On the importance of deoxyribonucleotide pools in the senescence of cultured human diploid fibroblasts

John E. Dick and Jim A. Wright

Departments of Microbiology and Biochemistry, University of Manitoba, and Manitoba Institute of Cell Biology, 100 Olivia Street, Winnipeg, Manitoba R3E OV9, Canada

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Previous studies have indicated that ribonucleotide reductase may participate in a mechanism of cellular senescence, which involves modifications in deoxyribonucleotide pools, the products of the reductase reaction. Since very little information about the levels of these pools in senescing cells is currently available, an analysis of deoxyribonucleotide levels was carried out in young, old and very old normal human diploid fibroblasts, as well as in a variant human fibroblast strain with an altered replicative life span. These studies indicated that there are marked age-related perturbations in these pools which imply that they may be fundamentally important in a process determining the nonproliferative or senescent state.

Deoxyribonucleotide *Ribonucleotide reductase* *Human fibroblast* *Senescence*

1. INTRODUCTION

An important step in the regulation of DNA synthesis in mammalian cells occurs at the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates; this reaction is carried out by ribonucleotide reductase, a complex and rate-limiting activity which appears to play an important role in the regulation of cell division and proliferation [1,2]. Alterations affecting this reaction influence a variety of cellular processes [1]. We have observed that there are significant changes in ribonucleotide reductase levels during the senescence of cultured human diploid fibroblasts [3] which imply an important role for the enzyme in a mechanism of cellular senescence. In support of this point, hydroxyurea, whose site of action is ribonucleotide reductase, was used as a selective agent to isolate variant human fibroblast strains with altered enzyme activity and reduced replicative life spans [4]. It is possible that alterations in ribonucleotide reductase may influence the aging process through modifications in deoxyribonucleotide pools, the eventual products of the reductase reaction. Very little information about deoxyribonucleotides in senescing cells is available. Therefore, to examine this important question in detail, deoxyribonucleotide pools in young, old, very old, and variant human fibroblasts were measured and compared. These studies indicate that there are significant age-related perturbations in these pools suggesting that their levels may be critically important in the establishment of the nonproliferative or senescent state.

2. MATERIALS AND METHODS

2.1. *Cells and growth conditions*

The fetal lung fibroblast strains, HSC172, kindly provided by Dr Buchwald, University of Toronto, was derived from a 12-week-old female fetus [5] and undergoes 95 ± 5 population doublings in culture [4,6]. In agreement with other studies (e.g., [5]), we have observed that the karyotype of these cells is normal 46,XX. The WC1-2 strain is a drug-resistant variant selected from HSC172 and exhibits a reduced lifespan of 76 ± 2 doublings in culture [4]. Cells were routinely maintained on 100-mm plastic tissue culture plates (Lux Scien-

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tific) in α-minimal essential medium (α-MEM; Flow Laboratories) supplemented with penicillin (100 units/ml), streptomycin sulfate (68 μg/ml) and 15% fetal bovine serum (Gibco) preselected for high plating efficiency characteristics. The cells were subcultured according to a rigid protocol using 1:4 split ratio, counting two mean population doublings each time [4]. Purified trypsin (Sigma) at 0.1% in phosphate-buffered saline (PBS) was used to remove cells from the surface of plates.

2.2. *Cell volume measurements*

Cell volumes were determined by optical measurement of cell diameters directly [7] and with a Coulter electronic particle counter [8]. In agreement with other studies [8,9] very similar results (within 5%) were obtained with the two techniques.

2.3. *Determination of nucleotide pools*

The levels of intracellular deoxyribonucleotide pools in wild-type and drug-resistant cells were measured by high-performance liquid chromatography (HPLC) using a modification of the procedures in [10,11]. We have described this procedure [4,12,13]. Briefly, cells were plated at a density of 2×10^6/150 mm tissue culture plate and grown for about 40 h. Cells were harvested and extractions were made at 4°C. The cells were removed with trypsin (0.2%), counted, and centrifuged in as short a time as possible. The cell pellets were resuspended at 5×10^7 cells/ml in PBS and a small aliquot of [^{3}H]thymidine was added to estimate the dilution factor. This resuspension was then transferred to glass test tubes with sufficient 70% perchloric acid to achieve a final concentration of 0.5 M, and the tubes were placed on ice for 30 min. Diethyl pyrocarbamate (100 μl/ml) and 2.2% tri-*N*-octylamine in Freon (2 vols to 1 vol. extract) were added to the extract and vortex-mixed for 2 min. The tubes were then centrifuged at low speed for 2 min and the supernatant was collected and immediately frozen at −65°C.

The separation and identification of the deoxyribonucleotides first required the destruction of ribonucleotides with periodate and methylamine and the extracts were separated by HPLC on an anion-exchange resin. A Beckman model 332 gradient liquid chromatograph consisting of two model 110A pumps coordinated by a model 420 system controller programmer was used. The nucleotides were detected with a Beckman model 160 detector at 254 nm, with maximum sensitivity of 0.001 AUFS (absorbance units full scale). A Partisil 10/25 SAX (Whatman) strong anion-exchange column (25 cm × 4.6 mm) preceded by a guard column (7.0 cm × 2.0 mm) was used in the separation process. Good resolution of the 4 deoxyribonucleotides was achieved using isocratic elution with 0.25 M KH_2PO_4 (Fisher, HPLC grade) and 0.25 M KCl (Fisher, ACS grade), pH 5.0 at a flow rate of 2 ml/min. All buffers were filtered through a 0.45 μm membrane filter and degassed prior to use. Identification was by retention times as compared to known standards. The peaks were quantitated on the basis of peak weight.

3. RESULTS AND DISCUSSION

The deoxyribonucleotide pools were determined in young (passage no.29), old (passage no.86), and very old (passage no.92) fibroblast cultures, as well as in variant human diploid fibroblasts which contain altered ribonucleotide reductase activity. The results of these studies are summarized in tables 1 and 2. The properties of the variant fibroblasts have been described [4]. In brief, 3 hydroxyurea-resistant clones were isolated and found to contain elevated CDP reductase activity, and a 20–40% reduction in replicative abilities, when compared to normal wild-type fibroblasts. The clone designated WC1-2 is one of these variant strains, and was examined after approx. 70 passages in culture, which is about 6 passage numbers from senescence for this strain [4]. Therefore, WC1-2 is situated between the old and very old cell cultures,

Table 1

Deoxyribonucleoside triphosphate pools (pmol/5 × 10^6 cells)

Cell strain	TTP	dCTP	dATP	dGTP
HSC172-29	468 ± 71	107 ± 15	140 ± 21	40 ± 12
HSC172-86	388 ± 92	89 ± 7	235 ± 32	31 ± 11
HSC172-92	248 ± 10	45 ± 10	180 ± 20	23 ± 15
WC1-2	282 ± 20	95 ± 5	223 ± 8	13 ± 5

The values are the averages of 3 determinations

Table 2

Deoxyribonucleoside triphosphate pools (taking cell volume into consideration)

Cell strain	TTP (μM)	dCTP (μM)	dATP (μM)	dGTP (μM)
HSC172-29[a]	27.8	6.3	8.3	2.4
HSC172-86[b]	7.9	1.8	4.8	0.8
HSC172-92[c]	3.1	0.6	2.2	0.3
WC1-2[d]	4.8	1.6	3.8	0.2

[a] Cell volume: 3.38 with an SD of 1.6
[b] Cell volume: 9.80 with an SD of 5.6
[c] Cell volume: 16.25 with an SD of 13.5
[d] Cell volume: 11.68 with an SD of 7.1

The values are the averages of 3 determinations

which are about 10 and 3 passage numbers, respectively, from senescence [4,6].

Data on deoxyribonucleotide pools can be expressed in two ways; either as pmol/cell number (as shown in table 1) or as μM (as shown in table 2). The latter measurement takes into account the large differences in cell volume which occur during senescence [7].

It is evident that important changes occur in deoxyribonucleotides as cells age. When the data were analyzed (table 1) with respect to cell number (5×10^6 cells), TTP declined by less than 20% between passages 29 and 86, and decreased by 40–50% in very old passage 92 cells and the variant WC1-2 fibroblasts. The dCTP pool declined by 17% of low passage fibroblasts in passage 86 and by 58% in passage 92 cells. Deoxy GTP levels were reduced by 22% in passage 86 cells, by 42% in passage 92 fibroblasts, and by 67% in WC1-2 cells. No reductions in dATP pools were observed when examined as pmol/cell number; instead, this pool was 1.7-fold higher in passage 86 cells, 1.3-fold higher in passage 92 cells, and 1.6-fold higher in WC1-2 cells when compared to low passage human fibroblasts.

When cell volume was taken into consideration, the pattern changed somewhat (table 2). In this case, TTP, dCTP and dGTP pools declined markedly to about 30% in old (passage 86) cells; about 10% in very old (passage 92) cells, and to 17, 25, and 8%, respectively, in WC1-2 variant cells. The dATP concentration varied between about 60% in old cells and 25% in very old fibroblasts. A general pattern emerged from these studies, in which old cells contained higher concentrations of all 4 deoxyribonucleotides than WC1-2 cells (intermediate between old and very old), and except for dGTP, the WC1-2 cells contained higher levels of deoxyribonucleotides than very old fibroblasts.

From these studies it is evident that in general, the deoxyribonucleotide pools are regulated in the same manner in aging and drug-resistant fibroblasts which contain altered ribonucleotide reductase; however, the aging process was accelerated in the variant cells [4]. Although very little is known about the biological effects generated by modifications in deoxyribonucleotide pools, it has been suggested that one or more of these compounds may be involved in a special way in triggering the synthesis of DNA [14], and it is known that perturbations in deoxyribonucleotide pools have mutagenic effects [15,16], and may modify differentiation capabilities of mammalian cells [12]. These changes in deoxyribonucleotide concentrations observed for the first time in this study (tables 1 and 2), may be fundamentally related to the finite proliferative abilities of cultured human diploid fibroblasts. When one or more of these specialized compounds of DNA synthesis decline below a critical threshold level required for DNA synthesis, the cells would lose their proliferative characteristics. For example, the control of cell cycle progression which is closely related to the process of senescence, has been described in terms of stochastic or random transition models [17–19]. In these models, the initiation of DNA synthesis is governed by the probability that a small number of initiation factors will rise above a critical threshold concentration, and the synthesis of DNA will begin. If these factors are not elevated above this threshold, the probability of cell cycle progression remains very low. The results of this study are in keeping with the hypothesis that the deoxyribonucleotides belong to this select group of initiators of DNA synthesis, and participate in a fundamental way, in a mechanism of cellular senescence.

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