T-Cell-Immunity-Based Inhibitory Effects of Orally Administered Herbal Medicine Juzen-taiho-to on the Growth of Primarily Developed Melanocytic Tumors in RET-Transgenic Mice

Yan Dai, Masashi Kato, Kozue Takeda, Yoshiyuki Kawamoto, Anwarul A. Akhand, Khaled Hossain, Haruhiko Suzuki, and Izumi Nakashima

Department of Immunology, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya, Japan

We examined the effect of oral administration of juzen-taiho-to, one of the most popular herbal medicines in Japan, on primary melanocytic tumor growth in RET-transgenic mice. There was virtually no difference between the lengths of tumor-free stages in the juzen-taiho-to-treated mice and the untreated littermate control mice. The rate of tumor growth in the juzen-taiho-to-treated mice, however, was greatly suppressed during the entire period after the initial tumor development. Correspondingly, the life span of juzen-taiho-to-treated transgenic mice was longer (over 6 mo in mean value) than that of control mice. We partially elucidated the mechanism of the antitumor effect of juzen-taiho-to. The addition of juzen-taiho-to at any of a wide range (50–1600 µg per ml) of concentrations to in vitro cultures of Mel-Ret cells, a malignant melanoma cell line derived from a RET-transgenic mouse, caused neither cell death nor cell cycle arrest directly. The addition of 50–400 µg per ml of juzen-taiho-to to cultures of murine spleen cells, however, promoted their DNA synthesis. More importantly, peritoneal exudate cells from the juzen-taiho-to-treated transgenic mice, in which the ratio and number of T cells were increased, displayed an antitumor immunity against Mel-Ret cells in vitro. Interestingly, the peritoneal-exudate-cell-associated antitumor immunity was further augmented by the addition of 200–400 µg per ml of juzen-taiho-to in vitro. This immunity, which was primarily conveyed by Thy-1+ T cells, was antigen (RET/melanoma) specific and cytotoxic. Amongst various chemical ingredients of juzen-taiho-to examined in this study, glycirrhizin displayed an action, partially replacing that of juzen-taiho-to, in promoting anti-Mel-Ret immunity when supplementarily added in vitro. These results suggest that juzen-taiho-to suppresses once-developed primary melanocytic tumors through potentiation of T-cell-mediated antitumor cytotoxic immunity in vivo. Key words: antitumor immunity/glycirrhizin/malignant melanoma. J Invest Dermatol 117:694–701, 2001

Malignant melanoma is one of the most aggressive human cancers, and the incidence of skin malignant melanoma in the world is increasing at a greater rate than that of any other human cancer (Gleason et al., 1993; Chen et al., 1996). Elucidation of the pathogenesis and establishment of an effective protocol for prevention and therapy of malignant melanoma are therefore needed urgently. We previously established an oncogene RET-transgenic mouse line, line 304/B6, in which skin melanosis, benign melanocytic tumor, and malignant melanoma develop stepwise (Kato et al., 1998b). A cell line of malignant melanoma, Mel-Ret, was also established from a RET-transgenic mouse (Taniguchi et al., 1992). In this animal model, we previously showed that the RET oncogene product or RET-induced tumor-specific antigen(s) can be the major target of antitumor T cell immunity (Ichihara et al., 1995; Kato et al., 1999). This antitumor T cell immunity developed in line 304 of RET-transgenic mice by repeated inoculation of alloantigen/RET-bearing melanocytic tumors for partial tumor growth suppression (Ichihara et al., 1995) and spontaneously in line 242 of RET-transgenic mice that are defective of immunologic tolerance to the RET antigen for complete growth inhibition (Kato et al., 1999). Another approach for the development of effective preventive/therapeutic protocols against tumor development in this model was the use of a herbal medicine, sho-saiko-to (SST), which has been widely used in clinics in Japan for treatment of chronic hepatitis and liver cirrhosis (Kato et al., 1998a). Oral administration of SST significantly prolonged the tumor-free stage in transgenic mice of line 304/B6 and suppressed the growth of once-developed tumors at the early stage of tumor development, although it barely suppressed the growth of fully developed tumors at the late stage. SST displayed direct cytotoxicity against tumor cells in vitro, and this activity seemed to be primarily involved in the mechanism of SST-mediated tumor growth suppression (Kato et al., 1998a). Corresponding to this...
result, SST induced apoptosis of tumor cells, at least in part, through the promotion of expression of cell surface fas and Fas-ligand and caused cell cycle arrest by regulating cyclin-dependent kinase activities (Liu et al., 1998). Baicalin and glycyrrhizin, two of the many ingredients of SST, were shown to be mainly responsible for the first (Kato et al., 1998a) and second (Liu et al., 1998) activities of SST, respectively. Saikosaponin-d and ginsenoside Rb1, two other ingredients of SST, as well as glycyrrhizin, have also been previously shown to display distinct actions for promoting the ligand (anti-CD3 or concanavalin A, Con A) stimulated T cell growth response (Zhang et al., 1992, 1993, 1995; Kato et al., 1994, 1995). H-2 class I antigen on lymphocytes (Zhang et al., 1990), and NO production by macrophages (Fan et al., 1995; Yi et al., 1996). Juzen-taiho-to (JTT), another well-known herbal medicine, has also been used widely in clinics in Japan for normalization of impaired immune activity and general cellular function in chronically diseased patients, including patients with malignant tumors for expectation of a recovery from anticancer-therapy-induced leukopenia and a potential increase in antitumor immunity (Ohnishi et al., 1990, 1996). Potential benefits of JTT for such patients have been indicated by experimental results demonstrating the actions of JTT in protection of mice from anticancer drugs or radiation toxicities (Haranaka et al., 1985; Sugiyama et al., 1995a, b), in increasing mitogen-induced growth and antibody responses of spleen lymphocytes (Hamada et al., 1998; Kiyohara et al., 1991), in promotion of the production of a number of cytokines such as interleukin-2 (IL-2), IL-4, IL-5, IL-6, G-CSF, granulocyte macrophage colony stimulating factor, tumor necrosis factor α, interferon γ (Kiyohara et al., 1993; lijima et al., 1999; Matsumoto and Yamada, 2000; Matsumoto et al., 2000), and in inhibition of the growth (Haranaka et al., 1985; Maruyama et al., 1993; Zhang et al., 1995; Ohnishi et al., 1996) and metastasis (Ohnishi et al., 1996) of transplanted tumors in mice, possibly through activation of macrophages/T cells (Maruyama et al., 1993; Zhang et al., 1995) and natural killer T cells (Ohnishi et al., 1996; Matsumoto et al., 2000). Despite the use of JTT for cancer patients in Japan, it still remains unclear whether JTT, possibly through its immunity-modifying effects, actually inhibits the growth of primarily developed tumors that less easily induce tumor-rejecting T cell immunity than transplanted tumors. In this study, we investigated the effect of oral administration of JTT on the growth of melanocytic tumors that had primarily developed in RET-transgenic mice. We demonstrated, for the first time, that JTT does in fact suppress the growth of primarily developed melanocytic tumors in RET-transgenic mice, not by prolonging the tumor-free stage after birth but by suppressing tumor growth during the entire period after initial tumor development. Unlike the previously reported antitumor activity of SST, JTT was found to suppress tumor growth primarily through promoting antitumor cytotoxic T cell immunity.

**MATERIALS AND METHODS**

**Reagents**

JTT and SST, the quality of which had been controlled by Japanese Pharmacopeia, were obtained from Tsumura, Tokyo, Japan. Mixtures of 10 kinds of herbs (Ginseng Radix, Glycyrrhiza Radix, Polygonum Radix, Cinnamomi Cortex, Rheumanniae Radix, Paeoniae Radix, Cnidii Rhizoma, Atractylodis Lanceae Rhizoma, Angelicae Radix, Hoelen) for JTT and of six kinds of herbs (Bupleuri Radix, Pinelliae Tuber, Stellariae Radix, Zizyphi Fructus, Zingiberis Rhizoma, Ginseng Radix, Glycyrrhizae Radix) for SST were added with water and extracted at 100°C over a period of 1 h (Ohnishi et al., 1990). The extracted solution was filtered and dried to obtain the extract powder. The blended powder was dissolved in distilled water for oral administration to mice or dissolved in tissue culture medium for in vitro use (see below). Some purified ingredients of JTT, such as paconflorin, liquiritin, glycyrrhizin, and ginsenoside Rb1 (Sakai et al., 1999) were purchased from Wako, Osaka, Japan.

**JTT treatment of RET-transgenic mice**

RET-transgenic mice of line 304/B6 (Kato et al., 1998b), which were established by crossing RET-transgenic mice of line 304 (originating from a BCF1 mouse; H-2k/d) with several times with C57BL/6 (H-2b) mice, were randomly divided into two groups after birth. One group (experimental group) was given drinking water containing 3–5 mg per d per mouse of JTT, which corresponds to the usual human dosage in conversion per weight, from 1 mo of age until death. The other group (control group) was given plain drinking water only. Each of the two groups consisted of several divided litters. For analysis of tumor volume and life span, 35 transgenic mice were used for each group. This study was approved by the Animal Care and Use Committee of Nagoya University School of Medicine.

**Cell line and culture**

Mel-Ret cells, which were previously established as a melanoma cell line from a tumor developed in a RET-transgenic mouse of line 304, were suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 U penicillin per ml, 100 μg streptomycin per ml, 2 mM l-glutamine, and 50 μM 2-mercaptoethanol (complete medium) in 60 × 15 mm tissue culture dishes (Coning, NY). JTT was dissolved in the complete medium to a concentration of 10 mg per ml. This stock solution was passed through 0.22 μm filters for sterilization. In this study, we also used a number of other cell lines for comparison, such as B16 (melanoma, originating from a C57BL/6 mouse; H-2b), EL-4 (lymphoma/leukemia, from a C57BL/6 mouse; H-2b), L1210 (leukemia, from a DBA/2 mouse; H-2d), and A431 (human epidermoid carcinoma) from laboratory stocks.

**Cell preparation**

Suspensions of spleen cells and peritoneal exudate cells (PEC) were prepared from 6- to 8-wk-old mice as described previously (Nakashima et al., 1982; Dai et al., 1994). For preparation of PEC, 1.5 ml of 10% proteose-peptone was inoculated intraperitoneally 3–4 d prior to collection of PEC. Cell type compositions of PEC were determined by flow cytometry after staining the cells with fluorescence-labeled monoclonal antibodies (PharMingen, San Diego, CA) specific to Thy-1 (CD95) for T cells, B220 (CD45R) for B cells, CD11b for macrophages, or Ly-6G (Gr-1) for granulocytes. For some experiments, PEC were treated with an anti-Thy-1 cytotoxic antibody (Nakashima et al., 1982) and a low-toxic rabbit complement (ICN Pharmaceuticals, Aurora) for elimination of Thy-1-bearing T cells or were incubated on a plastic dish for 2 h to elimate plastic-adherent cells, according to the techniques described previously (Nakashima et al.). In this study, the latter treatments reduced the ratios of T cells and macrophages measured by flow cytometry to less than 2% and less than 3%, respectively. For some other experiments, a T-cell-enriched fraction was obtained by passing PEC through a nylon wool column according to the technique described previously (Nakashima et al., 1982). The purity of this fraction, measured by flow cytometry after staining cells in the fraction with fluorescence-labeled anti-Thy-1 monoclonal antibody, was 86%–89%.

**Assay of antitumor immunity**

PEC-mediated antitumor immunizations for tumor growth inhibition and tumor cell lysis were measured as follows. For assays of immunity for tumor growth inhibition, we prepared cultures containing Mel-Ret cells (2 × 10⁴ cells per 200 μl per well) plus or minus PEC (1–5 × 10⁵) in 96-well plates for 2–3 d of incubation. For some experiments, JTT (50–1600 μg per ml) was added to the mixture of Mel-Ret cells and PEC at the time of starting the culture. ²H-Thymidine was added into the culture for the last 4 h of incubation, and uptake of radioactivity into the cells was assayed as described previously (Nagase et al., 1987).

The ³¹Cr-release test for measurement of tumor cell lysis was done as described previously (Nagase et al., 1987). Briefly, Mel-Ret cells, which were suspended in complete RPMI medium, were labeled with ³¹Cr by incubating them with 0.1 μCi Na³¹Cr per 10⁵ cells for 1 h. After washing four times with RPMI, the ³¹Cr-labeled target cells (1 × 10⁵ cells per well) were mixed with effector cells (PEC) at different E/T ratios in 200 μl of complete medium on 96-well plates and incubated in the presence of 200 μg per ml of JTT for 18 h. Radioactivity of 100 μl of culture supernatant was measured using an Auto Well Gamma System-ARC370M (Aloka, Tokyo, Japan). Maximal (100%) Cr release was measured by adding 0.2% Triton X-100 and spontaneous release was measured by adding the medium only. Cytotoxicity (%) was calculated using the following equation: cytotoxicity (%) = (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting**

SDS-PAGE and immunoblotting were performed as described elsewhere (Nakashima et al., 1991). In brief, cells were lysed by adding an equal volume of a 2-fold concentrated sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol), and proteins thus obtained were
subjected to SDS-PAGE on 8% gel. The proteins were then transferred to a polyvinylidenedifluoride membrane. Subsequently, the membrane was stained with an antipoly (ADP-ribose) polymerase (PARP) antibody (Upstate Biotechnology, Lake Placid, NY) followed by goat antirabbit IgG to horseradish peroxidase (Tago, Burlingame, CA). The protein bands were visualized by Western Blot Chemiluminescence reagent (DuPont NEN, Boston, MA) as directed by the manufacturer. The molecular sizes of the developed proteins were determined by comparison with prestained protein markers (New England Biolabs, Beverly, MA).

RESULTS

JTT suppresses growth of melanocytic tumors that spontaneously develop in RET-transgenic mice. Melanocytic tumors initially developed at about 2 mo after birth in all mice in both JTT-treated (n = 35) and untreated control (n = 35) groups, and there was no difference between the lengths of the tumor-free stages in JTT-treated (74.6 ± 29.8) and untreated control (79.6 ± 34.7) transgenic mice. All of the tumors that developed in JTT-treated and untreated control transgenic mice were melanocytic and appeared on the skin and/or eyes (one to three tumors developed per mouse, one of which usually became bigger than the other) invariably between the two groups. As shown in Fig 1, however, the rates of tumor growth assessed by measurements of tumor volumes, were remarkably reduced in the JTT-treated mice during the entire period after the initial tumor development, compared with those in nontreated littermate mice. Correspondingly, the life span of the JTT-treated mice (509 ± 182 d) was significantly longer (> 6 mo in mean value) than that of the untreated control mice (315 ± 127 d), as shown in Fig 2. Despite such obvious therapeutic effects of oral administration of JTT on growth rates of tumors, the histology of tumors at the terminal stage immediately before tumor death of the JTT-treated mice was unchanged (data not shown) from that of untreated control mice reported previously (Kato et al, 1998b). As shown in Fig 3, whereas 400 μg per ml of SST suppressed the growth of Mel-Ret cells during 3 d of culture, none of a wide range of concentrations (50–1600 μg per ml) of JTT showed this activity. This result suggests that, unlike SST, JTT does not suppress tumor growth through direct cytotoxic action on tumor cells. We next measured the effect of the addition of various concentrations of JTT into a culture of murine spleen cells on their growth in vitro. It was shown in this experiment that 50–400 μg per ml of JTT acted to promote DNA synthesis of cultured spleen cells, with a peak level in the culture to which 200 μg per ml of JTT had been added (Fig 4). These results suggested that JTT has an activity to nonspecifically upregulate the host-immune mechanism, partially agreeing with the results of earlier studies using other experimental systems (Kiyohara et al, 1991).

JTT promotes specific antitumor T cell immunity. As anti-T-cell immunity was previously shown to develop in RET-transgenic mice (Dai et al, 1994; Ichihara et al, 1995; Kato et al, 1999), we examined whether orally administered JTT promotes generation of antitumor T cell immunity. We prepared PEC from JTT-treated RET-transgenic mice and control untreated mice, and we made cocultures with Mel-Ret cells for examination of the level of tumor growth. As shown in Fig 5A, the levels of 3H-thymidine uptake in cultures of tumor cells mixed with 1 × 105/
$5 \times 10^5$ PEC from JTT-treated mice were significantly (p < 0.05/p < 0.01) lower than those in cultures mixed with the same number of PEC from untreated control mice. The former value might even be overestimated for the growth of tumor cells because it could have included uptake by potentially activated PEC. These results suggest that there was an increase in antitumor immunity for tumor growth inhibition in PEC from the JTT-treated transgenic mice. More importantly, the addition of 200 $\mu$g per ml of JTT into a mixed culture of Mel-Ret cells and PEC from JTT-treated RET-transgenic mice at the start of the culture further promoted the antitumor activity of the PEC from JTT-treated mice in vitro (Fig 5B). When the antitumor-immunity-promoting effects of a wide range of concentrations (50–1600 $\mu$g per ml) of JTT added in vitro were examined, 200–400 $\mu$g per ml of JTT was shown to be highly effective (data not shown). In this experimental protocol, however, it was difficult to exactly measure the tumor-mediated $^3$H-thymidine uptake separately from the activated-PEC-mediated uptake.

We therefore conducted experiments to directly measure antitumor activity of PEC to kill tumor cells in vitro by the standard $^{51}$Cr-release test. As shown in Fig 6(A), PEC from JTT-treated mice, but not those from nontreated control mice, displayed clear cytotoxicity (up to > 20% $^{51}$Cr release/target cell killing) against Mel-RET cells at E/T ratios of 10–90. The addition of 200 $\mu$M JTT into the mixture of PEC and tumor cells further intensified the antitumor cytotoxicity (up to > 30%) of PEC from the JTT-treated mice but did not change the cytotoxicity (around 10% or less) of PEC from control mice (Fig 6B). These results, together with the results presented in Fig 5, confirmed that antitumor cytotoxic immunity was increased in the PEC from JTT-treated mice.

We analyzed cell type composition of PEC by flow cytometry. The mean ratios of T cells, B cells, macrophages, and neutrophils in PEC from untreated control mice were 10.4%, 15.3%, 61.8%, and 15.3% ($n = 4$), respectively, and those in PEC from JTT-treated mice were 45.1%, 10.7%, 25.3%, and 20.3% ($n = 4$), respectively. The increase in the ratio of T cells and the decrease in that of macrophages in the latter PEC, compared with those in the former PEC, were statistically significant (p < 0.001/p < 0.005). Moreover, the total number of PEC recovered from JTT-treated mice ($1.1 \times 10^7$ cells per mouse as the mean of six mice) was significantly (p < 0.05) larger than that of PEC from nontreated control mice ($3.4 \times 10^6$ cells per mouse). This indicated that the number of T cells in the peritoneal cavity is increased in JTT-treated mice.

Figure 4. JTT stimulated murine spleen cells for DNA synthesis. The indicated concentrations of JTT were added to cultures of murine spleen cells, and the cultures were incubated for 3 d for measurement of $^3$H-thymidine uptake. Values of means ± SDs of triplicate cultures are presented. The experiment was repeated four times, and basically the same results were obtained. Representative data are presented. *Significantly different (p < 0.001) from the control.

Figure 5. PEC from the JTT-treated RET-transgenic mice displayed antitumor immunity to suppress the growth of Mel-Ret cells in vitro. (A) Littermate RET-transgenic mice were divided into two groups, one of which was given JTT by oral administration for 2 wk. We prepared mixed cultures of $2 \times 10^5$ Mel-Ret cells and indicated numbers of PEC from the JTT-treated or untreated control mice and incubated the cultures for 3 d for measurement of $^3$H-thymidine uptake. Values of means ± SDs of triplicate cultures from three mice per group are presented. The mean values in the cultures in which Mel-Ret cells alone were incubated were 3741 ± 88. The experiment was repeated four times, and basically the same results were obtained. Representative data are presented. *Significantly different (p < 0.001) from the control. (B) We prepared mixed cultures of Mel-Ret cells and PEC ($5 \times 10^5$) from the JTT-treated or untreated control mice as in (A) with or without addition of 200 $\mu$g per ml of JTT and incubated the cultures for 3 d for measurement of $^3$H-thymidine uptake. Values of means ± SDs of triplicate cultures are presented. The experiment was repeated four times, and basically the same results were obtained. Representative data are presented. *Significantly different (p < 0.001) from the control.
We next examined what cell type(s) conveyed the antitumor cytotoxic immunity that was associated with PEC from the JTT-treated mice. When we eliminated T cells from the PEC by treatment with a cytotoxic anti-Thy-1 antibody and low-toxic complement, the antitumor cytotoxicity of the PEC was totally lost (Fig 7), confirming the involvement of T cells as an essential element in the antitumor cytotoxicity. Depletion of adherent cells from PEC only partially decreased the level of antitumor cytotoxicity. These results suggested that the major part of the antitumor cytotoxicity is conveyed by cytotoxic T cells, although some cytotoxicity may be carried by plastic-adherent macrophages that are activated by antitumor T cells.

The antitumor cytotoxicity was further examined with the fraction of PEC that was enriched in T cells. As shown in Fig 8, the T-cell-enriched fraction of PEC from JTT-treated mice, which had been incubated with Mel-Ret cells in the presence of additional JTT in vitro, displayed high cytotoxicity (up to > 40%) against Mel-Ret cells at E/T ratios of 2:20. This level of cytotoxicity was significantly higher than the level of background cytotoxicity of the T-cell-enriched fraction of PEC from control mice. This finding proved that T-cell-dependent antitumor cytotoxic immunity was induced in PEC by oral administration of JTT to RET-transgenic mice.

Antigen specificity of the PEC-associated antitumor cytotoxic immunity was then examined. We prepared mixed cultures of each of five different cell lines (Mel-Ret, B16, EL-4, A431, and L1210) and PEC from JTT-treated or untreated mice, with addition of JTT in the culture. As shown in Fig 9, PEC from the JTT-treated transgenic mice, which displayed strong cytotoxicity against MHC-compatible Mel-Ret, showed significant but weaker cytotoxicity against another MHC-compatible melanoma cell line, B16, but barely showed cytotoxicity against any of the third-party MHC-compatible lymphoma EL-4 cells, MHC-incompatible leukemia L1210 cells, and human epidermoid carcinoma A431 cells. These results revealed that the immunity was basically Mel-Ret-specific with cross-reactivity to another MHC-compatible melanoma cell line, B16. It was concluded from all of these results that the cytotoxic immunity that developed in the peritoneal cavity of JTT-treated mice is principally conveyed by tumor antigen (RET/melanoma) specific cytotoxic T cells.

In order to partially identify the mechanism of antitumor cytotoxicity displayed by PEC from JTT-treated mice, we tested the ability of PEC after 3 d of incubation with Mel-Ret cells in vitro to induce caspase activation for degrading PARP as a cytotoxic ingredient of SST. The addition of JTT to a culture of murine spleen cells promoted their DNA synthesis. This result demonstrated the broad spectrum action mechanisms of the two herbal medicines. JTT is not completely action, as there were clear differences between the mode of suppression of tumor growth by JTT and that of the previously reported tumor growth inhibition by SST (Kato et al. 1998b) in the same animal model. First, SST (Kato et al. 1998) but not JTT (this study) prolonged the tumor-free stage. Second, JTT suppressed tumor growth during the entire period after the tumor had initially developed, whereas SST worked preferentially in the early and middle stages but had almost no effect in the late stage. Consequently, the life span of the JTT-treated transgenic mice (> 6 mo prolongation in mean value) was more extensively prolonged than was that of SST-treated mice (2.7 mo prolongation). This difference has been shown to be connected to distinct action mechanisms of the two herbal medicines.

Regarding the mechanism by which JTT inhibits tumor growth, we showed that, unlike SST, which was directly cytotoxic to tumor cells in vitro (Kato et al. 1998a; Liu et al. 1998), JTT displayed no demonstrable direct cytotoxicity against tumor cells in vitro, at any of the wide range of concentrations tested in this study. This might be mainly because JTT does not contain bicalin, which is the major cytotoxic ingredient of SST. The addition of JTT to a culture of murine spleen cells promoted their DNA synthesis. This result basically corresponds to earlier reports that JTT acts to stimulate T cell activities (Kiyohara et al., 1991). The most striking result in our study was the cytotoxicity in PEC from JTT-treated mice was abolished by previously eliminating T cells from PEC before cultivation (lane 3). These results suggest that the PEC-mediated T-cell-dependent antitumor immunity involves induction of apoptotic death of Mel-Ret cells.

Partial identification of ingredients of JTT that are responsible for enhancing the PEC-associated antitumor cytotoxic immunity We finally tested the actions of each of several known major ingredients of JTT, including glycyrrhizin, paoniflorin, gensenoside Rb1, and liquiritin, on the antitumor cytotoxic immunity of PEC from the JTT-treated transgenic mice. The results are shown in Fig 11. Amongst the four major ingredients of JTT tested, only glycyrrhizin significantly augmented the antitumor cytotoxicity of PEC when added in vitro, although its effect was weaker than that of the original JTT. This result suggests that glycyrrhizin is involved in the active component of JTT, although another undetermined ingredient(s) in JTT, possibly in cooperation with glycyrrhizin, is needed for complete action.

DISCUSSION

In this study, we demonstrated that daily oral administration of JTT from 4 wk after birth suppressed the growth of melanocytic tumors that spontaneously developed in RET-transgenic mice. This is the first report describing a clear antitumor effect of the herbal medicine JTT on primarily developed tumors and on malignant melanoma in an animal model. Our results encourage a future evidence-based usage of this herbal medicine in clinics for controlling malignant melanoma in humans. More interestingly, there were clear differences between the mode of suppression of tumor growth by JTT and that of the previously reported tumor growth inhibition by SST (Kato et al. 1998b) in the same animal model. First, SST (Kato et al. 1998) but not JTT (this study) prolonged the tumor-free stage. Second, JTT suppressed tumor growth during the entire period after the tumor had initially developed, whereas SST worked preferentially in the early and middle stages but had almost no effect in the late stage. Consequently, the life span of the JTT-treated transgenic mice (> 6 mo prolongation in mean value) was more extensively prolonged than was that of SST-treated mice (2.7 mo prolongation). This difference has been shown to be connected to distinct action mechanisms of the two herbal medicines.

Regarding the mechanism by which JTT inhibits tumor growth, we showed that, unlike SST, which was directly cytotoxic to tumor cells in vitro (Kato et al. 1998a; Liu et al. 1998), JTT displayed no demonstrable direct cytotoxicity against tumor cells in vitro, at any of the wide range of concentrations tested in this study. This might be mainly because JTT does not contain bicalin, which is the major cytotoxic ingredient of SST. The addition of JTT to a culture of murine spleen cells promoted their DNA synthesis. This result basically corresponds to earlier reports that JTT acts to stimulate T cell activities (Kiyohara et al., 1991). The most striking result in our study was the cytotoxicity in PEC from JTT-treated mice was abolished by previously eliminating T cells from PEC before cultivation (lane 3). These results suggest that the PEC-mediated T-cell-dependent antitumor immunity involves induction of apoptotic death of Mel-Ret cells.

Partial identification of ingredients of JTT that are responsible for enhancing the PEC-associated antitumor cytotoxic immunity We finally tested the actions of each of several known major ingredients of JTT, including glycyrrhizin, paoniflorin, gensenoside Rb1, and liquiritin, on the antitumor cytotoxic immunity of PEC from the JTT-treated transgenic mice. The results are shown in Fig 11. Amongst the four major ingredients of JTT tested, only glycyrrhizin significantly augmented the antitumor cytotoxicity of PEC when added in vitro, although its effect was weaker than that of the original JTT. This result suggests that glycyrrhizin is involved in the active component of JTT, although another undetermined ingredient(s) in JTT, possibly in cooperation with glycyrrhizin, is needed for complete action.

DISCUSSION

In this study, we demonstrated that daily oral administration of JTT from 4 wk after birth suppressed the growth of melanocytic tumors that spontaneously developed in RET-transgenic mice. This is the first report describing a clear antitumor effect of the herbal medicine JTT on primarily developed tumors and on malignant melanoma in an animal model. Our results encourage a future evidence-based usage of this herbal medicine in clinics for controlling malignant melanoma in humans. More interestingly, there were clear differences between the mode of suppression of tumor growth by JTT and that of the previously reported tumor growth inhibition by SST (Kato et al. 1998b) in the same animal model. First, SST (Kato et al. 1998) but not JTT (this study) prolonged the tumor-free stage. Second, JTT suppressed tumor growth during the entire period after the tumor had initially developed, whereas SST worked preferentially in the early and middle stages but had almost no effect in the late stage. Consequently, the life span of the JTT-treated transgenic mice (> 6 mo prolongation in mean value) was more extensively prolonged than was that of SST-treated mice (2.7 mo prolongation). This difference has been shown to be connected to distinct action mechanisms of the two herbal medicines.

Regarding the mechanism by which JTT inhibits tumor growth, we showed that, unlike SST, which was directly cytotoxic to tumor cells in vitro (Kato et al. 1998a; Liu et al. 1998), JTT displayed no demonstrable direct cytotoxicity against tumor cells in vitro, at any of the wide range of concentrations tested in this study. This might be mainly because JTT does not contain bicalin, which is the major cytotoxic ingredient of SST. The addition of JTT to a culture of murine spleen cells promoted their DNA synthesis. This result basically corresponds to earlier reports that JTT acts to stimulate T cell activities (Kiyohara et al., 1991). The most striking result in our study was the cytotoxicity in PEC from JTT-treated mice was abolished by previously eliminating T cells from PEC before cultivation (lane 3). These results suggest that the PEC-mediated T-cell-dependent antitumor immunity involves induction of apoptotic death of Mel-Ret cells.
study was that PEC from the JTT-treated RET-transgenic mice displayed a clear antitumor cytotoxicity against Mel-Ret cells in vitro, and this antitumor activity was further augmented by the addition of JTT into the culture. The antitumor cytotoxic immunity that had been primarily developed in JTT-treated mice and had been amplified by JTT added in vitro in the presence of Mel-Ret cells bearing tumor antigens was shown to be fully Thy-1-bearing T-cell-dependent. Moreover, the immunity specifically targeted Mel-Ret cells and, in part, MHC-compatible B16 melanoma cells, but not third-party MHC-compatible EL-4 lymphoma cells, MHC-incompatible L1210 leukemia cells, or human A431 epidermoid carcinoma cells. We previously revealed that RET antigen or RET-induced tumor-specific antigen(s) worked as a tumor transplantation antigen to induce both cytotoxic T cell immunity and T cell/macrophage-mediated immunity when alloantigen-containing RET-antigen-bearing tumor cells or recombinant RET antigens were inoculated into RET-transgenic mice (Dai et al., 1994; Ichihara et al., 1995) or into a line of RET-transgenic mice that were not neonatally tolerant to the RET antigen (Kato et al., 2000). In this study, we showed that immune peritoneal T cells attacked Mel-Ret cells, resulting in growth inhibition and cell death, partially involving the action of plastic-adherent cells. Additional experiments revealed that the rate and number of T cells were increased in the peritoneal cavity of the JTT-treated RET-transgenic mice and that nylon-wool-purified T cells from PEC of the JTT-treated mice displayed high cytotoxicity against Mel-Ret cells. Evidence that the JTT-promoted antitumor cytotoxic immunity involves activation of caspases in the target tumor cells was also obtained. Taken together, our results suggest that JTT assisted T cells in becoming effectively sensitized to RET and potentially RET-induced melanoma-common antigens for
generating mainly cytotoxic T cell immunity and partially T cell/macrophage-mediated immunity. Isolation of a tumor-specific cytotoxic T cell clone(s) for potential adoptive immune therapy for primarily developed melanoma is in progress as the second step of this series of studies.

JTT is composed of extracts from 10 herbs and contains various chemicals, including liquiritin, glycyrrhizin, paeni¯orin, and ginsenoside, as the major ever-known ingredients. These chemicals, including liquiritin, glycyrrhizin, paeni¯orin, and ginsenoside, are contained in the two medicines (although the quantities are different ± JTT contains more glycirrhizin than SST, for example), bicalin, which is one of the major components of SST and is cytotoxic to tumor cells (Kato et al., 1998), is not included in JTT. This suggests that a herbal medicine such as SST that displays direct cytotoxicity-based antitumor activity eliminates tumors most effectively in the early stage of tumor development, when the tumor is small, but has little effect in the late stage, when the tumor has become large. In contrast, a herbal medicine like JTT showing T-cell-immunity-based antitumor activity acts effectively after the tumor has developed to a size that is capable of stimulating T cell immunity. An appropriate stage-oriented combination of the two herbal medicines, using SST in the early stage and JTT in the later stage, for example, may provide a good therapeutic protocol.

Figure 10. Activated T cells that developed in a 3 d culture of Mel-Ret cells and PEC from the JTT-treated transgenic mice induced caspase activation in fresh Mel-Ret cells. We prepared mixed cultures of Mel-Ret cells (2 × 10⁶) and PEC (1 × 10⁶) from JTT-treated or nontreated control transgenic mice (see the legend of Fig 5) and incubated the cultures for 3 d in the presence of 200 µg per ml of JTT. Fresh Mel-Ret cells (1 × 10⁵) were then incubated for 2 d in the cultures containing fresh medium with (lanes 2–5) or without (lane 1: control) PEC from JTT-treated (lanes 2 and 3) or nontreated control (lanes 4 and 5) mice that had been incubated in the first-step 3 d culture. For lanes 3 and 5, T cells were eliminated by previously treating PEC with an anti-Thy-1 and complement before the start of the first culture. Cells collected after the second culture were lyzed by sample buffer and subjected to SDS-PAGE followed by immunoblotting with anti-PARP antibody. Positions of undegraded (116 kDa) and degraded (85 kDa) PARP molecules are shown on the right. The experiment was repeated four times and basically the same results were obtained. Representative data are presented.

Figure 11. PEC-associated antitumor-cytotoxic-immunity-promoting activities of ingredients of JTT. We prepared mixed cultures of PEC from the JTT-treated mice and Mel-Ret cells (E/T ratio = 90) in the presence ☰ or absence ☱ of each of the indicated ingredients at the concentrations contained in 200 µg per ml of JTT for measurement of ⁵¹Cr release. Values of means ± SDs of triplicate cultures are presented. The experiment was repeated three times, and basically the same results were obtained. Representative data are presented. †,‡Significantly different (†p < 0.01; ‡p < 0.05) from the control.

This study was supported in part by Grants-in-Aid for Scientific Research on Priority Areas and for Scientific Research (C) from the Ministry of Education, Science, Sports, and Culture of Japan, the Fund for Comprehensive Research on Aging and Health from the Ministry of Health and Welfare of Japan, and the Research Foundation for Oriental Medicine.
REFERENCES


INHIBITION OF MELANOMA GROWTH IN TRANSGENIC MICE