LOSS OF HEPATOMA RIBOSOMAL RNA DURING WARFARIN THERAPY

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SUMMARY. 40-50% decreases in cytoplasmic ribosomal RNA were observed in mouse hepatoma implants, but not livers, after 4-5 daily warfarin injections. Similar treatment greatly depressed rates of in vivo ^{14}C-orotate incorporation into hepatoma ribosomal RNA in the cytoplasm. Labelling of mature 18S and 28S RNA in the nucleus appeared to be unaffected. Possible mechanisms for this warfarin effect are briefly discussed.

Warfarin chemotherapy has frequently appeared to reduce the quantity of firm tissue in experimental mouse hepatomas, compared with controls (1). This observation suggested an interference with overall protein synthesis. Since the binding of warfarin to ribosomes in rat liver has been demonstrated (2), it seemed reasonable to expect similar binding in mouse hepatoma. If ribosomes of this malignant tissue were especially sensitive to warfarin, their function might be seriously impaired. This possibility was explored by searching for changes in the tumor ribosomes which could be ascribed to the in vivo action of warfarin. In this report we describe a gross warfarin-induced decrease in cytoribosomal RNA of mouse hepatomas, and the absence of a comparable effect in livers of the same animals.

METHODS

Subcutaneous BW7756 hepatomas in C57L/J mice (Jackson Laboratory, Bar Harbor, Maine), serially transplanted in 6-week-old males, same strain, were used for experiments 25-35 days after transplantation. Sodium warfarin, racemic, highest purity (Wisconsin Alumni Research Foundation) and/or orotic-6-^{14}C acid hydrate, 57.7 mCi/mmole (New England Nuclear), were injected as indicated. Livers and tumors (firm tissue only, fluids excluded) were homogenized in 0.25 M sucrose and centrifuged 4 minutes at 650 x g (av). Mitochondria were eliminated

by a procedure described previously for their preparation (3). Combined post-mitochondrial supernatants and washings were centrifuged 15 minutes at 19,600 x g (av) to clarify, and then 35 minutes at 122,000 x g (av) to obtain microsomes. Nuclei were prepared from the 650 x g pellets by a sucrose-calcium method (4).

Microsomes or nuclei were treated with Sodium dodecyl sulfate and Bentonite at pH 5.5 and extracted twice by cold H_2O-saturated phenol containing 0.1% 8-hydroxyquinoline. RNA was precipitated from 70% ethanol - 0.7% (w/v) potassium acetate, pH 5.2, washed, dried, dissolved in buffer used for gradients. 0.05 - 0.20 ml RNA were sedimented through linear gradients (5% - 22% sucrose in 100 mM KCl, 50 mM Tris-HCl, pH 7.0) at 25,000 RPM in a Spinco SW-41 Rotor, 2^{o}, for 15 hours (microsomes) or 12 hours (nuclei). Calibration was by the method of Noll and Stutz (5). A_{260} profiles were recorded via flow-cell. In some cases the effluent was passed to a fraction collector.

RNA was assayed as described by Fleck and Munro (6) except wavelengths were read from recorded spectra. Base composition of rat liver RNA was used in calculations. Radioactivity was measured in a Beckman liquid scintillation counter with ^{14}C-benzoic acid as internal standard. Protein assays were according to Lowry (7).

RESULTS

Experiment A, Table I, indicated that warfarin caused a large decrease in RNA content of microsomes from the tumors of injected mice, but had little or no effect on liver microsomal RNA. Experiment B was performed one month later. Fewer warfarin injections were given and the transplants were from an entirely different group of mice, but the same results were obtained. It was also determined that the RNA missing from the tumor microsome fraction could not be recovered in the post-microsomal supernatant. The balance sheet data in Table II point very clearly to a general deficiency of ribosomal RNA in the cytoplasm of warfarin treated tumors. All of these RNA's were subjected to sedimentation analysis. The profiles consisted of 5S, 18S, and 28S peaks with no significant

TABLE I

WARFARIN-INDUCED DECREASE OF RNA IN HEPATOMA MICROSOMES

Experiment	Warfarin Injections	Tissue	μg RNA/mg protein
A	none	tumor	152.2
		liver	81.3
	5	tumor	81.2
		liver	74.5
B	none	tumor	136.0
		liver	79.7
	4	tumor	75.1
		liver	82.6

(A) 3 mice received 5 daily injections of warfarin (10 mg/Kilo in 0.1 ml sterile saline). 4 hours after final injection microsomes were prepared from livers and tumors. Similar preparations were made from 4 control mice (no warfarin). RNA was extracted, assayed, and characterized by sedimentation analysis. (B) Same as (A) except warfarin was given only 4 days, there were 3 controls, and additional fractions were recovered (see Table II).

mitochondrial contamination. Minor species above 18S were minimal. Hence the RNA affected by warfarin, as reported here, is cytoribosomal.

The evidence above suggest that warfarin might: (1) inhibit cytoribosomal RNA synthesis; (2) block a step in the assembly or export of ribosomes; (3) cause production of defective ribosomes which are rapidly degraded; or (4) accelerate the degradation of normal ribosomes.

Pulse-labelling with ^{14}C-orotate (Table III) confirmed the preceding results and also provided some insight into possible mechanisms for the warfarin effect. The specific and total activities of liver microsomal RNA fluctuated considerably, but did not show a pattern of response to warfarin. In sharp contrast the radioactivity of tumor microsomal RNA was greatly depressed. Labelled high molecular weight RNA was also extracted from the nuclei of warfarin and control tumors in this experiment (Table III, 5 hour pulse). Sedimentation gave 6S, 18S, and 28S peaks. The specific activities of these species were compared with 18S and 28S microsomal RNA's of the same tumors, as indicated in

TABLE II

EXTRANUCLEAR DISTRIBUTION OF LIVER AND TUMOR CYTORIBOSOMAL RNA

Fraction	Tissue	Warfarin	PROTEIN mg	%	recovery %	RNA μg	%	recovery %	RNA/Protein μg/mg
Post-mitochondrial	liver		90.8	100	103.0	3225	100	104.2	35.5
Supernatant	liver	w	100.0	100	101.3	3595	100	93.7	36.0
	tumor		46.0	100	106.1	2626	100	99.8	57.1
	tumor	w	25.5	100	105.8	503	100	110.0	19.7
Microsomes	liver		25.5	28.1		2033	63.0		79.7
	liver	w	26.5	26.5		2192	61.0		82.6
	tumor		12.7	27.6		1722	65.6		136.0
	tumor	w	4.6	18.0		347	69.0		75.1
Post-microsomal	liver		68.0	74.9		1328	41.2		19.5
Supernatant	liver	w	74.8	74.8		1176	32.7		15.7
	tumor		36.1	78.5		898	34.2		24.9
	tumor	w	22.4	87.8		206	41.0		9.2

Additional data from Experiment B, Table I. High molecular weight RNA was also extracted from the post-mitochondrial and post-microsomal supernatants. All phenol layers were re-extracted, giving essentially quantitative yields.

TABLE III

DEPRESSED LABELLING OF MICROSOMAL RNA
IN HEPATOMAS TREATED BY WARFARIN

Tissue	Warfarin Injections	^{14}C-orotate pulse			
		3 hours		5 hours	
		(DPM)	(DPM/μg)	(DPM)	(DPM/μg)
liver	0	25000	14.4	37400	26.0
	4	32900	23.0	35500	18.0
tumor	0	10300	4.1	8800	6.5
	4	1100	2.0	1800	1.8

3 mice received 3 daily injections of warfarin (as in Table I), and on the fourth day they were given 10 μC ^{14}C-orotic acid (57.7 mCi/mmole) in 0.1 ml H_2O which also contained warfarin (10 mg/Kilo). 3 control mice were injected with ^{14}C-orotic acid only. At 3 hours liver and tumor microsomes were prepared from both groups. RNA was extracted quantitatively, assayed, counted, and characterized by sedimentation analysis. One week later the above was repeated, using a 5 hour pulse. In this experiment only, tumor nuclei were also recovered, high molecular weight RNA extracted, and treated as shown in Table IV.

TABLE IV

DIFFERENTIAL INFLUENCE OF WARFARIN ON LABELLING
OF HEPATOMA NUCLEAR AND CYTOPLASMIC RNA'S

RNA	Microsomes		Nuclei	
	Control	Warfarin	Control	Warfarin
	(CPM/μg)	(CPM/μg)	(CPM/μg)	(CPM/μg)
6S	-	-	19.3	18.4
18S	4.2	0.97	18.2	16.9
28S	4.5	0.87	12.7	11.1
Total*	6.1	1.5	36.6	44.7

*Unfractionated RNA, prior to sedimentation.

Nuclear RNA's from control and warfarin tumors (5 hour pulse, Table III) were assayed, counted, and also sedimented 12 hours (see Methods). A_{260} profiles were recorded and 36 fractions collected. 6S, 18S, and 28S species were recovered, then assayed and counted. Microsomal RNA's from the same tumors were similarly treated except sedimentation was 15 hours and only 18S and 28S were recovered.

Table IV. The results show that the specific activities of microsomal 18S and 28S RNA's were decreased in warfarin treated tumors, as expected. But the corresponding nuclear species, and also 6S, were affected little or not at all.

The unfractionated nuclear RNA was also counted prior to sedimentation and is compared with microsomal RNA at the bottom of Table IV. Again, the specific activity of this mixture of nuclear RNA's did not decrease in tumors of warfarin treated animals.

DISCUSSION

The foregoing experiments have shown that mouse hepatoma implants were largely deprived of cytoribosomal RNA during a 4-5 day series of warfarin injections, with most of the loss occurring in the microsome fraction (Tables I and II). The RNA-deficient microsomes were then found to be receiving new ribosomal RNA at a greatly diminished net rate (Table III). These highly significant changes in a malignant tissue (hepatoma) were seen to be absent or minimal in the related normal tissue (liver).

The mechanism by which warfarin brings about depletion of tumor cytoribosomal RNA remains to be explained. From the results presented, it appears that simple inhibition of RNA synthesis is not a satisfactory explanation. The specific radioactivities of hepatoma nuclear RNA and three of its components (Table IV) were not significantly reduced by warfarin therapy. Moreover, the persistent labelling of 18S and 28S species is a good indication that maturation of ribosomal RNA was not disturbed. It therefore seems probable that warfarin affected the assembly of viable ribosomes, or their survival in the cytoplasm.

The role of warfarin as vitamin K antagonist in the liver may be unrelated to the tumor RNA phenomenon described here. Recent work of Suttie (8) indicates that warfarin inhibits the K-mediated conversion of a prothrombin precursor, but not synthesis of the precursor itself. The warfarin level producing this inhibition in rats was 5 mg/Kilo. Presumably our use of 10 mg/Kilo in tumor-bearing mice also decreased the output of prothrombin, but without observable removal of RNA from the liver microsomes. Thierry *et al.* (2) have determined the *in vivo* binding of warfarin to organelles in rat liver. Affinity for the drug was at least threefold higher in ribosomes than in nuclei. These authors also found that *in vitro* binding of warfarin was much greater with liver ribosomes than

with those from spleen, kidney or heart, but point out that a microsomal protein could nevertheless be the true binding site. This alternative may be supported by our data, since a significant decrease in percent of microsomal protein was observed in warfarin treated tumors (Table II).

We have not yet determined the binding of warfarin in hepatoma organelles, but expect to find measurable affinities. Regardless of the quantity bound, however, the ribosomal or microsomal proteins of this malignant tissue may be exceptionally sensitive to warfarin. Defective ribosomes or faulty attachment to the endoplasmic reticulum might result, followed by early degradation of the RNA. Experiments are under way to test this possibility, and also to detect any minor losses of cytoribosomal RNA in organs other than liver.

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REFERENCES

1. Unpublished observations.
2. M. J. Thierry, M. A. Hermodson, and J. W. Suttie, Amer. J. Physiol., 219, 854 (1970).
3. J. J. Van Buskirk and W. R. Frisell, Biochim. Biophys. Acta, 143, 292 (1967).
4. H. Busch in "Methods in Enzymology" (Sidney P. Colowick and Nathan O. Kaplan, eds.) Vol. XII, Part A, p. 425. Academic Press, New York (1967).
5. H. Noll and E. Stutz in "Methods in Enzymology" (Sidney P. Colowick and Nathan O. Kaplan, eds.) Vol. XII, Part B, p. 129. Academic Press, New York (1967).
6. A. Fleck and H. N. Munro, Biochim. Biophys. Acta, 55, 571 (1962).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem., 193, 265 (1951).
8. J. W. Suttie, Science, 179, 192 (1973).