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WARFARIN INHIBITS BOTH PROCOAGULANT ACTIVITY AND METASTATIC CAPACITY OF LEWIS LUNG CARCINOMA CELLS

ROLE OF VITAMIN K DEFICIENCY

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Abstract—Chronic vitamin K deficiency, either dietary or pharmacologically induced with warfarin, depressed significantly the growth of lung secondaries in a spontaneously metastasizing murine tumor, the Lewis Lung Carcinoma. This effect was associated with a marked depression of the procoagulant activity of cancer cells, which could contribute to fibrin deposition around the tumor. Cellular anticoagulation may thus be an important mechanism in the antimetastatic effect of warfarin.

Blood clotting at the tumor–host interface has been suggested as playing a role in tumor dissemination and metastasis [1, 2]. Fibrin deposited around some experimental tumors has indeed been considered as a physical barrier protecting the cells from their host's immunological rejection, and as a natural substrate which stimulates tumor vascularization [3]. Similar protective mechanisms have been proposed to operate when tumor cells adhere to the endothelium of capillaries in target organs or tissues where tumor secondaries later grow. Fibrin layers have in fact been observed surrounding tumor cells attached to the endothelium [4].

Several types of cancer procoagulants have been described, which could contribute to fibrin deposition around tumor cells [2, 5–8]. In some experimental models, the amount of fibrin deposited has been found to correlate with the tumor cells' procoagulant activity [9].

On the basis of these observations, it is conceivable that, at least in some experimental models, inhibition of cancer procoagulant activity might be associated with reduction of metastatic growth.

We have studied here the Lewis Lung Carcinoma (3LL), a tumor which, upon i.m. implantation in syngeneic hosts, grows locally and gives spontaneous metastases selectively to the lungs [10]. Cells from this tumor and its metastatic nodules have a peculiar procoagulant activity which has been identified by clotting and amidolytic assays as a factor *X* activator [8, 11]. We report here that, in mice bearing the 3LL, vitamin K deficiency depresses both cancer cell procoagulant activity and lung metastasis growth.

MATERIALS AND METHODS

Tumor and animals. The tumor studied was the Lewis Lung Carcinoma (3LL) originated spontaneously in the lungs of a C57B1/6J mouse which, upon i.m. implantation to syngeneic hosts, gives spontaneous metastases selectively to the lungs [10]. Cells (1×10^5/mouse) from 3LL were injected in the hind leg of C57B1/6J mice. Primary tumor and metastasis growth were assessed 23 days after implantation as described [10].

Treatment schedules. Three groups of C57B1/6J mice (60 animals per group) were used and two different experimental conditions of vitamin K deficiency were studied. To the first group of animals racemic warfarin (Coumadin, Endo Laboratories, Garden City, NJ, U.S.A.) was given in drinking water (from day 7 to death) with the following treatment schedule: a loading dose of 7.5 mg/l. during the first 24 hr, then maintenance doses of 1.5–2 mg/l., depending on the Thrombotest values [12]. In the second group, vitamin K deficiency was induced by feeding animals a vitamin K deficient diet [13] starting 7 days before tumor cell implantation. Preliminary *ad hoc* expts had shown that treatment with the diet for 15 days was required to achieve a level of anticoagulation similar to that obtained with the warfarin schedule used. The third group received tap water and normal laboratory chow. Subgroups (30 animals) of each of the three treatment groups were given vitamin K_3 (Farmitalia-Carlo Erba, Milan, Italy) in drinking water (20 mg/l.) from the beginning of the pharmacological or dietary treatment (or the day of tumor implantation in untreated animals) until scheduled death. Treatment in each group was monitored by measuring the plasma prothrombin complex activity (Thrombotest) [12]. A last group (20 animals) was treated from day 7 to death with heparin (Liquemine, Roche, Basle, Switzerland) at a dosage (50 IU/kg b.w. twice daily i.p.) which prolonged the activated partial thromboplastin time of 2–3-fold the control values.

Prothrombin complex concentrate (PCC). Prothrombin complex concentrate (PCC) was prepared from C57B1/6J mouse plasma drawn into plastic syringes by intracardiac puncture and collected on

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Table 1. Effect of vitamin K deficiency on primary and metastatic 3LL growth, and on tumor cell procoagulant activity

Experimental group	Primary tumor (g)	Lung weight (mg)	Metastases		Thrombotest (sec)	Cancer cell procoagulant activity	
			Number	Weight (mg)		$A_{405\ nm/5\ min}$	Arbitary units
Control	9.2 ± 0.8	309 ± 28	17.5 ± 2.1	53 ± 9	24 ± 1.3	207 ± 15.6	102 ± 5.4
Control + Vit. K	10.2 ± 0.7	318 ± 14	18.3 ± 1.8	68 ± 11	23.2 ± 1.5	219 ± 14.8	106 ± 5.1
Warfarin	9.0 ± 0.5	260 ± 21	4.9 ± 0.6*	7.8 ± 1.7*	>180	39 ± 2.0	18 ± 1.6*
Warfarin + Vit. K	10.3 ± 0.5	360 ± 39	22.4 ± 3.0	77 ± 12	25 ± 1.6	198 ± 9.9	96 ± 4.6
Vit. K deficient	7.4 ± 0.7	250 ± 11	7.3 ± 0.9*	10.9 ± 1.3*	>180	46 ± 2.5	22 ± 1.4*
Vit. K deficient + Vit. K	8.2 ± 0.3	320 ± 26	20 ± 3.3	71 ± 11	26.6 ± 1.6	201 ± 13.4	103 ± 5.3

Means ± S.E. of 15–20 values per group.
* $P < 0.0001$ at Duncan's new multiple range test.

K-oxalate (0.25% final concentration). Platelet-poor-plasma pooled from 300 mice was adsorbed onto $BaSO_4$ and, subsequently, eluted as described [14]. The material so obtained was filtered and lyophilized. Just before use it was dissolved in sterile saline at a concentration of about 12–14 units/ml of prothrombin complex factors. The levels of the four factors of the prothrombin complex recovered in the concentrate were similar, not differing from each other by more than 20%. PCC (2.5 units per animal) or sterile isotonic saline were administered by slow injection into the tail vein. After 4 hr, the animals were tested for both Thrombotest and cancer cell procoagulant activity.

Assay of 3LL cell procoagulant activity. To determine 3LL cell procoagulant activity, animals were killed after 17 days, when tumors from both control and treated mice were macroscopically devoid of necrosis. Tumor masses were mechanically disgregated by gentle spilling and cell suspensions were obtained after repeated washing with phosphate-buffered saline. Macrophages infiltrating the tumor were eliminated after 45 min adherence in plastic dishes [15]. The procoagulant activity of cancer cells (1×10^7/ml) was measured by a two-stage amidolytic assay as described [11]. Factor *X* activating activity was expressed in arbitrary units, assuming as 1000 arbitrary units the activity of a 1:100,000 (w/v) dilution of Russel's Viper Venom (Stypven), a direct activator of factor *X*.

RESULTS

Table 1 shows that in warfarin-treated and vitamin K deficient animals there was a similar slight inhibition of primary tumor growth and a significant reduction of the number and weight of lung metastases. Concomitantly, in both groups of animals, a significant reduction of tumor cell procoagulant activity, measured by the two-stage amidolytic assay, was observed. Similar results were obtained with the clotting assay, using a one-stage recalcification time [8]. Administration of vitamin K to animals with pharmacologically-induced or dietary deficiency of vitamin K completely restored both the metastatic capacity and the procoagulant activity of tumor cells (Table 1). The injection of mouse prothrombin complex concentrate to warfarin-treated or vitamin K deficient mice normalized the activity of plasma prothrombin complex without correcting cancer cell procoagulant activity (Table 2).

When tumor-bearing mice were treated with heparin no changes were observed in either the procoagulant activity or the metastatic potential of the cells.

DISCUSSION

These data indicate that, in the Lewis Lung Carcinoma model, vitamin K availability modulates the metastatic capacity of tumor cells. Indeed, vitamin K deficiency, either dietarily or pharmacologically induced by warfarin, significantly depressed lung metastasis growth.

The antimetastatic activity of warfarin appears to be due to vitamin K antagonism rather than to any direct effect of this drug on tumor cell properties.

Table 2. Effect of replacement of prothrombin complex factors on 3LL cell procoagulant activity

	Thrombotest (sec)	Cancer cell procoagulant activity (arbitrary units)
Control	25 ± 1.3	118 ± 6.3
Control + PCC	21 ± 0.5	113 ± 5.6
Warfarin	>180	16 ± 1.1
Warfarin + PCC	27.1 ± 1.4	18 ± 0.9
Vit. K deficient	>180	21 ± 2.3
Vit. K deficient + PCC	28.4 ± 1.8	18 ± 2.1

Means ± S.E. of six values per group.

A similar mechanism had previously been suggested for the antimetastatic activity of phenprocoumon in the same experimental system [16]. The mechanism by which vitamin K deficiency exerts its antimetastatic activity is not defined. Vitamin K is required for post-ribosomal carboxylation of glutamic acid residues in different proteins, to form the structural moiety responsible for their specific calcium binding activity. Among these proteins, the four clotting factors of the prothrombin complex have first been identified. However, plasma anticoagulation resulting from impaired synthesis of these factors does not necessarily account for the antimetastatic effect of vitamin K deficiency. Hypocoagulability induced by other means (heparin or defibrinating enzymes) does not affect the metastatic behaviour of the same cells [17]. It is of interest in this context that vitamin K deficiency reduced not only the metastatic potential but also the procoagulant activity of the same cancer cells. This was not due to low plasma levels of vitamin K dependent clotting factors, since administration of mouse prothrombin complex concentrate normalized the activity of plasma prothrombin complex without correcting cancer cell procoagulant activity.

Fibrin deposition in the tumor cell microenvironment is likely to be a local phenomenon rather than the result of systemic activation of clotting [18]; the inhibition of cancer cell procoagulant activity (cellular anticoagulation) could, thus, be more important in this context than depression of circulating clotting factors (plasmatic anticoagulation).

In view of the proposed role of fibrin in tumor dissemination, the data reported here could offer fresh clues to the antimetastatic effect peculiar to warfarin, not shared by other anticoagulants. These data may be also of interest in view of the current debate about clinical use of warfarin in human cancer; warfarin treatment has indeed been recently reported, in a controlled clinical trial, to significantly prolong the survival of patients with small cell carcinoma of the lung and to delay the appearance of disease progression [19].

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