Manipulation of the expression of regulatory genes of polyamine metabolism results in specific alterations of the cell-cycle progression

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INTRODUCTION

The aliphatic polyamines putrescine (PUT), spermidine (SPD) and spermine (SPM) are necessary for normal and pathological cell growth and differentiation [1]. In the prostate gland these polyamines are present at high levels, and their metabolism is positively controlled by androgens [2–4]. In particular, all the regulatory proteins of polyamine metabolism are expressed in the epithelial cells of the gland ([3,4] and S. Bettuzzi, G. Guidetti and A. Corti, unpublished work). Increases in the levels of these polyamines are generally associated with cell proliferation and cell transformation induced by growth factors, carcinogens, viruses or oncogenes [5]. The level of expression of all the regulatory genes of polyamine metabolism is significantly higher in the neoplastic tissue than in the benign counterpart from the same human prostate specimen [6]. Polyamine metabolism is finely regulated by complex mechanisms that allow timely adjustments of their intracellular concentrations, as required by the changing cellular needs, and by the detrimental effects of both their overaccumulation [7,8] and depletion [9,10] within the cells.

Decarboxylation of L-ornithine by regulatory ornithine decarboxylase (ODC; EC 4.1.1.17) initiates the polyamine biosynthetic pathway. ODC is typically induced by growth-promoting factors; in the prostate gland it responds to the trophic actions of androgens [3]. ODC was shown to be critical in cell transformation, and thus suggested to be a proto-oncogene [11]. It undergoes a complex regulation, mostly based on the induction of a unique, non-enzymatic, regulatory protein named ornithine decarboxylase antizyme (OAZ) [12], which is induced by polyamine overaccumulation, binds and inhibits ODC activity, and accelerates ODC protein degradation by the 26 S proteasome system [13]. OAZ also prevents polyamine uptake from the extracellular compartment by inhibiting the specific polyamine transport system [14]. Recently, also OAZ was shown to play a pivotal role in the acquisition of the transformed phenotype in vitro and in vivo [15].

Increased levels of intracellular polyamines also lead to the induction of spermidine/spermine N1-acetyltransferase (SSAT; EC 2.3.1.57) [16], the regulatory enzyme of the polyamine degradative pathway (the ‘retroconversion’ pathway), which results in the excretion of polyamines in the forms of acetyl-SPD or PUT or degradation of PUT by diamine oxidase [16].

We have shown recently that all the regulatory proteins of polyamine metabolism undergo coordinate changes during cell-cycle progression in synchronized normal human fibroblasts, with ODC and OAZ overexpression occurring in the early S phase and OAZ down-regulation in the mid-S phase, followed by OAZ and SSAT overexpression in the G2/M phases [17]. This co-ordinate pattern of expression leads to cyclical phases of depletion and accumulation of the intracellular polyamines during the different phases of the cell cycle [17]. Since these polycations can effectively interact in vitro with DNA and cause specific modifications of chromatin structure [18–20], polyamines were hypothesized to affect both DNA synthesis and the chromatin folding/unfolding processes [21].

Strategies based on the use of inhibitors of polyamine biosynthesis for controlling pathological cell proliferation have been...

We have previously reported that cyclical phases of accumulation and depletion of polyamines occur during cell-cycle progression. Regulatory ornithine decarboxylase (ODC) catalyses the first step of polyamine biosynthesis. Ornithine decarboxylase antizyme (OAZ), induced by high polyamine levels, inhibits ODC activity and prevents extracellular polyamine uptake. Spermidine/spermine N1-acetyltransferase (SSAT) regulates the polyamine degradation/excretion pathway. Here we show that 24 h transient transfection of immortalized human prostatic epithelial cells (PNT1A and PNT2) with antisense ODC RNA or OAZ cDNA, or both, while effectively causing marked decreases of ODC activity and polyamine (especially putrescine) concentrations, resulted in accumulation of cells in the S phase of the cell cycle. Transfection with SSAT cDNA led to more pronounced decreases in spermidine and spermine levels and resulted in accumulation of cells in the G2/M phases. Transfection with all three constructs together produced maximal depletion of all polyamines, accompanied by accumulation of PNT1A cells in the S phase and PNT2 cells in the G2/G1 and G2/M phases. Accumulation of PNT1A cells in the S phase progressively increased at 15, 18 and 24 h of transfection with antisense ODC and/or OAZ cDNA. At 24 h, the DNA content was always reduced, as a possible outcome of altered chromosome condensation. A direct link between polyamine metabolism, cell proliferation and chromatin structure is thus proposed.

Key words: antisense, gene expression, prostate, transient transfection.
tried repeatedly, but they were frustrated in vivo by the remarkably efficient means by which cells maintain a strict homeostatic control over the intracellular content of these polyamines. These consist of prompt changes in: (i) the rate of biosynthesis; (ii) the rate of degradation/excretion of intracellular polyamines; and (iii) the rate of extracellular polyamine uptake [1,22].

PNT1A and PNT2 are two cell lines derived from human prostate epithelial cells immortalized with simian virus 40 (SV40) [23]. PNT1A cells are less differentiated than PNT2 ones. In the present work, by performing a series of transient transfections with different mammalian expression vector constructs, we caused specific alterations in the expression of the genes coding for polyamine regulatory proteins, leading to correspondent modifications in the intracellular contents of these amines. The possible alterations caused by the latter changes in cell-cycle progression were then investigated by cytofluorometric measurements.

EXPERIMENTAL

Cell lines

SV40-immortalized human prostate epithelial cell lines (PNT2 and PNT1A) were established and characterized previously [23,24]. PNT2 and PNT1A cells are derived from normal human epithelial prostate cells. PNT2 cells are more differentiated than PNT1A with respect to morphological features, the expression profile of cytoeratins and doubling time [23,24]. Cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% foetal bovine serum (FBS) and 1% l-glutamine, without antibiotics, in 75 cm² flasks (Nunc, Roskilde, Denmark) and incubated at 37°C in a 5% CO₂ atmosphere. Cell harvesting was performed routinely by trypsin/EDTA treatment.

Constructs

Three different plasmid constructs, all based on the same mammalian expression vector pOP-SVI-MCS containing the strong universal Rous sarcoma virus (RSV) promoter (Stratagene, La Jolla, CA, U.S.A.), were designed and cloned. A 304 bp cDNA fragment, sharing perfect homology with the region containing the first AUG codon (start site) of the human ODC mRNA, was ligated in the inverse orientation with respect to the promoter, in pOP/a.s.ODC, in order to generate an antisense RNA capable of specifically inhibiting human ODC mRNA translation (a.s.ODC). A full-length cDNA fragment coding for human OAZ was previously cloned by reverse transcriptase PCR and characterized by DNA sequencing [17], and then inserted into pOP-SVI-MCS to produce the pOP/OAZ expression vector. The full-length cDNA fragment coding for human SSAT, as described previously [17], was cloned in pOP-SVI-MCS to originate the pOP/SSAT expression vector.

Single-plasmid transfections

High-quality, endotoxin-free plasmid preparations were obtained routinely using the Jetstar Plasmid Maxi Kit (Genomed, GmbB, Bad Oeynhausen, Germany). Cells were transfected in 35 mm dishes (Nunc), in the presence of growth medium containing 10% FBS, using Fugene 6™ Transfection Reagent (Roche Diagnostics Corporation). The efficiency of transfection was assessed by transfecting cells with a CMV-EGFP mammalian expression vector and detecting, by laser confocal microscopy, the percentage of positively transfected cells, which were expressing enhanced green fluorescent protein (EGFP) under the control of the cytomegalovirus (CMV) promoter. Experiments were performed by adding the same amount of each plasmid to be tested, on an equimolar basis, to each cell culture for the time indicated. Controls were made by adding, under the same experimental conditions, the appropriate amount of the empty vector pOP-SVI-MCS alone to the cell cultures.

Co-transfections

Equimolar mixtures of two or three expression plasmids, with a total amount of plasmid DNA equal to that used for single-plasmid transfections, were added to cell cultures grown in 35 mm dishes, under the above transfection conditions.

Flow-cytometry analyses

The proliferation stages of the cells during cell-cycle progression were assessed by two-parameter FACS analysis, performed by dual staining with propidium iodide (red fluorescence) for total DNA content and with anti-mouse IgG FITC (green fluorescence), after 5-bromo-2-deoxyuridine (BrdU) incorporation into the newly synthesized cellular DNA and specific labelling of cells in the S phase by staining with mouse anti-BrdU. BrdU (10 μM; Sigma-Aldrich, GmbH, Steinheim, Germany) was added at the times indicated to transfected cells that were incubated at 37°C for 30 min; cells were then harvested by trypsinization and washed twice in 0.5% Tween-20 and 1% BSA in 1× PBS. DNA was denaturated by incubation with 2.0 M HCl/0.25%, Triton X-100 for 30 min at room temperature to produce single-strand molecules. After neutralization with 0.1 M Na₂B₄O₇ (pH 8.5), cells were incubated in mouse anti-BrdU (Becton-Dickinson, San Jose, CA, U.S.A.) diluted 1:20 in 0.5% Tween-20 and 1.0% BSA in 1× PBS, for 40 min at room temperature in the dark. After a further washing in Tween-20/BSA/PBS, cells were incubated with goat anti-mouse Ig FITC (Becton-Dickinson), diluted 1:12.5 in Tween-20/BSA/PBS, for 1 h at 4°C in the dark. Cells were washed again in Tween-20/BSA/PBS, and stained for DNA content for 30 min at 4°C in the dark after resuspension with 0.5 μg/ml propidium iodide/0.05% Nonidet P-40/0.1% tri-sodium citrate/5 μg/ml RNAse/0.25% Tween-20/0.5% BSA in PBS. Red and green fluorescence was measured immediately with a Coulter EPICS XL Flow Cytometer.

Enzyme assays

ODC activity was detected by measuring the release of ¹⁴CO₂ from [1-¹⁴C]ornithine by enzyme extracts [25]. SSAT activity was determined by assessing the label incorporation from [¹⁴C]acetyl-CoA into monoacetyl-SPD [25]. The protein concentrations in enzyme extracts were determined by using the Bio-Rad Protein Assay kit from Bio-Rad, Hercules, CA, U.S.A.

Polyamine determination

At the time indicated, 1 × 10⁶ cells were homogenized in perchloric acid and then centrifuged at 8000 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 HPLC [26].

Statistical analyses

Data were analysed by one-way ANOVA (completely randomized) followed by multiple comparisons using Dunnet’s test versus controls and Duncan’s test.
RESULTS

Preliminary experiments were conducted to find out the conditions for satisfactory transfection efficiency with PNT1A and PNT2 cells. The experimental protocol was based on the use of FUGENE 6* (Roche Diagnostics Corporation) as transfection reagent. To assess the overall efficiency, the percentage of positively transfected cells expressing the EGFP, under the control of the strong CMV promoter, was determined by laser confocal microscopy 24 h after transfection. As shown in Figure 1, the transfection efficiency was increased to more than 50% in PNT1A cells (out of the total cell population) and about 25% in PNT2 cells. It must be added that if about 50% of the PNT1A cells were clearly fluorescent green at the first observation, more cells (at least an extra 25%) showed a very faint but not artifactual green fluorescence (as compared with cells transfected with empty pCMV vector), which was detectable only by setting the laser confocal microscope on the maximum sensitivity. This suggests that transiently transfected PNT1A cells can express EGFP at different levels.

Figure 2 shows that in PNT1A cells transfected with the mammalian expression vector pOP/a.s.ODC, in which the RSV promoter drives the transcription of a 304 bp antisense RNA complementary to ODC mRNA (a.s.ODC), ODC activity was indeed negatively affected when measured in extracts from the whole cell population, being reduced by about 50%. The same result was obtained when cells were either transfected with pOP/OAZ, carrying the full-length cDNA coding sequence for OAZ (under the control of the same RSV promoter), or co-transfected with a mixture of the two plasmids, each at half the concentration used in single-plasmid experiments. On the contrary, SSAT overexpression obtained by transfection with pOP/SSAT (containing the full-length cDNA coding sequence for SSAT), produced an induction of ODC activity of about 50%, which can be viewed as a compensatory effect of polyamine depletion caused by SSAT over-induction. In cells co-transfected with all three plasmids, each at one-third the concentration used in single-plasmid experiments, ODC activity decreased by about 60%.

As expected, SSAT activity assayed in the same extracts was markedly enhanced in cells transfected with the SSAT construct (Figure 2), but was inhibited in cells transfected with a.s.ODC+OAZ, which could have been due to the decreased polyamine levels following the decreased biosynthetic activity. Co-transfection with all three constructs, while causing the reduction of ODC activity mentioned above, restored SSAT activity to levels comparable with the control (about 120% of control). This is an example of how the interplay between the activities of the latter regulatory enzymes, resulting in intracellular polyamine homeostasis, can be disrupted by gene manipulation. In fact, the induction of ODC by SSAT over-expression is prevented, under these conditions, by the converging inhibitory action of a.s.ODC and OAZ overexpression.

In Table 1 the intracellular polyamine levels in PNT1A cells, transfected as above, are shown. The expression of a.s.ODC or OAZ alone resulted in a significant decrease of intracellular PUT (of about 60%) and small decrements of SPD and SPM. A similar but more pronounced result was obtained when the cells were co-transfected with a mixture of both plasmids (same total amount of DNA). As expected, SSAT overexpression alone induced a significant (more pronounced) diminution of SPD and, to lesser extent, of SPM; however the PUT level was also very low under these conditions, suggesting that activation of the retroconversion pathway mostly resulted in acetyl-SPD and PUT excretion/oxidation rather than PUT accumulation within the cell. The combination of all three constructs caused the most

Figure 1 Transfection efficiency in PNT1A and PNT2 cells as assessed by laser confocal microscopy 24 h after transient transfection with a CMV-EGFP expression vector

(A and D) Phase-contrast photomicrographs; (B and E) autofluorescent EGFP-expressing cells; (C and F) digital superimposition of (A) and (B), and of (D) and (E), respectively.

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of 14CO2/h per mg of protein and 1270}

2001 Biochemical Society concentration decreased by 80 effective depletion of all intracellular polyamines in that PUT

cells were subjected to the same experimental conditions as described in the legend to Figure 2. PUT, SPD, SPM and total intracellular polyamine content (Total Cells were subjected to the same experimental conditions as described in the legend to Figure 1 Intracellular polyamine levels determined in 24 h transiently

determined in the S phase was again detected when a mixture of all three

and 50%, respectively. Figures 2 and 3(A) show that in PNT1A cells, 24 h after transient transfection with a.s.ODC, an accumulation of cells in the S phase occurred as measured by two-parameter flow cytometry (FACS) analysis; this was accompanied by a corresponding decrease in the G1/G0 and G0/M phases. Under the same

experimental conditions, a similar effect was obtained with OAZ overexpression. At variance with these results, when SSAT overexpression was induced, a significant accumulation of cells in the G2/M phases was evident, accompanied by a decrease in the number of cells in the S phase. Accumulation of PNT1A cells in the S phase was again detected when a mixture of all three

Figure 3(A) shows that in PNT1A cells, 24 h after transient transfection with a.s.ODC, an accumulation of cells in the S phase occurred as measured by two-parameter flow cytometry (FACS) analysis; this was accompanied by a corresponding decrease in the G1/G0 and G0/M phases. Under the same

<table>
<thead>
<tr>
<th>PUT</th>
<th>SPD</th>
<th>SPM</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>366 ± 22</td>
<td>646 ± 62</td>
<td>840 ± 63</td>
</tr>
<tr>
<td>a.s.ODC</td>
<td>142 ± 16**</td>
<td>515 ± 44*</td>
<td>701 ± 52</td>
</tr>
<tr>
<td>OAZ</td>
<td>114 ± 14**</td>
<td>581 ± 70</td>
<td>708 ± 90</td>
</tr>
<tr>
<td>a.s.ODC + OAZ</td>
<td>107 ± 5**‡‡</td>
<td>463 ± 37*</td>
<td>690 ± 47*</td>
</tr>
<tr>
<td>SSAT</td>
<td>96 ± 12**</td>
<td>366 ± 45**</td>
<td>501 ± 69</td>
</tr>
<tr>
<td>a.s.ODC + OAZ + SSAT</td>
<td>73 ± 4**§§</td>
<td>253 ± 32**</td>
<td>396 ± 42**§§</td>
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To confirm and further investigate these effects, we transfected the more differentiated PNT2 cells according to the same experimental protocol. Figure 3(B) shows that these cells also responded with an accumulation in the S phase when a.s.ODC or OAZ were overexpressed, accompanied by a corresponding decrease in the G1/G0 and G0/M phases; but again an accumulation of cells in the G2/M phases and a corresponding significant decrease in the number of cells in the S phase were detected when SSAT overexpression was induced. When the mixture of all three constructs was used in co-transfection experiments, the proliferative response of PNT2 was different from that of PNT1A cells, showing an accumulation in the G2/M phases, accompanied by a corresponding decrease in the number of cells in the S phase.
A time-course study was then performed with PNT1A cells by using either a.s.ODC or OAZ or both (Figure 4). At 15, 18 and 24 h after transfection, a progressive accumulation of cells in the S phase, accompanied by a corresponding progressive decrease in the percentage of cells in the G1/M phases, was detected during a.s.ODC (Figure 4B) or OAZ (Figure 4C) overexpression.

The combination of a.s.ODC + OAZ induced a different pattern of cell proliferation 24 h after transfection, in that the increased number of cells in the S phase was accompanied by an ac-
cumulation of cells in G$_2$/M (Figure 4D) and a marked decrease of the G$_S$/G$_1$ population.

The mono- and two-parameter FACS profiles for the PNT1A cells transfected as above for 24 h are shown in Figure 5. No significant signs of the typical morphological features of apoptosis could be seen in cell cultures. Widely dispersed sub-diploid peaks were not detected either, but a discrete sub-diploid peak was clearly evident in transfected cells (Figure 5).

**DISCUSSION**

Two lines of immortalized human prostate epithelial cells (PNT1A and PNT2) were efficiently transfected with three different constructs, the first one producing an antisense RNA complementary to ODC mRNA, the second one containing the full-length cDNA coding sequence for OAZ and the third one with the full-length cDNA coding sequence for SSAT. In both cells transfected with each of the above constructs at a time, and in cells co-transfected with two or three plasmids, changes in ODC and SSAT activities and polyamine concentrations were as expected based on the known homeostatic mechanisms that maintain the intracellular levels of the above compounds within the physiological range. For example, upon transfection with a.s.ODC and OAZ, inhibition of ODC activity was accompanied by a decrease in SSAT activity; vice versa, upon transfection with the SSAT construct, ODC was induced together with SSAT. These homeostatic responses, however, were not sufficient to prevent changes in polyamine concentrations. Disruption of polyamine homoeostasis was particularly evident in cells transfected with all three constructs (Figure 2); in fact, under these conditions: (i) polyamine biosynthesis was 2-fold inhibited (by a.s.ODC and OAZ); (ii) extracellular polyamine uptake was prevented (by OAZ) and (iii) polyamine degradation/excretion was activated (by SSAT). Indeed, this resulted in maximal polyamine depletion (Table 1), in spite of the exogenous polyamines present in FBS that was included in the standard media in which cells were grown and transfected. It is worth noting that the inhibition of ODC activity, which was consistently determined following transfection of PNT1A cells with pOAZ, demonstrates that OAZ was indeed expressed to effective levels, suggesting that, in this experimental model, OAZ expression is efficiently regulated also at the transcriptional level, and not only at the level of translation as reported previously [27]. This is also consistent with our observations that OAZ mRNA levels are significantly higher in human prostate cancer specimens as compared with contiguous benign tissue, and this induction correlates positively with tumour malignancy. Also, a significant correlation exists between OAZ and SSAT mRNA levels in advanced prostate cancers [6].

Changes in aliphatic polyamine concentrations and activities of their regulatory enzymes are known to take place during cell-cycle progression [17] and polyamine depletion results in cell-cycle phase perturbation, although the mechanism(s) by which this occurs remains unknown. Transfection with either a.s.ODC or OAZ, which caused a significant decrease of PUT and small diminution of SPD and SPM, produced accumulation of cells in the S phase of the cell cycle. These results are in agreement with previous findings [17] that increased PUT levels accompany the early S phase, and suggests that diminished availability of PUT may impair DNA duplication. Instead when, in both cell lines, SSAT was caused to be overexpressed, SPD and SPM (substrates of this enzyme) expectedly decreased to much lower levels than in cells transfected with a.s.ODC or OAZ. Under these conditions, accumulation in the G$_2$/M phases and decrease of cells in the S phase occurred (Figures 3A and 3B). In PNT2 cells maximal depletion of all three polyamines, by transfection with the three constructs, produced the same effect with the addition of cell accumulation in G$_2$/G$_1$, but in PNT1A cells the same distribution pattern obtained with a.s.ODC and OAZ resulted. This may depend on the different doubling times of the two cell lines, being 39 h for PNT2 and 30 h for PNT1A.

Time-course analysis of cell-cycle progression of transfected PNT1A cells confirmed that inhibition of the polyamine biosynthetic pathway produces a progressive accumulation of cells in the S phase (Figure 4), with no evident signs of apoptosis. This is in accord with a recent report in which polyamine depletion, obtained by administration of the ODC irreversible inhibitor a-difluoromethylornithine, induced growth arrest but not apoptosis [28]. The accumulation of cells in the S phase was accompanied by the appearance of peculiar mono- and bi-parametric profiles (Figure 5), suggesting that the total DNA content of the positively transfected cells was decreased, probably as a consequence of impaired DNA synthesis.

Altogether these data indicate that, by acting simultaneously on different regulatory steps of polyamine metabolism, specific perturbations in cell proliferation can be induced in two SV40-immortalized human prostate epithelial cell lines, resulting in alteration of cellular DNA content that could be the outcome of modifications in chromosome condensation, eventually leading to partial chromosome loss. Thus a direct link between polyamine metabolism, cell proliferation and chromatin structure is proposed. Also, based on many reports on the existence of interactions between polyamines and DNA [18,29,30], the hypothesis can be put forward that these polycations contribute in the correct DNA conformation in vivo, which would be required for the completion of the DNA duplication process.

It is well known that an increase in ODC activity, and consequently in PUT and higher polyamine production, is required for the progression of DNA replication during the S phase [31–33]. We show here that inhibition of PUT biosynthesis, not compensated by a correspondent extracellular polyamine uptake, prevents cells from completing the S phase. For the completion of mitosis (mostly during G$_2$ and probably also in M), intracellular polyamines undergo a relative and very rapid depletion [17], which is precisely obtained by SSAT induction [17]. This may be required for the correct folding of DNA into chromosomes. The sustained overexpression of SSAT induced here in PNT2 cells may interrupt the continuing fluctuations in polyamine content that are necessary for completion of the G$_2$ and M phases, thus preventing cells from entering a second round of replication.

The facts that genes regulating polyamine metabolism are expressed at significantly higher levels in human prostatic carcinoma [6] and correlate positively with tumour malignancy suggest that both polyamine biosynthesis and degradation are enhanced in transformed cells. This supports the hypothesis that a gene therapy strategy, aimed at targeting the polyamine-metabolism regulatory genes simultaneously, could interfere with transformed epithelial cell proliferation in prostate carcinoma.

We thank Dr O. Coussenot (Department of Urology, Hopital Saint Louis, Paris, France), Dr P. Berthon (Department of Urology, Hopital Saint Louis, Paris, France) and Dr N. J. Maitland (Department of Biology, York, U.K.) for making available the PNT2 and PNT1A cells, and Professor W. Vogel (Department of Medical Genetics, Ulm, Germany) for the suggestions given during our experimental work. These interactions were developed in the frame of the collaborative network BIOMED 2. This work was partially supported by MURST (Rome), Programma di Ricerca Scientifica di Rilevante Interesse Nazionale – “Studio di alcuni Parametri Biochimici del Carcinoma Prostatico”.

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Received 16 May 2000/1 November 2000; accepted 5 December 2000