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Life, Death and Genomic Change in Perturbed Cell Cycles

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Life, death and genomic change in perturbed cell cycles

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SUMMARY

HeLaS3 cells undergo apoptosis after 18–24 h of cell cycle stasis irrespective of the agent employed (colcemid, aphidicolin, cis-platin). At high drug concentrations apoptosis occurs in cells arrested in the cell cycle in which the drug is applied and at a cell cycle position dependent on the mechanism of drug action. At low concentrations (or short exposure times) cells undergo apoptosis after progressing through an aberrant mitosis and only after 18 h of cell cycle stasis in a 'pseudo G1/S' cell cycle position. Aberrent mitoses result in miltipolar mitoses, chromosomal breakage and interchromosomal concatenation events. We propose that the ability of cells to delay progression into aberrent mitosis, as well as their propensity to undergo apoptosis, are important determinants of clinical cytotoxicity. We also suggest that apoptosis plays an important role in preventing the generation of aneuploidy and recombination and rearrangement events commonly associated with cancer.

1. CELL CYCLE STASIS AND APOPTOSIS

We have analysed how various chemotherapeutic agents induce apoptosis, employing agents with different mechanisms of action: (i) colcemid (mitotic spindle blocking agent); (ii) aphidicolin (DNA synthesis inhibitor); and cis-platin (DNA damaging agent) which alter various aspects of cell cycle progression (Sherwood *et al.* 1994*a*, *b*). We have analysed HeLaS3 cells both in a long-term high dose exposure protocol, typical of cytotoxicity studies with cultured cells, as well as low dose or short term, high dose exposure protocols that are more comparable to those employed clinically, i.e. exposures of the order of 12–24 h duration.

2. HIGH DOSE–CONTINUOUS EXPOSURE

Figure 1 shows flow cytometric analyses of HeLaS3 cells exposed to concentrations of colcemid (figure 1*a*) and aphidicolin (figure 1*b*) that are completely inhibitory (see Sherwood *et al.* 1994*a*, *b*). In both instances, cells are 'arrested' at a cell cycle position consistent with the mechanism of drug action (early S-phase for aphidicolin, metaphase for colcemid). Cells undergo apoptosis only after a period of cell cycle stasis of 18 h. Apoptosis is discerned in flow cytometry by a reduction in cell size and an apparent decreased in DNA content per cell (arrows). Apoptosis has been documented by microscopic examination of such cells and the demonstration of DNA ladder formation. High dose cis-platin arrests cells in early S-phase from which they undergo apoptosis after an 18 h period of cell cycle stasis (data not shown).

3. LOW DOSE–CONTINUOUS EXPOSURE, OR HIGH DOSE–SHORT TIME EXPOSURE

With drug exposure protocols that more closely mimic clinical treatment régimes, cells undergo apoptosis only after progressing through mitosis. Such mitoses are highly abnormal, and cells remain in a 'pseudo G1/S' position for 18 h prior to commencing apoptosis. Figure 2 shows results with low continuous colcemid (figure 2*a*) or high colcemid for 12 h followed by its removal (figure 2*b*). In both cases (arrows) a population of microcells is generated with less than G1 content. These 'cells' when newly generated contain normal nuclear membranes, decondensed chromatin and exclude vital dyes. They are largely the result of multipolar mitoses as discussed below. Such microcells are unable to progress into a subsequent cell cycle and commence apoptosis 18 h after their generation (Sherwood *et al.* 1994*b*). Low doses of cis-platin likewise allow cells to undergo mitosis, but the resulting G1 cells are unable to progress through a subsequent cell cycle and undergo apoptosis 18 h following mitosis from an early S-phase position (Sherwood *et al.* 1994*b*).

The above results show that the detailed mechanisms of cytotoxicity differ based on the concentration of inhibitory agent employed. At high doses, cell cycle stasis occurs in the same cell cycle in which the agent is applied. Low dose or short exposures of a drug provokes aberrent mitotic events, the consequence of which is cell cycle stasis in G1 or early S-phase. In both instances apoptosis does not occur until cells have been in cell cycle stasis for 18 h.

Figure 3 shows HeLaS3 cells exposed to concentrations of aphidicolin that inhibit progression through

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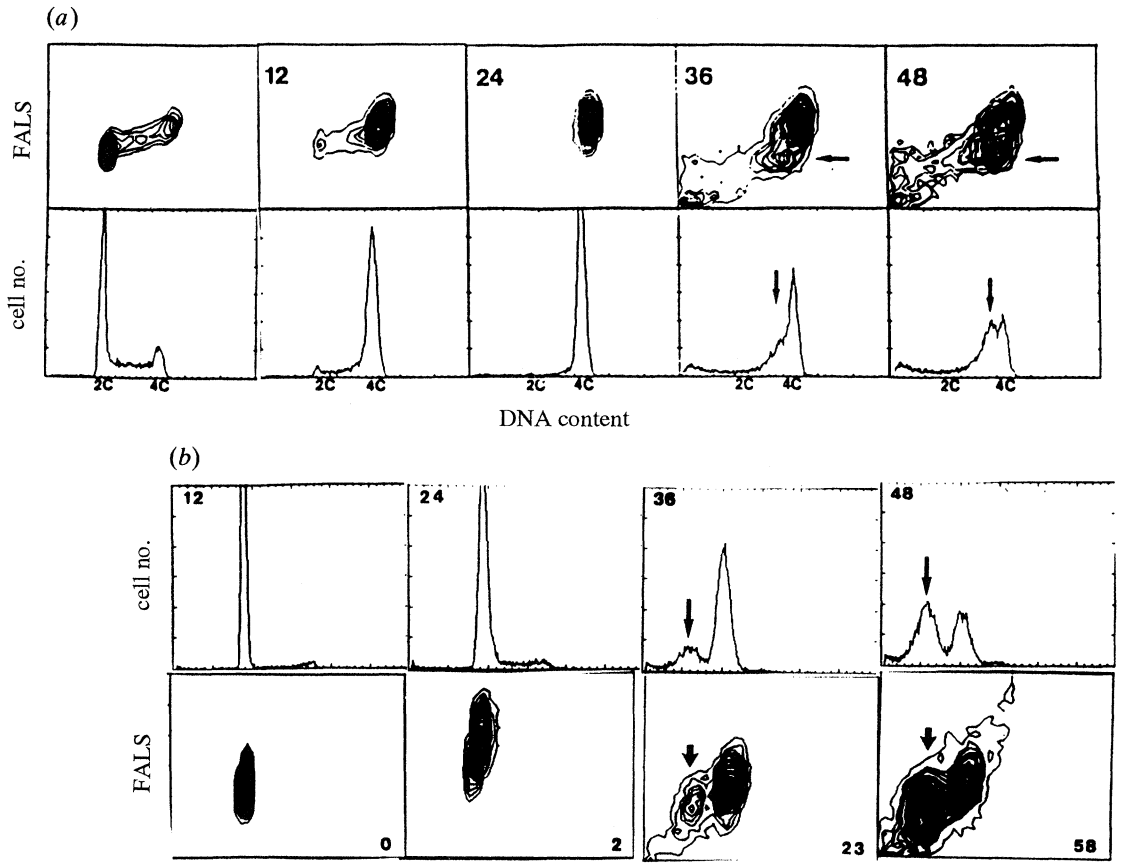

Figure 1. Cell cycle progression and apoptosis at (*a*) high colcemid and (*b*) aphidicolin doses. (*a*) Progression of asynchronous HeLaS3 cells (cell cycle time normally is 18 h) in the presence of 70 ng ml^{-1} colcemid (complete inhibition of mitotic spindle formation and metaphase arrest (Kung *et al.* 1990)). Apoptosis can discerned by a 'reduction' in DNA content per cell and a decrease in cell size-FALS (arrows). DNA is stained with propidium iodide. (*b*) Effects of 5 ng ml^{-1} aphidicolin (complete inhibition of DNA synthesis) in mitotically synchronized cells. Apoptosis is evident at 36 h by a reduction in cell size and DNA content (arrows). The numbers in the lower box indicate the number of apoptotic cells determined microscopically (condensed chromatin).

S-phase to different degrees. At 0.20 ng ml^{-1}, cells progress to a 4C DNA content with an extensive delay (normal HeLaS3 cell cycle time is 18 h) but eventually cells undergo mitosis (60 h) with extensive generation of microcells (small arrow). Although such > 2C 'particles', as detected by flow cytometry, appear similar to those generated at high aphidicolin concentrations (figure 1*b*), microscopic examination indicates high aphidicolin 'particles' are apoptotic cells (chromatin condensation, DNA degradation, permeable membranes) as opposed to microcells. Thus, microscopic examination is necessary to define the nature of > 2C 'particles' which are commonly observed by flow cytometric analyses as cells undergo apoptosis. At progressively higher aphidicolin concentrations (figure 3), the rate of progression through S-phase is proportionally delayed and progressively more cells undergo apoptosis (lower panel). Cells undergoing apoptosis at all concentrations constitute a population derived from early S-phase (large arrows). This finding suggests the existence of a critical early S-phase cell cycle position in which cells are particularly susceptible to undergoing apoptosis. If cells pass beyond this point, they progress, albeit slowly, through the remainder of S-phase without undergoing apoptosis. However, cells that 'escape' the early S-phase apoptotic position are subject to aberrent mitoses at such time as they undergo mitosis (beyond the time frame of this data) and may undergo apoptosis following an 18 h period of cell cycle stasis as a consequence of aberrent mitotic events (see below).

4. MITOTIC ABERRATIONS AND CELL CYCLE PERTURBATIONS

The consequence of mitotic aberrations is the potential generation of daughter cells with incomplete chromosome complements. Depending on time and concentration parameters, all agents studied (colcemid, aphidicolin, cis-platin) can result in such mitotic aberrations. Any cell, or cell population, may have combinations of such aberrations. Examples are shown in figure 4 and are all derived from cells treated with a low concentration of aphidicolin (0.3 ng ml^{-1}).

(*a*) *Multipolar mitoses*

Figure 4*a*,*b* shows mitotic HeLas3 cells stained with a beta-tubulin antibody or a centrosome antibody respectively (see arrows). Figure 4*a* shows the formation of multiple spindles, and figure 4*b* shows

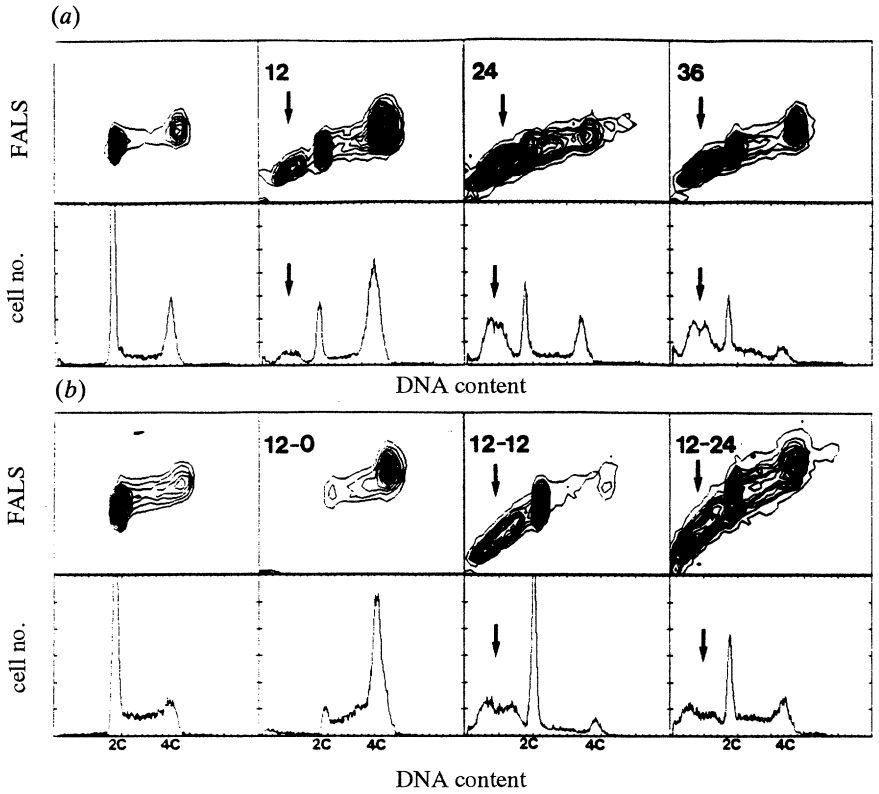

Figure 2. Cell cycle progression of asynchronous HeLaS3 cells in (*a*) continuous presence of 15 ng ml^{-1}, a concentration that does not completely disrupt spindle assembly, and (*b*) exposure to 70 ng ml^{-1} colcemid for 12 h, followed by its removal. Arrows indicate the generation of microcells (see text). Although such 2C 'particles' appear similar in flow cytometry to those generated with APC (figure 1*b*), they are not apoptotic when first generated (see text).

the aberrent metaphase positioning of condensed chromosomes with three centrosomes distributed such that this cell will undergo a tripolar mitosis following karyokinesis and cytokinesis. We suggest that during a slow progression through the cell cycle, centrosome replication/splitting and movement are altered to produce multipolar mitoses (Kreyer *et al.* 1984; Sluder *et al.* 1986).

(*b*) *Chromosomal interconcatenation and aberrent karyokinesis and cytokinesis*

Figure 4*c* shows a typical metaphase from aphidicolin-treated HeLaS3 cells in an attempted metaphase anaphase transition (no mitotic spindle blocking agent was employed). The abnormalities in this metaphase are many, of which we wish to emphasize the inability of chromosomes to separate (both chromatids as well as unrelated chromosomes) which will result in the inability of chromosomes to undergo proper segregation. We interpret this type of finding to be the result of interchromatid and interchromosome concatenation. Although the existence of interchromatid concentration has been recognized to occur during mitosis of cells treated with topoisomerase II inhibitors (Downs *et al.* 1991), we observe such a phenomenon with virtually any agent that inhibits cell cycle progression. In addition to interchromatid concatenation, concatenation also occurs between unrelated chromosomes. Interchromosomal concatenation has not been emphasized previously, although its existence has been reported (Schmid *et al.* 1983; Rose & Holm 1993). Our observations lead us to conclude that interchromosomal concatenation is a common result of perturbation of cell cycle progression that contributes to eventual cell death. This metaphase (figure 4*c*) also shows chromosomal breakage and the suggestion of attempted multipolar segregation. Thus, various aberrations of chromosome and chromatid integrity and spindle formation can occur in the same metaphase. Figure 4*d* shows a typical consequence of chromosome concatenation in G1 daughter cells following mitosis, where nuclear morphology is abnormal, cells contain micronuclei, and where daughter cells cannot separate completely because of concatenated DNA stretching (arrow) across of the cytokinetic furrow. Extensive concatenation can result in the inability of cells to undergo karyokinesis and cytokinesis during an attempted mitosis, or a variable karyokinesis such that most of the chromosomes are segregated into a single daughter cell. Such cells appear in flow cytometry as 4C cells which can progress into further cell cycles. Figure 4*e* shows such a cell which contains a large, abnormally lobulated nucleus. Note also that this cell contains micronuclei indicative of either chromosome loss or chromosome fragment loss from the nucleus following nuclear membrane reassembly.

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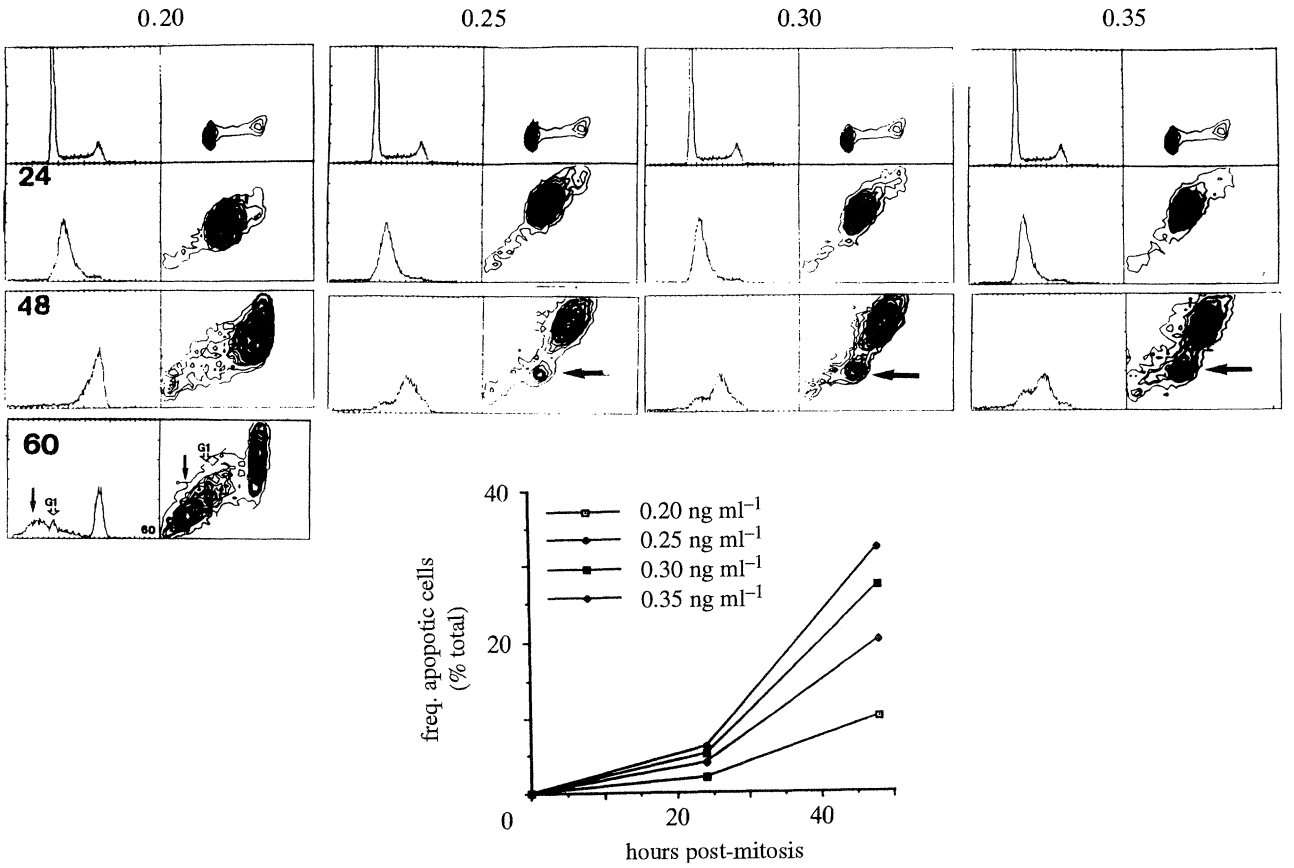

Figure 3. Flow cytometric analysis of cell cycle progression and apoptosis as a function of graded inhibition of DNA synthesis by varied aphidicolin concentrations. Asynchronous HeLaS3 cells were treated continuously with varying concentrations (ng ml^{-1}) of aphidicolin. The large arrows indicate apoptotic cells within the cell populations. The small arrow (at low APC concentration) constitutes predominantly microcells following a delayed mitoses. The lower panel shows quantification of apoptotic cells (chromatin condensation: apoptotic bodies visualized by light microscopy). Cell sorting confirmed that apoptotic cells are those indicated by the large arrows.

Such cells are not subject to apoptosis following mitosis (as opposed to those which go through karyokinesis and cytokinesis). We attribute this difference to the fact that such tetraploid or aneuploid cells contain a genome complement that can sustain further cell proliferation, whereas aberrent G1 and microcells do not have complete genome complements. We suggest that this process may be an important means of generating aneuploidy as it occurs in cancers.

(*c*) *Chromosome breakage*

Variable degrees of chromosome breakage or presence of extrachromosomal DNA are also characteristic findings of cells treated with various agents that result in slow progression towards and into mitosis. The metaphase of figure 4*c* shows chromosome breakages (see also Schimke *et al.* 1988; Sherwood *et al.* 1988). Although such breaks are typically attributed to the direct action of an agent, we suggest that chromosome breaks can also be accounted for as the result of aberrent progression into mitosis *per se* prior to completion of chromosome condensation and chromosome deconcatenation (see Kung *et al.* 1993).

5. THE IMPORTANCE OF THE MITOTIC CHECKPOINT IN CELL SURVIVAL AND IN GENOME STABILITY

Our results emphasize the role of mitosis in cell death and genomic stability, in particular when cells are subject to discontinuous and low exposure régimes. We suggest that transient exposures to carcinogenic agents may do likewise. Thus, the potential ability of cells to prevent aberrent mitoses may be important in clinical chemotherapy resistance and in maintaining genome stability (Schimke *et al.* 1991; Hartwell 1993). The mitotic checkpoint, as defined in yeast, involves the 'sensing' of completion of DNA synthesis, chromosome and mitotic spindle integrity to delay or prevent aberrent or lethal mitoses. In the absence of such 'sensors', yeast are reproductively non-viable following aberrent mitotic events (Weinert & Hartwell 1987; Murray 1992).

Checkpoint controls analogous to those of yeast exist in mammalian cells. Whereas a number of human cell lines, including HeLaS3, have mitotic checkpoint properties analogous to those of yeast, rodent cell lines often lack such mitotic checkpoint control properties (Kung *et al.* 1990, 1993; Schimke 1991). Perhaps most striking is cytotoxicity of CHO,

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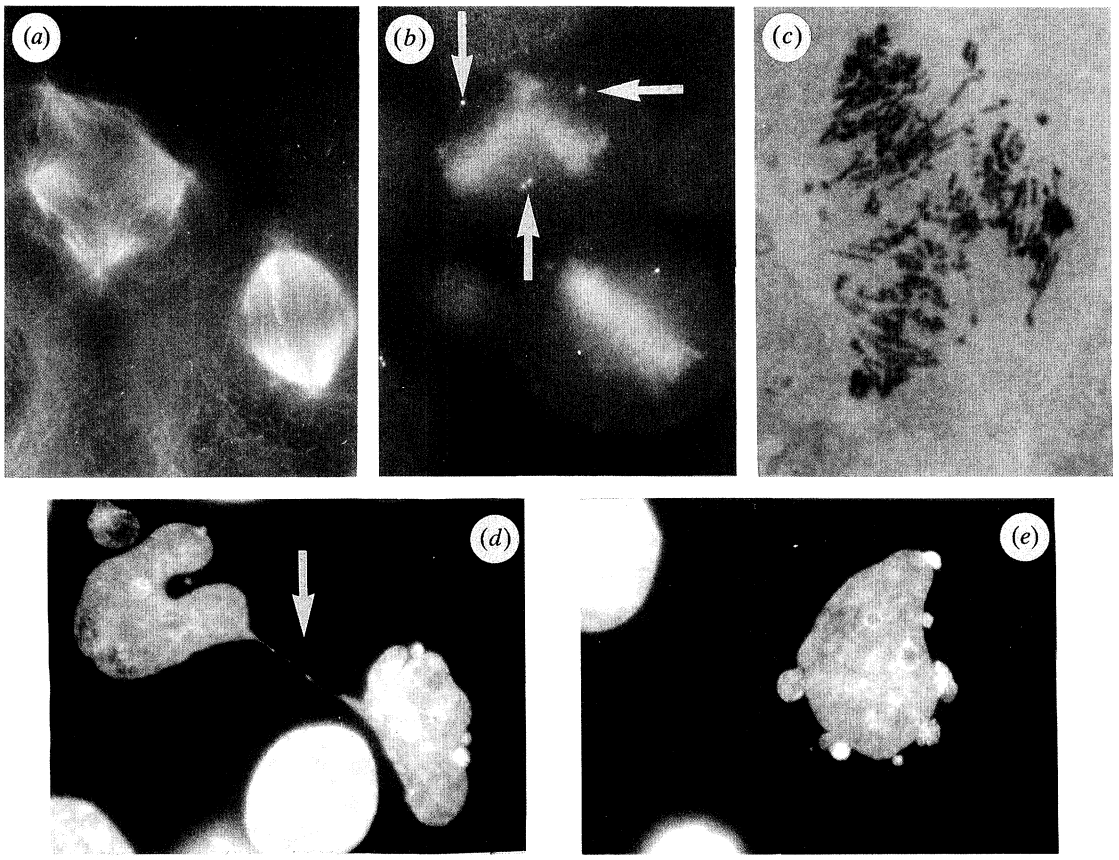

Figure 4. Mitotic aberrations resulting in HeLaS3 cells following delayed progression through a cell cycle. Cells were treated with 0.25 $\mu g\,ml^{-1}$ aphidicolin. (*a*) Mitotic cells stained with anti-tubulin, showing multiple centres of mitotic spindle nucleation in one of two cells. (*b*) Mitotic cell stained with an anti-centrosome antibody. The rhodamine stain shows aberrent chromosome positioning at the metaphase plate, as well as centrosomes placed abnormally (arrows). (*c*) A metaphase–anaphase transition (giemsa). This spread shows a composite of a number of abnormalities including: (i) inability of chromosomes to separate properly; (ii) lack of attachment of chromosomes to mitotic spindles; and (iii) extensive chromosomal breakage. (*d*) Daughter cells which have undergone incomplete karyokinesis and cytokinesis and which are attached through DNA strands (arrow) (Hoechsts 33342). The G1 nuclei are abnormal. Note also several micronuclei of varying sizes. (*e*) A HeLaS3 cell that has progressed through mitosis, but has failed to successfully undergo karyokinesis or cytokinesis (Hoechsts 33342). Note in this cell the presence of micronuclei, as well as a large and lobulated nucleus.

but not HeLaS3 cells, to an 18 h period of complete inhibition of DNA synthesis by aphidicolin. Death in CHO cells is a consequence of aberrent mitoses following removal of aphidicolin and the difference between HeLaS3 and CHO cells is attributed to the a down-regulation of overall protein synthesis in HeLaS3, but not CHO cells, during a period of inhibition of DNA synthesis. CHO cells accumulate cyclin B to mitotically competent levels during S-phase inhibition whereas HeLaS3 cells do not. As a consequence, CHO cells undergo aberrent (early) mitoses whereas HeLaS3 do not (Kung *et al.* 1993). Thus, the control of progression into an aberrent mitosis constitutes the lethal event. One consequence of aberrent mitoses that result from an 18 h exposure to high aphidicolin in CHO cells is the generation of extensive chromosomal breakage and an increased frequency of gene amplification in the clonogenic survivors of such a treatment (Sherwood *et al.* 1988; Schimke *et al.* 1988).

Intactness of the mitotic checkpoint is important in two contexts related to cancer biology based on our studies with cultured cells.

(*a*) *Cytotoxicity in relation to resistance*

When (if) cancers are exposed to short-term or minimally effective drug concentrations, aberrent mitoses are a central feature of cytotoxicity. Therefore the capacity of cells to 'withstand' entry into potentially lethal mitoses within the dose–time frame of clinical drug administration constitutes a mechanism(s) of resistance. We wish to point out that among such parameters of mitotic regulation are: (i) down regulation of cyclin B accumulation (Kung *et al.* 1993); (ii) intactness of a G2 (radiation) checkpoint (O'Conner & Kohn 1992); (iii) ability to deconcatenate DNA; and (iv) lack of centrosome replication or splitting during delayed cell cycle progression. These properties vary among cell lines we have studied (only HeLaS3 data have been presented herein), and likely vary in cancers as well. Thus, resistance mechanisms

as they occur in cancers may be determined, in part, by how cells respond to an inhibitory agent as opposed to current concepts of resistance mechanisms that equate resistance with mechanisms that prevent drug action. We note that the latter mechanisms have generally been elaborated in experimental cell culture systems subjected to high, continuous drug exposure protocols.

(*b*) *Genome instability*

We propose that when cells are slowed down in progression through a cell cycle, among the consequences is chromosome breakage and altered karyokinesis. Altered karyokinesis can result in aneuploidy, a central theme of many cancers. In addition, chromosomal breakage is proposed to be an initial event in recombinational processes resulting in gene amplification in many models (Schimke *et al.* 1988; Windle & Wahl 1992; Chi *et al.* 1993), another common theme in cancers. We have recently reported a correlation between the propensity for gene amplification in different cultured mammalian cell lines and their mitotic checkpoint properties (Sharma & Schimke 1994).

6. APOPTOSIS: THE ULTIMATE 'REPAIR' FOR PERTURBED CELL CYCLES

Within the context of cancer biology, all apoptotic deaths are desired (Kerr *et al.* 1972). We suggest that, in addition to mitotic checkpoint properties (see above), the inherent ability of cells to undergo apoptosis within clinical chemotherapy exposure times may be an additional, critical determinant in successful chemotherapy (Vaux 1993). We have found that cell lines commence apoptosis subsequent to metaphase arrest (colcemid) at times varying from 6 h to 30 h (L. Kim & L. Yilmaz, unpublished results). Interestingly, cell lines that undergo apoptosis most rapidly are predominately derived from bone marrow precursors (B and T cells) and embryonal cells. This finding suggests that success in cancer treatment may, in part, be a function of ability to initiate apoptosis within the constrained time of patient exposure to drugs and may be dependent on the expression state of genes affecting apoptosis, including Bcl-2 (Sentman *et al.* 1991) and p53 (Yonish-Rouach *et al.* 1991; Lowe *et al.* 1993).

In addition, we suggest that the ease of initiating apoptosis during cell cycle perturbation may also play a role in preventing genomic instability. In our studies with HeLaS3 cells, the treatment conditions most conducive to the generation of aneuploidy and chromosome breakage involve a slowing down of cell cycle progression. Although our studies have concentrated on use of aphidicolin, virtually all agents that interact with DNA (i.e. mutagenic agents) also slow down DNA synthesis and result in aberrent mitotic events. We note (see figure 3) an early S-phase cell cycle position from which cells undergo apoptosis readily. If cells progress past this cell cycle position, they can ultimately progress into aberrent mitoses and potentially generate aneuploidy and/or recombinational chromosome repair, including deletions, gene amplifications and translocations. The vast majority of such breakage and recombination events will be lethal or neutral. However, an occasional event may contribute to cancer progression. Thus, we suggest that the ability of cells to undergo apoptosis prior to entry into aberrent mitoses when cell cycle progression is altered by any variety of agents constitutes an important means of maintaining genome stability. By virtue of removing potentially 'dangerous' cells, apoptosis constitutes the ultimate 'repair' process for multicellular organisms where loss of any single proliferating cell is inconsequential to the organism. Whether p53 plays such a role in its facilitation of apoptosis under our conditions of cell cycle perturbation is under current study. It is intriguing to suggest that p53 may play a role in facilitating apoptosis at a G1/S cell cycle position where Kastan *et al.* (1991) have shown a role of p53, a cell cycle position which our data with aphidicolin suggests to be a critical position for apoptosis when cells are progressing slowly through a cell cycle.

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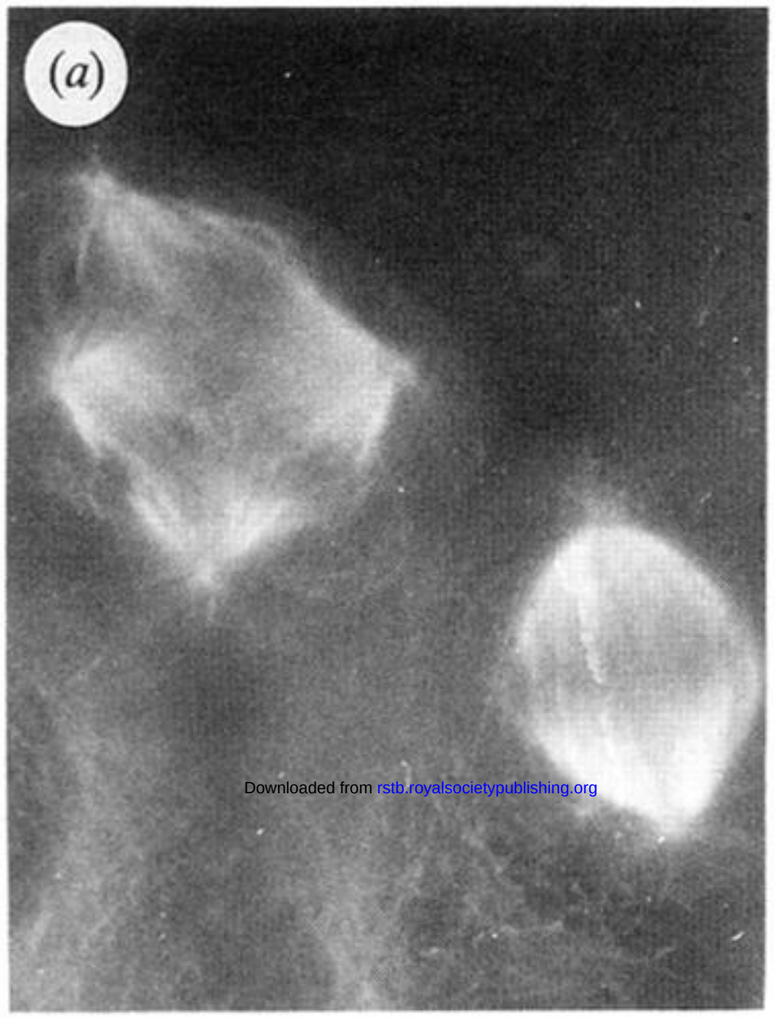

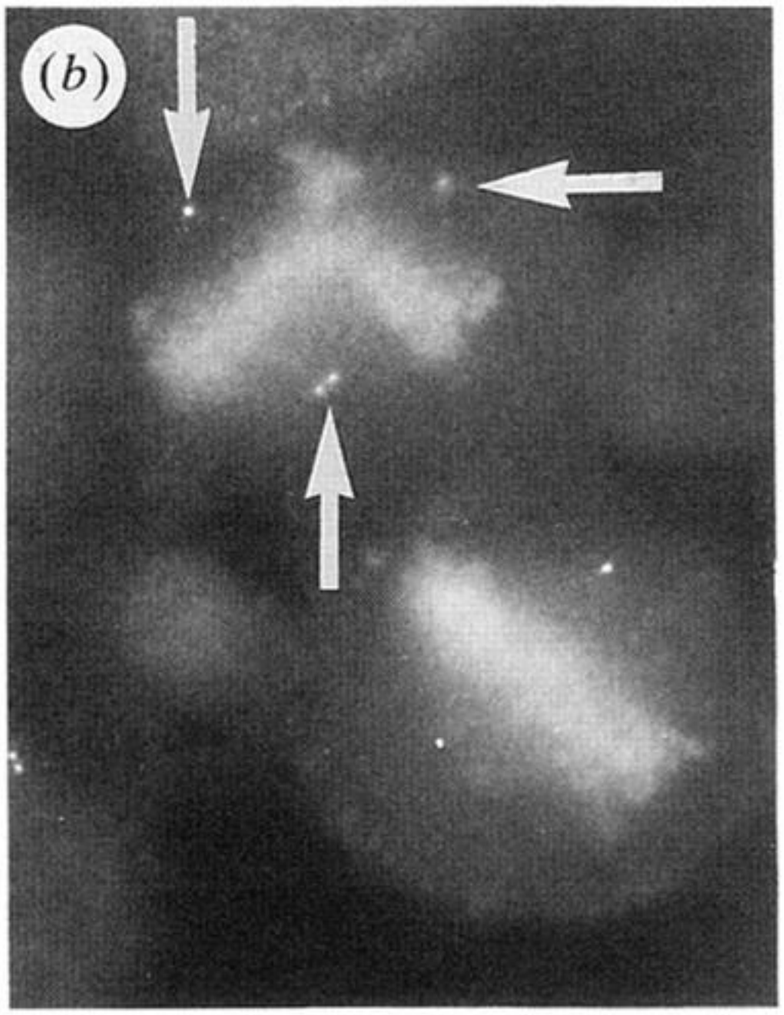

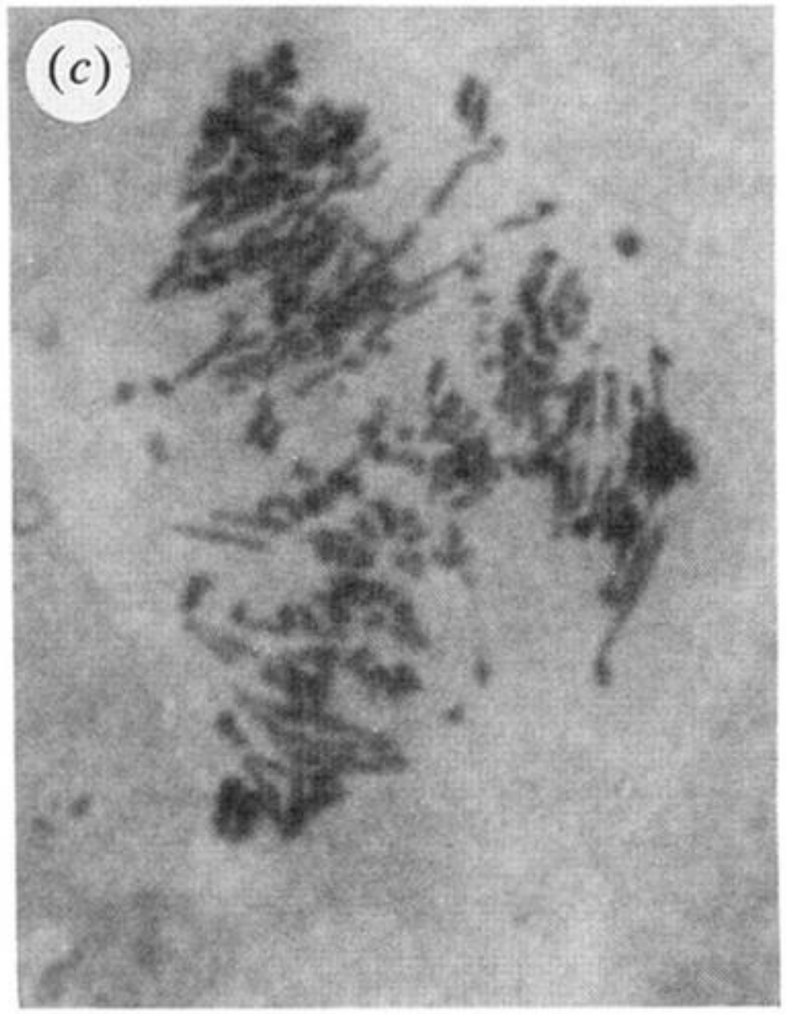

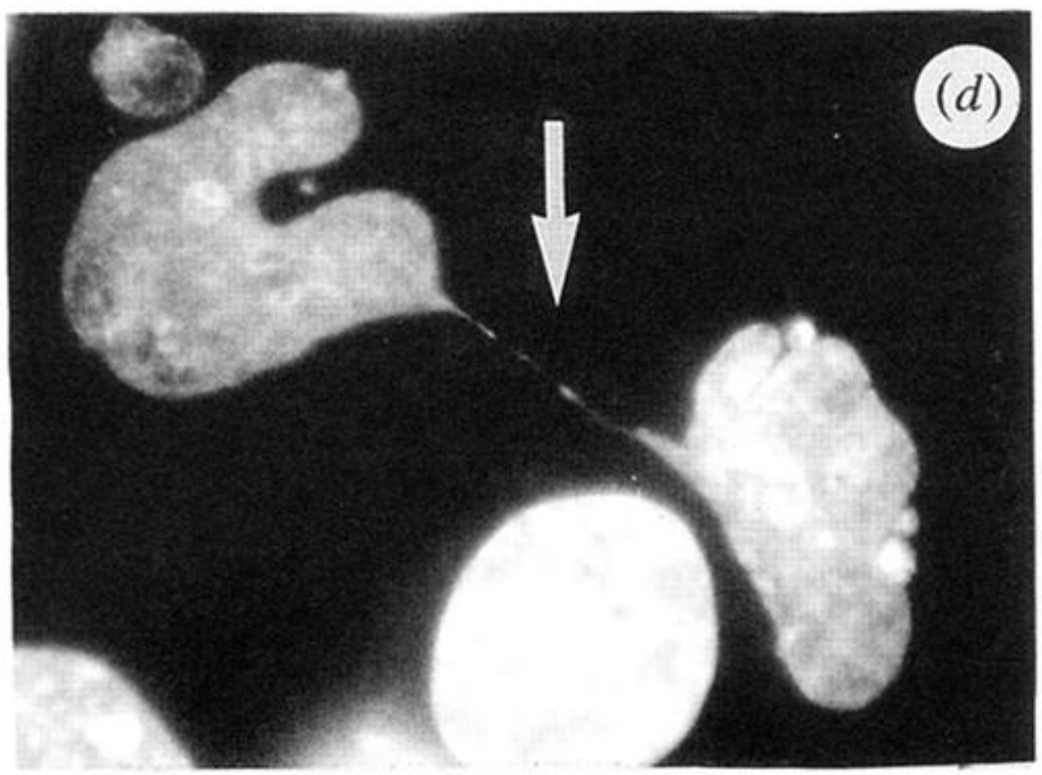

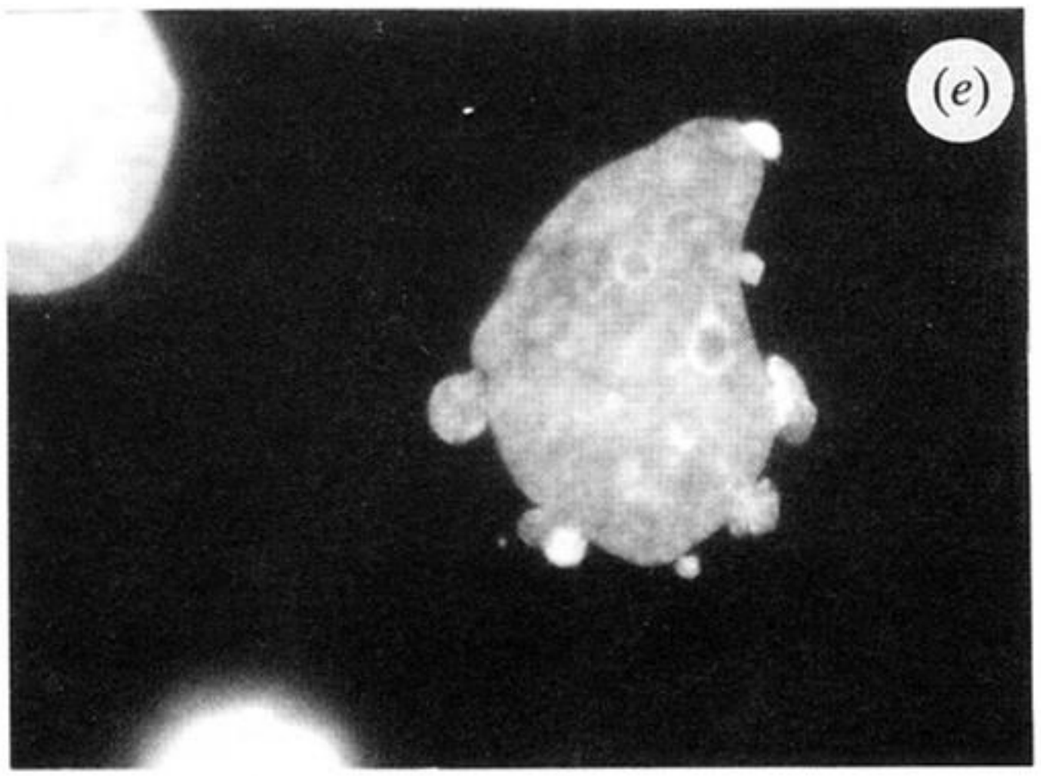

Figure 4. Mitotic aberrations resulting in HeLaS3 cells following delayed progression through a cell cycle. Cells were treated with 0.25 μg ml^{-1} aphidicolin. (*a*) Mitotic cells stained with anti-tubulin, showing multiple centres of mitotic spindle nucleation in one of two cells. (*b*) Mitotic cell stained with an anti-centrosome antibody. The rhodamine stain shows aberrent chromosome positioning at the metaphase plate, as well as centrosomes placed abnormally (arrows). (*c*) A metaphase–anaphase transition (giemsa). This spread shows a composite of a number of abnormalities including: (i) inability of chromosomes to separate properly; (ii) lack of attachment of chromosomes to mitotic spindles; and (iii) extensive chromosomal breakage. (*d*) Daughter cells which have undergone incomplete karyokinesis and cytokinesis and which are attached through DNA strands (arrow) (Hoechsts 33342). The G1 nuclei are abnormal. Note also several micronuclei of varying sizes. (*e*) A HeLaS3 cell that has progressed through mitosis, but has failed to successfully undergo karyokinesis or cytokinesis (Hoechsts 33342). Note in this cell the presence of micronuclei, as well as a large and lobulated nucleus.