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The therapeutic effects of an immunopotentiator, PS-K, on 3-methylcholanthrene-induced autochthonous tumors in C57BL/6 mice in combination with the surgical removal of primary sites*

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Summary. We investigated the therapeutic effects of an immunopotentiator PS-K on recurrent or metastatic tumors observed after the surgical removal of MCA-induced primary tumors in autochthonous C57BL/6 mice and on the survival time of treated mice. The MST of mice treated with PS-K at various times (59.8 ~ 63.4 days) was prolonged as compared with that of mice treated by surgery alone (48.6 days). Local recurrence of tumors was found in 36 of 66 mice (54.6%) treated by surgery alone, whereas it was inhibited significantly ($P<0.05$) when treatment with PS-K was started 1 day after the surgery and occurred in 22 of 64 mice (34.4%) when PS-K was given for 5 days in 1 week, or in 22 of 66 mice (33.3%) when PS-K was administered twice a week for 7 weeks. The MSTs of mice with local recurrence were also found to be prolonged as compared with those of mice treated by surgery alone (54.8 ~ 67.5 days vs 49.8 days). The MSTs of mice without tumor recurrence were also prolonged significantly ($P<0.05 \sim 0.001$) by combinations of PS-K at various times, although most of the mice died of metastatic tumors even in the groups of mice where a combined treatment with PS-K had been administered. The above findings suggest that the administration of PS-K inhibits the growth of recurrent or metastatic tumor cells in autochthonous mice after the surgical removal of the primary tumors.

Introduction

The therapeutic effects of various immunopotentiators on experimental tumors have been examined in conjunction with both chemotherapy [2, 8, 17, 21, 23, 25, 33] and surgical operation [4, 11, 15, 22, 31]. Although human cancers are autochthonous tumors in the primary host, only a few experiments make use of autochthonous tumors [11, 12, 16, 18, 26, 28, 32] and most experiments are carried out using transplanted tumors in syngeneic hosts. An exploration of the combined effects of surgery and immunopotentiators on autochthonous tumors in animals as compared with experiments on transplanted tumors is therefore likely to offer various suggestions for combination treatments of human cancer. With such an aim in mind, we investigated the efficacy of treatment with an immunostimulatory protein-bound polysaccharide, PS-K, in combination with a surgical operation on MCA-induced tumors in autochthonous mice, with special reference to the timing of their combination.

Materials and methods

Mice. Female C57BL/6 mice 6 weeks old were purchased from the Shizuoka Agricultured Coop. Assoc. for Laboratory Animals (Hamamatsu, Japan).

Tumors. 3-Methylcholanthrene (1 mg) (Sigma Chem. Co., St. Louis, Mo.) in 0.1 ml of olive oil was injected into the right hindlimb of 7 ~ 8-week-old mice according to the method reported by Wexler and Rosenberg [32]. Each mouse was examined to determine tumor development by measuring the difference in thickness between the right and left limbs once every week from 8 weeks after the MCA injection. Most tumors used in this experiment developed between 12 to 18 weeks after the MCA injection as determined by a 3 mm difference in thickness between the right and left limbs.

Treatment of mice. The initial treatment of either surgery, chemotherapy, or the administration of PS-K was carried out when the estimated tumor size had reached 8 mm in diameter. The right hindlimb was amputated at the hip under ether anesthesia to surgically remove the primary tumors. The mice were then divided into eight experimental groups, one of which was given no further treatment, while the others were treated with PS-K (Kureha Chem. Co., Tokyo, Japan) either before or after the surgery. PS-K was dissolved in a sterilized 0.85% NaCl solution (saline) and was administered i. p. at doses of 300 mg/kg per day 5 days a week for 1 week (300 mg/kg $\times 5/w \times 1$), 150 mg/kg per day 5 days a week for 2 weeks (150 mg/kg $\times 5/w \times 2$) or 300 mg/kg per day twice a week for 7 weeks (300 mg/kg $\times 2/w \times 7$). One group of mice was given PS-K p. o. at a dose of 1000 mg/kg per day for 5 days a week (1000 mg/kg per day $\times 5/w \times 1$) for 1 week before surgery. Thus, the moment when the tumors were surgically removed was delayed for a week, and in the cases where PS-K was given before surgery most tumors grew 1 ~ 2 mm larger in diameter during that time. Cyclophosphamide (Shionogi Pharm. Co., Osaka, Japan) was administered i. v. at a dose

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Abbreviations used: MCA; 3-methylcholanthrene, CY; cyclophosphamide, MST; mean survival time

of 150 mg/kg as chemotherapy when the tumors had reached 8 mm in diameter.

Observation of treated mice. All the mice were kept under observation until they died either of local recurrent tumors or metastases. The survival time in days for each mouse after the initial treatment was recorded in order to obtain the MST and the survival curve of each treated group. The mice which died were autopsied for estimation of recurrent and metastatic disease.

Statistical analysis. The incidence of local recurrent tumors between the two treated groups was compared by means of the χ^2 test. A significant difference in the MST and survival curves of treated mice from those of the control mice was established by the Student's *t*-test and the generalized Wilcoxon test respectively.

Results

Effects of a single treatment with surgery, chemotherapy, or immunotherapy on the survival times of mice bearing MCA-induced autochthonous tumors

The results of previous experiments which had been carried out in order to investigate the therapeutic effects on autochthonous tumors in mice are summarized in Table 1. We observed some beneficial effects on the survival times of the mice as a result of surgery and chemotherapy but not as a result of immunotherapy with PS-K alone. The survival times in days of 32 nontreated mice were found to fall over a broad range and their MST was 21.5 days with a standard error of 8.9 days. The MST (47.8 ± 15.6 day) of the mice was distinctly prolonged by surgical removal of the primary tumor, although 40 of 42 mice (95%) died of locally recurrent or metastatic tumor. Chemotherapy with CY (150 mg/kg, i. v.) brought about a slight prolongation of the MST (25.9 ± 8.5 days) yet immunotherapy with PS-K (300 mg/kg × 5/w × 1) alone seemed to have no effect at all. On the basis of the above results we proceeded with further experiments in order to explore the combined effects of surgery and immunotherapy on autochthonous tumors.

Table 1. Survival time of C57BL/6 mice bearing MCA-induced autochthonous tumors after treatment by surgery, chemotherapy, or immunotherapy

Treatment with[a]	Died/treated (%)	MST ± SD[b]
None	32/32 (100)	21.5 ± 8.9
Surgery[c]	40/42 (95)	47.8 ± 15.6[f]
Chemotherapy[d]	26/26 (100)	25.9 ± 8.5[g]
Immunotherapy[e]	33/33 (100)	19.2 ± 8.5

[a] The treatments were carried out when tumor sizes reached 8 mm in diameter after 1 mg of MCA had been injected i.m. into the right hindlimb of C57BL/6 mice
[b] Mean survival time in days and standard deviation after the treatment
[c] The primary site of tumors was removed by surgical amputation of the hindlimb at the hip
[d] Cyclophosphamide (150 mg/kg) was administered i.v. via the tail vein
[e] PS-K (300 mg/kg per day) was administered i.p. 5 times for 1 week
[f, g] Statistically significant difference compared with the nontreated group, f; $P<0.001$, g; $P<0.01$

Table 2. Prolongation of survival time of C57BL/6 mice by PS-K after surgical removal of MCA-induced autochthonous tumors

Protocol of PS-K[a] administration	Died/treated (%)	MST ± SD[b]
Surgery alone	64/66 (97.0)	48.6 ± 16.3
PS-K before surgery		
300 mg/kg × 5/w × 1, i.p.	66/66 (100)	63.4 ± 14.3[c]
150 mg/kg × 5/w × 2, i.p.	36/36 (100)	62.2 ± 15.1[c]
1000 mg/kg × 5/w × 1, p.o.	25/25 (100)	61.9 ± 16.8[c]
PS-K 1 day after surgery		
300 mg/kg × 5/w × 1, i.p.	62/64 (96.9)	60.5 ± 12.2[c]
300 mg/kg × 2/w × 7, i.p.	65/66 (98.5)	59.6 ± 11.5[c]
PS-K 8 days after surgery		
300 mg/kg × 5/w × 1, i.p.	39/39 (100)	60.6 ± 13.0[c]
300 mg/kg × 2/w × 7, i.p.	39/39 (100)	59.8 ± 13.2[c]

[a] The initial treatment with either PS-K or surgery was carried out when tumor sizes reached 8 mm in diameter after 1 mg of MCA had been injected i.m. into the right hindlimb of C57BL/6 mice
[b] Mean survival time in days and standard deviation after the initial treatment
[c] Statistically significant difference compared with the group treated by surgery alone, $P<0.001$

Table 3. Prolongation of survival time of C57BL/6 mice with recurrent tumors by PS-K after surgical removal of MCA-induced autochthonous tumors

Protocol of PS-K[a] administration	Recurred/treated (%)	MST ± SD[b]
Surgery alone	36/66 (54.6)	49.8 ± 17.2
PS-K before surgery		
300 mg/kg × 5/w × 1, i.p.	31/66 (47.0)	65.3 ± 15.0[c]
150 mg/kg × 5/w × 2, i.p.	14/36 (39.8)	58.1 ± 15.1
1000 mg/kg × 5/w × 1, p.o.	12/25 (48.0)	67.5 ± 16.2[c]
PS-K 1 day after surgery		
300 mg/kg × 5/w × 1, i.p.	22/64 (34.4)[e]	59.4 ± 13.2[d]
300 mg/kg × 2/w × 7, i.p.	22/66 (33.3)[e]	61.4 ± 11.8[d]
PS-K 8 days after surgery		
300 mg/kg × 5/w × 1, i.p.	14/39 (35.9)	59.6 ± 12.5[e]
300 mg/kg × 2/w × 7, i.p.	15/39 (38.5)	54.8 ± 11.7

[a] See the footnote to Table 2
[b] Mean survival time in days and standard deviation after the treatment
[c, d, e] Statistically significant difference compared with the group treated by surgery alone, c; $P<0.001$, d; $P<0.005$, e; $P<0.05$

Effects of the administration of PS-K on the survival times of mice after surgical removal of the primary tumors

The MSTs of mice treated with PS-K in conjunction with surgery were prolonged as compared with the MST of mice treated by surgery alone (Table 2). The administration of PS-K was found to be effective regardless of the timing of tis combination with surgery. Moreover, it is noteworthy that the MST was also prolonged even in a group of mice given PS-K (300 mg/kg × 5/w × 1) before surgery when the surgical removal of primary tumors was delayed for a week. Complete cure rates, however, could not be established by the combined use of PS-K with surgery.

Table 4. Prolongation of survival time of C57BL/6 mice which died of pulmonary metastatic tumors by PS-K after surgical removal of MCA-induced autochthonous tumors

Protocol of PS-K[a] administration	Died of metastasis without recurrence (%)	MST ± SD[b]
Surgery alone	28/30 (93.3)	47.0 ± 15.3
PS-K before surgery		
300 mg/kg × 5/w × 1, i.p.	35/35 (100)	61.8 ± 13.6[c]
150 mg/kg × 5/w × 2, i.p.	22/22 (100)	65.0 ± 16.4[c]
1000 mg/kg × 5/w × 1, p.o.	13/13 (100)	56.7 ± 16.2
PS-K 1 day after surgery		
300 mg/kg × 5/w × 1, i.p.	40/42 (95.2)	61.0 ± 11.7[c]
300 mg/kg × 2/w × 7, i.p.	43/44 (97.7)	58.6 ± 11.3[d]
PS-K 8 days after surgery		
300 mg/kg × 5/w × 1, i.p.	25/25 (100)	61.1 ± 13.4[c]
300 mg/kg × 2/w × 7, i.p.	24/24 (100)	62.9 ± 13.3[c]

[a] See the footnote to Table 2

[b] Mean survival time in days and standard deviation after the initial treatment

[c, d] Statistically significant difference compared with the group treated by surgery alone, c; $P < 0.001$, d; $P < 0.05$

Effects of the administration of PS-K on local recurrences

Further analyses of the therapeutic effects of PS-K revealed that recurrent tumors were found on the right hip joints in 36 of 66 mice (54.6%) treated by surgery alone and that their MST was 49.8 days. The incidence of recurrence significantly decreased in the two groups of mice when PS-K was given 1 day after surgery (Table 3). A similar tendency for a reduction in recurrence was observed, but not significantly, in other groups of mice treated with PS-K before or 8 days after surgery. These data suggest that immunotherapy with PS-K is effective in inhibiting the recurrence of local tumors after surgical removal of the primary tumor.

Effects of the administration of PS-K on metastases

Local recurrence of tumors was not observed in 30 of the 66 mice treated by surgery alone, although 28 mice (93.3%) eventually died of metastasis of tumors mainly in the lung (Table 4). A clear effect of PS-K was noted in the prolongation of survival times. The MSTs of mice which died of metastasis were significantly prolonged in all groups except for the group given PS-K p. o. before surgery as compared with the MST (47.0 ± 15.3 days) of mice treated by surgery alone. The effects of PS-K on the survival time of mice were also demonstrated by the survival curves (Fig. 1), e. g., as a result of analyses by the generalized Wilcoxon test ($P < 0.001$), the survival curves of mice in groups given PS-K (300 mg/kg × 5/w × 1) before, 1 day, or 8 days after surgery were statistically significant when compared with those of mice treated by surgery alone. It is noteworthy that the early death of mice was prevented by the combination of PS-K with surgery.

Discussion

We have demonstrated the combined effects of surgery and immunotherapy with PS-K on autochthonous tumors in C57BL/6 mice. We became aware that it requires complicated manipulation and considerable effort to use autochthonous tumors as a model for the experimental treatment of tumors. There may also be considerable differences between autochthonous and transplanted tumors if we take the following factors into consideration.

Firstly, autochthonous tumors may be composed of more heterogeneous populations of tumor cells than are transplantable tumors [3, 6, 7, 10, 13, 14, 24, 27]. Transplantable tumor cells become relatively homogeneous as a result of selections made over long periods during repeated transplantation in animals. It is necessary, therefore, to consider whether the results of experimental treatment of less heterogeneous tumor cells can be directly reproduced in the treatment of a human cancer which is, of course, an autochthonous tumor. Secondly, it has to be pointed out that a tumor-host relationship of transplanted tumors is brought about abruptly by the artificial manipulation of transplantation, while the relationship of an autochthonous tumor to its host is brought about gradually by the natural states of growth which have developed over a long period following the initiation of a single tumor cell. Such a difference in the tumor-host relationship will naturally affect the therapeutic effects of cancer treatments [1]. The therapeutic effects which we observed in autochthonous tumors in C57BL/6 mice are not fundamentally different from those seen in transplanted ones. These facts convince

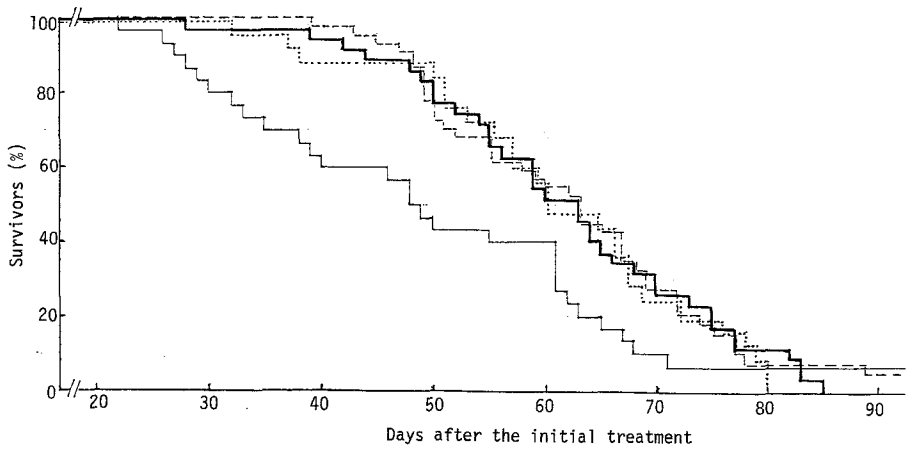

Fig. 1. Survival curves of mice which died of pulmonary metastasis after treatment with PS-K. MCA (1 mg) was injected into the right hindlimb of C57BL/6 mice and treatment was carried out when the tumor had grown to 8 mm in diameter by surgery alone (———), PS-K (300 mg/kg × 5/w × 1) before surgery (———), 1 day after surgery (– – –), and 8 days after surgery (. . . . .). A generalized Wilcoxon test gave a statistical difference in the curves of mice treated with PS-K compared with the curve of the group treated by surgery alone

us that we can expect a similar therapeutic efficacy with autochthonous tumors.

The effects of PS-K on experimental tumors [1, 2, 11, 17, 20, 29, 31] and on immune responses [5, 17, 19] have already been reported. We also reported that the therapeutic effects of PS-K alone were not strong enough to retard the growth of an established tumor [2, 17]. It is necessary therefore to combine this treatment with other treatments in order to minimize the tumor burden. PS-K alone was effective in curbing the recurrence and/or the metastasis after surgical removal of the primary tumor, and it may also be effective in dealing with the small number of tumor cells that remain after surgery. Necessarily, however, the effects on the autochthonous tumors have not been so clear as on transplanted tumors and have required the observation of more cases in each treated group before the therapeutic effects can be considered significant. It proved difficult to provide any complete cure, as has often been observed in previous experiments using transplanted tumors [2, 15]. This lack of curative effectiveness may be due to the heterogeneous sensitivity of the autochthonous tumors. The heterogeneity of tumor cells may cause the eventual growth of insensitive tumor cells, resulting in the death of most mice. This suggests the necessity of establishing further effective regimens against autochthonous tumors in the primary host. PS-K was found to enhance antiinfectious immunity [30]. Further investigation, therefore, is required to examine the role of the antiinfectious immunity of PS-K on the prolongation of survival time of mice.

With regard to the timing of PS-K administration, the authors have already reported that the combination timing of immunotherapy and chemotheraqy has a great influence on the therapeutic efficacy. For instance, immunotherapy with PS-K is more effective when it is given before chemotherapy with CY than when it is given after CY [2, 17]. In the present studies on the combination of PS-K with surgery, such a clear difference of therapeutic effects provided by the various timings of PS-K administration was not observed, although the incidence of local recurrence was reduced significantly by the administration of PS-K just after surgery, while the MST of mice with the metastasis was most prolonged by the administration of PS-K ($300\ mg/kg \times 5/w \times 1$) before surgery. It can therefore be stated that the administration of PS-K requires specific timing when used in conjunction with chemotherapy though not with surgery. There are three possible explanations for the above findings: (1) the administration of PS-K has been found to restore CY-induced suppression of antitumor immunity effectively when given before CY [17]. The immunosuppression brought about by the amputation of a limb may be slight as compared with that by CY chemotherapy, although it has been reported that a surgical operation such as a thoracotomy can lead to severe immunosuppresion [9]. It is not required therefore to administer PS-K at certain times to restore the antitumor immunity. (2) It should be observed that PS-K is effective not only on the host immune system. Therefore, PS-K may assist tumor cells to become sensitivie to CY chemotherapy. (3) PS-K may alter the activity of the drug-metabolizing enzymes which augment the antitumor activity of CY [34]. The last two explanations are not relevant to treatment using PS-K in combination with surgery.

Nevertheless, the fact that immunotherapy with PS-K was found to be effective in treating advanced autochthonous tumors in conjunction with surgery encourages us to investigate further the therapeutic effects of a newly developed regimen for cancer immunotherapy by using this MCA-induced tumor in autochthonous mice.

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