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Failure of Warfarin to Affect the Tissue Factor Activity and the Metastatic Potential of Murine Fibrosarcoma Cells*

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Abstract—*Vitamin K deficiency, either dietary or pharmacologically induced by warfarin, was unable to affect the metastatic capacity of cells from a benzopyrene-induced fibrosarcoma in C57BL/6J mice. The same cells had a procoagulant activity, of tissue thromboplastin type, which was also completely unaffected by vitamin K antagonism or deficiency. In another murine model of spontaneous metastasis we previously suggested that depression of a particular procoagulant such as a direct factor X activator might contribute to the antimetastatic activity of warfarin. The failure of vitamin K deficiency to affect both the procoagulant and the metastatic capacity of the model reported here offers strong negative support to the same concept.*

INTRODUCTION

COUMARIN anticoagulants have been reported to reduce growth in some experimental tumors [1]. We have recently shown that in the Lewis lung carcinoma (3LL) the antimetastatic effect of warfarin treatment was associated with depression of a particular procoagulant activity (PCA) of those cells, namely a factor X activator [2, 3]. This novel cellular effect of warfarin was proposed as one of the possible mechanisms of antimetastatic activity of this drug.

In order to investigate whether the metastasis reducing effect of warfarin was indeed associated with depression of a specific procoagulant, i.e. the activator of factor X, we have extended our study to another type of murine tumor, a benzopyrene-induced fibrosarcoma; this has been previously shown to trigger blood clotting not by direct activation of factor X but via the extrinsic pathway (tissue thromboplastin).

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MATERIALS AND METHODS

Animals and tumors

Male C57BL/6J mice weighing 20–25 g at the start of the experiment were obtained from Charles River, Calco, Italy. A total number of 180 animals were used.

The benzopyrene-induced mFS6 sarcoma was obtained as previously described [4]. Of the sublines derived from spontaneous lung nodules of this tumor, the highly metastatic M_4 was studied. Animals were killed on day 19 for primary tumor cell harvest before gross necrosis of the tumor mass occurred. Primary tumor weight and metastasis weight and number were recorded on day 24 after tumor implantation as previously described [5].

Treatments

Racemic warfarin (Coumadin, Dupont-de Nemours Inc., Garden City, NJ, U.S.A.) was given in drinking water from the time of tumor implantation until the animals were killed at the following treatment schedule: a loading dose of 7.5 mg/l, corresponding to approximately 1.5 mg/kg body wt, during the first 24 hr, then maintenance doses of 1–2.5 mg/l (corresponding to 0.2–0.5 mg/kg body wt), according to Thrombotest values. Vitamin K-deficient diet was administered to mice (housed in coprophagy-preventing cages) starting 15 days before tumor

cell implantation and continuously until scheduled death; this schedule was used on the basis of preliminary *ad hoc* experiments indicating that about 2 weeks of diet were required to obtain a stable anticoagulation level similar to that obtained with warfarin treatment. The anticoagulant effect of either dietary or pharmacologically induced vitamin K deficiency was monitored every second day by the Thrombotest (Immuno, Pisa, Italy) [6].

Cancer cell preparation and evaluation of PCA

At scheduled death necrosis-free tumor masses were disaggregated by exposure to 0.3% collagenase and 0.01% DNA in phosphate-buffered saline (PBS). The cells were washed twice with 50 ml basal medium Eagle (BME) and deprived of host macrophages by adherence on plastic dishes for 40 min at 37°C. Non-adherent cells were washed again (×3) with PBS, resuspended in the same buffer at a concentration of 10^7/ml and tested for PCA. In all cell suspensions viability assessed by the Trypan blue test gave values of at least 95%. Cell PCA was tested by a one-stage clotting assay using autologous plasma as the substrate. Clotting time was determined in duplicate in prewarmed plastic tubes using the following test system: 0.1 ml cell suspension or buffer, 0.1 ml plasma substrate and 0.1 ml 0.025 M $CaCl_2$. The PCA expressed by M_4 cells from warfarin-treated animals or from animals fed a diet with vitamin K was quantified by reference to that of M_4 cells from the respective controls as follows. Clotting tests were made with serial dilutions of cell suspensions. When clotting times were plotted against the corresponding cell concentrations on a double logarithmic scale the resulting straight lines were parallel. This enabled us to calculate the cell PCA of treated animals as a percentage of the controls. In experiments aimed at defining the type of PCA human plasma from normal subjects or patients with congenital deficiency factor X, factor VIII or VII was used as substrate. In some experiments PCA was assayed after exposure of M_4 cells from control or warfarin-treated animals to diisopropylfluorophosphate (DFP), an inhibitor of serine proteases which is known not to block the activity of tissue thromboplastin. DFP (Aldrich Chemical Company, Inc., U.S.A.) was resuspended in propanol at a concentration of 2.7 M and added to disrupted cells or thrombin (Topostasine, Roche, Milan, Italy) or rabbit brain thromboplastin (as controls) at a final concentration of 5 mM. The samples were kept at 37°C for 1 hr then dialyzed against PBS, pH 7.6, for 22 hr at 4°C and tested.

RESULTS

Table 1 shows that vitamin K deficiency, despite its ability to depress chronically the activity of the prothrombin complex, did not modify either the primary tumor or the metastatic growth of M_4 cells. In the same treatment conditions the PCA of the cells was also unaffected. When expressed as a percentage of the respective control, the PCA of M_4 cells from warfarin-treated animals and from animals fed the vitamin K-deficient diet ranged between 91 and 98% and between 82 and 89% respectively. Tumor cells of both treated and untreated mice showed a tissue thromboplastin-like activity, as indicated by the dependency of PCA on coagulation factor VII (Table 2). Moreover, DFP,

Table 1. *Effect of vitamin K deficiency on primary and metastatic M_4 growth and on cell PCA (means ± S.E. of 15–20 values per group)*

	Control	Warfarin	Vitamin K-deficient diet
Cell PCA (sec.)	24.5 ± 0.6	25.7 ± 0.6	27.0 ± 0.8
Thrombotest (sec.)	30.0 ± 0.9	>180	>180
Primary tumor palpable on day	15	14	15
Metastatic incidence (%)	100	100	100
Lung metastases (mg)	17.5 ± 4.3	12.8 ± 3.0	18.7 ± 8.8
Lung metastases (*n*)	12.5 ± 3.4	17.2 ± 3.7	11.0 ± 5.6

Table 2. *Effect of M_4 cells from control and warfarin-treated animals on the recalcification time of various human plasma substrates*

Cells (10^7/ml)	Plasma recalcification time (sec) Normal	Deficient—FVIII	Deficient—FVII	Deficient—FX
Control	112-135	109-144	241-273	>300
Warfarin-treated	118-143	109-138	234-271	>300
Buffer	>300	>300	>300	>300

The range of values was obtained from duplicate experiments on three different cell preparations. F = factor.

Table 3. Failure of DFP to inhibit the PCA of M_4 cells

Test material	Clotting time (sec) -DFP	+DFP
M_4 cells (control)	31	32
M_4 cells (warfarin)	30	31
Thromboplastin	32	32
Thrombin	35	145

a known inhibitor of serine proteases but not of thromboplastin, did not affect the PCA of M_4 cells (Table 3). This suggests that, in M_4, vitamin K deficiency leaves not only the amount but also the nature of the tumor cell PCA unaffected.

DISCUSSION

This study shows that vitamin K deficiency, induced either dietarily or pharmacologically, had no metastasis-reducing effect in the M_4 subline of mFS6 fibrosarcoma. This is apparently the first experimental model of spontaneous metastatic growth which is refractory to anticoagulant doses of warfarin [7, 8]. In this model not only the metastatic potential but also the procoagulant activity of the cells was unaffected by vitamin K deficiency. The reason why vitamin K deficiency does not influence the procoagulant activity of the M_4 fibrosarcoma whereas it markedly depresses the procoagulant activity of Lewis Lung Carcinoma cells [2, 3] remains to be established. The most obvious difference between the procoagulants of these different murine tumors is the nature of their intervention in the clotting cascade, the M_4 being of the tissue thromboplastin type and the 3LL a direct activator of coagulation factor X. One may speculate that only the latter would be a vitamin K-dependent activity. No evidence has been produced so far that tumor cell tissue factor is also modulated by vitamin K, although there are indications that some human normal cells (epithelial cells and mononuclear phagocytes) have reduced tissue factor during chronic warfarin anticoagulation [9, 10].

We previously suggested that depression of a particular procoagulant such as the direct factor X activator might contribute to the antimetastatic activity of warfarin [3]. The failure of warfarin to affect both the procoagulant and the metastatic capacity of M_4 cells reported here offers further support to the same concept.

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