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Mechanism of Lung Tumour Colony Reduction Caused by Coumarin Anticoagulation*

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Abstract—*Anticoagulation of syngeneic C57BL mice with the coumarin derivative phenprocoumon at various time intervals before and after the intravenous injection of Lewis Lung carcinoma cells reduced the number of tumour colonies in the lung. The results suggested that this effect was independent of the alteration of the clotting mechanism of the host. Restoration of blood coagulability by administration of human factor* IX *complex did not abolish the antimetastatic effects of coumarin medication. Since diet induced vitamin K deficiency appeared to be equally effective in reducing lung tumour colonies, it was concluded that coumarins did not act directly on the tumour cell, but that the antimetastatic effect was mediated by the drug induced vitamin K deficiency. A hypothesis of the possible mode of action is presented.*

INTRODUCTION

ULTRASTRUCTURAL studies have demonstrated that intravenously injected tumour cells, shortly after their arrest in lung capillaries, are surrounded by a fibrin and platelet thrombus, which disappears within a few hours of its formation [1, 2]. This early clot formation around embolic tumour cells was considered to be of pathogenic importance in the process of metastasis formation from blood-borne cancer cells [3]. Consequently, drugs interfering with blood coagulation have to be active during the early stages after tumour cell challenge if they are counteracting the development of lung tumour colonies. Various anticoagulants such as heparin, ancrod and coumarin-derivatives have been shown to reduce the number of lung colonies after intravenous tumour cell injection in numerous experimental systems, supporting the importance of fibrin formation in the initial stage of haematogenous metastases [4].

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There is, however, experimental evidence that other pharmacological actions of some of the anticoagulants—independent of their influence on blood coagulation—might be responsible for the effect on tumour dissemination [4]. The purpose of the present investigations was to further elucidate the mechanism of the antimetastatic effects of coumarins in a syngeneic tumour system. In order to compare these results with previous data [5–7] the Lewis Lung carcinoma (3LL) was used. The antimetastatic action of phenprocoumon (Marcoumar®) was investigated by means of various treatment schedules. In addition, substitution of impaired clotting factors and dietary vitamin K deficiency was investigated.

MATERIALS AND METHODS

Animals

Inbred C57BL/Rij male mice were used throughout the experiments I–VI and C57BL/6J-Han inbred males were used in the experiments VII–IX. Comparison of results is justified, since no biologic differences have been shown between the two strains. The weight range was 17–22 g. The animals were housed in macrolon cages and received standard laboratory diet and watter *ad libitum* unless changes were made corresponding to the experiments (see over).

Tumour

The Lewis Lung carcinoma (3LL), originally derived from a spontaneous lung carcinoma in a C57BL mouse in 1951, was obtained from Prof. K. Karrer (University of Vienna) and maintained by subcutaneous implants of approximately 5×10^6 tumour cells into syngeneic C57BL mice, with passage every 14 days. Tumour cell suspensions were prepared by exposing non-necrotic tumour fragments to a trypsin solution (Trypsin NBCO 0.25%) according to the method previously described [7]. The final viable tumour cell number to be injected was based on Eosin negative cells. Cells were injected in sterile, pyrogen-free saline.

Anticoagulation

Phenprocoumon (Marcoumar®, Hoffmann–La Roche, Basle) was administered in an initial dose of 2.5 mg/kg body weight intraperitoneally; the control animals received the same volume of saline. This was followed by a continuous oral dose added to the drinking water in concentrations ranging from 2 to 5 mg/l. Anticoagulation was monitored by the Thrombotest method: 0.003 ml of tail vein blood was added to 0.3 ml Thrombotest reagent (Nyegaard, Oslo) and the clotting time was recorded automatically at 37°C. The mean value in 35 untreated, healthy control animals was 49 sec (S.D. 3 sec). The therapeutic range of anticoagulation was considered to be a Thrombotest-clotting time of 250–400 sec. To monitor the Thrombotest-clotting time in the experiments, two to four animals from each test group were used in alternating sequence, in order to prevent excess blood loss.

Factor IX *complex*

Phenprocoumon treated animals with a prolongation of the Thrombotest-clotting time to 400 sec were substituted by the intravenous injection of human factor IX complex (Konyne®, Cutter Labs, Berkely) containing the vitamin K dependent clotting factors II, VII, IX and X. One unit Konyne® corresponded to the clotting factor activity of 1 ml normal human plasma. The animals were injected intravenously with 2.5 units of Konyne®/animal. Another group was injected with the same amount of inactivated Konyne® which was prepared by incubation of the solution for 10 min at 56°C. This procedure resulted in a complete destruction of the clotting activity of the factor IX complex.

Vitamin K deficiency

Vitamin K deficiency with prolongation of the animals Thrombotest-clotting time to the therapeutic range (250–400 sec) was induced by a semisynthetic, vitamin K deficient diet (Altromin® C 1020) containing 2 g of neomycin per kg dry food. In addition, the animals were housed in coprophagy preventing cages. The corresponding control diets contained 20 mg per kg dry food vitamin K_1 (Konakion, Hoffmann–La Roche, Basle).

Lung colony assay

After sacrificing the animals with ether, the number of lung colonies was evaluated by staining the lung through the trachea with 12% Indian ink according to Wexler [8]. The total number of macroscopically visible nodules on the surface of all lung lobes were recorded. Autopsies were performed on all animals to establish extrapulmonary tumour growth.

Experimental design

The treatment schedules with phenprocoumon, Konyne® and vitamin K deficient diet, as well as the blood coagulability of the treated animals at the time of tumour cell injection are shown in Table 1. The day of the intravenous tumour cell challenge is designated day 0. Control animals to experiment IX (vitamin K deficiency) were fed the vitamin K containing diet.

Statistical evaluation

The statistical analysis of the number of lung colonies in control and treated groups was performed with the U-test for two random variables according to Wilcoxon, Mann and Whitney.

RESULTS

The injection of 2.5 units Konyne® into 5 anticoagulated animals (Thrombotest-clotting time 400 sec) reduced the Thrombotest-clotting time back to normal values over a period of approximately 4 hr (Fig. 1). The injection of inactivated Konyne® did not alter the phenprocoumon-induced prolongation of the clotting time.

The results of the effects of various treatment schedules on the formation of lung tumour colonies in treated and control animals are summarized in Table 1. The intravenous injection of tumour cells into phenprocoumon anticoagulated animals

Table 1. Treatment schedules and average numbers of lung colonies in control and treated mice after intravenous tumour cell challenge at day 0

Group	Treatment	Duration (days)	Thrombotest clotting times* at time of tumour injection	Number of lung colonies						Significance (U-test)
				Control			Treated			
				Mean	S.D.	*n*	Mean	S.D.	*n*	
I	phenprocoumon	−4 to +1	320±25 sec	38.4	4.3	10	4.2	0.7	12	$P<0.01$
II	phenprocoumon	−4 to +10	332±28 sec	38.4	4.3	10	0.5	0.3	14	$P<0.01$
III	phenprocoumon	0 to +4	47±4 sec	23.5	2.5	11	8.3	1.0	15	$P<0.01$
IV	phenprocoumon	0 to +10	50±3 sec	23'5	2.5	11	7.2	1.0	17	$P<0.01$
V	phenprocoumon	+2 to +12	49±4 sec	13.1	1.4	13	6.5	0.8	14	$P<0.01$
VI	phenprocoumon	+4 to +14	48±4 sec	6.5	0.9	11	3.5	0.6	10	$P<0.01$
VII†	phenprocoumon	−4 to +10	35±2 sec	39.1	4.9	15	6.8	5.8	10	$P<0.01$
VIII‡	phenprocoumon	−4 to +10	295±18 sec	39.1	4.9	15	14.9	5.0	10	$P<0.01$
IX	vitamin K. deficient	−20 to +10	280±31 sec	39.1	4.9	15	3.3	1.2	13	$P<0.001$

*All corresponding control animals had normal Thrombotest clotting times (49±3 sec).
†Human prothrombin complex (Konyne®) was injected i.v. 10 min prior to the tumour cells.
‡Inactivated human prothrombin complex was injected i.v. 10 min prior to the tumour cells.

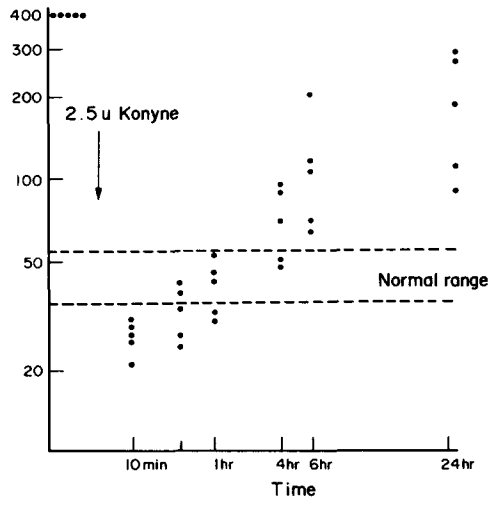

Fig. 1. Effect of intravenous injection of 2.5 *units human factor* IX *complex (Konyne®) on Thrombotest clotting time of phenprocoumon anticoagulated mice* (n=5).

resulted in a significant reduction of the number of lung colonies (groups I and II). If phenprocoumon treatment was started at the time of tumour cell challenge, at which time the animals were not anticoagulated, the reduction of the number of tumour colonies was still significant (groups III and IV). Beginning of the treatment as late as 4 days after tumour cell injection was able to diminish lung colony formation significantly (groups V and VI). Restoration of the phenprocoumon-induced clotting defect by human factor IX complex prior to tumour cell injection, did not influence the colony reduction (group VII), likewise the injection of inactivated Konyne® did not abolish the effect of phenprocoumon (group VIII). Vitamin K deficiency alone was equally effective in reducing the number of lung tumour colonies (group IX). In none of the groups extrapulmonary tumour growth was noted at autopsy.

DISCUSSION

Coumarin anticoagulation was found to be effective in reducing the number of lung colonies after intravenous tumour cell challenge [9–13] as well as the number of spontaneous metastases from experimental solid tumours [5, 9, 11, 14, 15].

Although antithrombotic effects were considered to be the main mode of action, other drug effects could not be ruled out. In some experimental systems a discrepancy between effects of different anticoagulants on tumour spread suggested a unique pharmacological property of the coumarins, not directly related to deceleration of blood coagulability [6].

The present experiments confirm earlier data: effective nontoxic anticoagulation [5] at the time of tumour cell challenge significantly reduced the number of lung colonies (group I). It is, however, remarkable that coumarin administration until day 10 after tumour injection further reduced the number of colonies if compared to short-term treatment (group II). If phenprocoumon was given at the time of tumour cell challenge (groups III and IV) or even two to four days later (groups V and VI) there was also a significant reduction of lung colonies. In these last experiments tumour cells were injected into animals which had no clotting defect at the time of injection. Thus the explanation of the antimetastatic effects of phenprocoumon by inhibited blood coagulability at the early stage after tumour cell arrest becomes questionable. In further experiments it was demonstrated (groups VII and VIII) that restoration of blood coagulability of phenprocoumon treated animals by human factor IX complex did not abolish the antimetastatic effects. It is unlikely that anticoagulation exerts its effect at any time after tumour cell challenge, since all treatment schedules, although not overlapping, were equally effective in reducing lung colonies. Unless one assumes two completely different modes of coumarin action on metastatic tumour growth, our data suggest that anticoagulation was not the main mechanism by which the reduction of lung colonies was brought about.

Alternatively, a direct effect of the drug could be discussed. However, diet induced vitamin K deficiency (group IX) had similar antimetastatic effects to coumarin induced vitamin K deficiency. These observations suggest that the effects of coumarin anticoagulation are mediated by vitamin K deficiency. This interpretation of the present data is in agreement with the results of Brown [12] who found that the administration of vitamin K to warfarin anticoagulated animals promptly abolished the antimetastatic effect of this treatment. In the rabbit ear chamber it was found that warfarin anticoagulation inhibited tumour cell locomotion; whereas the injection of vitamin K into the animal restored locomotion back to normal [16].

In conclusion, some experimental data would support the role of vitamin K deficiency in the antimetastatic effects of coumarin anticoagulation. Recent investigations using the spontaneously metastasising Lewis lung carcinoma have confirmed the metastases inhibiting action of vitamin K deficiency in long-term experiments [6]. The explanation for this phenomenon remains, however speculative. Vitamin K is required in a postribosomal synthetic step for carboxylation of glutamic acid to γ-carboxyglutamic acid [17]. The γ-carboxyglutamic acid containing clotting factors have strong calcium and phospholipid binding capacities which are responsible for the specific action of these enzymes within the clotting mechanism [18]. However, other proteins containing γ-carboxyglutamic acid with hitherto unknown biologic function have recently been identified [19]. Since the reduced clotting activity appears to be of minor importance in the antimetastatic effects of vitamin K deficiency, it is conceivable that other vitamin K dependent structures are responsible for the mode of action. Analogous with the physico-chemical properties of the vitamin K dependent clotting factors, the absence of calcium and phospholipid binding proteins could possibly alter some tumour characteristics, or the host's response to the tumour.

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