Advances in Brief

Different Effects of Staurosporine, an Inhibitor of Protein Kinases, on the Cell Cycle and Chromatin Structure of Normal and Leukemic Lymphocytes

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Abstract

Staurosporine, a microbial alkaloid, is a strong inhibitor of protein kinases. The effects of staurosporine on the cell cycle progression and nuclear morphology of normal human lymphocytes stimulated to proliferate by phytohemagglutinin were studied and compared with the effects of this drug on human lymphocytic leukemic MOLT-4 cells. Exposure of normal lymphocytes to either 5–10 or 50–100 ng/ml of staurosporine resulted in the preferential accumulation of cells in G1 or G2 phases of the cell cycle, respectively. In contrast, regardless of the concentration (5–100 ng/ml), staurosporine arrested MOLT-4 cells initially in G2; these cells then initiated additional rounds of DNA replication, without division. Staurosporine (5–100 ng/ml) induced severe changes in the nuclear morphology of MOLT-4 cells, manifested as nuclear elongation, deep invaginations of the nuclear membrane, extensive fragmentation, and micronucleation. At concentrations of 5–10 ng/ml, staurosporine had no apparent effect on the nuclear morphology of normal lymphocytes and at 50–100 ng/ml it produced minor changes in the nuclear shapes of these cells. The data indicate that the kinase(s) involved in the regulation of cell cycle exit from G1 and G2, respectively, in normal and leukemic lymphocytes may have different sensitivities to staurosporine, which suggests that the mechanisms controlling exit from G1 in these cells may be different. In MOLT-4 cells the staurosporine-sensitive kinase(s) appear to also be involved in phosphorylation of nuclear constituents essential for organization of gross chromatin structure. The different response of normal versus leukemic lymphocytes to staurosporine, if confirmed on clinical material, opens new strategies of tumor treatment.

Introduction

Recent findings indicate that a M, 34,000 serine-threonine protein kinase, a product of the cdc2 gene of the fission yeast *Saccharomyces pombe* (p34<sup>cdc2</sup>), appears to be one of the key molecules involved in regulation of the cell cycle (for review, see Ref. 1). Homologues of p34<sup>cdc2</sup> were identified in the budding yeast *Saccharomyces cerevisiae* (2), and also in a variety of animal (e.g., Refs. 3 and 4) and plant (5) species. Association of p34<sup>cdc2</sup> with the respective cyclins during G1 and G2 induces activation of this enzyme, resulting in phosphorylation of the substrates needed for triggering cell entrance to S phase and mitosis (M) (6, 7).

ST<sup>+</sup>, an alkaloid isolated from cultures of *Streptomyces*, is a potent inhibitor of a variety of protein kinases (8, 9). It was recently reported by Abe *et al.* (10) that, depending on concentration, ST can arrest cell cycle progression of normal rat fibroblasts either in G1 (1–10 ng/ml) or in G2 (100 ng/ml). Such data suggested that the kinase(s) involved in the regulation of cell exit from these phases may be the target of this drug and that their sensitivity to ST may be different in G1 as compared to G2. Recently, Crissman *et al.* (11) reported that, at 1–10 μg/ml, ST prevents arrested cells of several nontransformed lines from entering S phase whereas, at >10 μg/ml, ST arrested nontransformed cells in G1 and G2 and transformed cells in the G2 phase of the cell cycle. In the present study, we explored the sensitivity to ST of normal human lymphocytes stimulated to proliferation by PHA and compared it with the sensitivity of human lymphocytic leukemic cells (MOLT-4). The data show that there are significant differences between normal and leukemic lymphocytes, with respect to the specific points in the cell cycle in which cells are arrested by ST as well as the sensitivity of nuclear chromatin, which undergoes significant morphological changes as a result of treatment with the drug.

Materials and Methods

MOLT-4 cells, obtained from the American Tissue Culture Collection (Bethesda, MD), were grown in suspension in RPMI 1640, supplemented with antibiotics, L-glutamine, and 10% fetal bovine serum as described (12). Human peripheral blood lymphocytes, obtained from healthy volunteers by venipuncture, were isolated by lymphocyte separation medium (Teknika Corp., Durham, NC) density gradient centrifugation. Mononuclear cells from the interface were washed twice with buffered saline and resuspended in RPMI 1640 containing 10% bovine serum, at a density of 10<sup>6</sup> cells/ml. PHA (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 10 μg/ml. The stock solution of ST (Boehringer Mannheim, Indianapolis, IN) was prepared in ethanol (100 μg/ml) and stored at −20°C. The content of DNA and RNA in individual unstimulated and PHA-stimulated lymphocytes was measured by flow cytometry following cell staining with the metachromatic dye acridine orange, as described before (13). The DNA content of MOLT-4 cells was measured by flow cytometry following cell staining with DAPI, as described (14).

Fluorescence of lymphocytes stained with acridine orange was measured using the FACScan flow cytometer (Becton Dickinson, San Jose, CA). The red (RNA) and green (DNA) fluorescence emissions from each cell were separated using standard optics of the FACScan; the red emission was measured using the long-pass filter transmitting above 650 nm. The data were acquired and processed by the Consort 32 system (Becton Dickinson). The fluorescence of MOLT-4 cells stained with DAPI was measured with an ICP-22A flow cytometer (Ortho Diagnostics, Westwood, MA), as described (14). The data were analyzed using software provided by Phoenix Flow Systems (San Diego, CA). All experiments were repeated at least twice. Additional experimental details are given in the legends to the figures.

Results

The effects of ST on normal lymphocytes depend on the drug concentration (Fig. 1). Whereas lymphocytes exposed to 5 or 10 ng/ml of ST accumulate preferentially in G1, their progres-

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3 The abbreviations used are: ST, staurosporine; PHA, phytohemagglutinin; DAPI, diamidino-2-phenylindole.

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Inclusion through the cell cycle is halted in G1 and G2 at 50–100 ng/ml of the drug (Table 1). The first effects can be seen by 12 h after addition of ST and the arrest becomes more pronounced with time. The arrest does not appear to be complete because, even at high ST concentrations, some cells still remain in S phase after 24 or 48 h.

The response of MOLT-4 cells to ST is much different compared to that of lymphocytes. Namely, exposure of MOLT-4 cells to a wide range of ST concentrations (5–100 ng/ml), for periods of up to 6 h, results exclusively in cell arrest in G2 phase. At 50–100 ng/ml of ST, the arrest in G2 is rapid and complete. Thus, for example, more than 47% of the cells are in G2 and only 1% in G1 phase within 6 h of addition of 50 ng/ml of ST to MOLT-4 cultures (Fig. 2; Table 1). Treatment of MOLT-4 cells with ST for periods longer than 6 h results in a loss of cells with diploid DNA content and an accumulation of cells with DNA content above that of G2 cells. Analysis of DNA frequency histograms of MOLT-4 cells at various times of treatment with ST indicates that after 6 h cells enter higher DNA ploidy and then progress through the cycle at a tetraploid (Table 1) and, with time, even at an octaploid DNA level (not shown).

Observation of cells stained with the DNA-specific fluorochrome DAPI by UV light microscopy showed no significant changes in gross morphology of lymphocytes treated with 5 or 10 ng/ml of ST for up to 24 h. Exposure of proliferating normal lymphocytes to 50–100 ng/ml of ST resulted in moderate changes in nuclear morphology, characterized by elongated and somewhat irregular nuclear forms (Fig. 3, top row).

In contrast to normal lymphocytes, MOLT-4 cells treated with ST, even at low concentration (5 or 10 ng/ml), exhibited dramatic changes in nuclear morphology (Fig. 3, bottom row). The changes seen shortly (6 h) after addition of ST manifested in bizarre nuclear shapes. The nuclei were much larger and had elongated, cylindrical or “doughnut” shapes. Nuclear outlines were irregular, characterized by deep invaginations. The mitotic cells, occasionally seen in the ST-treated cultures, appeared to have disorderly arranged chromosomes, often less condensed than in control (not shown). Extensive nuclear fragmentation and micronucleation were apparent. The changes in chromatin, especially the depth of invaginations of the nuclear membrane, were more extensive at higher ST concentrations, although the nuclei were generally smaller at 50–100 ng/ml of ST than at lower concentrations of the drug. This was due to the fact that fewer cells entered higher DNA ploidy at higher ST concentrations (Table 1).

Discussion

We presently observe that the effects of ST on normal human proliferating lymphocytes and on lymphocytic leukemic cells are qualitatively different. Under the assumption that protein kinase(s) is the target of this alkaloid, the data suggest that the

Table 1: Effect of staurosporine on PHA-stimulated lymphocytes and MOLT-4 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Staurosporine conc. (ng/ml)</th>
<th>Treatment time (h)</th>
<th>Cell cycle distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>0</td>
<td>24</td>
<td>G1 57 S 30 G2M 13</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>10</td>
<td>24</td>
<td>78 15 7</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>50</td>
<td>24</td>
<td>63 9 28</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>0</td>
<td>6</td>
<td>34 58 8</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>5</td>
<td>6</td>
<td>1 42 47 10</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>5</td>
<td>24</td>
<td>10 15 28 47</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>10</td>
<td>6</td>
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<tr>
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</tr>
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<tr>
<td>MOLT-4</td>
<td>100</td>
<td>24</td>
<td>15 37 48</td>
</tr>
</tbody>
</table>

Fig. 1: Effect of ST on the cell cycle distribution of PHA-stimulated human lymphocytes. The scattergrams represent the RNA and DNA content of individual cell stained with the metachromatic dye acridine orange and measured by flow cytometry (12). A, control (unstimulated lymphocytes following 6 h incubation in medium alone); B, lymphocytes stimulated with PHA for 6 h; C, lymphocytes stimulated with PHA for 68 h the last 24 of which also included 10 ng/ml ST; D, same as C except that the ST concentration was 50 ng/ml. Inset, DNA frequency distributions. The G0, unstimulated lymphocytes with low RNA content (e.g., to the left of the dashed line in B), have been excluded from the DNA histograms B–D.
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Fig. 2. Changes in the cell cycle distribution of MOLT-4 cells untreated (A) and treated with 5 (B) or 50 (C) ng/ml ST for 6 h. Exponentially growing MOLT-4 cells, both untreated and exposed to ST, were harvested at 6 h and stained with the DNA-specific, UV-excited dye DAPI and analyzed by flow cytometry (13). The G1, S, and G2M phases of the frequency distribution histograms (B) were determined.

Fig. 3. Fluorescence photomicrographs of normal human lymphocytes stimulated with PHA (top row) and MOLT-4 leukemic lymphocytes (bottom row) exposed to varying concentrations of ST. PHA-stimulated lymphocytes were treated with 0 (A), 5 (B), or 50 (C) ng/ml ST for 24 h under conditions described in Fig. 1, followed by cytocentrifugation onto slides and fixation. The cell nuclei were stained with 1.0 μg/ml DAPI. MOLT-4 cells were treated for 24 h with either 0 (A), 5 (B), or 50 (C) ng/ml ST and stained as above. All photomicrographs were taken at the same magnification.

kinase(s) involved in the exit of cells from G1 is sensitive to ST in normal lymphocytes but not in the leukemic ones. In contrast, the kinase(s) associated with entrance of the cell to M appears to be more sensitive to ST in MOLT-4 cells compared to normal lymphocytes. Because the p34cdc2 protein kinase is considered to be responsible for triggering cell exit from G1 and entrance to M (1–7), it is likely that the observed effects may reflect differences in the sensitivity of this enzyme to ST. Namely, p34cdc2 in its active form, following association with the respective G1 or G2 cyclins, may exhibit different sensitivity to ST in normal and leukemic lymphocytes. However, since ST has inhibitory effects on a wide range of protein kinases (8, 15), involvement of kinases other than p34cdc2 in the observed effects cannot be excluded. It is also possible that cell exit from G1 in leukemic lymphocytes, in contrast to normal lymphocytes, may escape the regulatory mechanisms otherwise provided by ST-sensitive kinases.

Our results, with respect to normal lymphocytes, are in...
partial agreement with the data of Abe et al. (10), who observed that ST arrests normal rat fibroblasts in G1 at low concentrations (1–10 ng/ml) and in G2 at higher concentrations (50–100 ng/ml). These authors, however, did not compare normal cells with tumor cells. The present data also concur with the observations of Crissman et al. (11) who noted that ST preferentially arrests normal cells in G1 and transformed cells in G2.

ST induced dramatic changes in the gross chromatin structure of MOLT-4 cells, since significantly more subtle changes (seen only at high ST concentration) were observed in normal lymphocytes. Because p34cdc2, combined with cyclin B, is involved in phosphorylation of nuclear lamins, i.e., the proteins of the nuclear envelope (16), these changes are also compatible with the assumption that p34cdc2 protein kinase may be the target, or one of the targets, of the inhibitory action of ST.

As was recently emphasized by Baserga (17), the data obtained based on one cell line or type does not allow one to generalize to other types. Our present results, therefore, which show significant qualitative differences in the sensitivity to ST between normal lymphocytes and MOLT-4 leukemic cells, must be extended to other cell systems. If, however, differences are found between normal and tumor cells in the sensitivity of their protein kinase(s) involved in regulation of the cell cycle, as the data of Crissman et al. (11) suggest, new strategies for tumor treatment can be designed. For example, the extended combined treatment with an S-phase killing agent and ST is expected to be more selective towards eradication of tumor rather than normal cells, inasmuch as the latter, arrested in G1 by ST, will be refractory to the S-phase cytotoxic agent. This and other strategies were proposed by Crissman et al. (11).

Acknowledgments

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References


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