Nuclear Distribution of the Ki-67 Antigen during the Cell Cycle: Comparison with Growth Fraction in Human Breast Cancer Cells

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ABSTRACT

It has been claimed that the commercially available Ki-67 monoclonal antibody recognizes a nuclear antigen which is solely expressed in cycling cells. Therefore, at present, Ki-67 is increasingly used as a tool in evaluating growth fractions (GFs) of human tumors. Here we describe specific patterns in the expression and topological distribution of this antigen during the cell cycle in MCF-7 human breast cancer cells. Our results support earlier findings that the antigen belongs to a class of antigens associated with the structural organization of meta- and ana-phase chromosomes, and proteins located near the cortical regions of prenucleolar bodies and nucleoli. Using 5'-bromodeoxyuridine-labeling technology, we show that the expression may be undetectably low at the onset of DNA replication.

Comparison of Ki-67 fractions (KFs) and GFs as estimated from continuous 5'-bromodeoxyuridine-labeling curves revealed that KF was invariably higher than GF in exponentially growing MCF-7 human ovary cancer cells, KF was only 3.5% higher than GF; in MCF-7 cells, 11.3 ± 4.6%. In MCF-7 cultures either growing under suboptimal conditions or treated with 10^-6 M tamoxifen, the difference was more pronounced. Further, we observed a pronounced decrease of Ki-67-positive cells in nutritionally deprived and cell cycle-specific, drug-treated cultures. Since the results indicate that nonproliferating cells may retain the antigen for a considerable period of time, KF may not always be a reliable indicator of GF.

INTRODUCTION

An increasing number of studies underline the importance of tumor cell kinetics in the prediction of disease-free interval and short-term survival in breast cancer patients (1-3). Moreover, as cell kinetic parameters determine the effectiveness of chemotherapeutic treatment, an estimation of the fraction of tumor cells actively engaged in cell cycle traverse GF$^3$ or DNA synthesis (S-phase fraction) could be helpful in finding an optimal therapeutic approach in individual patients. Furthermore, cell kinetic data on lymph node metastases and the possible correlation with various characteristics of the primary tumors could be important with respect to chemotherapeutic and radiotherapy as an adjuvant to surgery.

Various methods to estimate GFs and S-phase fractions have been developed, including pulse- and continuous labeling with tritiated thymidine (4), biochemical analysis of DNA polymerase activity (5), and flow cytometric analysis of DNA and RNA content (6, 7). However, for routine clinical application these methods are either impractical, as they need in vivo administration of a radioactively labeled compound and many serial biopsies from possibly heterogenous tumors, or lack specificity and precision (8).

A promising recent development is the application of Moabs that recognize antigens associated with cell proliferation (9-15), therefore allowing an easy and rapid in situ evaluation of immunostained tissue samples. One of these Moabs, increasingly used in histopathology, is the commercially available Ki-67 Moab (16, 17). This mouse Moab, raised against crude nuclear fractions of a Hodgkin's lymphoma-derived cell line, has been used to determine GFs in a number of tumors, i.e., lymphomas (18, 19), and lung (20), brain (21), and breast carcinomas (22-26). Although the biochemical characteristics of the Ki-67 antigen have not yet been elucidated, it has recently been shown for human lung carcinoma and leukemia cells that the antigen is associated with the nucleolar cortex and the periphery of metaphase chromosomes (27, 28). In this paper we describe specific staining patterns in nuclei of MCF-7 human breast and MCF-7 ovario cancer and evaluate in MCF-7 monolayers the staining patterns of cells present in different phases of the cell cycle. We have studied the effects of cytostatic agents, such as MTX and TAM, on antigen expression and the correlation between the percent of Ki-67-positive cells and GFs as determined from continuous labeling with BrdUrd in MCF-7 cultures growing under various conditions.

We show that, although these patterns allow for a subdivision of the cell cycle, intercellular variability precludes an unambiguous determination of S-phase fractions. Furthermore, we discuss the observed discrepancy between Ki-67 positivity and GF in this cell line.

MATERIALS AND METHODS

Antibodies. Tissue culture supernatant of the mouse monoclonal antibody Ki-67 (IgG1; trade name, DAK-PC; code No. M722) was a gift from Dakopatts (Glostrup, Denmark). Anti-BrdUrd monoclonal antibody IU-4 was a gift from Dr. F. Dolbeare (Livermore, CA).

Cell Lines and Growth Conditions. The hormone-responsive human breast cancer line MCF-7 (29) was provided by Dr. M. Lippman (National Cancer Institute, Bethesda, MD). The MCF-7 ovarian cancer line (30) was a gift from Dr. P. Schrier (University Hospital, Leiden, The Netherlands). Cells plated in a density of about 0.1 x 10^6 cells/ml were grown as monolayers on glass slides in Leighton tubes at 37 °C in 5 ml of RPMI-1640 culture medium, supplemented with 5 to 10% FCS (Gibco, Paisly, Scotland), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (BDH Chemicals, Ltd., Dorset, England), 15 mM sodium bicarbonate (Baker Chem, Deventer, Holland), 100 IU/ml of penicillin, and 0.1 mg/ml of streptomycin sulfate. The culture medium was (unless stated otherwise) replaced by fresh culture medium every 3 days.

Hormone and Drug Treatments. The antierogenic compound TAM, a gift from ICI-Chicago, Macclesfield, England, was added at a final concentration of 10^-7 M in fresh culture medium to MCF-7 cultures 3 days after plating (when about 25% confluence was reached). Recruitment from a TAM-induced G1 to S phase was performed by washing the flasks twice with culture medium before adding fresh medium supplemented with 10^-7 M 17β-estradiol (Sigma, St. Louis, MO). TAM and 17β-estradiol were added as stock solutions in ethanol to the medium at the appropriate concentration, but such that the final concentration of ethanol in culture was less than 0.02%.
MTX (Pharmacie B.V., Haarlem, Holland) was used in final concentrations of $10^{-7}$ M.

BrdUrd-labeling and Fixation Procedure. Unless stated otherwise, cell cultures were labeled by adding BrdUrd (Sigma, St. Louis, MO) as a stock solution in PBS to the culture medium at a final concentration of 20 mM, 1 day after the cultures had received fresh culture medium. After various time intervals, cells were washed in PBS, fixed 2 times for 5 min in acetone at $-20^\circ$C and at RT subsequently, and air dried. Providing storage under dry conditions, storage of the slides at $-20^\circ$C after fixation did not affect the outcome of the staining procedure.

Other fixation protocols tested were: 4% neutral buffered formalin; Bouin’s fixative; 2.5% GA; methanol/acetic acid (3:1, v/v) (Carnoy’s fixative); 70% ethanol; 2% PF; and PLP.

Immunohistochemistry. In order to evaluate both Ki-67 and BrdUrd staining patterns in the same cell culture, a fraction of the area of the slides was incubated under a coverslip for 30 to 60 min at RT with Ki-67 Moab appropriately diluted in PBS with 1% BSA (Sigma, St. Louis, MO) and 0.1% Tween-20. Slides were washed in PBS (3 times, 5 min), and subsequently peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark), appropriately diluted in PBS with 20% normal human serum, was applied for 30 min. After washing 3 times for 5 min with PBS, peroxidase activity was detected with a substrate mixture of DAB (Sigma, München, FRG) and 0.05% H$_2$O$_2$ in PBS, resulting in the intranuclear deposition of brown pigment.

The protocol for the detection of incorporated BrdUrd has been described previously (31). In short, DNA was denatured by immersing the slides for 10 min in 0.07 M NaOH containing 70% ethanol, followed by dehydation in a series of ethanol and treatment with proteinase K (0.1 mg/ml, Boehringer, Mannheim, FRG) for 10 min at RT. These denaturation conditions did not affect the DAB stain already present in the area stained with Ki-67.

After a second dehydration step, a fraction of the slide surface (only partially overlapping the area stained with Ki-67) was incubated under a coverslip during 1 h at RT with IU-4, appropriately diluted in PBS with 1% BSA. Hereafter, the same protocol was followed as described above for the Ki-67 staining.

In additional experiments, BrdUrd incorporation was detected with 3-amino-9-ethylcarbazole (EAGchemie, Steinheim, FRG), resulting in a red stain and allowing an easy discrimination between BrdUrd-labeled replication sites and Ki-67 detected with the DAB technique.

Slides were finally counterstained with Mayer’s hematoxylin and mounted with Malinol.

Electron Microscopy. For electron microscopy, an MCF7 monolayer was fixed in PLP (2% paraformaldehyde, 15 min, RT), incubated during 15 min in 0.5 M NH$_4$Cl in PBS (3 times), 10 min in PBS with 1% BSA, and allowed to react for 1 h with Ki-67 in the same medium. After washing the slide with PBS (3 times, 10 min), a peroxidase-conjugated rabbit anti-mouse IgG antibody (Dakopatts, Glostrup, Denmark) was applied for 2 h, and cells were washed again in PBS and fixed with 2% GA in 0.1 M cacodylate buffer (pH 7.4). After another PBS wash step, peroxidase activity was detected with DAB (10 min), and cells were postfixed for 30 min with reduced OsO$_4$. After dehydration, a gelatin capsule was placed upon the monolayer, and cells were embedded in epoxy resin (Epon) and routinely processed for electron microscopy.

Morphometrical Analysis. Nuclear projection areas were measured by tracing the profiles of labeled nuclei on a graphic tablet (MOP-AMO3; Kontron, Echingen, FRG), using a Leitz microscope provided with a camera lucida drawing tube. About 25 to 50 nuclei of cells showing a specific Ki-67 staining pattern were measured.

Frequency distributions of staining patterns were determined by tracing the nuclei that traversed the image field while systematically scanning the slide until 250 to 500 nuclei were encountered.

Estimation of Growth Fractions. Growth fractions of both cell lines were estimated from continuous BrdUrd-labeling curves by extrapolating the more slowly rising component of each curve backwards to the end of the G$_2$ phase (see Fig. 7). The duration of G$_2$ was estimated by scoring the appearance of BrdUrd label in mitotic figures.

Flow Cytometry. Cells were carefully scraped from approximately one-fifth part of the area of the slides, frozen, stored in citrate buffer (pH 7.6) with 10% dimethyl sulfoxide, and prepared for flow cytometry by using the method as described by Vindelov et al. (32). After staining with propidium iodide (Sigma, St. Louis, MO), samples were measured on an ICP 22 flow cytometer (Ortho, Westwood, MA).

RESULTS

Fixation and Staining Conditions. In order to optimize intranuclear Ki-67 staining in MCF-7 monolayers, we evaluated the effects of different fixation protocols followed by a standard peroxidase staining. Results are summarized in Table 1. Immunoreactivity was not detectable after fixation with formalin, glutaraldehyde, or acid-containing fixatives. In contrast, intense staining was obtained after fixation with paraformaldehyde or acetone. Since most intense staining was obtained after fixation in cold acetone, this protocol was used in further experiments. We also compared a number of immunohistochemical staining protocols, namely, the peroxidase-antiperoxidase technique, incubation with biotinylated rabbit anti-mouse IgG followed by incubation with an avidine-biotin-peroxidase complex, and intensification of diaminobenzidine/H$_2$O$_2$ color development with cobalt chloride. Since these different protocols did not change the criteria with respect to the assessment of weakly positive cells (data not shown), the indirect immunoperoxidase assay as described in “Materials and Methods” was used as standard.

Morphology and Cell Cycle Distribution. The staining patterns as observed in nuclei of MCF-7 human breast cancer cells have tentatively been arranged in Fig. 1.

These highly characteristic patterns were also readily observed in cov362.c14 ovarian cancer cells (Fig. 2). Postmitotic daughter nuclei were easily recognized (Figs. 1 and 2, upper lanes); these nuclei were of relatively small size and always displayed many, intensely stained spots. Measurements of nuclear areas and frequencies of defined staining patterns in postmitotic nuclei of MCF-7 cells, summarized in Table 2, are suggestive of a remarkable rearrangement of the antigen during the telophase. As the heavily stained anaphase chromosomes decondense (and nuclei increase in size), they form a pattern of apparently homogeneously distributed speckles (Fig. 1B). The low frequency of this pattern suggests that the small spots quickly aggregate (Fig. 1C) to form larger, irregularly shaped clusters (Fig. 1D), eventually forming nucleoli-like structures (Fig. 1E). This staining seems more intense on the nucleolar rim, a phenomenon particularly evident in the relatively large nucleoli of cov362.c14 cells. The association of the antigen with nucleolar structures is also illustrated by electron micrographs (Fig. 3), showing an MCF-7 nucleus with many DAB deposits, which have a tendency to cluster around distinct regions (A), and two neighboring cells (B), in one of which the nucleoli are surrounded by a thin layer of electron-dense DAB.

| Table 1 Effect of various fixatives on Ki-67 immunoreactivity in MCF7 monolayers |
|----------------------------------------|-------------------------------|
| **Fixative**                            | **Immunoreactivity**          |
| Buffered formalin (4%, 10 min, RT)      | –                             |
| Bouin’s fluid (10 min, RT)              | –                             |
| Glutaraldehyde (2.5%, 10 min, RT)       | –                             |
| Carnoy’s fixative (10 min, RT)          | +/-                           |
| Ethanol (70%, 5 min –20°C, 100%, 5 min RT) | +                             |
| Methanol (100%, 5 min –20°C, 5 min RT) | –                             |
| PLP (2%, 10 min RT)                     | ++                            |
| Paraformaldehyde (2%, 10 min RT)        | +++                           |
| Acetone (100%, 5 min –20°C, 5 min RT)   | +++                           |

- Scored as negative (–), weakly positive in a limited number of cells (+/–), moderately positive (+), strongly positive (++), and very strongly positive (+++).
CELL CYCLE DISTRIBUTION OF Ki-67 ANTIGEN IN MCF-7 CELLS

Fig. 1. Ki-67 staining patterns in MCF-7 cells. The upper lane shows a putative sequence of cells during mitosis (A) and early G1 phase (B to F). In the middle lane nuclei are shown in which staining is absent (G) or confined to nucleoli (H to L). Finally, in the lower lane nuclei are shown in which the antigen is present in both nucleolar structures and the nuclear matrix. The last nucleus in this series represents a cell in prophase, characterized by chromosome condensation (R). × 500.

Fig. 2. Ki-67 staining patterns observed in cov362.cl4 cells. The antigen distribution is essentially the same as in MCF-7 cells (compare with Fig. 1), showing an association with chromosomes during mitosis (A), a clustering during telophase and early G1 phase (B and C), confinement to nucleoli in many cells (D), and the homogeneous darkening of the nucleus commensurate with a distortion and/or enlargement of the nucleolus (E and F). Mitotic cov362.cl4 cells frequently display many small spots around the clustered chromosomes (A and B, arrows). × 500.

In the largest fraction of exponentially growing MCF-7 cells, the expression of Ki-67 was restricted to a limited number of relatively small nucleoli (Fig. 1, middle lane). With increasing nuclear size, however (Table 2), staining became more intense and was found in the nuclear matrix as well (see also Fig. 1, lower lane). Also the nucleolar size increased dramatically, most often showing a highly distorted (worm-shaped) morphology. Occasionally we observed that the large, irregular clusters were granulated, possibly representing a disintegration of nucleoli (Figs. 1P and 2E). A relatively small fraction of nuclei, which were most intensely stained (Figs. 1, Q and R, and 2F), also appeared to have the largest mean nuclear projection area (Table 2). These nuclei probably represent the transition into prophase, in which the chromatin condenses into metaphase chromosomes (Fig. 1R).

Prevention of chromosomal segregation by Colcemid (an alkaloid which inhibits polar migration of centrioles) did not appear to affect the redistribution process of the antigen during telophase and early G1 phase (data not shown).

In order to evaluate the frequency and patterns of Ki-67 staining during S phase, we labeled MCF-7 cultures during 20 min with BrdUrd and subsequently performed an immunocytochemical double staining for Ki-67 antigen and DNA-incorporated BrdUrd. In a previous paper (33), we described that the characteristic patterns of incorporated BrdUrd reflect the spatiotemporal organization of DNA synthesis. Early S phase cells are characterized by the presence of 100 to 150 distinct replication sites, homogeneously distributed over the nuclear projection area, but with a low density close to the nuclear boundary and nucleoli (Fig. 4A). During mid-S phase, nuclei show a relatively large number of closely spaced spots (Fig. 4B), being particularly prominent in perinucleolar chromatin. In late S phase, spots are larger, irregularly shaped, and preferentially located close to the nuclear boundary and the nucleoli (Fig. 4C). The application of a double staining procedure enabled us to allocate Ki-67 staining patterns to these stages of DNA replication (Fig. 4, D to F; Table 3). Surprisingly, in a majority of cells in early S phase, the Ki-67 staining was either faint or undetectable, suggesting that, during late G1 phase, the antigen is expressed at a lower level or is even absent in actively proliferating cells. In contrast, in late S phase, a majority of cells showed large, intensely stained nucleoli of irregular shape and an increased nuclear matrix staining, therefore demonstrating a higher expression of Ki-67 antigen.

A different approach to evaluate the cell cycle distribution of the Ki-67 antigen is to analyze synchronized cell populations. For this purpose, MCF-7 cells were treated with TAM, resulting in a significant accumulation of cells in the G1 phase (Fig. 5, upper lane). Release of the G1 block by 17β-estradiol induced a wave of cells to enter the cell cycle, resulting in an accumulation in early S phase 17 h later. This was accompanied by a relative increase of lightly stained nuclei. Another 12 h later, cells had reached late S phase and G2 phase, evidently showing an increased expression of the antigen (Fig. 5, lower lane).
Treatment with TAM resulted in a decrease in the fraction of Ki-67-positive cells (Fig. 6A) and pronounced accumulation of weakly stained cells. As a result of replacement at 72 h with fresh culture medium with 20 mM BrdUrd was not different from that of unlabeled control cultures, suggests that BrdUrd at this concentration did not affect cell growth significantly (data not shown). Results of all continuous labeling experiments have been summarized in Table 4. In all experimental conditions, Ki-67 positivity appeared higher than the GFs as determined by the graphical method. This difference was only minor (3.5%) in the ovarian cancer cell line, but varied from 6 to 14.3% in the exponentially growing MCF7 cells. However, in MCF7 cultures growing under less optimal conditions, larger discrepancies were found. In contrast, a very high linear correlation was observed between GFs and S-phase fractions (Fig. 8).

**DISCUSSION**

In order to provide a reliable estimate of tumor GFs based upon immunohistochemical detection of proliferating cell-specific antigens in a single tissue sample, two requirements should be fulfilled. (a) The antigen should be continuously present during the cell cycle of all cell types. (b) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (c) The antigen should not be continuously present during the cell cycle of all cell types. (d) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (e) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (f) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (g) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (h) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (i) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (j) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (k) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (l) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (m) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (n) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (o) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (p) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (q) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (r) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (s) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (t) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (u) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (v) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (w) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (x) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (y) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled, (z) A transition to whatever type of nonproliferating state from any part of the cell cycle should be fulfilled.
Fig. 3. Transmission electron micrographs of Ki-67-stained MCF-7 cells. Nucleus showing various DAB spots dispersed over the nuclear matrix (A, arrows). Ki-67-negative and -positive cell with DAB staining at nucleolar boundaries (B, arrows). Counterstained with uranyl acetate and lead citrate; A, x. 8000; B, x. 1400.

Fig. 4. Patterns observed in MCF-7 cells in S phase, labeled with BrdUrd for 20 min. BrdUrd staining only (A to C), and BrdUrd staining in combination with Ki-67 staining. The BrdUrd patterns shown represent early S phase (A, D), mid-S (B, E), and late S phase (C, F), respectively. Ki-67-positive nucleoli are indicated (arrows). x 500.

Table 3 Ki-67 staining intensity of nucleoli in S-subphases of BrdUrd-labeled exponentially growing MCF-7 cells

<table>
<thead>
<tr>
<th>Ki-67 staining of nucleoli</th>
<th>Nuclear BrdUrd patterns*</th>
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<tbody>
<tr>
<td></td>
<td>Early S</td>
</tr>
<tr>
<td>Not detectable</td>
<td>36</td>
</tr>
<tr>
<td>Weakly stained</td>
<td>54</td>
</tr>
<tr>
<td>Medium, round</td>
<td>8</td>
</tr>
<tr>
<td>Medium, irregular</td>
<td>0</td>
</tr>
<tr>
<td>Strongly, round</td>
<td>0</td>
</tr>
<tr>
<td>Strongly, irregular</td>
<td>0</td>
</tr>
</tbody>
</table>

* See Fig. 4; 100 nuclei per BrdUrd pattern.

Fig. 5. Partial synchronization of MCF-7 cells. Top, flow cytometric DNA histograms of untreated control cells, cells treated during 72 h with 10^-6 M TAM, and cells recruited from the TAM-induced G0 state by treatment with 10^-7 M 17β-estradiol for 17, 29, and 45 h, respectively. Bottom, frequency distributions of staining patterns in Ki-67-positive nuclei in the following categories (see also Table 2): I, mitotic cells; II, nuclei in early G1 phase; III, antigen restricted to nucleolus, but not detectable in nuclear matrix; IV, large, intensely stained nucleoli in a stained nuclear matrix. At each time interval, 250 randomly chosen labeled nuclei were evaluated (control, 500 nuclei). Comparing the subsequent changes in the proportions of specific categories, all differences appeared statistically significant (P < 0.05), with the exception of differences between control and 72-h TAM in Categories I, II, and IV, and between 72-h TAM and 17-h 17β-estradiol in Category IV. E2, 17β-estradiol.

In postmitotic daughter nuclei, enlargement of the spots simultaneously with a decrease in their number is associated with an increase in nuclear size, therefore suggesting a continuous process of antigen clustering during chromosomal decondensation (preferably around prenucleolar bodies and nucleolar organizing regions). Neither in MCF-7 nor in cov362.c14 cell populations we could observe weakly stained early G1 nuclei, even if GF was significantly reduced as a result of starvation or treatment with TAM, indicating that cells tend to progress through this postmitotic phase before entering the G0 phase or becoming an end cell.

Ki-67-negative nuclei in exponentially growing MCF-7 cultures were relatively small, suggesting a G1 DNA content. However, double staining with anti-BrdUrd revealed that a certain fraction of these cells could be assigned to early S phase (Table 3), which has a frequency of about 3% in exponentially growing MCF-7 cells (33). It is possible therefore that all cells are negative at the onset of DNA replication, but then quickly start to express the antigen. However, an alternative explanation is possible as well. It has been found that mouse fibroblasts complete growth factor-dependent processes leading to commitment for proliferation during the first 3 to 4 h of G1 phase (denoted G1pm phase) and subsequently may either enter a state of quiescence (G0) or a growth factor-independent pre-
Cell cycle distribution of Ki-67 antigen in MCF-7 cells

Fig. 6. Effects on the frequency of Ki-67-positive cells after treatment of exponentially growing MCF-7 cultures with $10^{-7}$ M TAM (A), with $10^{-7}$ M MTX (B), and in unfed plateau-phase cultures (C). Depicted are the total fraction of positive nuclei stored (○) and the total fraction corrected for very weakly stained nuclei (●).

After 72 h in a series of TAM-treated cultures, tissue culture medium was replaced by medium containing $10^{-7}$ M 17β-estradiol (A, ●). Inset in B, flow cytometric DNA histogram after 58 h of MTX treatment. C, ●, fractions of BrdUrd-positive cells after a 20-min pulse labeling period. E2, 17β-estradiol. Bars, SD.

Fig. 7. A, continuous BrdUrd labeling curves of exponentially growing cov362.c14 cells (●) and MCF-7 cells (○). B, comparison of continuous labeling curves of MCF-7 cells growing in log phase (●), in 5% FCS (○), and $10^{-7}$ M TAM (●). C, Ki-67 positivity (●) during continuous labeling (●) of exponentially growing MCF-7 cells. □ in A and C, 20-min pulse labeling of MCF-7 cells at various time intervals (⋯⋯⋯⋯⋯⋯). –––––, linear extrapolation of the slowly rising part of the BrdUrd curves to the end of G2 phase; the intersect is used as an estimation of GF.

Table 4: Comparison of Ki-67 positivity with the GF as estimated from continuous BrdUrd-labeling curves of cov362.c14 cells and MCF-7 cells, growing under various conditions.

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>KF</th>
<th>Minus weakly stained GF</th>
<th>SF</th>
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<tbody>
<tr>
<td>cov362.c14, 10% FCS</td>
<td>99.5 ± 0.9</td>
<td>94.3 ± 3.1 (3)</td>
<td>96 50</td>
</tr>
<tr>
<td>MCF-7, 10% FCS</td>
<td>99.3 ± 0.3</td>
<td>96.8 ± 1.0 (2)</td>
<td>85 45</td>
</tr>
<tr>
<td>MCF-7, 5% FCS</td>
<td>97.7 ± 0.8</td>
<td>91.5 ± 2.0 (10)</td>
<td>84 44</td>
</tr>
<tr>
<td>MCF-7, 10% FCS</td>
<td>85.0 ± 4.2</td>
<td>71.0 ± 0.8 (2)</td>
<td>79 39</td>
</tr>
<tr>
<td>MCF-7, 10% FCS</td>
<td>98.5 ± 0.2</td>
<td>92.8 ± 1.2 (5)</td>
<td>62 32</td>
</tr>
<tr>
<td>MCF-7, 10% FCS</td>
<td>89.2 ± 1.1</td>
<td>72.6 ± 9.3 (2)</td>
<td>60 31</td>
</tr>
<tr>
<td>MCF-7, $10^{-7}$ M TAM</td>
<td>84.3 ± 10.8</td>
<td>55.9 ± 13.8 (4)</td>
<td>26 14</td>
</tr>
</tbody>
</table>

*a* Estimated by linear extrapolation of BrdUrd-labeled fractions at various time intervals departing from 24 h (see Fig. 7).

*b* SF, S-phase fraction; obtained by BrdUrd labeling during 20 min and corrected for progression of cells into G2 phase.

*c* Exponentially growing cell culture.

*d* Mean ± SD of percentages obtained at various time intervals within 24 h of starting continuous BrdUrd labeling.

*e* Numbers in parentheses, number of cultures evaluated.

*f* Confluent culture (see Fig. 7A). GF in this culture was estimated from the linear correlation between SF and GF (see Fig. 8).

DNA-synthetic part of G1 (G1ps), the duration of which shows a large intercellular variability (36). If Ki-67 antigen expression diminishes with a similar rate from cells which have entered G0 as from cells which have entered a G1ps phase, then the amount of nucleolar staining observed in early S phase may be determined by variation in the duration of the latter phase. In cancer types depending on exogenous hormones or growth factors as mitotic stimuli, it may then be impossible to discriminate between slowly cycling cells and cells arrested in G0 phase.

The level of antigen expression increases from an undetectable or low nucleolar staining in early S phase to intense staining of both nucleoli and nuclear matrix in late S phase and G2 phase (Table 3; Fig. 5). There is ample evidence suggesting that a number of constituents of the nucleolus may be involved in the process of condensation and decondensation of chromosomes (37, 41). It has been suggested recently that the antigen for Ki-67 might be type II DNA topoisomerase (27), an enzyme which is also a structural part of the chromosome scaffold (42), but although its presence in interphase nuclei has been confirmed, a nucleolar association has not been described (43). In contrast, the ribosomal S1 protein is both associated with the interphase nucleolus and distributed over chromo-
somal surfaces during mitosis (44). Moreover, in mitotic cells, distinct S1-containing granules have been described, which were scattered over the cell plasma and therefore resembling the granules we observed in cov36.2.c14 cells (Fig. 2, A and B, arrows).

However, within the scope of the potential application of PCN antigens in cell cycle kinetics, speculation about their nature seems relevant only if it gives us a better insight into the regulation of their expression in different cell types. Since cells may be arrested in all stages of the cell cycle (45), it is seems particularly important to study conditions and patterns of antigen reduction at various positions of the cycle.

In MCF-7 cultures, a small fraction of the nuclei, which had a size and nucleolar shape characteristic of G1 phase, appeared only moderately positive for Ki-67, therefore possibly representing G1-arrested cells.

Treatment of cultures with MTX prevented an increase in cell number (data not shown) and resulted in an accumulation of cells with an S-G2 DNA content (see Fig. 6B, inset). Although no significant change in the fraction of Ki-67-positive cells was observed up to 5 days of MTX treatment, after 2 days the total amount of antigen steadily decreased, as judged from an increase in the number of weakly stained nuclei (Fig. 6B). Further studies are needed to establish whether a decrease in the level of antigen expression in late S or G2 phase signifies an inevitable progression to cellular death or whether these cells are still able to restore antigen levels in order to enter prophase.

Treatment with the antiestrogenic compound TAM, used to block a majority of MCF-7 cells specifically in G1 phase (probably by preventing a hormonally induced commitment for proliferation in G1 pm), resulted after 3 days in a dramatic reduction in the fraction of cells with an S and G2 DNA content (as demonstrated by flow cytometric DNA analysis; Fig. 5). However, only a modest reduction in Ki-67 positivity was observed during this period (Fig. 6A). When cells were recruited from the TAM-induced G0 state by replacing the tissue culture medium by fresh medium containing 17β-estradiol, within 30 to 50 h the frequency distribution of Ki-67 positivity was completely restored. These data suggest that, in cells temporally blocked in G1 phase, the Ki-67 antigen expression only slowly decreases.

This discrepancy between the actual proliferation rate and the fraction of KF was further substantiated by BrdUrd labeling experiments. As stressed by Steel (34), in growing cell populations continuous labeling curves do not plateau at a level indicating GF, since if nonproliferating cells are continuously produced, they will originate also from the labeled compartment. Therefore, GFs were estimated by extrapolating the more slowly rising component of continuous labeling curves backwards to the end of G1 phase.

The values thus found showed a highly significant linear correlation with S-phase fractions measured at the beginning of the labeling experiments (Fig. 8). However, no such correlation was found between GFs and KFs. Furthermore, KF was invariably higher than GF, a difference being particularly large in cell populations growing under suboptimal conditions.

These results indicate that, in MCF-7 human breast cancer cells, it is not possible to unambiguously determine GFs by evaluation of nuclear Ki-67 staining patterns, since the rate of decrease of antigen expression in cells, which have entered a quiescent state, is slow compared to the proliferation rate. On the other hand, since antigen expression seems to be down-regulated during G1 phase, tumor cells with relatively long G1 ps durations may have an undetectably low expression of the antigen and therefore can easily be judged as noncycling. Further experiments are needed to evaluate whether or to what extent these phenomena are a general problem with respect to other cell types and/or other PCN antigens.

Sasaki et al. (46) have recently derived a (linear) relationship between KFs and BrdUrd labeling indices obtained from various tumor types pulse labeled in vitro. Although their statement that, in human solid tumors, GFs can be estimated from S-phase fractions (and vice versa) may be true to a certain degree, their a priori assumption that Ki-67 staining accurately reflects GFs (and therefore S-phase fractions) does not seem to be substantiated by our results. As shown particularly in perturbed cell populations, the discrepancy between KF and actual GF may be quite large.

One should bear in mind though that, especially in neoplasms like breast cancer, accurate data on GFs may have only limited meaning, as these tumors may consist of relatively small proliferative pools and display a broad range of intermitotic times among tumor cells (47).

Immunohistochemical detection of PCN antigens may then be important in characterizing the proliferative activity of primary tumors and lymph node metastases within a histological context. Particularly if combined with immunohistochemical staining of other proliferation markers, e.g., enzymes involved in DNA replication (48), this may provide a useful point-in-time estimate of the fraction of tumor cells assumed to be most sensitive to proliferation-dependent chemo- and radiotherapy.

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