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Original

Anti-Tumor Activity of Traditional Chinese Medicine, Ekki-Youketsu-Fusei-Zai, and Its Effects on Immunocyte Functions

2. Induction of Morphological Changes of Murine Macrophage Cell Line J774.1 and Enhancement of Their IL-12 Production by Traditional Chinese Medicine, Ekki-Youketsu-Fusei-Zai

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Abstract: Ekki-Youketsu-Fusei-Zai (EYFZ), one of the Traditional Chinese Medicines (TCMs) is a mixture of six kinds of crude drugs. It was already found that an oral administration of EYFZ had a life-prolonging effect on tumor-bearing mice and an inhibitory influence on tumor growth. To elucidate the mechanisms of these anti-tumor effects, we first investigated the effect of EYFZ on morphology and cytokine production of murine macrophage-like cell line, J774.1 cells. In this experiment, the addition of EYFZ (1.2mg crude drug/ml) to J774.1 culture induced the morphological change from round type to spread type, also the increase of their size. We next examined 6 kinds of cytokine mRNA expression by J774.1 cell in response to EYFZ-stimulation. RT-PCR revealed that both interleukin-12 (IL-12)p35 and IL-12p40 mRNA expression were induced by EYFZ treatment. Moreover, EYFZ promoted the secretion of IL-12 from J774.1 cells. These results strongly suggest that EYFZ with some significant anti-tumor effect induces the functional activation of J774.1 cells.

Keywords: murine macrophage cell line J774.1, Traditional Chinese Medicine (TCM), Ekki-Youketsu-Fusei-Zai (EYFZ), morphological change, IL-12 mRNA expression, IL-12 production.

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Introduction

Macrophages are known to be activated *in vitro* by a variety of agents, leading to an increase of metabolism, syntheses and release of lysosomal enzyme, phagocytosis, and anti-mitotic or anti-tumor effects. Many of these agents are generally potent immunological adjuvants or immunopotentiators, some of which are natural products called biological response modifiers (BRM)¹⁾ and possess functions affecting immune system and host defense mechanisms. BRM also alters the host biological responses²⁾, and is effective in suppressing tumor cells³⁾. Among these products, bacterial lipopolysaccharides (LPS) is one of the strongest activators of macrophage^{4,5)}. To verify the mechanisms underlying the LPS action on macrophage,

murine macrophage-like cell line J774.1 has been so far used in many investigations⁶⁻¹⁴⁾.

Though one of BRMs, Traditional Chinese Medicine (TCM), is an undefined crude extract from a mixture of many plants, animals and minerals, the prescription is defined for each TCM. There have been many reports describing the biological actions of TCMs on immune responses in mammals¹⁵⁻²¹⁾. Ekki-Youketsu-Fusei-Zai (EYFZ), one of the TCMs, is a mixture composed of extract of six kinds of crude drugs; *Astragali radix*, *Zizyphi fructus*, *Amomi semen*, *Angelica radix*, *Cervi parvum cornu*, and *Rehmanniae radix*. EYFZ has frequently been prescribed for the treatment of cancer patients in China. Recently, we demonstrated that an oral administration of EYFZ had a life-prolonging effect on tumor-bearing mice and an inhibitory influence on

tumor size²²). However, the biological action of EYFZ on murine immune response *in vitro* has not been investigated.

Previously, we tried to examine the effects of oral administration of EYFZ on the functions of peritoneal macrophage in tumor-bearing BALB/c mice, it was difficult to get some effects of EYFZ on macrophage functions. For example, first, mice got weak after the implantation of colon-26 cell, and it was difficult to collect enough cells of resting macrophage without thiolycollate-stimulation. Secondly, since the thiolycollate itself strongly stimulated the resting peritoneal macrophage in tumor-bearing mice, we could not find any difference between the EYFZ-administrated and control mice on functions of peritoneal macrophage. Thirdly, J774.1 macrophage-like cell line is originated from the BALB/c mice and can be used for study of macrophage function *in vitro* and were also reported to show the same cytokine production as the macrophages²³⁻²⁶. That's because, in the present study, we examined the effect of EYFZ on morphological changes and cytokine production of J774.1 *in vitro* to elucidate the mechanisms underlying the EYFZ action on immune responses.

Materials and Methods

Preparation of EYFZ EYFZ is a mixture of six kinds of crude drugs. All of the crude drugs were purchased from Tochimoto (Osaka, Japan). EYFZ (dose/person/day) was prepared as follows. First, a mixture of *Astragali radix* (10.0 g), *Zizyphy fructus* (5.0 g), *Amomi semen* (5.0 g), *Angelica radix* (8.0 g), *Cervi parvum cornu* (5.0 g), and *Rehmanniae radix* (10.0) was added to 200 ml distilled water (DW) and soaked for 20 min at room temperature. Then, it was boiled for 30 min for extraction of effective substances, and the solution was centrifuged at 3000 rpm for 20 min. Finally, the supernatant was condensed to 43 ml (1 g crude drug/ml) by evaporation, and diluted with DW to desired concentrations.

Cell culture The murine macrophage-like cell line, J774.1, was obtained from Riken Cell Bank (RCB, Japan). The cells were maintained in 75 cm² plastic culture flasks (Falcon) in RPMI-1640 medium (Roswell Park Memorial Institute) with kanamycin (5μg/ml, Sigma) and 5% fetal bovine serum. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Cells harvested by gentle scraping were passaged every 3-4 days by diluting 1:10 with fresh medium.

Observation of morphological changes of J774.1

J774.1 cells were suspended in culture medium at a concentration of 5 × 10⁴ cells/ml, and 5 ml of cell suspension is plated in a 25 cm² plastic culture flask (Falcon). The cells were incubated with 1.2 mg crude drug/ml of EYFZ, 10 ng/ml lipopolysaccharide (LPS) or culture medium only for 3, 6, 12 hrs at 37 °C in 5% CO₂ in air. After incubation, the cell morphology was observed under a phase-contrast microscope (model ND; Nikon, Tokyo, Japan), and photographed in random fields.

RT-PCR J774.1 cells were suspended in culture medium at a cell concentration of 5 × 10⁴ cells/ml, and 5 ml of the cell suspension was plated in a 25 cm² plastic culture flask (Falcon). Cells were cultured for 24 and 48 hrs in the presence of 1.2 mg crude drug/ml of EYFZ, 10 ng/ml LPS or culture medium only. After incubation, cells were harvested by scraping, and quickly frozen and stored at -80 °C. Total cellular RNAs were extracted from 1 × 10⁶ J774.1 cells by the use of RNasy Mini Kit (QIAGEN). The first strand cDNA synthesis was performed by incubating 0.5 μg of either RNA sample in a total reaction volume of 20 μl containing 2 μl of 10 mM dNTP mixture, 2 μl of 10mM oligo-(dT), 10X reaction buffer for AMV reverse transcriptase, and 4 units (U) of AMV reverse transcriptase XL (TOYOBO, Japan) at 42 °C for 1hr. The sequence of oligo-(dT) was 5'-GCTCTAGATTTTTTTTTTTTTTTTTTTTTTTT-3'. Two μl of the first strand synthesis production and 2 μl each of 10 mM oligonucleotide primers (Table 1) were added to the reaction mixture (100 μl) containing 8 μl of 10 mM dNTP mixture, 8 μl of 25 mM MgCl₂, 10X Ex Taq™ buffer, and 4 units (U) of Ex Taq™ (TAKARA, Japan). Each reaction was carried out as follows; IL-1β; denaturing at 94 °C for 5 min, 30 cycles of amplification (94 °C for 1 min, 58 °C for 2 min, and 72 °C for 3 min) and extension at 72 °C for 10 min. IL-12p40 and IFN-γ; denaturing at 94 °C for 5 min, 30 cycles of amplification (94 °C for 40 sec, 60 °C for 20 sec, and 72 °C for 49 sec) and extension at 72 °C for 5min. IL-12p35, TNF-α and β-actin; denaturing at 94 °C for 5 min, 30 cycles of amplification (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min) and extension at 72 °C for 7 min.

Measurements of IL-12 IL-12 production by J774.1 cells was assayed by using ELISA kits OptEIA™ Mouse IL-12 (p70) Set (PHARMINGEN) according to the protocols by manufacturer. Cells were cultured for 24, 48 and 72 hrs in the presence of 0.15 mg/ml, 1.2 mg/ml and 3.6 mg crude drug/ml of EYFZ, 10 ng/ml LPS or culture medium only. After incubation, the culture

supernatants were collected and stored at -80°C until use.

Statistical Analysis A statistical significance of difference between the control and experimental group was analyzed by Students t-test.

Results

Morphological changes caused by EYFZ To elucidate the effect of EYFZ on J774.1 cells, morphological changes were observed under a phase-contrast microscope after incubation with EYFZ for 3, 6, 12 hrs (Fig. 1). EYFZ at 1.2 mg crude drug/ml caused great changes in their size and shapes; while J774.1 cells in the presence of EYFZ became spread and/or elongated with numerous vacuoles and granules inside, the cells without EYFZ mostly remained round with some protrusions. We counted the number of spread and round cells in each condition (Fig. 2), and also measured the diameter of each cell (Fig. 3). As shown in Fig. 2, the number of round type cells in all these conditions after incubation for 3 hrs was over 70 % respectively, while the number of spreads types of cells with EYFZ and that with LPS for 12 hrs significantly increases compared with that of control. Similarly, the diameter of most cells after incubation with EYFZ and also with LPS for 3 hrs was below $20\ \mu\text{m}$ which was almost similar to that of the control, while after 12hrs over 70 % of the cells stimulated with EYFZ and also those with LPS had diameter over $21\ \mu\text{m}$. Because the

growth of J774.1 cells stimulated with EYFZ stopped, the number of the cells after incubation with EYFZ for 12hrs was smaller than the control. These results show that J774.1 cells change greatly in shape and size in response to EYFZ, suggesting that EYFZ induced the activation of J774.1 cells.

The effect of EYFZ on the expression of cytokine mRNAs The cytokine mRNA expression of J774.1 cells treated with or without EYFZ was investigated. The gel electrophoretic patterns of the RT-PCR products of IL-1 β , IL-12p35, IL-12p40, IFN- γ , TNF- α and β -actin are presented in Fig. 4. The expression level of IL-12p35 and IL-12p40 was induced in J774.1 cells after 12hrs and 24hrs of the treatment with EYFZ, though the expression of β -actin, a house keeping gene, was almost constant in each sample. We could not find any differences of other cytokine expression between treated cells and untreated cells.

EYFZ stimulates J774.1 cells to produce IL-12

We next examined whether this increased expression of IL-12 induced by EYFZ occurs at the protein level. After J774.1 cells were incubated with 0.15 mg/ml, 1.2 mg/ml and 3.6 mg crude drug/ml of EYFZ for 24, 48 and 72 hrs, IL-12 in the culture supernatants were assayed by ELISA. As shown in Fig. 5, IL-12 secreted from J774.1 cells stimulated with EYFZ in the culture supernatants was significantly higher in concentration compared to that of the control.

Table 1. Sequence of Primer Sets for Mouse Cytokine Genes

Cytokine		Sequence	Predicted size(bp) of cDNA
IL-1 β	sense	TGACGGACCCCAAAGATGAAG	658
	antisense	CTGCTTGTGAGGTGCTGATGTA	
IL-12p35	sense	GTGAAGACGGCCAGAGAAAA	308
	antisense	TCTGAAGTGCTGCGTTGATG	
IL-12p40	sense	ATGTGGGAGCTGGAGAAAGA	335
	antisense	ACAGAGACGCCATTCCACAT	
IFN- γ	sense	CATTGAAAGCCTAGAAAGTCTG	267
	antisense	CTCATGAATGCATCCTTTTTCG	
TNF- α	sense	ACAGAAAGCATGATCCGCGA	364
	antisense	TCTTTGAGATCCATGCCGTTG	
β -actin	sense	ATGGATGACGACATCGCTG	669
	antisense	CATGAGGTAGTCTGTCAGGT	

Discussion

In this study, we examined the activation of murine macrophage-like cell line J774.1 after EYFZ-treatment, and the results suggest that J774.1 cells were activated by EYFZ and expressed IL-12. We also showed that J774.1 cells stimulated with EYFZ secreted IL-12 in the culture supernatant. J774.1 cells are originated from BALB/c mice and have almost the same characterizations with the *in vivo* macrophages in the immunological function²³⁻²⁶), so it seems to be considered that these results will be applicable to the *in vivo* macrophages.

Macrophages are involved at almost all stages of the immune response and play a role in the initial response

to microbial infection before T- and B cell immunity is mobilized. Mechanisms by which macrophages act as effector cells in a host defense system include both intracellular and extracellular cytokine secretion activities. Thus, we investigated here whether EYFZ could stimulate the secretion of cytokine from J774.1.

As shown in this study, EYFZ was able to induce IL-12 production by J774.1. IL-12 has recently been brought into focus as an effector molecule to enhance immunity by murine macrophages²⁷⁻²⁹). Production of IL-12 by macrophages can be induced by interaction with activated T cells, which provide costimulatory signals via molecules such as CD40 ligand. These signals appear to be essential, because their inhibition can abrogate IL-12

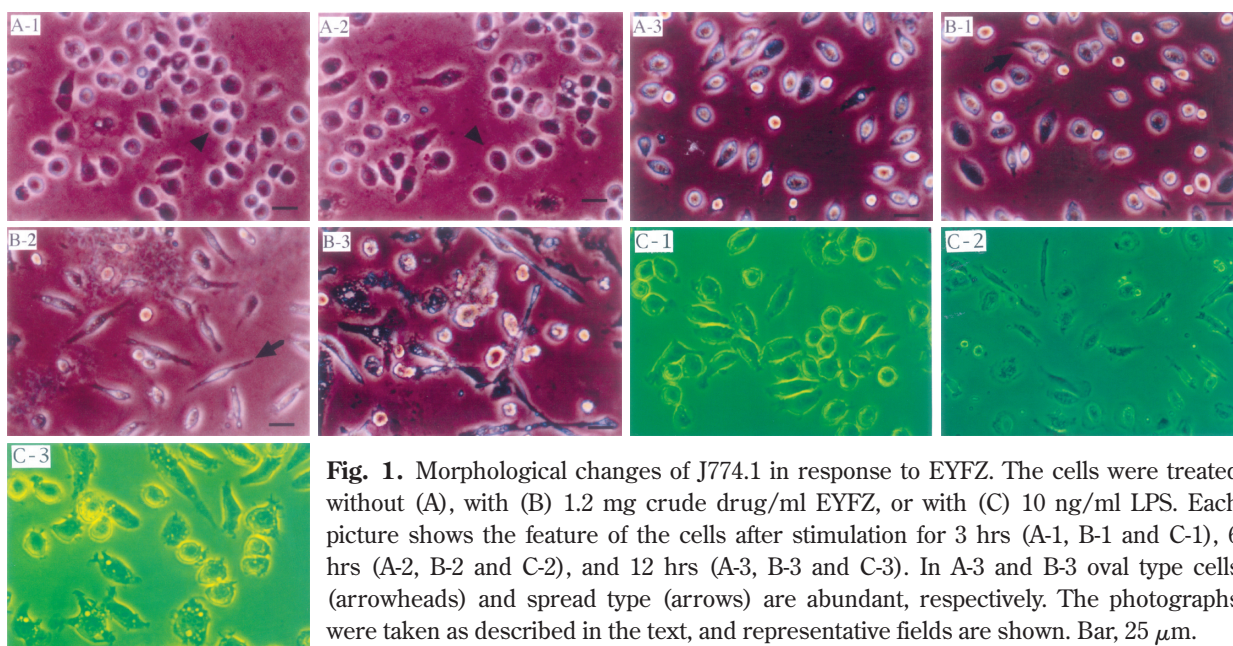


Fig. 1. Morphological changes of J774.1 in response to EYFZ. The cells were treated without (A), with (B) 1.2 mg crude drug/ml EYFZ, or with (C) 10 ng/ml LPS. Each picture shows the feature of the cells after stimulation for 3 hrs (A-1, B-1 and C-1), 6 hrs (A-2, B-2 and C-2), and 12 hrs (A-3, B-3 and C-3). In A-3 and B-3 oval type cells (arrowheads) and spread type (arrows) are abundant, respectively. The photographs were taken as described in the text, and representative fields are shown. Bar, 25 μ m.

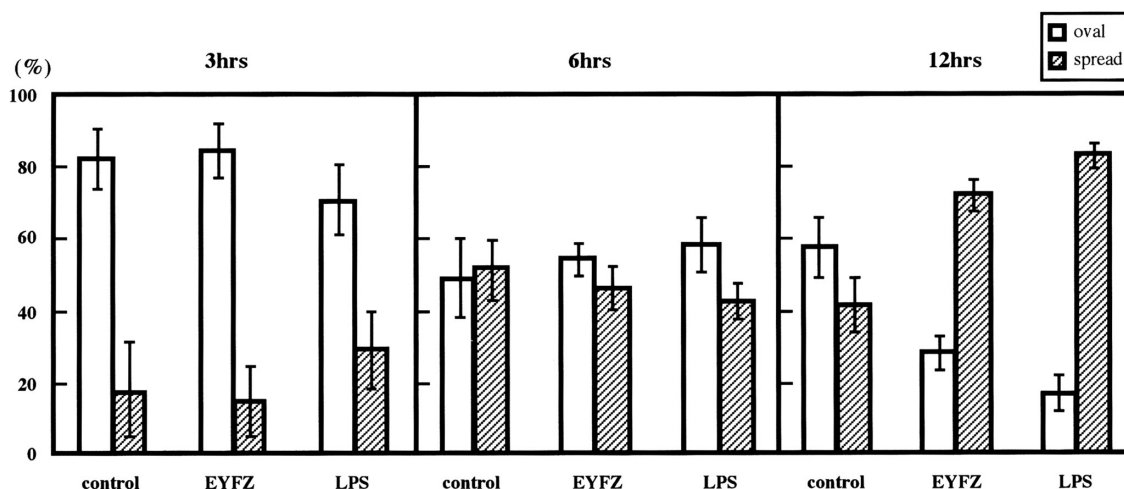


Fig. 2. The time course of morphological change after EYFZ stimulation.

Effects of EYFZ, LPS, and culture medium only on the morphology of J774.1 after incubation for 3, 6, and 12 hrs. Open bars show the % of the number of oval type cells (in Fig. 1) and hatched bars show the % of the number of spread type cells (in Fig. 1). Values represent the mean \pm S.D. of duplicate experiment.

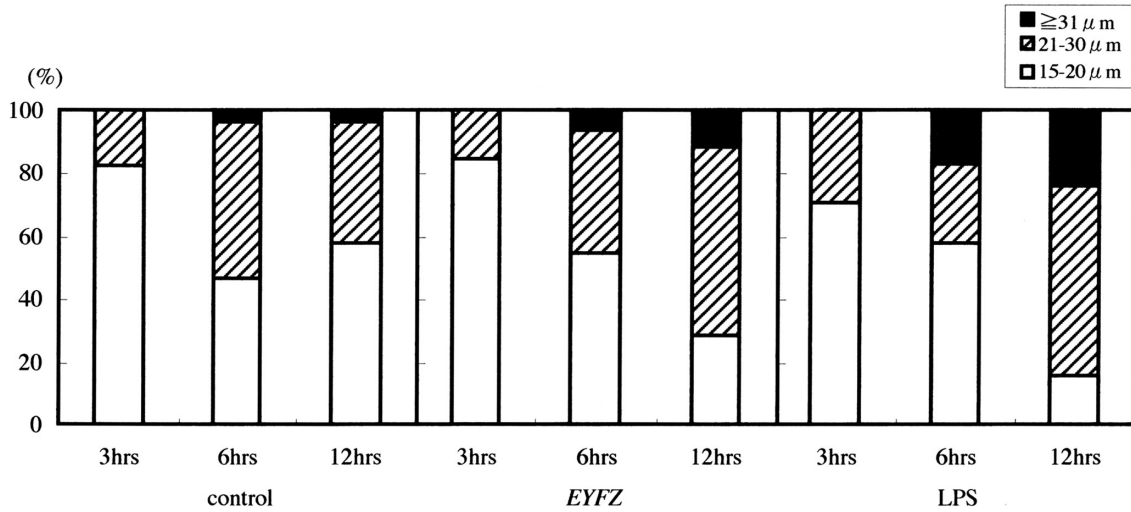


Fig. 3. The time course of size change after EYFZ stimulation. Effects of EYFZ, LPS, and culture medium only on the size of J774.1 incubation for 3, 6, and 12 hrs. Open bars show the % of the number of small cells (diameter was below 20 μm), hatched bars and solid bars show the % of the number of large cells (diameter were over 21 μm to 30 μm and over 31 μm, respectively).

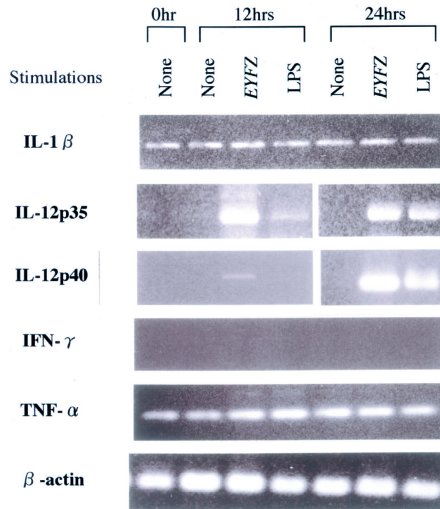


Fig. 4. The effects of EYFZ on the expression of cytokine mRNAs. J774.1 cells stimulated with EYFZ or LPS in culture were assayed for IL-1β, IL 12p35, IL-12p40, IFN-γ, TNF-α and β-actin mRNA by RT-PCR. The results are for a typical example of repeated experiments.

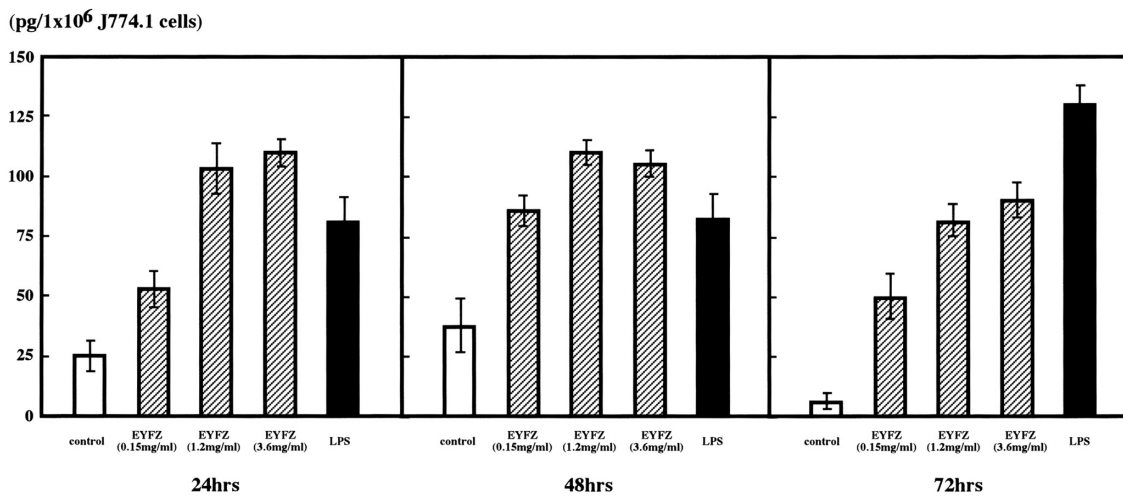


Fig. 5. The secretion of IL-12 from J774.1 stimulated with EYFZ. The secretion of IL-12 from J774.1 was examined after incubation for 24, 48, and 72 hrs. J774.1 cells (1×10^6 cells/ml) were cultured in the presence of EYFZ (0.15 mg/ml, 1.2 mg/ml and 3.6 mg crude drug/ml) (hatched bars), LPS (10 ng/ml) (solid bars), or control (culture medium only). Values represent the mean \pm S.D. of four independent experiments.

production. IL-12 exerts multiple effects on T and NK cells including the augmentation of IFN- γ production, proliferation, and cytotoxic activity, and also plays an important role to determine a Th1/Th2 balance³⁰. IL-12 is a heterodimeric cytokine composed of disulfide linked p40 and p35 subunits; both subunits have to be expressed within the same cell to produce biologically active p70 heterodimer³¹. It has been shown that p40 mRNA expression is up-regulated in the cells producing IL-12, whereas p35 mRNA is constitutively expressed in various cells³². As shown in Fig. 4, the expressions of IL-12p35 and IL-12p40 were induced similarly as assessed at 12 hrs and 24 hrs after the treatment with EYFZ. Moreover, our results of ELISA show that this increased expression of IL-12p35 and IL-12p40 induced by EYFZ occurs at the protein level.

The anti-tumor effects of EYFZ in colon-26 implanted mice were shown in our previous report²², and it was reported that IL-12 has anti-tumor effect³³⁻³⁶ and a powerful anti-tumor activity in mice against 17 different lines of transplantable murine tumors, including carcinomas, sarcomas, melanomas and lymphomas³⁷. IL-12 is much more effective in anti-tumor effect than are other cytokines, such as IL-2 and IFN- α , and is effective at the doses with much lower toxicity. In our study, relationship is unclear between the effects of EYFZ on anti-tumor and enhancement of NK function *in vivo* and activation of macrophage like cell line J774.1 *in vitro*. However, some papers showed that components of crude drugs of EYFZ include polysaccharide³⁸⁻⁴³, which can activate the function of macrophage. Thus, whether or not oral administration of EYFZ can induce macrophages to produce endogenous IL-12 is not demonstrated now. So, this elucidation needs more study in future.

Conclusively, we have found that EYFZ markedly activates J774.1 to produce IL-12, which is potentially an immune enhancer. The mechanism of this activating effect on J774.1 by EYFZ is still unclear. In order to study this mechanism, the isolation and chemical characterization of the active compounds from EYFZ are needed.

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中国の伝統的漢方薬「益気養血扶正剤」の抗腫瘍活性と免疫細胞機能に及ぼす影響

2. マウスのマクロファージ細胞株J774.1の形態変化の誘導とIL-12産生の促進

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中国の伝統的漢方薬である「益気養血扶正剤」(オウギ, ジュクジオウ, トウキ, ジュクシャ, タイソウ, ロクジョウの6種類から構成される:以下EYFZと略す)が抗腫瘍活性を示すことが担癌マウスの延命効果並びにNK活性促進から判明したので, 本研究では第2としてマクロファージ機能への影響を知る目的で, 同じBALB/c系マウス由来のマクロファージ細胞株J774.1細胞を用いてEYFZの影響をIn vitroで追究した. J774.1の培養液に種々の濃度のEYFZを添加した, 細胞形態の変化を細胞機能活性化の視点で経時的に顕微鏡観察するとともに, マクロファージ産生サイトカインであるインターロイキン-12 (IL-12) 並びに腫瘍

壊死因子 (TNF- α) の産生への影響をRT-PCR法及びELISA法によって調べた. 実験の結果, EYFZを添加すると, 非添加群よりも早く細胞の大きさが増し, 球状タイプよりも伸展タイプが増加することが判明した. 一方, RT-PCR法でサイトカインmRNA発現を調べると, 特にIL-12p35及びIL-12p40がEYFZによって強く誘導された. またJ774.1の培養液中IL-12産生をELISA法で検討した結果, 実際にIL-12がEYFZ存在下で強く産生されることがわかった. 以上の結果から, EYFZにはマウスのマクロファージ細胞株J774.1の機能を活性化する作用があり, 細胞性免疫機能を亢進することが強く示唆された.

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