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Effect of Moxibustion on Regulatory T cell Induction in Mouse CIA Model

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ABSTRACT

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.

KEYWORDS

Moxibustion; Collagen-induced arthritis; Regulatory T cell, TGF-β.

1. Introduction

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.

2. Methods

2.1 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℃ 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).

2.2 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. Twentyone 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).

2.3 Clinical Assessment of Arthritis

The disease severity in each limb of the 67 mice (notreated 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.

2.4 Moxibustion Application

Moxibustion was applied to the acupuncture point equivalent to GV 4 (Mingmen) located between the spinal process of the second lumber vertebra and the third lumber 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℃. 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 postimmunization (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.

2.5 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 (1×106 / 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).

2.6 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).

2.7 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 Fisher's PLSD test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 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 moxibustiontreated 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).

3.2 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).

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 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 moxibustiontreated 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 notreated CIA mice (Figure 4 A, B, C and Table 2).

3.3 Serum Cytokine Levels

The serum TGF-β1 levels were up-regulated in the notreated 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).

4. 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 study6). 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 abdomen13). 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 animals1,14). 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 spleen2,15). 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 CⅡ-sensitized lymphocytes through immunocytes at these sites.

In 1995, CD 4+CD 25+ regulatory T cells, which constitute approximately 10% of peripheral CD 4+ T cells, were identified as the T cells that suppress the immune response7), and some reports have revealed that these cells can regulate the pathology of CIA8-10). Therefore, we hypothesized that moxibustion might induce the upregulation of regulatory T cells, resulting in the suppression of autoimmunity. The results showed a significant increase in the percentage of CD 4+CD 25+ T cells in CD 4+ T cells in the PB, spleens and ILN taken from the moxibustion-treated CIA mice, in accordance with our expectations (Figure 4 A, B, C and Table 2). Later, forkhead box protein 3 (Foxp 3) 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 CD 4+CD 25+Foxp 3+ regulatory T cells by moxibustion and have observed similar results (data not shown). In present study, however, the population of CD 4+CD 25+ T cells in CD 4+ 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 IL10 and IL-3516,17). 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 induced18). 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 1719). 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 mice20). Taken together, in CIA mice, arthritis is exacerbated by the promoted differentiation 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 in creased production of TGF-β1 and decreased production of IL-6 (Figure 6).

5. 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.

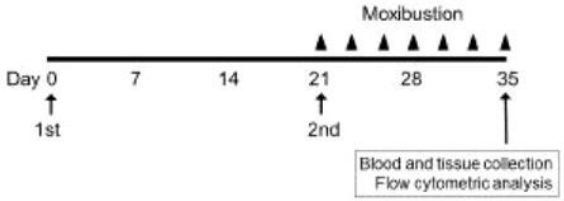

Figure 1.

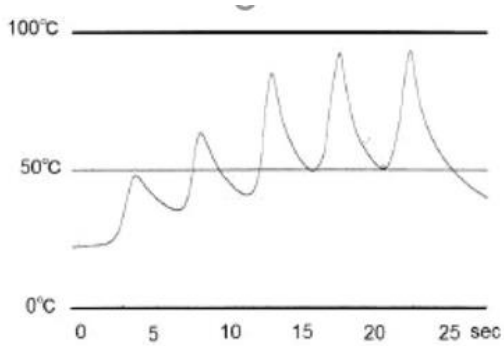

Figure 2.

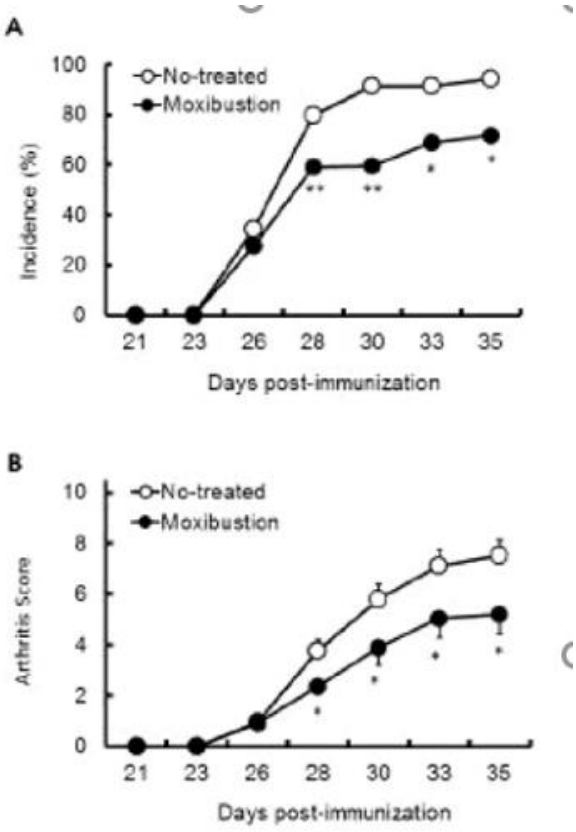

Figure 3.

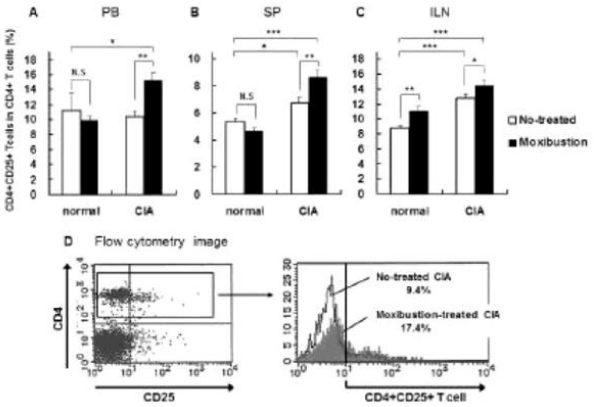

Figure 4. The no-treated normal mice and the moxibustion-treated normal mice

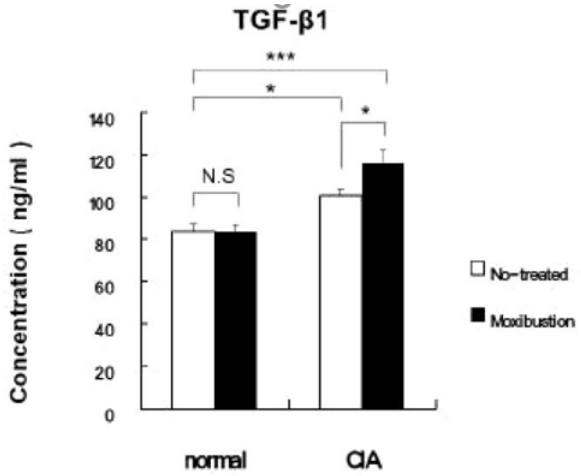

Figure 5. TGF-β1 alone promotes the differentiation of naive T cells to regulatory T cells

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