

Homocysteine Thiolactone Metabolism in Malignant Cells¹

Kilmer S. McCully

James Homer Wright Pathology Laboratories, Massachusetts General Hospital, and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02114

SUMMARY

Since abnormal homocysteine metabolism is associated with several disorders of growth, including neoplasia, the metabolic fate of homocysteine thiolactone was studied in cell cultures from several malignant tumors, established cell lines, cell lines transformed by oncogenic viruses, and normal skin cells. In all of the cultures of malignant cells, homocysteine thiolactone became incorporated in peptide linkage with cellular proteins (thiolation). Normal cells incorporated the sulfur of homocysteine thiolactone only as the sulfate groups of proteoglycans. An established cell line incorporated homocysteine thiolactone in peptide linkage, but homocysteine thiolactone was released by acid hydrolysis. The findings suggest the speculative possibility that malignant cells are deficient in a homocysteine thiolactone derivative that prevents thiolation of proteins by homocysteine thiolactone. This hypothetical substance may also catalyze the synthesis of methionine and release acrolein, a growth-regulatory substance, in normal cells. The growth characteristics and tumorigenicity of cultured cells may be related to depletion of the hypothetical substance, and its identification, synthesis, and administration to animals would be expected to affect growth of malignant neoplasms.

INTRODUCTION

Disorders of cellular and skeletal growth are associated with abnormalities of homocysteine metabolism in homocystinuria (13), experimental arteriosclerosis (10, 19, 20), scurvy (15), and hypophysectomized rats (7, 17). Cells cultured from individuals with cystathionine synthetase deficiency produce an aggregated sulfated proteoglycan matrix (14) that is associated with altered contact inhibition of cellular growth (16). Cultured malignant cells require methionine for growth in media containing homocystine, cobalamin, and folic acid, but normal cells have no such requirement (6, 9). Homocysteine thiolactone is a product of methionine metabolism (25), and certain synthetic derivatives of homocysteine thiolactone affect the growth of transplanted murine adenocarcinoma (18).

The abnormality of homocysteine metabolism in malignant cells was investigated by studying the metabolic fate of homocysteine thiolactone in cell cultures from malignant tumors, established cell lines, cell lines transformed by

oncogenic viruses, and normal skin cells. Malignant cells were found to incorporate homocysteine thiolactone into peptide linkage with cellular proteins (thiolation), whereas normal cells convert homocysteine thiolactone to sulfate esters of proteoglycans (15, 16). The results suggest the speculative possibility that malignant cells are deficient in a homocysteine thiolactone derivative that prevents thiolation of cellular proteins by homocysteine thiolactone. This hypothetical substance may also catalyze the synthesis of methionine and release acrolein, a growth-regulatory substance, in normal cells.

MATERIALS AND METHODS

Using conventional culture techniques, cell lines were established from a lymph node metastasis in a case of human renal cell carcinoma, KM; from a bladder carcinoma induced in rats by *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (21), RBT; from a transplanted murine mammary adenocarcinoma of spontaneous origin (22), A-10; and from a transplanted hamster sarcoma induced by SV40, T8AP. Established cell lines from baby hamster kidney, BHK, and mouse embryo, 3T3, were transformed *in vitro* by polyoma virus, PY-BHK, and SV40, SV-3T3, respectively. Diploid connective tissue cell strains were established from normal human skin biopsies, TR and Dunn.

Cells were passaged into Eagle's minimal essential medium containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) and 0.8 or 1.6 $\mu\text{mole } ^{35}\text{SO}_4$ per ml (1.0 $\mu\text{Ci/ml}$) or 0.1 μmole of L-[^{35}S]homocysteine thiolactone per ml (0.2 $\mu\text{Ci/ml}$) (Radiochemical Centre, Amersham, England). The cells were refed twice weekly until confluence was reached in 4 to 21 days. The media were removed and the cells were rinsed with cold 0.9% NaCl solution, scraped, and homogenized in 0.9% NaCl solution. Protein content and radioactivity of the homogenates were determined and the homogenates from cells grown in media with [^{35}S]homocysteine thiolactone were dialyzed, hydrolyzed in HCl, and chromatographed on a Beckman 120C amino acid analyzer, as described previously (16).

In order to study binding of homocysteine thiolactone, the dialyzed homogenates from cells grown in media with [^{35}S]homocysteine thiolactone were incubated with 5% TCA² at 100° for 10 min and filtered through Millipore filters. The homogenates were also incubated with 1 N mercaptoethanol at 37° for 1 hr followed by hot TCA with 1 N NaOH at 37° for 1 hr followed by hot TCA, or with 0.1% trypsin (Grand

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² The abbreviations used are: TCA, trichloroacetic acid; SV-3T3, simian virus-transformed 3T3 mouse embryo cells.

Island Biological Co.) and 0.1% Pronase (Worthington Biochemical Corp., Freehold, N. J.) at 22° for 18 hr followed by hot TCA and were filtered through Millipore filters. The radioactivity of the homogenates, filters, and filtrates was determined, and the percentage of hydrolysis was calculated for each incubation.

RESULTS

Malignant cells cultured from human metastatic renal cell carcinoma, KM, incorporated almost 4 times as much homocysteine thiolactone as did diploid connective tissue cells grown from normal human skin, TR (Table 1). Cultured hamster sarcoma cells, T8AP, incorporated 3 to 4 times as much homocysteine thiolactone as an established line of baby hamster kidney cells, BHK. The other 2 cultures of malignant cells, the murine mammary adenocarcinoma, A-10, and the rat bladder carcinoma, RBT, incorporated about the same amount of homocysteine thiolactone as did the normal skin cells, TR, or the 2 established cell lines, BHK and 3T3. The 2 cell cultures transformed by oncogenic viruses, PY-BHK and SV-3T3, incorporated only slightly more homocysteine thiolactone than the untransformed cells of origin, BHK and 3T3. The hamster sarcoma cells, T8AP, incorporated about 3 times as much homocysteine thiolactone as the hamster line transformed *in vitro* by oncogenic virus, PY-BHK.

The incorporation of $^{35}\text{SO}_4$ by malignant cells, normal cells, established cell lines, and transformed cells was approximately equal, except for RBT, which was somewhat lower (Table 1). Increased inorganic sulfate concentration in the medium had no effect on incorporation of homocysteine thiolactone, but the incorporation of $^{35}\text{SO}_4$ was in-

creased by increased inorganic sulfate in all cell lines tested.

Almost all of the homocysteine thiolactone bound to dialyzed homogenates of all 4 cultures of malignant cells was precipitated by hot TCA (Table 2). Cellular proteins are insoluble in hot TCA. Both proteolytic enzymatic hydrolysis and alkaline hydrolysis solubilized the bound homocysteine thiolactone in hot TCA, showing that the incorporated homocysteine thiolactone was bound to cellular proteins by peptide bonds. Incubation with mercaptoethanol did not solubilize the bound homocysteine thiolactone in hot TCA. Since mercaptoethanol breaks disulfide bonds, this result

Table 2

Hydrolysis of cell cultures grown in [^{35}S]homocysteine thiolactone

Cell cultures were grown to confluence in media with L- [^{35}S]homocysteine thiolactone and were scraped and homogenized in 0.9% NaCl solution. Homogenates from 6 culture dishes (diameter, 105 mm) for each line were pooled and dialyzed. Aliquots in triplicate for each cell line and for each condition were incubated with 5% TCA at 100° for 10 min, with 1 M mercaptoethanol at 37° for 1 hr, with 1 M NaOH at 37° for 1 hr, or with 0.1% trypsin and 0.1% Pronase at 22° for 18 hr, each was followed by hot TCA. After filtration through Millipore filters, radioactivity of filters and filtrates was determined in duplicate and the percentage of hydrolysis was calculated. Solubilization (%) = (cpm of filtrate)/(cpm of filter + cpm of filtrate) × 100.

Cell culture	Solubilization (%)			
	Hot TCA	Mercaptoethanol	NaOH	Trypsin and Pronase
A-10	10.4	12.2	86	97
RBT	10.8	11.3	90	98
T8AP	6.6			98
BHK	7.7	8.9	92	97

Table 1

Incorporation of [^{35}S]homocysteine thiolactone and $^{35}\text{SO}_4$ in cultured cells

Cells were grown to confluence in media containing [^{35}S]HCT^a or $^{35}\text{SO}_4$. The concentration of inorganic sulfate in the media was 0.8 $\mu\text{mole/ml}$ ($^{35}\text{SO}_4$ and ^{35}S -HCT) or 1.6 $\mu\text{moles/ml}$ ($^{35}\text{SO}_4$ + SO_4 and ^{35}S -HCT + SO_4). After the cultures reached confluence, the media were removed and the cells were homogenized in 0.9% NaCl solution. Duplicate dishes were used for each cell line and medium. Radioactivity and protein were determined for each homogenate in duplicate.

Cell culture	Origin	Incorporation at confluence (nmoles $^{35}\text{S}/\text{mg}$ protein)			
		^{35}S -HCT	^{35}S -HCT + SO_4	$^{35}\text{SO}_4$	$^{35}\text{SO}_4$ + SO_4
KM	Metastasis from human renal cell carcinoma	22.4	19.0	14.9	25.2
TR	Normal human skin	5.9	5.8	15.6	26.2
T8AP	Hamster sarcoma induced by SV40	9.7	7.6	10.0	
T8AP		8.8	11.5	14.0	
T8AP		11.8	14.6	9.7	
BHK	Baby hamster kidney	3.0	2.9	11.8	17.6
PY-BHK	Transformed by polyoma	3.7	4.0	16.7	35.4
3T3	Mouse embryo	2.5	2.9	11.3	20.4
Simian virus 3T3	Transformed by SV40	3.9	3.7	14.1	22.0
A-10	Murine mammary carcinoma	3.6	4.6	10.2	
RBT	Rat bladder carcinoma	5.3	5.5	7.1	

^a [^{35}S]HCT, L- [^{35}S]homocysteine thiolactone.

shows that the homocysteine thiolactone was not bound to cellular proteins by disulfide bonds.

Chromatography of the dialyzed homogenates that had been hydrolyzed by HCl at 110° showed that most of the bound homocysteine thiolactone was recovered as homocysteine or homocystine from the 4 cultures of malignant cells (Table 3). When special care was taken to exclude oxygen during hydrolysis and sample preparation, only homocysteine and no homocystine was recovered. Only small amounts of homocysteine thiolactone were recovered from the hydrolysates, except in the case of BHK cells, from which over one-half of the radioactivity was recovered as homocysteine thiolactone. No homocysteine, homocystine, or homocysteine thiolactone was recovered from the normal diploid skin cells, Dunn, but most of the radioactivity was eluted with inorganic sulfate. Simultaneous ninhydrin analysis revealed ninhydrin absorption peaks eluted at the position of radioactive homocysteine, homocystine, or homocysteine thiolactone recovered in the various experiments. In most experiments, a small unidentified peak containing ³⁵S was eluted just before (4 to 5 min) the position of authentic methionine sulfoxide.

DISCUSSION

The purpose of the study is to define the chemical nature of the abnormality in homocysteine metabolism found in malignant cells (6, 9). Our results show that 2 of the 4 cell cultures from malignant tumors bound larger amounts of homocysteine thiolactone than did cell cultures from normal skin, established cell lines, or cell lines transformed by oncogenic viruses. All of the 4 cultures of malignant cells incorporated homocysteine thiolactone into peptide bonds with cellular proteins, and large amounts of homocysteine or homocystine were found in acid hydrolysates of dialyzed cell culture homogenates by column chromatography

Table 3
Chromatography of hydrolyzed cell cultures grown in [³⁵S]homocysteine thiolactone

Cell cultures were grown to confluence in media containing L-[³⁵S]homocysteine thiolactone and were scraped and homogenized in 0.9% NaCl solution. Homogenates from 6 culture dishes (diameter, 105 mm) for each line were pooled and hydrolyzed with 6 N HCl at 110° for 18 hr under N₂. The radioactive amino acids and radioactive sulfate were separated by chromatography of an aliquot of each hydrolysate on a Beckman 120C amino acid analyzer. ³⁵S-labeled amino acids recovered (%) = (cpm in peak)/(total cpm recovered) × 100.

Cell culture	³⁵ S-labeled amino acids recovered (%)				
	Sulfate	Un-identified	Homocysteine	Homocystine	Homocysteine thiolactone
RBT	1.3	20.6	<0.1	76.1	<0.1
RBT	<0.1	5.8	<0.1	72.0	12.2
RBT	4.6	26.6	67.0	<0.1	2.8
T8AP	42.7	14.7	<0.1	41.1	<0.1
T8AP	<0.1	10.5	89.7	<0.1	<0.1
A-10	0.9	14.6	84.3	<0.1	1.2
BHK	8.3	15.7	23.0	<0.1	51.2
Dunn	73.0	27.0	<0.1	<0.1	<0.1

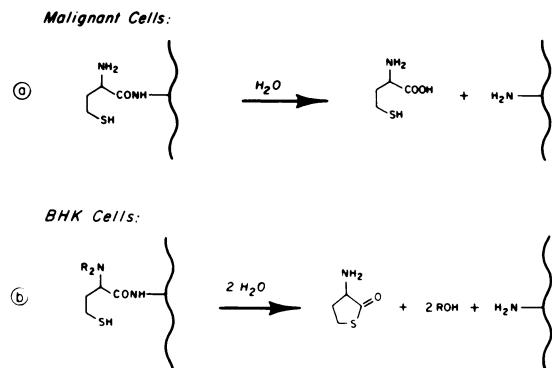


Chart 1. Hydrolysis of peptide-bound homocysteine from cellular proteins. The R groups are unidentified.

(Chart 1a). Peptide linkage of homocysteine to the free amino groups of proteins is known as thiolation. Under dilute alkaline conditions, homocysteine thiolactone spontaneously dimerizes, forming peptide bonds (8), and proteins such as immunoglobulin can be thiolated with homocysteine thiolactone under similar conditions (3, 12). Our results are the first to demonstrate that a similar thiolation of protein by homocysteine thiolactone occurs in malignant cells. Peptides containing homocysteine have been isolated from the urine of patients with homocystinuria, but the homocysteine was found to be linked to cysteine by disulfide bonds (24) rather than by peptide bonds.

The results with the established line, BHK, show that a homocysteine thiolactone derivative is bound to cellular proteins by peptide bonds and that homocysteine thiolactone is released by acid hydrolysis. Presumably, an *N*-substituted derivative of homocysteine thiolactone is hydrolyzed to yield the products shown in Chart 1b. The nature of the nitrogen substituents of the bound homocysteine thiolactone is presently unknown. *N*-Substituted homocysteine thiolactone thiolates proteins *in vitro*, yielding free sulfhydryl groups of *N*-substituted homocysteine bound to protein by peptide bonds (12). Several *N*-substituted derivatives of homocysteine thiolactone, including the arachidonoyl and oleoyl amides and the pyridoxal enamine derivative affect the growth of transplanted murine adenocarcinoma (18), possibly by altering the binding of homocysteine thiolactone to cellular proteins. Normal connective tissue cells from skin convert homocysteine thiolactone to the sulfate groups of proteoglycans (15, 16), and no evidence of thiolation of protein amino groups (Chart 1) was obtained with normal cells.

Although the cell cultures transformed by oncogenic viruses, PY-BHK and SV-3T3, were found to bind less homocysteine thiolactone than the hamster sarcoma cells, T8AP, further work is necessary to establish whether viral transformation affects cellular homocysteine metabolism.

Previous results have shown that the tRNA of malignant cells is more highly methylated than the tRNA of normal cells (4) and that malignant cells have more active tRNA methylases than normal cells (26). More recently, malignant cells have been shown to require methionine for growth in a medium containing homocystine, folic acid, and cobalamin, whereas normal cells have no such requirement (6, 9). A speculative possibility that explains our results is that

malignant cells may be deficient in a substance that prevents thiolation of cellular proteins by homocysteine thiolactone, presumably by catalyzing the formation of methionine from homocysteine thiolactone and methyl group precursors. Thus, if this formulation proves to be correct, methyl groups are transferred excessively to tRNA in malignant cells because of failure of methylation of homocysteine thiolactone. The consequent increase in homocysteine thiolactone synthesis presumably results in thiolation of the amino groups of cellular proteins of malignant cells.

The thiolation of cellular proteins by homocysteine thiolactone may in part be responsible for the widespread abnormalities found in cellular membranes of malignant cells, such as increased negative charge because of thiolated amino groups, immunological changes because of thiolated antigens, altered mitochondrial function, etc. (28).

In normal liver, homocysteine thiolactone is a precursor of homocysteic acid (15), a substance that promotes growth and releases somatomedin in hypophysectomized rats (7). The presumed increase in homocysteine thiolactone synthesis by malignant cells may result in abnormal homocysteic acid synthesis, affecting the growth potential of these cells.

The nature of the substance which prevents thiolation of cellular proteins and catalyzes synthesis of methionine from homocysteine, folic acid, and cobalamin in normal cells is presently unknown. Methionine and homocysteine yield acrolein on chemical degradation (2), and acrolein is believed to be a precursor in the prebiotic synthesis of methionine (27). A speculative possibility is that the deficient substance in malignant cells is an *N*-substituted homocysteine thiolactone derivative that is capable of releasing acrolein, a cytotoxic substance with growth-regulatory properties (1), under physiological conditions.

Elucidation of a chemically defined abnormality of homocysteine metabolism in cultures of malignant cells has implications for understanding the control of growth of malignant neoplasms *in vivo*. If the chemical nature of the *N*-substituents of the hypothetical homocysteine thiolactone derivative were established, chemical synthesis of the compound and its administration to animals would be expected to prevent or slow growth of spontaneous or transplanted neoplasms. Also, the growth of a malignant neoplasm, which is presumably depleted of the hypothetical compound, might be expected to diminish the concentration of the compound in normal tissues, producing systemic effects such as inhibition of liver cystathionase, cysteine sulphinic acid decarboxylase, and serine dehydrase (5). Thus administration of the hypothetical compound to an animal with a neoplasm would be expected to prevent systemic effects of the neoplasm and to slow its growth by enhancing the immunological responsiveness of the host and by correcting the metabolic defect of the neoplastic cells.

The finding of thiolation of cellular proteins in an established cell line suggests that the ability of cells to grow indefinitely in culture and that the tumorigenicity in animals of some established cell lines (23) may result from partial depletion of the hypothetical homocysteine thiolactone derivative during growth in culture. This possibility is supported by requirement of BHK cells for an increased con-

centration of folic acid for growth in media deficient in methionine and supplemented with homocysteine and cobalamin (11).

This study has shown that the metabolic block in malignant cells results in thiolation of cellular proteins by homocysteine thiolactone. This finding explains previously described abnormalities of methionine metabolism in malignant cells and predicts the existence of a homocysteine thiolactone derivative that is necessary for control of growth in normal cells.

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