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The modulation of serine metabolism in hepatoma 3924A during different phases of cellular proliferation in culture

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The activities of 3-phosphoglycerate dehydrogenase and serine hydroxymethyltransferase increased markedly during the transition of hepatoma cells from a resting non-proliferating culture into the proliferating growth phase. Activities declined as cells reached confluency and entered the plateau growth phase. This pattern was paralleled by changes in [^{14}C]serine incorporation into nucleic acids. The experiments support the hypothesis that the biosynthesis of serine is metabolically coupled to its utilization for nucleotide precursor formation in cancer cells.

INTRODUCTION

Serine is the most significant precursor of C_1 tetrahydrofolate cofactors for purine and pyrimidine nucleotide biosynthesis in mammalian cells. Furthermore, the enzyme which initiates this metabolism, serine hydroxymethyltransferase (EC 2.1.2.1), also produces glycine, which serves as both a carbon and a nitrogen source for purine-ring biosynthesis. An increased capacity for serine utilization via serine hydroxymethyltransferase has been demonstrated in proliferating lymphocytes after mitogenic stimulation [1,2], in proliferating fetal tissues [3,4], and in a variety of neoplastic tissues [4,5]. One of the most significant and striking reorientations of metabolism in neoplastic cells is an increased capacity for the biosynthesis *de novo* of purine and pyrimidine nucleotides, which favours the biochemical commitment of the cancer cell to continuous replication [6]. It is suggested that the specific enzymic diversion of serine metabolism into the provision of nucleotide precursors is a further manifestation of the genetic reprogramming that underlies this general biochemical strategy [5,7].

Previous work has suggested that the demands for serine utilization for nucleotide biosynthesis in neoplastic tissues might be metabolically coupled to an increased capacity for the synthesis *de novo* of serine from glycolytic precursors [4,5,7]. Such an integrated enzymic imbalance in serine metabolism should confer a selective growth advantage to cancer cells over normal cells in the tissue in which a tumour originates. In mammalian tissues the route of serine biosynthesis involves a series of phosphorylated intermediates in a pathway initiated by 3-phosphoglycerate dehydrogenase (EC 1.1.1.95) acting on the glycolytic intermediate, 3-phosphoglycerate [8]. 3-Phosphoglycerate dehydrogenase [5,9], as well as the other pathway enzymes, phosphoserine aminotransferase [4] and phosphoserine phosphatase [10], are increased in transplantable rat tumours *in vivo* compared with the normal tissue counterparts.

It should be emphasized that such enzymic changes are stable constitutive events which persist through repeated transplantations of the tumour line *in vivo*, and which can therefore be interpreted as being the result of a 'reprogramming of gene expression' in these cells [6]. Nevertheless there appears to be some plasticity in the metabolic response which results from this reprogramming, such that, if cells from a transplantable tumour are cultured *in vitro*, activities of enzymes and metabolic pathways are modulated in different growth phases of the culture. Thus hepatoma 3924A, a cell line derived from the solid tumour, shows marked increases in enzymes of purine and pyrimidine nucleotide biosynthesis which correlate temporally with increased precursor incorporation by the '*de novo*' pathways and expansion of the intracellular ribonucleoside and deoxyribonucleoside triphosphate pools during the early phase of cell growth in culture [11,12]. The transition of the resting, non-proliferating, cell culture of the rapidly growing hepatoma 3924A into the proliferating phase provides a valuable model system for investigating the sequence of metabolic events that is linked to the expression of neoplastic growth. In the present study this model system was used to elucidate the relation of the activities of the enzymes of serine biosynthesis (3-phosphoglycerate dehydrogenase) and utilization (serine hydroxymethyltransferase) to patterns of nucleotide biosynthesis. Measurements were also made of the incorporation of ^{14}C from [3-^{14}C]serine into intracellular nucleoside bases in the different growth phases of the cell culture. The results lend support to the hypothesis that the biosynthesis of serine in cancer cells may be metabolically coupled to its utilization for increased nucleotide formation.

MATERIALS AND METHODS

Materials

Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and ancillary enzymes were from Boehringer Corp. (Indianapolis, IN, U.S.A.). Cell-culture media and supplements were from Grand Island Biological Co. (Grand Island, NY, U.S.A.), and L-[3-^{14}C]serine (55 mCi/mmol) was obtained from Amersham International Co. (Arlington Heights, IL, U.S.A.).

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Cell culture

The hepatoma 3924A cell line was produced from the solid tumour and was obtained from the Tissue Center of this Laboratory. The cytogenetics, chromosomal karyotype and cell-cycle time have been reported previously [13,14]. The cells were cultured in McCoy's 5A medium supplemented with 10% (w/v) dialysed fetal bovine serum and penicillin (100 units/ml) and streptomycin (100 μg/ml), and grown in 25 cm^2 plastic tissue-culture flasks at 37 °C under a continuously monitored atmosphere of O_2/CO_2 (19:1). Plateau-phase cells were replated in fresh medium at a cell density of about 5×10^5 cells/5 ml and allowed to grow through lag, exponential and, finally, into the plateau phase of growth during a period of 96 h. Cell attachment occurred within 2 h of replating. The growth characteristics of the cultures are shown in Fig. 1(*a*) in terms of cell number. In the exponentially growing phase, cell death was zero and cells showed a generation time of about 14 h. Cell cultures were sampled at 0, 6, 24, 48, 72 and 96 h after plating, and were used for determinations of enzyme activities and radioactivity incorporation into nucleoside bases as detailed below. Cell numbers were determined in a Coulter counter after trypsin-mediated [1 ml of 0.125% trypsin dissolved in Dulbecco's phosphate-buffered saline (Grand Island Biological Co.), pH 7.4] release of the attached cells and resuspension in 5 ml of McCoy's 5A medium supplemented with 10% (w/v) dialysed fetal bovine serum.

[3-^{14}C]Serine incorporation

At the time periods detailed above, individual cell-culture flasks were sampled and the attached monolayer was washed once with 5 ml of phosphate-buffered saline at 37 °C. After aspiration of the washing medium, cells were incubated at 37 °C for 30 min in 1 ml of Eagle's basal medium with Earle's salts, 2 mM-L-glutamine and 25 mM-Hepes (Sigma) containing a final concentration of 0.25 mM-L-[3-^{14}C]serine (3.6 μCi/μmol). The concentration of L-serine used is the same as that originally present in the McCoy's 5A growth medium. At the end of the incubation period, the medium was aspirated and the cells were washed once with 5 ml of ice-cold phosphate-buffered saline. After aspiration of the medium, total nucleic acids were extracted by treating the cells with successive additions of 0.5 ml of 0.2 M-NaOH (to promote detachment) and 0.5 ml of 1 M-$HClO_4$. After sedimenting the cell pellet at 4 °C, it was washed once with 1 ml of 0.4 M-$HClO_4$, resedimented, and the acid-insoluble precipitate was resuspended in 1 ml of 12 M-$HClO_4$. Purine and pyrimidine bases were liberated from the nucleic acids in the acid-insoluble precipitate by hydrolysis for 1 h at 100 °C [15], conditions under which no hydrolysis of precipitated proteins occurs (as assessed by the absence of ninhydrin-positive material). After centrifugation, 0.5 ml of the supernatant was taken into Aquasol (Du Pont NEN Research Products, Boston, MA, U.S.A.) for determination of radioactivity by liquid-scintillation spectrophotometry.

Enzyme assays

Cell extracts were prepared after washing the monolayer of cells in the culture flasks twice, with phosphate-buffered saline. The cell suspension was subjected to three freeze–thaw cycles (using liquid N_2) and the supernatant was obtained by centrifugation for 30 min at 105000 ***g***. Rat liver homogenates prepared in phosphate-buffered saline and subjected to three freeze–thaw cycles showed no loss of enzyme activities. Serine hydroxymethyltransferase was assayed by trapping [^{14}C]formaldehyde in dimedone after incubation with [3-^{14}C]serine [16]. 3-Phosphoglycerate dehydrogenase was assayed in the direction of 3-phosphoglycerate formation by monitoring NADH oxidation spectrophotometrically [5]. Serine dehydratase and serine aminotransferase activities were assayed in an interrupted system, and the pyruvate and hydroxypyruvate formed were estimated enzymically by using lactate dehydrogenase and glyoxylate reductase respectively [17].

RESULTS AND DISCUSSION

Enzymes of serine metabolism during culture of hepatoma 3924A cells

We have previously shown that 3-phosphoglycerate dehydrogenase activity was elevated 75-fold in the solid hepatoma 3924A compared with normal liver [5]. The same tumour retained serine hydroxymethyltransferase (20% of control liver activity), whereas the competing enzymes of serine utilization, serine dehydratase and serine aminotransferase, were absent. This latter enzymic pattern, which determines a redirection of serine metabolism specifically towards the provision of purine and pyrimidine base precursors, was also expressed in the 3924A cell line in culture. At no phase of growth in culture after plating were serine dehydratase and serine aminotransferase activities detectable in cell extracts (with the limits of detection in the assays being 10 and 2 nmol/h per 10^6 cells respectively). Both these enzymes are hormonally inducible in rat liver by glucagon (acting via cyclic AMP), and glucocorticoid hormones show a permissive action in relation to the serine dehydratase induction (for review see [7]). In particular, serine dehydratase and serine aminotransferase are absent from fetal rat liver, but can be precociously induced by glucagon *in vivo* [17,18] and, in the case of serine dehydratase, by a combination of glucocorticoid and glucagon *in vitro* [19,20]. In contrast, in the present work, hepatoma 3924A cells at 72h after plating which were exposed for a further 24 h to glucagon (0.5 μM), dibutyryl cyclic AMP (0.5 mM) or dexamethasone (10 μM), alone or in combination with either of these agents, showed no detectable induction of serine dehydratase or serine aminotransferase. In these experiments, dexamenthasone alone or in combination with glucagon decreased 3-phosphoglycerate dehydrogenase activity to $56 \pm 3\%$ and $62 \pm 4\%$ of control cultures (means $\pm$ S.E.M. for four observations; $P < 0.001$ in both cases). Thus, although neither fetal rat liver nor hepatoma 3924A expresses serine dehydratase and serine aminotransferase, only in the former tissue is it possible to activate the relevant genes hormonally. This suggests that apparent phenotypic resemblances between cancers and fetal tissues [21,22] should be interpreted with caution, and may mask fundamental differences in the regulatory capabilities for gene expression in transformed and non-transformed proliferating cells.

During the different phases of 3924A cell culture, serine hydroxymethyltransferase and 3-phosphoglycerate

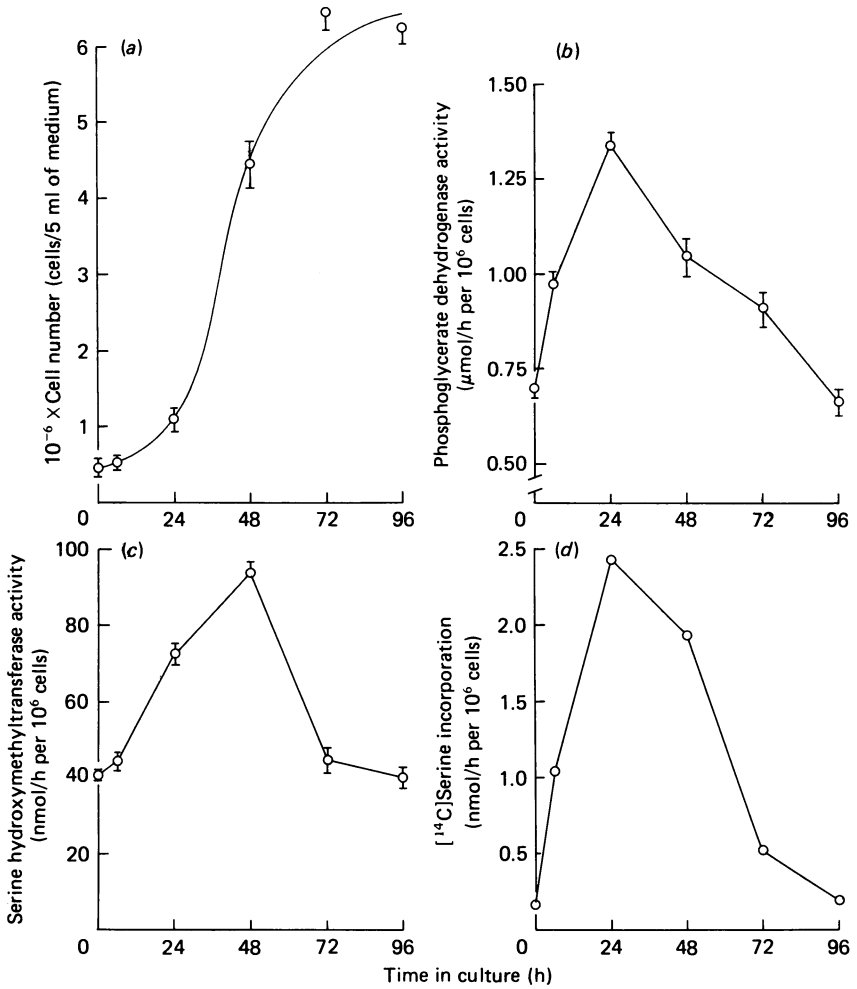

Fig. 1. Effect of growth phase of hepatoma 3924A cells in culture on (*a*) cell number, (*b*) 3-phosphoglycerate dehydrogenase activity, (*c*) serine hydroxymethyltransferase activity and (*d*) incorporation of radioactivity from [3-^{14}C]serine into nucleotide bases of total cellular nucleic acids

For the measurement of radioactivity incorporation, cells were sampled at the times indicated and incubated for 30 min with 0.25 mM-[3-^{14}C]serine (3.6 μCi/μmol) before isolation and hydrolysis of total cellular nucleic acids. Further details of the culture methods and the biochemical assays are given in the Materials and methods section. Enzyme activities are expressed as μmol or nmol/h per 10^6 cells; points represent the means (bars show S.E.M.) of three or more separate experiments. Radioactivity incorporation is the average of triplicate measurements from a single experiment and is expressed as nmol/30 min per 10^6 cells.

dehydrogenase activities increased rapidly to reach peaks at 24 h during the early exponential phase, and then declined as the cells reached the plateau phase of growth (Fig. 1). This temporal pattern is similar to that reported previously for enzymes involved in purine and pyrimidine biosynthesis and for intracellular ribonucleoside and deoxyribonucleoside triphosphate pools [11]. The rise in 3-phosphoglycerate dehydrogenase activity was significant at 6 h ($P < 0.001$), at which time there was no significant increase in cell number (Fig. 1*a*, cf. Fig. 1*b*). The increase in serine hydroxymethyltransferase did not reach statistical significance until 24 h, but this again preceded the exponential phase of cell growth (Fig. 1*a*, cf. Fig. 1*c*). Several other enzymes of pyrimidine biosynthesis show a delay of 6–12 h after cell plating before increased activity becomes evident [11]. Perhaps more significantly, the concentration of the intracellular dTTP pool shows a delayed rise of 6–12 h after cell plating and remains elevated until 48 h [11], in the same pattern as that shown here for serine hydroxymethyltransferase (Fig. 1*c*). In this connection it should be noted that serine hydroxymethyltransferase generates methylenetetrahydrofolate, which is the direct carbon donor for thymidylate synthesis. Previous work has shown that serine hydroxymethyltransferase activity increases in mitogenically stimulated human lymphocytes in culture before significant changes in cell number have occurred [1,2].

A relationship between the increased activity of 3-phosphoglycerate dehydrogenase (or the activity of any other enzymes of serine biosynthesis) and the transition from non-proliferating to proliferating cell growth phases in culture has not been reported. However, we have previously noted that 3-phosphoglycerate dehydrogenase activity is high in normal tissues of high cell-renewal capacity and markedly elevated in neoplastic tissues [5]. The temporal association between serine hydroxymethyltransferase and 3-phosphoglycerate dehydrogenase activities in 3924A cells at different phases of proliferation in culture

suggests that these cells have the capacity to increase serine synthesis and to channel serine preferentially into purine and pyrimidine biosynthesis. The provision of glycolytic intermediates for serine synthesis from glucose should also be enhanced, since glycolytic enzymes have been shown to be increased in exponential-phase cells compared with resting 3924A cells in culture [223].

Incorporation from [3-^{14}C]serine into nucleic acids during culture of hepatoma 3924A cells

Radioactivity incorporation from [3-^{14}C]serine into purine and pyrimidine bases hydrolysed from total cellular nucleic acids was measured at various times after plating of 3924A cells (Fig. 1*d*). Incorporation increased markedly so that at 6 h (the earliest time point investigated) the value was 5–6-fold greater than in resting cells, at a time when there was no increase in cell number. Incorporation peaked at 24 h after plating, and thereafter declined to 276% and 100% of resting cell values at 72 and 96 h. This pattern is similar to that observed for enzymes of the pathways of nucleotide biosynthesis and for intracellular nucleotide pools [11]. The pattern of incorporation from [3-^{14}C]serine (Fig. 1*d*) is also similar to that observed for serine hydroxymethyltransferase activity (Fig. 1*c*). Incorporation of radioactivity from [3-^{14}C]serine via serine hydroxymethyltransferase will result from label present in the methylenetetrahydrofolate product, but not glycine, which will directly label the pyrimidine nucleotide, thymidylate. However, purine nucleotides will also be labelled through the conversion of labelled methylenetetrahydrofolate into methenyl- and 10-formyl-tetrahydrofolate, which donate carbon for purine-ring biosynthesis. In the present study no attempt was made to distinguish between pyrimidine or purine biosynthesis from [3-^{14}C]serine. However, in other experiments it has been shown that formate (at 1–100 mM) effectively dilutes out the incorporation of [3-^{14}C]serine into purine bases, indicating that incorporation from [3-^{14}C]serine includes transfer of carbon via 10-formyltetrahydrofolate as an intermediate (Y. Natsumeda, unpublished work). In mitogenically stimulated human lymphocytes, it has also been shown that labelling of the purine nucleotide pools from [3-^{14}C]serine is substantial [24]. The apportionment of serine into purine and pyrimidine nucleotide biosynthesis in the hepatoma cells requires further investigation.

General conclusions

The present results show that serine metabolism at different phases of hepatoma-cell growth in culture contributes to the general biochemical strategy which determines the commitment to cell proliferation of these cancer cells. This commitment, and the underlying biochemical determinants, are already established in the genetic programme exhibited by solid hepatomas (including hepatoma 3924A) and other tumours. However, it is clear from the present and earlier [6,11–14,23] studies that, within limits, the relevant biochemical pathways can be modulated upwards to provide an increased cellular proliferation rate during the growth of hepatoma 3924A cells (and other cancer cell types; see [23]) in culture.

The present work confirms the pattern of serine metabolism established previously for cancer cells [4,5,7], namely that serine is preferentially directed towards the provision of nucleotide precursors through an enzymic imbalance (compared with normal tissues) in which serine hydroxymethyltransferase activity is retained or increased, whereas alternative enzymes of serine utilization, i.e. serine dehydratase and serine aminotransferase, are deleted. In this study, the supposition that the measured activity of serine hydroxymethyltransferase is indicative of metabolic flux from serine to nucleotide precursors has been validated by measuring the incorporation of radioactivity from [^{14}C]serine. Finally, evidence has been presented to support the hypothesis [7] that in cancer cells the utilization of serine for nucleotide precursor formation is coupled to an enhanced capacity for serine synthesis *de novo*. The key enzyme of serine biosynthesis, 3-phosphoglycerate dehydrogenase, showed parallel changes in activity during different growth phases to the utilization of serine, via serine hydroxymethyltransferase, for nucleotide synthesis. It appears that there is a co-ordinated regulation of gene expression in cancer cells which ensures that a metabolic coupling of these pathways of serine metabolism is established to confer a growth advantage on these cells.

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