Immunomodulating Activity of BL18 (Ganshu) Acupuncture on the Experimental Liver Metastasis Model of Mice

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We investigated that the immunomodulating activity of BL18 (Ganshu) acupuncture on the experimental liver metastasis model of mice. NA (non-acupoint)- and BL18-treatment enhanced the mitogenic activity of BALB/c whole splenocytes induced by various mitogenic stimuli. Acupuncture treatment tended to increase splenocytes differentiation even though did not show significance. Acupuncture treatment caused a marked increase of production of Th1 cytokine (IFN-γ) and Th2 cytokine (IL-4) by splenocytes and IL-12 and IFN-γ by macrophages. The increase of cytokine production on BL18-treated group was more pronounced compared to NA-treated group. The liver weight of NA- and BL18-treated group decreased compared to tumor group, but did not showed significant differences.

Key words : Acupuncture, BL18, Macrophages, Cytokine

Introduction

The basic theory of meridians in Oriental medicine is that the meridians connect the interior of the body with the exterior. Meridian theory says that working with points on the surface of the body will affect what goes on inside the body, because it affects the activity of the textures that are traveling through the meridians. The theory also says that each acupuncture point has a characteristic functional effect and shows a defined therapeutic action[1].

It was well known that several acupuncture stimulation evokes immune-activation. For example, acupuncture stimulation lessens symptoms of arthritis, promotes NK cell activity and IFN-γ production, enhances tyrosine protein kinases and so on[2,3]. Also, acupuncture stimulation suppresses relapse of cancer patients and reduces pains[4,5] and adverse effects of radiotherapy[6,7]. Recently, it was also reported that acupuncture stimulation on cancer patients enhanced cellular immunity[8]. Currently, as the use of alternative and complementary therapies is increasing, acupuncture treatment containing auricular acupuncture is commonly performed as the combination therapy for cancer patients.

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However, few studies have determined the effect of acupuncture on metastasis and immunomodulation. Therefore, we here investigated that the immunomodulating activity and anti-metastatic acitivity of BL18 acupuncture treatment on the experimental liver metastasis model.

Materials and Methods

1. Cell cultures

The liver metastatic cell line of the colon26-L5 carcinoma (colon26-L5) cells was kindly provided by Dr. Saiki (Institute of Natural Medicine, University of Toyama, Japan). Colon26-L5 cells were maintained as monolayer cultures in RPMI-1640 medium (GIBCO BRL, Life Technologies Inc., NY, USA) supplemented with 10% fetal bovine serum (FBS; INC Biomedicals Inc., CA, USA). Colon26-L5 cells were collected by brief treatment with EDTA, and then used for the experiments. All cultures were maintained at 37 ℃ in a humidified atmosphere of a 5% CO_2/95% O_2 air.

2. Animals

Six-week old, specific-pathogen-free female BALB/c mice were purchased from Daehan Biolinks Inc (Korea). The mice were maintained under specific pathogen-free conditions and used according to institutional guidelines.

3. Acupoints selection and treatment methods

Bilateral BL18 (Ganshu) were punctured perpendicularly

0.3 ㎝ with the reinforcing method, rotated the needles for 1 min, and let for 30 min. For NA (non-acupoint) treatment, non-acupoints of hip were selected and conducted the same stimulation as BL18 treatment. The two acupuncture groups were punctured once a day for 20 days. Animals were sacrificed one day after the last treatment.

4. Experimental liver metastasis of colon26-L5 carcinoma cells

Log-phase cell cultures of colon 26-L5 cells were harvested with 0.05% EDTA in phosphate-buffered saline (PBS), washed three times with serum-free RPMI, and resuspended at appropriate concentrations in PBS. BALB/c mice under ether anesthesia underwent laparotomy using an upper median incision, and the duodenal loop was exposed. An injection of colon26-L5 (1×10^4 cells/mice) cells was given into the portal vein through a 29-gauge needle attached to a 1-㎖ syringe. The mice were sacrificed 21 days after tumor inoculation and the liver weight was recorded to evaluate the tumor metastasis as previously described[9]. Mice were divided into four groups (n = 6 each): normal; cancer; cancer-NA; and cancer-BL18. Acupuncture treatment was conducted for 30 min from 1 day after tumor inoculation.

5. Preparation of mouse splenocytes and peritoneal exudates cells

Splenocytes were obtained by passing pieces of spleen through a stainless mesh, treated with a hypotonic solution to lyse erythrocytes, and washed three times with PBS. The viability of the splenocyte was more than 95%, as assessed by the trypan blue dye exclusion method. Whole splenocytes were suspended in RPMI-1640 medium supplemented with 10% FBS and then used for experiments.

6. Splenocyte proliferation assay in vitro

For the splenocyte proliferation assay, acupuncture treatment was conducted to BALB/c for 20 days and splenocytes were obtained 1 day after the last treatment of described above. Splenocytes (1×10^5 cells/100$\mu\ell$) suspended in RPMI-1640 medium supplemented with 10% FBS were cultured in 96-well U-bottom culture plates with or without concanavalin A (Con A; Sigma, MO, USA) or lipopolysaccharide (LPS; Sigma) for 72 hr at 37 ℃. This assay was performed using triplicate cultures. XTT assay (Sigma) was performed to measure cell proliferation.

7. Flow cytometry analysis

Isolated cells from spleen were stained with fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated monoclonal anti-CD3, CD19, CD4, CD8, MAC-1, and NK1.1 antibodies (Becton-Dickinson, CA, USA) in PBS for 20 minutes at 4 ℃. Stained cells were analyzed on flow cytometry (FACS Calibur; Becton-Dickinson) using a Cell Quest software. Differentiation of lymphocytes was determined using flowcytometric analysis of light scatter chamber characteristics relating size and granulation. Two-color flow cytometry was then performed to calculate the percentages of CD3, CD19, CD4, CD8, Mac-1, and NK1.1 positive cells in a subset of lymphocytes.

8. Induction of cytokine production

Interferon (IFN)-γ, interleukin (IL)-4 levels in the culture supernatants were evaluated using specific ELISA kits (BD Pharmingen, CA, USA) according to the manufacturer's instructions. Cell-free supernatant was prepared as follows. Splenocytes (1×10^6 cells/well) were prepared as described above and then cultured in 24-well culture plates with or without various concentrations of Con A for 24 or 48 hr at 37 ℃. The cell-free supernatant was collected from each well and stored at -80 ℃ until the ELISA assay.

9. Statistical Analysis

Representative data from each experiment are presented as mean values ± SD, as described in the figure legends. The statistical differences between the groups were determined by applying the Student's two-tailed t-test. The Dunnett's test was performed to decrease the multiplicity in comparisons of drug-treated groups with control group. Statistical significance was defined as a P value < 0.05.

Results

1. Effect on the proliferation of splenocytes

To clarify the biological properties of BL18 acupuncture stimulation, we investigated the mitogenic responses of mouse spleen cells. After acupuncture treatment for 20 days, splenocytes obtained from the normal, NA-treated or BL18 acupuncture-treated group were cultured with or without T cell mitogen (Con A) or B cell mitogen (LPS) for 72 hr. Both NA-treated and BL18-treated group resulted in an increasing tendency of cell proliferation with or without mitogenic stimuli (Fig. 1A). Specially, T cell mitogen caused a significant increase of cell proliferation. Acupuncture treatment did not show any apparent side effect such as a decrease of body weight (Fig. 1B).

2. Phenotypic characterization of lymphocytes

Next, we performed flow cytometric analysis to examine

lymphocytes differentiation by acupuncture treatment. The population of CD3, CD19, CD4, CD8, Mac-1 and NK1.1 positive cells was shown in Fig. 2.

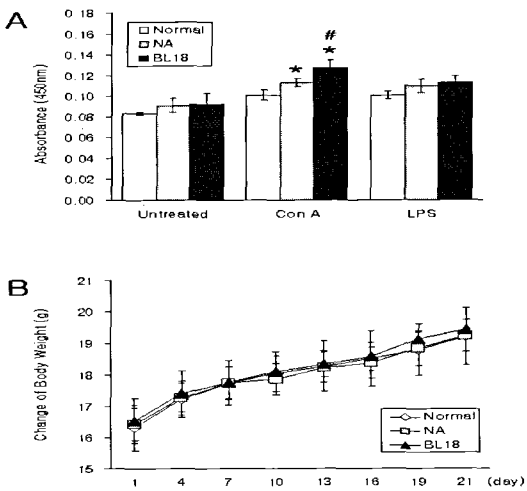

Fig. 1. Effect of BL18 (Ganshu) acupuncture stimulation on the proliferation of mouse splenocytes in response to various mitogenic stimuli. A. Female BALB/c mice were treated with BL18 or NA acupoint for 20 days. One day after the last treatment, mice were sacrificed and the splenocytes (1 × 10^5 cells/well) suspended in FPMI-1640 medium supplemented with 10% FBS were cultured with or without con A or LPS for 72 hr. XTT assay was conducted for evaluating cell proliferation. The data represent the mean S.D. *, $p < 0.05$ as compared to normal group. #, $p<0.05$ as compared to NA-treated group. B. The changes of body weight during expeirments.

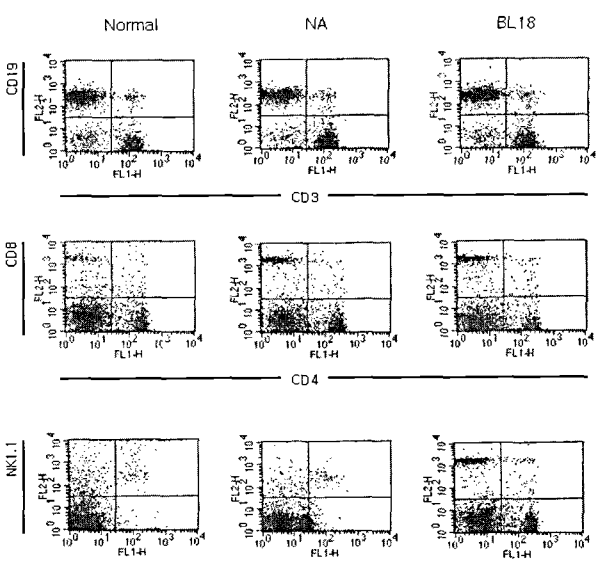

Fig. 2. Phenotypic characterization of lymphocytes by BL18 (Ganshu) acupuncture stimulation. Female BALB/c mice were treated with BL18 or NA acupoint for 20 days. One day after the last treatment, mice were sacrificed and the splenocytes(1 × 10^5 cells/well) were stained with FITC- and PE-conjugated monlclonal anti-CD3, CD19 CD4, CD8, Mac-1, and NK1.1 antibodies. Stained cells were analyzed on flow cytometry using a Cell Quest Software. A representative image of whole lymphocytes sorted using the light scatters.

3. Effect of cytokines production from splenocytes

We next investigated whether or not the BL18 acupuncture treatment can induce the production of Th1- and Th2-type cytokines by splenocytes. As shown Fig. 3, splenocytes from normal (untreated control), NA-treated and BL18-treated mice did not produce detectable amounts of Th1 cytokine (IFN-γ) and Th2 cytokine (IL-4) without Con A stimulation. (1 μg/mℓ). When splenocytes were incubated with Con A for 24 or 48 hr, detectable amounts of these cytokines were found in the cell-free supernatant. The NA and BL18 acupuncture treatment resulted in a significant enhancement of IFN-γ and IL-4 production as compared than untreated control.

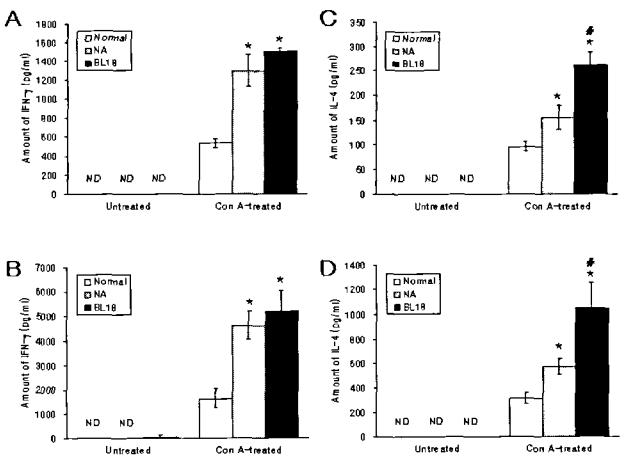

Fig. 3. Effect of BL18 (Ganshu) acupuncture stimulation on production of IFN-γ and IL-4 from splenocytes. Female BALB/c mice were treated with BL18 or NA acupoint for 20 days. One day after the last treatment, mice were sacrificed and the splenocytes (1 × 10^6 cells/well) suspended in RPMI-1640 medium supplemented with 10% FBS were cultured with or without con A for 24 or 48 hr. After the termination of culture, the cell-free supernatant was collected and the amount of IFN-γ and IL-4 was measured by ELISA kits. The data represent the mean ± S.D. *, $p < 0.05$ as compared to normal group. #, $p<0.05$ as compared to NA-treated group. ND, not detected. A and B, Amount of IFN-γ for 24 and 48 hr. C and D, Amount of IL-4 for 24 and 48 hr.

4. Effect of cytokine production from macrophages

Macrophages play an important role in the early stages of metastasis. Therefore, we examined whether or not the BL18 acupuncture treatment can induce the production of cytokines such as IL-12 and IFN-γ by macrophages in vivo. As shown Fig. 4, macrophages from NA-treated and BL18-treated mice released a high level of IL-12 and IFN-γ into the culture supernatant in response to LPS stimulation (1 μg/mℓ) for 24 or 48 hr. Also, BL18-treated mice have shown increased trends compared than NA-treated mice

5. Inhibition of experimental liver metastasis

To confirm the effect of acupuncture treatment on the liver metastasis, we conducted the experimental liver metastasis assay. The liver weight was measured on day 21 after tumor inoculation. The acupuncture treatment on NA and BL18 tended to decrease the metastasis liver weight but the liver weight was not significantly different between tumor, NA-treated and BL18-treated group.

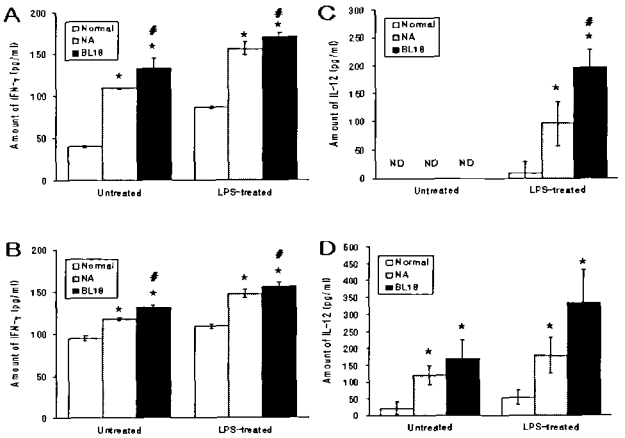

Fig. 4. Effect of BL18 (Ganshu) acupuncture stimulation on production of IFN-γ and IL-12 from macrophages. Female BALB/c mice were treated with BL18 or NA acupoint for 20 days. One day after the last treatment, mice were sacrificed and the macrophages (1 × 106 cells/well) suspended in RPMI-1640 medium supplemented with 10% FBS were cultured with or without con A for 24 or 48 hr. After the termination of culture, the cell-free supernatant was collected and the amount of IFN-γ and IL-12 was measured by ELISA kits. The data represent the mean ± S.D. *, $p < 0.05$ as compared to normal group. #, $p<0.05$ as compared to NA-treated group. ND, not detected. A and B, Amount of IFN-γ for 24 and 48 hr. C and D, Amount of IL-12 for 24 and 48 hr.

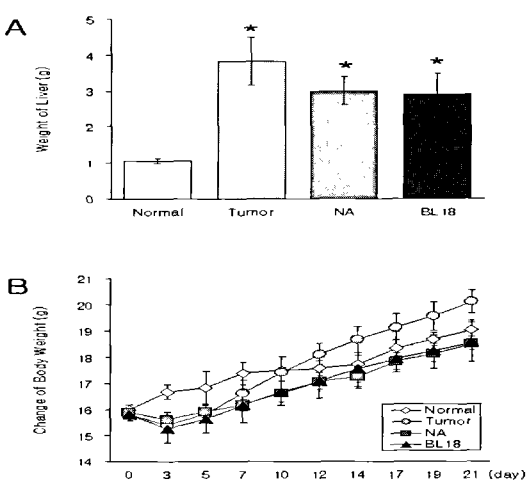

Fig. 5. Effect of BL18 (Ganshu) acupuncture stimulation on experimental liver metastasis. A. Female BALB/c mice (n=6) were inoculated intravenously with colon26-L5 cells (2×10^5 cells/mice). BL18 or NA acupoint were stimulated for 20 days. One day after the last treatment, mice were sacrificed and the liver weight was measured. The results represent the mean ± S.D. *, $p < 0.05$ as compared to normal group. B. The changes of body weight during expeirments.

Discussion

Complementary and alternative medicine (CAM) including acupuncture is considered a valid intervention for cancer patients with pain and adverse effect of radiation therapy[4-7,9]. Acupuncture is considered as a nonstandard therapy facilitating to fight cancer because the precise mechanism is unproven. Therefore, an effort has been made to elucidate the mechanism of acupuncture[10,11]. It is well known that BL18 acupoints implicate to the liver function and modulate liver-related diseases in Oriental medicine. It has been demonstrated that BL18 acupuncture adjusts aging by means of down-regulating the ratios of metallothioneins (MT)3 to MT1 to the normal range[12]. However, there are no reports have conducted the effect of BL18 on cancer metastasis and immune-regulating activity.

Therefore, we here investigated the effect of BL18 acupuncture treatment on the spleen cell proliferation in responses to mitogenic stimuli. The BL18 acupuncture treatment resulted in a marked augmentation of Con A-stimulated proliferation of splenocytes. LPS-stimulated proliferation tended to increase by BL18 acupuncture but did not show significance (Fig. 1). The differentiation of lymphocytes is important because it determines the activation of lymphocytes and immune responses to antigen. According to the flow cytometry analysis, Mac-1 positive cells increased significantly in the BL18-treated group. However, CD3, CD19, CD4, CD8 and NK1.1 positive cells showed increasing tendency without significance (Fig. 2). IL-12 is produced by phagocytic cells, B cells and other antigen-presenting cells in early stages of the immune responses. IL-12 possesses several biological activities, including generation of cytotoxic T lymphocytes, enhancement of NK cell-mediated cytotoxicity and anti-tumor and anti-metastatic activities[13,14]. As shown in Fig. 4, BL18 treatment enhanced IL-12 production by macrophages in response to LPS compared with normal and NA treatment. IFN-γ production by macrophages showed a similar pattern to IL-12, presumably because IL-12 can enhance the production of IFN-γ[15]. According to the increase of Mac-1 positive cells and the increment of IL-12 and IFN-γ production, BL18 acupuncture treatment might enhance the ability of macrophages.

It is generally accepted that the balance of helper T cell subsets (Th1-Th2 balance) in the host determines the various diseases, such as inflammation, allergy, autoimmune disease, cancer and so on[16,17]. Th1 cells, which produce IFN-γ, IL-2 and tumor necrosis factor-β (TNF-β), mediate cellular immune responses and are involved to inflammatory reactions. Th2 cells, which produce IL-4, IL-5, IL-10 and IL-13, affects humoral immune responses and are responsible for strong antibody responses, including IgE production and allergic reactions[18,19]. The BL18 acupuncture treatment caused the production of cytokines both Th1 and Th2 by splenocytes stimulated with Con A (Fig. 3). According to these results, BL18 acupuncture treatment could affect inflammatory diseases including cancer as well as induce allergic responses.

Metastasis is the major cause of mortality in cancer patients and the liver is the most common target of the metastasis of gastrointestinal tract cancer, especially colon cancer[9]. BL18 acupuncture stimulation did not show any

adverse effects compared with tumor and NA-treated group. However, the acupuncture treatment on NA and BL18 tended to decrease the metastasis liver weight but the liver weight was not significantly different between tumor, NA-treated and BL18-treated group (Fig. 5). The present study suggests that BL18 acupuncture stimulation increase immune responses of mice. However, the anti-metastatic effects between NA- and BL18-treated groups did not clear because NA treatment might stimulate immune system. Therefore, it would be needed to develop the proper control point or the proper stimulation method for control group.

Acknowledgements

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