Plasma and urine cyclic nucleotide levels in malignant disease and cirrhosis of the liver

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SUMMARY Plasma and 24-hour urinary cyclic AMP and cyclic GMP levels were determined by saturation analysis in specimens from normal subjects and from 101 patients with tumours of the gastrointestinal tract, breast, lung, bladder or prostate, or with cirrhosis of the liver. Relative to 46 control subjects, plasma cyclic GMP concentrations were significantly elevated in seven patients with gastric tumours, 20 patients with cancer of the breast, six patients with lung cancer, and 12 patients with cirrhosis of the liver. Urinary cyclic GMP/creatinine ratios were significantly increased in cirrhotic patients and in the lung and oesophageal cancer groups. In no cancer group were increases in plasma or urine cyclic GMP levels sufficiently consistent to be of value in the diagnosis of human malignant disease. Changes in extracellular fluid cyclic nucleotide levels in the cirrhotic group were very similar to those that have been reported for primary hepatoma patients.

Cyclic 3',5'-adenosine monophosphate (cyclic AMP) is a well-established second messenger for hormone stimulation of target cells. The exact role played by cyclic 3',5'-guanosine monophosphate (cyclic GMP) in the regulation of cell metabolism is still far from clear (Goldberg and Haddox, 1977). In some tissues, cyclic GMP can oppose the action of cyclic AMP in cell control processes. It is known from cell culture studies that increases in cyclic GMP concentrations are associated with cell proliferation, whereas high cyclic AMP levels inhibit cell growth (Goldberg *et al.*, 1975).

Cyclic GMP/cyclic AMP ratios in rat liver and kidney tumour tissue are high relative to uninvolved liver or renal tissue, the highest ratios being associated with the fastest growing tumours (Criss and Murad, 1977). Increased urinary excretion of cyclic GMP has been reported in rats bearing transplantable liver and kidney tumours (Criss and Murad, 1976). Cyclic GMP excretion in rats with Morris hepatoma 3924A correlated with tumour size and decreased in parallel with the efficacy of surgical, radiation, or drug therapy (Murad *et al.*, 1975).

There have been few reports to date of extracellular fluid cyclic GMP levels in human malignant disease. In a preliminary report, Neethling and

Shanley (1976) described significant elevation of urinary cyclic GMP excretion in three patients with primary hepatoma and suggested that extracellular fluid cyclic nucleotide measurements may be of value in the diagnosis and monitoring of malignancy.

This study was designed to investigate whether other types of human cancer are associated with altered plasma or urine cyclic nucleotide levels, and to determine if such changes could be correlated with tumour size or the presence of metastases. Patients with cirrhosis of the liver were also studied in an attempt to establish the specificity of the reported urinary cyclic GMP increases in primary hepatoma.

Patients and methods

The numbers and age ranges of the patients who were studied are listed in Table 1. Diagnoses were confirmed by histology of biopsy material or surgical specimens.

Patients with malignant disease were classified according to the type and size of the tumour and the presence of metastases. Of the 28 cases of colorectal cancer, two were classified as Duke's stage A, 11 as stage B, eight as stage C, and six as stage C₂ with hepatic metastases evident on tissue biopsy, isotopic scan, or ultrasound. Two of seven patients with gastric cancer were noted to have involved lymph nodes at surgery. Liver metastases were

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Table 1 Patients

Group	Plasma				24-hour urine			
	Total	M	F	Age range	Total	M	F	Age range
Normal subjects	46	12	34	19-60	36	10	26	24-60
Ca colon/rectum	28	14	14	29-75	25	12	13	29-75
Ca stomach	7	4	3	58-72	6	4	2	58-72
Ca oesophagus	9	6	3	48-79	8	6	2	48-79
Ca breast	20	—	—	35-65	18	—	—	35-63
Ca lung	6	4	2	42-70	5	4	1	42-60
Ca bladder	—	—	—	—	8	8	—	28-70
Ca prostate	4	—	—	60-75	2	—	—	68-75
Liver cirrhosis	12	10	2	40-68	13	10	3	40-68

evident in two of nine patients with oesophageal carcinoma. Widespread bony metastases were present in five of the 20 patients with carcinoma of the breast.

The liver cirrhosis group consisted of 10 patients with alcoholic cirrhosis and three patients with cirrhosis associated with α_1 -antitrypsin deficiency.

Control subjects were healthy volunteers or women who were found to have benign fibroadenoma of the breast on histological examination of breast biopsy tissue.

All plasma and urine specimens were collected from patients before the start of treatment or surgery. Aliquots of 24-hour urines were stored frozen until they were analysed. Blood (20 ml), obtained by venepuncture between 0900 and 1100, was rapidly added to two lithium heparin tubes (10 IU heparin/ml blood), one of which also contained EDTA (0.5 mol/l pH 7.4; 100 μ l per 10 ml of whole blood). Plasma from the tube containing EDTA was rapidly separated and stored at -20°C until analysed. Plasma separated from the tube containing heparin only was incubated at 37°C for 1 hour. In this way plasma cyclic nucleotides were degraded by endogenous cyclic nucleotide phosphodiesterase enzyme to give a 'blank' plasma sample for each patient.

Plasma and urine cyclic GMP levels were determined by direct radioimmunoassay (Wood and Marks, 1978). Plasma and urine cyclic AMP concentrations were measured by methods based on

the adrenal protein kinase binding assay of Brown *et al.* (1971) with the inclusion of cyclic AMP-free plasma in standardisation tubes to compensate for protein effects. Between-batch precisions for the plasma and urine cyclic nucleotide assays are set out in Table 2. Urine creatinines were determined by the alkaline picrate method on an AutoAnalyser.

The statistical significance of results was assessed using the non-parametric Mann-Whitney 'U' test. Very similar levels of significance were also obtained by log transformation of group data and comparison by Student's *t* test.

Results

The mean levels and 95% confidence limits for urine and plasma cyclic nucleotide results for control subjects are listed in Table 3.

Although several patients in the cancer and cirrhosis groups had very high plasma cyclic GMP concentrations (Fig. 1) there was considerable overlap with the control data. Plasma cyclic GMP levels in the stomach, breast, lung, and cirrhosis groups were significantly higher than those for the control subjects. Plasma cyclic GMP/cyclic AMP ratios showed a similar pattern (Fig. 2). Plasma cyclic AMP levels in the cancer patients (not shown) were not significantly different from normal levels, although in the cirrhotic patients plasma cyclic AMP levels were significantly higher than in the control group.

Table 2 Inter-assay precisions for cyclic nucleotide assays

		No. of assays		Mean result	CV (%)
Urine	cyclic AMP	QC1	7	3.9 pmol/tube	10.8
		QC2	7	5.0 pmol/tube	8.2
	cyclic GMP	QC1	12	0.9 pmol/tube	6.7
		QC2	12	4.7 pmol/tube	7.3
Plasma	cyclic AMP	QC1	10	5.0 nmol/l	10.9
		QC2	10	10.4 nmol/l	9.8
	cyclic GMP	QC1	10	1.0 nmol/l	12.3
		QC2	10	2.2 nmol/l	9.8

Table 3 Plasma and urine cyclic nucleotide levels—normal subjects

		N	Mean	95% Confidence limits*
Plasma	cyclic AMP	46	9.5 nmol/l	5.4-16.8 nmol/l
	cyclic GMP	46	2.7 nmol/l	1.1-6.5 nmol/l
	cyclic GMP/cyclic AMP	46	0.30	0.10-0.90
Urine	cyclic AMP†	36	2.6 µmol/g creat.	1.2-5.4 µmol/g creat.
	cyclic GMP†	36	0.36 µmol/g creat.	0.21-0.90 µmol/g creat.
	cyclic AMP/cyclic GMP†	36	0.14	0.04-0.45

*After log transformation of data

†24-hour collections

Fig. 1 Plasma cyclic GMP.

Fig. 2 Plasma $\frac{\text{cyclic GMP}}{\text{cyclic AMP}}$ ratios.

Urinary cyclic GMP/creatinine ratios were high in several patients (Fig. 3) although results were significantly higher than normal in only the oesophageal and lung cancer groups and the cirrhotic patients. The calculation of urinary cyclic GMP/cyclic AMP ratios (Fig. 4) did not improve the discrimination between control subjects and tumour patients. There were no significant differences in urinary cyclic AMP/creatinine levels in any of the patient groups compared with the control data.

The main findings of the study are summarised in Table 4 together with significance levels.

There was no correlation between plasma cyclic GMP and plasma cyclic AMP or between urine cyclic GMP/creatinine and urine cyclic AMP/creatinine ratios in patients in the groups investigated. There was no correlation between altered extracellular fluid cyclic nucleotide levels and tumour size or metastasis in any of the cancer patient groups.

Discussion

It is clear from the present study that plasma or urinary cyclic GMP levels or cyclic GMP/cyclic AMP ratios are of little value in the diagnosis of malignancy in spite of the fact that several individuals had strikingly high results. Although these individual high levels could reflect an increased output of cyclic GMP by tumour tissue, the finding from selected animal tumour lines that urinary cyclic GMP output is influenced by the presence and size of a tumour (Murad *et al.*, 1975) does not appear to be generally true for human malignant disease. The results of this study are compatible with the reported wide variation in total cyclic GMP/cyclic AMP ratios in a number of tumour tissues (Goldberg *et al.*, 1975) and are supported by the report of Murad (1978), who found wide variations in plasma and urine cyclic GMP levels

Table 4 Summary of results

Group	Plasma			24-hour urines		
	cAMP	cGMP	cGMP/cAMP	cAMP†	cGMP†	cGMP/cAMP
Ca colon/rectum	NS	NS	NS	NS	NS	NS
Ca stomach	NS	↑*	NS	NS	NS	NS
Ca oesophagus	NS	NS	NS	NS	↑*	↑*
Ca breast	NS	↑**	↑**	NS	NS	NS
Ca lung	NS	↑**	↑**	NS	↑***	NS
Ca bladder	—	—	—	NS	NS	NS
Ca prostate	NS	NS	NS	NS	NS	NS
Liver cirrhosis	↑*	↑***	↑**	NS	↑**	↑***

Significances: NS = no significant difference compared with control data
*0.05 > P > 0.02
(Mann-Whitney 'U' test) **0.01 > P > 0.001
***P < 0.001
†Results expressed as μmol/g creatinine.

Fig. 3 Urine cyclic GMP.

in a preliminary study of patients with malignant disease.

The observation that plasma and urinary cyclic AMP concentrations were unchanged in all of the malignancy groups relative to control subjects is supported in part by Bershtein and colleagues (1976), who found no differences in urinary cyclic AMP excretion between patients with breast or lung cancer and control subjects in a study of 150 people. Recently, Gennari *et al.* (1978) have reported a significant decrease in urinary cyclic AMP/creatinine ratios in patients with a variety of epithelial tumours. These same authors also reported abnormally high urinary cyclic GMP/creatinine ratios in 26 patients who were selected on the basis of a low cyclic AMP excretion. Such an inverse

Fig. 4 Urine $\frac{\text{cyclic GMP}}{\text{cyclic AMP}}$ ratios.

relationship between urinary cyclic AMP and cyclic GMP excretion was not established for any group in the present study, and the reason for this discrepancy is unclear.

Although it would be of interest to monitor changes in urinary cyclic nucleotide excretion after surgical removal of a tumour, the fact that trauma results in an increased excretion of cyclic AMP (Vitek *et al.*, 1976) and that urinary cyclic GMP levels may be raised for at least a week after surgery (P. J. Wood *et al.*, unpublished observations) complicates studies in the postoperative period.

The finding of raised plasma and urinary cyclic GMP levels in cirrhotic patients is particularly

interesting in view of the fact that these changes correlate closely with those reported by Neethling and Shanley (1976) and Murad (1978) in patients with primary hepatoma. It is possible, therefore, that the extracellular fluid cyclic nucleotide changes that have been reported for primary hepatoma patients are associated with a deranged liver metabolism rather than with the tumour tissue itself. The cause of increased plasma and urinary cyclic GMP content in cirrhosis is not clear, although possible contributing factors could be secondary proliferative changes within the liver, decreased hepatic uptake of circulating nucleotides, or changes secondary to a diminished catabolism of a hormone or other agent which can generate cyclic GMP within target cells. At present the picture of a moderate rise in plasma cyclic AMP in addition to a larger increase in plasma and urine cyclic GMP levels could best be interpreted in terms of diminished hepatic uptake of cyclic nucleotides, especially in the case of cyclic GMP. There is some evidence that the liver may take up cyclic GMP in preference to cyclic AMP (Exton *et al.*, 1971; Levine, 1973), and this could explain a possible greater influence of decreased hepatic uptake on extracellular fluid cyclic GMP levels. There was, however, no correlation between plasma alkaline phosphatase or γ -glutamyl transferase enzyme activities and plasma or urine cyclic GMP levels for the cirrhotic patients.

The results indicate that liver disease is associated with increases in plasma and urine cyclic GMP levels, and that further studies in this area may be worthwhile. Although the investigation of tissue cyclic nucleotide metabolism will continue to provide valuable information in the field of cancer research, this study has shown that the measurement of urinary or plasma cyclic GMP levels does not aid the diagnosis of malignancy or the detection of tumour metastases.

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