Immunomodulatory Effects of *Hominis Placenta* Extract Injection into an Acupuncture Point on the Experimental Subcutaneous Tumor Model of Mice

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Hominis placenta (HP) has been used as an agent for promoting physiological function in traditional asian medicine. The present study was performed to investigate whether HP acupuncture treatment in an experimental tumor mice model inhibit tumor growth through immunomodulatory effects. Mice were inoculated subcutaneously with colon26-L5 cells on the back. Three days after tumor inoculation, HP herbal acupuncture treatment was conducted on BL18 acupoint every other day for three weeks. HP herbal acupuncture treatment significantly suppressed the primary tumor growth and prolonged survival rate. To evaluate immunomodulatory effect of HP acupuncture, splenocytes proliferation assay, fluorescence-activated cell sorting (FACS) and ELISA for IFN-γ, and IL-4 cytokine level. HP herbal acupuncture enhanced the mitogenic activity of Balb/c whole splenocytes induced by various mitogenic stimuli and increased immune cell population such as T cell, B cell, Th cell, Tc cell and Macrophages. HP herbal acupuncture caused a marked increase of production of Th1 cytokine (IFN-γ,) and decrease of production of Th2 cytokine (IL-4). These results indicated that HP herbal acupuncture suppresses tumor growth through a mechanism leading to a Th1 dominant immune state.

Key words : Horminis placenta, herbal acupuncture, Th1 cytokine, colon26-L5 carcinoma

Introduction

Some chemotherapeutic agents have been used as adjuvants in combination with surgical treatment for malignant tumors. Unfortunately, most anti-cancer drugs are not sufficiently tumor-selective and sometimes cause hematopoietic disorders and resistance to the chemotherapeutic regimen[1]. Some natural herbal medicines including traditional Korean medicine are believed to show marked anti-cancer and anti-metastatic effects while possessing low toxicity for normal tissues and immunomodulating properties[2-4].

Cancer cells in the initial developmental stages are mainly scavenged by neutrophilic leukocytes, macrophages and other components of the primitive immune system. The immune response is decided depending on which sub-type of T cell is activated. The proper balance between Th1 and Th2 cells is essential in the treatment of tumors, which are generated when cellular immunity is affected by immuno-suppressing factors[5].

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In the traditional asian medicine, acupuncture is considered as one of the most effective treatments[6]. In a treatment with a herbal acupuncture, a fixed amount of the herbal extracts is injected into a specific point (acupoint) of the body, which has been proven effective in the treatment of specific disorders[7]. Also, it has been reported that several acupuncture or herbal acupuncture stimulation suppresses relapse of cancer patients and reduces adverse effects of radiotherapy through enhancing cellular immunity[8,9]. Hominis placenta (HP) is the dried placenta of a healthy women. The nature of HP is warm and its taste sweet-salty and HP has correspondence to the meridians of lung and kidney in Oriental medicine. In the recent studies, HP has been reported to exhibit a variety of abilities such as protecting osteoporosis[10], promoting the regeneration of injured nerve[11], alleviating arthritic symptoms[7] and asthma[12] and so on. The BL18 acupoint (Ganshu), located middle back part near the spine, is one of the effective acupuncture points with medical effects including anti-metastatic and immune promoting effects[8].

In the present study, it was investigated whether HP acupuncture treatment would suppress the primary tumor growth induced by murine colon 26-L5 cells. The effects of HP

acupuncture were clarified its anti-tumor mechanism with respect to its immunomodulating activities.

Materials and Methods

1. HP herbal acupuncture treatment

HP herbal acupuncture was purchased from Korean Institute of Herbal Acupuncture (KIHA) and then used for the experiments. HP herbal acupuncture treatment was conducted as follows. 100 $\mu\ell$ of HP herbal acupuncture was injected on each BL18 (Ganshu) acupoint of the mice every other day.

2. Animals

Specific pathogen-free female Balb/c mice, 6 weeks old, were purchased from Daehan Biolink Co. (Korea). Mice were acclimated for at least 1 week before experiments. Mice were maintained under specific pathogen-free conditions and used according to institutional guidelines.

2. Cancer cells

Colon26-L5 carcinoma cells were kindly provided by Dr. Saiki I (Toyama University, Japan)[2,13]. Colon26-L5 cells were maintained as monolayer cultures in RPMI-1640 medium (Gibco, NY, USA) supplemented with 10% FBS (JRH Biosciences, KS, USA) at 37℃ in a humidified atmosphere of a 5% CO_2/95% O_2 air.

3. In vivo tumor growth model.

Colon26-L5 cells were harvested, washed and resuspended in PBS. Mice were given subcutaneous injection on the back region with colon 26-L5 cells (5×10^5 cells/100 $\mu\ell$). Three days after tumor cells inoculation, HP herbal acupuncture treatment was conducted on BL18 acupoint every other day for three weeks and tumor sizes were also monitored during experiments. Tumor growth was assessed by measuring with a caliper square along the longer axes(a) and the shorter axes(b). Tumor volumes (mm^3) were calculated by the following formula. Tumor volume (mm^3)= $ab^2/2$.

4. Preparation of mouse splenocytes

HP herbal acupuncture treatment was conducted on BL18 acupoint of Balb/c mice every other day for two weeks and splenocytes were obtained 1 day after the last administration. Splenocytes were obtained by passing pieces of spleen through a cell strainer, treated with a hypotonic solution to lyse erythrocytes, and washed three times with PBS. The viability of the splenocytes 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.

5. Splenocytes proliferation assay

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-Aldrich, MO, USA) or lipopolysaccharide (LPS: Sigma-Aldrich) for 48 hr at 37℃. XTT assay was conducted for assessing cell proliferation[14].

6. FACS analysis

Phenotype characterization of splenocytes was carried out by flow cytometry using a FACS Caliber (BD Biosciences, CA, USA), and Cell Quest software (BD Biosciences). Staining was performed with the following fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labeled monoclonal antibodies (BD Biosciences) : FITC-CD3, PE-CD19, FITC-CD4, PE-CD8, FITC-Mac 1-α, PE-NK1.1.

7. Induction of cytokine production

IFN-γ and IL-4 levels in the culture supernatant were evaluated using specific ELISA kits (BD Biosciences) 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 Con A for 24 hr at 37℃. The cell-free supernatant was collected from each well and stored at -80℃ until the ELISA assay.

8. Statistical analysis

Values are expressed as the mean ± SD or SE, and differences between groups were examined using ANOVA test. Survival rates were calculated by the Kaplan-Meier method. Statistical anaylsis was performed using SPSS software 10.0 (SPSS Inc, IL, USA). A P value < 0.05 was considered statistically significant.

Results

1. Effect of HP herbal acupuncture on tumor growth

First, the effect of HP herbal acupuncture on tumor growth caused by the subcutaneous injection of colon 26-L5 carcinoma cells was examined. Colon26-L5 carcinoma cells were injected subcutaneously in the back part of mice and HP herbal acupuncture treatment was conducted on BL18 acupoint (100 $\mu\ell$/acupoint, 200 $\mu\ell$/mouse) every other day from 3 day after tumor cell inoculation. As shown in Fig. 1, treatment with

HP herbal acupuncture significantly suppressed the tumor growth compared with control group treated with PBS. HP herbal acupuncture did not show any adverse effects including body weight loss during the in vivo experiments (data not shown). These results suggest that HP herbal acupuncture has a potent inhibitory activity against the tumor growth in vivo.

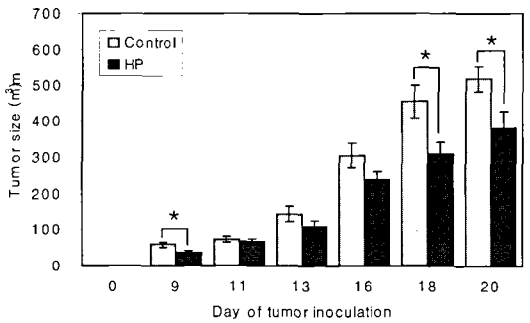

Fig. 1. Effect of HP herbal acupuncture on tumor growth induced by colon26-L5 cells. Mice were subcutaneously inoculated with colon26-L5 cells and treated with 100 ㎕ of HP herbal acupuncture on BL18 (Ganshu) acupoint every other day for 3 weeks from the 3 days after tumor cell inoculation. Tumor size were represented as the mean ± SE of 10 mice in each group. *, P < 0.05.

The effect of time difference on survival by HP herbal acupuncture treatment was also investigated. As shown in Fig. 2, HP herbal acupuncture treatment was significantly increased the overall survival rate (median survival time 33.00 ± 2.12 days) compared to control group (median survival time 24.00 ± 1.15 days).

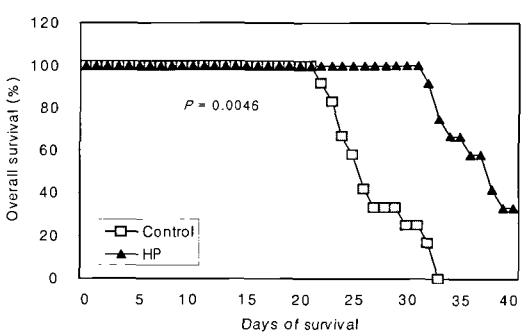

Fig. 2. Effect of HP herbal acupuncture on survival in the tumor growth model induced by colon26-L5 cells. Overall survival of mice with HP herbal acupuncture treatment compared with control group. P value were determined by a log-rank test of Kaplan-Meier survival curves.

2. Effect of HP herbal acupuncture on the proliferation of splenocytes

To clarify the biological properties of HP herbal acupuncture, mitogenic responses of moune spleocytes after treatment of HP herbal acupuncture were investigated. Splenocytes obtained from the control group or HP herbal acupuncture-treated group were cultured with or without T cell mitogen (Con A) or B cell mitogen (LPS) for 48 hr. As shown in Fig. 3, treatment with HP herbal acupuncture resulted in a significant increase of cell proliferation with or without mitogenic stimuli.

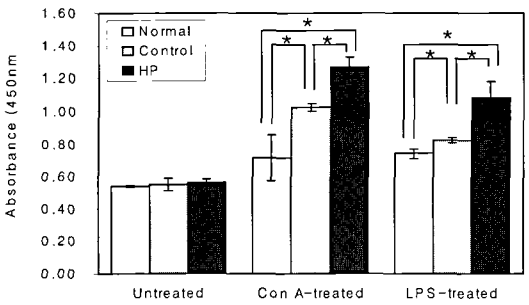

Fig. 3. Effect of HP herbal acupuncture on the proliferation of mouse splenocytes in response to various mitogenic stimuli. Female Balb/c mice were treated with 100 ㎕ of HP herbal acupuncture on BL18 (Ganshu) acupoint every other day for 2 weeks. One day after the last treatment, mice were sacrificed and the splenocytes suspended in complete medium were cultured with or without Con A (1 ㎍/ml) or LPS (1 ㎍/ml) for 48 hr. XTT assay was conducted for assessing cell proliferation. The data represent the mean ± SD of triplicate wells. *, P<0.05.

3. Flow cytometry analysis

Single-cell analysis was performed to verify the surface cell markers with or without HP herbal acupuncture treatment. Although both control and HP herbal acupuncture treatment caused the change of phenotype population, the change induced by HP herbal aucpuncture treatment was higher. HP herbal acupuncture treatment increased CD3, CD19, CD4, CD8 and Mac-1 phenotype of splenocytes. However, NK1.1 phenotype was decreased when control or HP herbal acupuncture was treated.

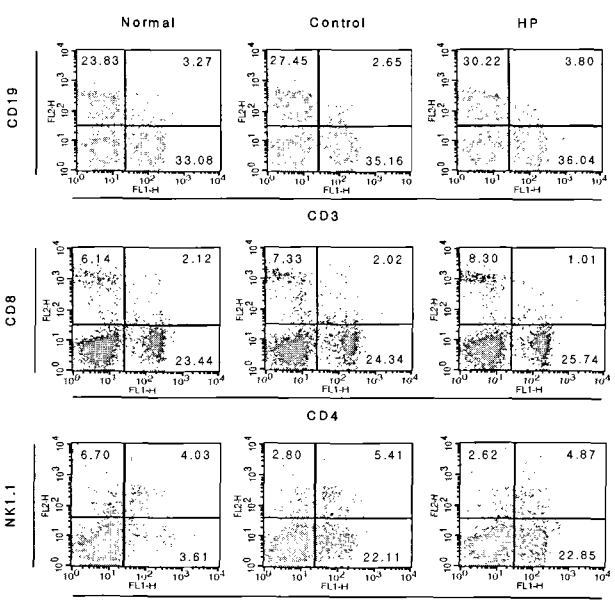

Fig. 4. Effect of HP herbal acupuncture on the expression of cell surface markers. Female Balb/c mice were treated with 100 ㎕ of HP herbal acupuncture on BL18 (Ganshu) acupoint every other day for 2 weeks. One day after the last treatment, mice were sacrificed and the splenocytes were stained with FITC- and PE- monoclonal antibodies. Cell surface antigen was analyzed using a FACS Caliber (BD Biosciences), and Cell Quest software (BD Biosciences). The number in each quadrant indicate the percentage of staining cells.

4. Production of Th1 and Th2 cytokines by splenocytes

The balance of Th1 an Th2 cells in their host is considered to be important for the regulation and induction of

immune functions[15,16]. Therefore, the effect of HP herbal acupuncture on the production of Th1- and Th2-type cytokines by splenocytes was investigated. As shown in Fig. 5, splenocytes from untreated control mice and HP-treated mice produced very little amount of Th1 cytokine (IFN-γ) or Th2 cytokine (IL-4) without Con A stimulation. When splenocytes were incubated with Con A for 24 hr, the amount of cytokine was increased remarkably. The treatment of HP herbal acupuncture resulted in a significant enhancement of IFN-γ and a significant decrease of IL-4 production as compared with that in untreated controls. Thus, HP herbal acupuncture mainly lead predominantly to the production of Th1-type cytokines.

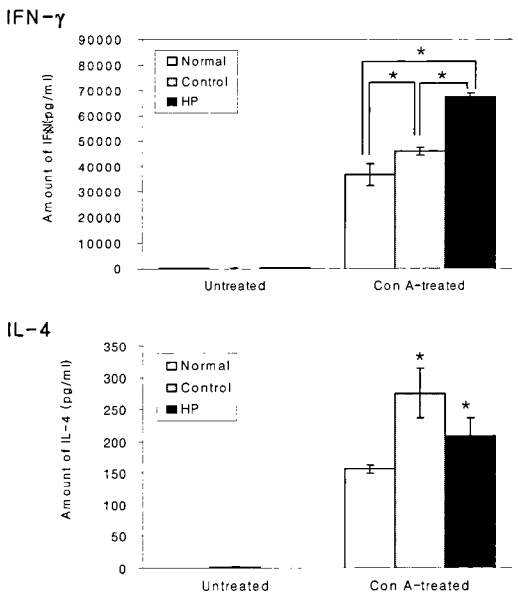

Fig. 5. Effect of HP herbal acupuncture on production of IFN-γ and IL-4 from splenocytes. Female Balb/c mice were treated with 100 μl of HP herbal acupuncture on BL18 (Ganshu) acupoint every other day for 2 weeks. Ond day after the last treatment, mice were sacrificed and the splenocytes suspended in complete medium were cultured in 24-well culture plate with or without Con A (1 μg/ml) for 24 hr. After the termination of culture, the cell-free supernatant was collected and the amount of IFN-γ or IL-4 was measured by ELISA kits. The data represent the mean ± SD of triplicate wells. *, $P < 0.05$.

Discussion

HP has been used as an agent for ameliorating physiological function of the body in many asian countries. Recently, it has been reported that HP herbal acupuncture therapy has various effects such as enhancing Qi (vital energy), nourishing blood and tonifying the essence. Also, HP herbal acupuncture therapy has shown marked therapeutic effects on many diseases such as asthma and rheumatoid arthritis[7,17]. In the present study, anti-tumor effect of HP herbal acupuncture and its mechanism from the viewpoint of immunomodulating activities were investigated. HP herbal acupuncture treatment caused a significant inhibition of the primary tumor growth induced by subcutaneous injection of colon26-L5 carcinoma cells (Fig. 1) without causing any adverse effects such as decrease in body weight (data not shown). Moreover, HP herbal acupuncture treatment significantly prolonged the survival rate of mice (Fig. 2). The median survival time was increased from 24.00 ± 1.15 days (control group) to 33.00 ± 2.12 days (HP herbal acupuncture treated group). These findings indicate that HP herbal acupuncture treatment may have a therapeutic effect on tumor-bearing mouse via preventing the primary tumor growth and extending the lifetime.

On the other hand, HP herbal acupuncture treatment resulted in a marked augmentation of mitogen-stimulated proliferation of splenocytes (Fig. 3). The increase of proliferation was found in both groups treated with T cell mitogen (Con A) and B cell mitogen (LPS) and these means HP herbal acupuncture could activate immune system both cellular and humoral immunity[3]. It is possible that the injected stimulation of herbal acupuncture treatment using 26-G needle attached to a 1-ml syringe could induce immunological reactions. However, the increase of proliferation was higher in HP herbal acupuncture treated splenocytes compared to control group. HP herbal acupuncture induced the differentiation of lymphocytes and increased CD3+, CD19+, CD4+, CD8+, Mac-1+ cells but not NK1.1+ cells (Fig. 4). Specially, the increase of Mac-1+ cells is prominent and then it can be thought HP herbal acupuncture treatment may induce Macrophage-related immune responses[17].

The balance of helper T cell subsets (Th1-Th2) in the host is considered to be important for the regulation and induction of immune functions[18,19]. The balance of Th1 and Th2 patterns of cytokines such as IL-2, IFN-γ, TNF-α, IL-4, IL-5 and IL-10 critically affects the expression of several immune responses. The activity of Th1 cells is related to cellular immunity, whereas Th2 cells are engaged mainly in regulation of humoral response. The deviation of Th1 or Th2 responses is regulated by the balance of IFN-γ/IL-12 and IL-4[20]. HP herbal acupuncture treatment caused the increased production of IFN-γ and decreased production of IL-4 (Fig. 5) by splenocytes stimulated with Con A compared with untreated controls. These results show that HP herbal acupuncture treatment can lead to Th1-dominant immune responses. Also, the anti-tumor activity of HP herbal acupuncture treatment could be explained because IFN-γ secreting Th1 cells are believed to be critical for long-lasting anti-tumor immunity[20].

In conclusion, this study has demonstrated that HP herbal acupuncture treatment may have a therapeutic potential for controlling tumor growth through enhancing its immunomodulating activities. Further study will be required to reveal the molecular mechanism and active contents of HP

herbal acupuncture.

Acknowledgements

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