Deoxycytidylate shortage is a cause of G1 arrest of ascites tumor cells under oxygen deficiency

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1. INTRODUCTION

In normal and malignant animal cells oxygen deficiency not only severely curtails mitochondrial energy supply, but also interrupts all O_2-dependent catabolic and anabolic processes; i.e., degradation of purines, polyamines and aromatic amino acids, and biosynthesis of cholesterol, unsaturated fatty acids, prostaglandines, tyrosine, hydroxyproline, porphyrine and uridine. We have demonstrated the importance of dihydroorotate oxidase (EC 1.3.3.1), an enzyme of UMP biosynthesis intimately connected to the respiratory chain, for growth cessation of anaerobically cultured Ehrlich ascites tumor (EAT) cells [1]. Such conditions result in accumulation of cells in the G1 phase of the cell cycle [2,3]. However, the observation that exogenously supplied uridine cannot at all relieve the anaerobic G1 arrest of EAT cells points to the possible existence of additional, oxygen-dependent reactions in nucleic acid synthesis. It has indeed become known that the main enzyme of deoxyribonucleotide biosynthesis, ribonucleoside diphosphate reductase (EC 1.17.4.1) requires aerobic conditions for generation of its active-site tyrosyl radical [4]. The study of characteristic changes in cell cycle distribution of proliferating cells after exclusion of oxygen was therefore extended to an analysis of intracellular deoxyribonucleotides, using EAT cells enriched in G1 phase by centrifugal elutriation. We now show that anaerobic arrest of mammalian cells at the G1/S border is specifically neutralized by addition of deoxycytidine, establishing a new type of oxygen involvement in DNA replication.

2. MATERIALS AND METHODS

All chemicals, buffers, culture media, and enzymes were of highest purity available and obtained from: Boehringer, Mannheim; Merck, Darmstadt; Serva, Heidelberg; and Sigma, München, respectively. Radioactive nucleotides came from Amersham-Buchler, Braunschweig. Horse serum was a gift from Behringwerke AG, Marburg, and microcillin from Bayer AG, Wuppertal.

Hyperdiploid EAT cells, strain Karzel, serially grown in female NMRI mice were made to grow in suspension culture using Eagle's medium containing 15% horse serum, 20 mM Hepes, microcillin (575 mg/l), and streptomycin (30 mg/l) [5]. Anaerobic culture conditions were achieved by continuous flushing with argon/CO_2 (95:5) which passed a heated oxysorb catalyst (R3-11M/3610, BASF, Ludwigshafen) and was then humidified by bubbling through a water column at 37°C. Absence of oxygen was controlled with a Clark electrode. Cell growth was routinely determined by enumeration in a hemocytometer, and as increase

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of protein content of the cultures [1]. Viability was checked by the nigrosin exclusion test or by phase-contrast microscopy. Proliferation activities were monitored by flow cytometry in an ICP-11 (Phywe, Göttingen) using the fluorescence from ethidiumbromide-stained DNA for discrimination of cells in the different cell cycle compartments [3,6]. Fractionation of asynchronous cells after a first in vitro passage was performed in Hanks' solution containing 15% horse serum, using a JE-6 elutriator rotor with standard chamber (Beckman, München) at 1600 rev./min constant rotor speed and a flow rate of elutriation medium increasing from 10–25 ml/min [7]. To obtain sufficient numbers of cells for parallel cultures a rather broad fraction of small cells (about 80% G1 + 20% early S phase) was collected, transferred into the second in vitro passage (2.5×10^7 cells/50 ml) and cultured under aerobic, or anaerobic conditions with or without addition of nucleosides as described below.

For analysis of deoxyribonucleotide pools, $\sim 2 \times 10^7$ cells were separated from the culture medium by centrifugation (2 min at $500 \times g$) and washed with Hanks' solution. They were then immediately suspended in 0.5 ml ice-cold H_2O, followed by addition of 2.5 ml prechilled (−20°C) 72% methanol; the samples were stored for 20 h at −20°C, centrifuged, and the clear supernatant was lyophilized. dATP, dTTP, or dCTP concentrations in the redissolved extract were determined by the enzymatic method [8,9], using 0.1 unit of *Escherichia coli* DNA polymerase, 1 μCi [*methyl*-^{3}H]dTTP, [8-^{3}H]dATP or [8-^{3}H]dGTP, respectively, and 1.5 nmol poly[d(A–T)] or poly[d(G–C)] as templates. The assays were run in duplicate, and standards with known amounts of dNTP were included in each series.

Preparation of a ribonucleotide reductase fraction from Ehrlich ascites cells and enzyme assays were done under the conditions in [10], combined with determination of deoxyribonucleotide product in our standardized liquid chromatography system [11]. Assay mixtures contained, in a 0.15 ml total vol. 0.1 M Na-phosphate buffer (pH 7.0), 3.7 μM (10 μCi) [5-^{3}H]cytidine diphosphate (spec. act. 21 Ci/mmol), 6 mM dithiothreitol, 2 mM ATP, 4 mM Mg-acetate, and 1–1.2 mg protein; incubation was for 45 min at 37°C. Protein was determined by the Lowry method.

3. RESULTS

DNA histograms of EAT cells, obtained by flow cytometry, are presented in fig.1. The first, left-hand histogram shows a typical cell cycle distribution after 15 h in the first in vitro passage, with the first peak representing G1 phase cells with 2c DNA and the second peak those with 4c DNA; i.e., G2 + M phase cells being indistinguishable from each other by their identical DNA content. S phase cells appear between the two peaks. After centrifugal elutriation the G2 and most of S cells are eliminated (second histogram). The third series of histograms, line B, demonstrates that lack of oxygen retards EAT cells in progressing out of the G1 compartment as compared to aerobic controls which pass S and G2 phase to enter cell division (line A). It is unknown why the G1 peaks of anaerobic cells broaden somewhat to higher channels but it is reasonable to assume that a few cells are able to utilize compounds for proliferation that are available in the culture medium from disintegrated cells or from the serum. Addition of 0.1 mM uridine, cytidine, or thymidine from the start of O_2-free cultures does not alter the cell cycle distribution shown in line B. In contrast, supplementation of cell cultures with 0.1 mM 2′-deoxycytidine specifically enables anaerobic EAT cells to move from G1 into S and to onset DNA synthesis (line C of histograms). Cell division was not detectable under these conditions. Obviously reprogression of cells in presence of deoxycytidine remains mostly limited to the S phase; further progression into G2 is difficult to assess from the histograms because of the overloading with cells in the S phase compartment.

Deoxyribonucleoside triphosphates were determined in Ehrlich ascites cells from aerobic, anaerobic, and anaerobic, deoxycytidine-stimulated cultures (table 1). The data represent 5 separate experiments, each set of cultures starting from one population of elutriated cells. dNTP pools in aerobic control cells varied up to 3-fold in the individual experiments but were all in the order of magnitude reported for EAT [12] and other proliferating mammalian cells [9,13,14]. In anaerobic cells the pyrimidine deoxyribonucleotides, in particular dCTP, were found reduced to 1/3 or 1/4 of the content of control cells whereas the dATP pools remained unchanged. Supplementation of

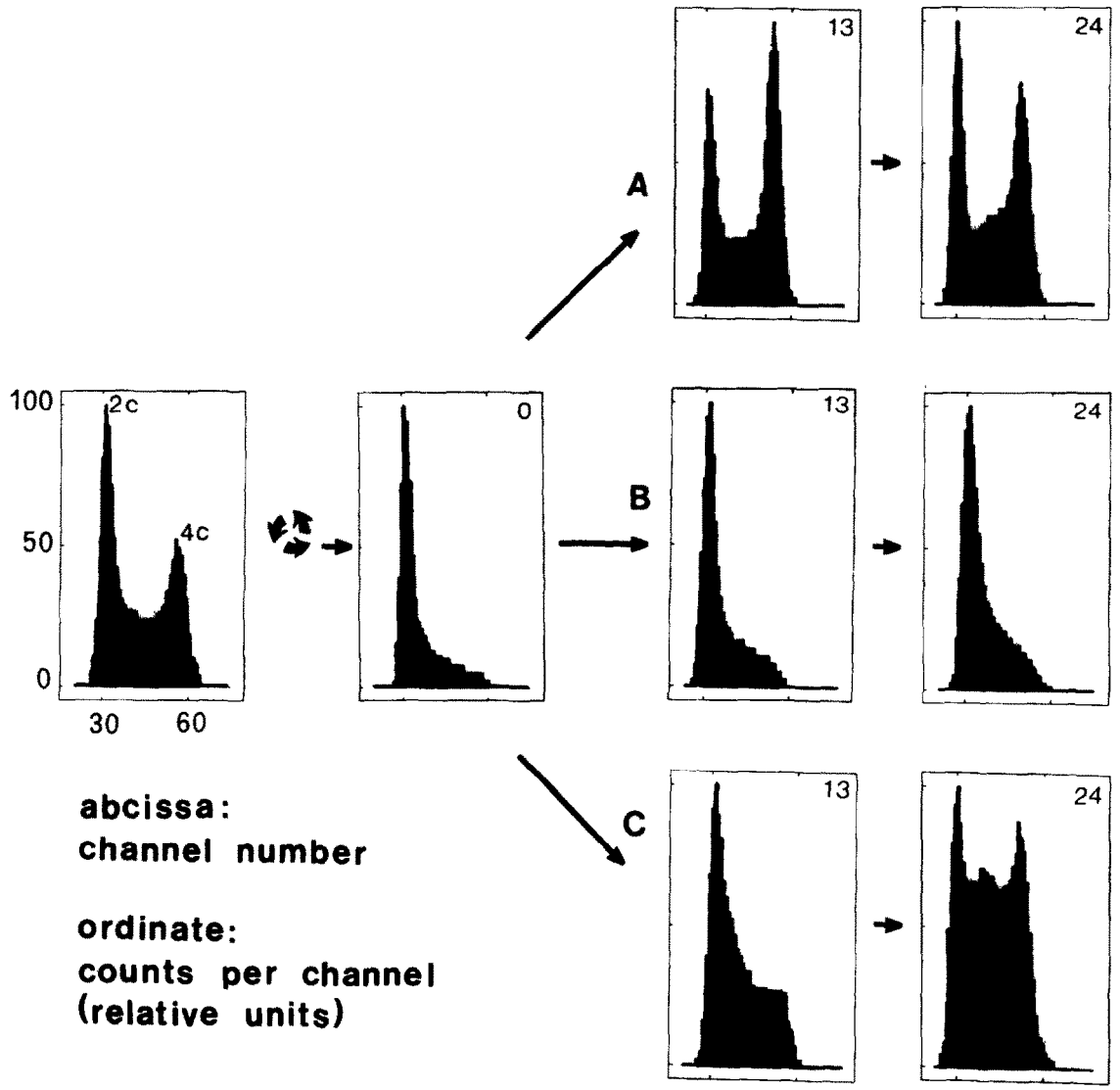

Fig.1. DNA histograms of Ehrlich ascites tumor cells measured by flow cytometry. Each histogram represents the typical cell distribution of one sample of asynchronous cells (left), G1 cells at 0 h after centrifugal elutriation (center), and of aerobic (A), anaerobic (B), and anaerobic, deoxycytidine-supplemented cultures (C) after 13 h or 24 h, respectively, as described in the text.

anaerobic cultures with deoxycytidine caused the dCTP concentration to triple and become almost normal, and led to an overproduction of dTTP; again, the dATP pool did not change. We have verified that these effects were not influenced by nucleosides or other low-M_r components of horse serum; the results were the same when the serum was dialyzed prior to use. From the correlation of DNA histograms and dNTP pools it is clear indeed that intracellular deoxycytidylate depletion is a specific cause for G1 arrest of the tumor cells in anaerobiosis.

Deoxycytidylate can only be synthesized by reduction of cytidylate. We have made an attempt to correlate the dCTP content of EAT cells with their ribonucleotide reductase activity after 10 h anaerobiosis. No significant difference was found in the amount of CDP reduction catalyzed by ex-

Table 1

Deoxyribonucleotide pools in Ehrlich ascites tumor cells after 8 h of in vitro culture

Culture conditions	dCTP	dTTP	dATP
Aerobic[a]	100%	100%	100%
Anaerobic	⩽25	⩽50	100
Anaerobic + 0.1 mM deoxycytidine	75	300	100

[a] 100% corresponds to 80–150 pmol dCTP/10^6 cells, 30–50 pmol dTTP/10^6 cells, and 100–200 pmol dATP/10^6 cells

tracts from anaerobic or from control cells (0.3 nmol CDP.h^{-1}.mg protein^{-1}). This result does not rule out differential enzyme activities in vivo. Oxygen-dependent generation of a tyrosyl free radical in the catalytic subunit of eukaryotic ribonucleotide reductase is a fast process [4] and could have occurred in the anaerobic sample although cell harvesting, work-up, and enzyme assays were performed under nitrogen; complete absence of oxygen was not guaranteed during the enzyme preparation.

4. DISCUSSION

From the determination of cell cycle distribution by flow cytometry and other growth parameters it could be concluded in previous work [1–3] that after establishing O_2-free culture conditions EAT cells still exit from G2, complete mitosis, and accumulate in G1, presumably at a point near the G1/S border. G1 phase cells did not enter S phase. A simple interpretation of these observations is complicated by the multitudinous effects of oxygen deficiency on cellular metabolism mentioned in section 1. It appears certain from these data showing a specific involvement of deoxycytidylate, and from the determination of virtually unchanged adenylate energy charge [15] that the block in cell cycle progression is not a consequence of impaired respiration in anaerobic ascites cells, which possess high glycolytic capacity. Switch-off of the respiratory chain does incapacitate dihydroorotate oxidase and leads to a lack of pyrimidine nucleotides. This defect could not be remedied by addition of 0.1 mM uridine or cytidine to anaerobic cultures of asynchronous or G1 cells, whereas the nucleosides were in fact capable of overcoming the cell growth limitation by a dihydroorotate oxidase inhibitor, dihydro-5-azaorotic acid [1]. Exogenous thymidine was found ineffective both in presence of the inhibitor or in absence of oxygen because it cannot replenish any other pyrimidine nucleotide pool necessary for RNA or for DNA synthesis.

The unexpected ability of deoxycytidine to establish cell cycle progression is explained by the depleted deoxycytidylate pool under oxygen deficiency (table 1). Exogenously supplied deoxycytidine raises the intracellular dCTP and, via deamination and thymidylate synthesis, dTTP concentrations. Moreover, its pyrimidine base, via salvage reactions, could also enter RNA precursor pools while UMP biosynthesis remains blocked.

Lack of oxygen for maintaining fully active, free radical-saturated ribonucleotide reductase species is the most likely explanation for the reduced amount of deoxycytidylate in EAT cells after prolonged anaerobiosis, although in vitro enzyme assays failed to demonstrate that point for the above reasons. The question then arises why dATP pools are unchanged under oxygen deficiency, since bacterial, or calf thymus ribonucleotide reductase is known to reduce both pyrimidine and purine ribonucleotides alike. However, during characterization of Ehrlich ascites cell ribonucleotide reductase it has been noted that CDP and ADP reduction may not be catalyzed by the same enzyme species but by proteins differing in their subunit composition [10,16]. It is not too unreasonable an assumption that radical stability, and hence modulation of enzyme activity by oxygen, are also somewhat different in these individual entities, resulting in decreased (but not zero) CDP reduction, and ADP reduction continuing like normal.

However its quantity is varied, deoxycytidylate appears the most critical deoxyribonucleotide for DNA replication; it not only serves as substrate but may provide the threshold concentration for initiation of replication [17,18]. This role is compatible with our observation of strong reduction of intracellular dCTP as well as with [19] that the initiation of new replicating units is suppressed under anaerobic conditions. On the other hand, deoxycytidylate shortage, while materially limiting DNA synthesis in G1 and S phase cells is probably

only one of several regulatory restraints as cell cycle progression remains far from normal in deoxycytidine-stimulated cells where DNA precursors are available and DNA is being produced (fig.1). Additional mechanisms may contribute to cell cycle control [15] independent of deoxyribonucleotide supply.

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