Immunological Mechanisms of Antitumor Activity of Some Kinds of Chinese Herbs: Meth A-Induced Delayed Type Hypersensitivity

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Abstract—In the present paper, we confirmed that a delayed type hypersensitivity response can be elicited against Meth A tumor (Meth A-DTH) in BALB/c mice bearing the primary tumor. This response was augmented by lipopolysaccharide. We examined the effects of 4 kinds of Chinese herbs including A. capillaris, S. doederleinii, A. macrocephala and S. subprostrata on the Meth A-DTH, and the results were compared with that of the herbs on picryl chloride-induced delayed type hypersensitivity (PC-DTH). All of the herbs examined augmented the Meth A-DTH 10 days after the primary tumor transplantation, and S. doederleinii, A. macrocephala and S. subprostrata prevented the decay of the response on the 20th day, but A. capillaris did not. On the other hand, none of the herbs affected the PC-DTH. When both DTH responses were caused simultaneously in the same mouse, Meth A-DTH decayed 20 days after the transplantation but PC-DTH did not. In this case, the effects of these 4 herbs on Meth A-DTH and PC-DTH were essentially the same as those seen in the case of separate experiments. The previous and present results suggest that A. capillaris shows antitumor activity mainly through a direct cytotoxicity, although this herb might have certain components to enhance Meth A-DTH, and the other herbs display the activity through the enhancement of T cell-mediated tumor immunity, particularly tumor specific DTH.

Some Chinese blended medicines have been used for diseases involving malignant tumors since more than 2000 years ago. Certain components of the Chinese blended medicines may have a tumoricidal action as a mechanism of antitumor activity. It is also likely that some other components stimulate the tumor immune response. With this thought in mind, we (1) previously examined the effects of 10 kinds of Chinese herbs on the growth of Meth A tumor in BALB/c mice and the survival of the syngeneic host. As a result, antitumor activity was seen in 4 kinds of Chinese herbs, Artemisia capillaris, Selaginella doederleinii, Atractylodes macrocephala and Sophora subprostrata. Furthermore, A. capillaris exhibited a significant inhibition of the growth of Meth A tumor inoculated in BALB/c-nu/nu mice, but the others did not, while all of the 4 herbs showed the antitumor activity against Meth A tumor in BALB/c-nu/+ mice. A. capillaris showed cytotoxicity against Meth A tumor and L-929 cells in the in vitro study. The concentration required for 50% inhibition of the tumor proliferation was 1.25–2.5 × 10^{-4} g/ml of A. capillaris. These results suggest that A. capillaris shows the antitumor activity mainly through a direct cytotoxic action, and the others display the activity through a host mediated mechanism.

It is generally accepted that T lymphocytes primed to tumor-associated antigens (TAA) play an important role in host defense mechanisms against tumor (2–19) as well as some
other effector mechanisms including natural killer cells (20, 21), antibody dependent cell-mediated cytotoxicity (22) and cytotoxic antibody (23). It is well-documented that BALB/c mice cause a potent concomitant immunity against Meth A fibrosarcoma, and T cells are required for the acquisition of the immune state (2, 3). It has also been pointed out that not only Lyt-1-2+ T cells (15-18) including cytotoxic T lymphocytes but also Lyt-1+2+ T cells (8-10, 12, 18) including delayed type hypersensitivity effector cells and amplifier/helper cells play a role in the host defense mechanisms. In the present paper, we confirmed that a DTH response can be elicited against Meth A tumor (Meth A-DTH) in the syngeneic host bearing the primary tumor and then examined the effect of the 4 kinds of Chinese herbs on the Meth A-DTH in comparison with the effect of the herbs on picryl chloride-induced contact delayed type hypersensitivity (PC-DTH).

Materials and Methods

Drugs: Chinese herbs employed in this study were as follows: Artemisia capillaris THUNB., Selaginella doederleinii HIERON., Atractylodes macrocephala KOIDE., and Sophora subprostrata CHUN et T. CHEN. These herbs were extracted with distilled water at 100°C for 2 hr and lyophilized to make the aqueous extracts. The following materials were also employed: benzylpenicillin potassium-treated and lyophilized cells of the Streptococcus pyogenes Su strain (A group, type 3) (OK-432, Chugai, Tokyo, Japan), cyclophosphamide (CY, Shionogi, Osaka, Japan), 5-fluorouracil (5-FU, Nakarai, Kyoto, Japan) and lipopolysaccharide E. coli 055:B5 (LPS, Difco, MI). The dose of OK-432 was expressed as units of KE (Klinische Einheit), where one KE contained 0.1 mg of dried whole cells of S. pyogenes. The following materials were dissolved or suspended in distilled water for p.o. administration, and in sterilized physiological saline for i.p. or i.v. administration.

Animals: BALB/c female mice and ddY male mice at 8 to 10 weeks of age were used. They were obtained from Shizuoka Experimental Animal Center (Hamamatsu, Japan) and maintained with free access to pellet food and water in laminar-air flow isolation cages at 21±1°C temperature and 60% humidity.

Tumor cells: Meth A tumor cells were obtained from Aichi Cancer Center (Nagoya, Japan). The cells were maintained by weekly passage in the peritoneal cavity of BALB/c mice in our laboratory and were collected from the ascites fluid by centrifugation followed by washing with Hanks’ solution for experiments. The size of Meth A tumor growing subcutaneously was determined with vernier calipers in terms of 2 diameters at right angles. Tumor size was expressed as volume (cm³) calculated as follows: \(4/3\pi \times (\text{long diameter}/2) \times (\text{short diameter}/2)^2\).

Immunization of mice with Meth A tumor: BALB/c mice were immunized with the s.c. transplantation of 10⁶ Meth A cells into their backs, followed by the excision of the growth 7 days later. The incisions were closed with stainless steel surgical clips.

Meth A tumor-induced delayed-type hypersensitivity (Meth A-DTH): Meth A-bearing BALB/c mice were challenged by the s.c. injection of 50 μl suspension of mitomycin C (MMC)-treated Meth A cells into their right hind footpad. Meth A cells used as the antigen were treated with 50 μg/ml of mitomycin C at 37°C for 30 min, then washed three times and resuspended at the appropriate cell density with Hanks’ solution. The dose of MMC-treated Meth A cells for challenge was 10⁶ cells/site unless otherwise indicated. Footpad swelling due to Meth A-DTH was evaluated as the difference in volumes measured just before and 24 hr after the challenge with a plethysmometer (model TK-101, Unicom, Yachiyo, Japan). Footpad swelling of normal mice was measured in every experiment and the mean value was in a range of 4 and 10 μl. The intensity of DTH was expressed as the difference in footpad swelling between DTH-induced mice and normal mice.

Picryl chloride (PC)-induced delayed type hypersensitivity (PC-DTH): Mice were sensitized by painting 0.1 ml of 1% PC dissolved in ethanol on the skin of their abdomens, according to Asherson and Ptak (24). Mice were challenged by painting 15 μl of 1% PC in olive oil/each face of both their ear lobes. DTH elicited was evaluated by the increase of ear thickness 24 hr after the challenge. Ear thickness was measured by a dial thickness...
gauge (Ozaki Mfg. Co. Ltd., Tokyo, Japan).

Statistics: For all data shown in the figures and tables, the values were expressed as the mean±S.E.M. Wilcoxon's rank sum test (U-test) was employed to analyze significant difference between 2 groups in the data on tumor size. Student's or Welch's two tailed t-test after the F-test was used to analyze significance of difference for data on parameters other than tumor size.

Results

Induction of Meth A-DTH and effect of CY and LPS on it: BALB/c mice were transplanted s.c. with $10^6$ Meth A cells into their flanks. After 10 days, MMC-treated Meth A cells were injected s.c. in varying doses into their footpads to provoke the reaction. Since the biggest difference of the footpad swelling between normal mice and tumor bearing mice was seen in the group injected with $10^6$ cells as an antigen (Table 1), the dose of $10^6$ MMC-treated Meth A cells was used to elicit Meth A-DTH in the following experiments. Time courses of tumor growth and Meth A-DTH in BALB/c mice and the effects of CY and LPS on them are shown in Fig. 1, where the animals were transplanted s.c. with $10^6$ Meth A cells into their flanks. CY was given i.p. in a dose of 150 mg/kg 3 days before the transplantation. LPS was given i.v. in a dose of 50 μg/animal into the tail vein 8 days after the transplantation. Animals in each group were divided into 4 groups for eliciting Meth A-DTH on the 5th, 10th, 15th and 20th days, respectively (Fig. 1B). The Meth A-DTH in the control was obviously detected on day 5 and 10, then decreased slightly on day 15, and decayed completely on day 20, where the increase in footpad volume was less than that of normal mice. CY pretreatment showed a tendency to suppress the Meth A-DTH on day 5 and 10, but tended to augment it on days 15 and 20. On the other hand, LPS enhanced the Meth A-DTH significantly on day 20. The growth of Meth A was suppressed slightly by CY treatment, and it was suppressed significantly by LPS treatment, where the tumor was regressed in 4 out of 7 mice until day 20 (Fig. 1A).

Meth A-DTH in immunized mice and effect of 5-FU on it: BALB/c mice were immunized with Meth A tumor by the excision of the 7 days' growth of Meth A tumor, and they were divided into 2 groups. Five days after the excisions, both groups of mice as well as normal mice were transplanted with $10^6$ Meth A cells s.c. into their flanks. One group of the immunized mice was treated with 5-FU (26 mg/kg, p.o.) for 14 days from 4 days before the transplantation. The tumor growth was clearly suppressed compared with that in non-immunized mice; 5 out of 7 animals in the immunized group showed regression of the growth (Fig. 2A). The potent response of Meth A-DTH was observed throughout the experimental period without the decay that was seen in the case of non-immunized mice (Fig. 2B). 5-FU treatment suppressed the tumor growth in the immunized mice, but abrogated the response of Meth A-DTH almost completely.

Effect of Chinese herbs on Meth A-DTH: Mice were transplanted s.c. with $10^6$ Meth A cells and administered p.o. with 250 mg/kg of Chinese herbs for 24 days from 4 days

Table 1. Footpad swelling in BALB/c mice by challenge with varying doses of mitomycin C (MMC)-treated Meth A cells

| No. of cells | No. of mice | Footpad swelling (μl)a) | DTH-intensityb) (μl) |
|--------------|-------------|------------------------|---------------------|
|              | Normal      | Tumor-bearing          |                     |
| $5 \times 10^6$ | 5           | 1.6±2.02               | 4.5±1.08            |
| $1 \times 10^6$ | 5           | 4.2±2.35               | 9.9±1.43            |
| $2 \times 10^6$ | 5           | 9.3±3.93               | 10.3±1.55           |

Animals were transplanted s.c. with $10^6$ cells of Meth A tumor into their flanks. Ten days later, MMC-treated Meth A cells were injected s.c. into their footpads for challenge. Footpad swelling was evaluated by the increase in footpad volume 24 hr later. a: mean and S.E.M. b: Difference between normal and tumor-bearing mice.
Fig. 1. Time courses of tumor growth and Meth A-induced delayed type hypersensitivity (DTH) in BALB/c mice and effects of cyclophosphamide (CY) and lipopolysaccharide (LPS) on them. Animals were transplanted s.c. with $10^6$ Meth A cells into their flanks. CY (150 mg/kg) was given i.p. 3 days before the transplantation. LPS (50 μg/animal) was given i.v. 8 days after the transplantation. DTH was induced in their footpads by the s.c. injection of MMC-treated Meth A cells (10^6 cells/site). Intensity of DTH was evaluated by the increase in footpad volume 24 hr after the challenge as compared to footpad volume of normal mice. Each column represents the mean±S.E.M. of 7-9 animals. □: Control, ◯: CY-treated mice, ◯: LPS-treated mice. *, †: Statistically significant difference from the control at P<0.05 and P<0.01, respectively.

before the tumor transplantation. DTH was induced on days 10 and 20 after the transplantation (Fig. 3). The DTH on day 10 was augmented significantly by A. capillaris, S. doederleini, A. macrocephala and OK-432, which was used as a comparative compound, and showed a tendency to be augmented by S. subprostrata. A decay of DTH in the control was seen on day 20, where the level of footpad swelling is less than that of normal mice in accord with the results shown in Figs. 1 and 2. A. capillaris did not affect the decay, while S. doederleini and S. subprostrata recovered the swelling to the level of normal mice. A. macrocephala and OK-432 augmented the response significantly. Meanwhile, the tumor growth on day 10 was significantly inhibited by S. doederleini and showed a tendency to be inhibited by the others. On day 20, S. doederleini and A. macrocephala showed a significant inhibition of the tumor growth on day 20 and OK-432
showed a tendency to inhibit it.

Effect of Chinese herbs on PC-DTH: PC was applied to ddY mice for sensitization. The Chinese herbs (250 and 500 mg/kg) were given p.o. for 10 days from 4 days before the sensitization. OK-432 (0.5 and 1 KE/animal) was given i.p. for 6 days from the sensitization. Mice were challenged 6 days after the sensitization. No appreciable effect of the Chinese herbs on PC-DTH was seen as shown in Fig. 4. OK-432 also did not affect the DTH.

Effects of Chinese herbs on Meth A-DTH and PC-DTH induced in the same mouse: BALB/c mice were sensitized twice with PC in an interval of 7 days. Seven days after the final sensitization, they were transplanted with $10^6$ Meth A cells s.c. into their flanks. Chinese herbs (250 mg/kg) were given p.o. for 24 days from 4 days before the transplantation, and OK-432 (0.5 KE/animal) was given i.p. for 10 days from the transplantation. PC-DTH and Meth A-DTH were simultaneously elicited on days 10 and 20 after the transplantation (Fig. 5). A. macro-
cephala and OK-432 augmented Meth A-DTH on day 10, and the others showed a tendency to augment the DTH. On day 20, A. capillaris, S. doederleinii and A. macrocephala restored the level of footpad swelling to that of normal mice, and S. subprostrata tended to restore it. The level of swelling was not recovered by OK-432 on day 20. On the other hand, PC-DTH did not decay on day 20 in contrast to the case of Meth A-DTH. None of the Chinese herbs showed an appreciable effect on the PC-DTH on both days 10 and 20.

Discussion
Recent studies (8–10, 12, 18) have revealed the importance of the Lyt-1+2− T cell subset as an effector cell in host resistance against tumors. Lyt-1+2− effector cells are known to elicit a delayed type hypersensitivity response. There are some reports (2, 4) concerning Meth A-DTH in BALB/c mice immunized with the tumor, although it is not well-documented about Meth A-DTH in non-immunized mice bearing the primary tumor. In our initial survey (Fig. 2), it was examined whether Meth A-DTH can be elicited in the syngeneic mice bearing the primary Meth A tumor. The most potent Meth A-DTH was elicited 5 days after the inoculation, then the response slightly declined until 15 days and
Fig. 4. Effect of A. capillaris (A), S. doederleinii (B), A. macrocephara (C), S. subprostrata (D) and OK-432 on picryl chloride (PC)-induced delayed type hypersensitivity (DTH) in ddY mice. Animals were sensitized with 1% PC in ethanol on day 0. Chinese herbs (250 and 500 mg/kg) were administered p.o. for 10 days from 4 days before the sensitization. OK-432 (0.5 and 1 KE/animal) was given i.p. for 6 days from the sensitization. DTH was induced in ears by painting them with 1% PC in olive oil 6 days after the sensitization. Each column represents the mean±S.E.M. of 6–8 animals.

Fig. 5. Effects of A. capillaris (A), S. doederleinii (B), A. macrocephara (C), S. subprostrata (D) and OK-432 on Meth A-induced DTH and PC-induced DTH in BALB/c mice bearing Meth A tumor. Chinese herbs (250 mg/kg) were given p.o. for 24 days from 4 days before the transplantation, and OK-432 (0.5 KE/animal) was given i.p. for 10 days from the transplantation. The intensity of Meth A-DTH in non PC-sensitized and tumor-bearing mice was 2.6±1.29 μl 10 days after the transplantation and -3.38±1.37 μl 20 days after, respectively. The intensity of PC-DTH in PC-sensitized and non-tumor-bearing mice was 6.8±0.58×10^-3 cm 10 days after the day of transplantation and 6.5±0.54×10^-3 cm 20 days after, respectively. Each column represents the mean±S.E.M. of 8 animals. *, †: Statistically significant difference from the control at P<0.05 and P<0.01, respectively.
disappeared completely by 20 days in inverse proportion to the tumor growth. This response was augmented by LPS treatment with the suppression of tumor growth in agreement with the reports (2, 3) that LPS has an antitumor activity through T cell mediated immunity. It is generally accepted that treatment with CY before antigen increases immune response by eliminating the precursors of suppressor T cells (19, 25). CY pretreatment in the present study, however, showed only a slight suppression of the tumor growth and a tendency to stimulate the Meth A-DTH 15 and 20 days later (Fig. 2). Further investigation will be necessary to prove a poor participation of CY-sensitive suppressor T cells in this syngeneic host-tumor system, because North (25) reviewed CY-sensitive suppressor T cells which down regulate the anti Meth A tumor immune response in BALB/c mice. In mice immunized with Meth A tumor by the excision of 7 days' growth of the tumor, a potent Meth A-DTH was observed without any decay with a regression of the retransplanted Meth A tumor. Treatment with 5-FU, an antitumor and immunosuppressive agent, abrogated this DTH response almost completely. These results indicate that Meth A-DTH can be detected in BALB/c mice bearing the primary tumor in spite of lesser intensity than in the immunized mice.

There is still much debate about whether delayed type hypersensitivity effector T cells (9–12, 18) or cytotoxic T cells (13–18), or perhaps even both of them, play a more important role in host defense mechanisms against tumors. Here, we have studied the effect of Chinese herbs on DTH responses as one aspect of this problem. All of the examined herbs augmented the Meth A-DTH 10 days after the primary tumor transplantation, and S. doederleinii, A. macrocephala and S. subprostrata prevented the decay 20 days after, but A. capillaris did not (Fig. 3). On the other hand, none of the Chinese herbs affected the PC-DTH. There is, therefore, a discrepancy between the effects of the Chinese herbs on Meth A-DTH and PC-DTH. Indeed, this discrepancy is supported in the latter experiment (Fig. 5). When these two types of DTH responses were caused simultaneously in the same mouse, Meth A-DTH decayed 20 days after the tumor transplantation in inverse proportion to the tumor growth, but PC-DTH did not. These results mean that the progressive decay of delayed type immune response associated with the growth of primary tumor is restricted to the Meth A-DTH. This also indicates that the competence of inflammatory cells including macrophages remains intact with respect to the reactivity to lymphokines, and the inflammatory process thereafter is not altered even 20 days later. In this experiment, all of the Chinese herbs tested also showed an enhancing activity on Meth A-DTH, but hardly influenced the PC-DTH. Therefore, it is likely that enhancement of Meth A-DTH by the 4 herbs was not dependent on the augmentation of the inflammatory process.

Previously, we reported that A. capillaris inhibited the growth of Meth A tumor inoculated in BALB/c-nu/nu mice, but the other 3 herbs not, whereas all of the herbs showed the antitumor activity in BALB/c-+ mice. Furthermore, S. doederleinii, A. macrocephala and S. subprostrata augmented Meth A tumor neutralizing activity (Winn's assay) in the spleen of BALB/c mice bearing the tumor, but A. capillaris did not (26). It was also shown (1) that A. capillaris had a direct cytotoxic activity in vitro. These findings and the results described in this study suggest that S. doederleinii, A. macrocephala and S. subprostrata display their activity through the enhancement of T cell-mediated tumor immunity, particularly tumor specific DTH. Our previous study (1, 26) suggested that A. capillaris showed the antitumor activity mainly through a direct cytotoxicity. However, A. capillaris augmented Meth A-DTH 10 and 20 days after the tumor transplantation (Figs. 3 and 5). It seems, therefore, that A. capillaris has certain components to enhance Meth A-DTH, which are other than the components exerting cytotoxic activity.

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