Mitotic Cell Death by Chromosome Fragmentation

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Abstract

Cell death plays a key role for both cancer progression and treatment. In this report, we characterize chromosome fragmentation, a new type of cell death that takes place during metaphase where condensed chromosomes are progressively degraded. It occurs spontaneously without any treatment in instances such as inherited status of genomic instability, or it can be induced by treatment with chemotherapeutics. It is observed within cell lines, tumors, and lymphocytes of cancer patients. The process of chromosome fragmentation results in loss of viability, but is apparently nonapoptotic and further differs from cellular death defined by mitotic catastrophe. Chromosome fragmentation represents an efficient means of induced cell death and is a clinically relevant biomarker of mitotic cell death. Chromosome fragmentation serves as a method to eliminate genomically unstable cells. Paradoxically, this process could result in genome aberrations common in cancer. The characterization of chromosome fragmentation may also shine light on the mechanism of chromosomal pulverization. [Cancer Res 2007;67(16):7686–94]

Introduction

The process of cellular death is clinically important for prevention and treatment of cancer and in development. Traditionally, the processes of apoptosis and necrosis have been the most frequently noted forms of cell death (1). Increased understanding of the process of cell death has led to exploitation of the direct mechanisms of cell death, although its application in clinical treatment has yielded only limited progress. Recently, there has been an increased emphasis on nonapoptotic paths, including mitotic catastrophe and autophagy (2–8).

Cell cycle specificity of different types of deaths is also important to understand as knowledge of cell cycle specificity of cell death could lead to new chemotherapeutic targets (9). Apoptosis typically takes place during interphase (1, 10), whereas mitotic catastrophe seems to take place during the G_2-M phase but precise information of the timing is lacking. By effectively targeting only M-phase cells, elimination of rapidly dividing tumor cells could be enhanced by avoiding the nonspecific killing of slow dividing or noncycling normal cells. To this end, there have been reports detailing increased efficacy when cancer cells treated with microtubule inhibitors in combination with chemotherapy or radiation combinations, but few of these reports involve detailed characterization of regulated elimination of mitotic cells and the effects of the treatment on the chromosome (2, 11–13).

The chromosome represents not only the vehicle in which genes are carried but also serves as a genetic framework that controls all genes in a systemic manner (14, 15). The karyotypic-defined genome is the driving force for cancer evolution and for other types of organismal evolution (14–20). Clinical cytogenetic analysis is commonly done in many types of cancers and often serves as a diagnostic and prognostic marker of cancer progression. Clonal events are recorded, but recent evidence shows that nonclonal events provide the diversity that is required for tumor progression and survival in the extremely harsh environment of a tumor (16, 17, 19). Change on the chromosomal level has the capability of directly altering the regulation of hundreds or possibly thousands of genes. Chromosomal change, including translocations, deletions, duplications, inversions, defective mitotic figures, and fragmentation of chromosomes can serve to increase diversity that is required for cancer progression and evolution (14, 16–19).

Here, we describe a new type of mitotic cell death, termed chromosome fragmentation, which is a consequence of certain cellular stressors such as inherited genomic instability or chemotherapeutic treatment in M phase, and a pathologically related process that results in the breakdown of the chromosomes, elimination of genetic material, and subsequent death of cells (18, 21). This form of cell death is different from typical apoptosis and mitotic catastrophe. It is caspase independent, does not exhibit the typical oligosomal DNA degradation of apoptosis, and is not inhibited by overexpression of Bcl-2. In the case of mitotic castastrophe, classic methods of inducing mitotic catastrophe (5, 22) do not increase levels of chromosome fragmentation detectable by cytogenetic analysis. Chromosome fragmentation, although morphologically similar to, is distinct from S-phase premature chromosome condensation (and chromosome pulverization) as chromosomes undergoing fragmentation are from mitotic, nonreplicating cells. Chromosome fragmentation offers insight into the basis for chromosome pulverization in cases where the genome has been destabilized. Chromosome fragmentation represents a clinically relevant form of cell death, which unlike other types of cell death is clinically identifiable using standard cytogenetic analysis that is commonly done on tumors, due to its defined morphologic features. Chromosome fragmentation is an important form of non-clonal chromosome aberrations (NCCAs), which are linked to cancer progression (15–19). It can serve as a much needed biomarker of induced cell death and genome instability.

Materials and Methods

Cell culture. Cell lines were used, including HeLa, HCT116, HCT116 *p53−/−*, *HCT116 14-3-3s−/−*, H460-*neo*, H460-*Bcl-2* (23), and H1299-v138.

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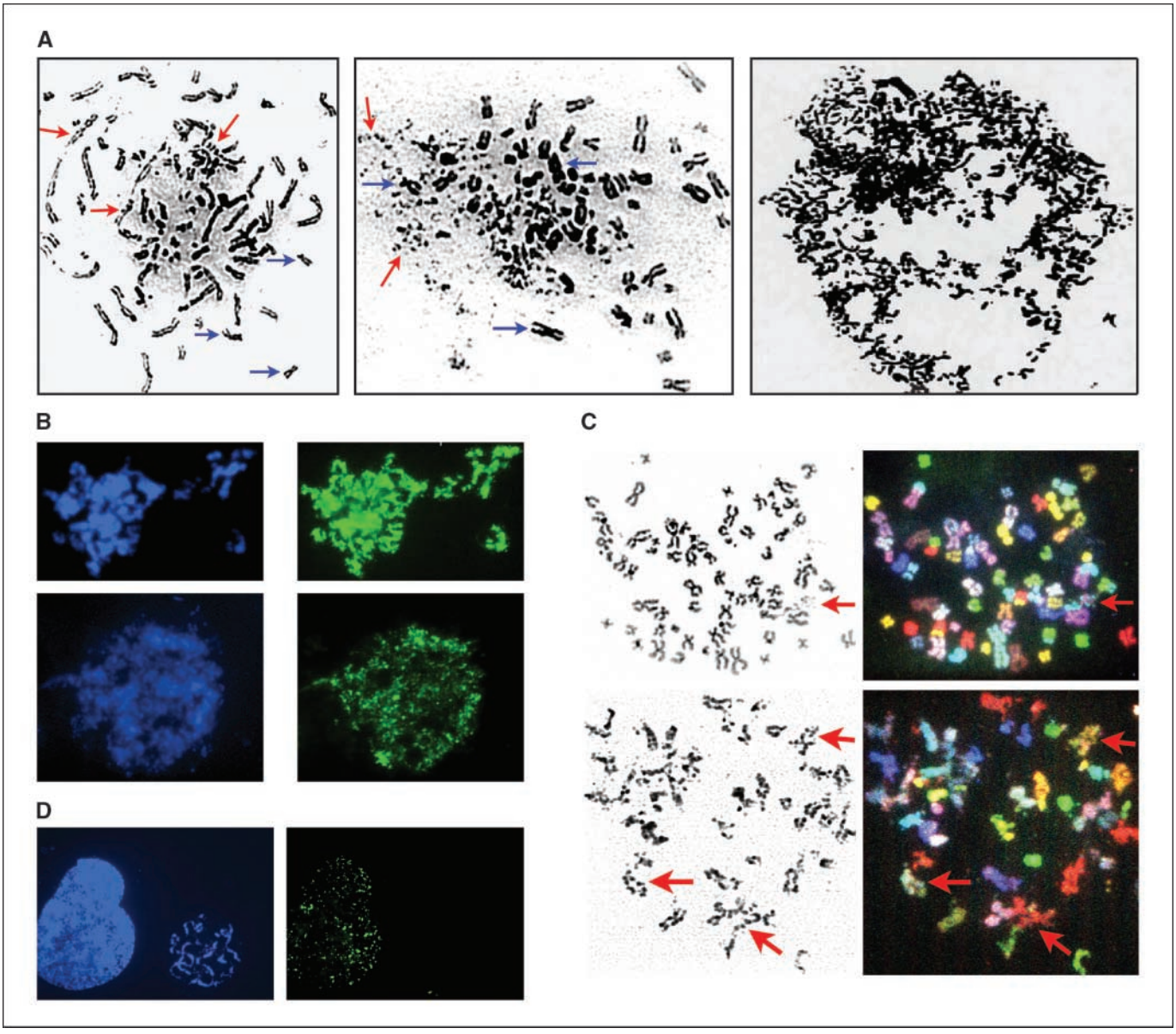

Figure 1. Morphologic features of chromosome fragmentation. Chromosomes undergoing fragmentation display many breaks and often seem frayed. *A,* Giemsa staining shows chromosome fragmentation is a progressive process with early stages showing few fragmented chromosomes [*left,* chromosome fragmentation (*red arrows*); intact chromosomes (*blue arrows*)], mid stage with approximately half of the chromosomes fragmented (*middle*), and late stage with nearly all chromosomes except for one at the top showing degradation (*right*). *B,* evidence illustrating that chromosome fragmentation occurs in condensed mitotic chromosomes. Cells undergoing chromosome fragmentation stain positive (*left* DAPI, *right* FITC) for phosphorylated H3 (Ser^{10}) both in early (*top*) and late (*bottom*) stages. *C,* spectral karyotyping images (inverted DAPI *right*, SKY *left*) indicate that chromosomes are condensed before fragmentation as fragmented chromosomes retain their chromosomal domains in early and later stages of fragmentation (*top* and *bottom*). Additionally, spectral karyotyping images show that single chromosomes can be eliminated via chromosome fragmentation (*bottom, arrow*). *D,* chromosome fragmentation is distinct from S-phase premature chromosome condensation as chromosomes from S-phase cells forced to undergo premature chromosome condensation should show BrdUrd uptake (DAPI *left*, anti-BrdUrd-FITC *right*). Cells undergoing chromosome fragmentation (*cell on right*) do not show incorporation of BrdUrd, whereas S-phase cells (*left cell*) from the same treatment do.

HCT116 *14-3-3*s−/− cells were grown in McCoy's 5A medium; others were grown in RPMI 1640. G418 (400 μg/mL) was used as necessary.

Induction of chromosome fragmentation. After 3 to 8 h treatment of colcemid, mitotic cells were gently shaken off and resuspended in culture medium containing colcemid. Cells were then treated with doxorubicin or methotrexate at 1 μg/mL for various times.

Cytogenetic examination. Cells were harvested and cytogenetic slides were prepared using standard protocols (24). Slides were stained by Giemsa for scoring nuclear structures (24) or stored at −20°C for further characterization including spectral karyotyping (SKY) and antibody staining. The fragmentation index was determined by dividing the number of cells displaying chromosome fragmentation by the total number of mitotic cells, including cells undergoing chromosome fragmentation.

Spectral karyotyping analysis. SKY was done on mitotic spreads as described (25, 26). Briefly, cytogenetic slides were denatured and hybridized with human painting probes. After washing and spectral karyotyping detection, mitotic structures were captured using a charge coupled device camera.

Bromodeoxyuridine incorporation and antibody staining. Cells were pretreated for 3 h with colcemid and collected via mitotic shake off. Cells were treated for 6 h with 1 μg/mL doxorubicin, colcemid, 100 μmol/L deoxycytidine, 100 μmol/L bromodeoxyuridine (BrdUrd), and harvested for cytogenetic analysis.

Antibody staining was done as described (26, 27). Cell suspensions were dropped on ice-cold microscope slides followed by washes in cold buffer [10 mmol/L Na_2HPO_4, 0.15 mol/L NaCl, 1 mmol/L EGTA, and 0.01% NaN_3] and buffer [1.0 mmol/L triethanolcomine-HCl (pH 8.5), 0.2 mmol/L NaEDTA (pH 8.0), 25 mmol/L NaCl, 0.05% TweenR20, and 0.1% bovine serum albumin]. Slides were then air dried and primary antibodies were loaded.

Viability assessment. Viability was assessed using a LIVE/DEAD assay (Invitrogen) according to manufacturer's protocol.

Terminal deoxyribonucleotide transferase–mediated nick-end labeling staining. Terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) staining was done using a kit supplied by Roche. Briefly, mitotic cells were induced to undergo chromosome fragmentation washed thrice in PBS, attached to slides using a Shandon cytospin at 500 rpm for 5 min, fixed in 4% paraformaldehyde, and subjected to TUNEL.

Caspase inhibition. Caspase activity was inhibited by treatment of HCT116 cells for 12 h with 20 mmol/L z-vad-fmk followed induction of chromosome fragmentation (by concurrent treatment with colcemid and 1 μg/mL doxorubicin) for 6 or 12 h in the presence of or after removal of z-vad-fmk. Following treatment, cytogenetic slides were made and scored.

Caspase-3 activity. Caspase-3 activity was measured using the caspase-3 colorimetric assay kit from R&D Systems as described (28). HCT116 cells were treated for 8 h with colcemid with and without z-vad-fmk. Mitotic cells were then shaken off and treated with doxorubicin for an additional 6 and 12 h.

Western blotting. Analysis was done as described (28).

Induction of genomic instability. For induction of genomic instability, H1299-v138 cells were grown at 39°C for 2 weeks and then grown at 32°C where cells were photographed and collected daily and cytogenetic preparations were made and analyzed for chromosome fragmentation.

Induction of mitotic catastrophe. Mitotic catastrophe was induced as described (5, 22). HCT116 *14-3-3σ−/−* cells were treated for up to 72 h with 2 μg/mL doxorubicin and HCT116 *p53−/−* cells were treated with 1 μg/mL aphidicolin for up to 96 h were each harvested every 24 h. Cytogenetic preparations and cytospin preparations were made allowing for chromosomal and morphologic analysis.

Results

Chromosome fragmentation represents a unique phenotype of chromosome aberration. To define chromosome fragmentation, we first characterized its morphology. Fragmented chromosomes are distinct from normal chromosomes prepared by cytogenetic techniques. Chromosomes that are progressively cut into smaller pieces and fragmented chromosomes often show lighter density of Giemsa or 4′,6-diamidino-2-phenylindole (DAPI) staining than normal chromosomes stained in parallel indicating the loss of chromosomal material. Mitotic figures displaying chromosome fragmentation can be grouped into at least three groups, early fragmentation where few chromosomes are broken, midstage fragmentation where a significant number of the chromosomes have been fragmented, and late stage where all or most of the chromosomes have been fragmented, suggesting a progressive process (Fig. 1*A*). Chromosome fragmentation is not the result of

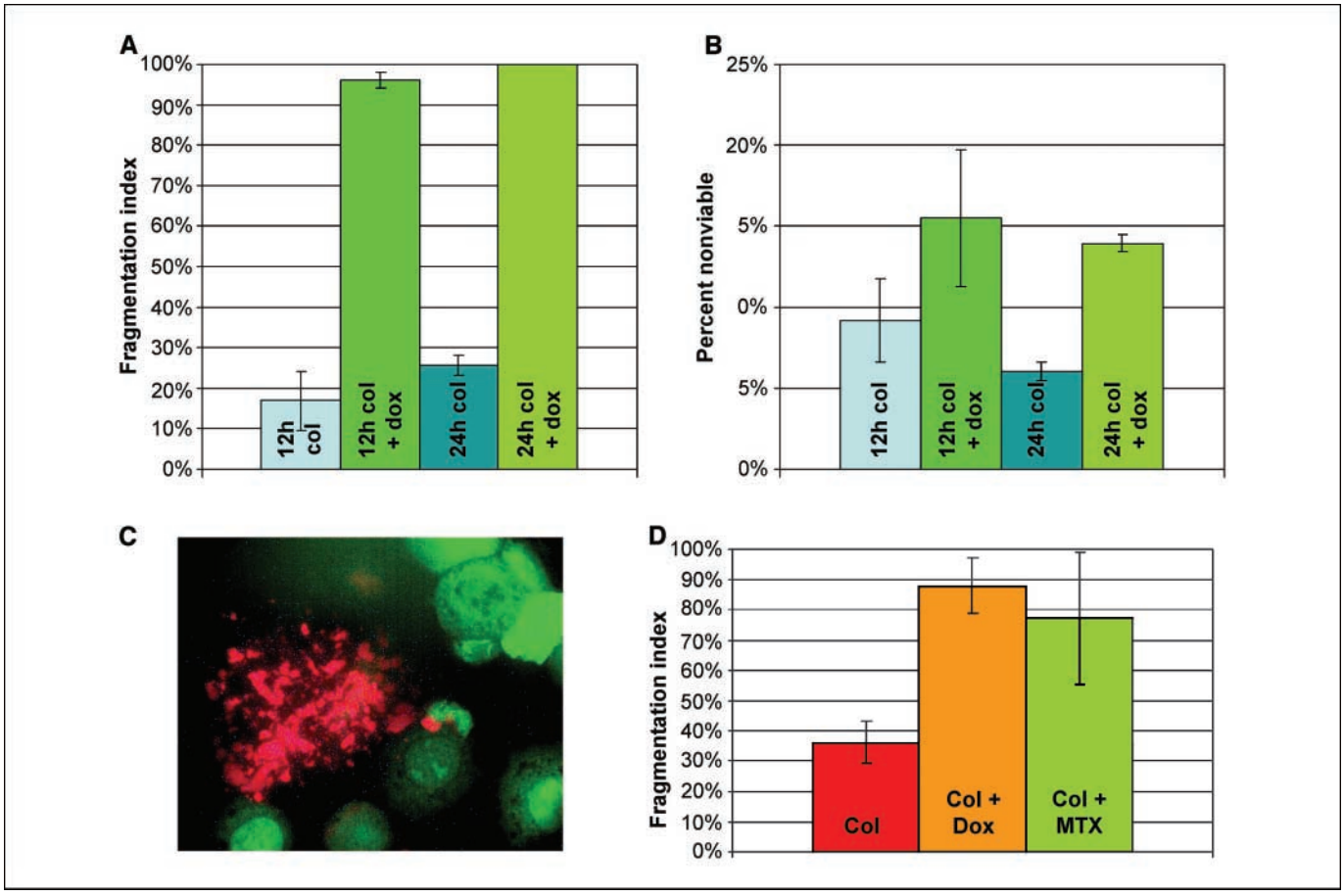

Figure 2. Chromosome fragmentation results in cell death. As the rate of chromosome fragmentation increases (*A*), cellular viability decreases (*B*). Viability staining shows that cells undergoing chromosome fragmentation display loss of membrane integrity as ethidium homodimer is able to enter the cell and stain chromosome fragments red (*C*). Viable cells show only green staining. Chromosome fragmentation is induced more efficiently if combinations of chemotherapeutics and microtubule inhibitors are used (*D*). *Col,* colcemid; *dox,* doxorubicin; *MTX,* methotrexate.

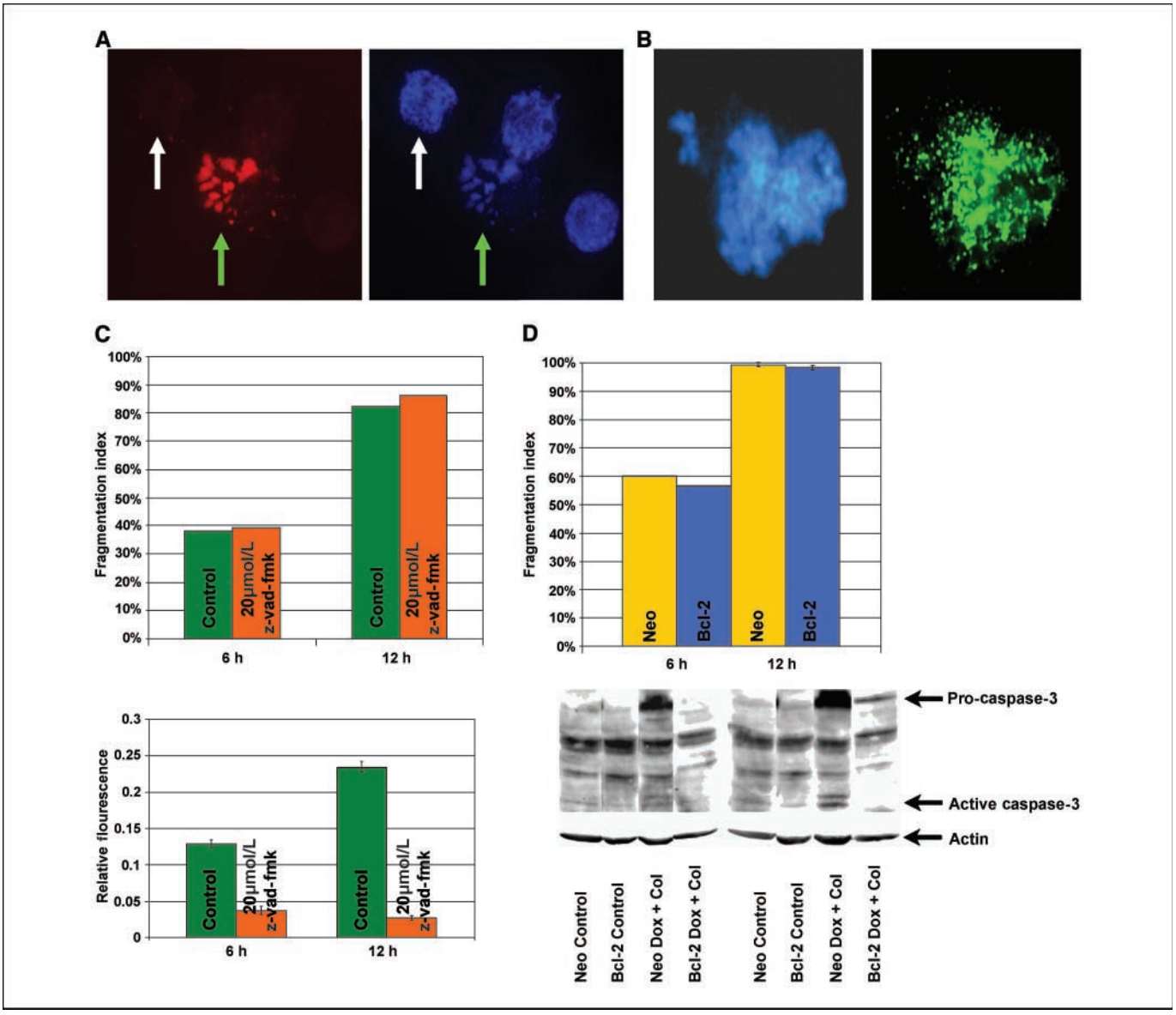

Figure 3. Chromosome fragmentation is not apoptotic. Fragmented chromosomes show negative TUNEL staining (*A, white arrows*, DAPI *right*, TUNEL *left*). Typical apoptotic bodies display positive TUNEL results (*green arrows*) as do positive controls (data not shown). Chromosome fragmentation results in intense γ-H2AX staining (DAPI *left*, FITC *right*), which is an indicator of strand breaks (22), indicating that despite negative TUNEL staining, there is indeed strand breakage (*B*). Caspase inhibition does not inhibit chromosome fragmentation (*C*). Cells pretreated with broad-spectrum caspase inhibitors and then treated to induce chromosome fragmentation in the continued presence of, or after removal of, caspase inhibitors show similar frequencies of chromosome fragmentation at 6 and 12 h of treatment (*top*). Despite no change in the frequency of chromosome fragmentation, levels of caspase-3 activity significantly (6 h, $P = 0.003479$; 12 h, $P = 0.00007$) differed between the cells treated with z-vad-fmk and those without (*bottom*), indicating that activation of the initiator caspase, caspase-3, is not required for chromosome fragmentation. Interestingly, treated cells in the presence of caspase inhibitors tend to exhibit later stages of fragmentation compared with cells removed from caspase inhibition after 12 h of treatment, although total chromosome fragmentation frequencies remain similar. Chromosome fragmentation frequencies were unaltered in H460 cells that overexpress the apoptosis inhibitor Bcl-2 when compared with H460 cells carrying an empty neo vector when chromosome fragmentation is induced once again, indicating that caspase activation and/or mitochondrial membrane permeabilization are not required for chromosome fragmentation to occur (*top*). Levels of pro– and active caspase-3 are higher in the H-460 *neo* control compared with H460-*Bcl-2* overexpressing cells when fragmentation is induced for 6 and 12 h, as indicated by Western blotting (*bottom*).

the slide preparation, as under identical conditions both normal and fragmented chromosomes coexist within the same cell (Fig. 1*A*). In fact, the variable frequencies of fragmented chromosomes are determined by the specific cell line used and drug treatment before the making of slides (Fig. 2*D*).

A mitosis-specific event. Chromosome fragmentation was observed at low levels (typically <5% of mitotic cells, depending on specific cell line) in a number of cell lines without any drug treatment. To show that chromosome fragmentation is generated directly from mitotic cells and not from cells in interphase, the population of mitotic cells was increased via shaking off non-adherent mitotic cells and treating with colcemid to arrest them in mitosis and concurrently treating them with the topoisomerase II inhibitor, doxorubicin. Nearly 100% of these mitotic cells showed a degree of chromosome fragmentation within 12 h of this treatment (Fig. 2*D*). When interphase cells were treated with doxorubicin and colcemid that was preceded by a double thymidine block, no chromosome fragmentation was observed for up to 24 h.

BrdUrd incorporation was monitored during induction of chromosome fragmentation and no BrdUrd incorporation was observed (Fig. 1*D*), suggesting that despite the morphological similarity of chromosome fragmentation and PCC, they are in fact distinct processes.

Additional indications that chromosome fragmentation takes place exclusively in mitotic cells include assessment of the phosphorylation status of histone H3 at serine 10 (29). Immunofluorescent staining of phosphorylated H3 (Ser^{10}), revealed the majority of mitotic cells, fragmented or not, show positive H3 staining, suggesting that these fragments are indeed derived from condensed mitotic chromosomes (Fig. 1*B*). Multiple color spectral karyotyping was then done to examine the relationship between fragmented and normal chromosomes as spectral karyotyping can precisely identify individual chromosomes (26). Results from spectral karyotyping analysis (Fig. 1*C*) show that even highly fragmented chromosomes are condensed and have a grouping and localization similar to what would be expected if that chromosome was intact.

Chromosome fragmentation results in cell death. Fragmented mitotic figures with extensive chromosomal damage would seemingly be incompatible with survival. To test the direct link between fragmentation and cell death, we treated mitotic cells to induce chromosome fragmentation and followed this with calcein AM and ethidium homodimer staining to assess viability. Following treatment, cells were collected and analyzed for viability and the frequency of chromosome fragmentation. It was found that chromosome fragmentation does result in cell death as chromosome fragmentation increases (Fig. 2*A*) as viability decreases (Fig. 2*B*). Additionally, cells were observed that displayed extensive ethidium homodimer labeling of fragmented chromosomes (Fig. 2*C*), further illustrating that chromosome fragmentation directly results in loss of membrane integrity and loss of viability. Therefore, chromosome fragmentation is a form mitotic cell death.

Differs from typically described apoptosis. Next, we sought to determine if chromosome fragmentation is a typical apoptotic process. TUNEL staining done on cells undergoing chromosome fragmentation shows negative staining of cells displaying chromosome fragmentation (Fig. 3*A*) in agreement with previous reports (30). Cells with typical apoptotic morphology (round clusters of DNA) from the same slides, however, show positive TUNEL staining (Fig. 3*A*) as did positive controls (data not shown). Also, apoptotic oligosomal DNA ladders were not evident after induction of fragmentation (data not shown). Chromosome fragmentation, however, involves strand breaks as evidenced by positive γ-H2AX staining along chromosomes (Fig. 3*B*). This has been implicated in apoptosis and damage repair signaling (31).

Mitotic cells were pretreated with the caspase inhibitor z-vad-fmk or with a negative control of z-vad-fmk for a short period then treated with colcemid and doxorubicin and analyzed for frequency of chromosome fragmentation. As shown in Fig. 3*C* (*top*), chromosome fragmentation frequencies were not significantly

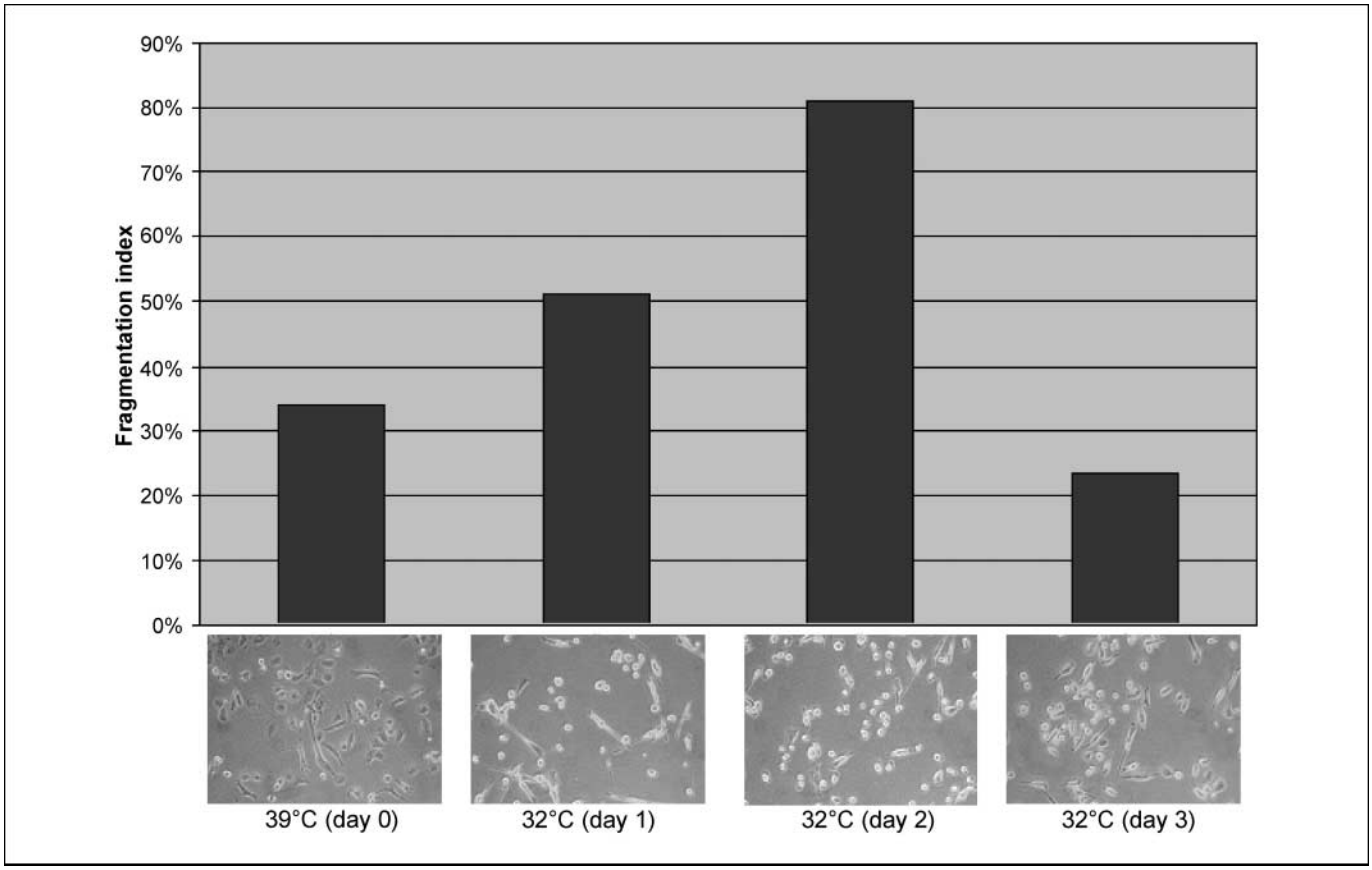

Figure 4. Chromosome fragmentation rate is associated with genomic instability. The H1299 v-138 cell line contains a temperature-sensitive p53 mutation with the restrictive temperature being 39°C and the restrictive temperature being 32°C (43). H1299 v-138 cells were grown at 39°C and then shifted to 32°C. During the shift, the cells progressively detached and died over a period of 5 d until few viable cells remained in agreement with previous reports (43). Upon temperature shift, spontaneous chromosome fragmentation rates increased, then declined, as the number of dying cells and overall genomic instability decreased.

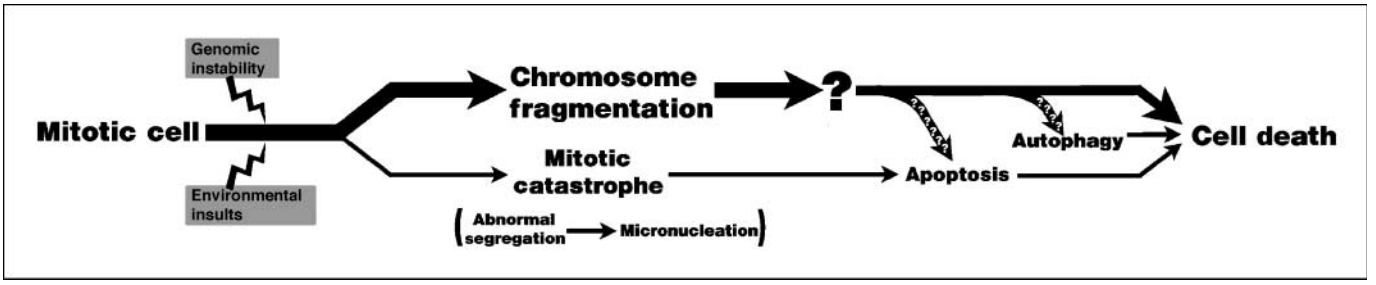

Figure 5. Model illustrating the proposed relationship of chromosome fragmentation with other types of cell death. Chromosome fragmentation differs from mitotic catastrophe in that cells undergoing chromosome fragmentation commit to death during mitosis, whereas cells dying via mitotic catastrophe die after mitosis (5).

altered upon caspase inhibition. Furthermore, Fig. 3*C* (*bottom*) shows that caspase-3 reactivity is largely inhibited when 20 μmol/L zvad-fmk is added; however, levels of caspase-3 activation increase from 6 to 12 h of treatment in the uninhibited cells. Bcl-2 overexpression was also found not to inhibit chromosome fragmentation. Using two variants of the non–small cell lung cancer cell line, H460, the first overexpressing Bcl-2 and the second carrying an empty neo vector, chromosome fragmentation was induced. Both lines showed similar frequency of chromosome fragmentation as indicated in Fig. 3*D* (*top*), whereas levels of pro–caspase-3 and activated caspase-3 were increased in the treatments on cells containing the empty neo vectors and levels and activation were decreased or eliminated in cells overexpressing Bcl-2 (Fig. 3*D, bottom*). This indicates that unlike apoptosis, Bcl-2 overexpression does not influence frequency of chromosome fragmentation.

Differs from typically described mitotic catastrophe. If chromosome fragmentation lacks the classic hallmarks of apoptosis perhaps it was mitotic catastrophe. During treatment, cells in culture became large and detached as reported (5). Cytogenetic analysis coupled with phosphorylated H3 antibody staining indicated that the cells that were undergoing mitotic catastrophe may not be typical mitotic cells. Cells were harvested after 24, 48, and 72 hours and prepared for cytogenetic analysis or as described (5). Chromosome fragmentation was not observed, indicating that this model of mitotic catastrophe is distinct from chromosome fragmentation. Mitotic shake off was then done on the HCT116 *14-3-3*$\sigma^{-/-}$ cell line and accumulated mitotic cells were treated to induce fragmentation. Interestingly, HCT116 *14-3-3*$\sigma^{-/-}$ seemed to be resistant to chromosome fragmentation, as very few mitotic figures displayed fragmentation, whereas the parental strain of HCT116 shows drastically increased frequency of fragmentation when the same treatment was applied.

A second model of mitotic catastrophe induced by aphidicolin treatment in *p53* null HCT116 cells was assessed for chromosome fragmentation (22). A mitotic fraction of 3.2% at 72 h [compared with over 20% by three-dimensional fluorescence-activated cell sorting (FACS) analysis of DNA content and H3 phosphorylation; ref. 22] and 5.4% at 96 h was observed via our conventional cytogenetic analysis. Chromosome breaks were evident in the majority of chromosome spreads at 96 h. These breaks in contrast to chromosome fragmentation were more regular in size. Most cells do not show extensive breaks as typical chromosome fragmentation does, and when viewed at ×20 magnification these mitotic spreads appear normal. In the case of chromosome fragmentation, normal mitotic spreads are typically discernible from chromosome fragmentation at ×20 magnification. A minority of mitotic cells show some chromosome fragmentation; however, even the total mitotic fraction does not represent the proportion of cells reported as undergoing "mitotic catastrophe." Further previous reports have failed to show mitotic specific proteins such as MPM2 or phosphorylated H3 (5). Mitotic chromosomes with and without breaks stained intensely positive for phospho-H3 (Ser^{10}); however, a number of cells with no apparent chromosomal condensation showed slight H3 staining indicating that those cells previously identified as mitotic by FACS analysis are not actually mitotic.

Discussion

A true mitotic cell death. Chromosome fragmentation is an event that takes place in stressed cells and results in death directly from mitosis, seemingly unlike what has been termed mitotic catastrophe. Chromosome fragmentation seems to be a form of programmed cell death although the possibility remains that chromosome fragmentation is a form of necrosis. Chromosome fragmentation is not a form of apoptotic cell death; it lacks the morphologic and biochemical markers of apoptosis.

The process of chromosome fragmentation results in cell death as evidenced by loss of viability in cells undergoing chromosome fragmentation (Fig. 2*A* and *B*). Further, chromosome fragmentation cannot be induced from cells in other stages of the cell cycle. When cells were blocked in S phase with a double thymidine block, chromosome fragmentation was not apparent, although massive cell death occurred likely due to G2 arrest and subsequent apoptosis induced by the doxorubicin (32, 33). Furthermore, the lack of BrdUrd incorporation in cells undergoing chromosome fragmentation shows that the chromosomes are not induced to condense during S-phase. Thus, chromosome fragmentation does not represent premature chromosome condensation during S-phase. Rather, chromosome fragmentation is a phenomenon that takes place during mitosis and occurs when chromosomes are damaged either by drug treatment or inappropriate passage of damaged cells through the G_2 checkpoint. It should be noted that previous reports have shown spontaneous fragmentation in the ATR knockout model, most likely due to cells entering mitosis with DNA damage and ineffective G_2 checkpoint activation (34, 35). Therefore, chromosome fragmentation is not a unique phenotype of the *ATR−/−* genotypes, but rather the general feature of unstable genomes.

Based on the morphologic characterization, chromosome fragmentation might offer insight into the mechanism of the long-described phenomenon of chromosome pulverization (36). Frequently observed after viral infection and thought to occur from cell fusion, chromosome pulverization has often been linked to the prematurely condensed chromosomes and thus was thought of as occurring in interphase nuclei. The lack of BrdUrd incorporation

noted in chromosome fragmentation (Fig. 1*D*) challenges this notion and shows that fragmentation is the likely mechanism of chromosome pulverization in cases of drug treatment or viral infection.

Nonapoptotic cell death. Chromosome fragmentation lacks many of the hallmark features of apoptosis, including the characteristic pattern of DNA fragmentations detectable by the TUNEL reaction. Caspase activation is also a hallmark of apoptosis (10). As caspase activation is intimately involved in apoptosis, we sought to determine if chromosome fragmentation was also dependent on caspase activity. These results were not due to cells already undergoing apoptosis as cells were rinsed to remove detached dying or mitotic cells and cultured for 15 h in the presence of the caspase inhibitor, shaken off, and then treated with colcemid and doxorubicin in the continued presence of z-vad-fmk or with z-vad-fmk removed. Although there was strong repression of caspase activation in the presence of z-vad-fmk (Fig. 3*C, bottom*), the fragmentation index was not significantly altered, indicating independence of caspase activation in chromosome fragmentation.

Bcl-2 is an antiapoptotic protein that is commonly overexpressed in tumors (37, 38). Bcl-2 overexpression inhibits mitochondrial pore opening, cytochrome *c* release, and subsequent apoptosis (37, 38). Bcl-2 overexpression was shown not to alter the frequency of chromosome fragmentation when it was induced by drug treatment of mitotic cells, despite the repression of caspase-3 expression and activation detectable in the Bcl-2–overexpressing cells compared with cells expressing an empty neo vector only. Chromosome fragmentation therefore lacks many of the major hallmarks of apoptosis, DNA strand breaks, sensitivity to caspase inhibition, and sensitivity to Bcl-2 overexpression. However, chromosome fragmentation does display double strand breaks as noted by positive γ-H2AX staining (Fig. 3*B*), which can serve as an early apoptotic sign.

Differs from previously described mitotic catastrophe. Mitotic catastrophe has been loosely defined as cell death that results from aberrant mitoses (7, 8). It has been contradictorily suggested to be apoptotic and nonapoptotic (3, 39), but seems to typically be linked with segregation abnormalities (8). Not only do the definitions of mitotic catastrophe differ but there is also a lack of morphologic characterization of chromosomes from cells undergoing mitotic catastrophe. It has been strongly suggested that more descriptive terms for mitotic cell deaths be used to avoid the vagueness and confusion of the term "mitotic catastrophe" (8). Due to the confusion of mitotic catastrophe, and its apparent importance, chromosome fragmentation was assessed in two representative systems of mitotic catastrophe. Cells were induced to undergo mitotic catastrophe according to published reports (5, 22). Half of the cells were prepared using reported protocols and half were prepared according to standard cytogenetic procedure (24) to score chromosome fragmentation frequency.

One of the first models of mitotic catastrophe described in human cells is the HCT116 cell line without 14-3-3σ function (5). Cells treated with a low dose of doxorubicin undergo mitotic catastrophe, which was described as a near-steady population of 2N cells, a declining population of 4N cells, and an increase in sub-2N content (5). Cells were subjected to the same treatment as reported collected and processed by conventional cytogenetic protocols but chromosome fragmentation was not evident although irregularly shaped small nuclei that seem to have condensed chromatin due to their uniform dark staining are quite regular. When cells were stained for phosphorylated H3, only a very small fraction of cells show any positive signal, leading to the conclusion that mitotic catastrophe described in such a system (22) may not truly be mitotic.

It is known that there is not a consensus on the exact role of 14-3-3σ in mitotic catastrophe. On one hand, 14-3-3σ is described as a regulator of the G_2 checkpoint that functions with cdc25 to sequester cdc2 in the cytoplasm (5), whereas other reports suggest that 14-3-3σ does not take part in the G_2 checkpoint, but rather may serve as an antiapoptotic protein much like survivin (6, 40). Nevertheless, the mitotic catastrophe shown by the HCT116 14-3-3$\sigma^{-/-}$ model is distinct from chromosome fragmentation.

It is reported that by 72 h of aphidicolin treatment in the HCT116 p53−/− system, more than 20% of cells were mitotic as determined 4N content and positive phospho-H3 staining, whereas ~40% of the cells died (22). We did not observe a similar number of mitotic figures upon cytogenetic analysis, although a portion of cells in interphase showed phosphorylation of histone H3 and may represent a portion of the mitotic population described by Nitta et al. (22) by FACS analysis.

Mitotic catastrophe is commonly defined as cell death after abnormal or failed segregation. In fact, the mitotic catastrophe generally is not considered a form of cell death, but rather an irreversible trigger of cell death (41). Further, the Nomenclature Committee on Cell Death realizes the ambiguity of mitotic catastrophe and suggests use of descriptive terms such as "cell death at metaphase" and "cell death preceded by mutinucleation" (8). Neither model of mitotic catastrophe displays typical chromosome fragmentation in concordant levels to the portion of the cell population dying by mitotic catastrophe. This is likely due to chromosome fragmentation resulting in death directly during mitosis, whereas the majority of cell death due to mitotic catastrophe in the two systems examined takes place after the completion of an abnormal mitosis. Although the mechanistic differences between chromosome fragmentation and mitotic catastrophe are yet to be addressed in depth, importantly they are distinct based on the fact that one is a typical mitotic event and the other does not directly seem to be. Nevertheless, further study is warranted regarding the molecular links between mitotic catastrophe and chromosome fragmentation.

Linked to genomic instability. Interestingly, it was observed that the frequencies of chromosome fragmentation correlate to levels of genomic instability of a given cell line. Elevated chromosome fragmentation was often detected from cell lines with high level of instability. Such correlation is nicely reflected by the dynamics of a given cell population in that cells are more likely to undergo chromosome fragmentation during the transition from periods of instability to stability. During cancer progression, there are distinct transitional stages of genomic stability as evident by patterns of clonal and nonclonal karyotypic progression (15–20). The cell line H1299 v-138 is a line that expresses a temperature-sensitive p53 mutation. This system is genomically unstable at the restrictive temperature (data not shown; ref. 42). Culturing cells at the restrictive temperature results in rapid growth and increased nonclonal chromosome aberrations (NCCA), indicating the presence of an unstable phase. When there is a shift to the permissive temperature, there is a major die off as the cells regain relative genomic stability. As the cells die off, death by chromosome fragmentation is elevated (Fig. 4), indicating that chromosome fragmentation is involved in the elimination of genomically unstable cells. When cells are continuously cultured at the same temperature, there is a dramatically decreased rate of chromosome fragmentation that is highlighted at the permissive temperature

when p53 function is restored. The transiently increased frequencies of chromosome fragmentation and NCCAs can be achieved by additional drug treatment or even by switching from the restrictive to permissive temperature, demonstrating that genomic instability and the mitotic cell killing are tightly linked possibly by evolutionary selection. When both internal and environmental conditions change (either by dysfunction of p53, or simply by switching the temperature or carcinogen treatment), genomic instability, reflected by increased NCCAs, will lead to cell death reflected by increased chromosome fragmentation frequency, which will in turn reduce the heterogeneity for a given cell population. According to this analysis, the frequency of chromosome fragmentation should be used to monitor the *in vivo* and *in vitro* response of chemotherapeutics. We have observed that the high frequencies of fragmentation often occur after chemotherapy.[7]

Paradoxically, the process of fragmentation could also introduce additional heterogeneity. This may occur through the differential elimination of certain chromosomes leading to aneuploidy, a form of NCCA that serves as the driving force of cancer progression (16–19). We have frequently observed mitotic figures in which only one or two chromosomes were fragmented (Fig. 1*C*). These cells are expected to survive but display aneuploidy. In addition, if a cell is undergoing chromosome fragmentation and is not able to complete the process of death, these fragments can be lost and form micronuclei. These can be "stitched" together by various repair complexes, or can result in double minute chromosomes if they are retained and replicated. Thus, incomplete chromosome fragmentation can potentially lead to genomic instability, which, in turn, could lead to genome complexity that drives cancer progression (15–19).

We have shown that during chromosome fragmentation, there is intense γ-H2AX staining along all chromosomes (Fig. 3*B*). Phosphorylation of H2AX is one of the initial events in the recruitment of repair complexes (31). If chromosome fragmentation is halted during the process, it is conceivable that repair complexes could be recruited, fragments rejoined, and karyotypically complex chromosomes could be formed. Thus, chromosome fragmentation is a clinically important phenomenon that can result in DNA damage, change in chromosome number, and changes in NCCA levels that increase population diversity. The degree of chromosome fragmentation could also serve as a measurement of induced and spontaneous mitotic death and transient genomic instability. For example, for some patients, chromosome fragmentation has been observed in peripheral lymphocytes even months after chemotherapy is withdrawn. High frequencies of fragmentation have also been observed from the blood of brain tumor patients without any chemotherapy. Obviously, determining the clinical significance of these observations requires more research.

Clinical significance. What is the clinical significance of chromosome fragmentation? First, all chemotherapeutics tested to this point can generate increased frequencies of chromosome fragmentation. For example, doxorubicin and methotrexate, which exert significantly different effects and cause death by different mechanisms (32, 43), can induce chromosome fragmentation. This indicates that the initial pathway to trigger the cell death process could be different, but as long as the killing happens in mitotic cells, chromosome fragmentation is a common form of cell death. Second, limited clinical samples were also examined from lymphocyte cultures of cancer patients and chromosomal preparations of primary tumors. Elevated fragmentation is often detected particularly in individuals under chemotherapy (data not shown). Further studies of the effects of drug treatment and the frequencies of chromosome fragmentation and its clinical outcome are urgently needed.

Although the mechanism of the relationship of chromosome fragmentation with other forms of cell death requires further characterization, we propose a model showing the interconnectivity of various forms of cell death (Fig. 5). It is possible that chromosome fragmentation represents progressive degradation of DNA from chromosomes to chromosome fragments to smaller DNA fragments or that it is an initial event that results in the formation of smaller chromosomal fragments that are easier for cells to further digest possibly via apoptotic pathways as the cell dies. An additional possibility is that chromosome fragmentation could represent a form of autophagy, as it has been shown that chromosomes can be enveloped by autophagic vesicles upon intense free radical formation (2). In fact, for the early fragmented mitotic figures, often one or a few chromosomes were fragmented indicating such a connection. Another challenge is to understand the molecular mechanism of chromosome fragmentation, which molecules are involved in digestion, and how they are activated when the genome is unstable or when the cell is insulted.

Conclusion

Chromosome fragmentation is a clinically relevant mode of mitotic cell death that results in the progressive degradation of chromosomes during mitosis. Chromosome fragmentation is apparently not apoptotic and differs from models of mitotic catastrophe. Interestingly, chromosome fragmentation is intimately linked with genomic instability, serving to remove cells that display an altered genomic system stability, which is commonly seen in cancer progression (15–17, 19). Furthermore, as it represents one form of NCCA, chromosome fragmentation could lead to increased genome stability if the process is compromised, which could lead to the increased genome complexity noted in cancer (15–17, 19). Although questions still exist about the mechanisms of chromosome fragmentation, this form of cell death needs to be monitored especially in cancer where genome stability plays such a key role in disease progression. In closing, as chromosome fragmentation is a new form of mitotic cell death that is of great importance to cancer treatment, follow-up experiments are being done to further define the molecular mechanism and potential clinical implications.

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[7] J.B. Stevens, et al., unpublished observations.

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