Biochemical Pharmacology, Vol. 35, No. 19, pp. 3239–3242, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

INCREASED SUSCEPTIBILITY TO THE ANTICOAGULANT EFFECT OF WARFARIN IN MICE BEARING THE LEWIS LUNG CARCINOMA

ROLE OF VITAMIN K DEFICIENCY

LUCIA PEZZI, MARCO GERNA, MARIA CARLA RONCAGLIONI, CEES VERMEER* and MARIA BENEDETTA DONATI

Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy and *Department of Biochemistry, University of Limburg, Maastricht, The Netherlands

(*Received* 27 *December* 1985; *accepted* 17 *March* 1986)

Abstract—This study reports an increased susceptibility to warfarin anticoagulation in mice bearing an experimental tumour, the Lewis lung carcinoma. In these animals, following a single i.v. injection of warfarin, the prothrombin complex activity decreased normally but recovered far slower than in controls, while the rate of degradation of the clotting factors was not modified.

At the level of the vitamin K-dependent liver carboxylase, it was possible to demonstrate an increase in the endogenous substrate (reflecting an impairment of the carboxylase vitamin K dependent system). This abnormality was reversed by vitamin K administration and can be reasonably ascribed to a vitamin K deficiency in association with tumour growth.

Oral anticoagulants of the coumarin type have been repeatedly reported to reduce metastasis formation in experimental animals [1, 2]. However, the mechanism and the optimal conditions for this effect have not yet been thoroughly investigated. In fact, very little is known about the pharmacokinetics and pharmacodynamics of warfarin in mice, the animal species in which the antimetastatic effect has been reported.

The aim of this study was to evaluate whether the growth of the tumour would influence the host's response to warfarin anticoagulation. As a model, the Lewis lung carcinoma has been chosen as a murine tumour in which the antimetastatic effect of warfarin has been characterized in more detail. For this purpose we had first to study the hitherto unknown pharmacokinetics of warfarin in mice.

We show here an increased susceptibility to the anticoagulant effect of warfarin in tumour bearing mice, probably due to a partial vitamin K deficiency.

MATERIALS AND METHODS

Animals and tumours. Male C57 Bl/6J mice weighing 20–25 g at the start of the experiment were obtained from Charles River (Calco, Italy), 10^5 Lewis lung carcinoma (3LL) cells were implanted intramuscularly in the hind leg of the animals on day 0. A loading dose of warfarin (Coumadin R, Endo Laboratories, Garden City, NY) was given by dissolving 7.5 mg/l of the drug in the drinking water 24 hr before the start of the experiment. Maintenance doses of 2 mg/l were given for the duration of the experiment. After 20 days from the implantation, animals were killed, primary tumours weighed and metastasis counted; the livers were removed and immediately frozen at −80°. In this experiment the effect of warfarin on 3LL development was as follows: primary tumour weight 8 g ± 1.5 (controls) vs 7.2 g ± 1.2 (treated); lung metastasis number: 20 ± 3 vs 6 ± 2.8 ($P < 0.01$); lung metastasis weight: 120 mg ± 11.5 vs 25 mg ± 6.5 ($P < 0.01$).

In some experiments on liver carboxylase activity, vitamin K (Konakion Roche, Milano, Italy) was supplemented in the drinking water for 2 days at a dose of 20 mg/l.

Pharmacokinetics and pharmacodynamics of warfarin. The anticoagulant effect of warfarin was checked by measuring the plasma prothrombin complex activity using Thrombotest (Immuno s.p.a., Pisa Italy). (^{14}C)-Warfarin (10–15 μCi/mmol, Radiochemical Centre, Amersham, U.K.) was injected i.v. in the tail vein at the dose of 1.5 mg/kg b.w. In each mouse, at different time intervals (2, 4, 6, 8, 15, 25, 33, 50, 75, 96 hr), blood was collected from the retro orbital plexus and the Thrombotest was immediately performed. The clot was subsequently dissolved in a scintillation mixture (Instagel R 11, Packard Instruments, Illinois, USA) and ^{14}C radioactivity was counted in a liquid scintillation counter (TRI-CARB 300, Packard Instruments, Italy).

In order to establish the distribution of warfarin in the liver, the drug was administered i.v. at the dose of 1.5 mg/kg in 16 control and 16 tumour bearing mice. The animals were sacrificed after 2 hr and 15 hr, and warfarin was measured in blood and liver by a HPLC method [3].

To evaluate the rate of degradation of the prothrombin complex activity (K_d), six tumour-bearing mice (day 20) and six normal mice were injected with 12 mg/kg i.v. of warfarin. At that dose synthesis of the functional vitamin K-dependent clotting factors is completely blocked. Thrombotest in capillary blood of the retro orbital plexus was measured at several intervals (2.5, 5, 8, 15 hr) after warfarin and

the K_d was determined as described by Nagashima *et al.* [4].

Liver carboxylase measurement. The microsomal fractions of the livers were prepared as described for bovine livers [5] and carboxylase was solubilized by adding 10% (v/v) of Triton X-100 to the preparation at a final concentration of 0.5%. It was checked that for all the preparations this was the optimal concentration to solubilize mouse liver carboxylase. Carboxylase activity was measured at 25° in a reaction mixture (0.25 ml) containing 800 mM KCl, 4 mM Phe-Leu-Glu-Glu-Leu (Vega Biochemical, Tucson, AZ), if added, 0.2 mM vitamin K hydroquinone and 0.01 mCi $NaH^{14}CO_3$ (Amersham International, U.K.).

After different incubation times at 25° the reaction was stopped by adding 2 ml of 5% (w:v) trichloroacetic acid. Traces of non-bound label were removed by gentle boiling for 1 min, before 10 ml of Insta-gel R11 scintillation mixture were added. The samples were counted in a liquid scintillation counter (TRI-CARB 300, Packard Instruments, Italy).

The amount of carboxylase in liver was quantified in the presence of an excess of endogenous substrate; since under these conditions all reaction components are present in excess, the initial reaction rate is proportional to the respective amounts of carboyxlase [5].

Carboxylation of endogenous substrate (presumably precursors of the vitamin K-dependent clotting factors) was measured as incorporation of labelled $^{14}CO_2$ in the absence of pentapeptide during 60 min incubation. The results are expressed as cpm incorporated by a fixed concentration of microsomal protein (20 mg/ml). Protein concentration was determined using Bradford's assay [6].

Data analysis. The blood curves of radioactivity concentration vs time were analysed following a one compartment open model system after i.v. administration.

Experimental points were fitted by a non-linear regression iterative program [7] on a HP-85 desk computer (Hewlett-Packard, U.S.A.). The radioactivity counted in blood was converted to ng/ml of warfarin on the basis of specific activity and count efficiency. In this experimental system we were not able to distinguish the metabolites of warfarin. Statistical analysis was performed following a statistical program developed for a HP-85 desk computer [8].

RESULTS

Figure 1 shows the time course of the prothrombin complex activity following a single i.v. injection of

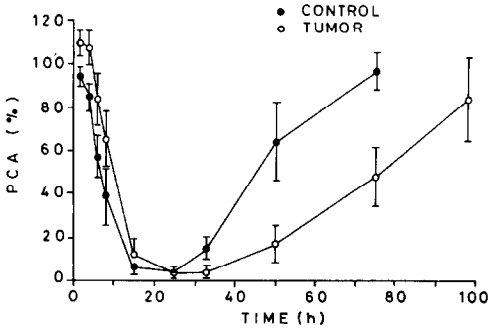

Fig. 1. Time course of prothrombin complex activity (PCA) after a single injection of warfarin (1.5 mg/kg). Curves are the average of 12 curves from 12 single animals.

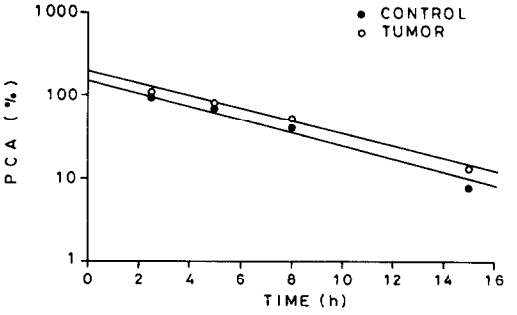

Fig. 2. Disappearance of prothrombin complex activity after a single i.v. dose of 12 mg/kg of warfarin. Each curve is the average of 6 curves from 6 single animals. (V_d = volume of distribution, Cl = clearance).

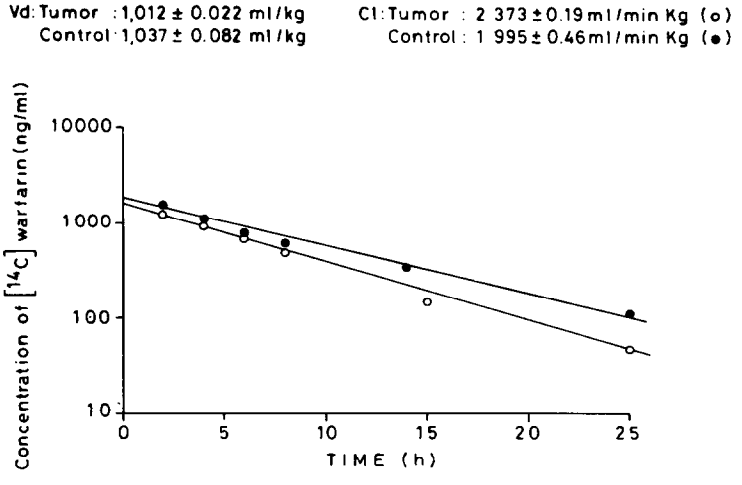

Fig. 3. Disappearance of blood radioactivity after a single i.v. injection of 1.5 mg/kg of ^{14}C warfarin. Each curve is the average of 12 curves from 12 single animals.

Table 1. Warfarin levels in blood and liver of control and tumour bearing mice after i.v. administration of 1.5 mg/kg b.w.

	Controls		Tumour bearing	
	2 hr	15 hr	2 hr	15 hr
Liver (μg/g)	3.89 ± 0.71	0.66 ± 0.27	3.53 ± 0.72	0.72 ± 0.31
Blood (μg/ml)	1.13 ± 0.21	0.19 ± 0.08	1.02 ± 0.41	0.21 ± 0.07

No significant difference in warfarin levels, in blood and liver between controls and tumour bearing animals were found, either at 2 or 15 hr after drug administration (Student's t test).
Each value is the mean of 8 animals ± SD.

Table 2. Endogenous substrate and amount of carboxylase in the microsomal fraction of liver from normal and tumour bearing mice

	Endogenous substrate (cpm/20 mg protein)		Carboxylase (cpm/20 mg protein min)	
	Normal mice	Tumour bearing mice	Normal mice	Tumour bearing mice
	1590 ± 600	3805 ± 90*	190.5 ± 28.0	491.5 ± 164.5†
+ Warfarin	10,915 ± 928	10,363 ± 828	553.3 ± 28.1	659.2 ± 57.8
+ Vitamin K	1177 ± 136	1242 ± 300	162.4 ± 26.7	282.4 ± 10.1

The endogenous substrate is quantified as the amount of $^{14}CO_2$ (cpm) that was incorporated into endogenous substrate after 60 min incubation. The amount of carboxylase is expressed as the amount of $^{14}CO_2$ (cpm) which was incorporated into endogenous + exogenous substrate per minute. Livers from 10 mice for each group were pooled before the preparation of the various microsomal fractions. The results are expressed as the mean of 4 different experiments.
Warfarin and vitamin K were supplemented in the drinking water as described in the Material and Methods section.
* $P < 0.01$ vs normal mice.
† $P < 0.05$ vs normal mice.

1.5 mg/kg of radiolabelled warfarin in both tumour-bearing and normal mice. The Thrombotest level of tumour-bearing mice at the start of the experiment was within the normal range and even higher than that of controls (110 ± 7.0 vs 95 ± 5.0, $P < 0.05$). The decay of prothrombin complex activity was similar in both groups, with values around 10% after 13 hr, and the lowest level (5%) after 25 hr. In contrast, recovery of prothrombin complex activity differed markedly between the two groups. Indeed the time required to reach 50% of activity was 45.7 hr for control animals vs 71.9 hr for tumour-bearing mice ($P < 0.01$ Student's t test).

Figure 2 shows the degradation time-course of the prothrombin complex activity in normal and tumour-bearing mice. The K_d was not significantly different in the two groups ($K_d = 3.9 \pm SD\ 0.37$ in normal mice vs 3.27 ± SD 0.61 in tumour-bearing mice).

Figure 3 shows the disappearance of radioactivity from the blood of control and tumour-bearing animals. No significant differences were found in the pharmacokinetic parameters of radioactivity in the two groups.

Table 1 shows warfarin levels in blood and liver at 2 hr and 15 hr. There was no significant difference in liver and blood warfarin levels between normal and tumour-bearing mice.

Table 2 shows the presence of endogenous substrate for carboxylase and the total amount of carboxylase activity in the livers of non-treated controls and those of non-treated tumour-bearing mice. A significantly higher amount of endogenous substrate was found in the livers of the tumour-bearing mice. Administration of vitamin K to the tumour-bearing mice decreased the level of endogenous substrate to normal, whereas the level in the controls remained unaffected by the vitamin. As might be expected, the warfarin-induced accumulation of hepatic substrate was several-fold higher in the controls than in tumour-bearing mice, with a final level which was comparable in both groups.

Similar trends were observed when the amounts of hepatic carboxylase were compared in the two groups. In agreement with the observations of several other laboratories, the *in vivo* administration of warfarin (or the dietary vitamin K deficiency) induces an apparent increase of the microsomal carboxylase activity [9]. It is striking, however, that the amount of hepatic carboxylase in tumour-bearing mice was high, whether the animals had received warfarin or not. Also this effect was almost completely corrected by vitamin K administration.

DISCUSSION

In this study mice bearing an experimental metastatizing tumour showed increased susceptibility to the anticoagulant effect of warfarin. This may be concluded from experiments in which the time course of prothrombin complex activity after warfarin treatment was studied: a parallel drop was observed, but the recovery was much slower in tumour-bearing mice. It is worth mentioning that the basal level of prothrombin complex activity was not decreased in tumour-bearing animals but slightly increased to show statistical significance. The latter finding is in

agreement with previous data of normal clotting and fibrinolysis overall tests in the mice bearing the Lewis lung carcinoma [10].

We attempted to ascertain whether the increased sensitivity to warfarin anticoagulation was due to pharmacokinetic or pharmacodynamic mechanisms. No differences were found in the disappearance and volume of distribution of radioactivity from control or tumour-bearing mice, despite the presence of a 6–8 g tumour mass (representing $\frac{1}{3}$ of the body weight). Similarly no differences were found in the levels of warfarin in liver and blood when the drug was measured by an HPLC method. The HPLC method is specific to warfarin, therefore it further validates the data obtained in blood following administration of ^{14}C warfarin.

The lack of change in distribution volume in tumour-bearing mice could be due to the relatively low blood flow through the tumour, especially in its necrotic part. We have previously measured the percentage of cardiac output distributed to the Lewis lung carcinoma tissue by a radioactive microsphere technique and found that the amount of blood reaching tumour tissue during the final days of tumour development was about 1/10 of that arriving when the tumour is still vegetating [11]. This could markedly limit access of warfarin to the tumour area.

We subsequently studied whether the metabolic fate or the synthesis of prothrombin complex factor was changed in tumour-bearing mice.

The rate of degradation of prothrombin complex activity was not modified, which rules out the possibility of increased proteolysis or consumption by tumour-released enzymes. The delayed recovery of prothrombin complex activity observed in tumour-bearing mice after warfarin administration suggests that some interference occurs on the synthesis of functionally active prothrombin complex factors.

We thus explored the liver microsomal system which is involved in carboxylation of glutamic acid residues of prothrombin complex factors. It is well known that vitamin K is required as a cofactor for carboxylase in this reaction [12]. As a consequence, vitamin K-antagonists such as coumarin anticoagulants prevent the formation of carboxylated clotting factors. Administration of these drugs induces the accumulation of coagulation factor precursors (as an endogenous substrate for carboxylase) in the liver microsomes.

We have found that in liver microsomes from tumour-bearing animals, the carboxylase-dependent vitamin K-system was impaired as indicated by the accumulation of the endogenous carboxylase substrate. This abnormality was not due to a defective enzyme activity since in the presence of a synthetic substrate the activity is not decreased but even enhanced. Moreover, the addition of vitamin K was able to completely abolish the substrate accumulation.

It thus appears that vitamin K deficiency is involved in the observed accumulation of endogenous substrate in liver microsomes from tumour-bearing animals and presumably in their increased susceptibility to warfarin anticoagulation. Increased susceptibility to warfarin anticoagulation has been recently reported in rats bearing Walker 256 carcinoma [13]. The pathogenesis of such an effect in rats was not clarified; however, the hypothesis of vitamin K deficiency playing a role in rat hypersensitivity to warfarin cannot be excluded.

The mechanism of vitamin K deficiency in tumour-bearing mice is not clear; the possibility that reduced food intake during the last phase of tumour growth would somewhat favour vitamin K deficiency cannot be ruled out. However, this hypothetical mechanism could only partially account for vitamin K deficiency; indeed, withdrawal of vitamin K from the diet in tumour-bearing animals gives only after several weeks signs of vitamin K deficiency [14]. In any case, it is of interest that the vitamin K deficiency observed here does not involve a decrease in the basal level of prothrombin complex factors. Presumably, this deficiency is partial and just borderline; it is thus detectable only in the liver, where an increase in both carboxylase activity and substrate levels could lead to normal circulatory clotting factor levels. The same vitamin K deficiency does, however, mediate an increased susceptibility to the anticoagulant effect of warfarin. It is not yet known whether this may imply a lower requirement of warfarin in order to achieve the same degree of anticoagulation in tumour-bearing animals.

Acknowledgements—This work was partially supported by Associazione Italiana per la Ricerca sul Cancro, by Gustavus and Louise Pfeiffer Foundation (U.S.A.) and by Italian National Research Council (CNR contract n°85.00531.04). Judy Baggot, Felice and Vincenzo de Ceglie, Gustavus Pfeiffer Library and Ivana Garimoldi helped to prepare this manuscript.

REFERENCES

1. M. B. Donati and A. Poggi, *Br. J. Haematol.* **44**, 173 (1980).
2. P. Hilgard, in *Malignancy and the Hemostatic System* (Eds. M. B. Donati, J. F. Davidson and S. Garattini), p. 103. Raven Press, New York (1981).
3. M. J. Fasco, L. J. Piper and L. S. Kaminsky, *J. Chromatogr.* **131**, 365 (1977).
4. R. Nagashima, R. A. O'Reilly and G. Levy, *Clin. Pharmac. Ther.* **10**, 22 (1968).
5. C. Vermeer, B. A. M. Soute, M. de Metz and H. C. Hemker, *Biochim. biophys. Acta* **714**, 361 (1982).
6. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
7. G. Sacchi-Landriani, V. Guardabasso and M. Rocchetti, *Comput. Programs Biomed.* **16**, 35 (1983).
8. M. Recchia and M. Rocchetti, *Comput. Programs Biomed.* **15**, 111 (1982).
9. D. V. Shah and J. W. Suttie, *Archs. Biochem. Biophys.* **191**, 571 (1978).
10. A. Poggi, N. Polentarutti, M. B. Donati and G. de Gaetano and S. Garattini, *Cancer Res.* **37**, 272 (1977).
11. E. Raczka, A. Quintana, A. Poggi and M. B. Donati, *Eur. J. Cancer Clin. Oncol.* **19**, 1021 (1983).
12. J. W. Suttie, *CRC Crit. Rev. Biochem.* **8**, 191 (1980).
13. C. A. Owen, Jr., *Proc. Soc. exp. Biol. Med.* **169**, 1 (1982).
14. M. Colucci, F. Delaini, G. De Bellis Vitti, D. Locati, A. Poggi, N. Semeraro and M. B. Donati, *Biochem. Pharmac.* **32**, 1689 (1983).