Advances in Brief

Cell Cycle-related Expression of p120 Nucleolar Antigen in Normal Human Lymphocytes and in Cells of HL-60 and MOLT-4 Leukemic Lines: Effects of Methotrexate, Camptothecin, and Teniposide

Wojciech Gorczyca, Silvia Bruno, Myron R. Melamed, and Zbigniew Darzynkiewicz

Cancer Research Institute and Department of Pathology, New York Medical College, Valhalla, New York 10595

Abstract

Expression of the proliferation-associated nucleolar antigen p120 was studied by flow cytometry in human quiescent and phytohemagglutinin-stimulated lymphocytes, as well as in human lymphocytic (MOLT-4) and promyelocytic (HL-60) cell lines. Bivariate analysis of p120 and DNA content data made it possible to correlate p120 expression with cell position in the cycle. Proliferating lymphocytes and MOLT-4 and HL-60 cells had a similar pattern of p120 expression. Populations of G1 cells, during progression through S phase, and the antigen was maximally expressed in these cells. The p120/DNA content ratio, however, was highest in early G2 cells (G2a) and was declining during S and G2. The data thus suggest that p120 may be degraded during mitosis and that the postmitotic cells inherit little, if any, of this protein; the antigen then accumulates predominantly during G1, and must reach a threshold level to enable the cells to enter S phase. Antigen p120 could not be detected in noncycling lymphocytes nor in HL-60 cells induced to myeloid differentiation by growth in the presence of dimethyl sulfoxide. Treatment of MOLT-4 cells with pharmacological concentrations of methotrexate, camptothecin, or teniposide induced cell arrest in S or G2 phase expression of p120 in the arrested cells was unchanged from that of untreated MOLT-4 controls at the same phase of the cycle. The level of p120 was minimal in MOLT-4 or HL-60 cells arrested in S phase by vinblastine, but vinblastine had no effect on p120 fluorescence of interphase cells. Camptothecin or teniposide induced apoptosis selectively in S phase of HL-60 cells; apoptotic cells from camptothecin-treated cultures, however, despite the marked nucleolysis, still expressed p120. The data on the drug-treated cells indicate that the p120 level in tumors of patients may be used as a marker of tumor/malignancy even in clinical samples obtained during treatment.

Introduction

There is an extensive body of evidence that the nucleolus of proliferating tumor cells is morphologically, biochemically, and functionally different from that of normal quiescent or benign cells (1, 2). Attempts have been made to develop antibodies to nucleolar constituents, which could then be used as a marker of proliferating cells (3, 4). Several monoclonal antibodies recognizing different proteins localized in nucleoli were recently developed, and some of them were successfully used to identify proliferating cells or as tumor prognostic markers in the clinic (5–8).

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2 To whom requests for reprints should be addressed, at The Cancer Research Institute, New York Medical College, Valhalla, NY 10595.

Busch and coworkers (5, 6) have developed monoclonal antibodies to a nucleolar protein of an apparent molecular weight of 120,000, which is localized in the fibrillar network of this organelle. The molecular structure and nature of the epitope of p120 antigen have been recently characterized (9, 10). In a variety of cell types, expression of this protein correlates with cell proliferation (5, 11–13), and its prognostic value has been demonstrated in human breast carcinoma (8). Standard immunochemical methods, or measurements in bulk, were used in most of these studies, which made it impossible to measure the expression of this protein per cell or to correlate its expression with the cell cycle position.

In the present study expression of p120 in normal quiescent and stimulated human lymphocytes as well as in the human lymphocytic (MOLT-4) and promyelocytic (HL-60) leukemic cell lines, untreated and treated with antitumor drugs, was measured by flow cytometry. The drugs chosen either suppress DNA replication (MTX3), arrest cells in mitosis (VIN), or are DNA topoisomerase I and II inhibitors (CAM and TN, respectively). Bivariate analysis of cells with respect to their p120 and DNA content made it possible to correlate the expression of this antigen with the cell position in the cycle.

Materials and Methods

Cells. Human peripheral blood lymphocytes, obtained from healthy volunteers by venipuncture, were isolated by density gradient centrifugation as described (14). The cells were washed twice with buffered saline and resuspended in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum and antibiotics at a density of 106 cells/ml (14). PHA (Sigma Chemical Co., St. Louis, MO) was added at a final concentration of 10 ìg/ml. HL-60 and MOLT-4 cell lines were maintained as described before (15). All experiments were performed on cells during their exponential growth, unless otherwise indicated.

Drugs. CAM or VIN (both from Sigma), dissolved in DMSO at 3 mM or 1 mg/ml concentrations, respectively, was stored at —72°C. MTX (Sigma), 1 mM in H2O, was stored at 4°C. TN (Bristol-Myers Co., Wallingford, CT) was dissolved at 10 mg/ml in RPMI 1640 at 4°C. Prior to use the drugs were diluted in RPMI 1640 to the desired concentrations. The cells were treated for the time period equivalent of about 25% of their respective doubling times, i.e., 3 h (MOLT-4) and 6 h (HL-60). Control cultures were treated with solvent (DMSO) alone, which, up to the highest dose (0.1%), had no detectable effect on any of the parameters measured.

Immunocytochemistry. Several methods of cell fixation were tried involving the use of acetic acid, ethanol, and formaldehyde. The data presented in this paper were obtained on cells that were fixed in 0.5% paraformaldehyde for 15 min on ice, rinsed in PBS, and treated with 0.1% Triton X-100 for 5 min, also on ice (15, 16). After addition of 5

The abbreviations used are: MTX, methotrexate; VIN, vinblastine; CAM, camptothecin; TN, teniposide; PHA, phytohemagglutinin; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; PI, propidium iodide; IgG1, immunoglobulin G1.
ml of PBS and centrifugation, the cells were incubated for 30 min at room temperature in the presence of the primary antibody p120 (kindly provided by Dr. H. Busch and Dr. R. K. Busch of the Baylor College of Medicine, Houston, TX), diluted 1:40 in PBS containing 1% BSA. Cells were then washed and incubated with the FITC-conjugated goat anti-mouse IgG antibody (Sigma) diluted 1:40 in PBS containing 1% BSA. The cells were washed again, resuspended in 10 μg/ml of PI and 0.1% RNase A in PBS, and incubated at room temperature for 30 min prior to measurements (15). Controls were prepared identically as described above, except that the isotype-specific antibody (mouse IgG1; Sigma) was used instead of p120 antibody. All experiments were repeated at least 3 times, and results essentially identical to those shown in Figs. 1 to 3 were obtained from each experiment. Fluorescence of cells was measured using the FACScan flow cytometer (Becton Dickinson, San Jose, CA). The red (PI) and green (FITC) emissions from each cell were separated and measured using standard optics of the FACScan. Other experimental details were presented before (14, 15, 17) or are included in the legends to figures.

Results

Expression of p120 in Normal Human Lymphocytes. Unstimulated lymphocytes showed no presence of p120 (Fig. 1). The antigen became detectable 24 h after addition of PHA, and maximal expression was seen after 48 h, when most lymphocytes were progressing through the cell cycle. A decrease in p120 expression was observed after 72-h stimulation with PHA (not shown).

In cultures of proliferating lymphocytes (Fig. 1D) the cell population with diploid DNA content of (G0 and G1 cells) was very heterogeneous, some cells expressing p120 at levels severalfold higher than the others. Cell entrance to S phase followed accumulation of a threshold level of p120. It was possible, therefore, to discriminate G0 cells expressing p120 below the threshold level (G1a cells) from those having a p120 content similar to that of early S-phase cells (G1b).

Cell progression through S phase was paralleled by an increase in the expression of p120. The cells with tetraploid DNA content (G2 + M) expressed p120 maximally. It was apparent under UV light microscopy, however, that p120 fluorescence of mitotic cells was low (not shown). Thus, the tetraploid cells with high p120 fluorescence were G2 cells. Expression of p120 during the cell cycle was recalculated per unit of DNA (Table 1). The data show that the p120/DNA ratio, which was highest in late G1 cells (G1b), decreased during S and G2 and was minimal in M. The increase in cellular DNA content during S, reflecting the extent of DNA replication at a given point in the S phase, thus exceeded the increase in p120.

Expression of p120 in MOLT-4 Cells and Effects of Treatment with CAM, TN, MTX, and VIN. The distribution of p120 during the cell cycle of MOLT-4 cells (Fig. 2A) resembled that of cycling normal lymphocytes. High heterogeneity of the G1 population with respect to p120 content was apparent, as was the threshold of p120 content in G1, discriminating G1a from G1b cells. Expression of p120 increased during S phase and was highest in G2 phase. The p120/DNA ratio, however, as in the case of lymphocytes, also decreased during cell progression through S (Table 1). Mitotic cells expressed p120 minimally; the latter was confirmed by UV light microscopy (not shown). The experiment in which cells were treated with VIN (Fig. 2E) provided further confirmation that M-phase cells have a low p120 content. Namely, exposure of MOLT-4 cells to VIN for 3 h resulted in the arrest of approximately 40% cells in M phase. As is evident from Fig. 2E, the cells arrested in M had minimal p120 fluorescence. Actually, some M-phase cells had p120 fluorescence at the level of isotype control.

Treatment of MOLT-4 cells with MTX, CAM, or TN resulted in significant perturbation of their cycle: a loss of cells from G1 and increased proportions of S and G2 cells were evident in the frequency distribution histograms (Fig. 2, insets). Deconvolution of these histograms revealed that MTX or CAM arrested cells predominantly in S phase, whereas TN inhibited progression through S and G2. As is evident from the contour maps in Fig. 2, the expression of p120 in the arrested cells was not significantly changed by any of these drugs. Likewise, exposure of cells to VIN, although resulting in an accumulation of M-phase cells with low p120 content, did not alter expression of p120 in the interphase cells.

G1a cells were absent in all the drug-treated cultures, i.e., when cycle progression was halted in S, G2, or M by the respective drugs, preventing cells from reentering G1. This indicates that G1a cells were postmitotic, early G1 cells, which during the time of exposure to the drugs (3 h) underwent transition to the G1b compartment, i.e., synthesized p120 up to, and possibly above, the threshold level.

Expression of p120 in HL-60 Cells. Distribution of p120 in HL-60 cells during the cell cycle was similar to that of MOLT-4 cells or lymphocytes. The heterogeneity of G1 cells, the presence of a p120 threshold in G1, and low expression of this protein in M-phase cells were all evident in HL-60 cells (Fig. 3A). In HL-60 cultures treated with 1.5% DMSO for 5 days, most of the cells arrested in the G1 phase (Fig. 3B). This was paralleled by the near total loss of p120; the p120 fluorescence of these cells was at the level of the isotype control.

Treatment with MTX, CAM, or TN had a different effect on the cell cycle distribution of HL-60 compared with MOLT-4 cells (Fig. 3, D and E). The former responded to CAM and TN by rapid, selective loss of S-phase cells. However, the remaining
cells (predominantly G1, also some G2 cells in TN-treated cultures) expressed p120 at a level comparable to that of the untreated cells. Simultaneous with the loss of S-phase cells, cells with fractional DNA content appeared in the CAM- and TN-treated cultures (Ap). As shown before (18), these are apoptotic cells; CAM and TN selectively induce apoptosis of S-phase HL-60 cells (18). Whereas p120 was still expressed in most apoptotic cells in cultures treated with CAM, this antigen was absent in apoptotic cells from the TN cultures.

More HL-60 cells remained in S phase following the treatment with MTX than after CAM or TN. Exposure to MTX for 6 h resulted in some cells losing their entire stainability with the p120 antibody. Most cells, however, still showed significant, although a somewhat reduced, expression of this protein (compared with the control).

Discussion

Detection of p120 combined with measurement of cellular DNA, followed by bivariate analysis of the correlated data, made it possible to study expression of this protein in relation to cell position in the cell cycle, without the necessity of cell synchronization. Confirming earlier observations from Dr. Busch’s laboratory (5), we noticed that quiescent normal lymphocytes, as well as HL-60 cells, induced to myeloid differentiation did not express p120.

During progression through the cycle, expression of p120 was generally similar in normal lymphocytes and in MOLT-4 and HL-60 cells. Thus, in all three cell types the population of G1 cells was very heterogeneous with respect to p120 content. Also, in each case, the p120 threshold in G1 was observed; cells entering S phase expressed p120 above the threshold level. Expression of p120, per cell, increased during S and peaked in G2. A rapid drop in p120 was observed during mitosis. Mitotic cells, especially when mitosis was prolonged by VIN, had no detectable p120 fluorescence. The similarity of the pattern of p120 expression in normal proliferating cells and in two leukemic lines of different lineage seen in the present study, its presence in proliferating cells of other types (6, 11–13), and its absence in nonproliferating normal lymphocytes and differentiated HL-60 cells all suggest that this protein is associated with cell proliferation and is ubiquitous to all cell types, regardless of lineage.

The present data show that p120 antigen is barely detectable late in mitosis. This would indicate that this protein is either degraded during mitosis, or its epitope in mitotic cells is in an altered conformation, unreactive or inaccessible to the antibody. Our preliminary observation on cells in which protein synthesis was inhibited by cycloheximide4 indicates a rapid turnover of p120, which would suggest that this antigen may indeed be degraded during mitosis, i.e., when the overall protein synthesis is halted. Postmitotic cells, thus, inherit little, if any, p120, which is then synthesized in G1, S, and G2. To enter S phase the cells have to accumulate the threshold amount of p120.

The increase of p120 during S phase was of a lesser magnitude than the rate of DNA replication, so that the p120/DNA ratio was declining (Table 1). Because all cell constituents double during the cycle, the ratio of a particular constituent per DNA (15) or per total protein content (16) is a more accurate representation of specificity of its expression at a given phase of the cycle.

Antitumor drugs, especially DNA topoisomerase inhibitors, alter the morphology and function of the nucleolus and, most prominently, induce its segregation (1). Interestingly, although the topoisomerase I inhibitor, CAM, and topoisomerase II inhibitor, TN, perturbed progression through the cycle, they had no significant effect on p120 expression. The content of this protein was unchanged, even after prolonged arrest of MOLT-4 cells in S or G2 by these drugs. The findings on p120

Table 1 Changes in p120 expression in normal lymphocytes and in MOLT-4 and HL-60 cells during the cell cycle

<table>
<thead>
<tr>
<th>Cell type (n = 3)</th>
<th>G1A</th>
<th>G1B</th>
<th>Early S</th>
<th>Mid-S</th>
<th>Late S</th>
<th>G2</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>1.00</td>
<td>1.97 ± 0.08*</td>
<td>1.65 ± 0.05</td>
<td>1.48 ± 0.07</td>
<td>1.38 ± 0.03</td>
<td>1.35 ± 0.04</td>
<td>0.88 ± 0.01</td>
</tr>
<tr>
<td>HL-60</td>
<td>1.00</td>
<td>2.08 ± 0.09</td>
<td>1.68 ± 0.1</td>
<td>1.45 ± 0.1</td>
<td>1.56 ± 0.08</td>
<td>0.69 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>MOLT-4</td>
<td>1.00</td>
<td>1.89 ± 0.2</td>
<td>1.80 ± 0.1</td>
<td>1.51 ± 0.07</td>
<td>1.54 ± 0.01</td>
<td>1.52 ± 0.09</td>
<td>0.69 ± 0.08</td>
</tr>
</tbody>
</table>

*Number of experiments.

± Mean ± SE.

4 W. Gorczyca et al., unpublished observation.
expression by the drug-treated cells are also of relevance in the clinic. Namely, the agents studied represent different classes of antitumor drugs that are in common use. Since tumors from the treated patients may be tested for p120 for prognostic reasons, e.g., by needle biopsy, the possible loss of this protein as a result of treatment may complicate interpretation. Clearly, this cannot be the case, at least with the drugs tested in the present study. The exception is the M-phase cells that accumulate in the presence of VIN.

DNA topoisomerase inhibitors cause selective apoptosis of S-phase cells in the HL-60 line (18). The apoptotic cells (Ap) having partially degraded DNA were identified by flow cytometry as having fractional DNA content (18, see Fig. 3). Interestingly, some Ap cells following CAM treatment were still p120 positive, despite a significant degree of nucleolysis. Thus, p120 appears to be preserved, at least initially, during apoptosis. In TN-treated cultures apoptosis appeared to be more advanced and, after 6 h of treatment, Ap cells were p120 negative.

The minimal activity of the nucleolus, which is the site of rRNA synthesis, in noncycling cells (1), is a reflection of the low turnover of ribosomes. Noncycling cells also have a markedly lowered number of ribosomes, compared with their cycling counterparts (19), and recruitment of cells to the cycle is associated with nucleolar activation. The regulation of cell growth in size (20) or accumulation of rRNA (21) and the commitment to enter S phase appear to be coupled in the cycle. Thus, various parameters related to activation of the nucleolus (e.g., also activity of the nucleolar organizer regions; Ref. 22) as well as the rRNA content itself are markers of cell proliferation and, being predictive of tumor growth rate and often of the response to the cell cycle-specific drugs, may have prognostic value in the clinic (reviewed in Ref. 23).

MTX, CAM, AND TN EFFECTS ON p120 NUCLEOLAR ANTIGEN

Fig. 3. Expression of p120 in HL-60 cells in relation to their position in the cell cycle. A, exponentially growing cells; B, cells from the culture treated with 1.5% DMSO for 5 days; C to F, cells from the cultures treated for 6 h with 1 μM MTX, 0.15 μM CAM, 1 μM TN, and 0.05 μg/ml of VIN, respectively. The position of the mean values of green fluorescence of the isotypic control is shown in C. Insets present the respective DNA frequency histograms.

References


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