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Original Paper

Homocysteine Accumulation in Human Ovarian Carcinoma Ascitic/Cystic Fluids Possibly Caused by Metabolic Alteration of the Methionine Cycle in Ovarian Carcinoma Cells

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The major role of the high-affinity folate-binding protein (FBP) is to regulate cellular folate homeostasis by increasing folate uptake in case of extracellular and intracellular folate deficiency. On this basis, we hypothesised that the overexpression of FBP in ovarian carcinoma might be physiologically associated with folate deficiency in the extracellular fluids, where ovarian carcinoma cells develop *in vivo*, or it might be the result of a reduced intracellular regeneration of the 5-methyltetrahydrofolate (5-CH_3H_4 folate). To test these hypotheses, we determined the bioavailability of folate in serum and in ascitic/cystic fluids of ovarian carcinoma patients ($n = 36$). The intracellular shortage of 5-CH_3H_4 folate was evaluated in the extracellular fluids by measuring the concentration of homocysteine (Hcy), which is a useful marker of intracellular folate deficiency. Patients with ascites from malignant and benign non-ovarian pathologies were used as controls ($n = 30$). We found no folate shortage in the serum and ascitic/cystic fluids of ovarian carcinoma patients. The folate concentration was within the normal range and superimposable on that observed in serum and ascites of control patients. However, the ascitic/cystic Hcy concentration was significantly higher ($P < 0.005$) than the corresponding serum concentration in a large fraction of ovarian carcinoma patients (72%, 26/36), whereas it was higher only in a small fraction of patients with non-ovarian malignant ascites (24%, 4/17), and in no patient with benign ascites. Hcy accumulation in ovarian carcinoma patients was not associated with a defect in the catabolic pathway of Hcy to cysteine, but was consistent with an impaired remethylation process of Hcy to methionine caused by an intracellular shortage of 5-CH_3H_4 folate. This suggests a possible association between FBP overexpression and a biochemical defect of the cellular folate metabolism involved in the methionine cycle. © **1997 Published by Elsevier Science Ltd.**

Key words: enzyme defect, folate, folate-binding protein, 5,10-methylenetetrahydrofolate reductase, methylation process

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INTRODUCTION

APPROXIMATELY 80% of human non-mucinous ovarian carcinomas and 100% of adenomas overexpress the high-affinity folate-binding protein α (FBPα) [1–3], a glycosylphosphatidylinositol-linked membrane protein relevant to cellular folate uptake [4, 5]. The mechanism(s) by which FBP expression is elevated in ovarian carcinomas does not appear to involve amplification or structural alteration(s) of the gene, but may be ascribed to gene overexpression [2].

Previous studies have demonstrated that FBPα expression, both in normal and tumour cell lines, is regulated by intracellular and extracellular folate concentration [6–9]. Cells physiologically respond to folate shortage by raising FBPα expression in order to increase the folate uptake from

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extracellular fluids. Therefore, FBPα overexpression in ovarian carcinoma cells may be related to folate deficiency in the tumour's environment or it may mirror an altered folate metabolism in the tumour cells. Determination of the folate concentration in the serum and ascitic/cystic fluids of ovarian carcinoma patients can shed light on the possible shortage of availability of extracellular folates. If FBPα overexpression is consequent to altered intracellular folate metabolism, this may be due to a lack of cellular 5-methyltetrahydrofolate (5-CH_3H_4 folate), since this coenzyme is the natural ligand of FBPα, representing the predominant seric form of folate coenzymes (more than 90% of the overall seric folates, under physiological conditions). Moreover, a shortage of intracellular 5-CH_3H_4 folate because of impaired intracellular reconversion from other folate coenzymes uniquely affects the functionality of the methionine (Met) cycle (Figure 1), and its consequences on cellular metabolism can be obviated by increasing the cellular uptake of 5-CH_3H_4 folate and Met from the extracellular fluids. If this occurs, the increased uptake of extracellular Met necessary to meet the cellular requirement of methyl groups for the transmethylation processes [10] may result in an increased production of the Met catabolite, homocysteine (Hcy), which may possibly be released by the cells. To evaluate the presumed shortage of 5-CH_3H_4 folate in ovarian carcinoma cells *in vivo*, it seems warranted to determine the catabolic release of Hcy by ovarian carcinoma cells. We assumed that higher Hcy concentrations in ascitic/cystic fluids than in serum may be indicative of a source of Hcy in ascitic/cystic compartments where ovarian carcinoma cells grow and, possibly, excrete part of their catabolites. Our experimental data seem to support the existence of the supposed defect in the remethylation of Hcy to Met in a large fraction of human ovarian carcinomas.

MATERIALS AND METHODS

Materials

Hcy, cysteine (Cys), and cysteine–glycine (Cys–Gly) were obtained from Sigma Chemical Co. (St Louis, Missouri, U.S.A.). Ti-*n*-butylphosphine, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), and trichloroacetic acid (TCA) were purchased from Fluka Chemie AG (Buchs,

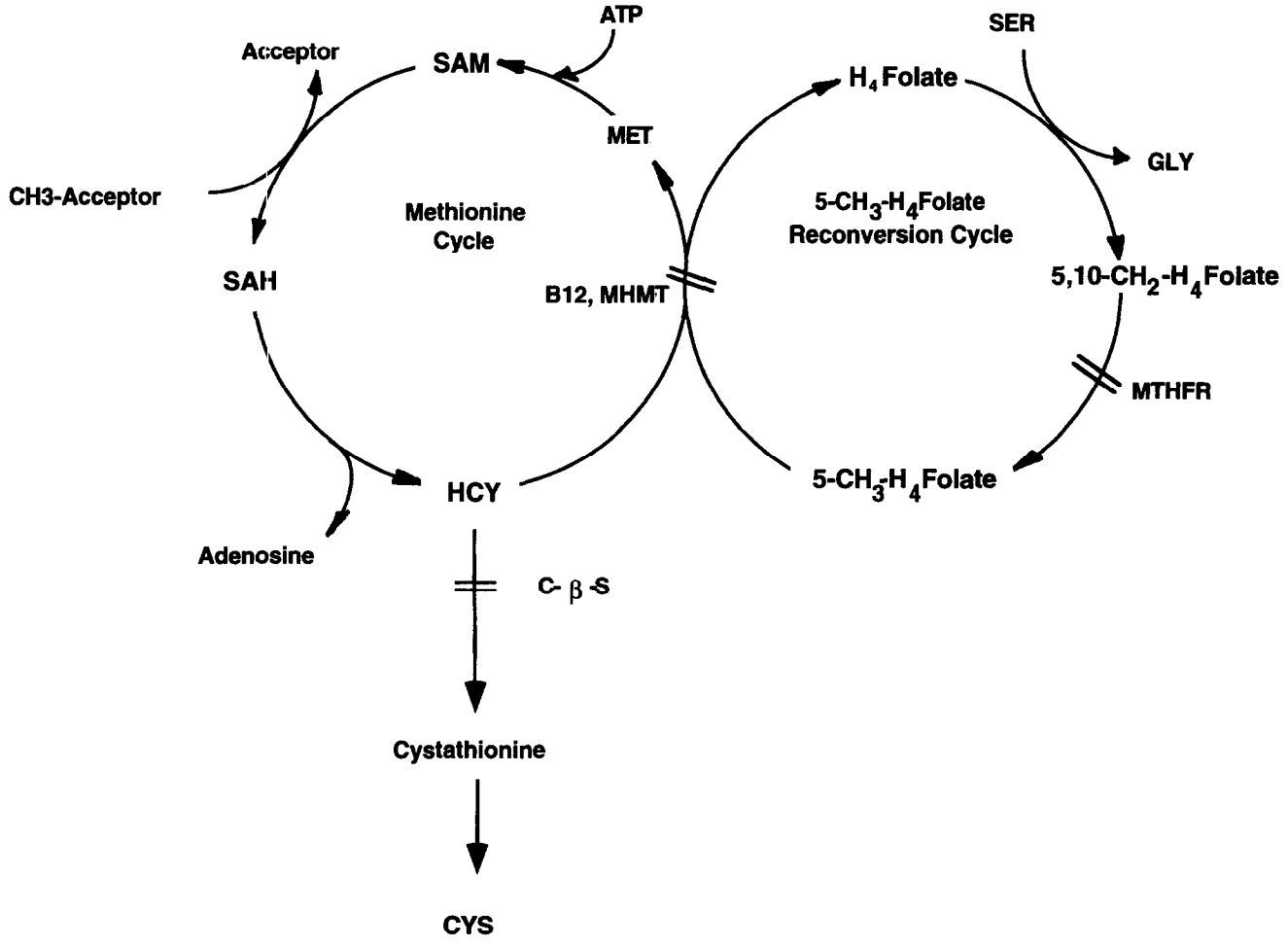

Figure 1. Pathways of Hcy metabolism. Hcy formation derived exclusively from the demethylation process of methionine: Hcy is either catabolised to cysteine by a series of reactions, called the *trans*-sulphur pathway, in which the first step is catalysed by cystathionine β synthetase, or remethylated to methionine in the transmethylation process by a cobalamin-dependent reaction catalysed by 5-methyltetrahydrofolate:Hcy methyltransferase. B12-MHMT, cobalamin dependent homocysteine methyltransferase (methionine synthetase); C-β-S, cystathionine β synthetase; Cys, cysteine; 5,10-CH_2H_4 folate, 5,10-methylenetetrahydrofolate; 5-CH_3H_4 folate, 5-methyltetrahydrofolate; Gly, glycine; H_4 folate: tetrahydrofolate; Hcy, homocysteine; Met, methionine; MTHFR, 5,10-methylenetetrahydrofolate reductase; SAH, *S*-adenosylhomocysteine; SAM, *s*-adenosylmethionine; Ser, serine; || Possible enzyme defect involved in cellular Hcy overproduction.

Switzerland). HPLC-grade methanol was from Merck AG (Darmstadt, Germany) and all other chemicals, analytical grade, were from Sigma Chemical Co. The reversed-phase HPLC Novapack C18 column (300 × 3.9 mm), equipped with a micro-guard column (10 × 3.9 mm), packed with the same stationary phase, was obtained from Waters (Milford, Massachusetts, U.S.A.).

Serum and ascitic/cystic fluid samples

Peripheral blood and ascitic fluid were collected simultaneously by abdominal paracentesis from 66 fasting patients who had not been treated with chemotherapy for at least 1 month. The patients, who presented with ascites originating from different causes, were admitted to the Division of Medical Oncology, Centro di Riferimento Oncologico, Aviano, or to the Division of Internal Medicine III, Pordenone Hospital. Cystic fluid from some patients with ovarian carcinoma was also collected by syringe at the time of surgery.

On the basis of the final diagnosis, histologically assessed by previous laparoscopy or surgery biopsy, the patients were divided into two groups. Group A included patients with non-mucinous ovarian carcinoma ($n = 36$; mean age 61 ± 14 years, range 20–78) and group B included patients with non-ovarian pathologies ($n = 30$; mean age 66 ± 22 years, range 49–89). Group B in turn was divided into two subgroups: patients with ascites related to malignant neoplasms (hepatocarcinoma, $n = 6$; colon carcinoma, $n = 4$; breast carcinoma, $n = 3$; gastric carcinoma, $n = 1$; lung carcinoma, $n = 1$; melanoma, $n = 1$; carcinoma of unknown origin, $n = 1$) and patients with ascites related to non-neoplastic diseases (hepatic cirrhosis, $n = 13$).

The procedures of sample collecting were in accordance with the standards of the Ethical Committee of our centre. Samples were collected according to the recommendation of Fiskerstrand and associates [11] in order to improve reproducibility of total thiol determination.

Immediately after clot retraction, coagulated blood and ascitic/cystic fluids were stored on ice. Within 1 h of sampling, they were centrifuged at 3000***g*** for 10 min at 4°C. Serum, ascitic and cystic cell-free supernatants were aliquoted and stored at −80°C until use. For each patient, serum and ascitic/cystic fluids were simultaneously analysed for Hcy, Cys and Cys–Gly concentrations, and total folate and cobalamin concentrations. The seric creatinine concentrations were obtained from routine clinical analysis.

Thiol determination

Total (free plus protein-bound) Cys, Hcy, and Cys–Gly concentrations were determined using the HPLC method described by Araki and Sako [12], with slight modifications. In brief, 100 µl of serum or ascitic fluid were added to 10 µl of 10% tri-*n*-butylphosphine in dimethylformamide and incubated for 30 min at 4°C in order to obtain the reduced form of thiols. The sample solutions were then deproteinised by adding 100 µl of 10% TCA and 1 mM ethylenediaminetetraacetic acid (EDTA) under vigorous vortexing, after centrifugation for 10 min at 4000***g*** at 4°C. For thiol derivatisation, an aliquot of 100 µl of the clear supernatant was mixed with 200 µl of 2.5 M potassium borate buffer, 4 mM EDTA (pH 10.5) and 100 µl of 2.5 µM SBD-F in 2.5 M potassium borate buffer (pH 9.5).

The mixture was incubated for 60 min at 60°C. The solution was then cooled and 50 µl were subjected to HPLC analysis. Chromatographic analysis was performed according to the procedure described by Ubbink and associates [13], with modifications. The SBD derivatives were eluted isocratically from the reversed-phase column by 4% methanol in 0.1 M KH_2PO_4 as a mobile phase (pH 6.0), with a flow rate of 1 ml/min and detected with a fluorescence detector using excitation and emission wavelengths of 385 nm and 515 nm, respectively. Under these conditions, the retention times for Cys, Hcy, and Cys–Gly were 3.4 min, 5.6 min, and 7.7 min, respectively. The lowest limit of concentration detected by this method was 0.5 µM for Hcy and Cys–Gly and 2 µM for Cys, with a response linearity up to 1000 µM. The method was validated for precision and recovery: intra-anol inter-assay coefficients of variation (CV) for Hcy, Cys, and Cys–Gly were less than 3% and the analytical recovery evaluated by spiking a known amount of thiol to control serum or ascites was in the range of 90–100% for each thiol.

Folate and cobalamin determination

Seric, ascitic, and cystic folate and cobalamin concentrations were simultaneously determined using a solid-phase dual count kit radioassay (Diagnostic Products Corporation, Los Angeles, California, U.S.A.). The reference ranges for a healthy population were 7–39 nM for serum folate and 110–650 pM for serum cobalamin, according to the manufacturer.

Statistical analysis

In assessing statistical significance, the non-parametric Mann–Whitney test and the Wilcoxon test were used for unpaired and paired samples, respectively. The Spearman's correlation coefficients (Rs) were calculated to test for the monovariate relationships between two independent variables. The P value was expressed as two-tailed and a P value < 0.05 was considered significant.

RESULTS

Seric, ascitic and cystic folate concentration

The means ± S.D. of serum, ascitic, and cystic folate concentrations for all groups of patients are reported in Table 1. When statistically analysed by the Wilcoxon test, the ascitic folate concentration in group A patients ($n = 36$; mean 10.78 ± 4.48 nM, range 5.45–20.50 nM) was significantly lower than the serum folate concentration ($n = 36$, mean 13.76 ± 5.65 nM, range 2.88–25.28) ($P = 0.005$), whereas the folate concentration in the cystic fluid ($n = 7$; mean 12.98 ± 4.75 nM, range 7.35–20.05 nM) was not significantly different from that in the serum ($n = 7$; mean 15.48 ± 5.45 nM, range 9.23–22.70 nM). Analogously, the mean ascitic folate concentration in patients with non-ovarian pathologies ($n = 30$; mean 8.39 ± 4.91 nM, range 3.28–21.49 nM) was significantly lower than serum folate concentration ($n = 30$; mean 14.40 ± 9.63 nM, range 1.45–37.01 nM) ($P < 0.0005$). Although the ascitic folate concentrations were lower than serum folate concentrations in both groups of patients, such differences were not indicative of a specific folate deficiency in the ascitic compartment of ovarian carcinoma patients. Moreover, the Mann–Whitney test showed that ascitic folate concentrations in ovarian carcinoma patients were significantly higher ($P < 0.05$) than asci-

Table 1. Folate and cobalamin concentrations in serum and ascitic/cystic fluids of patients with ovarian carcinoma and patients with non-ovarian pathology

	Patients (n)	Folate (nM)			Cobalamin (pM)		
Group A (ovarian carcinoma)	36	Serum 13.76 ± 5.65 (2.88–25.28)	Ascitic 10.78 ± 4.48* (5.45–20.50)	P 0.005	Serum 264.05 ± 100.50 (123.06–647.39)	Ascitic 194.92 ± 79.25 (91.46–472.64)	P 0.0001
	7	Serum 15.48 ± 5.45 (9.23–22.70)	Cystic 12.98 ± 4.75 (7.35–20.05)	P NS	Serum 269.38 ± 58.22 (195.29–346.47)	Cystic 208.78 ± 35.89 (166.56–269.16)	P NS
Group B (non-ovarian pathology)	30	Serum 14.40 ± 9.63 (1.45–37)	Ascitic 8.39 ± 4.91 (3.28–21.49)	P <0.0005	Serum 352.89 ± 158.61 (158.61–640.10)	Ascitic 169.16 ± 81.53 (57.72–371.17)	P 0.0001

Significance was evaluated by the Wilcoxon test by comparing ascitic/cystic and serum concentrations. *$P < 0.05$, significant difference from ascitic folate concentration in group B patients by the Mann–Whitney test. The ranges are indicated in parentheses.

tic folate concentrations in patients with non-ovarian pathologies. However, such differences did not appear to be associated with a differential serum folate concentration in the two groups of patients.

Hcy, Cys and Cys–Gly in serum

Serum Hcy, Cys and Cys–Gly concentrations for all groups of patients are reported in Figure 2. The mean ± S.D. of serum Hcy concentration for the patients in groups A and B was 12.52 ± 6.03 μM (range 3.08–32.84 μM) and 15.91 ± 11.29 μM (range 5.02–52.35 μM), respectively. The difference between the two groups was not significant by the Mann–Whitney test. Mean Hcy serum levels for both groups of patients were no different from that observed in healthy subjects of the same age [14].

Ten out of 36 patients with ovarian malignant carcinoma, 5 patients with non-ovarian malignant pathology and 3 patients with hepatic cirrhosis had a serum Hcy concentration greater than 15 μM, which may represent the upper normal limit [15]. It has been reported that high Hcy serum concentrations may be associated with folate and/or cobalamin seric deficiency or altered renal function [15]. Only in 2 patients was such an association shown: a patient with melanoma (Hcy > 35.00 μM) presented a low serum folate concentration (1.5 nM) and a patient with breast carcinoma (Hcy > 40.00 μM) had high serum creatinine (>320 μM). The other patients with serum Hcy over the normal upper limit had serum folate, cobalamin and creatinine levels within the normal range. Mean values of folate and cobalamin concentrations for all groups of patients are reported in Table 1 (data for creatinine are not shown).

The mean serum concentrations of Cys, an amino acid belonging to the catabolic pathway of Hcy (Figure 1), were similar for both groups of patients (mean 241.61 ± 103.51 μM, range 169.00–452.59 μM and mean 281.53 ± 95.30 μM, range 88.77–501.77 μM for group A and group B, respectively). The mean serum concentrations of Cys–Gly, a dipeptide associated with cellular glutathione metabolism but not directly with Hcy metabolism were not significantly different between the two groups of patients (mean 32.98 ± 20.70 μM, range 9.28–136.42 μM and mean 33.98 ± 25.42 μM, range 10.81–148.40 μM for group A and group B, respectively). Moreover, no significant correlation was found between Cys/Cys–Gly and folate and cobalamin serum concentrations for all groups of patients.

Hcy, Cys and Cys–Gly in ascitic and cystic fluids

In ovarian carcinoma patients, the total Hcy concentration in ascitic fluid ($n = 36$; mean 15.64 ± 7.96 μM, range 6.56–45.11 μM) as well as in cystic fluid ($n = 7$; mean 17.92 ± 7.41 μM, range 6.62–27.17 μM) was significantly higher than the corresponding serum level when the data were statistically analysed by the Wilcoxon test ($P < 0.005$ and $P < 0.05$, respectively) (Figure 2(a)). In contrast, in the patients with non-ovarian pathology, the total Hcy ascitic concentration ($n = 30$; mean 12.88 ± 10.87 μM, range 3.42–49.06 μM) was significantly lower ($P < 0.01$) than the corresponding serum concentration (Figure 2(b)). Considering the two subgroups of patients with non-ovarian pathology, the total Hcy ascitic concentration in patients

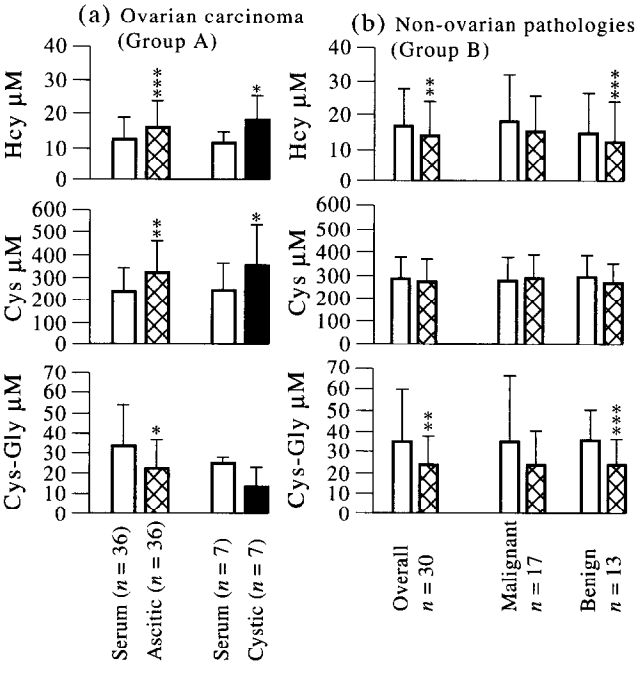

Figure 2. (a) Hcy, Cys and Cys–Gly concentration in serum ($n = 36$; $n = 7$), in ascitic fluid and in cystic fluid of patients with ovarian carcinoma (group A). (b) Hcy, Cys and Cys–Gly concentrations in serum and ascites of all patients with non-ovarian pathology ($n = 30$) (group B), and divided into those with malignant and benign disease. Bars ± S.D. Significance: ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

with malignant diseases ($n = 17$; mean 14.48 ± 10.09 μM, range 3.48–38.08 μM) was not significantly different to the corresponding serum concentration ($n = 17$, mean 17.50 ± 10.72 μM, range 5.11–41.14 μM), whereas the total Hcy ascitic concentration in patients with hepatic cirrhosis ($n = 13$, mean 10.79 ± 11.90 μM, range 3.42–49.06 μM) was significantly lower than the total serum Hcy concentration ($n = 13$; mean 13.83 ± 12.09 μM, range 5.0–52.3 μM) ($P < 0.005$).

26 of 36 patients (72%) with ovarian carcinoma showed a total ascitic/cystic Hcy concentration higher than the corresponding serum concentration, whereas only 4 out of 17 patients (24%) with non-ovarian malignant disease (1 hepatocarcinoma, 1 breast carcinoma, 1 colon carcinoma and 1 melanoma) and none of the patients with hepatic cirrhosis ($n = 13$) had ascitic Hcy concentrations higher than the corresponding serum concentration.

Like Hcy, Cys ascitic accumulation was observed only in patients with ovarian carcinoma. In fact, in these patients, the ascitic and cystic Cys concentrations were significantly higher (ascitic: $n = 36$; mean 321.74 ± 132.50 μM, range 104.15–709.05 μM; $P < 0.01$; cystic: $n = 7$, mean 344.86 ± 187.41 μM, range 29.38–661.16 μM, $P < 0.05$) than the corresponding serum concentration. Moreover, in these patients, ascitic but not cystic Cys accumulation was significantly correlated (Rs = 0.598, $P < 0.001$) with ascitic Hcy accumulation (Figure 3). In contrast, no significant difference was observed between ascitic and serum Cys concentration in patients with non-ovarian carcinoma pathology ($n = 30$; mean 274.44 ± 92.60 μM, range 85.44–534.51 μM), even when the two subgroups of patients were considered separately (Figure 2(b)).

The Cys–Gly ascitic concentration was significantly lower than the corresponding serum concentration in all groups of patients, both in the ovarian carcinoma patients ($n = 36$; mean 22.43 ± 13.63 μM, range 9.01–56.6 μM) ($P < 0.05$) and in non-ovarian carcinoma patients ($n = 30$; mean 22.77 ± 14.93 μM, range 3.24–68.21 μM) ($P < 0.01$). The Cys–Gly cystic concentration in ovarian carcinoma patients was not significantly different from the corresponding serum concentration ($n = 7$; mean 12.87 ± 9.67 μM, range 4.27–28.40 μM).

Relationship between Hcy and folate/cobalamin levels in serum and ascitic/cystic fluids

In ovarian carcinoma patients the serum Hcy concentration showed a significant negative correlation with serum cobalamin concentration (Rs = – 0.371, $P < 0.05$), whereas no significant correlation was observed with serum folate concentration (Rs = – 0.269, $P > 0.1$). In the same patients, ascitic Hcy showed a significant negative correlation with the corresponding ascitic folate concentration (Rs = – 0.578, $P = 0.001$) (Figure 4), but not with the ascitic cobalamin concentration (Rs = – 0.15, $P = >0.1$). No significant correlation was found between cystic Hcy and folate and cobalamin. In the non-ovarian carcinoma patients, no significant correlation was observed between Hcy and folate/cobalamin concentrations both in serum and ascitic fluids (data not shown).

DISCUSSION

FBPα overexpression in normal and tumour cell lines is a physiological cellular response to folate shortage at the extracellular or intracellular levels [6–9]. Our experimental data on folate bioavailability in serum and ascitic/cystic fluids of ovarian carcinoma patients seem to rule out the possibility that these patients have a systemic defect in folate metabolism. These patients, in fact, present serum and ascitic/cystic folate concentrations that are at the lower end of the normal range. Therefore, FBPα overexpression in ovarian carcinoma cells cannot be a consequence of folate shortage in their environment. Alternatively, FBPα overexpression may be consequent to an alteration(s) in the folate metabolism in ovarian carcinoma cells. Since FBPα is the natural ligand of 5-CH_3H_4 folate, Hcy seems to be a suitable marker for intracellular 5-CH_3H_4 folate availability since it derives uniquely from the 5-adenosylmethionine-dependent transmethylation reactions and is physiologically remethylated to Met in a 5-CH_3H_4 folate-dependent reaction catalysed by the 5-CH_3H_4 folate:Hcy methyltransferase (Figure 1). Overproduction of Hcy is, therefore, consequent to a defective Met cycle. Patients with an inborn error(s) at the Met cycle present with elevated levels of serum and urinary Hcy [16–18]. Moreover, plasma Hcy is highly increased in rats [19] and in patients [20] treated with methotrexate, a drug that causes intracellular depletion of

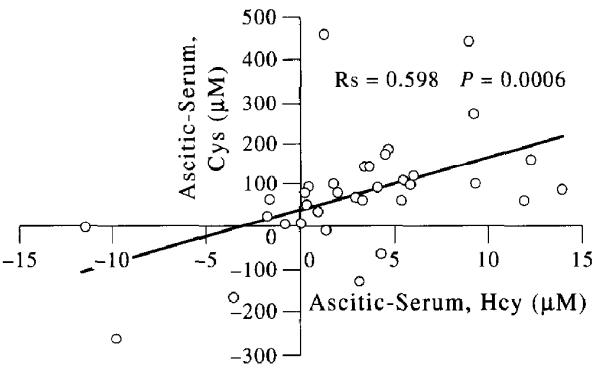

Figure 3. Correlation between Cys and Hcy accumulation in the ascites of patients with ovarian carcinoma. Cys and Hcy accumulation is expressed as the difference between the ascites values and the corresponding serum values. Rs, Spearman's correlation coefficients; *P* = significance.

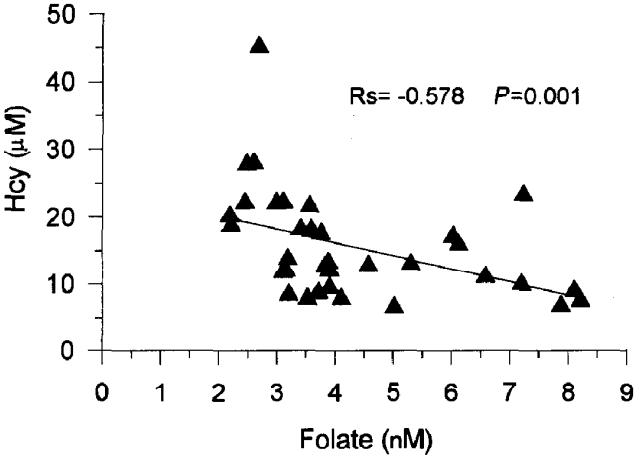

Figure 4. Relationship between Hcy concentration and folate concentration in the ascites of patients with ovarian carcinoma. Rs, Spearman's correlation coefficients; *P* = significance.

the methyl-donor 5-CH_3H_4 folate required for the remethylation of Hcy to Met. To assess a presumed defect in remethylation of Hcy to Met in human ovarian carcinomas *in vivo*, we assumed that higher Hcy concentrations in ascitic/cystic fluids than in serum of ovarian carcinoma patients may be indicative of an elevated source of Hcy in the peritoneal/cystic compartments, where these tumour cells grow and possibly excrete their catabolites. Because the *in vivo* kinetics of Hcy exchange from carcinoma cells to ascitic/cystic fluids and from these fluids to serum could not be determined, the rate of catabolic production of Hcy by ovarian carcinoma cells was not assessed.

In spite of these limitations, our data seem to support the existence of a defective Met cycle in ovarian carcinoma cells *in vivo* and, more precisely, of an ineffective remethylation of Hcy to Met. In fact, Hcy and its derivative compound Cys (Figure 1) were present at significantly higher concentrations in ascitic/cystic fluids than in the serum of ovarian carcinoma patients (Figure 2(a)). In contrast, the concentrations of the same amino thiol compounds were slightly lower in the ascitic fluid than in the serum of patients with non-ovarian pathologies, both malignant and benign (Figure 2(b)). Moreover, Cys–Gly, a thiol compound derived from cellular glutathione metabolism but not related to Met metabolism, was present at higher concentrations in serum than in ascitic/cystic fluids of ovarian carcinoma and control patients (Figure 2). Therefore, the finding of higher Hcy and Cys concentrations in ascitic/cystic fluids than in serum is not a phenomenon merely associated with ascitic formation, but may be consequent to specific local situations existing in the peritoneal and cystic cavities of human ovarian carcinoma patients. In fact, an active source of Hcy and Cys, possibly identifiable with ovarian carcinoma cells, could release these Met catabolites in the surrounding peritoneal fluids.

Because of the lack of neoplastic specimens at the time of ascites and serum harvesting, we were unable to assess whether the ovarian carcinoma cases without Hcy accumulation in the ascitic/cystic fluids (approximately 20% of the cases) had tumours that did not overexpress FBPα, and whether the few cases ($n = 4$) of ascites with Hcy accumulation associated with non-ovarian neoplastic pathologies were tumours overexpressing FBPα. In fact, FBPα overexpression is observed in approximately 80% of ovarian carcinomas [1–3], but is also present in other tumour histotypes, although at a much lower frequency [1, 21]. Therefore, further studies are needed to assess the possible interdependence between FBPα overexpression and Hcy overproduction.

Hcy overproduction may be the result of defective activities of the following cellular enzymes: (1) cystathionine β synthase; (2) 5,10-methylenetetrahydrofolate reductase (MTHFR) and (3) 5-CH_3H_4 folate:Hcy methyltransferase (Figure 1). Defective enzymatic activity of cystathionine β synthase in carcinoma cells can be excluded by the fact that the higher Hcy concentration in ascitic/cystic fluids was concomitant with a higher concentration of Cys, indicating an active conversion of Hcy to Cys. Similarly, defective enzymatic activity of the 5-CH_3H_4 folate:Hcy methyltransferase causes an accumulation of intracellular 5-CH_3H_4 folate and, consequently, a hypoexpression of the FBPα receptor molecules instead of the observed overexpression of the receptor. By contrast, defective enzymatic activity at the MTHFR can explain both FBPα overexpression and Hcy overproduction. In fact, cells with this enzymatic defect can make up for the shortage of the methyl group needed for transmethylation processes. They can do this by increasing 5-CH_3H_4 folate uptake from extracellular fluids by means of FBPα overexpression or by increasing the uptake of exogenous Met, which results in overproduction of the catabolite Hcy. High levels of Hcy may also derive from an increased need for exogenous methionine in order to keep an adequate intracellular pool of *S*-adenosylmethionine, which is needed to maintain the abnormally elevated rates of transmethylation frequently found in cancer cells *in vitro* [22, 23]. However, in the context of this investigation, it is worth noting that a significant inverse relationship exists in ovarian carcinoma patients between the bioavailability of 5-CH_3H_4 folate in ascitic fluid and the level of Hcy accumulation. This suggests that ovarian carcinoma cells may overcome the shortage of intracellular methyl groups, consequent to the defect in 5-CH_3H_4 folate reconversion from 5,10-methylenetetrahydrofolate, by using exogenous 5-CH_3H_4 folate rather than exogenous Met. The existence of a possible biochemical alteration affecting the activity of MTHFR in ovarian carcinoma cells is also supported by our previous results [24], which showed a high frequency (approximately 50% of human ovarian carcinomas) of allelic losses at the MTHFR locus and a concomitant decrease in MTHFR activity in these tumours.

In conclusion, our data seem to support the existence in ovarian carcinoma cells of an altered functionality of the Met cycle that causes increased requirement for exogenous 5-CH_3H_4 folate and Met by tumour cells. These metabolic requirements, which are not present in normal cells, which physiologically derive methyl groups from serine (Figure 1), might be the basis for specific and selective therapeutic treatments of human ovarian carcinomas, as previously suggested for the so-called "methionine-dependent tumours" [25, 26].

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