Acetylcholine Suppresses Release of Interleukin-6 in Fibroblast-Like Synoviocytes in Rheumatoid Arthritis

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Received Date: April 15, 2017, Accepted Date: June 28, 2017, Published Date: July 05, 2017.
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Abstract

Objective: To investigate to which degree acetylcholine (ACh) can modulate Tumor necrosis factor (TNF)-induced production of interleukin (IL)-6 and/or other cytokines in primary human fibroblast-like synoviocytes (FLS).

Design: Primary human FLS from a patient with Rheumatoid arthritis (RA) were cultivated and used in experiments where TNF and ACh were added to the cell medium in various concentrations alone or in combinations. The cell medium concentration of IL-6 was measured after four and 24 hours using a commercially available bead array multiplex assay.

Results: IL-6 release increased after four and after 24 hours of incubation with one or 10 ng/mL TNF. The IL-6 release was decreased after incubation with 10 nmol/L ACh for 24 hours. After four hours of incubation co-stimulation with TNF 10 ng/mL and ACh 10 nmol/L produced a decrease of IL-6 release with 65% compared to the IL-6 concentration with TNF 10 ng/mL stimulation.

Conclusions: ACh decreases the native as well as the TNF-induced IL-6 release from FLS in RA. The results thereby support a possible mechanism by which the autonomic nerve system may modulate the local immune system.

Key words: Acetylcholine; Rheumatoid arthritis; Fibroblast; Tumor necrosis factor; IL-6

Introduction

The central role of cytokines in the pathophysiology of rheumatoid arthritis (RA) has been thoroughly recognized. RA commonly involves the temporomandibular joint (TMJ) resulting in cranio-mandibular pain, jaw functional limitations and risk of anterior open bite. Due to destruction of cartilage and bone tissue in the joint with sometimes debilitating results for the individual [1]. There is a need for advance knowledge in mechanisms behind the RA disease process in the TMJ with the hope of improving future diagnostic methods and treatments.

TNF is considered as one of the most important cytokines in regulating the production of other pro-inflammatory cytokines in RA synovial tissues [1]. TNF also promotes activation of immunocompetent cells like fibroblast-like synoviocytes (FLS). These can modulate the bone tissue metabolism, eg. via the osteoclasts and joint cause destruction [2]. TNF is detectable in 33% of TMJ synovial fluid samples from patients with RA or psoriatic arthritis. Synovial fluid levels of TNF exceed plasma levels indicating local release of TNF in the TMJ [3]. Synovial fluid TNF levels are higher in individuals with TMJ pain than in those without such pain [3]. Also high synovial fluid TNF levels are associated with tenderness to TMJ palpation. Which corresponds to sensitization of synovial tissues and other tissues surrounding the TMJ [4].

TNF modulates IL-6 release and; IL-6 plays a crucial role in the inflammatory process in diseases like RA. For example synovial fluid IL-6 levels in RA patients are elevated and also associated with the degree of joint destruction [5]. IL-6 has been found to be increased in synovial fluid of RA patients not responding to the conventional anti-TNF treatment [6]. Targeting IL-6 in the RA synovium is therefore promising and could improve RA treatment [1,5,7,8].

Acetylcholines (ACh) decrease the production of inflammatory cytokines such as IL-6 and thereby reduce the inflammation via the α7 nicotinic receptor (α7R). Such receptors have been found on FLS surfaces in the human synovium [9]. Therefore Targeting α7R on FLS might improve clinical outcomes in inflammatory arthritis with minimal suppression of the systemic immunity. Indeed the α7R can be a likely therapeutic target for inflammatory diseases such as RA [9,10]. FLS also called synovial fibroblasts are resident mesenchymal cells of synovial joints. FLS in RA exhibits an aggressive, invasive phenotype commonly associated with transformed cells [11,12]. RA FLS can not only grow in an anchorage-independent manner in soft agarose [13]. FLS can be isolated from synovial tissue and grown in culture for prolonged periods of time. These cells seem to serve as a practical tool to further understand the pathogenesis of RA. The aim of the present study was to investigate to which degree ACh can modulate TNF-induced production of IL-6, IL-15 and IL-17 or other cytokines in primary human RA FLS.

Materials and Methods

Primary Human Fibroblast-Like Synoviocytes

FLS from a patient with RA (age 65 years) (FLS-RA; Health Protection Agency Culture Collections, Salisbury, UK) were isolated and characterized the cells. The FLS which were obtained from synovial tissues of the knee were plated in 75 cm2 culture flasks containing 15 ml of synoviocyte growth medium (SGM). Cells were grown in culture for prolonged periods of time. These cells seem to serve as a practical tool to further understand the pathogenesis of RA. The aim of the present study was to investigate to which degree ACh can modulate TNF-induced production of IL-6, IL-15 and IL-17 or other cytokines in primary human RA FLS.

FBS, Invitrogen AB, Stockholm; Sweden), Fetal bovine serum (FBS, Invitrogen AB, Stockholm, Sweden), Phosphate buffered saline (pH 7.2; Nitrogen AB, Stockholm, Sweden) and trypsin (0.25%; Invitrogen AB, Stockholm; Sweden).
Agents

In the experiment, various concentrations of human TNF (Humankine™ Tumor Necrosis Factor human, Sigma-Aldrich, Stockholm; Sweden) and ACh (Acetylcholine chloride; Sigma-Aldrich, Stockholm; Sweden) were used to modulate the release of the investigated cytokines.

Experimental Procedures

The cells were seeded in 6-well culture plates previously prepared with SGM. After reaching 80% confluences, the medium was changed to serum-free SGM. The wells were then allocated to controls (3 wells) or experiments (3-6 wells in each experiment) in which the following media and agents were added to a total volume of 4 ml. The plates were incubated in a humidified incubator with 5% CO₂. Samples (100 µL) of the medium were collected after four and 24 hours and stored in -80°C until assayed. All experiment was repeated to a total of three times to confirm the results.

Experiment 1: Stimulation with TNF or ACh

Medium (serum-free SGM) and cells (control)
1 ng/mL TNF in serum-free SGM
10 ng/mL TNF in serum-free SGM
10 nmol/L ACh in serum-free SGM
100 nmol/L ACh in serum-free SGM

Experiment 2: simultaneous stimulation with TNF and ACh

Medium (serum-free SGM) and cells (control)
1 ng/mL TNF and 10 nmol/L ACh in serum-free SGM.
1 ng/mL TNF and 100 nmol/L ACh in serum-free SGM.
10 ng/mL TNF and 10 nmol/L ACh in serum-free SGM.
10 ng/mL TNF and 100 nmol/L ACh in serum-free SGM

Immuoassay

The concentration of IL-6 in the cell medium was determined using a Bioplex assay (Bio-Rad Laboratories, Hercules, CA, USA). Commercially available bead array multiplex assay kits (Milliplex HSCYT0-60SK, St. Charles, MI, USA) were used. 50 µL of the sample was used in the assay. The detection limits for IL-6 was 0.9 pg/mL. Cross-reactivity between the antibodies or with any of the other analytes in this panel was non-detectable or negligible.

Statistics

Non-parametric statistics were used. For descriptive statistics median 25th and 75th percentile are reported. The IL-6 concentration in the medium was normalized to the IL-6 concentration in the medium of unstimulated cells. Mann-Whitney U-test was performed in STATA 12 SE. A probability level of less than 0.05 was considered as significant.

Results

Stimulation with TNF or Ach Separately

There was a significant increase of IL-6 release after four hours of incubation with one or 10 ng/mL TNF compared to the unstimulated IL-6 release in the control cells ($p = 0.039$ and $p = 0.020$, respectively; Table 2). After 24 h. incubation with one or 10 ng/mL TNF increased the IL-6 release ($p = 0.039$ and $p = 0.020$, respectively; Table 2).

The IL-6 concentration was not significantly changed compared to that of the unstimulated cells when stimulated with ACh 10 nmol/L or ACh 100 nmol/L after four hour or 24 hour or with 10 ng/mL TNF for four hours (Table 2). The IL-6 concentration was undetectable in the medium where the cells had been incubated with 10 nmol/L ACh for 24 hours (Table 1).

Stimulation with TNF and ACh in Combinations

After four hours of incubation, co-stimulation with TNF 10 ng/mL and ACh 10 nmol/L produced a decrease of IL-6 release with 65% compared to the IL-6 concentration with TNF 10 ng/mL stimulation in one of our experiments ($p = 0.049$). After 24 hours there was no decrease in IL-6 concentration in the medium.

Discussion

In this study we investigated a possible anti-inflammatory effect by ACh, which have shown an attenuation of systemic inflammatory response evoked by endotoxin in experimental animals stimulation of nicotinic receptors on splenic macrophages. The current study

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>0.9</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.1</td>
</tr>
<tr>
<td>IL-12</td>
<td>7.4</td>
</tr>
<tr>
<td>IL-15</td>
<td>1.2</td>
</tr>
<tr>
<td>IL-17</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Table 1: Detection limit concentrations of different cytokines in the Luminex (Millipore) assay.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>4 hour incubation Median</th>
<th>25%</th>
<th>75%</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF 1 ng/mL</td>
<td>7.4</td>
<td>3.8</td>
<td>10</td>
<td>0.039</td>
</tr>
<tr>
<td>TNF 10 ng/mL</td>
<td>12</td>
<td>10</td>
<td>16</td>
<td>0.020</td>
</tr>
<tr>
<td>ACh 10 nmol/L</td>
<td>1.8</td>
<td>1.0</td>
<td>2.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>ACh 100 nmol/L</td>
<td>2.8</td>
<td>2.3</td>
<td>3.3</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Table 2: IL-6 levels (relative to unstimulated cell media concentrations) in cell media with RA fibroblast-like synoviocytes incubated for four or 24 hours with tumor necrosis factor (TNF) and acetylcholine (ACh), separately or in combinations. (n.d. = undetectable concentrations; n.s. = not significant).
suggests that ACh decreases TNF-stimulated IL-6 release from RA FLS, i.e. intraarticular cells. Our finding could point to one pathway of how ACh exerts its previously shown anti-inflammatory effects in RA [14].

Stimulation of FLS by ACh has been shown to reduce production of IL-6, which supports our results [9]. In RA, TNF, IL-1β and IL-6 are pivotal cytokines and an effect of ACh on IL-6 production and release from synoviocytes suggests an important role for ACh in local regulation of the inflammatory activity in RA. However, IL-6 can both promote and suppress arthritis by various actions on different cell types, depending on the cell surface expression of its receptor and the receptor attachment protein gp130. On the one hand, IL-6 promotes chemokine expression of T and B-cells that activate osteoclasts for bone resorption. On the other hand, IL-6 has anti-inflammatory actions by not only suppression of production of other cytokines like IL-1, TNF and IL-12 but also by induction of IL-1ra and protease inhibitors in RA [5].

ACh, released by the vagus nerve as a part of the anti-inflammatory cholinergic pathway [14], can reduce the inflammatory activity in RA [14]. This anti-inflammatory action seems to in part be due to ACh modulating the production and release of cytokines such as TNF and IL-6 from immunocompetent cells through the α7 nicotinic acetylcholine receptor subunit [15,16]. Fibroblasts respond to cholinergic stimulation via the α7 nicotinic acetylcholine receptor subunit to potentially inhibit the production of pro inflammatory cytokines in RA patients [16]. RA patients have elevated heart rate variability, a marker of decreased vagus nerve activity. In turn, an impaired vagus nerve activity may promote increased production of the cytokines TNF and IL-6 [17]. Consequently, impaired cholinergic anti-inflammatory modulation of the immune system may be accompanied by an increased incidence or severity of arthritis, including pain and joint tissue destruction [14].

In conclusion the current study suggests that ACh decreases native and TNF-stimulated IL-6 release from RA FLS. This finding could point to one pathway of how ACh exerts its previously shown anti-inflammatory effects, which in turn points to an important role for ACh in local regulation of the inflammatory activity in RA.

Acknowledgements

The study was financially supported by an unrestricted grant from Swedish Consultation AB, Stockholm, Sweden.

Conflict of Interest

No perceived or actual conflicts of interest exist.

References


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Received Date: April 15, 2017, Accepted Date: June 28, 2017, Published Date: July 05, 2017.