Unscheduled Expression of Cyclin B1 and Cyclin E in Several Leukemic and Solid Tumor Cell Lines

Jianping Gong, Barbara Ardelt, Frank Traganos, and Zbigniew Darzynkiewicz

The Cancer Research Institute, New York Medical College, Valhalla, New York 10595

Abstract

Normal, nontumorous cells express cyclin proteins in an orderly, scheduled fashion, at a given phase of the cell cycle. Thus, cyclin B1 is synthesized during G2 and abruptly degraded during mitosis. The onset of cyclin E synthesis takes place in mid-G1, its maximal expression is at the time of cell entrance to S, and its degradation occurs during cell progression through S phase. In the present study, multiparameter flow cytometry was used to correlate expression of cyclin B1 or cyclin E with cell cycle position (estimated by cellular DNA content) in normal human proliferating lymphocytes as well as in T-cell MOLT-4 leukemia; promyelocytic HL-60 leukemia; histiocytic U937 lymphoma; MCF-7, T-47D, and HS 587T breast carcinoma; Colo 320DM colon carcinoma; and the T-24 transitional cell carcinoma cell line. The scheduled expression of both cyclins, namely of cyclin B1 restricted to G2 + M cells and of cyclin E restricted to late G2 and early S cells, was observed only in normal lymphocytes and MOLT-4 cells. The cells of HL-60, U937, T-47D, and HS 587T lines expressed both cyclins in an unscheduled ("ectopic") fashion, i.e., unrelated to cell cycle position. Colo 320DM cells showed unscheduled expression of cyclin E (i.e., during G2) but expression of cyclin B1 in this line was generally restricted to G2 + M cells. There were relatively few (10-12%) cells in MCF-7 and T-24 cell lines that expressed cyclin B1 or E in an unscheduled manner. It may be expected that the unscheduled expression of cyclins in tumor cells may lead to a loss of the regulatory mechanisms of cell cycle progression and that such feature of the tumor may be of prognostic value. There is a need, therefore, to conduct similar studies in primary tumor cells.

Introduction

Research of the past several years led to identification of the key components of the complex machinery propelling the cell through the cell cycle (for reviews, see Refs. 1-6). Among these components, cyclins appear to be of special interest. They are expressed selectively in different phases of the cycle and their role is to activate one of the several cell cycle-specific kinases, each of which phosphorylates a distinct group of proteins. Phosphorylation of these proteins is essential for advancement of the cell through a particular stage of the cell cycle. For example, expression of cyclin B1, a member of the class of G2 cyclins, is required for the entrance of the cell into mitosis. Onset of synthesis of this protein can be traced to an early portion of G2, its expression peaks during the G2 to M transition, and its degradation occurs at the completion of mitosis. The enzymatic activity of the heterodimer of cyclin B1 and the serine/threonine-specific protein kinase p34cdc2 is regulated by sequential phosphorylations and dephosphorylations of threonine-14 and tyrosine-15 of p34cdc2, the site of ATP binding. Phosphorylation of p34cdc2 on threonine-161, in turn, facilitates its binding to cyclin B1. Cyclin A, discovered in marine invertebrates, shows a pattern of expression somewhat similar to that of type B cyclins except that onset of its expression occurs prior to that of cyclin B1, namely relatively early in S phase (7).

The most prominent of the class of G1 cyclins is cyclin E. Onset of synthesis of cyclin E takes place in mid-G1, its expression peaks at the G1-S transition, and its stepwise degradation occurs during cell progression through S phase (8, 9). This cyclin associates with p33cdk2, and the complex phosphorylates histone H1 and several other proteins late in G1 and early in S phase (for reviews, see Refs. 5 and 6). Cyclin D, like cyclin E, belongs to the G1 class of cyclins and forms a complex with p33cdk2, as well as with cdk4 and cdk5 kinases. The onset and peak of its expression, however, are prior to those of cyclin E (10, 11). Furthermore, whereas stimulation of quiescent cells by growth factors (11) or mitogenic activation of lymphocytes (12) induce early expression of cyclin D, this protein remains invariant (11) or at a very low level (12) in normal cells that proliferate continuously. It has been suggested, therefore, that unlike the periodically expressed E, A, and B cyclins, D type cyclins are induced in response to mitogens or growth factors and may not be integral parts of the cell cycle progression machinery (5).

The availability of antibodies to cyclins, reactive with native proteins within the permeabilized cell, made it possible to measure their expression in individual cells immunocytochemically (12-16). Bivariate analysis of DNA content of individual cells versus cyclin expression, by flow cytometry, provides a simple and direct assessment of the cell cycle phase specificity of cyclin expression, without the need for cell synchronization or physical separation which generally disturbs cell cycle progression. During analysis of a variety of cell types, we observed that in several tumor cell lines, the pattern of expression of cyclins in relation to the phase of the cycle was unexpected and was quite different compared to that seen in nontumor cells. In these cell lines, the cyclins were expressed in an unscheduled ("ectopic") fashion; namely the class G2 cyclins were expressed not only in G2 and M cells but also in G1 cells, while G1 cyclins, in addition to G1 and S, were present in G2+ M cells. Examples of such expression are presented in this paper. One may expect that the unscheduled expression of cyclins may correspond to loss of mechanisms regulating cell progression through particular stages of the cycle.

Materials and Methods

Cells. Human PBL, obtained from healthy volunteers by venipuncture, were isolated by density gradient centrifugation (12, 17). The cells were washed twice with buffered saline, resuspended in RPMI 1640 (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum and antibiotics at a density of 10⁶ cells/ml, and cultured in the presence of 10 μg/ml of phytohemagglutinin (Sigma Chemical Co., St. Louis, MO), as described before (17). With the exception of the human promyelocytic HL-60 cell line, which

Received 6/2/94; accepted 7/5/94.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
1 This work was supported by National Cancer Institute Grant CA 28704. J. G., on leave from the Department of Surgery, Tongji University, Wuhan, China, was supported by a Fellowship from the "This Close" for Cancer Research Foundation.
2 To whom requests for reprints should be addressed, at the Cancer Research Institute, New York Medical College, 100 Grasslands Road, Elmsford, NY 10523.

3 The abbreviations used are: PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PI, propidium iodide.

4285
was originally obtained from Dr. Harry A. Crissman of the Los Alamos National Laboratory. Most cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, as described (17, 18). All medium, supplements, and serum were obtained from GIBCO BRL. The cultures were passaged by dilution to a cell concentration of 2 x 10^5/ml to maintain asynchronous, exponential growth. The cultures were periodically tested for mycoplasma infection by staining of cytocentrifuge preparations with the DNA fluorochrome 4',6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR). Other details concerning culture conditions are described in earlier publications (13, 17-19).

**Immunocytochemistry.** Several methods of cell fixation and permeabilization have been tested, as described elsewhere (13, 15, 16). The optimal fixation for detection of cyclin B1 or E and simultaneous DNA content analysis was achieved when the cells were rinsed with PBS and suspended in 80% ethanol at −20°C for 2 h or overnight. The samples were then centrifuged, washed with PBS, and treated with 0.25% Triton X-100 in PBS 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 B1 or E (PharMingen, San Diego, CA; clones GNS-1 and HE 12, respectively) which were diluted 1:400 or 1:100 in PBS containing 1% bovine serum albumin, respectively. Cells were then washed and incubated with a fluorescein 5-isothiocyanate-conjugated goat anti-mouse IgG antibody (Sigma) diluted 1:40 in PBS containing 1% bovine serum albumin, for 30 min. 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 20 min prior to measurement (13, 16). The control was prepared as described above, except that an isotype-specific antibody (mouse IgG1; Sigma) was used instead of the cyclin B1 or E antibody. Cellular fluorescence was measured using the FACScan flow cytometer (Becton Dickinson, San Jose, CA). The red (PI) and green (fluorescein 5-isothiocyanate) emissions from each cell were separated and quantitated using the standard optics of the FACScan.

**Results**

Fig. 1 presents the bivariate cyclin B1 versus DNA content distributions of normal human PBL stimulated to proliferation by phytohemagglutinin, as well as of the cell populations of several tumor cell lines. These contour maps clearly indicate that the expected expression of cyclin B1, namely to be confined to G2 + M cell population, was observed for PBL as well as MOLT-4 cells. The cells in G1 and in early S phase, in these cell lines, were cyclin B1 negative. A similar pattern of cyclin B1 expression, although with evidence of a few G1 cells expressing cyclin B1, was apparent in MCF-7, T-24, and Colo 320DM lines.

However, the pattern of cyclin B1 expression in U937, HL-60, Hs 578T, and T-47D lines was much different. In these lines, cyclin B1 was detected in cells residing not only in G2 + M but also in other phases of the cell cycle, including G1 (Fig. 1).

Expression of cyclin E in relation to the position of the cells in the cell cycle is shown in Fig. 2. In normal lymphocytes, cyclin E was maximally expressed in late G1 (G1a) cells and its content progressively declined during S phase. Lymphocytes residing in G2 + M were virtually cyclin E negative. Expression of cyclin E in MOLT-4, T-24 and MCF-7 cell lines were, in essence, similar to that seen in lymphocytes.

Compared with normal lymphocytes or MOLT-4 cells, the pattern of cyclin E expression was different in U937, Hs 587T, T-47D, HL-60, and Colo 320DM lines. In the latter cell lines, expression of this protein was distinctly less correlated with the cell cycle position, inasmuch as there were many cyclin E-positive cells residing in G2 + M or in late S phase.

Quantitative analysis of the experiments (the raw data of which are presented in Figs. 1 and 2), presenting the percentage of cells residing in G1 that express cyclin B1 and the cells residing in G2 + M that express cyclin E, is shown in Fig. 3. As is evident, less than 1% of normal G1 lymphocytes expressed cyclin B1 while less than 3% of G2 + M cells were cyclin E positive. Likewise, fewer than 4% MOLT-4 cells showed unscheduled expression of these proteins. Approximately 10% of the cells in the MCF-7 and T-24 cell lines expressed cyclins in an unscheduled fashion, which was at the border of statistical significance. Unscheduled expression of these proteins, by 20–50% of the cells, seen in HL-60, U937, T-47D, and Hs-587T,
Fig. 2. Bivariate distributions of cyclin E expression in proliferating PBL 72 h after stimulation with phytohemagglutinin, and in different tumor cell lines. In the PBL culture, expression of cyclin E is maximal in late G, and early S phase and declines during cell progression through S. Cyclin E is similarly expressed in MOLT-4, HL-60, T-24, and MCF-7 cell lines. The pattern of cyclin E expression, however, is different in the case of U937, Hs 587T, T-47D, and Colo 320DM cell lines. The latter lines display a lesser degree of cell cycle phase specificity in expression of this protein.

was statistically significant (P < 0.001) compared with lymphocytes or MOLT-4 cells.

Discussion

The bivariate analysis of particular cyclins versus cellular DNA content, as offered by multiparameter flow cytometry, provides a unique opportunity to relate expression of these cell cycle phase-specific proteins with the actual cell cycle position of the individual cell. In contrast to the analysis by the standard methods of molecular biology (e.g., Western blotting), this approach does not require prior cell synchronization and, as a result, can be applied to asynchronous populations growing exponentially. Furthermore, because individual cells are measured, the method allows one to assess the intercellular variability in the population, detect subpopulations sharing similar features, and identify rare cells with exceptional features. Thus, the method is optimally designed to detect unscheduled ("ectopic") expression of cell cycle phase-specific proteins, such as cyclins.

Several control experiments indicate that the observed in situ reactivity of cyclin E and B1 antibodies represents their interaction with the respective cyclins. Therefore, the measured fluorescence of the secondary antibody is indeed attributable to expression of these proteins. Thus, the cyclin measurements for each cell type were compared with their respective control, utilizing isotype IgG staining for the otherwise identically treated cells. Furthermore, essentially identical cyclin distributions were observed when the cells were fixed in 70% ethanol, in a mixture of ethanol:acetone (which, however, caused a higher degree of cell aggregation), or in 1% paraformaldehyde (which resulted in somewhat inferior DNA stainability with PI). Because of the different chemistry of the fixatives (ethanol and acetone precipitate and reversibly denature proteins while formaldehyde is a cross-linking agent) it is unlikely, therefore, that the results were affected by a fixative-induced change in the cyclin epitope, which theoretically could alter its reactivity with the antibody. The experiments were repeated (with some cell lines 4–6 times), yielding essentially identical patterns of cyclin distribution in each instance, in each cell line. The monoclonal antibodies used in this experiment have been reported to be specific to these cyclins in Western blotting and immunoprecipitation assays (8, 9, 19, 20). The observed expected (scheduled) expression of both cyclin E and cyclin B1 in normal lymphocytes provides additional evidence of specificity of detection of the respective cyclins.

The present data thus can be interpreted as indicating that several tumor cell lines express cyclin B1 and/or cyclin E in an unscheduled fashion. Thus, the ductal breast carcinoma lines Hs 578T and T-47D,

---

Fig. 3. Percentage of cells expressing cyclin E or B1 in unscheduled fashion in proliferating human PBLs (72 h after stimulation with phytohemagglutinin) and in eight tumor cell lines. The percentage of cells in G, phase expressing cyclin B1 (G) and percentage of cells in G + M expressing cyclin E (E) was calculated by gating the cells (based on differences in DNA content) in the respective phases of the cycle and estimating the percentage of cells with the cyclin-associated (green) fluorescence values exceeding by 3 SD the mean value of each respective control (isotypic IgG). Standard error bars are included when three or more estimates were done. Cell lines are indicated as in the text except for Hs 578T (578T), Colo 320DM (Colo), and MOLT-4 (MOLT).
as well as histiocytic lymphoma U937 and promyelocytic leukemia HL-60, displayed both a G2-cyclin (cyclin B1) in the G2 phase of the cell cycle and a G1-early S cyclin (cyclin E) in G1 + M. On the other hand, colon carcinoma Colo 320DM cells displayed unscheduled expression of cyclin E, while their cyclin B1 expression pattern appeared normal. In all of these lines, the number of cells showing unscheduled expression of the respective cyclins ranged between 20 and 50% (Fig. 3). Between 10 and 12% of the cells expressing cyclins in an unscheduled fashion were seen in cultures of the transitional cell carcinoma T-24 and breast carcinoma MCF-7 lines.

The unscheduled expression of cyclins, as presently observed in most of the tumor cell lines studied, may indicate either that proteolytic degradation of these proteins is impaired or that their synthesis is not limited to a particular phase of the cycle. Regardless of the mechanism, such unscheduled expression may have significant consequences with respect to regulation of cell cycle progression. These proteins, when combined with the respective cell cycle-specific kinases, are the motors driving the cell through a particular stage of the cell cycle (1–6). Normally, they undergo degradation at the end of each stage at which they function, whereas levels of their partner kinases remain invariant throughout the cell cycle. Therefore, when particular cyclins are expressed in an unscheduled fashion; namely, they remain present in the cell throughout the whole cell cycle, they may interact with their respective partner kinases also in an unscheduled pattern, phosphorylating a variety of substrates regardless of the phase of the cycle. Such activation is expected to drive the cell through the cycle, bypassing some control mechanisms.

Among eight tumor cell lines studied, only one (T-cell leukemia, MOLT-4) had expression of both, cyclin E and B1 in a fashion similar to that observed in normal lymphocytes (Fig. 3). In our ongoing study of expression of other cyclins, however, we have noticed that MOLT-4 expressed cyclin D in an unscheduled way, compared to normal, proliferating human lymphocytes. It is possible, therefore, that tumor transformation may invariably be associated with altered content of one or more of the cyclins. While the present study was performed on tumor cell lines adapted to culture, it will be important to determine if unscheduled cyclin expression also occurs in primary tumor cells. Such studies are currently in progress.

The unscheduled expression of a particular cyclin is expected to be manifested as an elevated amount ("overexpression") when measured in bulk, e.g., by Western blotting, due to its expression in a larger proportion of cells, compared to the expression of that cyclin in only a fraction of cells, i.e., at a given phase of the cell cycle. The oncogenic role of cyclins (21, 22) and the increased levels of these proteins reported in some human tumors to be associated with poor prognosis (23, 24) may, therefore, be associated with their unscheduled expression.

In an earlier study, we reported that the expression of cyclin B1 can be used as a marker to discriminate G2 + M cells from G1 cells having the same DNA content (13). This was demonstrated using MOLT-4 cells as an example. As the present data indicate, MOLT-4 cells are rather exceptional, in terms of the scheduled expression of both B1 and E cyclins. Clearly, the discrimination between cells having the same DNA content but at different phases of the cycle based on cyclin expression is limited to cells that express these cell cycle phase-specific proteins in a scheduled fashion. Likewise, the use of cyclin restriction points as landmarks to map the cell cycle, in order to more accurately localize the point of action of a particular drug (25), is also applicable only to cells characterized by the scheduled order of expression of these proteins.

The diverse patterns of unscheduled expression of particular cyclins in different tumor cell lines, as presently observed (actually, each cell line could be recognized from the bivariate DNA content/cyclin E and B1 distributions), support the cautionary note voiced by Baserga (26), that particular features of abnormality of the cell cycle, detected in a given tumor cell line or type, cannot be assumed to be generic to all tumors.

References


* Unpublished observations.
Unscheduled Expression of Cyclin B1 and Cyclin E in Several Leukemic and Solid Tumor Cell Lines

Jianping Gong, Barbara Ardelt, Frank Traganos, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/16/4285

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.