Synchronization in the cell cycle by inhibitors of DNA replication induces histone H2AX phosphorylation: an indication of DNA damage

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Abstract. Several methods to synchronize cultured cells in the cell cycle are based on temporary inhibition of DNA replication. Previously it has been reported that cells synchronized this way exhibited significant growth imbalance and unscheduled expression of cyclins A and B1. We have now observed that HL-60 cells exposed to inhibitors of DNA replication (thymidine, aphidicolin and hydroxyurea), at concentrations commonly used to synchronize cell populations, had histone H2AX phosphorylated on *Ser*-139. This modification of H2AX, a marker of DNA damage (induction of DNA double-strand breaks; DSBs), was most pronounced in S-phase cells, and led to their apoptosis. Thus, to a large extent, synchronization was caused by selective kill of DNA replicating cells through induction of replication stress. In fact, similar synchronization has been achieved by exposure of cells to the DNA topoisomerase I inhibitor camptothecin, a cytotoxic drug known to target S-phase cells. A large proportion of the surviving cells ‘synchronized’ by DNA replication inhibitors at the G₁/S boundary had phosphorylated histone H2AX. Inhibitors of DNA replication, thus, not only selectively kill DNA replicating cells, induce growth imbalance and alter the machinery regulating progression through the cycle, but they also cause DNA damage involving formation of DSBs in the surviving (‘synchronized’) cells. The above effects should be taken into account when interpreting data obtained with the use of cells synchronized by inhibitors of DNA replication.

INTRODUCTION

Often, there is a need to obtain populations of cells synchronized in the cell cycle, and numerous approaches have been developed to accomplish this goal (reviews: Grdina *et al.* 1987; Merril

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1998; Davis *et al.* 2001; Amon 2002). Each of these methods offers some advantages but also has limitations. One of the classical and widely used approaches is based on selection of mitotic cells that detach from flasks during culturing (Terasima & Tolmach 1963). Its advantage stems from the fact that the procedure does not perturb cell progression through the cycle. However, the technique is applicable to relatively few cell lines, for example, HeLa or Chinese hamster ovary (CHO) cells, that grow attached in cultures. A further technique that selects mitotic cells has been designed for cells that otherwise grow in suspension; their enforced attachment to polylysine-coated membranes leads to production of daughter cells that detach from the membranes during mitosis (Thornton *et al.* 2002; Edward *et al.* 2004). This method has only recently been introduced and it is unknown whether it can be applied to wide range of cell lines as several lines do not strongly attach to polycation surfaces (Huang *et al.* 2005a). Some cell lines can be synchronized in G₁ by inhibitors of protein farnesylation and geranyl-geranylation such as statins (Jakóbsiak *et al.* 1991), or protein kinase inhibitors such as staurosporine (Crissman *et al.* 1991; Bruno *et al.* 1992). These techniques also are not universally applicable because many cell lines do not respond to statins or kinase inhibitors by arrest in G₁.

Non-tumour cells can be synchronized in G₀ or early G₁ by withdrawal of either growth factors ('serum starvation') (Griffin 1976), or a particular amino acid (Tobey *et al.* 1990), or by contact inhibition (Holley & Kiernan 1968). These approaches generally fail to synchronize most tumour lines. Furthermore, the metabolism of cells synchronized this way is often perturbed which affects their rate of progression through the cycle (Pardee & Keyomarsi 1992). It should also be noted that when cell populations are synchronized in early G₁ or at mitosis they lose synchrony during progression through G₁ and therefore become less synchronous while in S or G₂.

Centrifugal elutriation offers the possibility to select uniformly sized cells, and because cell size generally correlates with cell cycle stage, these cells are synchronized with respect to their position in the cycle (Mitchell & Tupper 1977). This procedure, like mitotic detachment, also does not perturb cell cycle progression. It requires, however, rather complex and expensive instrumentation and an experienced operator. Furthermore, the synchrony of cell populations obtained through elutriation is not always sufficiently narrow. Density gradient centrifugation has several problems and generally yields cell populations less synchronized than the elutriation procedure (Cymerman & Beer 1980). Once again complex and expensive instrumentation (a cell-sorting flow cytometer) is needed to select cells based on differences in their DNA content after staining with Hoechst 33342 (Juan *et al.* 2002). The dye Hoechst 33342 elicits long-term toxicity (Zhang *et al.* 1999), and undergoes redistribution from labelled to unlabelled cells in mixed cell populations (Mohorko *et al.* 2005).

Synchronization of cell populations at mitosis by using mitotic spindle poisons such as colchicines, vinca alkaloids or nocodazole has been widely used (Samake & Smith 1996; Harper 2005). The advantage of these methods is simplicity, low cost and high degree of synchrony when one desires to obtain mitotic or immediately post-mitotic cell populations (Darzynkiewicz *et al.* 1982). As mentioned, however, the populations become less synchronous after progression through G₁. Cytotoxicity and other undesirable effects of such synchronization procedures, including growth imbalance, have been reported (Urbani *et al.* 1995; Chou & Chou 1999; Piotrowska *et al.* 2000).

The use of agents that inhibit DNA replication such as hydroxyurea (HU), high concentration of thymidine (TH), methotrexate or aphidicolin (AP) are the most commonly used approaches to obtain populations of synchronized cells, particularly in S phase (Tobey & Crissman 1972; Vogel *et al.* 1978; Fox *et al.* 1987; Matherly *et al.* 1989). A combination of these agents with other treatments that synchronize cells is also widely used (Kues *et al.* 2000; Harper 2005). The virtue of cell synchronization by DNA replication inhibitors is, similar to mitotic blockers, their

simplicity and low cost. A plethora of undesirable side effects, however, plague this methodology. The major drawback of all methods utilizing DNA synthesis inhibitors is the induction of unbalanced growth (Cohen & Studzinski 1967; Urbani *et al.* 1995). While cells exposed to these inhibitors are arrested in their progression through S, their growth, in terms of RNA and protein synthesis, continues which leads to severe perturbation of metabolic functions. Cell cycle regulatory machinery of cells arrested in this way is also severely perturbed, as reflected by unscheduled expression of cyclin proteins (Gong *et al.* 1995a).

We have recently observed that inhibitors of DNA replication such as hydroxyurea and aphidicolin induce phosphorylation of histone H2AX on Ser-139 in S-phase cells (Kurose *et al.* 2006). Phosphorylated H2AX is defined as γ H2AX and this modification is considered to be an indicator of DNA damage that involves formation of DNA double-strand breaks (DSBs) (Rogakou *et al.* 1998; Sedelnikova *et al.* 2002). The aim of the present study was to explore in more detail the effects of HU, AP, and TH at concentrations commonly used in synchronization protocols, on histone H2AX phosphorylation. For comparison, we also treated cells with the DNA topoisomerase I inhibitor camptothecin (CP), an agent which selectively induces apoptosis of S-phase cells leaving the live subpopulation of cells 'synchronized' in G₁ (Del Bino *et al.* 1992). Multiparameter cytometry was used to correlate the induction of DSBs with the cell's position in the cycle. The data indicate that not only DNA replicating cells but also the cells arrested at the G₁/S boundary by these inhibitors have phosphorylated histone H2AX.

MATERIALS AND METHODS

Cell lines and culture conditions

HL-60 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). They were grown in 25-ml Falcon flasks (Becton Dickinson Co., Franklin Lakes, NJ, USA) in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine (all from Gibco/BRL Life Technologies, Inc., Grand Island, NY, USA) at 37 °C in an atmosphere of 5% CO₂ in air. At the onset of the experiments, there were fewer than 5×10^5 cells per millilitre in culture and the cells were at an exponential and asynchronous phase of growth. HeLa cells were obtained from ATCC and were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine (Gibco/BRL) at 37 °C in an atmosphere of 5% CO₂ in air. The cultures were diluted and re-plated every 4 days to maintain them in an asynchronous and exponential phase of growth. For experiments, the cells were trypsinized and seeded in 2-chambered Falcon Culture Slides (Beckton Dickinson Labware).

Evaluation of DNA damage after using synchronizing drugs

HL-60 cells were incubated with various concentrations of HU, AP, TH or CP (Sigma Chemical Co., St. Louis, MO, USA) for different time intervals, as described in the figure legends. The cells were then fixed with 1% methanol-free formaldehyde (Polysciences, Inc., Warrington, PA, USA) in PBS for 15 min on ice followed by suspension in 80% ethanol in which they were stored at -20 °C for 2–24 h.

G₁/S cell synchronization of HeLa cells using double thymidine block

HeLa cells were grown in 2-chambered Falcon Culture Slides to about 40% confluency. TH was added to a final concentration of 2 mM and the cells were incubated for 19 h. Cells were then

washed twice with PBS, and incubated with regular medium for 9 h before a second incubation in 2 mM TH for 16 h. The cells were then fixed on slides with 1% methanol-free formaldehyde in PBS for 15 min on ice followed by suspension in 80% ethanol, where they were stored at -20°C for 2–24 h.

Immunocytochemical detection of γH2AX phosphorylation

The fixed HL-60 cells were washed twice in PBS and suspended in 0.2% Triton X-100 (Sigma) in a 1% (w/v) solution of bovine serum albumin (BSA; Sigma) in PBS for 30 min to suppress non-specific antibody (Ab) binding. The cells were then incubated in 100 μl of 1% BSA containing 1 : 100 diluted antiphospho-histone H2AX (Ser-139) mAb (Upstate, Lake Placid, NY, USA) for 2 h at room temperature, washed twice with PBS and resuspended in 100 μl of 1 : 30 diluted FITC-conjugated F(ab')₂ fragment of goat anti-mouse immunoglobulin (DAKO, Carpinteria, CA, USA) for 30 min at room temperature in the dark. The cells were then counterstained with 5 $\mu\text{g/ml}$ PI in the presence of 100 $\mu\text{g/ml}$ of RNase A (Sigma) for 30 min. The fixed HeLa cells were washed twice in PBS and suspended in 0.2% Triton X-100 (Sigma) in a 1% (w/v) solution of BSA in PBS for 30 min. The cells were then incubated in 100 μl of 1% BSA containing 1 : 100 diluted antiphospho-histone H2AX (Ser-139) mAb, and were then incubated for 2 h at room temperature. The cells were washed twice in PBS and incubated with 1 : 30 diluted FITC-conjugated F(ab')₂ fragment of goat anti-mouse immunoglobulin for 30 min at room temperature in the dark. The cells were then counterstained with 1 $\mu\text{g/ml}$ 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR, USA) in PBS for 10 min.

Fluorescence measurements

Cellular green (FITC) and red (PI) fluorescence of HL-60 cells in suspension were measured using a FASCCan flow cytometer (Becton-Dickinson, San Jose, CA, USA). The red (PI) and green (FITC) fluorescence from each cell were separated and quantified using standard optics and CELLQuest software (Becton-Dickinson). Ten thousand cells were measured per sample. Cellular green (FITC) and blue (DAPI) fluorescence of HeLa cells attached to slides was measured using an iCys laser scanning cytometer (LSC) (CompuCyte, Cambridge, MA, USA) utilizing standard filter settings; fluorescence was excited with 488-nm argon ion and violet diode lasers, respectively. The intensities of maximal pixel and integrated fluorescence were measured and recorded for each cell. At least 3000 cells were measured per sample. All experiments were repeated at least three times. Other experimental details are presented elsewhere (Huang *et al.* 2004, 2005a,b; Kurose *et al.* 2006).

RESULTS

Figure 1 illustrates the effect of HU at 0.1–3.0 mM concentration on expression of γH2AX in HL-60 cells. It is evident from this set of γH2AX IF versus DNA content bivariate distributions that exposure of cells to HU led to apoptosis preferentially of S-phase cells. Apoptotic cells, as shown previously (Huang *et al.* 2004, 2005b), were characterized by markedly increased intensity of γH2AX IF reflecting phosphorylation of this histone on Ser-139 (Rogakou *et al.* 1998; Sedelnikova *et al.* 2002), and in the cultures treated with HU for 2 h had an S-DNA content. The presence of apoptotic cells in these cultures was confirmed visually by fluorescence microscopy of DAPI-stained cells (not shown). Apoptosis of S-phase cells was also reflected by the decline in percentage of mid-S-phase cells among the non-apoptotic cell population, from 12.9% in the

Figure 1. Changes in expression of γ H2AX in relation to cell cycle phase in HL-60 cells treated with 0.1, 1.0 or 3.0 mM HU for 2 or 4 h. Subpopulations of cells in G₁, S and G₂M phases are distinguished based on differences in their DNA content, as shown in the left panel (control). Apoptotic cells (Ap), identified based on their very high intensity of γ H2AX IF (Huang *et al.* 2004, 2005b), are located within the oval dashed gates. The dashed-line horizontal threshold shows the upper limit of γ H2AX immunofluorescence (IF) for the 95% of G₁ cells in the untreated culture (control). The cells with a G₁-DNA content in the HU-treated cultures that express γ H2AX IF above this threshold are located within the rectangular dashed gates. The percentage of cells within the mid-S DNA-content gate (DI = 1.3–1.7) as marked in control sample by the vertical dashed lines (g) was calculated among the non-apoptotic cell population for each culture (shown at the bottom of panels). The inset shows DNA content histogram of the cells in untreated culture.

untreated culture to 0.5% at 3 mM HU after 4 h of treatment. Due to progressive DNA fragmentation and partial extraction of the fragmented DNA (Gong *et al.* 1995b), some apoptotic cells had a sub-G₁ DNA content, which was apparent after 4 h. Also, as a result of exposure to HU, the remaining non-apoptotic S-phase cells, as well as some cells with a G₁ DNA content (i.e. arrested at G₁/S boundary), showed an increased degree of H2AX phosphorylation. Thus, a distinct subpopulation of cells with a G₁-DNA content and expression of γ H2AX higher than that of G₁ cells in untreated specimens (control) culture was apparent in the HU-treated cultures (Fig. 1; within rectangular dashed boxes).

The effect of cell treatment with AP on H2AX phosphorylation, in relation to the cell cycle phase of HL-60 cells, is presented in Fig. 2. Exposure of cells to AP led to an increase in γ H2AX IF and to the appearance of apoptotic cells. The AP-induced increase in γ H2AX IF was most pronounced for early S-phase cells, though, compared with cultures treated with HU, the frequency of apoptotic cells was lower in AP-treated cultures. Following the treatment with AP, as in cultures treated with HU, a subpopulation of cells that had higher intensity of γ H2AX IF than G₁ cells of the untreated culture was present at the G₁/S boundary.

Exposure of HL-60 cells to TH also led to apoptosis of S-phase cells and accumulation of cells with a G₁ DNA content having increased content of phosphorylated H2AX (Fig. 3). After 24-h exposure to TH nearly all cells at G₁/S boundary had γ H2AX IF higher than G₁ cells from the untreated culture. The two right panels in Fig. 3 present γ H2AX IF versus DNA content bivariate distributions of HeLa cells, control, and those subjected to double thymidine block, used to synchronize them at G₁/S phase. Unlike HL-60 cells that were measured in suspension by flow cytometry, HeLa cells were grown on microscope slides and their fluorescence was measured by LSC. The absence of apoptotic HeLa cells in the scattergrams reflects their selective detachment in cultures, known to occur at an early stage of apoptosis (Darzynkiewicz *et al.* 2001). It is quite evident that the double thymidine block was successful in 'synchronizing' HeLa cells at G₁/S interphase, with rather few cells remaining in S. However, it is also apparent that a large percentage of these 'synchronized' cells had increased levels of phosphorylated H2AX.

Figure 2. Expression of γ H2AX in relation to cell cycle phase in HL cells treated with 1 or 4 μ M AP for 2 or 4 h. Subpopulations of G₁, S and G₂M phase cells are identified as shown in the control. The presence of apoptotic cells (Ap) is apparent at 4 μ M AP. As in Fig. 1, the dashed-horizontal threshold defines the upper limit of γ H2AX IF for cells in G₁ in control. Note the presence of cells with a G₁ DNA content having γ H2AX IF increased above this threshold in AP-treated cultures. The inset presents DNA content histogram of the cells in untreated culture. See legend to Fig. 1 for further explanation.

Figure 3. Expression of γ H2AX in relation to the cell cycle phase in HL cells treated continuously with 2 mM TH, for 4, 8 or 24 h (4 left panels), and in HeLa cells synchronized in G₁ by double TH block (TH DB, 2 right panels). See legend to Fig. 1 for further explanation. The dashed-horizontal thresholds mark the upper limit of γ H2AX IF for untreated cells. The presence of cells with a G₁ DNA content having γ H2AX IF increased above this threshold (gated in rectangular windows) is apparent in all TH-treated cultures. The insets show DNA content histograms in the respective cultures. Fluorescence of HL-60 cells was measured by flow cytometry (γ H2AX IF plotted on log scale), of HeLa cells, which were growing on slides, by LSC (γ H2AX IF plotted on linear scale).

There was a striking similarity in the pattern of response of HL-60 cells to the DNA topoisomerase I inhibitor CP (Fig. 4), as compared to the changes induced by HU (Fig. 1). Namely, concurrent with phosphorylation of H2AX in S-phase cells, subsequent loss of S-phase cells within the non-apoptotic cell population, and with the appearance of apoptotic cells, there was induction of H2AX phosphorylation in cells arrested at G₁/S boundary.

DISCUSSION

The present data show that exposure of cells to concentrations of HU, AP and TH, at which these agents are being generally used to synchronize cells in the cell cycle, induced phosphorylation

Figure 4. Effect of CP at 50 or 250 nM concentration on H2AX phosphorylation in HL-60 cells. See legend to Fig. 1 for further explanation. Note the increase in expression of γ H2AX in S-phase cells as well as in the cells 'synchronized' at G_1/S boundary (gated in rectangular windows), concurrent with the appearance of apoptotic cells (AP), in the CP-treated cultures.

of histone H2AX on Ser-139. Most affected were the S-phase cells, as well as the cells that arrested at the G_1/S boundary. S-phase cells underwent apoptosis, and thus the 'synchronization', to a large extent, was generated by a selective kill of DNA replicating cells and by the arrest of cells at the entrance to S phase. Of all four inhibitors used in the present study, the frequency of apoptosis was lowest in cultures treated with AP, while HU and CP were the most effective killers of S-phase cells. The above findings are consistent with our earlier observation that HU and AP induces H2AX phosphorylation, followed by apoptosis, preferentially affecting S-phase cells (Kurose *et al.* 2006). The novelty of the present findings is that histone H2AX also is phosphorylated in cells 'synchronized' by these agents at the G_1/S boundary. Because H2AX phosphorylation is considered a specific marker of induction of DSBs (Rogakou *et al.* 1998; Sedelnikova *et al.* 2002), the data indicate that the synchronization procedure causes DNA damage that involves formation of DSBs in the population of synchronized cells. In fact, the exposure of cells to CP, a cytotoxic agent which targets DNA topoisomerase I, produced a response similar to that observed following cell treatment with DNA replication inhibitors. Therefore, inhibitors of DNA replication not only selectively kill DNA replicating cells (Kurose *et al.* 2006), induce growth imbalance (Cohen & Studzinski 1967; Urbani *et al.* 1995), and alter machinery regulating progression through the cycle (Gong *et al.* 1995a), but also cause DNA damage involving formation of DSBs in the surviving ('synchronized') cells. The damage to DNA in all probability triggers the signalling pathways activating the G_1/S DNA damage checkpoint (Lisby & Rothstein 2005). Cells synchronized by DNA replication inhibitors, thus, are significantly altered and in many respects do not represent cells at the G_1/S boundary from untreated cultures. These changes should be taken into an account when interpreting data obtained with the use of cell populations synchronized by these compounds.

Although different agents were used in the present study, the pattern of cell response in terms of H2AX phosphorylation and apoptosis vis-à-vis cell cycle phase was similar. HU is an inhibitor of ribonucleotide reductase, the enzyme which by converting ribonucleotide diphosphates to deoxyribonucleotide diphosphates, is essential for the *de novo* synthesis of all the DNA precursors (Yarbro 1992). By altering the ribo- and deoxyribonucleotide diphosphate pools, HU stalls DNA replication. AP is a specific inhibitor of DNA polymerase α (Ikegami *et al.* 1978). TH at the concentrations used to synchronize cells blocks DNA replication by altering the pool of deoxynucleotides. CP is a DNA topoisomerase I inhibitor (Thomas *et al.* 2004). The common trait of

all these agents was inhibition of DNA replication. As shown by Ward & Chen (2001), replicative stress, such as caused by DNA replication inhibitors, induces DNA damage involving formation of DSBs, which is reflected by phosphorylation of H2AX. In the case of CP, the collisions of DNA replication forks with the DNA-topoisomerase complexes stabilized by CP lead to formation of DSBs (Deptala *et al.* 1999; Liu *et al.* 2000). As the present data indicate, DNA was damaged not only in the cells that were already progressing through S phase at the time of administration of these agents but also in the cells that were initiating DNA replication but become arrested at the G₁/S boundary.

Our team has previously shown (Deptala *et al.* 1999) that the G₁ checkpoint is activated in MCF-7 cells arrested by CP, as manifest by induction of p53 and subsequent up-regulation of p21^{WAF1/CIP1}, which led to prolongation of cell progression arrest at the boundary. In the present study we have observed that the cells arrested at G₁/S by CP were entering S phase, but only 4 h after removal of the drug, when H2AX was already de-phosphorylated (data not shown). Apparently this was the minimal time during which DNA was repaired to the point that the cells could be released from checkpoint arrest. One would expect therefore that the cells with damaged DNA arrested at G₁/S boundary by HU, AP or TH, if they have a functional G₁/S checkpoint, also will be delayed before they resume progression through S. Indeed, such a delay was observed following prolonged cell arrest by HU or AP (Borel *et al.* 2002).

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REFERENCES

- Amon A (2002) Synchronization procedures. *Meth Enzymol.* **351**, 457–467.
- Borel F, Lacroix FB, Margolis RL (2002) Prolonged arrest of mammalian cells at the G₁/S boundary results in permanent S phase stasis. *J. Cell Sci.* **115**, 2829–2838.
- Bruno S, Ardelt B, Skierski JS, Traganos F, Darzynkiewicz Z (1992) Different effects of staurosporine, an inhibitor of protein kinases, on the cell cycle and chromatin structure of normal and leukemic lymphocytes. *Cancer Res.* **52**, 470–473.
- Chou LF, Chou WG (1999) Dna-end binding activity of Ku in synchronized cells. *Cell Biol. Int.* **23**, 663–670.
- Cohen LS, Studzinski GP (1967) Correlation between cell enlargement and nucleic acid and protein content of HeLa cells in unbalanced growth produced by inhibitors of DNA synthesis. *J. Cell. Physiol.* **69**, 331–339.
- Crissman HA, Gadbois DM, Tobey RA, Bradbury EM (1991) Transformed mammalian cells are deficient in kinase-mediated progression through the G₁ phase of the cell cycle. *Proc. Natl Acad. Sci. USA* **88**, 7580–7584.
- Cymerman U, Beer JB (1980) Some problems in using density gradient centrifugation for synchronization of L5178Y-S cells. *Neoplasma* **27**, 429–436.
- Darzynkiewicz Z, Bedner E, Traganos F (2001) Difficulties and pitfalls in analysis of apoptosis. *Methods Cell Biol.* **63**, 527–559.
- Darzynkiewicz Z, Crissman H, Traganos F, Stainkamp J (1982) Cell heterogeneity during the cell cycle. *J. Cell Physiol.* **112**, 465–474.
- Davis PK, Ho A, Dowdy SF (2001) Biological methods for cell-cycle synchronization of mammalian cells. *Bio-Techniques* **30**, 1322–1326.
- Del Bino G, Bruno S, Yi PN, Darzynkiewicz Z (1992) Apoptotic cell death triggered by camptothecin or teniposide: the cell cycle specificity and effects of ionizing radiation. *Cell Prolif.* **25**, 537–548.
- Deptala A, LiX, Bedner E, Cheng W, Traganos F, Darzynkiewicz Z (1999) Differences in induction of p53, 21^{WAF1/CIP1} and apoptosis in relation to cell cycle phase of MCF-7 cells treated with camptothecin. *Int. J. Oncol.* **15**, 861–871.

- Edward KL, Van Ert MN, Thornton M, Helmstetter CE (2004) Cyclin mRNA stability does not vary during the cell cycle. *Cell Cycle* **3**, 1057–1061.
- Fox MH, Read RA, Bedford JS (1987) Comparison of synchronized Chinese hamster ovary cells obtained by mitotic shake-off, hydroxyurea, aphidicolin, or methotrexate. *Cytometry* **8**, 315–320.
- Gong J, Traganos F, Darzynkiewicz Z (1995a) Growth imbalance and altered expression of cyclins B1, A, E and D3 in MOLT-4 cells synchronized in the cell cycle by inhibitors of DNA replication. *Cell Growth Differ.* **6**, 1485–1493.
- Gong J, Traganos F, Darzynkiewicz Z (1995b) A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal. Biochem.* **218**, 314–319.
- Grdina DJ, Meistrich ML, Meyn RE, Johnson TS, White RA (1987) Cell synchrony techniques. A comparison of methods. In: Gray JW, Darzynkiewicz Z, eds. *Techniques in Cell Cycle Analysis*. Humana Press, Clifton, New Jersey, pp. 367–402.
- Griffin MJ (1976) Synchronization of some human cell strains by serum and calcium starvation. *In Vitro* **12**, 393–398.
- Harper JV (2005) Synchronization of cell populations in G₁/S and G₂/M phases of the cell cycle. *Methods Mol. Biol.* **296**, 157–166.
- Holley RW, Kiernan MA (1968) 'Contact inhibition' of cell division in 3T3 cells. *Proc. Natl Acad. Sci. USA* **60**, 300–304.
- Huang X, Dai W, Darzynkiewicz Z (2005a) Enforced adhesion of hematopoietic cells to culture dish induces endomitosis and polyploidy. *Cell Cycle* **4**, 801–805.
- Huang X, Halicka HD, Traganos F, Tanaka T, Kurose A, Darzynkiewicz Z (2005b) Cytometric assessment of DNA damage in relation to cell cycle phase and apoptosis. *Cell Prolif.* **38**, 223–243.
- Huang X, Okafuji M, Traganos F, Luther E, Holden E, Darzynkiewicz Z (2004) Assessment of histone H2AX phosphorylation induced by DNA topoisomerase I and II inhibitors topotecan and mitoxantrone and by DNA crosslinking agent cisplatin. *Cytometry* **58A**, 99–110.
- Ikegami JA, Taguchi T, Ohashi M, Oguro M, Nagano H, Mano Y (1978) Aphidicolin prevents mitotic cell division by interfering with activity of DNA polymerase- α . *Nature* **275**, 458–460.
- Jakóbsiák M, Bruno S, Skierski J, Darzynkiewicz Z (1991) The cell cycle specific effects of lovastatin. *Proc. Natl Acad. Sci. USA* **88**, 3628–3632.
- Juan G, Hernandez E, Cardon-Cardo C (2002) Separation of live cells in different phases of the cell cycle for gene expression analysis. *Cytometry* **49**, 170–175.
- Kues WA, Anger M, Carnawath JW, Paul D, Motlik J, Niemann H (2000) Cell cycle synchronization of porcine fetal fibroblasts: effects of serum deprivation and reversible cell cycle inhibitors. *Biol. Reprod.* **62**, 419.
- Kurose A, Tanaka T, Huang X, Traganos F, Dai W, Darzynkiewicz Z (2006) Effects of hydroxyurea and aphidicolin on phosphorylation of ATM on Ser 1981 and histone H2AX on Ser 139 in relation to cell cycle phase and induction of apoptosis. *Cytometry* **69A**, 212–221.
- Lisby M, Rothstein R (2005) Localization of checkpoint and repair proteins in eukaryotes. *Biochimie* **87**, 579–589.
- Liu LF, Desai SD, Li TK, Mao Y, Sun M, Sim SP (2000) Mechanism of action of camptothecin. *Ann. N. Y. Acad. Sci.* **922**, 1–10.
- Matherly LH, Schuetz JD, Westin E, Goldman ID (1989) A method for the synchronization of cultured cells with aphidicolin: application to the large-scale synchronization of L1210 cells and the study of the cell cycle regulation of thymidylate synthase and dihydrofolate reductase. *Anal. Biochem.* **182**, 338–345.
- Merrill GF (1998) Cell synchronization. *Methods Cell Biol.* **57**, 229–249.
- Mitchell BF, Tupper JT (1977) Synchronization of mouse 3T3 and SV40 3T3 cell by way of centrifugal elutriation. *Exp. Cell Res.* **106**, 351–355.
- Mohorko N, Kregar-Velikonja N, Repovs G, Gorenssek M, Bresjanac M (2005) An *in vitro* study of Hoechst 33342 redistribution and its effects on cell viability. *Hum. Exp. Toxicol.* **24**, 573–580.
- Pardee AB, Keyomarsi K (1992) Modification of cell proliferation with inhibitors. *Curr. Opin. Cell Biol.* **4**, 186–191.
- Piotrowska K, Modlinski JA, Korwin-Kossakowski M, Karasiewicz J (2000) Effects of preactivation of ooplasts or synchronization of blastomeres nuclei in G₁ on preimplantation development of rabbit serial nuclear transfer embryos. *Biol. Reprod.* **63**, 677–682.
- Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.* **273**, 5858–5868.
- Samake S, Smith LC (1996) Synchronization of cell division in eight-cell bovine embryos produced *in vitro*: effects of nocodazole. *Mol. Reprod. Dev.* **44**, 486–492.
- Sedelnikova OA, Rogakou EP, Panuytin IG, Bonner W (2002) Quantitative detection of ¹²⁵IUdr-induced DNA double-strand breaks with γ -H2AX antibody. *Radiation Res.* **158**, 486–492.
- Terasima T, Tolmach LJ (1963) Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. *Exp. Cell Res.* **30**, 344–362.
- Thomas CJ, Rahier NJ, Hecht SM (2004) Camptothecin: current perspectives. *Bioorg. Med. Chem.* **12**, 1585–1604.

- Thornton M, Edward KL, Helmstetter CE (2002) Production of minimally disturbed synchronous cultures of hematopoietic cells. *BioTechniques* **32**, 10598–10605.
- Tobey RA, Crissman HA (1972) Use of flowmicrofluorimetry in detailed analysis of effects of chemical agents on cell cycle progression. *Cancer Res.* **32**, 2726–2732.
- Tobey RA, Oishi N, Crissman HA (1990) Cell cycle synchronization: Reversible induction of G₂ synchrony in cultured rodent and human diploid fibroblasts. *Proc. Natl Acad. Sci. USA* **87**, 5104–5108.
- Urbani L, Sherwood SW, Schimke RT (1995) Dissociation of nuclear and cytoplasmic cell cycle progression by drugs employed in cell synchronization. *Exp. Cell Res.* **219**, 159–166.
- Vogel W, Schempp W, Sigwarth I (1978) Comparison of thymidine, fluorodeoxyuridine, hydroxyurea, and methotrexate blocking at the G₁/S phase transition of the cell cycle, studied by replication patterns. *Hum. Genet.* **45**, 193–198.
- Ward IM, Chen J (2001) Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J. Biol. Chem.* **276**, 47759–47762.
- Yarbro JW (1992) Mechanism of action of hydroxyurea. *Semin. Oncol.* **19**, 1–10.
- Zhang X, Chen J, Davis B, Kiechle F (1999) Hoechst 33342 induces apoptosis in HL-60 cells and inhibits topoisomerase I *in vivo*. *Arch. Pathol. Lab. Med.* **123**, 921–927.