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Coordinate changes of polyamine metabolism regulatory proteins during the cell cycle of normal human dermal fibroblasts

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Abstract In human dermal fibroblasts, brought to quiescence (G_0) by serum starvation, the S phase peaked 24 h and G_2/M phases 36 h after serum re-addition. Under the same conditions, ornithine decarboxylase mRNA peaked at 12 h, decreased markedly in S phase and remained low until 48 h. Conversely, ornithine decarboxylase antizyme transcript dropped to its lowest level at 12 h, while reaching its highest values between 24 and 48 h. Ornithine decarboxylase activity followed essentially the pattern of its mRNA, but relative changes were much greater. *S*-Adenosylmethionine decarboxylase transcript and enzyme activity also peaked at around 12 h, decreasing thereafter. Spermidine/spermine N^1-acetyltransferase mRNA and activity reached the highest values at 36–48 h. Putrescine concentration increased up to 18 h and fell dramatically in the S phase, remaining low thereafter. Both spermidine and spermine reached peaks at 18 h and decreased in the S phase, but not nearly as much as putrescine. We discuss how this comprehensive study may help to understand the involvement of polyamines in the control of cell proliferation.

Key words: Ornithine decarboxylase; Ornithine decarboxylase antizyme; *S*-Adenosylmethionine decarboxylase; Spermidine/spermine N^1-acetyltransferase; Polyamine; Cell cycle

1. Introduction

The aliphatic polyamines, spermidine (SPD) and spermine (SPM), and the diamine putrescine (PUT), are natural compounds ubiquitously present, in their polycationic form, in both eukaryotic and prokaryotic cells. Many data suggest that they may have different functions in cell physiology, however it is their obligatory participation in normal or pathological cell growth and cell differentiation that has been unequivocally demonstrated [1,2]. Both their depletion and overaccumulation may be lethal to the cell, thus a complex and intricate regulatory system has evolved for the rigorous control of their intracellular concentrations [2,3].

The biosynthetic pathway of polyamine metabolism starts with the decarboxylation of L-ornithine by ornithine decarboxylase (ODC) (EC 4.1.1.17), the first and rate-limiting step, leading to formation of the diamine PUT. *S*-Adenosylmethionine decarboxylase (AdoMetDC) (EC 4.1.1.50) is the second enzyme, also a regulatory one, catalyzing the production of decarboxylated adenosylmethionine. Transfer of an aminopropyl moiety from the latter to PUT, by SPD synthase, results in the production of SPD. Transfer of a second aminopropyl group from the same source to SPD, by SPM synthase, produces SPM. The degradative route of polyamine metabolism is peculiar in that it transforms polyamines back into their precursors (retroconversion pathway). In fact, spermidine/spermine N^1-acetyltransferase (SSAT) produces acetylspermine which is oxidized to SPD by constitutive polyamine oxidase (PAO). In turn, SPD can be acetylated by SSAT and the acetylspermidine produced can be either excreted or oxidized to PUT by PAO. PUT can be excreted or oxidized by diamine oxidase. SSAT is the regulatory enzyme of this pathway and is induced by increased intracellular polyamine concentrations [4]. The regulatory mechanisms of both the biosynthetic and degradative routes are mostly based on changes of regulatory protein concentrations, which are modulated at the transcriptional and translational level and/or through changes of mRNA and enzyme protein half-lives [1,2]. A unique mechanism plays a crucial role in the regulation of ODC activity, which is based on the 26.5 kDa protein ornithine decarboxylase antizyme (OAZ). This non-enzymatic protein is induced by polyamine overaccumulation; OAZ binds to ODC, inactivates it and accelerates its degradation through the 26 kDa cytosolic proteasome system [5].

Although several specific aspects of polyamine metabolism have been investigated in relation to the different phases of the cell cycle in various cell types [6–8], no study has been reported in which the known intracellular regulatory mechanisms of polyamine metabolism are evaluated together and in relation to the intracellular polyamine levels in normal human cells. We considered such an investigation to be a prerequisite for a conclusive definition of the role played by these amines in the control of cell cycle progression.

Thus, the levels of the transcripts encoding ODC, AdoMetDC, SSAT and OAZ, the enzymatic activities of the first three proteins, and the intracellular content of PUT, SPD and SPM were measured during the cell cycle progression of normal human dermal fibroblasts in primary culture. Cells were brought to quiescence by serum starvation, stimulated by se-

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Abbreviations: ODC, ornithine decarboxylase; OAZ, ornithine decarboxylase antizyme; AdoMetDC, *S*-adenosylmethionine decarboxylase; SSAT, spermidine/spermine N^1-acetyltransferase; PUT, putrescine; SPD, spermidine; SPM, spermine; PAO, polyamine oxidase; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle medium; BrdU, 5′-bromo-2′-deoxyuridine; PI, propidium iodide; RT-PCR, reverse-transcription polymerase chain reaction; H-gas1, human growth arrest specific protein 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

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rum re-administration and studied during their first synchronized round of cell replication.

2. Materials and methods

2.1. Cell cultures and flow cytometry analysis

1.2×10^6 dermal fibroblasts isolated from skin biopsies of healthy subjects were plated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics in 75 cm^2 flasks [9]. To study the proliferative response, confluent cultures were serum-starved for 14 days in DMEM containing 1% FCS and stimulated by the addition of fresh medium supplemented with 20% FCS. The proliferation stages of the cells at different times during cell cycle progression were assessed by two-parameter flow cytometry analysis. This was performed after dual staining: with propidium iodide (PI) (red fluorescence) for total DNA content, and with anti-5′-bromo-2′-deoxyuridine (BrdU)/FITC (green fluorescence) after BrdU incorporation into the newly synthesized cellular DNA, for the specific labelling of cells in the S phase. In the latter instance, 10.0 μM BrdU (from Sigma-Aldrich, Italy) was added to the cultures at the indicated times, followed by incubation at 37°C for 30 min; cells were then harvested by trypsinization and washed twice in 1×PBS, and the DNA was denatured by incubation with 2.0 N HCl/Triton X-100 for 30 min at room temperature to produce single-strand molecules. After neutralization with 0.1 M $Na_2B_4O_7$ pH 8.5, followed by resuspension in 0.5% Tween 20 plus 1.0% BSA in 1×PBS, 10 μl of anti-BrdU/FITC (Caltag Laboratories, Burlingame, CA, USA) per 1×10^6 cells were added, and incubated for 30 min at room temperature. After a further washing in Tween/BSA/PBS, the cells were stained for DNA content with 5.0 μg/ml of PI and immediately analyzed by flow cytometry.

2.2. Northern hybridization

At the times indicated, total cellular RNA was extracted from cell cultures grown under the above conditions using RNAfast (Molecular Systems, San Diego, CA, USA). Ten μg aliquots were electrophoresed in a 1% agarose-formaldehyde gel, blotted onto Hybond-N nylon membranes (Amersham Italia) and then hybridized to the specific ODC or GAPDH cDNA probes, which were purified and labelled by random priming [^{32}P]dCTP incorporation as previously described [10]. For the detection of the SSAT mRNA, a 643 bp cDNA fragment, containing the entire coding region for SSAT, was excised from the plasmid vector pBluescript II KS [11] by digestion with *Hin*dIII and purified from an agarose gel by using the Jetquick Gel Extraction Kit (Genomed GmbH, Germany). The human growth arrest specific protein 1 (h-gas1) cDNA, kindly donated by Dr G. Del Sal [12], and the histone H3 full-length cDNA, kindly donated by Dr G. Stein [13], were purified, labelled and used as specific probes following the same procedures.

The full-length cDNAs encoding human AdoMetDC and human OAZ were obtained by reverse-transcription polymerase chain reaction (RT-PCR) from total RNA extracted from normal human dermal fibroblasts. For the amplification of the AdoMetDC cDNA, the following primers were used: GGC GGC AGC GGC GGG AGA AGA GGT TGA ATT CAG TTG ATT T (forward primer); CAC TGC CCC CAG CAT CGA CAT CTA GAA AGA ATT CAC CAC C (reverse primer). For the amplification of the OAZ cDNA, the following primers were used: GGA GGT TTT CCT GAA TTC GGA CCC CAG CGG (forward primer); TGG CCC CAG GGG CCG AAT TCC CAG CCC CGA (reverse primer). The amplification products obtained were then purified and cloned in pGEM 7z. The identity of the products was confirmed by restriction analysis and sequencing. The full length cDNAs were then excised from the amplification vectors, purified from an agarose gel by using the Jetquick Gel Extraction Kit and used directly as radioactive probes after random priming [^{32}P]dCTP incorporation. Quantitation of the autoradiograms was obtained by densitometric scanning (LKB Ultrascan XL densitometer).

2.3. Enzyme assays

ODC and AdoMetDC activities were detected by measuring the release of $^{14}CO_2$ from [1-^{14}C]ornithine and *S*-adenosyl-L-[carboxy-^{14}C]methionine, respectively, by enzyme extracts [14,15]. SSAT activity was determined by assessing the label incorporation from [^{14}C]acetyl-CoA into monoacetyl spermidine [15].

2.4. Polyamine determination

The method used for the determination of intracellular polyamine levels was as previously described [16]. Briefly: at the time indicated, 1×10^6 cells were homogenized in perchloric acid and then centrifuged at $8000\times g$ for 15 min. Aliquots of the supernatant were then neutralized and dansyl chloride was added. The dansyl derivatives were then extracted with benzene and separated by high performance liquid chromatography [17].

2.5. DNA and protein determination

The protein concentrations were determined by using the Bio-Rad Protein Assay kit from Bio-Rad Laboratories (Hercules, CA, USA). The DNA assay was performed according to Labarca and Paigen [18].

3. Results

At 2 and 3 days after plating, 14 days after serum starvation (0 h) and at various time intervals after serum re-addition (from 1 h to 48 h), the percentages of total cell populations in G_0/G_1, S and G_2/M phases were detected by two-parameter flow cytometry analysis (Fig. 1). The highest percentage of cells in S phase was found 24 h after serum addition, while the G_2/M phase peaked at 36 h. In parallel with the flow cytometry analysis, the timing of the cell cycle phases was confirmed by assessing the abundance of the transcripts coding for h-gas1, a marker of the G_0 phase [12], histone H3, a marker of the S phase [13] and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping enzyme (Figs. 2 and 3a). As expected, the h-gas1 mRNA increased markedly on the third day after plating (cells at confluence), continued to rise up to 1 h after serum addition, decreased rapidly until 6 h, then started to increase again slowly; at 48 h, when cells returned to confluence following proliferation, it returned to a level as high as on the third day after plating. The concentration of the transcript coding for H3 dropped dramatically in cells at confluence on the third day after plating, remained undetectable until 12 h after serum re-addition, reached its highest value at 24 h and returned to a very low level at 48 h. GAPDH mRNA exhibited a marked decrease at G_0, in-

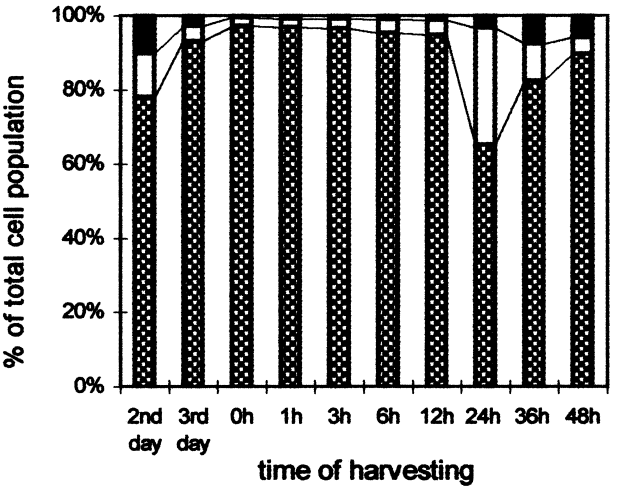

Fig. 1. Representative histogram, obtained by two-parameter flow cytometry analysis, showing the relative distribution (percentage) of normal human dermal fibroblasts in the different phases of the cell cycle. Cells were harvested on the second and the third day (cells at confluence) after plating, 14 days after serum starvation (quiescence state, 0 h) and during the first synchronized round of cell replication, at the indicated times after serum re-addition. Hatched bars: G_0/G_1 phases; white bars: S phase; black bars: G_2/M phases.

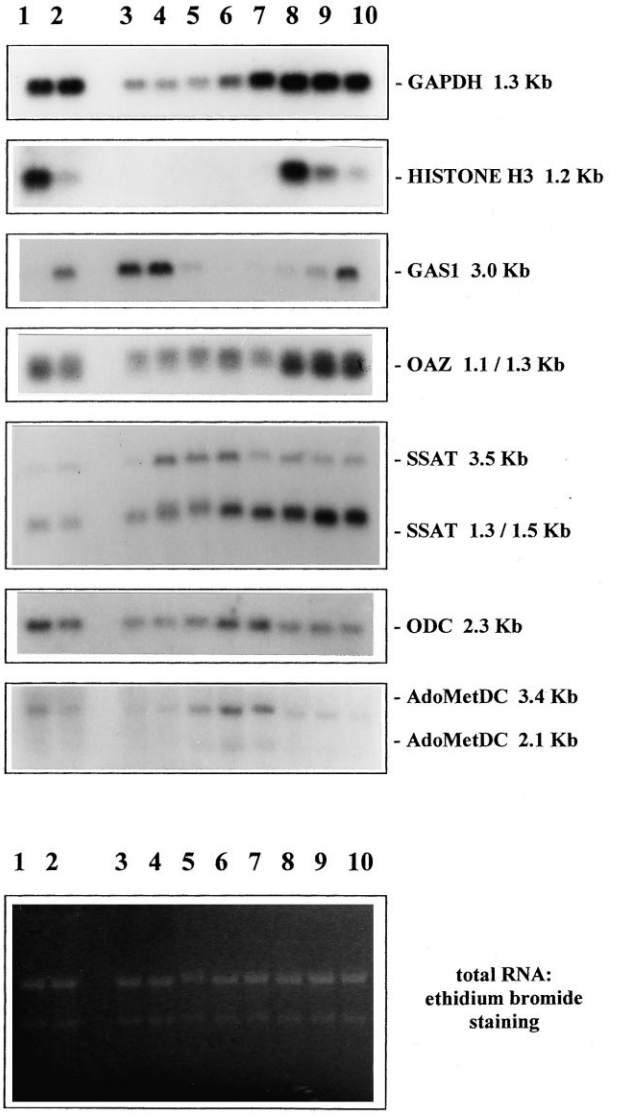

Fig. 2. Representative Northern blot experiments on total RNA extracted from cells harvested as follows: lane 1, second day after plating; lane 2, third day after plating (cells at confluence); lane 3, 14 days after serum starvation (quiescent state); lanes 4–10, 1 h, 3 h, 6 h, 12 h, 24 h, 36 h and 48 h, respectively, after serum stimulation. The specific radioactive probes used for hybridization and the size of the specific mRNAs detected are indicated. The ethidium bromide staining of the same RNA specimens, separated on agarose gel, is also shown for comparison (10 μg of total RNA was loaded on each lane).

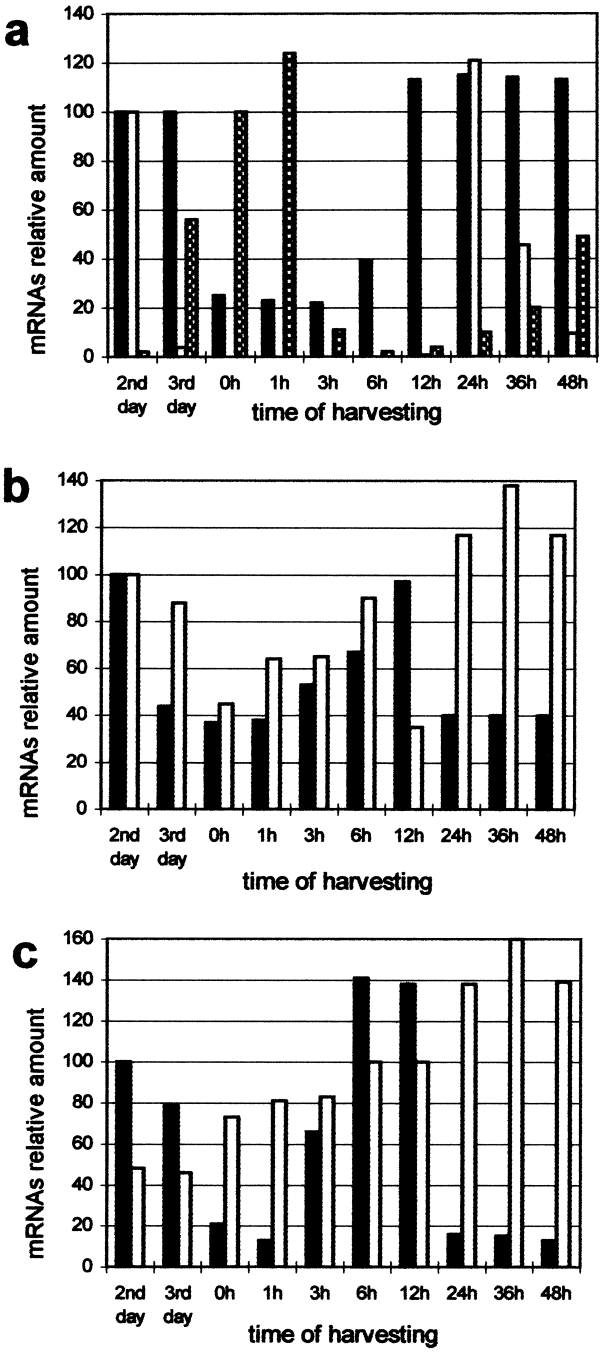

Fig. 3. Quantitation, by densitometric scanning, of the autoradiograms shown in Fig. 2. *Y*-axis: relative optical density in arbitrary units. a: Histone H3 mRNA (white bars); GAPDH mRNA (black bars); h-gas1 mRNA (hatched bars). For H3 and GAPDH data have been normalized by setting at 100% the values obtained from cells harvested on the second day after plating; for h-gas1 normalization was against levels from cells harvested after induction of the quiescent state (0 h). b: OAZ mRNA (white bars); ODC mRNA (black bars): data have been normalized with the values obtained from cells harvested on the second day after plating. c: SSAT mRNA (white bars); AdoMetDC mRNA (black bars): for AdoMetDC data have been normalized with the value obtained on the second day after plating; for SSAT from quiescent cells. The data shown are representative of four determinations obtained in separate sets of experiments.

creased gradually up to 12 h and remained constant afterwards. This pattern was parallel to that exhibited by the total RNA recovered from cell cultures under the same conditions (data not shown), suggesting that the changes in GAPDH mRNA accumulation are the consequence of the expected changes in the general transcriptional activity of the cells.

Variations in the abundance of the transcripts coding for the three regulatory enzymes of polyamine metabolism plus OAZ are shown in Fig. 3b,c. The concentration of ODC transcript diminished in fibroblasts at confluence (third day after plating); it decreased further in quiescent cells, reached the highest level in response to serum stimulation at 12 h, dropped back to the level of G_0 at 24 h (S phase) and remained constant thereafter. The mRNA coding for OAZ de-

creased in the fibroblasts progressing from the second day after plating to quiescence. It increased upon serum supplementation, reaching a peak at 6 h; it dropped dramatically at 12 h, when ODC mRNA and activity (Fig. 4a) were still high, and increased steeply to 24 h (in correspondence to the fall in ODC mRNA and activity, Fig. 4a), maintaining this high level at subsequent time points. The transcript coding for AdoMetDC decreased progressively while cells proceeded through the second day after plating to G_0 phase (Fig. 3c); it started to increase 3 h after serum addition, reaching the highest levels at 6 h and 12 h. A dramatic decrease occurred when cells progressed to S phase (24 h) and this low value was maintained through the remaining time points. Accumulation of SSAT mRNA exhibited no changes between 2 and 3 days after plating; it increased from G_0 phase onward, showing the highest levels from 24 h to 48 h.

The activities of the three regulatory enzymes of polyamine metabolism during the cell cycle of normal human dermal fibroblasts are shown in Fig. 4a. ODC activity was very low on the second and third day after plating and became almost undetectable in quiescent cells. A marked enhancement of this

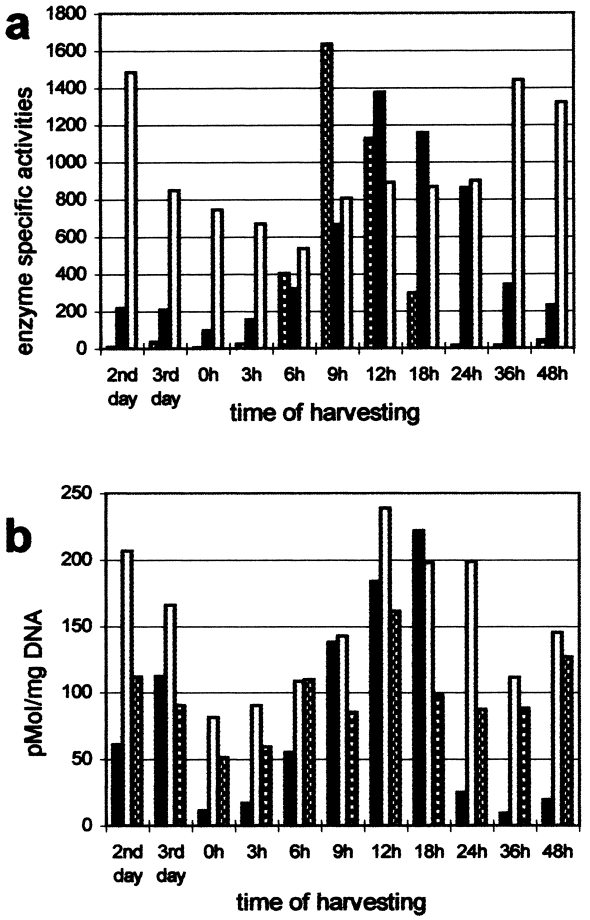

Fig. 4. a: Activities of the regulatory enzymes of polyamine metabolism. b: Polyamine content in normal human dermal fibroblasts harvested at the indicated times. a: *Y*-axis: pmol $^{14}CO_2$/h/mg protein for ODC (hatched bars) and for AdoMetDC (black bars); pmol of N^1-acetylspermidine formed/h/mg protein for SSAT (white bars). b: PUT (black bars); SPD (white bars); SPM (hatched bars). Data are expressed as pmol of polyamine/mg of DNA. The data shown are representative of three determinations obtained in separate sets of experiments.

activity occurred upon serum stimulation, the highest value being attained 9 h later; at 12 h the activity started to decrease, becoming again almost undetectable from 24 h up to 48 h. AdoMetDC activity did not change significantly between the second and third day after plating; it was halved in quiescent cells and, similarly to ODC, started to increase upon serum re-addition, reaching a peak at 12 h, and decreased gradually thereafter. Note that increases and decreases in AdoMetDC activity at different phases during the cell cycle proceeded gradually without abrupt changes, in contrast to ODC (see ODC activity variations at 3–6 h, 12–18 h, 18–24 h). SSAT activity decreased markedly on the third day after plating and continued to diminish slowly until 6 h after serum addition; it remained approximately at the level of G_0 phase from 9 h to 24 h and increased at 36–48 h.

As far as polyamine concentrations are concerned (Fig. 4b), the diamine PUT decreased rapidly upon serum starvation. Re-addition of serum resulted in a progressive increase in the concentration of PUT that reached a maximum at 18 h; at 24 h (S phase) the concentration of the diamine fell by almost 90% and remained that low thereafter. SPD concentration exhibited a gradual decrease while cells progressed to G_0 phase. Serum re-addition was followed by a steady increase in SPD culminating at 12 h. At 24 h (S phase), a small diminution occurred, that became larger at later times. SPM concentration exhibited the same changes shown by SPD concentration, forming a peak at 12 h. Note that during growth broadly corresponding to G_1/early S phases of the cell cycle (9–12–18 h), the concentrations of PUT overtook those of SPM, while the latter was always less than SPD.

4. Discussion

Changes in aliphatic polyamine concentrations and in the activities of their regulatory enzymes are known to take place during the cell cycle. However, these changes have not been found to be uniform across the various cell types studied [6,7,19–23]; additionally, alterations in specific polyamine concentrations may not correlate with changes in the activities of their biosynthetic enzymes [7]. Polyamine depletion results in cell cycle phase perturbations, but the mechanism by which these polycations exert their action is unknown [8,19,24–26]. We show here that in normal human cells the levels of the polyamines SPD and SPM, and especially of the diamine PUT, undergo considerable fluctuations during the cell cycle. All three amines increase progressively from the G_0 phase, reaching a peak when cells are mostly in late G_1/early S (12–18 h) phase (Fig. 4b). At mid S phase (24 h) SPD and SPM exhibit a modest decrease, but PUT drops dramatically. The modest changes in the two higher polyamines may result from the fact that most of the intracellular SPD and SPM molecules are bound to intracellular structures and do not essentially change during the life of the cell. The polyamine component undergoing rapid concentration changes comprises the unbound fraction, which represents a minor portion of the entire intracellular pool. Since the two fractions cannot be measured separately, the changes in free SPD and SPM concentrations tend to be masked by the stability of the bound component and thus are probably underestimated under our experimental conditions. It is conceivable however, that in specific cellular compartments, polyamine levels reach concentrations similar to those that have been demonstrated

to affect DNA and chromatin structures [27], which has been proposed as a possible mechanism for the effect of these polycations on cell growth.

The timing of polyamine peaks (late G_1, Fig. 4b) strongly suggests that they are required for the cell to traverse the restriction point at which cells become committed to enter the S phase and complete the cell cycle. The pattern of PUT concentrations, characterized by a dramatic and almost linear increase from G_0 to late G_1, ending with a sudden drop at 24 h, suggests that this diamine may have a direct effect on the mechanisms that drive the cell through the restriction point.

Variations in the polyamine concentrations correlate fairly well with changes in the activities of their biosynthetic enzymes (Fig. 4a), while the relationship with the alterations in SSAT activity is less clear. The patterns of accumulation of the transcripts coding for these enzymes (Fig. 3b,c), in turn, correlate qualitatively but not quantitatively with the patterns of enzyme activities (Fig. 4a). This is unsurprising since it is well known that transcriptional and post-transcriptional modifications cooperate in the regulation of these enzymes. A pivotal role in regulating polyamine levels in cycling human fibroblasts appears to be played by the ODC regulating protein OAZ. In fact, the level of OAZ mRNA, after increasing in parallel with the ODC transcript between G_0 and 6 h (Fig. 3b), drops markedly at 12 h, when the ODC mRNA reaches its highest level, and increases again to maximal values from mid S phase (24 h) onward, when the ODC transcript is at its lowest concentration. Similar results were obtained by others when detecting the OAZ activity in Ehrlich ascites tumor cells [28]. This suggests that when growing fibroblasts approach the restriction point at late G_1, unknown factors may come into play triggering temporarily the repression of the OAZ gene and the simultaneous induction of the ODC gene, leading to the peaks of polyamine accumulation (Fig. 4b) that may be required for the cell to pass the restriction point. This situation would be rapidly reversed as cells enter the S phase, with repression of the ODC gene and simultaneous induction of the OAZ gene (Fig. 3b). The almost complete disappearance of ODC activity at 24 h (Fig. 4a) would thus result from the converging effects of ODC gene repression and ODC enzyme inhibition/degradation by OAZ.

The dramatic fall in PUT concentration occurring at 24 h is probably due not only to ODC inactivation, but also to the fact that AdoMetDC activity decreases at a rate much lower than that of ODC (Fig. 4a), thus maintaining the availability of the aminopropyl group donor (decarboxylated adenosylmethionine). The latter favours conversion of PUT to SPD and SPM, which indeed decrease much less than PUT at this time point (Fig. 4b).

The simultaneous detection of all the parameters controlling the steady state of polyamine metabolism reveals an integrated pattern of finely coordinated changes, which besides suggesting a central involvement of these compounds in the process of mitosis, could allow us to assess more precisely the stage of cell cycle progression in normal, and possibly malignant, human cells.

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