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Inhibition of spontaneous and experimental tumor metastasis by the calcium antagonist verapamil

Takashi Tsuruo, Harumi Iida, Fusao Makishima, Takao Yamori, Hironori Kawabata, Shigeru Tsukagoshi, and Yoshio Sakurai

Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Kami-Ikebukuro Toshima-ku, Tokyo 170, Japan

Summary. *Verapamil, a calcium antagonist, inhibited both experimental (IV inoculation of tumor cells) and spontaneous metastasis (SC inoculation) of the highly metastatic B16 melanoma and colon adenocarcinoma 26 cell lines. Verapamil treatment resulted in a maximum 80% inhibition of metastases, the degree of inhibition varying among the different metastatic systems. Verapamil inhibited platelet aggregation induced by these tumor cell lines, the patterns of inhibition being different for B16 melanoma and colon adenocarcinoma. The inhibition of platelet aggregation induced by tumor cells is proposed as a mechanism by which the calcium antagonist exerts its antimetastatic effect. These results, together with our previous findings that calcium antagonists can increase the cytotoxicity of drugs in tumor cells with induced or inherent drug resistance by inhibiting outward transport of the drug, indicate that calcium antagonists have potential as a new class of adjuvant agents in the field of cancer chemotherapy.*

Introduction

The ability to inhibit tumor metastasis effectively would be an important step forward in cancer treatment, as current chemotherapy approaches for the management of metastatic disease and immunotherapy intended to delay or inhibit onset of metastasis are most often not successful. The search for agents with this capacity therefore has been undertaken.

A growing body of evidence suggest that platelet aggregation plays an important role in tumor metastasis [1–4, 6, 9–11, 24]. Interactions between tumor cells and platelets causes enhanced tumor cell localization in the microvasculature and/or tumor cell adhesion to the blood vessel walls, thereby facilitating metastasis. Thus, it is not surprising that several inhibitors of platelet aggregation have been found to retard metastasis in several tumor models [1, 4, 6, 10, 11, 24].

Platelet aggregation encompasses a complicated series of steps (see reference [23] for review), and both intracellular and extracellular calcium are considered to be involved in this process [23]. Calcicum antagonists inhibit the calcium slow channel in smooth muscle cells, which results in decreased cellular calcium. In addition to the classic role of calcium antagonists, recent findings from this laboratory indicate that various calcium channel blockers can inhibit the efflux of some chemotherapeutic agents from resistant tumor cells, and thereby reverse drug resistance in these cells [15–17, 19]. Although the mechanisms of the phenomenon are not known, it is speculated that the calcium antagonists, as a part of this process, might modify the cellular calcium environment of the tumor cells [17, 19]. As calcium antagonists have been shown to inhibit platelet aggregation in a variety of experimental systems [12], it then seems possible that calcium antagonists also could inhibit tumor metastasis by virtue of their potential to modify cellular calcium environment of tumor cells and thereby change the tumor-platelet interaction. We have examined the effect of verapamil, a calcium antagonist, on various models of tumor metastasis. Verapamil inhibited the tumor metastasis in both spontaneous (after SC inoculation of tumor cells) and experimental (after IV inoculation of tumor cells) models, and the drug also inhibited the platelet aggregation induced by tumor cells.

Materials and methods

Animals and tumor cells. Adult (12- to 16-week old) male Balb/c and C57BL/6J mice were obtained from Charles River Japan Inc., Tokyo, Japan. The metastatic variant (B16 BL-6) of B16 melanoma origin [5] was kindly provided by Dr J. Fidler (M.D. Anderson Hospital & Tumor Institute). Spontaneous axillary lymph node and lung metastases occur after the inoculation of these tumor cells into a front footpad [5, 22], and experimental metastasis occurs after IV inoculation of tumor cells [21]. The metastatic colon cancer cell clones C26 NL-17 and C26 NL-22 used in this study were isolated from a metastatic variant of colon adenocarcinoma 26 (C26) in this laboratory [20]. Experimental lung metastasis occurs after the inoculation of C26 NL-17 IV [20, 21] and spontaneous lung metastasis occurs after the inoculation of C26 NL-22 into a front footpad [20, 22]. All tumor lines were maintained in culture in minimal essential medium (MEM) containing 10% calf serum.

Tumor metastasis models and drug administration. Logarithmically growing B16 BL-6 cells (5×10^4), harvested from culture dishes by trypsin were inoculated IV into C57BL/6J mice (10 mice/group) [20, 21]. Verapamil formulated as previously described [19] was given IP once a day for 6 days beginning 2 days prior to tumor inoculation. Lung metastasis was examined 25 days after the tumor inoculation [20, 21].

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B16 BL-6 cells (2.5×10^5) were inoculated SC into the right front footpads of C57BL/6J mice (10 mice/group) [20, 22]. Verapamil was given once a day for 11 days as above, starting from day 5 after the tumor inoculation. On day 17 the right foreleg, including the original tumor, was amputated. Lymph node and lung metastasis were examined on day 38 [20, 22].

C26 NL-17 cells (5×10^4) were inoculated IV into Balb/c mice (10 mice/group) [20, 21]. Verapamil was given IP for 6 days as above, beginning 2 days before tumor inoculation. Lung metastasis was examined on day 23 [20, 21].

C26 NL-22 cells (1×10^6) were inoculated SC into the right front footpads of Balb/c mice (10 mice/group) [20, 22]. Verapamil was given IP once a day for 7 days starting at day 6 after inoculation. The right foreleg, including the original tumor, was amputated on day 13, and spontaneous lung metastasis was examined on day 29 after tumor inoculation [20, 22].

Inhibition of tumor cell-induced platelet aggregation by verapamil. Platelet rich plasma (PRP) was prepared by centrifugation at room temperature (230 *g*, 7 min) of fresh blood drawn from Balb/c or C57BL/6J mice (matched to the tumor) with a 21 gauge needle and a heparin solution (10 u/ml) blood ratio of 1 : 9 (v/v) [2, 10, 13]. Platelet-poor plasma (PPP) was obtained by centrifuging the remaining blood at 1,500 *g* for 10 min at room temperature. The platelet count in PRP was adjusted to $1-3.5 \times 10^6/mm^3$ (depending on mouse strain) by adding PPP. The platelet count for C57BL/6J was usually two to three times higher than that for Balb/c mice. Platelet aggregation was measured photometrically by an NSS HEM TRACER I (Niko Bioscientific, Tokyo). PRP (0.2 ml) was incubated in a cuvette at 37° C for 5 min under constant stirring in the spectrophotometer. Verapamil was dissolved in Ca/Mg-free Hank's balanced salt solution (HBSS), after which 10-µl aliquots of diluted verapamil solution were added to the cuvette and the incubation continued a further 2 min under the constant stirring. Logarithmically growing tumor cells, which were collected from culture dishes by trypsin, washed with growth medium by centrifugation, and suspended in Ca/Mg-free HBSS containing 10 m*M* Hepes buffer, were added to the cuvette (1.5×10^5 cell in 10 µl). Changes in absorbance were monitored for 15–22 min after the addition of verapamil.

Results

Inhibition of experimental and spontaneous metastasis of B16 BL-6 by verapamil

Verapamil given before and after the IV inoculation of B16 BL-6 cells inhibited the formation of lung metastases in C57BL/6J mice (Table 1). Although dose-dependent inhibition was not clear, verapamil 30–50 mg/kg inhibited lung metastasis by 50%–70%. This extent of inhibition is almost equal to that obtained previously with 5-fluorouracil and adriamycin [21, 22]. In the spontaneous metastasis system, verapamil could not inhibit the axillary lymph node metastasis of B16 BL-6 (Table 2), but it again inhibited spontaneous (through the hematological route) metastasis of B16 BL-6 to the lung (Table 2). Approximately 25%–80% inhibition was observed in this experiment. Tumor size, determined by the thickness of the front footpad amputated on day 17, was also slightly decreased in the treated groups.

Inhibition of experimental and spontaneous metastasis by verapamil

Verapamil inhibited experimental lung metastasis of C26 NL-17 (Table 3). Approximately 80% inhibition occurred with verapamil at doses of 60–75 mg/kg. Verapamil also inhibited the spontaneous metastasis of C26 NL-22 (Table 4). The effect was smaller than that observed for C26 NL-17 in the experimental metastasis system, however, as approximately 50%–60% inhibition was observed with C26 NL-22. Again a slight inhibition of primary tumor growth as determined by monitoring tumor size (thickness) was observed in C26 NL-22. This phenomenon, however, was not so striking as the antimetastatic effect.

Inhibition of tumor cell induced platelet aggregation by verapamil

The major mechanism for inhibition of plumonary metastasis by calcium antagonist probably involves the drug's inhibitory effect on platelet aggregation. We have examined whether verapamil could inhibit platelet aggregation induced by tumor cells in vitro. Verapamil at 0.5–100 µg/ml was an effective antagonist to platelet aggregation induced by the three types of metastatic tumor lines used in these studies (Fig. 1–3). Inhibition was dependent on the concentration of verapamil in all three tumor lines, but the modes of inhibition were different

Table 1. Inhibition of experimental pulmonary metastasis of B16 BL-6 by verapamil

Dose (mg/kg)	No. of pulmonary nodules		
	Mean ± SD	Range	Percent of
30	5.0 ± 1.7	3– 9	29[a]
40	9.8 ± 6.8	1–19	56[a]
50	8.2 ± 4.3	2–13	46[a]
Control	17.5 ± 9.3	4–33	100

[a] Significant ($P < 0.05$) by *t*-test

Table 2. Inhibition of spontaneous axillary lymph node and pulmonary metastases of B16 BL-6 by verapamil

Dose (mg/kg)	Weight of lymph node (mg)			No. of pulmonary nodules			Tumor size (mm) (Mean ± SD)
	Mean ± SD	Range	Percent of control	Mean ± SD	Range	Percent of control	
30	437 ± 322	101–1,186	143	12.3 ± 6.5	3–21	75	4.5 ± 0.9
40	358 ± 206	104– 762	117	5.8 ± 3.7[a]	1–12	35	4.2 ± 0.7
50	450 ± 306	0– 977	148	3.5 ± 4.3[a]	0–12	21	3.9 ± 0.4
Control	305 ± 162	30– 488	100	16.5 ± 17.1	1–42	100	4.8 ± 0.8

[a] Significant ($P < 0.05$) by *t*-test

Table 3. Inhibition of experimental pulmonary metastasis of C26 NL-17 by verapamil

Dose (mg/kg)	No. of pulmonary nodules		
	Mean ± SD	Range	Percent of control
50	79.3 ± 74.8	0–200+	94
60	13.1 ± 31.1	0– 96	15.6[a]
75	18.6 ± 24.5	0– 67	22.1[a]
Control	84 ± 71.3	5–200+	100

[a] Significant ($P < 0.05$) by *t*-test

Table 4. Inhibition of spontaneous pulmonary metastasis of C26 NL-22 by verapamil

Dose (mg/kg)	No. of pulmonary nodules			Tumor size (mm) (mean ± SD)
	Mean ± SD	Range	Percent of control	
50	33.1 ± 14.6	12– 55	59	8.7 ± 0.9
60	23.5 ± 8.5	10– 41	42[a]	8.5 ± 0.5
75	27.8 ± 21.8	7– 74	50[a]	8.8 ± 0.5
Control	55.9 ± 31.8	22–126	100	9.3 ± 0.6

[a] Significant ($P < 0.05$) by *t*-test

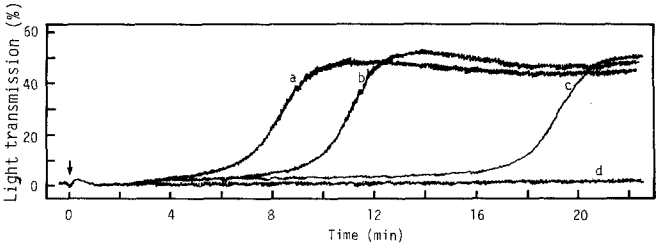

Fig. 1. Inhibition by verapamil of platelet aggregation induced by B16 BL-6. Platelet-rich plasma (PRP) from C57BL/6J mice containing 7×10^8 platelets in 0.2 ml was incubated in the cuvettes at 37° C for 5 min. Verapamil dissolved in 10 µl Ca/Mg-free Hank's balanced salt solution was added to the cuvettes at final concentrations of 0 *(a)*, 0.2 *(b)*, 5 *(c)* and 50 *(d)* µg/ml, and the whole incubated further for 2 min. B16 BL-6 cells (1.5×10^5) suspended in 10 µl Ca/Mg-free HBSS containing 10 m*M* Hepes buffer was added *(arrow)* and the changes in absorbance were monitored

between B16 melanoma and colon 26 adenocarcinoma. In the case of B16 BL-6, the onset of inhibition was delayed by verapamil; nonetheless the aggregation ultimately reached control levels in the presence of 5 µg/ml of drug (Fig. 1), while at 50 µg/ml no aggregation was observed during the course of the experiment. In the case of C26 NL-17 and NL-22, inhibition occurred immediately after the addition of tumor cells, with the extent of inhibition dependent on the concentration of verapamil (Figs. 2 and 3). A more efficient inhibition was observed for C26 NL-17 than for C26 NL-22, as the inhibition at 0.5, 5, and 50 µg/ml of verapamil was 61%, 76%, and 95%, respectively, for C26 NL-17, and the inhibition at 5, 50 and 100 µg/ml of verapamil was 58%, 77%, and 91%, respectively, for C26 NL-22.

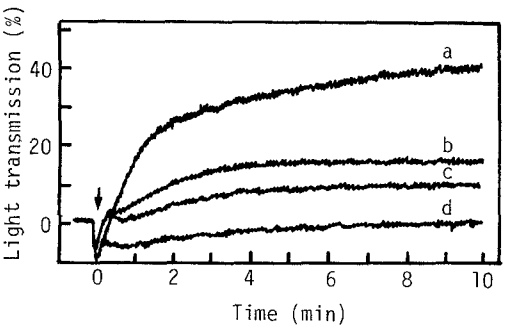

Fig. 2. Inhibition of platelet aggregation induced by C26 NL-17 by verapamil. A similar experiment to that illustrated in Fig. 1 was carried out with C26 NL-17 (1.5×10^5 cells), except that platelets (2.2×10^8/cuvette) of Balb/c mouse origin were used

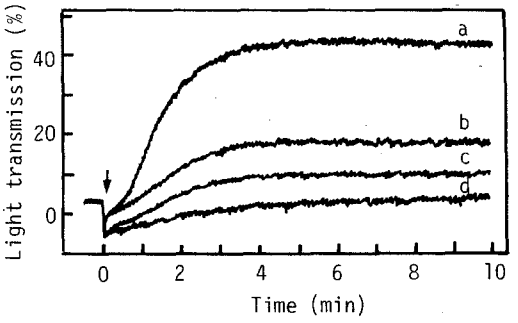

Fig. 3. Inhibition by verapamil of platelet aggregation induced by C26 NL-22. A similar experiment to those illustrated in Figs. 1 and 2 was carried out with C26 NL-22 (1.5×10^5 cells), except that the final verapamil concentrations were 0 *(a)*, 5 *(b)*, 50 *(c)*, and 100 *(d)* µg/ml

Discussion

Verapamil inhibited the formation of experimental and spontaneous metastasis of two different types of tumors, B16 melanoma and colon adenocarcinoma 26. Although further studies are needed to optimize schedule and dosage, the inhibition by verapamil is impressive compared with the efficacy of the chemotherapeutic agents 5-fluorouracil and adriamycin. These antitumor agents inhibited tumor metastasis in the experimental models described by approximately 80% at their most effective dosages [21, 22]. Thus, calcium antagonists such as verapamil are good candidates for clinical antimetastatic agents.

We have reported that calcium channel blockers, including dihydropyridine type (nifedipine, nicardipine, niludipine, etc.), phenylalkylamine type (verapamil), bezothiazepine type (diltiazem), and diphenylalkylamine type (prenylamine), overcame the pleiotropic drug resistance in various murine and human tumor systems by inhibiting the outward transport of the drugs in resistant tumor cells [15–17, 19]. Calcium channel blockers also increased the sensitivity of non-drug-resistant or inherently drug-resistant tumor cells [15, 18]. The possibility that calcium antagonists could serve dual roles in treatment, i.e., augment standard-agent cytotoxicity and inhibit metastasis (this report) makes them attractive candidates for inclusion in innovative clinical trials.

The mechanisms underlying the antimetastatic phenomena reported here are not well defined. It is most plausible that

platelet aggragation induced by tumor cells might be inhibited by calcium antagonists in vivo, and thus decrease the likelihood of occlusion of the microvasculature, an accepted mechanism of hematogenous spread of malignant cells [2, 3, 9, 11]. Our finding that verapamil could not inhibit the nonhemological lymph node metastasis of B16 BL-6 (Table 2) might support the above assumption. Recently, Honn et al. [7, 8] and Bando et al. [1] reported similar results with other types of calcium antagonists, such as nifedipine, nimodipine, and diltiazem. They also speculate that the inhibition of tumor-induced platelet aggregation by calcium antagonists is the mechanism by which these agents inhibit tumor metastasis.

The inhibition patterns of platelet aggregation by verapamil were different in B16 melanoma and colon 26 adenocarcinoma, as shown in Fig. 1–3. We used a higher platelet concentration in the experiment with B16 BL-6 cells, because platelet counts of C57BL/6J mice were usually higher than those of Balb/c mice. The aggregation assay was carried out with optimal platelet numbers as described in each legend. These different patterns were reproducible and we believe that the mechanisms of inhibition of platelet aggregation might be different between different tumor cells. Others have reported that different tumors have different aggregation mechanisms [13, 14]. Further studies will hopefully clarify the mechanisms by which these potentially clinically important antimetasatic effects are produced.

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