Original Article

Effects of *Cirsium japonicum var. ussuriense* Extract on Tumor Immunity

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Objective : The present study is aimed to elucidate the effects of *Cirsium japonicum var. ussuriense* on immunomodulation and the potential as an herbal remedy for cancer treatment.
Method : It was performed through measurement of effects *Cirsium japonicum var. ussuriense* extract (CJE) on NO production, NK cell cytotoxicity and cytokine gene expressions related with macrophage and NK cell activity.
Result : 1. CJE did not show any direct cytotoxic effects on 7250, HT1080, Hep G2 and CT-26 cells.
2. CJE activated macrophages partially to product NO and up-regulated gene expressions for iNOS in RAW 264.7 cells.
3. CJE promoted cytotoxicity of NK cells against YAC-1 cells at higher concentration than 200 $ug/m\ell$.
4. CJE up-regulated gene expressions for IL-1β, IL-2, iNOS, IFN-γ and TNF-α in mice splenocytes.
5. CJE inhibited lung tumor metastasis induced by CT-26 cell transplantation compared with the control group.
Conclusion : It could be concluded that CJE is an effective herbal drug for immune modulating and anti-cancer treatment by promoting activity of macrophages and NK cells.

Key Words : *Cirsium japonicum var. ussuriense*, Tumor Immunity

Introduction

Recently, in accordance with rapid progress in the understanding of tumor immunology, a variety of immunotherapeutic strategies has shown that immune manipulation can induce the regression of established cancer in humans.

However, an effective anti-tumor immune response requires an complicated interaction between cancer and immune cells, although it is not still perfectly understood

In this point of view, though enormous efforts were devoted to the research of tumor immunity, it was not successful in clinical use up to now.

On the other hand, many people have used natural products for the treatment of tumor.

The immune modulating and anti-tumor activity of various herbal plants has been investigated extensively and reported all over the world.

These herbs are known to inhibit the tumor incidence or growth, and prolong the survival of the tumor bearing rodent in various experimental models[4-6].

From the viewpoint of Oriental medicine, *Cirsium japonicum var. ussuriense*, a well-known herbal plant, has efficacy of the removing heat to cool blood and stopping blood. Therefore, *Cirsium japonicum var. ussuriense* has been used as a

potentiating hemostatic agent for a long time.

Also, it has been frequently prescribed for patients suffering tumor-related syndrome by some oriental doctors. To date, many studies have been conducted to explore effects of *Cirsium japonicum var. ussuriense* in the various diseases except cancer[7-9]. Therefore, there is strong demand of the characterization of *Cirsium japonicum var. ussuriense* for scientific evaluation of it for anti-cancer properties before clinical usage.

To achieve this, initially, the present study is aimed to elucidate the effects of *Cirsium japonicum var. ussuriense* on immunomodulation and the potential as a potential herbal remedy for cancer treatment. Here, it was performed through measurement of effects *Cirsium japonicum var. ussuriense* extract (CJE) on NO production, NK cell cytotoxicity and cytokine gene expressions related with macrophage and NK cell activity.

Materials and Methods

1. Materials

Cirsium japonicum var. ussuriense is received from Daejeon Oriental Medical Hospital. Fifty grams of *Cirsium japonicum var. ussuriense* was mixed with 2 L of distilled water and left for 1 h at room temperature, and the whole mixture was then boiled twice for 2 h. Then it was filtered and then lyophilized for getting the CJE. The yield CJE was 18.8% (w/w) in terms of the dried medicinal herbs. M-MLV RT, taq. polymerase, dNTP and 5X TBE buffer were obtained from Promega (Madison, USA). Other chemicals were purchased from Sigma (St louis, USA).

2. Experimental animals

Specific pathogen-free BALB/c were obtained from commercial animal breeder (Daehan BioLink,

Korea). The animals were housed under normal laboratory conditions (23 ± 2℃ and 40-60% relative humidity) with 12 h light/dark cycle with free access to standard rodent food and water.

3. Cell culture

RAW 264.7 and HT1080 cell were obtained from Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Hep G2, CT-26 cell were obtained from Korean Cell Line Bank (Seoul, Korea) and human fibroblast, 7250 cell was obtained from National Cancer Institute (USA). The cells were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 units streptomycin and 100 units penicillin.

4. Measure of cytotoxicity

HT1080, Hep G2, 7250, and CT-26 cells (1×10^3) were seeded into 96-well plate and cultured overnight. The cells were treated with CJE (0, 50, 100, 200 ㎍/㎖). CCK-8 (20 ㎕) were added into each well. Three hour later, 150 ul medium was harvested to determine optical density. Cell proliferation was determined on day 0, 2, 3, and 6.

5. NO assay

RAW 264.7 cells were cultured with DMEM containing 10% FBS. RAW 264.7 (5×10^5 cells) were plated in 24-well plates (BD, NJ, USA) and treated with CJE (0, 2, 20, 200 ㎍/㎖) and LPS (1 ㎍/㎖) and incubated at 37℃ with 5% CO2. NO formation was measured as the stable end product nitrite ($NO2-$) in the culture supernatant with Griesse reagent. Briefly, an aliquot of culture supernatant (100 ㎕) was added to each well of 96-well plates and mixed with the same volume of Griesse reagent (1:1[v/v]; 0.1% N-[1-naphth yl]ethylenediamine dihydrochloride in H2O, 1%

Table 1. Oligonucleotide sequences of primers

Gene	Primer	Sequence	Product(bp)
IL-1β	Sense Antisense	5'-AAG CTC TCA CCT CAA TGG A-3' 5'-TGC TTG AGA GGT GCT GAT GT-3'	302
IL-10	Sense Antisense	5'-TCC TTG GAA AAC CTC GTT TG-3' 5'-TCT CTT CCC AAG ACC CAT GA-3'	389
iNOS	Sense Antisense	5'-TGG TGG TGA CAA GCA CAT TT-3' 5'-CTG AGT TCG TCC CCT TCT CTC C-3'	229
TNF-α	Sense Antisense	5'-CTC CCA GGT TCT CTT CAA GG-3' 5'-TGG AAG ACT CCT CCC AGG TA-3'	195
β-actin	Sense Antisense	5'-ACC GTG AAA AGA TGA CCC AG-3' 5'-TCT CAG CTG TGG TGG TGA AG-3'	285

sulfanilamide in 5% H2PO4), and then the A540 was read with microplate leader (Molecular device, USA). Nitrite concentration was determined by using dilutions of sodium nitrite in culture medium as standards. By adding CJE to standard solutions of sodium nitrite, it was confirmed that CJE did not interfere with the nitrite assay.

6. Gene expression of IL-1, IL-10, TNF-α, iNOS in RAW 264.7 cells

RAW 264.7 cells (4×105) were plated into 6 well and treated with various concentration of CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) and incubated for 12 and 24 h at 37℃ with 5% CO2. Total RNA was isolated by the EasyblueℝR reagent and all process of first strand cDNA and polymerase chain reaction were done according to the manufacturer's instructions. The used primers were described in Table 1.

7. NK cell ^{51}Cr release assay

^{51}Cr release assay was performed as described previously with modifications[15]. Spleen cell suspensions were prepared in ice-cold DMEM from BALB/c mice. After adjusting to final concentration (1×10^7 cells/mℓ), 100 $\mu\ell$ of cell suspension (4×10^6, 2×10^6 and 1×10^6 cells/well)

were plated onto the round bottom 96 well plate (4 well per group) with various concentration of CJE (0.2, 2, 20, 200 μg/mℓ) and IL-2 (300 U/mℓ). These cells were incubated for 14 h at 37℃ with 5% CO2 and prepared as effector cells.

On the other hand, YAC-1 cells (5×10^6) were cultured for using as target cells of NK cell. After labeling the target cells by incubating for 2 h (37 ℃, 5% CO₂) with 51Cr (200 μ Ci), washing and lysis of unhealthy cell, the labelled target cells were centrifuged for 5 min at 400 ×g, and adjusted to 2×10^5 cell/mℓ. Fifty microliter cell suspension (1×10^4 cells) was added to effector cells and incubated for 4 h. Maximum leased groups were added with 150 $\mu\ell$ of 2% NP-40, and spontaneous leased group with 150 $\mu\ell$ of complete medium. After 4 h, the cells were concentrated by centrifugation at 500 ×g for 10 min, and cell-free supernatant were harvested from each well for assessment of radioactivity. Then gamma irradiation from each well was assessed in a scintillation counter (Packard Instruments). The percentage of specific lysis was calculated by the following equation:

$$\text{Specific killing activity (\%)} = \frac{\text{CJE release - spont. release}}{\text{max. release - spont. release}} \times 100$$

Table 2. Oligonucleotide sequences of primers

Gene	Primer	Sequence	Product(bp)
IL-1β	Sense Antisense	5'-AAG CTC TCA CCT CAA TGG A-3' 5'-TGC TTG AGA GGT GCT GAT GT-3'	302
IL-2	Sense Antisense	5'-TGC TCC TTG TCA ACA GCG-3' 5'-TCA TCA TCG AAT TGG CAC TC-3'	391
IL-4	Sense Antisense	5'-TCA ACC CCC AGC TAG TTG TC-3' 5'-TGT TCT TCA AGC ACG GAG GT-3'	254
IL-10	Sense Antisense	5'-TCC TTG GAA AAC CTC GTT TG-3' 5'-TCT CTT CCC AAG ACC CAT GA-3'	389
TNF-α	Sense Antisense	5'-CTC CCA GGT TCT CTT CAA GG-3' 5'-TGG AAG ACT CCT CCC AGG TA-3'	195
IFN-γ	Sense Antisense	5'-GGA TAT CTG GAG GAA CTG GC-3' 5'-GAG CTC ATT GAA TGC TTG GC-3'	250
TGF-β	Sense Antisense	5'-TGA GTG GCT GTC TTT TGA CG-3' 5'-TTC TCT GTG GAG CTG AAG CA-3'	310
iNOS	Sense Antisense	5'-TGG TGG TGA CAA GCA CAT TT-3' 5'-CTG AGT TCG TCC CCT TCT CTC C-3'	229
β-actin	Sense Antisense	5'-ACC GTG AAA AGA TGA CCC AG-3' 5'-TCT CAG CTG TGG TGG TGA AG-3'	285

8. Gene expression of IL-1, IL-2, IL-4, IL-10, IL-12, TNF-α, IFN-γ, TGF-β, iNOS in splenocytes

BALB/c mice were sacrificed and spleen were removed to PBS. After RBC lysis, the cells were washed twice with PBS. The splenocytes (2×10^7 cells) were treated with various concentration of CJE (0, 0.2, 2, 20, 200 $\mu g/m\ell$) or LPS (1 $\mu g/m\ell$) in 6 well plate and incubated for 6 and 12 h at 37°C with 5% CO_2. Total RNA was isolated by the Easyblue[R] reagent (Intron, Korea) and all process of first strand cDNA and polymerase chain reaction were done according to the manufacturer's instructions.

Briefly, PCR amplification was carried out in the thermal cycler using a protocol of initial denaturing step at 95°C for 10 min; then 27 cycles for β-actin and 35 cycles for other genes at 95°C for 1 min, 60°C for 40 seconds and 72°C for 40 seconds. The PCR products were run on a 1% agarose gel in 0.5×TBE buffer. The used primers were described in Table 1.

9. Cytokine expression of IL-2, IL-10, TNF-α, IFN-γ in splenocytes

BALB/c mice were sacrificed and spleen were removed to PBS. After RBC lysis with lysing buffer, the cells were washed twice with PBS. The splenocyts (5×10^6 cell) were treated with CJE (0, 2, 20, 200 $\mu g/m\ell$), Con A(0.5 $\mu g/m\ell$), or LPS (0.5 $\mu g/m\ell$) in 24 well plate and incubated for 24 and 48 h at 37°C with 5% CO_2. Supernatant was harvested and cytokines were determined by ELISA kit (BD, USA).

10. Splenocyte proliferation

BALB/c mice were sacrificed and spleen were removed to PBS. After RBC lysis with lysing buffer, the cells were washed twice with PBS. The splenocytes (1×10^6 cells) were seeded into 96 well plate and treated with CJE (0, 2, 20, 200 $\mu g/m\ell$) with 3 $\mu g/m\ell$ Con A (T cell mitogen), or 3

μg/mℓ LPS (B cell mitogen). The plate was incubated for 72 h at 37℃ with 5% CO_2. Cellular proliferation was determined by using CCK-8.

11. Pulmonary colony assay

Mice were divided into 2 groups (10 mice per each group). The mice were treated with 200 mg of CJE per kg body weight and the mice in control group were administrated with distilled water. CJE were treated from 7 days before CT-26 injection to 7 days after CT-26 injection. CT-26 cells (2×10^4) were inoculated to tail vein. The mice were sacrificed 14 days after tumor inoculation and lungs were removed. Lung tumor colonies were counted.

12. Histopathological observations

For the histomorphological evaluation, a portion of lung tissue was removed and fixed in 10% phosphate buffered formalin. The paraplast-embedded lung section (4 μm in thickness) was stained with hematoxylin & eosin (HE) for histopathological examination.

13. Statistical analysis

Results were expressed as the mean ± SD. Statistical analysis of the data was carried out by Student's t-test. A difference from the respective control data at the levels of $p < 0.05$, $p < 0.01$ $p < 0.001$ and $p < 0.0001$ was regarded as statistically significant.
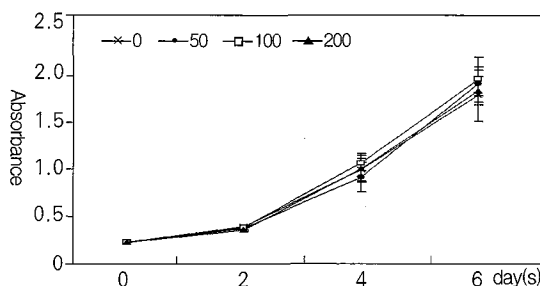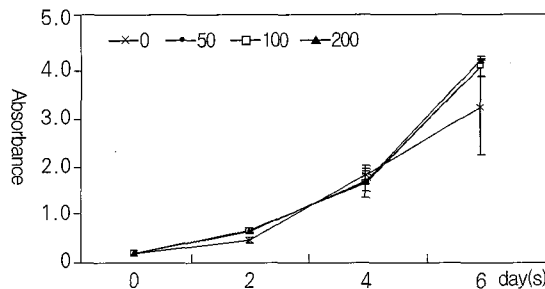

Fig. 1. Effect of CJE on cytotoxicity of 7250 cell. Fibroblast cell, 7250 cells (1×10^3) were seeded into 96 well plate and cultured overnight. The cells were treated with CJE (0, 50, 100, 200 μg/mℓ). OD450-560 was determined at 3 h after CCK-8 addition.
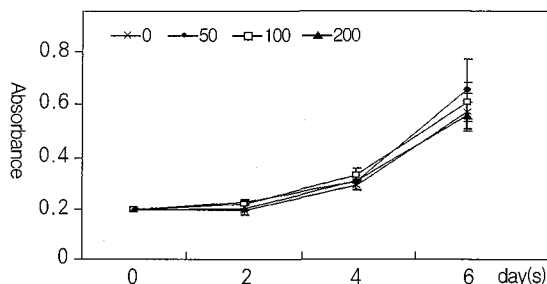

Fig. 2. Effect of CJE on cytotoxicity of HT1080. HT1080 cells (1×10^3) were seeded into 96 well plate and cultured overnight. The cells were treated with CJE (0, 50, 100, 200 μg/mℓ). OD450-560 was determined at 3 h after CCK-8 addition.
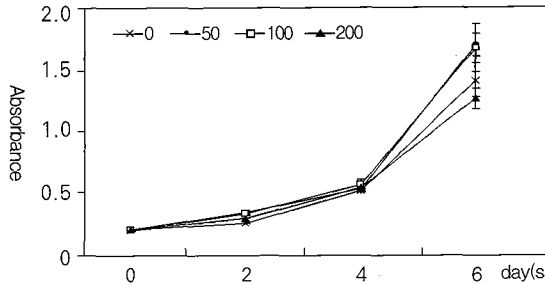
Fig. 3. Effect of CJE on cytotoxicity of Hep G2. Hep G2 cells (1×10³) were seeded into 96 well plate and cultured overnight. The cells were treated with CJE (0, 50, 100, 200 $\mu g/m\ell$). OD450-560 was determined at 3 h after CCK-8 addition.


Fig. 4. Effect of CJE on cytotoxicity of CT-26. CT-26 cells (1×10³) were seeded into 96 well plate and cultured overnight. The cells were treated with CJE (0, 50, 100, 200 $\mu g/m\ell$). OD450-560 was determined at 3 h after CCK-8 addition.

Results

1. Effect of CJE on cytotoxicity

7250, HT1080, Hep G2, CT-26 cells (1×10³) were seeded into 96-well plate and cultured overnight. The cells were treated with various concentration of CJE (0, 50, 100, 200 $\mu g/m\ell$). As shown in Fig. 1-4, any direct affect of CJE on each cell growth was not observed.
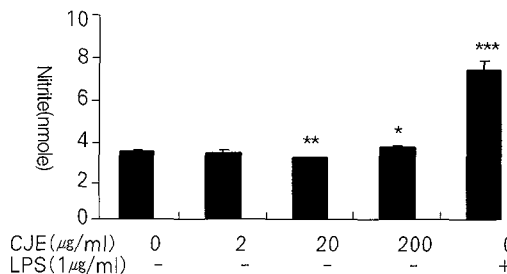
Fig. 5. Effect of CJE on NO production. RAW 264.7 (5×10⁵) were treated with CJE (0, 2, 20, 200 $\mu g/m\ell$) and PBS (control). Each data represents the mean ± SD. Statistically significant value compared with control by T-test. (***: p<0.001, ****: p<0.0001).

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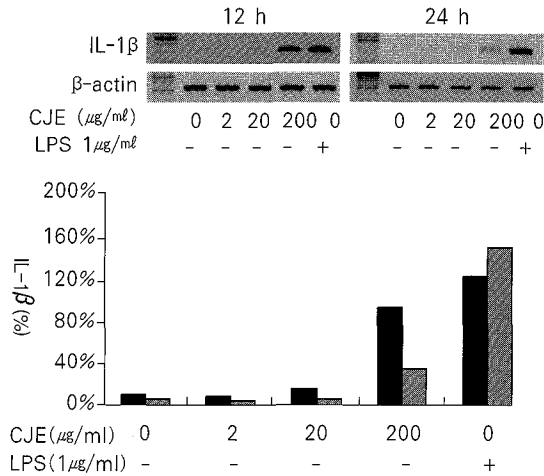
Fig. 6. IL-1β gene expression in RAW 264.7 cells. RAW 264.7 cells were treated with CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 12(■) and 24 h(▒). Total RNA was isolated and RT-PCR was performed. Each data represents as the percentage of IL-1β to β-actin.

2. Effect of CJE on NO production

NO production was measured after treatment of various concentration of CJE (0, 2, 20, 200 μg/mℓ). NO production was significantly decreased at 20 μg/mℓ of CJE. However, CJE increased slightly NO production at the concentration

below 200 μg/mℓ (Fig. 5).

3. Effect of CJE on gene expression in RAW 264.7 cells

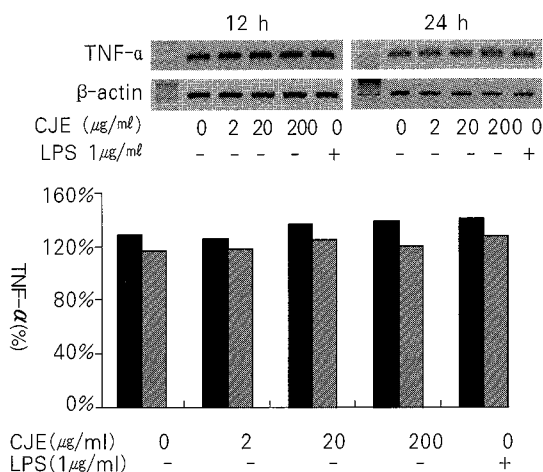To investigate gene expression of cytokines in RAW 264.7, macrophage cell line, RAW 264.7
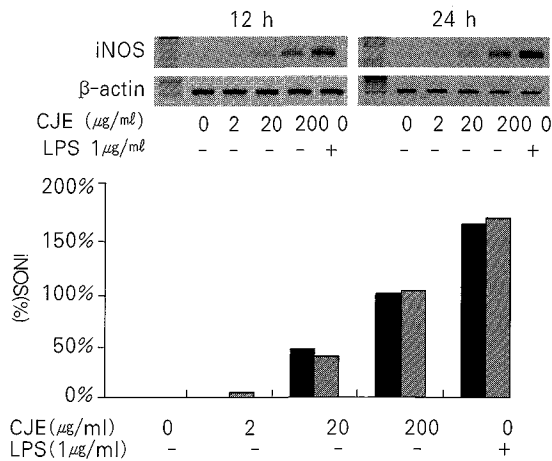
Fig. 7. iNOS gene expression in RAW 264.7 cells. RAW 264.7 cells were treated with CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 12(■) and 24 h(▒). Total RNA was isolated and RT-PCR was performed. Each data represents as the percentage of iNOS to β-actin.
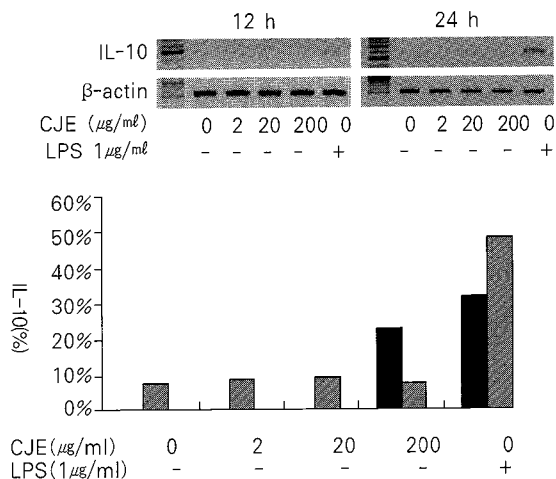
Fig. 8. IL-10 gene expression in RAW 264.7 cells. RAW 264.7 cells were treated with CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 12(■) and 24 h(▨). Total RNA was isolated and RT-PCR was performed. Each data represents as the percentage of IL-10 to β-actin.

cells were treated with various concentration of CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 12 and 24 h. As shown in Fig. 6-9, IL-1β and iNOS mRNA expression were up-regulated significantly by CJE treatment on dose-dependent manner.

After 12 h, IL-1β relative mRNA expression level was increased by 95 % at 200 μg/mℓ CJE whereas 11% at control. CJE up-regulated slightly IL-10 gene expression within 12 h. However, CJE did not affect TNF-α gene expression at all

Fig. 9. TNF-α gene expression in RAW 264.7 cells. RAW 264.7 cells were treated with CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 12(■) and 24 h(▨). Total RNA was isolated and RT-PCR was performed. Each data represents as the percentage of TNF-α to β-actin.

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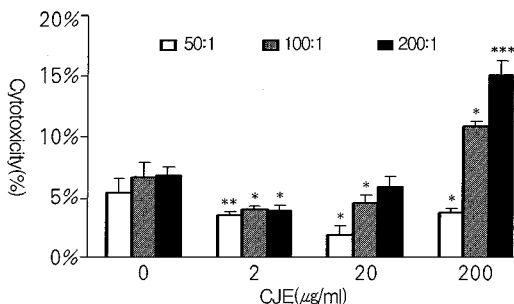
Fig. 10. The Effect of CJE on NK cell activity. Spleen cells (effector cell) were treated with CJE (2, 20, 200 μg/mℓ) and IL-2 (300 U/ml) for 14 h. Yac-1 cells (target cell) labeled with ^{51}Cr were mixed to effector cells for 4 h. Cell-free supernatant containing released ^{51}Cr was counted by using gamma scintillating counter. Each data represent the mean±SD. Statistically significant value compared with control by T-test.(*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

concentrations of CJE.

3. Effect of CJE on NK cell activity

CJE showed a significant effect on NK cytotoxic activity compared with control at 200 μg/mℓ CJE at ratio of 100:1 and 200:1 (effector cell to target cell). AT ratio of 200 (effector cell) : 1 (target cell) and 100:1, CJE (200 μg/mℓ) increase the NK cytotoxic activity significantly by 11% and 16%

respectively whereas 7.2% in control. However, there is not effect at ratio of 50:1 at all concentration of CJE.

4. Effect of CJE on gene expression in splenocytes

To investigate gene expression of cytokines in murine splenocytes, splenocytes were isolated from BABL/c and treated with various concentration
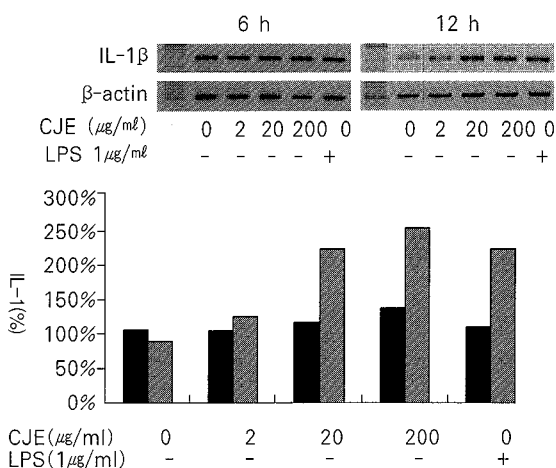
Fig. 11. IL-1β gene expression in splenocytes. Splenocytes were treated with CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▨). The gene expression was shown as the percentage to β-actin.
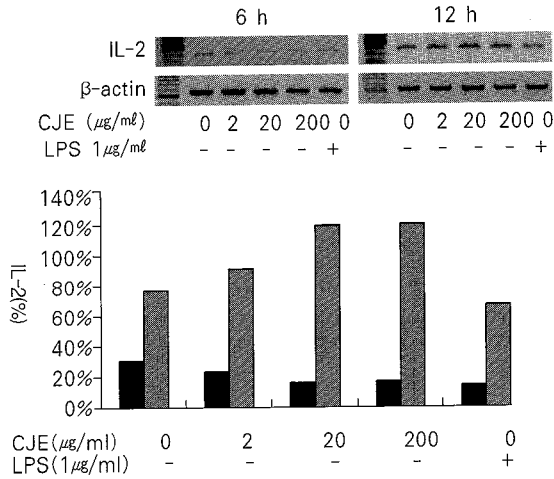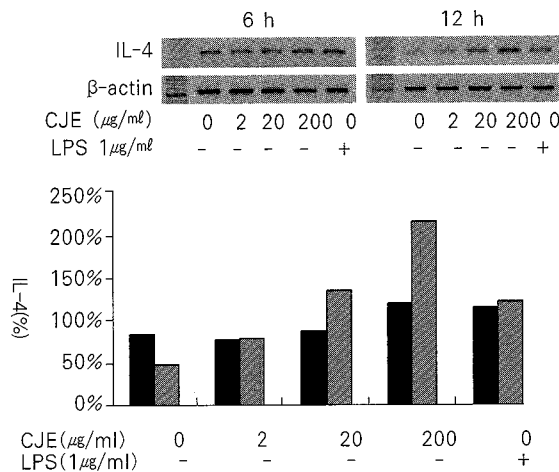
Fig. 12. **IL-2 gene expression in splenocytes**. Splenocytes were treated with CJE (0, 2, 20, 200 $\mu g/m\ell$) or LPS (1 $\mu g/m\ell$) for 6(■) and 12 h(▨). The gene expression was shown as the percentage to β-actin.

of CJE (0, 2, 20, 200 $\mu g/m\ell$) or LPS (1 $\mu g/m\ell$) for 6 and 12 h. IL-1β and IL-2 mRNA expression were up-regulated on dose-dependant manner at 12 h after CJE treatment. IL-4 gene expression also increased slightly by CJE at all concentrations of CJE. IL-6, and IL-10 were slightly up-regulated by CJE. iNOS and TNF-α were up-regulated at 12 h.

CJE up-regulated IFN-γ gene expression remarkably and dose-dependantly. CJE treatment did not altered IL-12 and TGF-β gene expression.

5. Effect of CJE on cytokine production in splenocytes
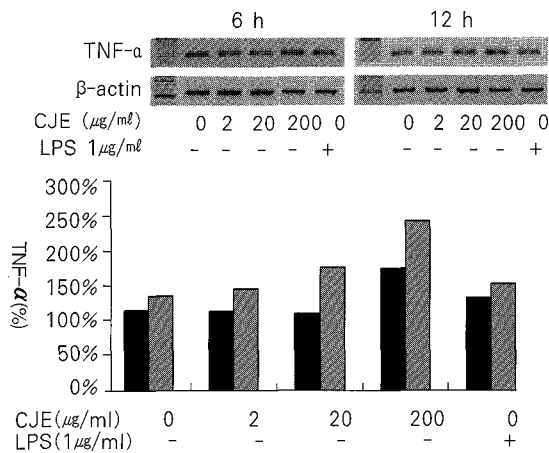
To investigate protein expression in mouse

Fig. 13. **IL-4 gene expression in splenocytes**. Splenocytes were treated with CJE (0, 2, 20, 200 $\mu g/m\ell$) or LPS (1 $\mu g/m\ell$) for 6(■) and 12 h(▨). The gene expression was shown as the percentage to β-actin.
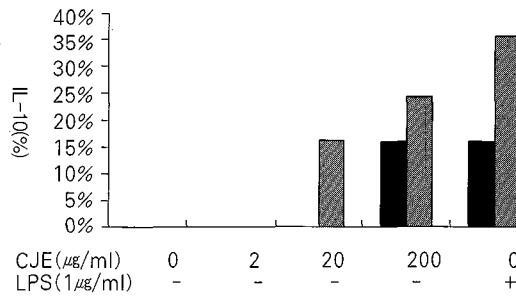
Fig. 14. IL-10 gene expression in splenocytes. Splenocytes were treated with CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▨). The gene expression was shown as the percentage to β-actin.
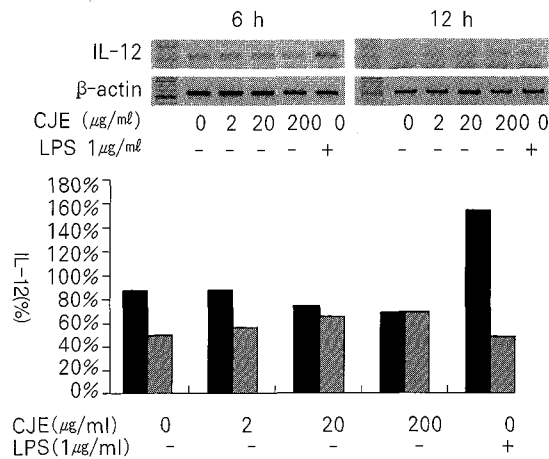
Fig. 15. IL-12 gene expression in splenocytes. Splenocytes were treated with CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▨). The gene expression was shown as the percentage to β-actin.

Fig. 16. TNF-α gene expression in splenocytes. Splenocytes were treated with CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▨). The gene expression was shown as the percentage to β-actin.
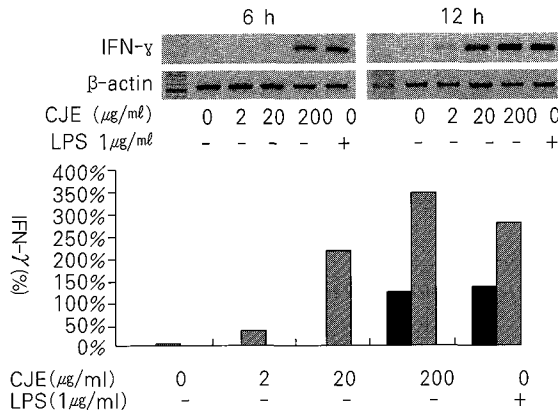
Fig. 17. IFN-γ gene expression in splenocytes. Splenocytes were treated with CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▨). The gene expression was shown as the percentage to β-actin.
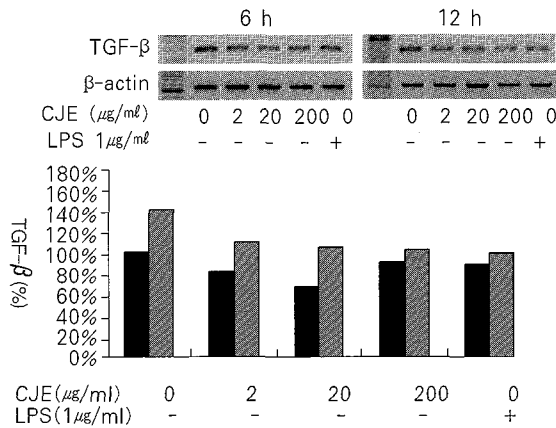

Fig. 18. TGF-β gene expression in splenocytes. Splenocytes were treated with CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▨). The gene expression was shown as the percentage to β-actin.
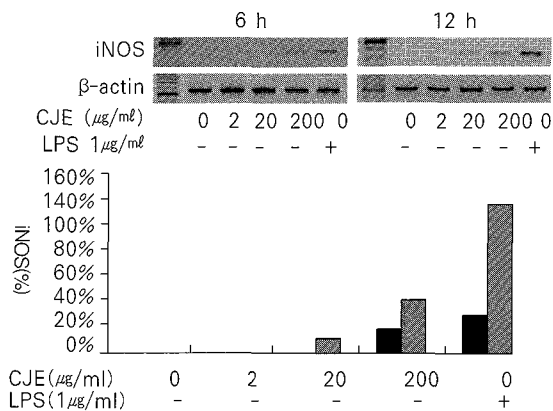

Fig. 19. iNOS gene expression in splenocytes. Splenocytes were treated with CJE (0, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 6(■) and 12 h(▨). The gene expression was shown as the percentage to β-actin.
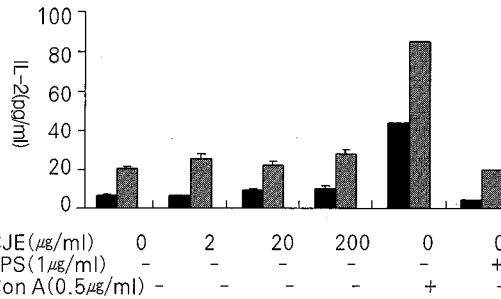
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Fig. 20. IL-2 protein expression in splenocytes. Splenocytes were treated with CJE (0, 2, 20, 200 μg/mℓ), Con A (0.5 μg/mℓ) or LPS (0.5 μg/mℓ) for 24(■) and 48 h(▨). The supernatant were used for analysis of protein expression. The results were expressed as mean ± SD.
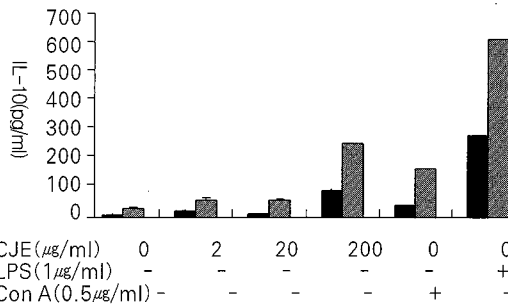
Fig. 21. IL-10 protein expression in splenocytes. Splenocytes were treated with CJE (0, 2, 20, 200 μg/mℓ), Con A (0.5 μg/mℓ) or LPS (0.5 μg/mℓ) for 24(■) and 48 h(▨). The supernatant were used for analysis of protein expression. The results were expressed as mean ± SD.

splenocytes, splenocytes were treated with various concentration of CJE (0, 2, 20, 200 μg/mℓ) and LPS (1 μg/mℓ) for 24 h and 48 h. Protein expression was determined by ELISA kit. IL-2 expression was not altered by CJE treatment. CJE increased IL-10 expression continuously within 48 h and dose-dependantly. TNF-α expression also increased by CJE treatment only at 200 μg/mℓ. IFN-γ
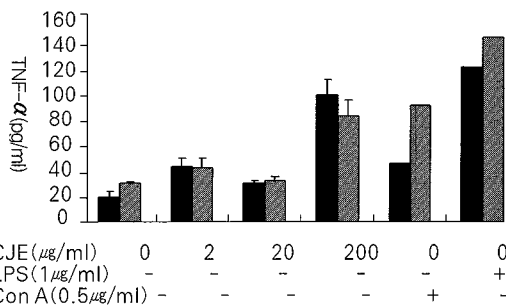

Fig. 22. TNF-α protein expression in splenocytes. Splenocytes were treated with CJE (0, 2, 20, 200 μg/mℓ), Con A (0.5 μg/mℓ) or LPS (0.5 μg/mℓ) for 24(■) and 48 h(▨). The supernatant were used for analysis of protein expression. The results were expressed as mean ± SD.

Fig. 23. IFN-γ protein expression in splenocytes. Splenocytes were treated with CJE (0, 2, 20, 200 μg/mℓ), Con A (0.5 μg/mℓ) or LPS (0.5 μg/mℓ) for 24(■) and 48 h(▨). The supernatant were used for analysis of protein expression. The results were expressed as mean ± SD.
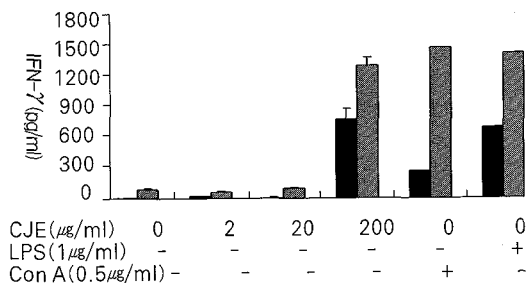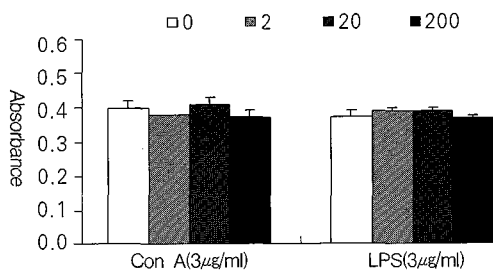
expression also increased significantly. Six hours and 12 h after CJE (200 μg/mℓ) treatment, IFN-γ level increased by 795 and 1340 pg/mℓ respectively whereas 13 and 91 pg/mℓ in control.

6. Effect of CJE on splenocyte proliferation

To understand other immunomodulatory activity of CJE, we investigated the effect of CJE on lymphocyte proliferation. As shown Fig. 24. CJE

Fig. 24. Effect of CJE on cell proliferation. Splenocytes (1×10^6) were seeded into 96 well plate and treated with CJE (0, 2, 20, 200 μg/mℓ) with Con A (T cell mitogen), or 3 μg/mℓ LPS (B cell mitogen). The plate was incubated for 72 h at 37℃ with 5% CO2. Cellular proliferation was determined by using CCK-8.
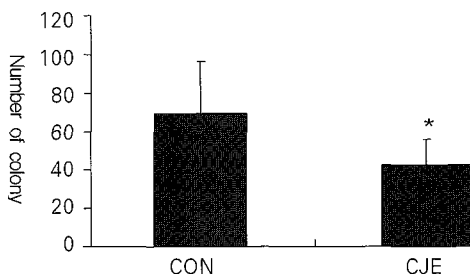
Fig. 25. Pulmonary colony assay. Each group were administered with water (CON), or CJE (200 mg/5ml/kg) from 7 days before CT-26 (2×10^4 cells) injection to 7 days after CT-26 injection. The mice were sacrificed 14 days after tumor inoculation, and lungs were removed and tumor colonies were counted. The results were expressed as mean ± SD. Statistically significant value compared with control by T-test.(*: $p < 0.05$).

did not affect T and B cell proliferation.

7. Effect of CJE on inhibition of pulmonary colony

The mice were treated with 200 mg of CJE per kg body weight and the mice in control group were administrated with distilled water. Pulmonary colonization was observed on day 14 after CT-26 inoculation. In CJE treatment group, tumor implantation was inhibited by 46% compared with control group.
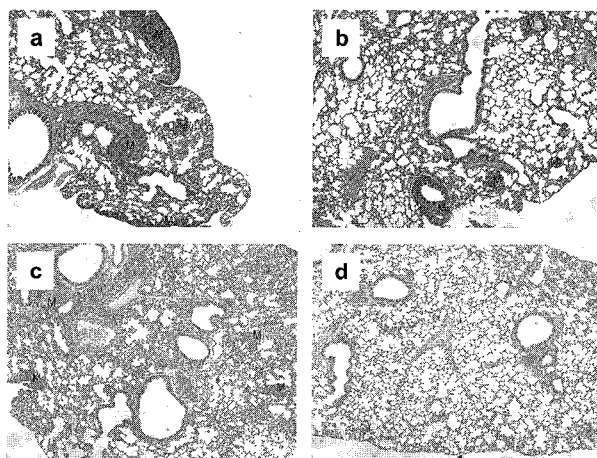
8. Histopathological finding

After 14 days of tumor inoculation, metastatic foci in lung were observed in both control and CJE treated group. In CJE treated group, the number of metastatic foci was decreased compared with control group and the size of metastatic foci also smaller than that of control group.

Discussion

Cirsium japonicum var. ussuriense has been used as a medicinal plant showing clinical efficacy as a hemostatic, and anti-inflammatory or analgesic remedies for a long time. Recently, it has been frequently prescribed to control tumor-related syndrome with expectation of the anti-tumor effects. But, there is no scientific data proving anti-cancer properties of *Cirsium japonicum var. ussuriense* so far, which is demanding the characterization of *Cirsium japonicum var. ussuriense* for rational prescription and further research forward anti-cancer drug development.

First of all, it is likely that studying immune activities of *Cirsium japonicum var. ussuriense* especially affecting macrophage, NK cell and cytokines secretion is essential for immune-based characterization of this plant. This is proposed by the fact that among immune system, innate immune system is more important than others, because most of tumors have multiple mechanisms for evading immune surveillance and specific immune system of host dose not response tumor cell effectively[10-11].

Fig. 26. Histopathological examination of lung of mice inoculated CT-26. Each group were administered water (upper), or CJE (under) from 7 days before CT-26 (2×10^4 cells) injection to 7 days after CT-26 injection. The mice were sacrificed 14 days after tumor inoculation, and lungs were removed and fixed in 10% phosphate buffered formalin. The paraplast-embedded lung section (4 μm in thickness) was stained with hematoxylin & eosin (H&E). M is the metastasis region of CT-26.

Recently, substantial attention has been paid to the role of NK cell and macrophages in regulating the innate immune response, because they are the most important non-specific effector cells contributing on anti-tumor process against generally less immunogenic cancer cells without previous notice[12-16].

Therfore, in this present study, we aimed at exploring *Cirsium japonicum var. ussuriense* extract(CJE) with its immune activities related macrophage and NK cells, and cytokines connected with them.

First, to know the direct effect of CJE on inhibition of cancer cell growth including normal cell, we measured cytotoxicity for 7250, HT1080, Hep G2 and CT-26 cells. CJE didn't affect on each cell growth.

Next, we evaluated the effects of CJE on activation of macrophage by detection of NO release from RAW 264.7 cells treated with CJE. Activated macrophage expresses inducible nitric oxide synthase (iNOS), whose product (NO) has major role in bactericidal and anti-tumor function[17-19]. NO production was significantly increased only at 200 $\mu g/m\ell$ of CJE. Although CJE did not affect on NO production in concentration lower than 20 $\mu g/m\ell$, CJE enhanced iNOS gene expression on dose-dependant manner.

In gene expression of cytokines in macrophage (RAW 264.7 cells), TNF-α gene expression were almost not affected by CJE treatment, but, IL-1β mRNA expression was up-regulated significantly at 20 and 200 $\mu g/m\ell$ of CJE. IL-1, secreted by activated macrophage, usually prone to act for pro-inflammatory contrary to IL-10 acting generally as a immunosuppressive mediators[17-19].

For know the effects of CJE on activation of NK cell, we next measured cytotoxicity against Yac-1 cell, and gene expression of cytokines with whole splenocytes. Also some of cytokines were checked at the level of secreted proteins from total splenocytes after treatment by CJE.

CJE significantly promoted NK cell activity to lysis the Yac-1 cells especially at 200 $\mu g/m\ell$ at ratio of 100:1 and 200:1 (effector cell to target cell). In gene expression, TGF-β and IL-12 were not affected by CJE. TGF-β has been know as bi-directed, tumor suppressive at early stages and tumor promotor at stage of invasiveness or metastasis respectively[20-22], and IL-12 leads to eradication of the tumor through a macrophage and NK-dependent interactions partially connected with IFN-γ secretion[23]. IFN-r can be secreted by NK cell or activated Th1 cell, and strongly activate macrophage and NK cell itself, which mainly roles in tumor cell destroying. IFN-γ production is enhanced most clearly in the presence of IL-1 and IL-2 and in the presence of IL-2 and IL-12[24-26].

CJE showed a significant increase IL-1 and iNOS in splenocytes same as in RAW 264.7 cells. Also, CJE up-regulated IL-2 gene expression on dose-dependant manner partially consistent with secreted IL-2 protein. Among measured cytokines, IL-10 and TNF-α gene expression were up-regulated in protein levels, too. Especially, CJE up-regulated IFN-γ gene expression remarkably and dose-dependantly. CJE did not affect T and B cell proliferation activated by LPS or Con A.

These results above indicate that CJE may have anti-tumorigenic property by promotion of the NK cell activity connected with activation of IL-1, IL-2, TNF-α and IFN-r.

Finally, CJE significantly inhibited pulmonary colonization of CT-26 cells compared with control group in vivo. In CJE treated group, the number and size of metastatic foci were decreased compared with control group in histopathological observation

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after 14 days of tumor inoculation.

From the results above, it could be concluded that CJE has significant properties to activate macrophages and NK cells by changing the quantity of cytokines like IL-1, IL-2, IFN-r, iNOS and TNF-α especially at high concentration around 200 $\mu g/m\ell$. It can be a candidate for anticancer drug development, and it is remained to investigate their mechanisms of action and therapeutic potential in clinical practice.

Conclusion

This study was carried out to evaluate immune modulating and anti-tumor activity of CJE related with macrophage and NK cell activity through analysis of NO production, NK cytotoxicity and gene expressions of cytokine connected with them.

The results were as follows.

1. CJE didn't show any direct cytotoxic effects on 7250, HT1080, Hep G2 and CT-26 cells.

2. CJE activated macrophages partially to product NO and to upregulate gene expression for iNOS in RAW 264.7 cells.

3. CJE promoted cytotoxicity of NK cell against YAC-1 cells at higher concentration than 200 $\mu g/m\ell$.

4. CJE up-regulated gene expression for IL-1β, IL-2, iNOS, IFN-γ and TNF-α in mice splenocytes.

5. CJE inhibited lung tumor metastasis induced by CT-26 cell transplantation compared with control group.

From above results, it could be concluded that CJE is an effective herbal drug for immune modulating and anti-cancer by promoting activity of macrophages and NK cells.

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