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DECREASED ACTIVITY OF CYTIDINE 3':5'-MONOPHOSPHATE (CYCLIC CMP) PHOSPHODIESTERASE IN THE FAST-GROWING MORRIS HEPATOMA 3924A, BUT NOT IN THE SLOW-GROWING MORRIS HEPATOMA 9618A

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SUMMARY: The activity level of the newly-identified cyclic CMP phosphodiesterase in the fast-growing Morris hepatoma 3924A was found to be much lower than the control (normal or host) liver. Its level in the slow-growing Morris hepatoma 9618A (a minimal deviation tumor), on the other hand, was the same as the host liver. The level of cyclic AMP phosphodiesterase was higher, whereas that of cyclic GMP phosphodiesterase was lower, in hepatoma 3924A than the control liver. In comparison, the levels of the two enzymes were both depressed in hepatoma 9618A. These findings suggest that depression of cyclic CMP phosphodiesterase may be related to the process and the rate of malignant growth, and that metabolism of cyclic CMP may be more crucial than that of cyclic AMP or cyclic GMP in the neoplastic cell proliferation.

We have reported recently the occurrence of cyclic CMP phosphodiesterase in a number of mammalian tissues (1), and its decreased activity levels in the normal tissues undergoing rapid cell proliferation, exemplified by regenerating liver (1,2) and several fetal tissues of guinea pigs (1-3). These observations are in line with the hypothesis that cyclic CMP is a putative positive effector of cell proliferation (4-6), with its cellular concentration being regulated either by the hydrolytic enzyme cyclic CMP phosphodiesterase (1-3,7) or by the synthesizing enzyme cytidylate cyclase (8,9), or both. We suspected that a decreased activity of cyclic CMP phosphodiesterase may be also associated with the malignant growth of tissues. Our experiments suggest that this is indeed the case. We found that the enzyme activity was greatly decreased in the fast-growing Morris hepatoma 3924A, but its activity in the extremely slow-growing Morris hepatoma 9618A remained unaffected.

EXPERIMENTAL PROCEDURE

Materials: Cyclic [5-^{3}H]CMP was purchased from Amersham; cyclic [G-^{3}H]AMP and cyclic [G-^{3}H]GMP were from New England Nuclear. The ACI/c strain rats (female,

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100-140 g) were from Laboratory Supply Co., Indianapolis, Indiana; the Buffalo strain rats (female, 100-140 g) were from Simonsen Laboratories, Gilroy, California.

Methods: Hepatoma 3924A (2 weeks after subcutaneous transplantation on ACI/c rats) and hepatoma 9618A (12-16 months after transplantation on Buffalo rats) were dissected free of necrotic tissue. The liver from the non-tumor bearing rats (normal liver) and the liver from the tumor bearing animals (host liver) served as controls. The tissue samples were quickly homogenized in 3 vol. of ice-cold 50 mM Tris-HCl buffer (pH 7.5) containing 3 mM 2-mercaptoethanol using glass-Teflon homogenizers. The homogenates were used directly as the enzyme sources. The assay methods for cyclic AMP and cyclic GMP phosphodiesterase activities were the modifications (10) of that originally described by Thompson and Appleman (11), using 40 mM Mg^{2+} to activate the enzymes and using 1 μM of cyclic AMP and cyclic GMP as substrates. The cyclic CMP phosphodiesterase activity was assyed by the method recently described (1-3), using 10 mM $FeSO_4 \cdot 7H_2O$ to activate the enzyme and using either 1 μM or 1 mM cyclic CMP as the substrate. Appropriate amounts (2.2-10 mg protein) of tissue homogenates and incubation times (1-10 min) were used in all experiments so that 10-25% of the cyclic nucleotides added were hydrolyzed under the assay conditions. The activity values reported were corrected for blank values obtained in the absence of added enzyme (homogenates). Protein was determined by the method of Lowry et al (12). DNA was determined by the diphenylamine method of Richards (13).

RESULTS AND DISCUSSION

The phosphodiesterase activity for cyclic CMP from the normal liver and hepatoma 3924A homogenates, assayed using either 1 μM or 1 mM substrate, was linear in the presence of at least up to 13 mg of tissue and about 10 min of the incubation time (Fig. 1). In subsequent experiments reported herein, all assay conditions were such that the rates of hydrolysis of cyclic CMP, cyclic AMP and cyclic GMP were linear with respect to the tissue (enzyme) amount and the incubation time.

The activity of cyclic CMP phosphodiesterase from the homogenates of hepatoma 3924A was found to be much lower than the normal liver or the host liver (Table I), in agreement with the data shown in Fig. 1. This was true regardless whether the enzyme was assayed using 1 μM or 1 mM substrate, or the data were expressed on the basis of either tissue weight, total tissue protein or DNA. The decrease in the enzyme activity, however, was less pronounced when the data were expressed on the protein basis; this was due to the fact that the protein content of hepatoma 3924A is only 63% of that of the liver (either the normal or the host). The phosphodiesterase activities for cyclic AMP and cyclic GMP in the same tissue samples were also assayed for comparison. They were assayed using only 1 μM substrates, since the low Km form of the enzymes has been considered physiologically more im-

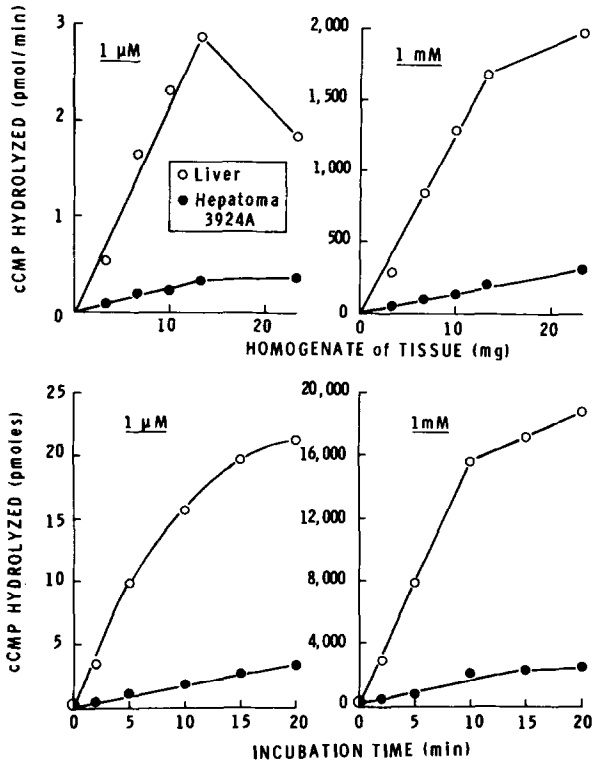

Fig. 1. Dose-dependence and time course of the hydrolysis of cyclic CMP by phosphodiesterase in homogenates of the normal liver from ACI/c rats and hepatoma 3924A. Conditions and procedures were as described under Experimental Procedure. For the dose-dependent hydrolysis (upper panel), the incubation time used was 10 min for 1 μM and 1 mM of cyclic CMP. For the time course of the hydrolysis (lower panel), the quantities of the homogenates used were 6.7 and 10.0 mg of fresh tissues for 1 μM and 1 mM of cyclic CMP, respectively.

portant and perhaps is the more predominant species of the enzymes as well. It was noted that hepatoma 3924A had a higher activity of cyclic AMP phosphodiesterase, and conversely a lower activity of cyclic GMP phosphodiesterase, confirming our earlier findings (14) and providing a tentative explanation for the greatly elevated (over 200-fold) cyclic GMP in this hepatoma reported by Thomas et al (15). A decrease in the cyclic GMP phosphodiesterase activity, however, was not obvious if the value was expressed on the basis of protein.

The double-reciprocal plots of kinetic data reveal that cyclic CMP phosphodiesterase from both the normal liver and the hepatoma had an identical Km of 4.2

TABLE I

Comparison of activity levels of phosphodiesterases for cyclic CMP, cyclic AMP and cyclic GMP in homogenates of the normal liver and the host liver from the ACI/c strain rats, and of the fastest-growing Morris hepatoma 3924A.

The enzyme activities in homogenates of the liver and the hepatoma were assayed using 1 μM or 1 mM of the respective cyclic nucleotides as substrates, as indicated. Data are presented as means ± standard errors of the means from three normal livers, three host livers and six hepatomas. One unit (u) of the enzyme activity is defined as one pmole cyclic nucleotide hydrolyzed per min.

Substrate concentration	Tissue	Levels of phosphodiesterase activities		
		u/g fresh tissue	u/mg protein	u/mg DNA
cCMP (1 μM)	Normal Liver	162±6	1.11±0.03	61±4
	Host Liver	150±5	1.06±0.04	52±1
	Hepatoma 3924A	24±2*	0.26±0.03*	8±1*
cCMP (1 mM)	Normal Liver	87,400±3,517	601±16	32,564±1,588
	Host Liver	78,267±3,254	556±33	26,978± 294*
	Hepatoma 3924A	14,633±1,578*	161±20*	4,750± 502*
cAMP (1 μM)	Normal Liver	8,966± 359	62± 1	3,344± 187
	Host Liver	8,505± 256	60± 2	2,939± 127
	Hepatoma 3924A	12,248± 212*	134± 6*	4,005± 158*
cGMP (1 μM)	Normal Liver	4,759± 216	33± 2	1,768± 62
	Host Liver	5,805± 289*	41± 3	2,000± 50
	Hepatoma 3924A	2,784± 130*	31± 1	914± 67*

* Significantly different from the normal liver ($p < 0.025 - 0.0005$)

mM for cyclic CMP (Fig. 2), probably indicating the hepatoma enzyme is the same molecular species as the normal liver enzyme. As reported earlier for the liver (1), only the high Km species (with the saturating substrate concentration in the mM range) of the enzyme was detected for the hepatoma (data not shown). Since the same amount of the tissue homogenates was used for the assay, the relative Vmax values obtained clearly indicate that the cyclic CMP-hydrolyzing enzyme was much lower in the hepatoma, agreeing with the data shown in Fig. 1 and Table I. The possibility that the low cyclic CMP phosphodiesterase activity found in hepatoma 3924A may be due to the presence of an endogenous inhibitor appears to be eliminated, since additions of aliquots of the hepatoma homogenate to the liver homogenate did not result in a reduced activity of the liver enzyme.

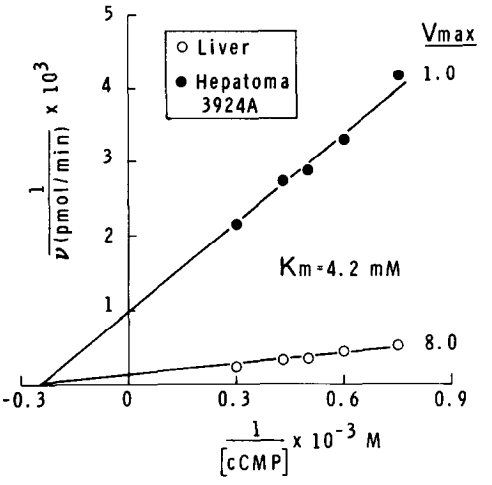

Fig. 2. Double-reciprocal plots of cyclic CMP hydrolysis by phosphodiesterase in homogenates of the normal liver from ACI/c rats and hepatoma 3924A. The amount of the homogenates used contained 10 mg of fresh tissues. The incubation was carried out for 10 min. The concentrations of cyclic CMP used ranged from 1.3 to 3.3 mM.

Compared to the fast-growing Morris hepatoma 3924A shown above, the cyclic CMP phosphodiesterase level in the extremely slow-growing Morris hepatoma 9618A remained unaltered (Table II). The enzyme level from hepatoma 9618A, however, was found to be slightly higher than the host liver when the value was expressed on the DNA basis. The levels of both cyclic AMP and cyclic GMP phosphodiesterase were lower in hepatoma 9618A than the host liver. It should be mentioned that hepatoma 9618A (a minimal deviation and the slowest-growing tumor), as opposed to the fast-growing hepatoma 3924A, has been shown to have the same chromosomal number and karyotype as the normal liver (16). The present studies thus provide evidence for the first time to indicate that a decreased cyclic CMP phosphodiesterase activity may be more closely related to the process and the rate of malignant growth than the metabolism of cyclic GMP and cyclic AMP. This relationship seems to prevail also in cell proliferation of a more physiologic nature, as in the cases of regenerating liver (1,2) and fetal tissues (1-3).

When accurate and sensitive assay methods for cyclic CMP become available in the future, it is important to determine whether the rapidly proliferating

TABLE II

Comparison of activity levels of phosphodiesterases for cyclic CMP, cyclic AMP and cyclic GMP in homogenates of the host liver from the Buffalo strain rats, and of the slowest-growing Morris Hepatoma 9618A.

The enzyme activities in homogenates of the liver and the hepatoma were assayed using 1 μM or 1 mM of the respective cyclic nucleotides as substrates, as indicated. Data are presented as means ± standard errors of the means from three host livers and three hepatomas. One unit (u) of the enzyme activity is defined as one pmole cyclic nucleotide hydrolyzed per min.

Substrate concentration	Tissue	Levels of phosphodiesterase activities		
		u/g fresh tissue	u/mg protein	u/mg DNA
cCMP (1 μM)	Host Liver	315±10	1.8±0.03	141± 7
	Hepatoma 9618A	310±16	1.9±0.20	190± 7*
cCMP (1 mM)	Host Liver	146,067±12,969	822±69	65,301±6,770
	Hepatoma 9618A	119,600± 4,980	808±31	73,549±3,818
cAMP (1 μM)	Host Liver	7,620± 416	43± 2	3,386± 71
	Hepatoma 9618A	4,965± 422*	33± 3*	3,029± 29*
cGMP (1 μM)	Host Liver	7,425± 213	42± 2	3,313± 161
	Hepatoma 9618A	3,525± 299*	24± 2*	2,173± 225*

* Significantly different from the host liver ($p < 0.01 - 0.0005$)

tissues indeed have higher concentrations of cyclic CMP. If this should be the case, then the contention that cyclic CMP is a positive effector of cell proliferation (4-6,8,9) and our assumption that decreased cyclic CMP phosphodiesterase activity is related to elevated cellular cyclic CMP (1-3) would be supported. A large body of data accumulated based upon experiments using the normal and trans formed cells in culture and tumors *in vivo*, although sometimes controversial and even contradictory, suggest a positive role for cyclic GMP and a negative role for cyclic AMP in cell proliferation (see Ref. 17 and 18 for reviews). The exact nature of the intricate balance and interrelations possibly existing among the effects of cyclic CMP, cyclic AMP and cyclic GMP with respect to the molecular events triggering cell proliferation in general and dictating normal *vs.* malignant growth in particular, remains to be further elicidated.

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