Inhibitory effects of interleukin–1 receptor antagonist transfection by sonoporation on synovial cell inflammatory reactions

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Abstract

Administration of an exogenous IL–1 receptor antagonist (IL–1ra) is effective for reducing inflammatory reactions mediated by IL–1. In the present study, I transfected a plasmid containing IL–1ra into synovial cells using sonoporation and investigated the anti-inflammatory effects on inflammatory reactions induced by lipopolysaccharide (LPS) in vitro. IL–1ra–transfected synovial cells produced extracellular IL–1ra, with a remarkable increase seen when the cells were cultured with LPS for 48 hr. Interestingly, though treatment with LPS enhanced the release of IL–1β in the culture supernatant of IL–1ra–transfected cells, a significant decrease of PGE2 release was observed when the cells were cultured with LPS for 60 hr. These results indicate that IL–1ra has a valuable role in regulating inflammatory reactions by synovial cells and suggest that administration of IL–1ra by sonoporation is useful for controlling inflammation due to arthritis.

Key words: Gene therapy/Sonoporation/Synovial cell/IL–1/IL–1ra

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory reaction of the joints and about 30–50 % of patients with RA experience symptoms in the temporomandibular joint (TMJ). The equilibrium among pro-inflammatory cytokines, including interleukin–1 (IL–1) and tumor necrosis factor alpha (TNF–α), as well as their inhibitors are disrupted in the localized regions of RA and TMJ arthritis, and the levels of pro-inflammatory cytokines, especially IL–1β, are dramatically increased. Pro-inflammatory cytokines are produced by
monocytes, macrophages, and synovial cells, which subsequently induce prostaglandin E$_{2}$ and collagenase$^{3,4}$. Thereafter, cytokines and pro-inflammatory mediators accumulate in synovial fluid, resulting in destruction of cartilage and bone$^{5}$.

It is known that TMJ arthritis is caused by different pathogenic factors including mechanical problems, such as trauma, stress, and posterior disk attachment, which induce the production of inflammatory cytokines, with IL–1$\beta$, IL–6, and TNF–$\alpha$ especially noted to play important roles in TMJ arthritis$^{9,10}$. Moreover, an imbalance between pro-inflammatory cytokines and cytokine antagonists in RA has been reported$^{10}$. We also found an imbalance between IL–1$\beta$ and IL–1ra in synovial fluid samples from patients with TMJ arthritis$^{10}$. These results indicate that restoration of the balance between IL–1$\beta$ and IL–1ra in RA and TMJ arthritis via the administration of IL–1ra protein or gene therapy with IL–1ra cDNA would be effective for treating these patients.

In general, gene therapy using a viral vector is used to treat various conditions, though few techniques have been reported for experimental TMJ arthritis$^{12,13}$, because of adverse reactions, such as immune response$^{16}$ and insertional mutagenesis$^{15}$. On the other hand, it has been reported that non–viral methods, including electroporation, microinjection, and lipofection, are effective for the treatment of TMJ disorders$^{10}$. We previously showed that gene transfer of an IL–1ra plasmid by electroporation in vivo inhibited inflammatory reactions in adjuvant–induced arthritis. However, these methods have several problems, and additional studies are necessary for development of an effective and safe therapeutic regimen. Recently, ultrasonication techniques (sonoporation) have been used in vivo and in vitro to load anti–cancer agents, anti–inflammatory cytokines, and plasmid DNA into cells, as well as for other efficient non–viral methods$^{15,18}$. In the present study, we attempted to establish a new gene therapy using sonoporation for TMJ arthritis and examined the effects of transfection with an IL–1ra plasmid into synovial cells using sonoporation in vitro.

**Materials and Methods**

I  Cell line and reagents

HIG–82, a rabbit synovial cell line, was maintained in Dulbecco’s modified Eagle’s/F–12 medium (DMEM/F–12) (GIBCO BRL, Grand Island, NY, USA) with 10 % heat–inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml) at 37 °C in 5 % CO$_{2}$. SonoVue$^{TM}$ (Bracco, Milan, Italy), a microbubble–based ultrasound contrast agent that consists of 59 mg of sulfur hexafluoride gas (SF$_{6}$) and 25 mg of a freeze–dried white powder in a vial, was also used. Five milliliters of sterile saline was added to the vial and then it was shaken for several sec, which led to the generation of phospholipid–stabilized microbubbles filled with sulfur hexafluoride with a diameter of less than 8 $\mu$m (mean, 2.5 $\mu$m).

II  Plasmid preparations

Human IL–1ra complementary DNA (cDNA) was obtained by reverse transcription–
polymerase chain reaction (RT–PCR) from phorbol 12–myristate 13–acetate–differentiated U937 cells. In brief, pCI-neo–IL–1ra was constructed by inserting the amplified cDNA into the multiple cloning region of a pCI-neo mammalian expression vector (pCI-neo–empty) (Promega, Madison, WI, USA). pVIVO–1–GFP/LacZ was purchased from InvivoGen (San Diego, CA, USA). Competent Escherichia coli DH5α was transformed with these plasmids. The plasmids were prepared with a Qiagen Maxi kit (Qiagen, Chatsworth, CA, USA) following the manufacturer's protocol. Agarose gel electrophoresis was performed to verify the identity and purity of the plasmid DNA.

III In vitro sonoporation

Separate transfection of the pCI-neo–IL–1ra and pVIVO–1–GFP/LacZ genes was performed by sonoporation using SonoVue™. HIG–82 cells were trypsinized, washed twice in phosphate–buffered saline (PBS; pH 7.2), and resuspended at 1.0 × 10⁶ cells/600 µl of FBS–free DMEM/F12 medium in a 48–well plate. pCI-neo–IL–1ra or pVIVO–1–GFP/LacZ was added to a SonoVue™ solution, then mixed and kept for 30 sec, after which pCI-neo–IL–1ra and SonoVue™ solution (600 µl; 1:50) were added to the cell suspension, for a final concentration of pCI-neo–IL–1ra of 332 µg/ml. After the mixed solution was added to the wells, the cells were exposed to sonoporation for 20 sec at room temperature using an ultrasonication transducer (Sonitron 2000, Rich Mar Inc, Inola, OK, USA) at 1 MHz, with an output intensity of 2.0 W/cm² and a 10 % duty cycle for the transfection of pCI-neo–IL–1ra or pVIVO–1–GFP/LacZ. Finally, the head of the transducer was directly immersed into the cell suspension, and the ultrasonication probe and well plates were firmly fixed to avoid dislocation during sonoporation exposure.

IV X–gal staining

HIG–82 cells were fixed with 4 % paraformaldehyde and stained with X–gal (5–bromo–4–chloro–3–indoly–β–d–galactosidase) (WAKO, Osaka, Japan) in PBS containing 5 mM K₃Fe (CN)₆, 5 mM K₄Fe (CN)₆, and MgCl₂. The reactions were carried out at 37 °C for 24 hr.

V RT–PCR assay

Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. To reduce DNA contamination, the RNA samples were treated with a Turbo DNase kit (Ambion, Austin, TX, USA) for 30 minutes at 37 °C, and first–standard DNA was synthesized from DNase–treated total RNA with Oligo–dT primer and Superscript II (Invitrogen, Carlsbad, CA, USA). Reverse transcribed total RNA was subjected to PCR amplification with AmpliTaq GOLD DNA polymerase (Applied Biosystems, Foster, CA, USA) using specific PCR primers. Oligonucleotide primers used in the PCR amplification are indicated in Table 1. RT–PCR was performed under the following conditions: 1 cycle at 95 °C for 9–12 minutes, 94–95 °C for 30–40 sec, and 51–63 °C for 30–40 sec, for 35–40 cycles. The amplified DNA fragments were subjected to 8 % polyacrylamide gel
Table 1  Primers and RT-PCR conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Primer (F=forward; R=reverse) (5’-3’)</th>
<th>Annealing temperature (°C)</th>
<th>Cycle</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1ra</td>
<td>X52015</td>
<td>F GCAAGATGCAAGCGCTTCAAGAATCTGGG R GCTGGTCAGCTTCATCGCTGTGCA</td>
<td>63</td>
<td>35</td>
<td>314</td>
</tr>
<tr>
<td>GAPDH</td>
<td>M32599</td>
<td>F ACCACAGTCCATGCCATCAC R TCCACCCCATGGCTGTGTA</td>
<td>51</td>
<td>40</td>
<td>452</td>
</tr>
</tbody>
</table>

electrophoresis and visualized with GelRed™ (Biotium, Hayward, CA, USA).

VI  Immunocytochemical staining

HIG-82 cells were suspended on LAB-TEK™ chamber slides (NUNC, Roskilde, Denmark) and incubated in FBS-free DMEM/F-12 medium, followed by 48 hr of incubation in medium containing 10% FBS. The samples were then fixed in 4% paraformaldehyde for 1 hour and any endogenous peroxidase was blocked with 3% H2O2 in methanol. An immunocytochemical reaction was then performed using an Elite ABC kit (Vector Laboratory, Burlingame, CA, USA). Briefly, the cells were incubated for 30 minutes with normal rabbit serum at room temperature and incubated overnight with 1 μg/ml of goat anti-human IL-1ra (R&D Systems Inc, Minneapolis, MN, USA) at 4°C. The cells were incubated for 30 minutes in 5 μg/ml of biotinylated rabbit anti-goat IgG, then incubated for 30 minutes in avidin–biotin–peroxidase complex. Diaminobenzidine (KPL Laboratory, Gaithersburg, MD, USA) was used as a chromogen. Counter-staining was performed using hematoxylin.

VII  Measurement of IL-1β, IL-1ra, and PGE2

The cells were treated with 25 μg/ml of lipopolysaccharide (LPS), which was purified from Aggregatibacter actinomycetemcomitans ATCC 29523 as described previously19. The amounts of IL-1β, IL-1ra, and PGE2 in the culture supernatants of the transfected cells were measured using enzyme–linked immunosorbent assay (ELISA) kits (R&D Systems Inc, Minneapolis, MN, USA), according to the manufacturer’s instructions.

VIII  Statistical analysis

Data are expressed as the mean ± SD. The significance of differences between comparable groups was determined using Student’s t-test.

Results

I  Optimal conditions for sonoporation

The delivery of pVIVO1–GFP/LacZ into HIG-82 cells was performed by sonoporation with SonoVue™. The cells were cultured with various concentrations of SonoVue™ at 2.0 W/cm².
and a 10% duty cycle for the indication of pVIVO1–GFP/LacZ. A large number of β–galactosidase positive cells were detected by the addition of 250 μg/ml of SonoVue™ (Fig. 1a). Next, the cells were cultured at various intensities with a concentration of 250 μg/ml of SonoVue™ and a 10% duty cycle, and the intensity of positive cells was dose-dependently increased (Fig. 1b). It was found that the optimal conditions for sonoporation into HIG–82 cells was 250 μg/ml of SonoVue™ at 2.0 W/cm² and a 10% duty cycle (Fig. 2).

II Expression of IL–1ra

I investigated the expression level of IL–1ra mRNA in pCI-neo–IL–1ra–transfected HIG–82 cells by RT–PCR. The results revealed that delivery of pCI-neo–IL–1ra into HIG–82 cells enhanced IL–1ra mRNA expression for at least 24 hr after sonoporation (Fig. 3). In addition, I confirmed IL–1ra protein expression by immunocytochemistry and ELISA. HIG–82 cells transfected with pCI-neo–IL–1ra expressed IL–1ra for 24 hr after transfection, as shown by

![Fig. 2 β–galactosidase staining. HIG–82 cells were transfected with pVIVO1–GFP/LacZ using sonoporation at an output intensity of 2.0 W/cm², frequency of 1 MHz, and a 10% duty cycle for 20 sec. β–galactosidase–positive cells were observed by microscopy. (a), (b), (c) ×40; (d), (e), (f) ×100. (a), (d) untreated HIG–82 cells. (b), (e) HIG–82 cells transfected with pVIVO1–GFP/LacZ by sonoporation in the absence of SonoVue™. (c), (f) HIG–82 cells transfected with pVIVO1–GFP/LacZ by sonoporation in the presence of SonoVue™. Arrowheads indicate β–galactosidase–positive cells.]

![Fig. 1 In vitro gene transfer of pVIVO1–GFP/LacZ into HIG–82 cells. Transfection of pVIVO1–GFP/LacZ was performed using sonoporation in the presence of various concentrations of SonoVue™ at 1 MHz with a 10% duty cycle of 20 sec. The figures show the in vitro transfection ratios of pVIVO1–GFP/LacZ by sonoporation (a. SonoVue™ concentration. b. Intensity). The number of β–galactosidase positive cells was counted and the results are expressed as the mean ± S.D. (n=3). *P<0.05. **P<0.01.]

Fig. 3 Detection of IL-1α mRNA in pCI-neo-IL-1α transfected cells. Expression of GAPDH (452 bp) was detected in untreated HIG-82 cells, pCI-neo-IL-1α and pCI-neo-empty transfected cells. Expression of IL-1α mRNA (314 bp) was detected only in pCI-neo-IL-1α transfected cells by RT-PCR.

Fig. 4 Detection of IL-1α protein in pCI-neo-IL-1α transfected cells. Detection of IL-1α protein by immunocytochemistry. (a), (d) untreated HIG-82 cells. (b), (e) cells transfected with pCI-neo-IL-1α. (c), (f) cells transfected with pCI-neo-empty. Nuclei were stained with hematoxylin, as described in Materials and Methods. Normal goat IgG was used as the primary antibody for the negative control in (d), (e), and (f). Arrowheads indicate IL-1α-positive cells.

immunocytochemical staining (Fig. 4). The release of IL-1α into the culture supernatant was increased in a time-dependent manner after sonoporation with pCI-neo–IL-1α (Fig. 5a).

III Expression of IL-1α and IL-1β following LPS stimulation

Next, we investigated the release of IL-1α and IL-1β from the transfected cells by ELISA. The release of IL-1α from pCI-neo–IL-1α–transfected HIG-82 cells was sequentially
increased for 98 hr in LPS–stimulated and non–stimulated cells (Fig. 5a). On the other hand, the release of IL–1β was increased for 48 hr only in LPS–stimulated cells (Fig. 5b).

IV PGE₂ production

I also examined the effect of pCI–neo–IL–1ra transfection on the production of PGE₂ by HIG–82 cells. Production peaked at 58.5±0.8 ng/ml at 48 hr after LPS stimulation and remained enhanced until 60 hr. In the cells transfected with pCI–neo–empty, the production of PGE₂ was 43.8±3.9 ng/ml at 48 hr after LPS stimulation. In contrast, in cells transfected with pCI–neo–IL–1ra, PGE₂ production was dramatically decreased at 48 hr (19.3±0.5 ng/ml) and the decrease was maintained at 60 hr (Fig. 6).

Discussion

In the present study, a plasmid containing IL–1ra was transfected into HIG–82 cells using sonoporation. Since sonoporation is a non–viral technique and minimally invasive, therapy with this method is expected to decrease the burden of patients. However, it is also known that sonoporation has a low level of transfection efficiency. Therefore, we used SonoVue™, an echo contrast agent similar to Optison, Levovist, and Sonazoid, to enhance the efficiency of gene transfer (Fig. 2).

In our previous study, we used Optison for transfer of a cdtB–expression plasmid into Ca9–22 cells, a human gingival squamous carcinoma cell line, by sonoporation. Optison is an albumin–shelled US contrast

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**Fig. 5** Effects of IL–1β and IL–1ra release by transfected cells following stimulation with LPS. Cells were treated with or without 25 μg/ml of LPS for the indicated time periods. IL–1β (a) and IL–1ra (b) concentrations in the supernatant were determined using ELISA, as described in Materials and Methods. The results are expressed as the mean ± S.D.

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**Fig. 6** PGE₂ production following LPS stimulation in transfected cells. Cells were treated with or without 25 μg/ml of LPS for the indicated time periods. PGE₂ concentrations in the supernatants following LPS stimulation were determined using ELISA, as described in Materials and Methods. The results are expressed as the mean ± S.D.
agent sized from 2–4.5 μm in diameter and filled with octaperfluoropropane\textsuperscript{10}. In contrast, SonoVue\textsuperscript{TM} is a lipid–shelled US contrast agent filled with sulfur hexafluoride gas with an average diameter of 2.5–6.0 μm, though 90 \% are smaller than 6.0 μm\textsuperscript{10}. We found that the efficiency of gene transfection into Ca9–22 cells with Optison and SonoVue\textsuperscript{TM} was nearly the same (data not shown).

In the local region of TMJ arthritis, large amounts of pro-inflammatory cytokines and mediators, such as IL–1, IL–6, TNF–α, and PGE\textsubscript{2}, are produced in synovial fluid\textsuperscript{4,9}. Notably, IL–1 produces inflammatory cytokines including TNF–α, IL–6, IL–8, PGE\textsubscript{2}, and metalloproteinases, which induce destruction of cartilage and bone by activating chondrocytes, osteoblasts, osteoclast precursors, synoviocytes, and fibroblasts. LPS is known to bind to Toll-like receptor–4, resulting in activation of the MyD88 signal pathway, which subsequently promotes the translocation of NF–κB from cytosol to nuclei, producing various pro-inflammatory cytokines\textsuperscript{21}. Further, IL–1β promotes the production of PGE\textsubscript{2} by enhancing cyclooxygenase–2 (COX–2) expression and increases the expression of the IL–1 receptor (IL–1R) through the autocrine action of PGE\textsubscript{2}\textsuperscript{20}.

It has been reported that IL–1 knock–out mice have a decrease in the onset of arthritis by about half, whereas IL–1ra knock–out mice spontaneously develop arthritis\textsuperscript{31,30}. In addition, Fernandes et al. directly injected an IL–1ra plasmid with a lipid component into rabbit knee joints as a non–viral in vivo method and found it promising for treatment of osteoarthritis\textsuperscript{30}. In the present study, LPS stimulation remarkably induced IL–1β in synovial cells, while the overexpression of IL–1ra dramatically decreased the production of PGE2, even when HIG–82 cells were stimulated with LPS.

In summary, I found that in vitro sonoporation–mediated transfer of an IL–1ra plasmid into synovial cells had a remarkable effect on the production of PGE\textsubscript{2} as an inflammatory mediator. These results suggest that application of gene therapy by sonoporation with IL–1ra is a promising method for treatment of inflammation in temporomandibular joint arthritis.

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**References**


滑膜細胞に対するIL-1ra超音波遺伝子導入の抗炎症作用

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抄録

関節症患者の関節滑液中ではInterleukin-1β（IL-1β）発現が増強し、対称的にその抑制剤であるIL-1 receptor antagonist（IL-1ra）が減少していることが報告されているが、組織内にIL-1raを増加させることができれば、この炎症性変化の制御に有効だと考えられる。そこで今回、関節炎を制御する安全で非侵襲的な方法を確立するために、IL-1ra遺伝子を滑膜細胞に超音波遺伝子導入し、抗炎症効果が発現するかを検討した。

ウサギ膝関節由来滑膜細胞株HIG-82を5％CO₂、37℃にて培養し、実験に供した。超音波振動装置（Sonitron 2000™）を用いて、IL-1ra cDNAをHIG-82に導入した。遺伝子導入の際に、導入効率増強を目的にマイクロパブル（Sonovue™）を併用した。遺伝子導入細胞中のIL-1ramRNAの発現をRT-PCRで、またIL-1raタンパクの発現を免疫染色にて確認した。また、HIG-82にIL-1ra遺伝子を導入後、細胞をLPSで刺激し、IL-1β、IL-1raとPGE₂の産生をELISAにて検討した。

予備実験としてHIG-82に対してIL-1ra遺伝子を超音波遺伝子導入する際の超音波ならびにSonovue™の濃度の至適条件の検索を行ったが、IL-1ra遺伝子の導入効率が最も優れていたのは超音波強度；2.0 W/cm²、周波数；1 MHz、Duty比；10％、照射時間30秒条件であった。また、Sonovue™は細胞懸濁液中で5％の濃度で最も遺伝子の導入効率が優れていた。HIG-82にIL-1raを超音波遺伝子導入することにより、RT-PCRにてIL-1ra mRNAの発現が確認され、免疫染色においてはIL-1ra陽性細胞が確認された。また、遺伝子導入細胞にLPS刺激を行うと、炎症反応が惹起され、コントロール群、IL-1ra導入群ともにIL-1βの発現が誘導された。両群間に有意な差は認められなかった。しかし、コントロール群ではIL-1β発現に続いて緩時にPGE₂の産生が亢進したに対し、IL-1ra遺伝子導入群ではPGE₂発現は有意に抑制されていた。

この研究で、in vitroにおける滑膜細胞へのIL-1raの遺伝子導入による抗炎症効果が確認され、関節炎の炎症性変化に対する遺伝子治療の可能性が示唆された。

キーワード: 遺伝子治療/超音波遺伝子導入/滑膜細胞/IL-1/IL-1ra