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ORIGINAL ARTICLE

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Ameliorative effect of ozone on cytokine production in mice injected with human rheumatoid arthritis synovial fibroblast cells

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Abstract Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by invasion of hyperplastic synovial cells and progressive joint destruction. Ozone therapy has been proposed as an immunomodulator and cellular metabolic activator which shows long-term anti-inflammatory effects and serves to reduce further the proinflammatory factors. We purified RA synovial fibroblast cells (RA-SFc) from patients and avoided contaminating macrophages by flow cytometry, then treated them with ozone. Following the observable decreased production of proinflammatory factors TNF-α, IL-1β, and IL-6 from RA-SFc, we infused the cultured RA-SFc into joints of severe combined immunodeficiency mice. The mRNA and protein levels of the RA-SFc exposed to 3% and 5% ozone were the same. As a result, 3% and 5% ozone applied externally ameliorated the inflammatory reaction of RA without toxicity or serious side effects. Therefore, ozone injected into the knees of RA patients could become a valuable treatment, and we confirm the interactive mechanism between ozone and RA-SFc.

Keywords Decreased cytokine secretion · Ozone therapy · Rheumatoid arthritis · Synovial fibroblast cells

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Introduction

Rheumatoid arthritis (RA) is chronic inflammatory disease characterized by the invasion of hyperplastic synovial cells and progressive joint destruction. Due to the unclear etiology of this disease, it has proven exceedingly difficult to treat. The pathogenesis of joint destruction in RA has been attributed to the phenotype of rheumatoid synovial cells transformed to proliferate abnormally [1, 2, 3]. These hyperplastic synovial cells invade bone and cartilage by producing elevated amounts of proinflammatory cytokines [4] and inducing osteoclast formation and activation [5].

No matter what pathogenesis is caused by RA, recent evidence suggests that cytokines are important pathologic mediators in the disease. In the inflamed synovium, monocytes/macrophages have been shown to produce large amounts of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β in local activation leading to joint destruction and interaction with synoviocytes to produce inflammation mediators such as IL-6, which play a pivotal role in the pathology of RA [6, 7]. This cytokine production may drive or control many of the characteristic features of rheumatoid inflammation and contribute to the arthritogenic potential of RA synovial fibroblast cells (RA-SFc) in severe combined immunodeficiency (SCID) transfer models [8, 9].

Ozone is a gas constituting an ozone and oxygen mixture. It is obtained by means of an electrical discharge through pure oxygen, achieving ozone concentrations between 0.05% and 5% of volume [8]. Ozone therapy has been proposed as an antioxidant enzyme system activator, immunomodulator, and cellular metabolic activator. The gas is a potent oxidizing agent that induces specific enzymes and short-lived peroxides which have been injected into cells and scavenged by glutathione peroxidase [10, 11].

Free radicals generated from the cascade and oxygenated bimolecular activity that results from reaction

with ozone may mediate the effects of ozone. Peroxides created by ozone administration show long-term anti-RA effects which serve further to reduce proinflammatory factors [11]. In addition, ozone failed to induce the release of cytokines and fibronectin in vitro in human alveolar macrophages [12], indicating that macrophages may not be the initiating factor in the ozone-induced inflammatory cascade.

To investigate whether ozone has a healing capability in RA, we performed in vitro and in vivo experiments. We purified RA fibroblasts from primary culture, and treatment with ozone observably decreased the production of TNF-α, IL-1β, and IL-6 by RA-SFc. Our animal model with SCID mice also demonstrated the same efficacy. In other words, ozone applied externally can ameliorate the inflammatory reaction of RA without toxicity or uncomfortable side effects. Therefore, ozone directly injected into knees of RA patients could become a valuable treatment, and we confirm an interactive mechanism between ozone and RA-SFc.

Materials and methods

Patients

Synovial tissue was obtained by joint surgery on patients with RA ($n = 16$) who met the American College of Rheumatology (1987) revised criteria for the classification of RA and were positive for RA-SFc.

Preparation of human synovial cells

Primary rheumatoid synovial cells were obtained from the joints of patients with RA. After washing three times and transferring to 25-cm^2 plastic flasks, cells were cultured with Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal calf serum (FCS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cellular outgrowths from the joints were observable after several days and continued for up to 2 weeks. Subcultured synovial fibroblasts that were free of macrophages and nonspecific esterase were identified by phase-contrast microscopy, indirect immunofluorescence staining, and immunohistochemistry with avidin-biotin peroxidase complex staining (data not shown). Mycoplasma contamination was checked with a Mycotect kit in all cells (Life Technology, Gaithersburg, Md., USA), and all human synovial cells used were free of mycoplasma contamination.

Negative isolation from primary culture

Typsinized and washed RA-SFc from the primary culture (10^7 cells/ml) were incubated with 4×10^7/ml of M-450 CD14 Dynabeads (clone RM052) (Dynal) in phosphate-buffered solution (PBS) containing 2% FCS at 4°C under bidirectional rotation. Nine milliliters of PBS containing 2% FCS were then added, and the confected cells were collected using a Dynal magnetic particle concentrator. Magneto-bead-conjugated cells and unconjugated cells were collected and washed twice in PBS containing 2% FCS; and cell composition and phenotypes were analyzed by flow cytometry using the antibodies presented in Table 1. For comparison with fourth-passage cells obtained by conventional isolation, negatively isolated RA-SFc were then passaged four times by culture in complete RMPI-1640 containing 10% FCS, with a 1:3 split of confluent cells in each passage [11, 13].

Conventional isolation of RA-SFc by repeated passage

Synovial cells were obtained by trypsin/collagenase digestion of the RA synovium as already described. The cells were subsequently cultured with four passages in RPMI-1640 containing 10% FCS containing the aforementioned additives by splitting confluent cells in each passage at a ratio of 1:3 [13].

Skin fibroblast isolation

Skin fibroblasts were obtained either by Dispase II/collagenase (Roche) digestion or normal skin samples (primary-culture fibroblasts from normal skin). The phenotype and composition of isolated/passaged fibroblasts were characterized by flow cytometry in a fluorescence-activated cell sorter (FACS), using saturating amounts of the monoclonal antibodies [11].

Table 1 Composition of rheumatoid arthritis synovial fibroblasts (RA-SFc) and normal SFc as determined by flow cytometry for various numbers of patients/donors

Composition	Mean ± SE positivity in percent	Mean ± SE fluorescence intensity
Primary-culture RA-SFc		
Thy-1 (CD90)	69 ± 13.22	199 ± 35
CD14	15.87 ± 7.55	288 ± 46
CD11b	0.9 ± 0.02	65 ± 11
CD68	0.3 ± 0.0	34 ± 11
RA-SFc (negative isolation from primary culture)		
Thy-1 (CD90)	88.33 ± 9.22	130 ± 38
CD14	0.88 ± 0.11	74 ± 14
CD11b	0.21 ± 0.05	50 ± 12
CD68	0.2 ± 0.0	155 ± 22
Fourth-passage RA-SFc		
Thy-1 (CD90)	82.45 ± 7.88	65 ± 13
CD14	4.88 ± 1.1	306 ± 88
CD11b	0.23 ± 0.1	73 ± 13
CD68	0.8 ± 0.2	56 ± 28
Normal skin Fc		
Thy-1 (CD90)	92.45 ± 5.33	175 ± 24
CD14	2.7 ± 0.23	79 ± 22
CD11b	0.23 ± 0.1	66 ± 10
CD68	0.77 ± 0.24	91 ± 28

Flow cytometric analysis

One×10^7 cells/ml were suspended in Hank's balanced salt solution (HBSS) buffer containing 10% FCS, 10 mmol/l hydroxyethylpiperazine ethanesulfonic acid, and 0.02% Na-azide for FACS stain, and the antibodies were conjugated in 50 µl for 30 min at 4°C and then washed three times with PBS, fixed in 2% paraformaldehyde in phosphate-buffered saline (pH 7.3), and analyzed for cell surface markers with a FACScan flow cytometer (Becton Dickinson). Data analysis was performed using Cell-Quest software (Becton Dickinson) and the Windows Multiple Document Interface flow cytometry application.

Cell surface marker analysis

The cells were washed twice with ice-cold PBS and resuspended at 1×10^7 cells/ml in assay buffer. A total of 1×10^6 cells were incubated for 10 min with human immunoglobulin (Ig)G to block fibroblast cell receptors and prevent the nonspecific binding of antibodies. Cells (1×10^6) were stained with 20 µl of fluorescein isothiocyanate (FITC)-conjugated mouse antihuman CD14 (M5E2) antibodies (BD Biosciences) and/or 20 µl of phycoerythrin-conjugated mouse antihuman CD11b/Mac-1 (ICRF44) (BD Biosciences) and/or 20 µl of FITC-conjugated mouse antihuman CD68 (KP1) antibodies (DAKO) and/or 20 µl of mouse antihuman CD90 (Thy-1) (FibAS02) antibodies (Dianova, Hamburg, Germany) [12]. After 30 min of incubation, the cells were washed twice, fixed with 2% paraformaldehyde, and kept overnight at 4° to be analyzed by flow cytometry the following day [14].

In vitro ozone exposure

The system used for in vitro ozone exposure was described previously [15]. The Ozonsan-α generator (Iffezheim, Germany) delivers precisely controlled flow of gases to the exposure vessels with precise ozone concentrations and appropriate humidification. Cells were exposed to either N_2 or 1%, 3%, 5%, or 8% of ozone for 1 h a day for at least 2 weeks. After ozone exposure, the cells were gently rinsed and immediately prepared for cell viability assay by trypan blue or kept in culture for various periods of time in the presence of RPMI-1640 with 10% FCS for the purpose of assessing cell viability, cell surface marker expression, and cytokine expression at later time points after exposure [13].

Measurement of cytokine production in RA-SFc culture by enzyme-linked immunosorbent assay

The RA-SFc (2×10^4 cells/well) were first cultured in a 96-well plate for 24 h. Gas mixtures of 1%, 3%, 5%, and 8% ozone (14 µg/ml, 42 µg/ml, 70 µg/ml, and 112 µg/ml ozone, respectively) or N_2 were applied individually by Ozonsan-α generators three times, and the RA-SFc were then further incubated in a 5% CO_2/air humidified atmosphere at 37°C for another week. The supernatants were then collected and assayed for IL-1β, IL-6, and TNF-α concentrations by human or mouse IL-1β, IL-6, and TNF-α enzyme-linked immunosorbent assay kits (Biotrak, Amersham, UK).

Animal model

Female C.B.17/IcrCrl SCID mice 8–10 weeks of age (Charles River Wiga, Sulzfeld, Germany) were acclimatized for at least 1 week prior to the experiments in laminar-flow filter cabinets in the local animal care facility under stable conditions (27°C, 80% air humidity). The murine immunoglobulin levels were examined according to Kuhn et al. Mice with immunoglobulin levels >10 µg/ml were not included in these experiments.

Fibroblast injection into knee joints and ozone treatment

Cells from 16 RA patients and two controls (skin fibroblasts and healthy synovial fibroblasts) were trypsinized and washed three times in PBS. Fibroblasts and control cells (2×10^6/20 µl PBS) were separated and injected intra-articularly into the mouse knee joints from an anterolateral position [16]. Seven days after cell injection, 0.3 ml of ozone were poured into the swelling joints at concentrations of 3% or 5% three times a week.

Monitoring and assessment of arthritis activity

Joint swelling was monitored every week by measuring knee joint diameter with calipers until the mice were killed after 5 weeks of the ozone treatment. The knee and hip joints, elbows, and hind paws were frozen for preparation of serial cryostat sections or fixed in 4% paraformaldehyde (Sigma) for preparation of serial paraffin sections, respectively. The extent of joint destruction was assessed histologically in Giemsa-stained cryostat sections and hematoxylin/eosin-stained paraffin sections according to published criteria [17].

Histology

The frozen mouse joints were embedded in glycerol gelatin (Sigma) for cryostat cutting (Leica, Wetzlar, Germany). Sections 8 µm thick were fixed to transparent tape (Uhu, Bühl, Germany) immediately after cutting to avoid disintegration of articular structures, then air-dried and stored at −80°C until staining. Histologic

staining was performed with Giemsa reagent (Merck, Darmstadt, Germany).

Extraction of total cellular RNA

The total cellular RNA was extracted from the RA-SFc by a method described previously [17]. The 5×10^6 cells were cultured for 7 days and treated with 15 ml of 1%, 3%, 5%, and 8% ozone or N_2 by Ozonsan-α generators three times a week. The RA-SFc were trypsinized and washed by cold Tris-saline containing 25 mmol/l of Tris (pH 7.4), 130 mmol/l of NaCl, and 5 mmol/l of KCl and then suspended in sodium deoxycholate dextran buffer. After centrifugation (10,000 rpm at 4°C for 15 min), the supernatants were extracted with a phenol-chloroform mixture (v:v = 1:1). The extracted RNA was precipitated with 1 ml of 100% cold ethanol. The total cellular RNA was pelleted by centrifugation at 4°C, 10,000 rpm for 30 min, and redissolved in 50 µl of diethyl pyrocarbonate-treated H_2O to prevent RNA from degradation, and its concentration was determined at optical densities of 260 nm and 280 nm.

Primer selection

Reverse transcription polymerase chain reaction (RT-PCR) was performed to generate and amplify cDNA sequences from cellular mRNA for IL-1β, IL-6, and TNF-α. The glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene served as an internal control for the mRNA expression level and cDNA synthesis in each treatment condition. Primer 3 software (provided by Steve Rozen) was used to design optimal PCR primers that would amplify at similar temperatures and magnesium concentrations. We used the following primers: for TNF-α, 5' primer-aaagaggcactggcagaaaa, 3' primer-gaggtgccca tgctacattt-452 bp; for IL-1β, 5' primer-aagaattcaaactg gggcct, 3' primer-gaggaaggcctaaggtccac-171 bp; and for IL-6, 5' primer-AGCGGCAGGCAGGCAGTATCA CCGGCGACCCATCATCCAGTTG, 3' primer-TCT GCTTGTGAGGTGCTGATGTTTGACCGCCTTCT GCTTGTTGC-396bp; and GADPH, 5' primer-AAG-G TCGGTGTGAACGGATTTG, 3' primer-TTGGGGG TAGGAACACGGAAGG–99 bp as an internal control.

The amplifiers had similar G-C contents to avoid large differences in reaction efficiencies. All primers were compared with GenBank and EMBL nucleic sequence libraries to ensure that they would not hybridize to any other known nucleic acid sequences under the conditions used.

Reverse transcription and polymerase chain reaction

Reverse transcription of RNA and PCR amplification of cDNA were carried out in the same test tube according to a Perkin-Elmer-Cetus protocol (Norwalk, Ct., USA). Briefly, $MgCl_2$, 10× PCR buffer, an RNase inhibitor, reverse transcriptase, deoxynucleoside triphosphates, oligo d(T), and RNA were mixed in a total volume of 25 µl. The thermal cycler (GeneAmp PCR system 9600) (Perkin-Elmer-Cetus) program for the RT step was 30 min at 42°C, 5 min at 95°C, and 5 min at 5°C.

Polymerase chain reaction of each cDNA sequence was performed with 1 µg of mRNA (concentration determined by spectrophotometry), 2.5 U of Taq polymerase (GeneAmp PCR kit) (Perkin-Elmer-Cetus), and 5 mM Mg^{2+} in a total volume of 100 µl. All samples were amplified simultaneously with specific primers using a master mixture containing all components of the PCR reaction. Negative controls routinely used for each set of primers included water control and control without template. An RT-PCR-positive kit control was included for each reaction.

Programmable temperature cycling was performed with the following cycle profile: 94°C for 1 min and then 35 cycles each comprising denaturation for 30 s at 94°C, annealing for 45 s 55°C, and extraction for 45 s at 72°C. After 35 cycles, the reaction tubes were kept for 5 min at 72°C and then at 4°C. The samples were electrophoresed in gels containing 2% agarose (FMC, Rockland, Me., USA). The sequencing as described above authenticated the PCR bands.

Western blotting

For preparation and quantitation of protein extracts, 5×10^5 synovial cells were grown to subconfluence for 24 h. After starvation with 10 ml/L FCS, synovial cells were poured with 15 ml of ozone at different concentrations (1%, 3%, 5%, or 8%) or N_2, and serum-free RPMI-1640 was used as the control. Cells were washed with cold PBS and lysed by the addition of 100 µl of lysis buffer (20 mmol Tris-HCl [pH 8.0], 1% [v:v] NP-40, 150 mmol/l NaCl, 1 g/l [v:w] NaN_3, 5 mg/l aprotinin, 1 mmol/l PMSF, 1 mmol/l ethylenediamine tetra-acetate, 1 mmol/l Na_3VO_4, 25 µmol/l PNP', 1 µmol/l pepstatin A, and 1 µmol/l leupeptin), then scraped off and kept on ice for 20 min. Insoluble material was removed by centrifugation at 15,000 *G* for 15 min at 4°C. The supernatant was saved, and protein concentration was determined using a Bio-Rad DC protein assay kit.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting

Thirty micrograms of protein for each lysate were subjected to 100 g/l sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred to a nitrocellulose membrane. The membrane was blocked overnight with 50 g/l of skimmed milk in PBS at 4°C, washed in phosphate-buffered saline with Tween (PBST) three times, and incubated at room temperature for 3 h with a 1:2000 dilutions of mouse anti-IL-1β anti-

body, mouse anti-TNF-α antibody, and mouse anti-IL-6 antibody. The membrane was washed in PBST immediately and incubated with a 1:2000 dilution of goat anti-mouse or rabbit IgG antibodies coupled with horseradish peroxides. An enhanced chemiluminescence system (Amersham) was used to detect the expression of IL-1β, TNF-α, and IL-6 in ozone-treated RA-SFc. The membranes were sequentially exposed to X-Kodak film for 15 s, and the latter was processed.

Statistical analysis

Histological scores values were expressed as means ± SE. Statistical significance was tested by Student's *t*-test. Differences were considered statistically significant if the two-sided *P* value was < 0.05.

Results

Composition of isolated/passaged fibroblasts

All cell preparations were highly enriched with fibroblasts, as determined by positivity for CD90 (monoclonal antibody AS02) (Table 1). Contamination with CD14+ and/or CD68+ macrophages was routinely below 2%, with the exception of primary culture.

The cell viability of RA-SFc after treatment with ozone

After ozone exposure, the viability of RA-SFc was determined by Trypan blue exclusion. At the first 3 days, the viability of RA-SFc from the 5% concentration of ozone was higher than from 3%. At the same time, the cell viability of control samples treated with N_2 was as good as with ozone (Fig. 1). The viability and levels of CD14 and CD11b expression showed no significant difference between cells exposed to 5% ozone or N_2. Cell death was pronounced at the 3rd h after ozone exposure at the concentration of 8%. Approximately 50% of RA-SFc exposed in 8% ozone became inflated and then burst. The results indicated that cells exposed in lower than 8% ozone had no cytotoxicity.

Quantitation of cytokine protein production in RA-SFc

To study whether the impairment of RA-SFc was related to cytokine production, the cells were poured with four concentrations of ozone three times for a week. Cell supernatants were collected, and IL-6, IL-1β, and TNF-α productions were assayed by enzyme-linked immunosorbent assay (ELISA). the stimulated production of IL-1β, TNF-α, and IL-6 in RA-SFc was significantly suppressed by ozone treatment at 3% and 5% concentrations. Furthermore, the inhibitory activities of preinflammatory cytokines by ozone were concentration-dependent. At 5%, the stimulated production of IL-1β, TNF-α, and IL-6 in RA-SFc was greatly decreased by ozone, returning to almost the same levels as those produced in N_2. The RA-SFc secreted similar amounts of IL-1β, TNF-α, and IL-6, and protein expression was significantly higher than IL-6 and TNF-α (Fig. 2). We also found fifth-passage RA-SFc cytokine secretion markedly lower for IL-1β, TNF-α, and IL-6 (data unpublished).

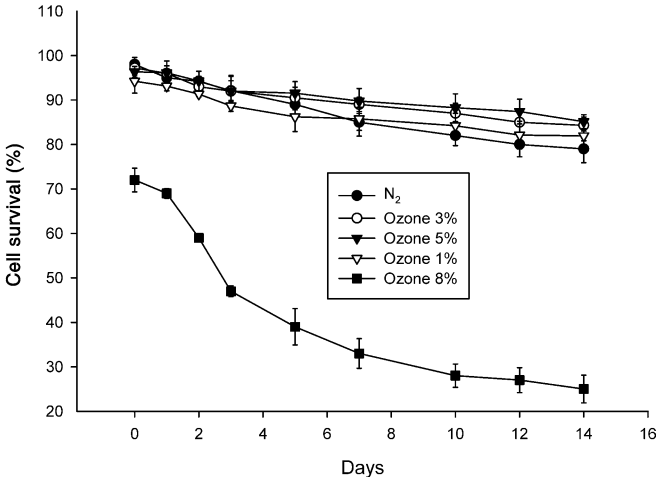

Fig. 1 Percentages of cells surviving N_2 or ozone exposure. At the first 3 days, the viability of RA-SFc was higher at the ozone concentration of 5% than at 3%. Cell viability of control samples treated with N_2 was the same as with 5% ozone, displaying no significant difference. Approximately 50% of RA-SFc exposed to 8% ozone inflated to bursting

Induction and time course of knee arthritis by RA synovial fibroblasts

Injection of 2×10^6 RA-SFc or control cells into the left knee joints of the SCID mice led to the development of arthritis, as demonstrated by joint swelling (Fig. 3). The initial reaction to intra-articular injection of RA-SFc or control fibroblasts (skin or normal synovial cells) was general, with increased joint diameter of approximately 0.5 mm at week 1 (Fig. 4). Nevertheless, as the RA-SFc injections increased, the associated swelling in controls increased (Fig. 3). At the 1st week of 3% and 5% ozone therapy for RA-SFc-induced swelling, the swelling decreased approximately 50% and persisted slowly, reducing with continued ozone exposure. At the end of the ozone therapeutic treatment, joints exposed to 3% and 5% ozone showed detumescence of about 60% and 80%, respectively.

Histopathological configuration of RA-SFc-induced arthritic joint erosion

The infused joints showed erosion of cartilage and bone (see Fig. 3a for the most extreme case), in contrast to the noninvolved contralateral knee joints (Fig. 4C).

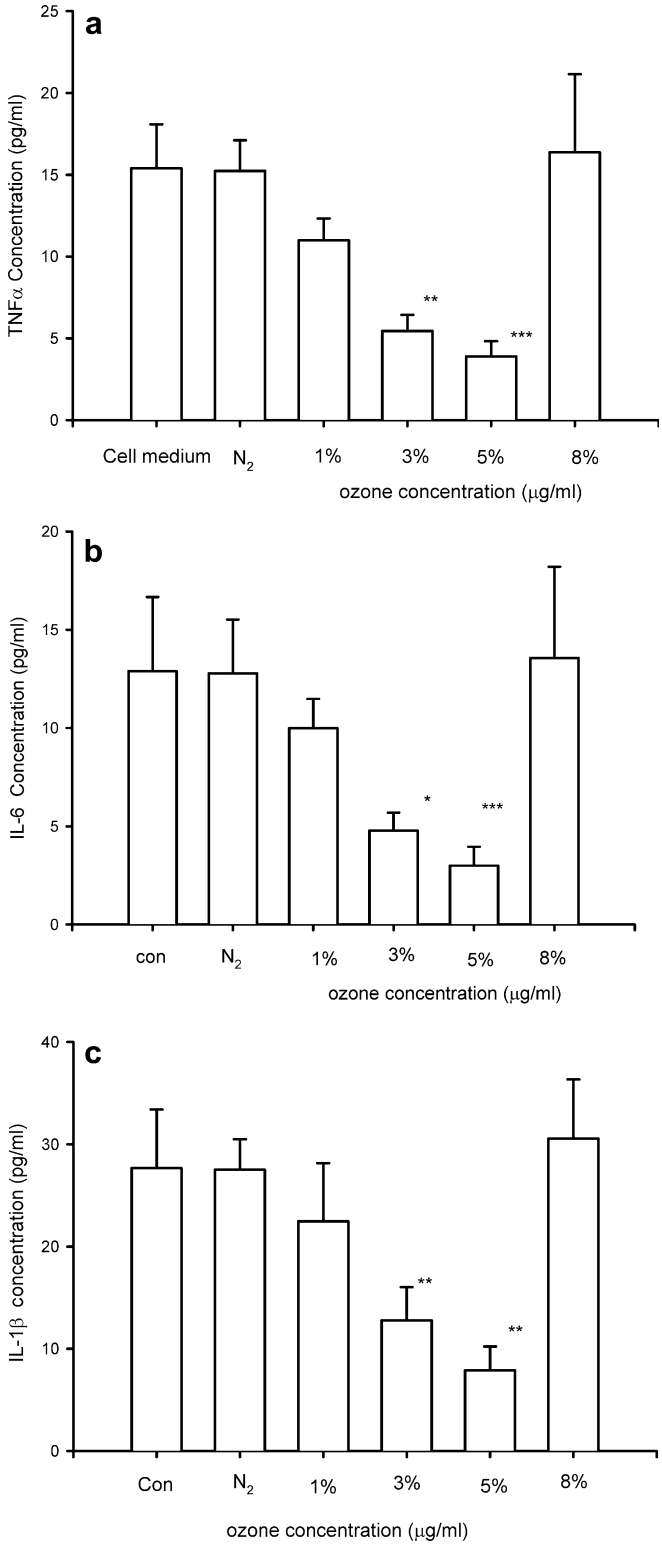

Fig. 2a–c Effect of ozone on RA-SFc cytokine expression. Exposure to 1% ozone and N_2 had no significant influence, but the production of TNF-α (**a**), IL-6 (**b**), and IL-1β (**c**) in RA-SFc cells was suppressed by 3% and 5% ozone. Data show means ± SE. $*P < 0.05$, $**P < 0.01$ vs culture medium

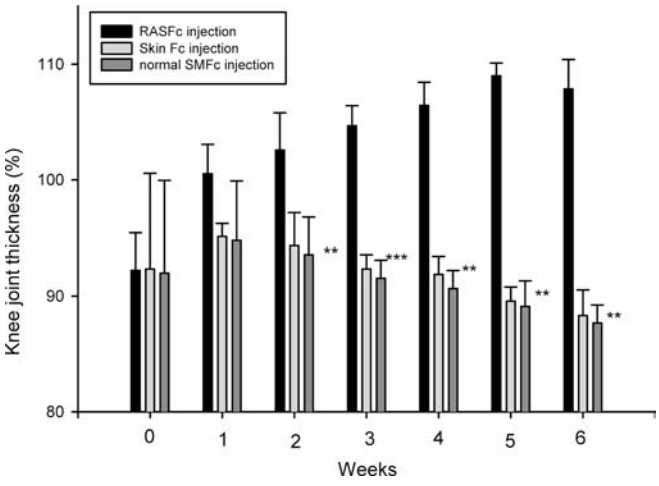

Fig. 3 Course of joint swelling over 6 weeks, showed as percentage changes in diameter of left knee (mean ± SE) compared to pre-ejection levels. Knees infused with RA-SFc kept swelling for at least 6 weeks. Control fibroblast injection had no influence. Injection of RA-SFc and control fibroblast cells became statistically significant from the 2nd week. $**P < 0.01$, $***P < 0.005$

Infusion of skin or normal synovial fibroblasts did not result in any histological damage (Table 2).

Cytokine mRNA expression after poured ozone

Total cellular RNA and cytokine were extracted from synoviocytes of RA-SFc-treated SCID mice after ozone treatment. To determine the ameliorative effect on cytokine by ozone poured to RA-SFc, the IL-1β, TNF-α, and IL-6 concentrations in conditioned medium were measured by ELISA (Fig. 5). Then the mRNA levels of these three cytokines in ozone-treated swelling joints were detected by RT-PCR analysis. Their concentrations in conditioned medium treated by 3% and 5% ozone decreased, approaching 50% of the level of synoviocytes in nontreated joints. The results showed a dramatic decrease in mRNA levels of IL-1β, TNF-α, and IL-6 in synovial cells treated with 3% and 5% ozone (Fig. 6).

IL-6, IL-1β, and TNF-α production after ozone treatment as viewed by Western blotting

Ozone treatment to the RA-SFc-infused SCID mice reduced the expression of IL-1β, TNF-α, and IL-6 as shown by Western blotting. After 3% or 5% poured ozone treatment, the IL-1β, TNF-α, and IL-6 from swelling portions of the mouse knee joints were significantly lower (Fig. 7).

Discussion

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by chronic inflammation of the synovial tissue in multiple joints that leads to joint destruction

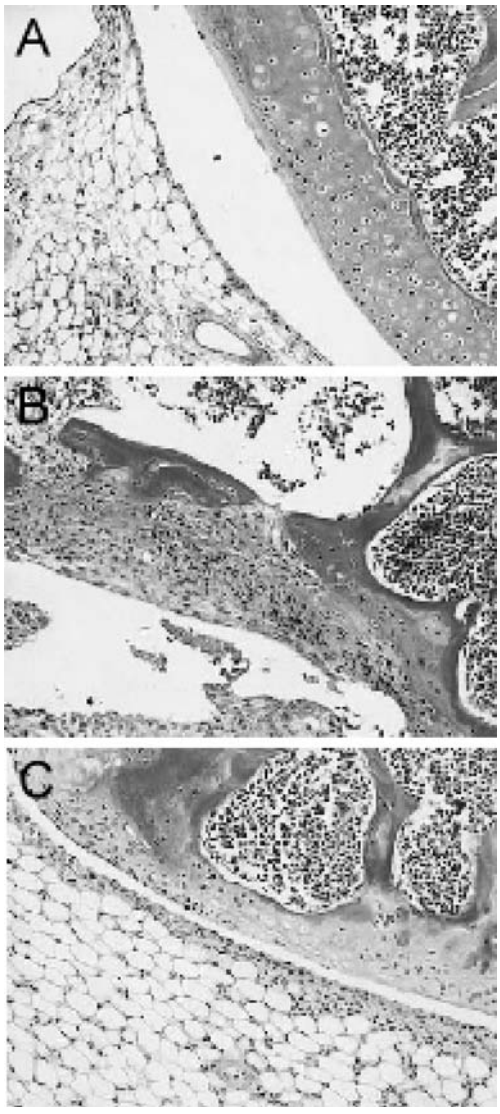

Fig. 4a–c Histopathological examination of knee joint changes 6 weeks after intra-articular injection of 2×10^6 RA-SFc stained with Giemsa and hematoxylin (×100). (**A**) Control mice with no cell injection. No features of synovitis were detected (grade 0). (**B**) Mice infused with RA-SFc developed inflammation with dense infiltration, synovial hyperplasia, and neovascularization of the joint space. Erosion of cartilage and bone in the knee joint was visible within the pannus but generally outside the area of destruction (grade 4). (**C**) Mice infused with RA-SFc and poured with 5% ozone showed only limited synovial lining cell hyperplasia (grade 2)

[18]. Rheumatoid arthritic synovitis is characterized by cell interaction between bone marrow-derived cells such as monocytes, T and B cells, and resident mesenchymal cells, namely synoviocytes. It is clear that monocyte-derived cytokines such as IL-1β and TNF-α interact with synoviocytes, leading to the production of other cytokines such as IL-6 and granulocyte-macrophage-CSF [19].

The cytokines IL-1β, IL-6, and TNF-α have been determined in synovial fluid from RA patients [20]. Cytokine expression and regulation in rheumatoid synovial cells has led to the hypothesis that TNF-α and IL-1β regulate the production of other proinflammatory cytokines such as IL-6. Therefore, these cytokines could be potential targets for ozone therapy.

The proinflammatory cytokines IL-1β and TNF-α are authentic products of monocyte/macrophage, allowing these cells to contribute to synovial inflammation and produce IL-6, which is mainly produced by synovial macrophages and fibroblasts. The detection of high levels of IL-6 mRNA and protein agrees with previous observations in passaged SFc/synoviocytes. The production of these cytokines was significantly lower in repeated-passage RA-SFc, which reflected either loss of macrophage and/or in vitro alteration of SFc due to withdrawal of cytokine stimulation [16].

Several studies have described different types of fibroblast populations; separation protocols and cultured conditions seem to achieve different functional changes [17]. To characterize and describe the phenotype of the fibroblasts obtained by the separation protocol and used in this study, fibroblasts were defined by the expression pattern of several surface markers detected by flow cytometry (Table 1). The detection of CD11b and CD68 at the surface of fibroblasts distinguished fibroblasts from macrophages. In this study, we found a high percentage of RA-SFc positive for fibroblast markers, CD90, and contamination of $<2\%$ of other monocytes. By negative isolation from primary culture and repeated-passage culture, CD14 indicated a limited contribution of contaminating cells to cytokine expression.

Medically, ozone at higher concentrations (3% and 5% ozone in oxygen) exhibits the effect of allaying inflammation by oxidative destruction [21]. The present study demonstrates that the proinflammatory cytokines

Table 2 Histological scores for RA-SFc injection and ozone treatment in SCID mouse knee joints. *SFc* synovial fibroblasts, i.a. intra-arterial injection

Group	Histology scores of mice at day 42						
	Therapy	0	1	2	3	4	Mean ± SE
Knee joint i.a. RA-SFc	No treatment	–	–	–	2	6	3.75 ± 0.1637
Knee joint i.a. RA-SFc	Treated with 3% ozone	2	5	1	–	–	0.875 ± 0.2266
Knee joint i.a. RA−SFc	Treated with 5% ozone	4	2	2	–	–	0.75 ± 0.3134
Knee joint i.a. skin fibroblast	No treatment	5	3	–	–	–	0.375 ± 0.183
Knee joint i.a. normal SFc	No treatment	6	2	–	–	–	0.25 ± 0.163
None joint i.a.	No treatment	8	–	–	–	–	0 ± 0

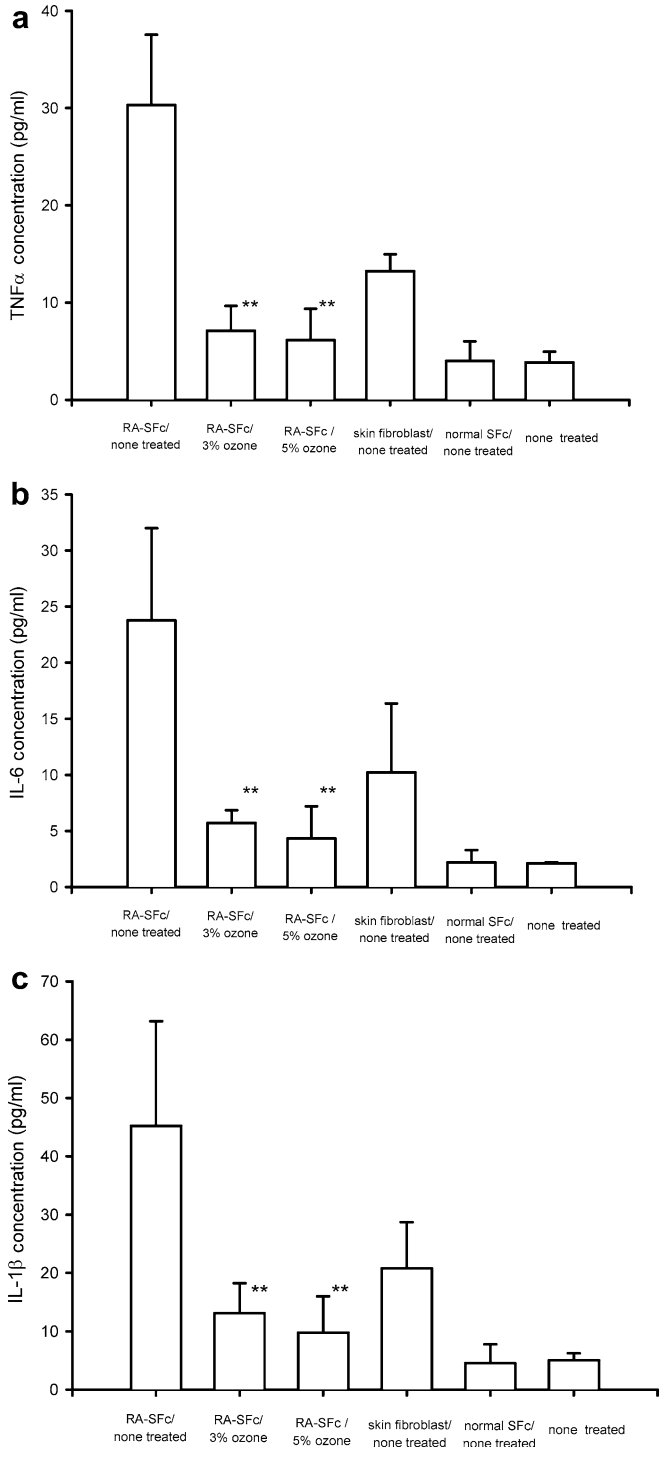

Fig. 5a–c Effect of cytokine secretion by ozone treatment on RA-SFc or control cell injection. RA-SFc or control cells caused swelling in the left knee joints of SCID mice. The production of TNF-α (**a**), IL-6 (**b**), and IL-1β (**c**) was suppressed in the treated mice by ozone at 3% and 5% concentrations. The data show means ± SE. $*P < 0.05$, $**P < 0.01$ vs culture medium

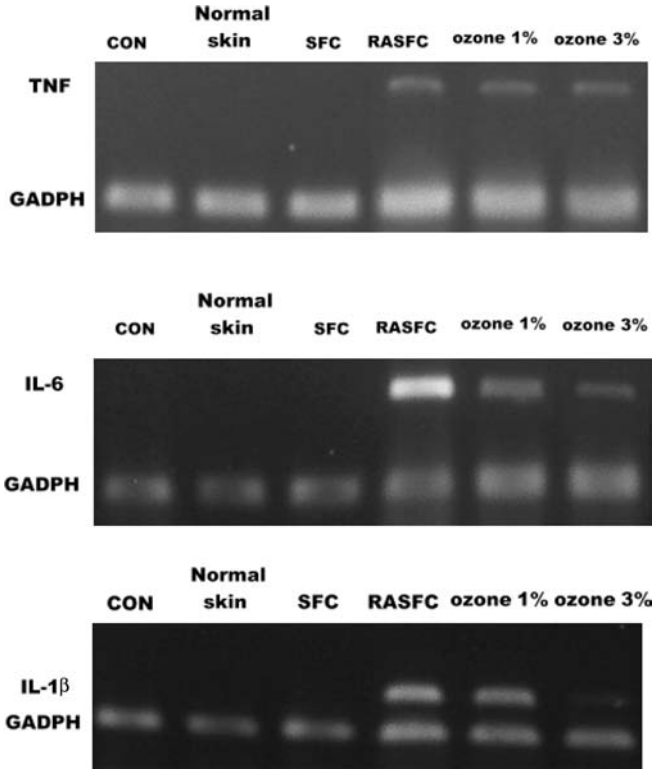

Fig. 6 Reverse transcription polymerase chain reaction analysis of the expression of synovial TNF-α, IL-6, and IL-1β. Ozone at 1% and 3% applied to the swelling portions of SCID mouse knee joints significantly decreased these cytokines' expression

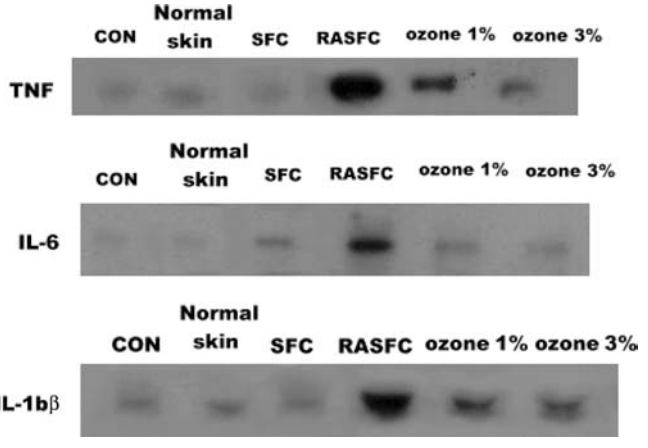

Fig. 7 After pouring of 1% or 3% ozone, the TNF-α, IL-6, and IL-1β protein levels from swelling portions of RA-SFc-infused SCID mouse knees were significantly decreased as seen with Western blotting

IL-1β, IL-6, and TNF-α in cultured RA-SFc were significantly decreased. As a result, we suggest that ozone is able to induce the specific immunological interaction of synoviocytes to retard the RA-SFc-sustained transformation to hyperplasia.

After ozone exposure, the viability of RA-SFc was found to be not significantly different between ozone concentrations of 3% and 5%. In contrast to the exposure of ozone or N_2 on RA-SFc, there was no morphological difference. However, microscopy showed that cells exposed to 8% ozone expanded to bursting due

to physical pressure from the excessive ozone entering the cells. We speculate that cytokine structures were not destroyed by the permeation of 8% ozone. Therefore, the antibodies could be recognized with an ELISA kit and the cytokines could be detected. We suggest that ozone therapy be kept below the concentration of 8%.

Rheumatoid arthritic SFc can induce chronic arthritis in the human/murine SCID model [22]. This is consistent with the view that RA-SFc, which play a decisive role in the joint destruction of arthritis, are generally mediated by alterations in cytokine synthesis and secretion, matrix synthesis, and cell adhesion molecules [17]. The initial reaction was general from intra-articularly injected RA-SFc or control fibroblasts. While swelling associated with the injection of fibroblasts decreased, the swelling of RA-SFc-injected joints kept increasing. After pouring 3% and 5% ozone into the swelling areas for at least 3 weeks, the swollen joints were predominantly restored. This efficacy was greater at the ozone concentration of 5% than at 3%. We speculate that the further decrease in cytokine synthesis and secretion was due to the pouring of ozone into the inflamed and infiltrated area via oxidation and eliminating the hyperplastic synovial fibroblasts.

In the results from this SCID mice model, the arthritic process was observed only at injected knee joints, suggesting that post-transcriptional or post-translational regulation may be associated with mRNA and protein expression [23]. Such regulation (at the level of mRNA stability, translational efficiency, stabile production of protein, or other post-translational modifications) has been demonstrated for IL-1β, IL-6, and TNF-α.

Medical ozone is an effective and extensive therapy without uncomfortable side effects or cytotoxicity [24]. This conclusion is supported by recent evidence of the improved efficacy of combination therapies in experimental models of arthritis and in human RA [21, 24, 25]. We recommend study on other cytokines secreted in vivo such as IL-2, IL-4, IL-10, and IL-13 to learn more about the effect of ozone on swelling induced by RA-SFc. Furthermore, we shall continue studying the interactive mechanism between ozone and RA-SFc.

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