Abstract. Expression of cyclin E is believed to be a critical factor promoting cell entry into the S-phase and cell proliferation. Indeed, normal proliferating cells and most tumor cell lines are characterized by the existence of a minimal cyclin E threshold level in the G1-phase, and only those cells expressing cyclin E over this threshold enter into the S-phase of the cell cycle. However, through studying clinical tumor tissue specimens, we recently observed that some cancer cells can enter into the S-phase with minimal levels of cyclin E expression. In an effort to establish an in vitro cell model system for studying the mechanisms underlying this phenomenon, we treated MOLT-4 lymphocyte leukemia cells with 50 mM caffeine and found that the levels of cyclin E expression were decreased markedly in these cells following 2 to 4-h exposure to caffeine. Quite unexpectedly, we observed that the percentage of the cells progressing through the S-phase increased despite the reduced levels of cyclin E, as analyzed for the cellular DNA contents, expression of nuclear-bound PCNA, immunolabelling with Ki-67 antibody and incorporation of BrdU. In fact, these cells entered into the S-phase with a level of cyclin E well below the threshold level for untreated cells, thus suggesting that lower levels of cyclin E expression are associated with cell proliferation under certain circumstances. We speculate that caffeine may enhance MOLT-4 cell entrance into the S-phase through activation of Cdc25, which in turn activates cyclin-dependent protein kinases (CDKs) including CDK2 and drives the cell cycle progression; while degradation of cyclin E by the ubiquitin/proteasome pathway may account for the decreased levels of cyclin E in these cells. Our findings from both the MOLT-4 cell line and patients' cancer tissues may help decipher the mystery of the deregulation of cell cycle progression and carcinogenesis in some malignant tumors.

It is generally accepted that defective regulation of the cell cycle is one of the most characteristic features of cancer. The key components of the cell cycle regulatory machinery are cyclins and cyclin-dependent protein kinases (CDKs). The activity of CDKs is modulated by their periodic phosphorylation and by interaction with cyclin-dependent kinase inhibitors (CDIs) (1,2). In normal proliferating cells and in some cultured cell lines, the expression of cyclin E is discontinuous during the cycle and is maximal at the G1-S transition. At that time, cyclin E enters into active complexes with its catalytic partner CDK2 (3). In fact, during G1 there is a progressive accumulation of cyclin E and only those cells with cyclin E expression above a distinct threshold level enter the S-phase of the cell cycle (4). There is evidence in the literature showing that a high level of cyclin expression promotes cell proliferation. However, others show that low levels of cyclin expression may not necessarily be associated with a decrease in cell proliferation rate (5). Interestingly enough, while studying clinical specimens of malignant tumor tissues, we unexpectedly observed that, despite low levels of cyclin expression (including cyclin E), or even an apparent lack of their expression, the cell cycle progression was unaffected (unpublished observations).

In an attempt to explore the mystery of this phenomenon, in the current project we established an in vitro cell model system for studying the mechanisms in the process. Toward...
this end, we lowered the level of cyclin E expression below the threshold by treating the exponentially and asynchronously growing acute lymphocyte leukemic cell line MOLT-4 with caffeine and investigated whether such low levels of cyclin E could affect cell proliferation. We assessed cell proliferation by measuring the S-phase cell population using three independent cytometric methods: expression of Ki-67/DNA (6) and PCNA (7), and incorporation of BrdU using the SBIP (DNA strand break induction by photolysis) methodology (8, 9). As expected, we observed that the reduced levels of cyclin E expression were correlated with the increased rather than decreased rate of cell proliferation in our model system.

Materials and Methods

Cell line and cell culture conditions. The acute lymphocyte leukemia cell line MOLT-4 (ATCC, Manassas, VA, USA) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. All media, supplements and sera were obtained from GIBCO (Grand Island, NY, USA). The cultures were split by dilution to a cell density of 2-8 x 10^5/ml to maintain asynchronous and exponential growth, unless otherwise indicated.

Cell treatment. Caffeine (Sigma Chemicals, Inc., St. Louis, MO, USA) was dissolved in RPMI 1640 medium and added to the cultures at a final concentration of 50 mM. The aliquots of cells were collected at 0 h, 2 h, or 4 h after administration of caffeine.

Immunocytochemistry. (i) Ki-67 versus DNA content and cyclin E versus DNA content bivariate analysis: Cells were washed with PBS and then fixed in suspension in 80% ethanol at -20°C for at least 2 h. The samples were then centrifuged, washed with PBS and treated with 0.25% Triton X-100 for 5 min on ice. After addition of 5 ml of PBS and centrifugation, the cells were incubated overnight at 4°C in the presence of the mouse monoclonal antibody to human cyclin E or Ki-67 (Pharmingen, San Diego, CA, USA), which was diluted 1:100 in PBS containing 1% BSA. The cells were then washed again, resuspended in 10 μg/ml of propidium iodide (PI) and 0.1% RNase A in PBS and incubated at room temperature for 20 min prior to measurement. The control was prepared as described above, except that the isotype-specific antibody (mouse IgG1; Sigma) was used instead of the cyclin E or Ki-67 antibody.

(ii) PCNA versus DNA content analysis: Cells collected from the cultures as outlined above were treated with lysing buffer (0.5% Triton X-100, 0.2 μg/ml EDTA and 1% BSA) on ice for 15 min, then fixed in 80% ethanol at -20°C for 10 min. The cells were then centrifuged, washed with PBS and 1% BSA in PBS and incubated overnight at 4°C in the presence of the mouse monoclonal antibody against human PCNA (Pharmingen), which was diluted 1:100 in PBS containing 1% BSA. The cells were then washed and incubated with a FITC-conjugated goat anti-mouse IgG antibody.

Table I. The percentage of cells expressing cyclin E and the emended threshold values at different times following caffeine (CAFF) treatment.

<table>
<thead>
<tr>
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<th>CAFF/0 h</th>
<th>CAFF/2 h</th>
<th>CAFF/4 h</th>
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<tbody>
<tr>
<td>Cyclin E (%)</td>
<td>47.71</td>
<td>44.21</td>
<td>36.66*</td>
</tr>
<tr>
<td>Cyclin E threshold</td>
<td>1.777</td>
<td>0.632**</td>
<td>0.481**</td>
</tr>
</tbody>
</table>

* p<0.05 versus the control, untreated cells (0 h).
** p<0.01 versus the control, untreated cells (0 h).

Figure 1. The effect of caffeine on the expression of cyclin E in MOLT-4 cells. A marked reduction in the expression of cyclin E is apparent at 2 h after administration of caffeine, while a moderate decrease is seen between 2 and 4 h following the treatment of cells with caffeine. Note that in the caffeine-treated cultures, cells enter the S-phase with much lower levels of cyclin E (lower threshold than the untreated cells).
(DAKO) diluted 1:20 in PBS containing 1% BSA, for 30 min. The cells were washed again, resuspended in 10 µg/ml PI and 0.1% RNase A in PBS and incubated at room temperature for 20 min prior to measurement.

(iii) BrdU labelling with SBIP:
Cells were cultured in the presence of 20 µM BrdU for 20 or 40 min in the absence and presence of caffeine and the samples were collected as described above. The cells were washed with PBS and briefly treated with 0.2% formaldehyde followed by 70% ethanol at -20°C for 4 days. The cells were exposed to UV light at 300 nm wavelength for 5 min and washed with PBS. The samples (per 10⁶ cells) were incubated in the buffer (50 µl) containing 0.2 M potassium cacodylate, 2.5 mM Tris-HCl (pH 6.6), 2.5 mmol cobalt chloride, 0.25 mg/ml BSA, 5 units of terminaldeoxynucleotidyl transferase (TDT) and 50 µM BrdUTP, at 37°C for 60 min. After washing with PBS, the cells were treated with 0.25% Triton X-100 in PBS and then incubated with a FITC-conjugated goat anti-mouse BrdU antibody (DAKO) diluted 1:100 in PBS containing 1% BSA, for 30 min. The cells were then washed again, resuspended in 10 µg/ml of PI and 0.1% RNase A in PBS, and incubated at room temperature for 20 min prior to measurement.

**Table II. The percentage of the cells in the S-phase expressing PCNA, Ki-67 and SBIP at different times following caffeine (CAFF) treatment.**

<table>
<thead>
<tr>
<th></th>
<th>CAFF/0 h (%)</th>
<th>CAFF/2 h (%)</th>
<th>CAFF/4h (%)</th>
</tr>
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<tbody>
<tr>
<td>PCNA</td>
<td>43.99</td>
<td>46.29*</td>
<td>45.77</td>
</tr>
<tr>
<td>Ki-67</td>
<td>27.46</td>
<td>35.78*</td>
<td>32.36</td>
</tr>
<tr>
<td>SBIP</td>
<td>49.87</td>
<td>57.91*</td>
<td>50.32</td>
</tr>
</tbody>
</table>

* *p* < 0.05 versus the control, untreated cells (0 h).

**Figure 2. The bivariate distributions (scatter-plots) of cellular DNA content versus PCNA expression (Fig. 2A), DNA content versus Ki-67 expression (Fig. 2B) and DNA content versus BrdU incorporation (assayed by the SBIP methodology; Fig. 2C) at different time points following the addition of caffeine to the cultures.**

Flow cytometry analysis. Cellular fluorescence was measured using a FASort flow cytometer (Becton Dickinson, San Jose, CA, USA). The red (PI) and green (FITC) emissions from each cell were separated using the standard optics of the FASort and quantified by separate photomultipliers.
Results

Figure 1 shows the bivariate distribution of cellular DNA content versus expression of cyclin E at different time points after addition of caffeine to the cultures of exponentially growing MOLT-4 cells. Three levels (A, B, and C) of cyclin E were marked, where the levels A and C represent the lowest and highest expression of cyclin E of the G1 cell population, respectively. Level B indicates the characteristic threshold level of cyclin E expression during the G1-phase and the untreated cells expressing cyclin E below this level do not enter to the S-phase. In order to avoid experimental mistakes occurring in the same sample due to the different photomultiplier tube (PMT) voltages used, we worked out an emended value for the threshold level of cyclin E expression using the formula \( B^2/A^C \). The threshold expression of cyclin E in MOLT-4 cells calculated by the formula \( B^2/A^C \) was invariable at different voltages (10). In Table I, we calculated the percentage of the MOLT-4 cells expressing cyclin E at different times after the addition of caffeine. The threshold level of cyclin E expression decreased dramatically following administration of caffeine in the first 2 h, and the decrement of cyclin E levels was moderate between 2 h and 4 h after treatment of the cells with caffeine.

The gating analysis (CellQuest) was performed by selecting the cells expressing PCNA (Figure 2A) above the control value (above the isotype IgG) and measuring the percentage of the cells expressing this protein (Figure 2A). Similarly, the percentage of the S-phase cells (R1) was determined based on immunostaining with Ki-67 antibody (Figure 2B) and incorporation of BrdU (Figure 2C). As is evident, the percentage of cells expressing PCNA, or positive for Ki-67 with an S-DNA content, or with BrdU incorporation, increased significantly during the first 2 h following the treatment with caffeine and remained at a relatively high percentage for up to 4 h (Table II).

Discussion

It is generally accepted that normal cells (such as fibroblasts and mitogen-stimulated lymphocytes) and some tumor cells, when growing exponentially in cultures, enter the S-phase of the cell cycle with maximal cyclin E expression (3). In fact, a minimal cyclin E threshold level is often seen, as the cells with cyclin E expression below this threshold would not enter the S-phase (3). Quite surprisingly, through studying clinical specimens of patients’ tumor tissues by multi-parameter flow cytometry, we found that some cancer cells entered into the S-phase with very low or minimal levels of cyclin E expression. Therefore, the aim of this study was: (i) to confirm whether a deliberate decrease in the expression of cyclin E alters the cell’s ability to enter the S-phase in this system. MOLT-4 leukemia lymphocytes were used as a model.

We showed that the levels of cyclin E expression were decreased when MOLT-4 cells were treated with caffeine in culture and that this decrease in cyclin E expression was paralleled by an increased rather than decreased rate of cell entry into the S-phase. These results are consistent with our observation from patients’ cancer tissue specimens, and confirm the concept that low levels or even no apparent expression of cyclin E occur in some proliferating cells of malignant origins.

Several independent methods were used to assess and confirm whether treatment with caffeine did indeed lead to the increased rate of cell entry to the S-phase. One of these assays made use of PCNA expression. PCNA is an assistant protein of DNA polymerase, directly participating in DNA replication during the S-phase. PCNA is minimally expressed in resting cells, while in proliferating cells it peaks in late in G1 and in S-phase, and then significantly decreases during the G2/M-phase. During the S-phase, PCNA is a nuclear-bound part of the multi-complex assembly associated with DNA replication. Thus, when the cells are fixed in the presence of detergent, as was the case in the present study, only the S-phase-associated PCNA is detected, because the unbound, cytoplasmic PCNA is lost during sample preparation (7). The PCNA-positive cells, detected (Figure 2A) thus represent the cells actively involved in DNA replication.

The antigen detected by Ki-67 antibody is a multifunctional protein, expressed exclusively in proliferating cells of human origin during the late G1-, S-, G2-, or M-phases of the cell cycle, but absent in the G0-phase. Ki-67/DNA multi-parameter flow cytometry is widely applied to cell proliferation analysis (6). In the present study, we determined the percentage of Ki-67-positive cells with an S-phase DNA content.

Another method here used to evaluate the percent cells replicating DNA directly measures incorporation of the thymidine analogue BrdU (11). The incorporation of BrdU was detected using the SBIP methodology, which is based on selective photolysis of DNA that incorporated the precursor, followed by labelling the resulting DNA strand breaks (8,9).

All the three above methods provided evidence that, with a decrease in expression of cyclin E following caffeine treatment, the percentage of cells replicating DNA was increased. In fact, in the caffeine-treated cultures, cells entered the S-phase with a cyclin E level well below the minimal threshold that was otherwise required to enter the S-phase in the control, untreated cells (Figure 1).
As already mentioned, we observed low levels of cyclin E expression in many clinical specimens of human tumors in which the tumor cells were strongly proliferating. Thus, in analogy to the present findings, tumor cells can enter the S-phase and proliferate even at low levels of cyclin E expression. Our data thus indicate that, if phosphorylation of pRB is essential for cell entry to the S-phase, then its phosphorylation may be carried out at a low level of cyclin E (or even in the absence of cyclin E). The presence of cyclin E-CDK2 complex activity thus appears not to be the absolute prerequisite for cell entry to the S-phase.

The mechanism through which caffeine promotes cell proliferation is unknown at this point. However, it may be associated with inhibition of ATM kinase, which is involved in regulation of the G1 checkpoint (12). ATM is not only a surveyor of occasional damage such as DNA damage, induced by γ-irradiation, but also a firmly integrated component of cellular physiology (13,14). It is possible that, caffeine, at the concentration used in our experiments, may enhance phosphorylation of Cdc25 via inhibiting phosphorylation of Chk1, the protein that phosphorylates the CDKs (including CDK2), and then urge the cells to enter the S-phase (13,15,16). Cell entry to the S-phase, driven by this mechanism, appears to be more efficient than that otherwise regulated by cyclin E-CDK2 kinase activity (17). On the other hand, the observed decreased levels of cyclin E may occur through degradation by the ubiquitin/proteasome pathway (18). This hypothesis may explain, at least in part, the minimal expression of cyclin E existing in our clinical specimens of proliferating tumors.

In conclusion, we provided the first evidence from both clinical cancer tissues and cancer cell lines to support the notion that cancer cells can enter the S-phase with low or minimal levels of cyclin E expression under certain circumstances. Further investigations will be required to evaluate the significance of our findings in carcinogenesis of some malignant carcinomas and to elucidate the underlying mechanisms in these diseases. Understanding the regulation and deregulation of cell cycle progression in physiological and pathophysiological conditions could lead not only to a better understanding of the tumorigenesis of some tumors, but also to the definition of new therapeutic approaches for cancer management.

Acknowledgements

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