Prevention of Lung Metastasis by Intra-tumoral Injection of Cepharanthin and Staphylococcal Enterotoxin B in Transplantable Rat Osteosarcoma

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The antitumor effect of intra-tumoral injection of Cepharanthin, a biscoclaurin alkaloid extracted from Stephania cephalanta Hayata, and staphylococcal enterotoxin B was evaluated using F344 male rats bearing transplantable rat osteosarcoma, S-SLM. A macroscopic lung metastatic nodule of tumor was transplanted into the subcutaneous back space, and 0.5 mg of Cepharanthin and 2 pg of staphylococcal enterotoxin B were injected into the tumor on days 12, 13 and 14. On day 28, all animals were killed with an overdose of pentobarbital sodium, and the transplanted tumors and lungs were examined. The wet weight of the lungs of the rats treated with Cepharanthin and staphylococcal enterotoxin B was significantly lower, and apoptosis in the lung metastatic nodules was significantly higher than that of the control or that of rats treated with only Cepharanthin or staphylococcal enterotoxin B. In the transplanted tumors, infiltration of TRAP (tartrate-resistant acid phosphatase)-positive multinucleated giant cells was prominent in the rats treated with Cepharanthin and staphylococcal enterotoxin B. These findings indicate that intra-tumoral injection of Cepharanthin and staphylococcal enterotoxin B induced infiltration of TRAP-positive multinucleated giant cells within the transplanted rat osteosarcoma, and reduced lung metastasis.

Key words: Cepharanthin — Apoptosis — Osteosarcoma — Lung metastasis — Enterotoxin

It was reported that Cepharanthin, a biscoclaurin alkaloid extracted from Stephania cephalanta Hayata, inhibits proliferation of cancer cells as a biological response modifier.1, 2) Ebina et al. demonstrated that intra-tumoral administration of Cepharanthin prevented metastasis in their model (the double grafted tumor system), in which mice first received simultaneous intradermal inoculation of tumor cells in both right and left flanks, and were then injected with 0.5 mg of Cepharanthin in the right tumor. The intra-tumoral administration of Cepharanthin in the double grafted tumor system inhibited the growth of not only the right, but also the left tumor.3) We examined the effect of intra-tumoral injection of Cepharanthin in transplantable rat osteosarcoma, S-SLM, which has high lung metastatic potential.3) This was not sufficient to prevent metastasis, although infiltration of macrophages was induced around the metastatic foci in the lung.

Superantigens including staphylococcal enterotoxin B are strong activators of macrophages.4) Biological antitumor effects of activated macrophages have occasionally been reported,4, 5) and several reports have mentioned the direct antitumor effect of staphylococcal enterotoxin B.5)

Staphylococcal enterotoxin B may enhance the antitumor effect of Cepharanthin in vivo. The purpose of this study was to examine the combined effect of Cepharanthin and staphylococcal enterotoxin B on a transplantable rat osteosarcoma, S-SLM.

MATERIALS AND METHODS

Animals Specific-pathogen-free male Fisher 344 rats were purchased from Shizuoka Laboratory Co., Shizuoka. Rats were used at 4 weeks of age. The weight of the rats ranged from 60 to 70 g. The animals were housed in an air-conditioned room at 22°C, and given a standard diet (CE-2, Clea Japan Inc., Tokyo) and water. The protocols for the animal experiments described in this paper were previously approved by the Animal Research Committee, Akita University School of Medicine; all subsequent animal experiments adhered to the “Guidelines for Animal Experimentation” of the University.

Chemicals Cepharanthin was kindly provided by Kaken Pharma Co., Ltd., Tokyo. Staphylococcal enterotoxin B was purchased from Sigma Chemical Co., St. Louis, MO. All reagents except staphylococcal enterotoxin B were free of endotoxin as determined by “E-TOXATE” (Sigma Chemical Co.).

Tumor and transplantation Transplantable rat osteosarcoma was kindly supplied by Dr. Y. Mii, Nara Medical School, Kashihara, Nara. The tumor S-SLM, derived from a spontaneous osteosarcoma, was established by serially
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transplanting lung metastatic lesions into subcutaneous tissue and has high lung metastatic potential. All procedures were performed under aseptic conditions.

**Experimental protocol** A lung metastatic nodule, approximately 5 mm in diameter, was transplanted into the subcutaneous back space of each rat. Rats were divided into 4 treatment groups, as follows: group CR, Cepharanthin 0.5 mg and saline 0.1 ml; group SEB, staphylococcal enterotoxin B 2 pg and saline 0.1 ml; group CR+SEB, Cepharanthin 0.5 mg and staphylococcal enterotoxin B 2 pg; control, saline 0.2 ml. In each group, Cepharanthin and staphylococcal enterotoxin B were injected into the tumor on days 12, 13 and 14 after the tumor transplantation. Each group consisted of 8 rats. On day 28, all animals were killed with an overdose of pentobarbital sodium, and the transplanted tumor and bilateral lungs were examined.

**Evaluation and tissue preparation** The wet weight of the transplanted tumor and the bilateral lungs was measured. For histological sections, a viable portion of the transplanted tumor was selected by macroscopic examination, since the central portion of the tumor was necrotic. The left lung was cut on the plane parallel to the main bronchus. Tissues were fixed in neutral formalin and embedded in paraffin as usual. Three-point-five-micrometer sections were cut on silane-coated glass slides (Muto Pure Chemicals, Tokyo). Three histological slides were obtained from each paraffin block. Hematoxylin and eosin stain, TUNEL method stain by “Apoptag” (Dako Japan, Tokyo), and TRAP (tartrate-resistant acid phosphatase) stain were applied to each block. The ratio of the size of the metastasis in the left lung to that of the lung was measured using a computerized image-analysis unit (Cosmozone 1S, Nikon, Tokyo) in the hematoxylin and eosin-stained section. In the TUNEL method stain and TRAP stain, positive cells were counted in the transplanted tumor and metastatic foci of the lung. The average numbers were calculated based on 50 random high-power fields, and statistically analyzed by the ANOVA test. Differences between groups were considered significant when the P value was 0.05 or smaller.

**RESULTS**

**Wet weight of the transplanted tumor and bilateral lungs** The wet weight of the tumors was 17.7±3.6 g (mean±SD), 19.3±4.3, 14.2±2.9, 21.6±5.9, in the groups CR, SEB, CR+SEB, and the control, respectively. The wet weight of the group CR+SEB was significantly lower than that of the control (P=0.0018). In macroscopic evaluation, lung metastasis was reduced in the rats treated with Cepharanthin and staphylococcus enterotoxin B (Fig. 1A).

The wet weight of the bilateral lungs was 2.5±0.7 g (average±SD), 2.8±0.5, 1.1±0.3, 2.9±0.5 in the groups CR, SEB, CR+SEB, and the control, respectively. The wet weight of bilateral lungs in the group CR+SEB was significantly lower than those of other groups (P<0.0001, Fig. 1B).

**Histological features of the metastatic tumor and the ratio of the size of the metastatic tumor to that of the lung** Histological examination of the left lung showed prevention of metastatic lesions in the group CR+SEB (Fig. 2, A and B). The ratio of the metastatic tumor to the lung was 21.3±4.0% (mean±SD), 26.3±4.5, 11.5±4.2, and 34.1±3.6 in the groups CR, SEB, CR+SEB, and the control, respectively. The ratio of the size of the metastasis of the left lung to that of the lung in the group CR+SEB was significantly lower than those of the groups CR, SEB, and the control (P<0.0001), and those of the groups CR

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**Fig. 1.** (A) Gross findings of the lungs in the group CR+SEB (treated with Cepharanthin and staphylococcal enterotoxin B) and the control. Lung metastases were reduced in the group CR+SEB. (B) The wet weight of bilateral lungs in group CR+SEB was significantly lower than those of groups CR (treated with Cepharanthin), SEB (treated with staphylococcal enterotoxin B), and control.
and SEB were significantly lower than that of the control ($P<0.0001$, $P=0.0007$). Infiltration of macrophages with foamy cytoplasm was observed around the metastatic nodules in the groups CR, SEB, and CR+SEB. This infiltration was prominent in the group CR+SEB (Fig. 2C).

The number of TUNEL-positive cells (“Apoptag” stain) The number of positive cells in the transplanted tumors was $2.0 \pm 0.6$ (mean$\pm$SD), $0.9 \pm 0.3$, $3.6 \pm 1.0$, and $0.8 \pm 0.4$ in the groups CR, SEB, CR+SEB, and the control, respectively. The number of positive cells in the
transplanted tumor in the group CR+SEB was significantly higher than those of the groups CR, SEB, and the control (\(P<0.0001\)), and that of the group CR was significantly higher than those of the group SEB and the control (\(P=0.0020, P=0.0007\)). In the metastatic foci, apoptotic cells were marked in the group CR+SEB (Fig. 3A). The number of positive cells in the metastatic foci in the group CR+SEB was significantly higher than those of the groups CR, SEB, and the control (3.8±0.8 vs. 1.7±0.5, 0.9±0.2, and 0.7±0.3, \(P<0.0001\)), and that of the group CR was significantly higher than those of the group SEB and the control (\(P=0.005, P=0.0004\), Fig. 3B).

**Number of cells positive to TRAP stain** TRAP-positive cells were diffusely observed in the transplanted tumors in the group CR+SEB. Almost all of these TRAP-positive cells were multinucleated giant cells (Fig. 4A). These TRAP-positive cells were also observed in the groups CR and SEB, but were sparse in the control. The number of TRAP-positive cells in the transplanted tumors was 1.8±0.5 (mean±SD), 3.0±0.7, 7.1±3.1, 0.7±0.3 in the groups CR, SEB, CR+SEB, and the control, respectively. The number of TRAP-positive cells in the group CR+SEB was significantly higher than those of the other groups (\(P<0.0001\)), and that of the group SEB was significantly higher than that of the control (\(P<0.0097\), Fig. 4B). In the lung metastatic foci, no TRAP-positive cell was observed.

**DISCUSSION**

Cepharanthin has been used in the treatment of several diseases, such as allergy, leucopenia associated with radiation therapy, and malignancies with multidrug resistance,\(^8,9\) and has many antitumor effects.\(^10-12\) The effects of Cepharanthin are thought to be derived from its potent membrane-stabilizing effect,\(^13\) and its effectiveness in increasing the number of T-cells,\(^14\) in increasing the cell activity of lymph nodes,\(^11\) in increasing the activity of spleen-derived T-cells,\(^3\) and in blocking the traverse of tumor cells through the S phase.\(^10\) Prevention of transplanted tumor proliferation was pronounced when Cepharanthin was administered into the tumor, but subcutaneous administration was not effective.\(^1,11\) In our study, intra-tumoral injection of only Cepharanthin showed a mild, but not significant, inhibitory effect on tumor growth. This inhibitory effect was enhanced by the combined injection of Cepharanthin and staphylococcal enterotoxin B. Similar effects are evident in the prevention of lung metastasis in the experimental animal model S-SLM. Osteosarcoma is the most frequent malignant bone tumor, and has a high metastatic potential to the lung. In the treatment of osteosarcoma, therefore, prevention of lung metastasis is one of the most important goals. The S-SLM used in our study is a transplantable rat osteosarcoma with high metastatic potential to the lung, and is considered useful as an experimental model to study the biological behavior of this type of malignancy.

Furusawa et al. demonstrated that apoptosis of P388 Doxorubicin-sensitive and -resistant cells occurred after exposure to Cepharanthin for 24 h.\(^2\) Rat osteosarcoma treated with Cepharanthin only showed apoptosis within the transplanted tumor, and this apoptosis was more prominent after combined administration of Cepharanthin and staphylococcal enterotoxin B. Interestingly, apoptosis in the lung metastatic foci in the rats treated with Cepharanthin and staphylococcal enterotoxin B was also frequently observed. Intra-tumoral injection of Cepharanthin appeared
to induce apoptosis not only in the primary tumor but also in the metastatic foci, and staphylococcal enterotoxin B, which was administered with Cepharanthin, played an important role in enhancing the effect of Cepharanthin.

A major function of macrophages is to participate in host defense. It is well known that macrophages are able to recognize tumor cells specifically in vivo, and inhibit growth of solid tumors by a process of infiltration and cytotoxic effect.\(^5\) Several patterns of macrophage infiltration have been noted.\(^5,\)\(^6\) Fidler showed that several agents, including lymphokines, microorganisms, and enterotoxins, activated the antitumor effect of macrophages.\(^6\) In our study, infiltration of TRAP-positive multinucleated giant cells within the transplantable rat osteosarcoma was induced by intra-tumoral injection of staphylococcal enterotoxin B, and this effect was markedly enhanced by the combined injection of Cepharanthin and staphylococcal enterotoxin B. Since TRAP was found in activated macrophages and osteoclasts,\(^7,\)\(^7,\)\(^8\) these TRAP-positive multinucleated giant cells were probably macrophages. The diffuse infiltration of TRAP-positive multinucleated giant cells within the tumor may be associated with the inhibition of tumor growth and the prevention of metastasis. Newell et al. showed that systemic administration of staphylococcal enterotoxin B activates T-cells expressing the \(V\beta\) gene products 7 or 8.1–3, and increased interleukin 2 receptor expression and production of interferon-\(\gamma\).\(^8\) Dohlsten et al. reported that chemical conjugates of superantigen and colon carcinoma-reactive monoclonal antibodies mediate T-cell dependent destruction of colon carcinoma cells lacking MHC class II molecules.\(^9\) In addition, Cepharanthin alone has some effect on the function of host immune cells such as NK, T cells,\(^1\)\(^1,\)\(^11,\)\(^14\) and a characteristic histological feature of this experiment is infiltration of macrophages within or around the tumor. Considering the above findings, T cell-associated or direct activation of macrophages is probably the most important mechanism of metastasis inhibition in the combination therapy of Cepharanthin and staphylococcal enterotoxin B.

Newell used intraperitoneal administration of 5 to 250 \(\mu\)g of staphylococcal enterotoxin B at the time of inoculation of mice with tumor fragments, and found a statistically significant decrease in the frequency of tumor outgrowth.\(^6\) However, we found that only 2 \(\mu\)g of staphylococcal enterotoxin B administered 3 times was enough to enhance the anti-tumor effect of Cepharanthin. It should be stressed that the combined injection of Cepharanthin and low-dose staphylococcal enterotoxin B could induce intra-tumoral infiltration of macrophages. The use of smaller doses of staphylococcal enterotoxin B has the clinical advantage of preventing side effects. Since intratumoral injection of antitumor agents would be easy in tumors occurring in the extremities and on the surface of the body, osteosarcoma or other soft tissue sarcomas would be good targets. When we tried to culture these multinucleated cells, however, the results were poor. If these tumor-associated multinucleated giant cells can be cultured, they might be useful in tumor treatment via several administration routes. The clinical use of enterotoxin in the treatment of tumors should be further investigated.

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Rare-type Mutations of MMAC1 Tumor Suppressor Gene in Human Glioma Cell Lines and Their Tumors of Origin

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A total of 10 glioma cell lines were examined to evaluate the status of the MMAC1 gene, a candidate tumor suppressor gene. Six cell lines showed mutations with presumed loss of heterozygosity and 1 cell line showed no mRNA expression. The 6 mutations consisted of 3 3-bp deletions (codons 17, 101 or 199), 1 missense mutation (codon 252) and 2 truncation mutations (1 nonsense mutation at codon 233 and 1 2-bp insertion at codon 241). Among them, the 3-bp deletions, which are a rare type of mutation in MMAC1 gene, were located in the N-terminal half (codons 1–212) of the coding region, which is considered important in MMAC1 function. The missense mutation was located unusually in the C-terminal half (codons 212–403), but it was in a small region in which some other reported missense mutations are clustered. Thus, these 4 mutations were suggested to have functional effects on the MMAC1 activity, like the other 2 mutations with predicted protein truncations. By sequence analysis of cDNA clones, we confirmed that all the mutations including these 4 rare ones were in the MMAC1 gene, not in the PTH2 pseudogene. In 2 cases, we also examined the primary glioma tissues from which the cell lines had been derived and found the same mutations as in the cell lines in both cases. This suggested that the mutations in these cell lines were derived from the primary glioma tissues, but not from artifacts arising during long-term in vitro cultivation.

Key words: MMAC1 — PTEN — Tumor suppressor gene — Pseudogene — Glioma

The MMAC1 gene (also called PTEN and TEP1), a candidate tumor suppressor gene which is located on chromosome 10q23, contains 9 exons and encodes 403 amino acids.1–3 The proximal half of the protein is homologous to phosphatases and cytoskeleton-associated proteins, tensin and auxilin,1–3 and its phosphatase activity has been demonstrated in in vitro assays.1–4 MMAC1 gene alterations have already been examined in various tumors including malignant glioma, prostate carcinoma and endometrial carcinoma.1, 2, 7–10 Among them, malignant gliomas have revealed frequent alterations in both primary tumor tissues and cell lines,1, 2, 7–14 consistent with the previous LOH (loss of heterozygosity) studies that showed frequent deletion of regions of chromosome 10 in malignant gliomas.20–22 MMAC1 gene alterations in malignant gliomas included small deletions, small insertions, splicing mutations, nonsense mutations and missense mutations. However, despite the considerable number of MMAC1 gene alterations reported, the entire profile of the alterations in malignant gliomas and other tumors as well has not been fully detailed.

In this study, we examined a total of 10 glioma cell lines for alterations of the MMAC1 gene and its mRNA and found mutations in 6 cell lines and no mRNA expression in 1 cell line. All the mutations, which included 4 rarely reported ones, were confirmed to be in the MMAC1 gene itself, but not in the PTH2 pseudogene (also called pPTEN),23, 24 and were analyzed in connection with previous results in various malignancies. In 2 cases, we also examined primary glioma tissues from which the cell lines had been derived and detected the same mutations as in the cell lines.

MATERIALS AND METHODS

Glioma cell lines Ten human glioma cell lines were used. Nine (cases 1–6 and 8–10) had been established in our institute25–28 and the other was U-251MG (case 7).29 The pathological diagnosis of the primary tumors from which the cell lines were established was anaplastic glioma (WHO grade III) in 8 cases and glioblastoma (WHO grade IV) in 2 cases.25 Brief summaries of the clinical and pathological data of the patients are given in Table I.

DNA isolation DNA was isolated from glioma cell lines and frozen primary glioma tissues as described.25

RNA isolation Total RNA of glioma cell lines was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method.30

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