52–77 years; 3 males and 7 females) undergoing synovectomy or joint replacement. Individual participants in this study provided informed consent. The study protocol was approved by the local ethics committee (number of ethics proposal 19-111 ex 07/08; Ethical Committee of the Medical University of Graz, Graz, Austria). Synovial membrane tissue was cut into 1-mm slices, rinsed several times with phosphate-buffered saline (PBS), and then digested with 0.2% collagenase B (Roche Diagnostics, Mannheim, Germany) in high glucose Dulbecco’s-modified Eagle’s medium (DMEM-HG; GIBCO® Invitrogen, Darmstadt, Germany) containing 10% foetal bovine serum (FBS; GIBCO® Invitrogen), 1% l-glutamine (GIBCO® Invitrogen), 100 units/ml penicillin (GIBCO® Invitrogen), 100 μg/ml streptomycin (GIBCO® Invitrogen), and 0.25 μg amphotericin B (PAA Laboratory, Pasching, Austria). After overnight incubation at 37 °C, the cell suspension was filtered with a nylon membrane (Cell Strainer 40 μm; BD Biosciences, Franklin Lakes, NJ, USA) and plated in T75 culture flasks (TPP, Trasadingen, Switzerland). Cells were cultured in growth medium at 37 °C in a humidified atmosphere with 5% CO₂, for expansion. Each biological sample was cultured separately, and the biological replicates were obtained from different cultures and different patients. The medium was replaced every third day, and cells were passaged after reaching 70–90% confluence. Cells were cultured from three to a maximum of five passages in order to establish homogeneity.

*Characterization of FLS using fluorescence-activated cell sorting (FACS) analysis*

1 · 10⁶ cells were counterstained with 1 μg/ml of propidium iodide (PI; Molecular Probes, Invitrogen, Vienna, Austria), and nonviable cells were excluded from living cells. Commercial monoclonal antibodies, CD44PE, CD14FITC, CD90APC, and CD68PE (BD Biosciences, San Jose, CA, USA), and nonviable cells were excluded from living cells. Commercial monoclonal antibodies, CD44PE, CD14FITC, CD90APC, and CD68PE (BD Biosciences, San Jose, CA, USA), were applied for characterization of FLS, and each experiment contained isotype-matched control antibodies. The multicolour flow cytometric analysis was performed on a BD LSR II system (BD Biosciences). Data were acquired using BD FACS DivaTM software (BD Biosciences). The daily consistency of measurements was checked with a cytometer set-up and tacking beads (BD Biosciences). To exclude debris, a forward scatter (FSC) versus side scatter (SSC) gate was used and analysed on a linear scale. FLS were defined by their phenotype (CD90⁺, CD44⁺, CD68⁺, CD14⁻) and analysed on a logarithmic scale (according to Rosengren et al., 2007).

*Human MMP Fluorokine® MAP*

The cell culture supernatant was five-fold diluted with PBS. All subsequent steps were carried out in the same way. One hundred μl of calibrator diluent RD%-37 were added and mixed thoroughly. RA-FLS from ten RA patients (n = 10)
were pre-treated in 6-well plates with the MMP-inducing agent IL-1β at 10 ng/ml for 2 h in 2 ml of culture medium and then co-stimulated with 100 μM resveratrol (R5010; Sigma-Aldrich, Vienna, Austria) for 24 h [in accordance to the dosages used by Byun et al. (2008)]. The samples for the test with the human MMP base kit were prepared according to the manufacturer’s instruction (R & D Systems, Vienna, Austria). The microparticles were detected using the Luminex® analyzer (BioPlex TM dual laser; BioRad, Munich, Germany) within 90 min. One laser classified microparticles and determined the detected MMPs which were detected, and the second laser determined the magnitude of the signal derived from the streptavidin conjugated to phycoerythrin (PE), which was in direct proportion to the amount of bound MMPs.

qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to determine the relative expression of TNFSF11 [tumour necrosis factor (ligand) superfamily, member 11; RANKL] and TNFRSF11B (tumour necrosis factor receptor superfamily, member 11b; OPG). FLS from six RA-patients (n = 6) were pre-treated in 6-well plates with 10 ng/ml IL-1β for 2 h and co-stimulated with 100 μM resveratrol for 24 h. Total RNA was obtained from resveratrol-treated RA-FLS and control cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. Total RNA was reverse transcribed using the high-capacity RNA-to-cDNA Master Mix (Applied Biosystems, Carlsbad, CA, USA). TaqMan® gene expression assays with pre-designed, validated, gene-specific primers and probes help to perform quantitative gene expression in the 7900HT real-time PCR system (Applied Biosystems). The data were normalized to the expression of the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Statistical analysis

All values are presented as median and first and third quartiles (Q1, Q3). The significance of changes was determined using the Wilcoxon signed-rank test. We considered p < 0.05 as significant. Data were analysed using PASW 18 software (SPSS Inc., Chicago, IL, USA). The average outcome of the obtained data was recorded.

Results

Phenotypical characteristics

Fluorescence-activated cell sorting (FACS) analysis was performed for surface antigen expression of FLS by their phenotype. For each experiment, 10,000 events were analysed, and (93 ± 6.2)% viable cells were gated on forward scatter (FSC) versus side scatter (SSC). Fig. 1 shows representative examples of three independent FACS experiments. In the analysis, the negative staining for CD68 [(0.45 ± 0.49)%; Fig. 1A] and CD14 [(0.1 ± 0.14)%; Fig. 1C], which are both macrophage markers (Edwards, 2000), verified the absence of synovial macrophages, while positive staining for CD44 [(99.7 ± 0.14)%; Fig. 1B] and CD90 [(90.2 ± 6.73)%; Fig. 1A] verified the presence of FLS in our in vitro culture.
CD90 is specifically expressed on FLS in the synovial membrane (Palmer et al., 1985; Zimmermann et al., 2001), and CD44 is highly expressed on both cultured FLS and synovial macrophages (Johnson et al., 1993). Since macrophages were excluded, it can be assumed that FLS were cultured.

**Resveratrol decreases different MMP expression levels in RA-FLS**

Resveratrol reduced the expression of IL-1β-induced MMP-1, MMP-3, and MMP-9 production (Fig. 2) in the cell culture supernatant. MMP-2, -8, -12, and -13 expression was low and was not reduced further. Median fluorescence intensity (MFI) of MMP-1 decreased by 30.9% (Fig. 2A) after the treatment with 100 μM resveratrol for 24 h (p = 0.013). Fluorescence intensities of MMP-3 and MMP-9 decreased by 9.9% (Fig. 2B; p = 0.021) and 28.6% (Fig. 2C; p = 0.028), respectively. Table I offers detailed information.

**qRT-PCR of RANKL and OPG expression**

To investigate the effect of resveratrol on TNFSF11 (RANKL) and TNFRSF11B (OPG) expression, we conducted a qRT-PCR. Stimulation with IL-1β resulted in a significant increase of RANKL and OPG expression in RA-FLS (data not shown).

Expression of the RANKL gene (Fig. 3A) was reduced by 91% after co-treatment with resveratrol (p = 0.018), while under these conditions expression of the OPG gene (Fig. 3B) decreased by 82% with a statistical significance of p = 0.012.

**Discussion**

Chronic exposure to inflammatory cytokines and growth factors are likely key factors mediating the transformation of mesenchymal cells or their progenitors into stably activated RA-FLS (Keffer et al., 1991).

MMPs are primarily produced in RA-FLS and are highly elevated in the serum of patients with early RA (Fiedorczyk et al., 2006). They partici-

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**Table I. Additional data of the effect of resveratrol on expression levels of different MMPs.**

<table>
<thead>
<tr>
<th>Group</th>
<th>NK</th>
<th>IL</th>
<th>IL+RES</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Q1</td>
<td>Median</td>
<td>Q3</td>
</tr>
<tr>
<td>MMP-1</td>
<td>10</td>
<td>3846.9</td>
<td>9521.6</td>
<td>27822.9</td>
</tr>
<tr>
<td>MMP-3</td>
<td>9</td>
<td>8955.5</td>
<td>8955.5</td>
<td>26368.6</td>
</tr>
<tr>
<td>MMP-9</td>
<td>10</td>
<td>118.3</td>
<td>268.5</td>
<td>987.3</td>
</tr>
</tbody>
</table>

NK, control group, unstimulated cells. IL, cells stimulated with interleukin-1β to increase the MMP expression level. IL+RES, on an interleukin-1β stimulation followed a co-treatment with resveratrol. Median, the average of the middle two in a set with an even number of values. Q1, Q3, the two ends of the rectangles represent the first quartile (Q1) and the third quartile (Q3).
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...pate in irreversible proteolytic degradation and remodelling of the extracellular matrix. According to their substrate specificity, primary structure, and cellular localization, MMPs can be classified into five main groups: collagenases, gelatinases, stromelysins, matrilysins, and membrane-bound MT-MMPs (Murphy et al., 2002). MMP-1 (collagenase) and MMP-3 (stromelysin 1) are the major enzymes involved in tissue destruction (Curran and Murray, 1999). High levels are detected in synovial fluids of RA patients (Yoshihara et al., 2000). Moreover, MMP-1 preferentially degrades fibrillar collagens, whereas MMP-3 degrades a broad array of extracellular matrix substrates (Curran and Murray, 1999).

MMP-9 (gelatinase B) is markedly elevated in the sera and joints of RA patients, and correlates positively with disease progression and severity (Gruber et al., 1996). MMP-9 cleaves denatured collagen (gelatins) and type IV collagen, which is the major component of basement membranes (Ram et al., 2006).

MMP inhibition through the action of various components has been described in the literature, e.g. ajulemic acid – a synthetic nonpsychoactive cannabinoid acid – inhibited the release of MMP-1, MMP-3, and MMP-9 from TNF-α-stimulated human RA-FLS in vitro (Johnson et al., 2007). Also the flavonoid nobiletin down-regulated the production of pro-MMP-1 and -3 in human FLS, while on the other hand it up-regulated the expression of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) (Lin et al., 2003).

The results of our MMP multiplex study showed that resveratrol has a tendency to suppress the IL-1β-induced production of MMP-1, -3, and -9 by RA-FLS. This signifies a cartilage-protective effect of resveratrol. Also Elmali et al. reported in 2005 that resveratrol reduced the severity of cartilage lesions in experimentally induced osteoarthritis in rabbits.

This study also investigated the effect of resveratrol on RANKL and its decoy receptor OPG. RANKL is a key molecule in the pathogenesis of the osteolytic process in RA (Gravallese et al., 2000). OPG is the natural negative regulator of RANKL, which competes with RANKL for binding to RANK as a decoy receptor. The relative levels of RANKL and OPG are possibly key factors in the determination of the rate of osteoclast formation in vitro (Bucay et al., 1998). Our qRT-PCR analysis revealed that resveratrol suppresses the IL-1β-induced expression of RANKL and OPG expression.

It has previously been demonstrated that resveratrol analogues inhibit the differentiation and bone-resorbing activity of osteoclasts and promote the formation of osteoblasts from mesenchymal precursors in cultures (Mizutani et al., 1998; Boissy et al., 2005; Kupisiewicz et al., 2010). Resveratrol also enhanced the proliferation and osteoblastic differentiation in human mesenchymal stem cells via ER-dependent ERK1/2 activation (Dai et al., 2007).

Our in vitro study showed resveratrol affects human RA-FLS by reducing MMPs, RANKL, and inflammatory cytokines. This could provide a further explanation for the in vivo findings of Elmali et al. (2005) and Wang et al. (2012) which established the cartilage protective potency of resveratrol in OA. This study could be the basis for further examinations of the influence of resveratrol on OA- and RA-FLS.
Conclusion

Our results indicate that resveratrol may provide a new therapeutic target for further studies and an alternative approach to conventional drugs against RA by virtue of its inhibition of the overproduction of MMPs and RANKL which cause chondrocyte degeneration and pathological bone resorption in RA.

Acknowledgements

This work was supported by the Association for Orthopaedic Research (AFOR) scientific fund and by the Jubilee Fund of the Austrian National Bank (OENB 12953). The funding agencies had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors would like to thank Heike Kaltenegger and Bettina Turner for excellent technical assistance with cell cultures, Alexandra Novak for perfect technical assistance with the flow cytometric analysis, and Wolfgang Karl for perfect technical assistance in qRT-PCR. The authors would also like to thank Jenny Chapman for language editing.


Nachdruck – auch auszugsweise – nur mit schriftlicher Genehmigung des Verlages gestattet
Satz und Druck: AZ Druck und Datentechnik GmbH, Kempten