Original Research

Therapeutic Effect of Moxibustion on Collagen-induced Arthritis in Mice by Induction of Regulatory T Cells

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Abstract

[Objective] Moxibustion has been reported to suppress murine collagen-induced arthritis (CIA); however, the responsible mechanisms for this action are unclear. Regulatory T cells are known to suppress the immune response and to be involved in the suppression of CIA. Therefore, we investigated the effects of moxibustion on regulatory T cell induction in a murine CIA model.

[Methods] DBA/1 J mice were immunized with bovine type II collagen (CII) twice. Direct moxibustion using 1 mg of cone-shaped moxas was applied to the acupuncture point GV 4 (Mingmen) three times a week from day 21 to day 35 post-immunization. On day 35 post-immunization, we collected blood, spleens and inguinal lymph nodes and measured the populations of CD 4⁺CD 25⁺ regulatory T cells using flow cytometry and the serum levels of Transforming Growth Factor-β1 (TGF-β1) using ELISA.

[Results] The levels of CD 4⁺CD 25⁺ regulatory T cells in peripheral blood, spleens and inguinal lymph nodes and the concentrations of TGF-β1 in the sera were observed to significantly increase in moxibustion-treated CIA mice.

[Conclusions] TGF-β is known to promote differentiation to regulatory T cells if it affects naive T cells by itself. These results suggest that moxibustion suppresses CIA through the induction of regulatory T cells.

Key words: moxibustion, collagen-induced arthritis, regulatory T cell, TGF-β

I. Introduction

Direct moxibustion is a technique in traditional oriental medicine that applies heat stimuli to the skin surface by burning moxa corn thereby inducing a biological defense response. One of the efficacies of moxibustion is to regulate immune disorders, and this technique has been clinically used to treat diseases such as Hashimoto's thyroiditis, systemic lupus erythematosus and rheumatoid arthritis (RA)¹⁻³. Of all the immune-related diseases, we focused on rheumatoid arthritis, a high prevalence disease. The type II collagen-induced arthritis (CIA) model, an established animal model of human rheumatoid arthritis, has been experimentally utilized for efficacy evaluation of acupuncture and moxibustion⁴⁻⁶. Fang et al. reported that direct moxibustion applied to the acupuncture point GV 4 (Mingmen) suppresses the incidence and severity of murine CIA⁶. Although it has been suggested that moxibustion ameliorates pathological changes in the joints and decreases the levels of anti-type II collagen antibodies, the underlying mechanisms of these beneficial effects remain unclear.

On the other hand, CD 4⁺ T cells expressing CD 25⁺ regulatory T cells have been identified as T cells that suppress the immune response and play an important role in peripheral immune tolerance⁷, and have been called "CD 4⁺CD 25⁺ regulatory T cells". Failures in the function of regulatory T cells can be re-
sponsible for the development of various autoimmune diseases. In CIA, depletion of the regulatory T cells by antibody treatment leads to increased disease severity\(^8\,^9\), however, transferring pre-activated CD\(^4^+\) CD\(^25^+\) T cells from healthy mice ameliorates disease severity\(^8\,^9\). These studies suggest that regulatory T cells play a crucial role in regulating the pathology of CIA. Although relationships between the suppressive effects of various therapies on CIA and regulatory T cells have been reported\(^11\,^12\), there is no report concerning moxibustion.

In the present study, to investigate whether regulatory T cells are involved in the suppressive effects of moxibustion on CIA, we measured the populations of regulatory T cells in the peripheral blood, spleens and inguinal lymph nodes using flow cytometric analysis and the concentration of Transforming Growth Factor-β1 (TGF-β1), which is involved in the differentiation and induction of regulatory T cells, in the serum using ELISA.

II. Methods

Animals and experimental groups

Seven-week-old male DBA/1 J mice were purchased from Sankyo Labo Service (Tokyo, Japan). They were bred in rooms kept at a temperature of 25 ± 2°C and a relative humidity of 55 ± 5% under a 12-hour light-dark cycle. They were allowed free access to tap water and a standard diet. The mice were randomly assigned to four groups: no-treated normal, moxibustion-treated normal, no-treated CIA and moxibustion-treated CIA. This study was approved by the Ethics Committee of Showa University for Animal Experiments (No.00010).

Induction of CIA

Bovine type II collagen (CII) solution (Collagen Research Center, Tokyo, Japan) was dissolved in a 0.01 M acetic acid solution at 4.0 mg/ml and emulsified with an equal volume of complete Freund’s adjuvant (CFA) (Difco Laboratories, Detroit, MI, USA). Male DBA/1 J mice were injected intradermally at the base of the tail with 100 μl (200 μg CII) of the emulsion. Twenty-one days after primary immunization, the mice were boosted with the same amount of bovine CII emulsified with incomplete Freund’s adjuvant (IFA) (Difco Laboratories, Detroit, MI, USA) (Figure 1).

Clinical assessment of arthritis

The disease severity in each limb of the 67 mice (no-treated CIA: n=35, moxibustion-treated CIA: n=32) was recorded every two to three days from day 21 to day 35 post-immunization as follows: 0 = normal, 1 = erythema and swelling of one digit, 2 = erythema and swelling of more than two digits or mild erythema and swelling of the entire paw, 3 = progressively more severe erythema and swelling of the entire paw, 4 = severe swelling and erythema with lack of flexibility. Each limb was graded, thus giving a maximum possible score of 16 per animal.

Moxibustion application

Moxibustion was applied to the acupuncture point equivalent to GV 4 (Mingmen) located between the spinal process of the second lumbar vertebra and the third lumbar vertebra. The mice were shaved in an area measuring approximately 4 cm\(^2\) on the back in advance, and 1 mg of cone-shaped moxa was placed directly on the skin surface of the acupuncture point GV 4 and then ignited. Five moxa cones were applied consecutively at
intervals of approximately five seconds. A temperature curve of the heat stimulation produced by the moxibustion measured using a platinum electrode thermometer and a recorder (Memory Hicorder 8840, Hioki EE Corp., Nagano, Japan) is shown in Figure 2. The mean maximal temperature of five applications of moxibustion was 73.6 ± 14.2°C. During the moxibustion treatment, the mice were lightly restrained in a human hand. The no-treated mice were restrained in the same way for 30 seconds. This treatment was conducted every two to three days from day 21 to day 35 post-immunization (Figure 1).

Body weight, white blood cell count, spleen weight and lymphocyte number of lymph nodes

To investigate the influence of moxibustion on the general condition of the mice, the body weight (BW), white blood cell (WBC) count, spleen weight and lymphocyte number of bilateral inguinal lymph nodes (ILN) were measured in 30 mice (no-treated normal: n=5, moxibustion-treated normal: n=5, no-treated CIA: n=10, moxibustion-treated CIA: n=10). After measuring BW, the mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg: Dainippon Sumitomo Pharma Co., Osaka, Japan) on day 35 post-immunization. In each mouse, a blood sample was obtained from the abdominal aorta and the WBC count was measured using the Particle Counter Model PCE-210 (ERMA Inc., Tokyo, Japan). At the same time, the spleen and bilateral ILN were harvested. The spleen was weighed on an electronic balance. The ILN were pressed through steel mesh and suspended in 1 ml of physiological saline, and the lymphocyte number in the suspension was measured using the Particle Counter Model PCE-210.

Flow cytometric analysis

On day 35 post-immunization, the CD 4+ CD 25+ T cell populations in the peripheral blood (PB) (n=5 per group), spleens (n=5 per group) and ILN (n=10 per group) were measured using flow cytometric analysis. The blood was hemolyzed with Lysing Buffer (BD Pharmingen, San Diego, CA, USA) and washed twice, and the spleens and bilateral ILN were pressed through steel mesh to make a single cell suspension. The cells (1×10^6 / ml) were stained with FITC-conjugated anti-CD 4 and PE-conjugated anti-CD 25 mAb (BD Pharmingen, San Diego, CA, USA) or respective isotype controls. Flow cytometry was performed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA), and the analysis was performed using the CellQuest software program (Becton Dickinson, San Jose, CA, USA).

Cytokine assays

The levels of TGF-β1 in sera (n=5 per group) were assessed with an ELISA Kit (R&D System Inc., Minneapolis, MN, USA) according to the manufacturer’s recommended procedures, and the absorbances were measured with a microplate reader (Immuno-Mini NJ-2300, Inter Med Co., Tokyo, Japan).

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Comparisons between two values were performed using unpaired Student’s t-test. Multiple groups were compared using ANOVA followed by Fish-

![Figure 2. A temperature curve of the heat stimulation induced by moxibustion. Five moxa cones were ignited consecutively at intervals of approximately five seconds. The mean maximal temperature of five applications of moxibustion was 73.6±14.2°C.](image)
er’s PLSD test. A value of \( p < 0.05 \) was considered statistically significant.

### III. Results

#### Incidence and severity of CIA

The disease incidence on day 35 was 94.3% (33/35) in the no-treated CIA mice and 71.8% (23/32) in the moxibustion-treated CIA mice. There was a significant difference (\( p < 0.05 \)) in the incidence of arthritis between the no-treated CIA and moxibustion-treated CIA mice (Figure 3 A). Disease severity progressively increased in the no-treated CIA mice from day 26 onward and peaked on day 35; however, in the moxibustion-treated CIA mice, the increase in disease severity was inhibited significantly starting on day 28. The mean peak disease severity (day 35) was 7.5±0.6 in the no-treated CIA mice and 5.2±0.8 in the moxibustion-treated CIA mice (Figure 3 B).

#### Changes in general condition

BW did not change among any group of mice. The WBC counts and spleen weights were increased in the no-treated CIA mice, and moxibustion treatment tended to suppress these increases. The lymphocyte numbers of ILN were increased in the no-treated CIA mice, and moxibustion treatment triggered further increases in these values (Table 1).

#### CD 4^+ CD 25^+ T cell population in the peripheral blood, spleen and lymph nodes

To obtain the percentage of CD 4^+ CD 25^+ T cells in CD 4^+ T cells, histograms were created from all flow cytometry images of CD 4^+ CD 25^+ T cells. Figure 4 D

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**Table 1. The effects of moxibustion on the general condition of the mice.**

<table>
<thead>
<tr>
<th></th>
<th>BW (g)</th>
<th>WBC (×10^3/μL)</th>
<th>SP weight (mg)</th>
<th>Lymphocyte number of ILN (×10^3/ILN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (N=5)</td>
<td>22.8±0.7</td>
<td>2.8±0.2</td>
<td>72.3±3.0</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>Normal+Moxibustion (N=5)</td>
<td>22.2±0.3</td>
<td>3.4±0.2</td>
<td>79.8±2.9</td>
<td>3.4±0.7</td>
</tr>
<tr>
<td>CIA (N=10)</td>
<td>21.5±0.4</td>
<td>5.8±0.3**</td>
<td>122.8±3.6***</td>
<td>6.2±1.1***</td>
</tr>
<tr>
<td>CIA+Moxibustion (N=10)</td>
<td>21.9±0.4</td>
<td>5.0±0.5**</td>
<td>109.9±6.2***#</td>
<td>14.9±0.4***##</td>
</tr>
</tbody>
</table>

BW: body weight, WBC: white blood cell count, SP weight: spleen weight, Lymphocyte number of ILN: lymphocyte number of bilateral inguinal lymph nodes. The data represent the mean ± SEM of 5 mice (No-treated normal and moxibustion-treated normal mice) or 10 mice (No-treated CIA and moxibustion-treated CIA mice). Significant differences are indicated with *\( p<0.05 \), **\( p<0.01 \) and ***\( p<0.001 \) versus the no-treated normal mice, and /\( p<0.05 \), ###\( p<0.01 \) and ####\( p<0.001 \) versus the no-treated CIA mice.
shows one example. The percentage of CD $4^+\ CD\ 25^+\ T$ cells in CD $4^+\ T$ cells in the no-treated normal mice was 11.2±2.3% in PB, 5.4±0.2% in SP and 8.7±0.4% in ILN, respectively (Table 2). The percentage of CD $4^+\ CD\ 25^+\ T$ cells in CD $4^+\ T$ cells significantly increased in the spleens and ILN of the no-treated CIA mice compared with that observed in the no-treated normal mice (Figure 4 B, C and Table 2). The percentage of CD $4^+\ CD\ 25^+\ T$ cells in CD $4^+\ T$ cells in the ILN of the moxibustion-treated normal mice was significantly increased in comparison with that observed in the no-treated normal mice (Figure 4 C). The percentages of CD $4^+\ CD\ 25^+\ T$ cells in CD $4^+\ T$ cells in the PB, spleens and ILN of the moxibustion-treated CIA mice were significantly increased in comparison with those observed in the no-treated CIA mice (Figure 4 A, B, C and Table 2).

Figure 4. The effects of moxibustion on the population of CD$4^+CD25^+$ T cells in CD$4^+\ T$ cells in the peripheral blood (A), spleen (B) and inguinal lymph nodes (C). The white bars indicate the no-treated normal mice or the no-treated CIA mice. The black bars indicate the moxibustion-treated normal mice or the moxibustion-treated CIA mice. The data represent the mean ± SEM of 5 mice (A and B) or 10 mice (C) per group. Significant differences are indicated with *p<0.05, **p<0.01 and ***p<0.001, respectively. D, A flow cytometric image of the whole CD$4^+CD25^+$ T cells (left) was created on CellQuest software. A histogram (right) was created to obtain the percentage of CD$4^+CD25^+$ T cells in CD$4^+\ T$ cells. This is the representative data of peripheral blood taken from a no-treated CIA mouse (blank) and a moxibustion-treated CIA mouse (gray).

Table 2. The percentage of CD$4^+CD25^+$ T cells in CD$4^+\ T$ cells in the peripheral blood (PB), spleen (SP) and inguinal lymph nodes (ILN).

<table>
<thead>
<tr>
<th>Group</th>
<th>PB (%)</th>
<th>SP (%)</th>
<th>ILN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>11.2±2.3</td>
<td>5.4±0.2</td>
<td>8.7±0.4</td>
</tr>
<tr>
<td>Normal+Moxibustion</td>
<td>10.0±0.5</td>
<td>4.7±0.2</td>
<td>11.2±0.6</td>
</tr>
<tr>
<td>CIA</td>
<td>10.3±0.8</td>
<td>6.7±0.4*</td>
<td>12.8±0.5***</td>
</tr>
<tr>
<td>CIA+Moxibustion</td>
<td>15.2±1.1*##</td>
<td>8.7±0.5***##</td>
<td>14.5±0.7###***</td>
</tr>
</tbody>
</table>

The data represent the mean ± SEM of 5 mice (PB and SP) or 10 mice (ILN) per group. Significant differences are indicated with *p<0.05, **p<0.01 and ***p<0.001 versus the no-treated normal mice and #p<0.05, ##p<0.01 and ###p<0.001 versus the no-treated CIA mice.
Serum cytokine levels

The serum TGF-β1 levels were up-regulated in the no-treated and moxibustion-treated CIA mice and significantly increased in the moxibustion-treated CIA mice compared with those observed in the no-treated CIA mice (Figure 5).

IV. Discussion

In the present experiments, we confirmed that direct moxibustion applied at GV 4 using 1 mg of moxa cone decreases the incidence and severity of arthritis (Figure 3 A and B). These results are consistent with those of a previous study. In our previous study, however, no suppressive effects on CIA were observed when moxibustion was applied at the acupuncture point CV 12 (Zhongwan), which is located in the abdomen. The acupuncture point GV 4, a well-known point in the Governor Vessel meridian, has been applied in the treatment of human immune disorders and the modulation of the immune system in animals. The effects of moxibustion are not consistent and differ depending on the acupuncture points used.

In this study, the application of moxibustion in CIA mice tended to decrease the WBC count and the spleen weight augmented by CIA and enhance increases in the lymphocyte number of ILN (Table 1). If moxibustion affects the weight and cell counts of these tissues, it might affect the cell subpopulations inside these tissues. In fact, it has been reported that direct and indirect moxibustion changes the lymphocyte subpopulations in the PB and spleen. As ILN is the common regional lymph node in both sites examined in this study (the lower lumber area where moxibustion was applied and the base of the tail where C II was injected), moxibustion stimulation might affect C II-sensitized lymphocytes through immunocytes at these sites.

In 1995, CD4+CD25+ regulatory T cells, which constitute approximately 10% of peripheral CD4+ T cells, were identified as the T cells that suppress the immune response, and some reports have revealed that these cells can regulate the pathology of CIA. Therefore, we hypothesized that moxibustion might induce the up-regulation of regulatory T cells, resulting in the suppression of autoimmunity. The results showed a significant increase in the percentage of CD4+CD25+ T cells in CD4+ T cells in the PB, spleens and ILN taken from the moxibustion-treated CIA mice, in accordance with our

Figure 5. The effects of moxibustion on cytokine production.
The concentrations of TGF-β1 in sera were measured using ELISA. The data represent the mean± SEM of 5 mice per group. Significant differences are indicated with *p<0.05, **p<0.01 and ***p<0.001.
expectations (Figure 4 A, B, C and Table 2). Later, forkhead box protein 3 (Foxp3) was identified as the master regulatory gene of regulatory T cells and has become the specific molecular marker for these cells. Currently, we are confirming the induction of CD4+CD25+Foxp3+ regulatory T cells by moxibustion and have observed similar results (data not shown). In present study, however, the population of CD4+CD25+ T cells in CD4+ T cells in the PB and spleen was similar in both the no-treated normal mice and the moxibustion-treated normal mice (Figure 4 A and B). It is thought that moxibustion adjusts immunity if necessary, although this has not been certified experimentally. It therefore seems that remarkable changes are not observed when moxibustion is applied to healthy mice.

Regulatory T cells are known to inhibit the proliferation of effector cells when cultured with effector cells and to secrete immunosuppressive cytokines such as IL-10 and IL-35. Regulatory T cells induced by moxibustion might suppress CIA as a result of inhibiting the proliferation of effector cells such as T helper 17 cells (Th 17) and secreting immunosuppressive cytokines. In order to investigate the effects of moxibustion on the production of cytokines involved in the differentiation to regulatory T cells, we measured the concentrations of TGF-β1 in sera. The serum TGF-β1 levels were significantly increased in the CIA mice compared with those observed in the normal mice and were further increased in the moxibustion-treated CIA mice compared with those observed in the no-treated CIA mice (Figure 5). IL-2 and TGF-β1 are known to be cytokines involved in the differentiation and induction of regulatory T cells. In particular, TGF-β1 is necessary for the differentiation to inducible regulatory T cells that are peripherally differentiated and induced. Furthermore, TGF-β1 is also involved in the differentiation to Th 17, which act as an effector cell in autoimmune diseases such as rheumatoid arthritis. As shown in Figure 6, TGF-β1 alone promotes the differentiation of naive T cells to regulatory T cells, and TGF-β1 in the presence of IL-6 promotes differentiation to Th 17. In our previous study, the serum IL-6 levels were increased in CIA mice compared with those observed in normal mice; however, they were decreased in moxibustion-treated CIA mice compared with those observed in no-treated CIA mice. Taken together, in CIA mice, arthritis is exacerbated by the promoted dif-

![Figure 6. The effects of moxibustion on the differentiation to regulatory T cells.](image)

TGF-β alone promotes differentiation of naive T cells to regulatory T cells (Treg), and TGF-β1 in the presence of IL-6 promotes differentiation to Th17. Moxibustion (GV4) induces differentiation to regulatory T cells by increasing the secretion of TGF-β and decreasing the secretion of IL-6.
ferentiation of Th 17 as a result of the increased production of TGF-β1 and IL-6. On the other hand, when moxibustion is applied in CIA, both the incidence and severity of arthritis are suppressed by the promoted differentiation to regulatory T cells as a result of the increased production of TGF-β1 and decreased production of IL-6 (Figure 6).

V. Conclusion

We herein demonstrated that direct moxibustion applied to the acupuncture point GV 4 suppresses incidence and severity of CIA through the differentiation and induction of regulatory T cells. This is the first report to provide evidence that moxibustion affects the regulatory T cell population in murine CIA.

References

