CYCLIC AMP AND CYCLIC GMP CONTENT AND BINDING IN MALIGNANCY*

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SUMMARY: 3',5'-cyclic AMP and 3',5'-cyclic GMP concentrations in several strains of Morris hepatomas as well as the ability of the hepatoma cytosol to bind the cyclic nucleotides were measured. The tumors were found to have levels of cyclic AMP either equal to or depressed below those of the host livers. Hepatomas with cyclic AMP levels equal to those of livers disclosed at least one of the following characteristics: low cyclic AMP binding capacity, high cyclic GMP concentration, high cyclic GMP binding capacity. The tumors with high cyclic GMP levels were particularly fast-growing. We suggest that malignancy is associated with a low effective level of cyclic AMP and that the effective level of cyclic AMP is a function not only of its concentration but also of the level of the various proteins which react with cyclic AMP and also the activity of its antagonists, such as cyclic GMP.

A number of recent studies have pointed to an important role for 3',5'-cyclic AMP (cyclic AMP) in malignancy. However, several findings appear to be contradictory. For some years, scientists have known that many malignant, cultured mammalian cells can be restored to seeming normality by adding cyclic AMP (or its derivatives) to the culture media (1-4), and that in whole animals, the administration of cyclic AMP or inhibition of diesterases greatly curbed or halted the growth of a variety of malignant tumors (5-7). In accordance with these findings, several early papers (8-11) reported that cyclic AMP concentration in malignant cells is lower than in comparable normal cells, and that low cyclic AMP levels are associated with viral transformation (12). However, it was soon discovered that a number of malignant cells contain

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normal or even supranormal levels of cyclic AMP (13, 14) and adenyl cyclase (15-18). Surprisingly, some neoplastic cells with apparently normal levels of cyclic AMP could be restored to normal function by the addition of exogenous cyclic AMP. Such restoration to normality by the cyclic nucleotide, however, does not invariably occur (19).

We speculated that biologically effective concentrations of cyclic AMP were not necessarily the same as the total concentration in the cell, and the amount of actually operative cellular cyclic AMP might depend upon factors other than simple concentration of the cyclic nucleotide. That is, there might be a protein deficiency in those proteins which interact (i.e., bind) with cyclic AMP; or, the level of cyclic GMP, which, in general, acts in opposition (20, 21) to cyclic AMP, might be elevated. In the only previous report on cyclic nucleotide binding in neoplasia, Granner (22) found a decrease in cyclic AMP binding capacity in a cultured malignant cell line. It is surprising that there have been no studies on cyclic GMP content or binding in malignant tissue.

MATERIALS AND METHODS

Reagents for cyclic AMP assay were obtained from Schwarz-Mann, Orangeburg, N.Y.; those for cyclic GMP assay were obtained from Collaborative Research, Inc., Waltham, Mass. The various strains of Morris hepatomas were maintained by intramuscular transplantation into the thighs of female rats.

Animals were decapitated and their tumors or livers (tumor-free) were either frozen immediately in liquid nitrogen (for cyclic nucleotide assay) or chilled in grinding media (0.25 M sucrose, 0.05 M KCl, 0.001 M $MgCl_2$, 0.05M Tris, pH 7.4, 0.004 M thioglycerol) for the cyclic nucleotide binding assay.

The cyclic nucleotides were extracted from the frozen tissue by homogenization with 1% perchloric acid (1ml/g) containing tracer

amounts of the radioactive cyclic nucleotides. After centrifugation, the precipitate was assayed for protein (23). The supernatant, neutralized with potassium hydroxide to ∿pH 7, was first separated into cyclic AMP and cyclic GMP fractions by passage through a Dowex-1 formate column. To remove materials which react in the cyclic GMP assay but are not destroyed by cyclic nucleotide diesterase, we followed a suggestion of N. D. Goldberg and passed the cyclic GMP fractions successively (24) through columns of Sephadex QAE-25 (formate form) and Dowex-50 (hydrogen form). The resultant extracts were assayed by the method of Steiner (25). Standard deviations were never greater than 15% and averaged 10%.

For binding studies, fractionation of tumors and livers was performed as described by Walton and Garren (26). The binding affinity was examined by slightly modifying the procedures of Gilman (27) and of Daniel et al. (28). Extracts (100 µg of protein for the cyclic AMP assay, 200 µg for the cyclic GMP assay) were incubated at 0^{o} for 1 hr in 200 µl of binding mix composed of 0.05 M sodium acetate buffer (pH 4.0), 0.007 M theophylline, and 0.5-1.0 X 10^{-6}M tritium-labeled cyclic nucleotide (cyclic AMP, 28 Ci/mmole; cyclic GMP, 3.4 Ci/mmole). After incubation, 5.0 ml of 0.02 M potassium phosphate were added; the reaction mix was allowed to stand for 5 min at room temperature. Protein-bound cyclic nucleotide was then collected on Millipore filters (0.45-µm pore size) and washed with the same phosphate buffer, and the radioactivity was measured. Standard deviations were never greater than 10%.

In both content and binding studies, tissues from three rats were combined in each case. Values were obtained in triplicate and confirmed using separate sets of rats.

RESULTS

If we compare cyclic AMP contents of the first three tumors (Nos.

TABLE I. Cyclic nucleotide content and binding capacity in cytoplasm of liver and hepatomas*

Tumor growing in rat	Tissue examined	Cyclic nucleotide binding (pmole/μg protein) Cyclic AMP	Cyclic GMP	Cyclic AMP binding tumor/ cyclic AMP binding liver	Cyclic GMP binding tumor/ cyclic GMP binding liver	Cyclic nucleotide concentration in tissue (pmole/mg protein) Cyclic AMP	Cyclic GMP
None	Liver	7.4	0.95	---	---	6.9	0.24
7800	Liver	6.5	0.23	0.4	1.5	2.1	0.82
	Tumor	2.5	0.34			1.3	0.89
7787	Liver	5.8	1.2	0.6	0.1	2.7	0.36
	Tumor	3.7	0.11			6.3	0.68
7316A	Liver	3.8	0.62	0.7	0.3	16.5	0.92
	Tumor	2.5	0.16			11.1	0.42
3924A	Liver	5.5	0.50	1.1	1.4	6.5	0.37
	Tumor	6.2	0.70			5.7	2.6
9098	Liver	4.8	0.73	1.2	1.8	19.5	0.24
	Tumor	5.7	1.3			18.6	0.33

*Cyclic nucleotide content and binding were determined as described in Materials and Methods.

7800, 7787, and 7316A) (Table I) to the levels in the animals' livers, we see that there is no consistency in respect to cyclic AMP or cyclic GMP levels. If, however, we examine the binding capacity for cyclic AMP, and especially the ratio of the binding capacity of tumor cytoplasm for cyclic AMP (preliminary experiments revealed no binding differences in cellular organelles) to the binding capacity of liver cytoplasm, we note that the binding capacity of tumor cytoplasm is reduced to about half that of liver from the host animal. These results are in complete accord with the previously mentioned studies of Granner (22).

Let us now inspect these aspects in the other two tumors, Nos. 3924A and 9098. These neoplasms were studied because they are particularly fast-growing. In tumor No. 3924A, cyclic AMP binding is slightly elevated and the content of cyclic AMP is minimally depressed compared to that of the host's liver. If we examine the neoplasm for cyclic GMP, we find that the binding capacity for cyclic GMP is considerably heightened, and even more interestingly, the content of cyclic GMP is enormously augmented (7X) over that of the liver. Turning to the data on tumor No. 9098, we see that cyclic AMP binding is higher for the tumor than for the liver. Furthermore, the tumor content of cyclic AMP is not significantly lower than in the liver. Yet if we examine cyclic GMP, the tumor content is 140% in excess of the liver content, while the binding capacity of cyclic GMP is twice as large in the tumor as in the liver. Now let us re-examine the previously discussed tumor, No. 7800, one of the first three mentioned. Among the original three studied, this tumor is the one wherein cyclic GMP binding is considerably higher than that of the liver and it is the fastest-growing of them.

DISCUSSION

In agreement with a previous report by Butcher *et al.* (13), apparently in rat liver hepatomas, as in several other tissues, malignancy

is not inevitably associated with a low concentration of cyclic AMP. The limited results presented here would suggest (at least in rat hepatomas) that particularly fast tumor growth is accompanied by elevated content or binding of cyclic GMP. Especially pertinent findings of other workers are that while cyclic AMP acts to inhibit cell division, cyclic GMP either stimulates mitosis or is present in high concentrations at sites where cells are vigorously dividing (29-35).

The notion that simple cyclic AMP concentration is not a measure of effective cyclic AMP concentration is corroborated by other observations: In tissues from adrenalectomized animals, cyclic AMP manifests activity only at concentrations far above those needed by normal animals, but tissues become sensitive to cyclic AMP by treatment with glucocorticoids (36-39). In another example, Drezner et al. (40) described a syndrome wherein the patient appears hypoparathyroid, but in actuality, the tissues do not react to increases in the level of cyclic AMP caused by parathyroid hormone. Similarly, Bricker and Cevey (41) and Sharma and Bush (42), studying the metabolism of several tumors, found a number of metabolic pathways very unresponsive to cyclic AMP compared to normal cells.

On the basis of these observations, our own work, and the binding studies of Granner (22), we suggest that malignancy is associated with a low effective level of cyclic AMP and that the effective level of cyclic AMP is a function not only of its concentration per se, but also a function of several other factors: the level of the various proteins which react with cyclic AMP, and the activity of the antagonists of cyclic AMP -- such as cyclic GMP.

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