Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death

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Disrupted passage through mitosis often leads to chromosome missegregation and the production of aneuploid progeny. Aneuploidy has long been recognized as a frequent characteristic of cancer cells and a possible cause of tumorigenesis. Drugs that target mitotic spindle assembly are frequently used to treat various types of human tumors. These lead to chronic mitotic arrest from sustained activation of the mitotic checkpoint. Here, we review the linkage between the mitotic checkpoint, aneuploidy, adaptation from mitotic arrest, and antimitotic drug-induced cell death.

Almost 100 years ago, Theodor Boveri, working with sea urchin embryos containing aberrant numbers of spindle poles, observed that the progeny produced contained abnormal numbers of chromosomes, a condition described as aneuploidy (Boveri, 1914). At that time it was already well known that aneuploidy was a common characteristic of tumors, and Boveri proposed that aneuploid progeny produced from a disrupted mitosis become the progenitor cells of tumors. This original hypothesis remains untested. Nevertheless, a link between mitosis, aneuploidy, and cancer has been widely assumed.

Contrary to its potential for initiating tumorigenesis, mitosis has also served as a successful antitumor target. Indeed, drugs that arrest cells in mitosis, known as antimitotics, are common treatments for a variety of human tumors, including breast, ovarian, and non-small-cell lung cancer. However, the mechanism linking long-term mitotic arrest to cell death has remained almost completely unexplored. The paper published in this issue by Tao and colleagues (Tao et al., 2005) represents some of the first evidence on the clinically relevant linkage between mitotic arrest, the mitotic checkpoint whose action is responsible for that arrest, and cell death. In light of this evidence, we review here what is currently known about the mitotic checkpoint and its role in tumorigenesis and cell death, and highlight some of the fundamental questions that remain unanswered.

The mitotic checkpoint: The primary defense against aneuploidy

The mitotic checkpoint, also known as the spindle assembly checkpoint, is the major cell cycle control mechanism in mitosis. It is responsible for the production of genetically identical daughter cells by ensuring accurate chromosome segregation. Proper chromosome segregation requires that one copy of each pair of replicated sister chromatids be delivered to each daughter cell. This is accomplished by organizing the sister chromatids on a bipolar mitotic spindle composed of microtubules (Figure 1). The chromosomes connect to spindle microtubules through their kinetochores, protein-rich structures that assemble and disassemble every mitosis at sites of centromeric DNA, located at the primary constriction of the chromosome. Unattached kinetochores generate diffusible checkpoint complexes that comprise a "wait anaphase" signal, which delays the irreversible process of sister chromatid separation until each and every kinetochore has become productively attached to the mitotic spindle. This ensures the faithful segregation of sister chromatids and the prevention of aneuploidy.

At a molecular level, the mitotic checkpoint prevents advance to anaphase by producing an inhibitor(s) of the anaphase-promoting complex (APC). The APC is an E3 ubiquitin ligase which ubiquitinates mitotic substrates whose subsequent proteosome-mediated destruction is necessary for anaphase onset. APC inhibition is accomplished by recruiting checkpoint proteins, including Bub1, BubR1, Bub3, Mad1, and Mad2 to unattached kinetochores (Figure 2A). There, they are converted into one or more inhibitors of Cdc20, the specificity factor that APC requires to recognize mitotic substrates, including securin and cyclin B. The identity of the in vivo inhibitor(s) remains unknown, but may include activated forms of Mad2 or BubR1, or a complex of Cdc20, Mad2, BubR1, and Bub3 (Figures 1B and 2A) (Fang, 2002; Fang et al., 1998; Sudakin et al., 2001; Tang et al., 2001). After all kinetochores have properly attached (metaphase; Figure 1C), signal generation is silenced and the APC^{Cdc20} inhibitors decay through an ill-defined mechanism that may include the action of the Mad2 binding factor Cmt2 (Habu et al., 2002). APC^{Cdc20}-mediated ubiquitination of securin leads to activation of its binding partner separase, which cleaves the cohesins that maintain the linkage between sister chromatids, leading to sister chromatid separation and anaphase onset (Figure 1D). Ubiquitination and degradation of cyclin B inactivates Cdk1, thereby permitting exit from mitosis (reviewed in Wasch and Engelbert, 2005). In this fashion, the mitotic checkpoint prevents aneuploidy by permitting unattached kinetochores on chromosomes that would be missegregated to delay the irreversible transition from metaphase to anaphase until they become appropriately attached.

The mitotic checkpoint was initially recognized 15 years ago in experiments using antimitotic drugs that depolymerize microtubules (Hoyt et al., 1991; Li and Murray, 1991). These microtubule poisons cause all kinetochores to become unattached and, therefore, a maximal mitotic checkpoint signal is generated. In the succeeding years, testing for the ability to arrest in response to microtubule poisons has been commonly used as the sole test for checkpoint competence. This approach fueled a view still held by some that the checkpoint is either "on" or "off," depending on whether or not cells accumulate in mitosis in response to spindle disruption. This view is incorrect. The primary role of the mitotic checkpoint is to protect against misseg-

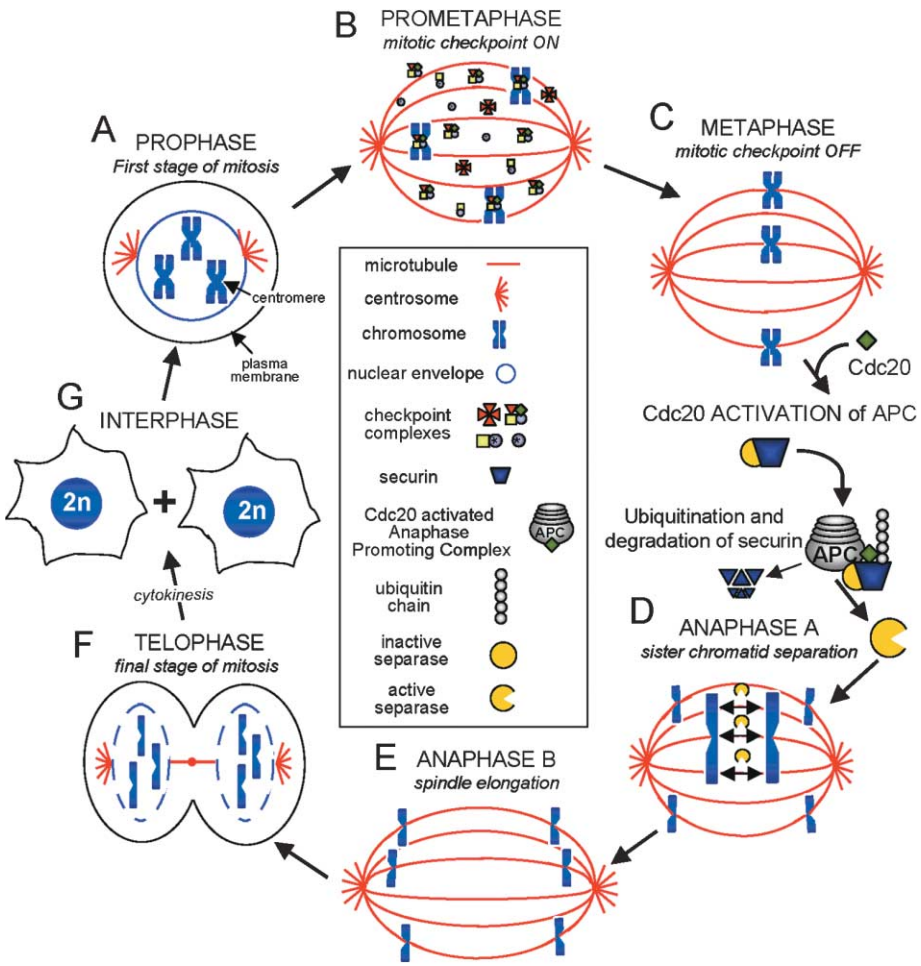

Figure 1. The stages of mitosis

Chromosomes enter mitosis as pairs of replicated sister chromatids that are linked by proteins known as cohesins.

A: The chromatids condense during prophase and are released into the cytoplasm by nuclear envelope breakdown, which marks the transition into prometaphase and also represents the first irreversible transition into mitosis.

B: During prometaphase, the initially unattached chromatids make connections to the microtubules of the mitotic spindle and the mitotic checkpoint is active, which means that the kinetochores assembled at the centromeres of unattached chromosomes generate a diffusible "wait anaphase" inhibitor. Antimitotic drugs delay cells in prometaphase by producing unattached kinetochores.

C: At metaphase, every chromosome has made proper attachments to the mitotic spindle and has congressed to a central position. Production of the diffusible "wait anaphase" inhibitor has been silenced by stable kinetochore-microtubule interactions. As the checkpoint inhibitors decay, the anaphase promoting complex (APC), an E3 ubiquitin ligase, becomes active and recognizes securin and cyclin B, provoking their degradation.

D: Loss of securin activates the protease, separase, that cleaves the cohesins, triggering sister chromatid separation and chromosome segregation during anaphase A.

E: At anaphase B, the spindle elongates.

F: At telophase, the now segregated chromosomes begin decondensing and the nuclear envelopes reform.

G: Cytokinesis separates the nuclei into two daughter cells that re-enter interphase.

regation of single chromosomes, a role that requires the ability to produce a robust checkpoint response in the presence of even a single unattached kinetochore.

Genetic evidence has now demonstrated that cells with weakened checkpoint machinery cannot prevent single chromosomes from being missegregated during an otherwise unperturbed mitosis, although they can and do arrest when all kinetochores are signaling after spindle disruption. For example, cells with diminished levels of CENP-I/Mis6 (Liu et al., 2003), Aurora B (Ditchfield et al., 2003; Hauf et al., 2003), CENP-E (Weaver et al., 2003), Bub1 (Johnson et al., 2004), or the adenomatous polyposis coli gene (Fodde et al., 2001; Kaplan et al., 2001), which is frequency mutated in colon cancers, arrest in response to microtubule depolymerization, but produce aneuploid progeny due to missegregation of one or a few chromosomes per division. The molecular mechanism of this differential checkpoint response has been investigated in four of these five examples. In each case, the signals of two or more essential kinetochore proteins were diminished, but not eliminated. While complete mislocalization or deletion of an essential checkpoint component leads to an inactive checkpoint, partial loss or mislocalization of these components leads to weakened signal generation at individual unattached kinetochores (Figure 2C). Thus, cells that recruit suboptimal levels of checkpoint proteins to kinetochores need larger numbers of unattached kinetochores to create the threshold of inhibitor necessary to block anaphase onset.

Mitotic checkpoint inactivation is lethal: A weakened checkpoint generates chromosomal instability

It has long been recognized that missegregation of large numbers of chromosomes results in rapid cell death (Boveri, 1914), and it is self-evident that a minimum number of genes and/or chromosomes must be required for viability. More recently, mitotic checkpoint genes have been shown to be required for viability in flies (Basu et al., 1999) and mice (Baker et al., 2004; Dobles et al., 2000; Kalitsis et al., 2000; Wang et al., 2004), presumably due to chromosome missegregation. Moreover, direct evidence has emerged from two groups that the mitotic checkpoint is absolutely essential for viability in vertebrates (Kops et al., 2004; Michel et al., 2004). Cells (including human cancer cells exhibiting chromosomal instability) in which the mitotic checkpoint is completely inactivated by siRNA-mediated depletion of Mad2 or BubR1 speed through prometaphase and missegregate large numbers of chromosomes during the subsequent premature anaphase (Kops et al., 2004; Meraldi et al., 2004; Michel et al., 2004). This results in rapid cell death that is dependent on chromosome loss: Mad2- or BubR1-depleted cells that do not undergo cytokinesis remain viable through continued cycles of DNA replication up to at least 32N (Kops et al., 2004; Michel et al., 2004).

On the other hand, cells and organisms with a weakened checkpoint remain viable. Mice heterozygous for essential checkpoint genes are viable and fertile, but have an increased

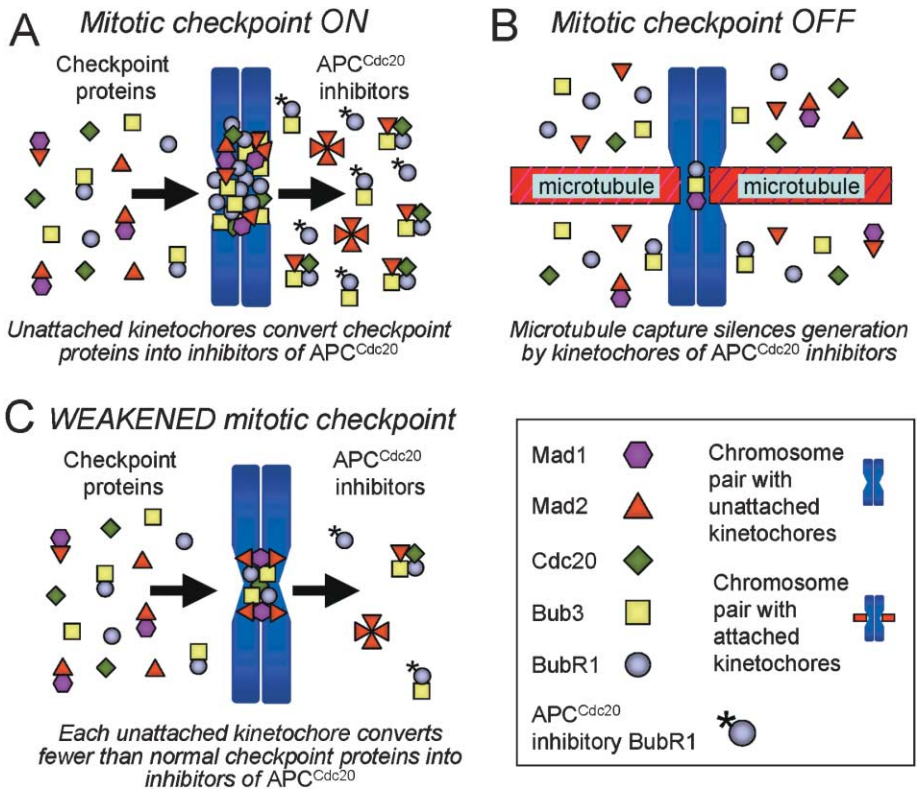

Figure 2. Mitotic checkpoint signaling

A: Unattached kinetochores are the signal generators of the mitotic checkpoint. They recruit mitotic checkpoint proteins, including Mad1, Mad2, BubR1, and Bub3, and convert them into inhibitors of APC^{Cdc20}.

B: Once all kinetochores have made productive attachments to spindle microtubules, production of the APC^{Cdc20} inhibitors is silenced.

C: Unattached kinetochores assembled from mutated components or in the presence of lower concentrations of checkpoint proteins generate a weakened mitotic checkpoint signal and produce fewer APC^{Cdc20} inhibitors.

frequency of aneuploidy (Baker et al., 2004; Babu et al., 2003; Dai et al., 2004; Michel et al., 2001; Putkey et al., 2002). The usual number of chromosomes in these aneuploid cells is actually quite close to diploid (±4), with ±1 being the most common karyotype, indicating that these aneuploid cells most likely arose from the missegregation of only one or a few chromosomes due to a weakened checkpoint. Thus, a completely inactive checkpoint results in cell autonomous lethality, but cells with a weakened checkpoint survive and exhibit chromosomal instability.

Tumors are associated with weakened mitotic checkpoints

All of the evidence obtained to date linking tumorigenesis with the mitotic checkpoint implicates a weakened mitotic checkpoint in the transformation process. Mice with a weakened checkpoint due to heterozygous loss of Mad2 (Michel et al., 2001) or hypomorphic alleles of BubR1 (Baker et al., 2004) develop spontaneous tumors with enhanced frequency (27% and 6%, respectively), and mice heterozygous for Bub3 (Babu et al., 2003) or BubR1 (Dai et al., 2004) exhibit elevated rates of tumorigenesis when treated with carcinogen (70% in Bub3 heterozygotes versus 50% in wild-type and 11% in BubR1 heterzygote versus 0.4% in wild-type). BubR1 heterozygosity also accelerates intestinal tumor formation and progression in $Apc^{Min/+}$ mice, which form colon tumors by 3 months of age (Rao et al., 2005).

In human cancers, a report of heterozygous point mutations in the Bub1 and BubR1 genes in 2 of 19 colorectal cancer cell lines (Cahill et al., 1998) stimulated a broad search for mutations in mitotic checkpoint genes in a variety of tumors and tumor cell lines. Recently, a small number of human patients have been identified with the rare recessive disorder mosaic variegated aneuploidy (MVA), which is characterized by an increase in aneuploidy (>25% of cells exhibit near-diploid aneuploidy) and childhood cancers (Hanks et al., 2004). Five of eight MVA patients were found to have mutations in both alleles of the BubR1 gene. One allele contained a mutation giving rise to a truncated or absent product, while the second allele carried a missense mutation in either the Cdc20 binding domain or in the essential kinase (Mao et al., 2003; Tang et al., 2001). However,

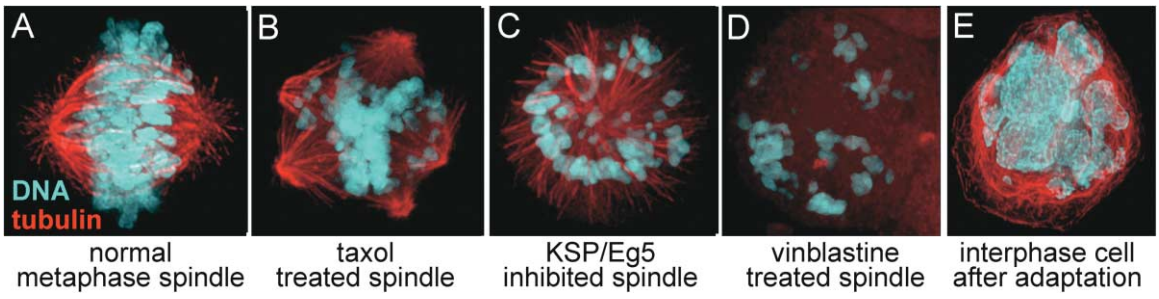

Figure 3. Spindle defects produced by antimitotic drug treatments

A: The normal bipolar spindle of an untreated cell at metaphase.
B: Taxol-stabilized microtubules produce multipolar spindles.
C: Monastrol, a KSP/Eg5 inhibitor, prevents spindle pole separation, resulting in monopolar spindles.
D: Vinblastine depolymerizes microtubules, preventing spindle assembly.
E: Cells that have undergone adaptation exit from mitosis despite the continued presence of antimitotic drugs and assemble abnormal interphase nuclei after aberrant mitotic exit without cytokinesis.

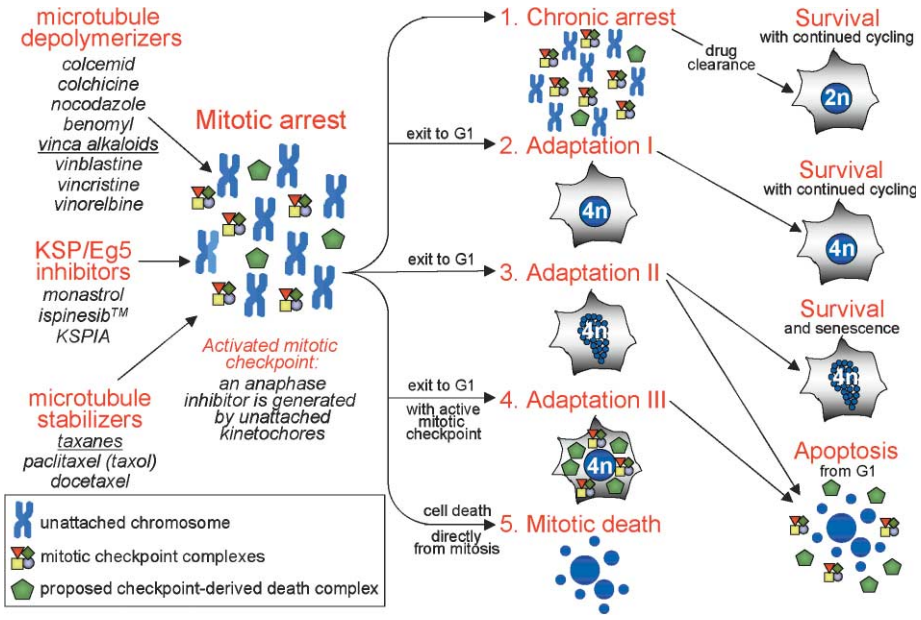

Figure 4. Possible outcomes of prolonged treatment with antimitotic drugs

Long-term treatment with spindle poisons (including microtubule depolymerizers, stabilizers, or KSP/Eg5 inhibitors) can have several outcomes: (1) chronic arrest in mitosis until the drug is removed; (2–4) adaptation from mitosis into G1 without cytokinesis despite the continued presence of drug, leading to tetraploidy and (2) continued cycling, (3) senescence, or (3 and 4) apoptosis; and (5) death directly from mitosis. Ispinesib, a KSP inhibitor produced by Cytokinetics (South San Francisco, CA), is currently in phase II clinical trials.

strikingly few mutations in mitotic checkpoint genes have been found in other human cancers, though altered transcription of checkpoint genes may be more common (Wang et al., 2000).

What has been found as a frequent feature of human tumor cells and cell lines is the inability to sustain mitotic checkpoint signaling, as judged by a reduced mitotic index after long-term drug-mediated spindle disruption. While this outcome has been alternately reported as reflecting a checkpoint that is "defective" (Wang et al., 2000) or "robust" (Tighe et al., 2001), in all of these cases, a checkpoint signal is produced but not sustained. The unifying message is that many tumors and tumor cell lines, including chromosomally unstable ones, have a weakened checkpoint signal that is sufficient for maintaining a viable population of cells but allows them to missegregate small numbers of chromosomes per division, resulting in aneuploidy and chromosomal instability.

Chronic activation of the mitotic checkpoint is a common chemotherapeutic strategy

The taxanes (paclitaxel/taxol, docetaxel), and vinca alkaloids (vinblastine, vincristine) have been used in the treatment of human cancer for decades. However, their mechanism of antitumor action remains unresolved. Taxanes stabilize microtubules, inducing multipolar spindles (Figure 3B), while the vinca alkaloids inhibit microtubule assembly (Figure 3D). Both types of drugs cause cells to accumulate in prometaphase as a consequence of mitotic checkpoint signaling from unattached kinetochores. However, the length of time for which intracellular drug concentrations persist at levels sufficient to sustain a checkpoint response remains an unanswered question. Additionally, though it has long been known that continuous treatment with such microtubule poisons often ultimately leads to cell death, the mechanism coupling mitotic delay to subsequent death remains almost completely unexplored.

Possible outcomes of chemotherapeutic activation of the mitotic checkpoint are outlined in Figure 4. The most obvious possibility of short-term drug treatment is sustained mitotic arrest until the drug is cleared. This mechanism of action would be cytostatic, with renewed cell cycling after drug removal. A second outcome is known as adaptation (Rieder and Maiato, 2004), in which cells exit long-term mitotic arrest while still in the presence of drug, fail cytokinesis, and enter G1 (Figure 3E). Whether or not mitotic checkpoint silencing is a prerequisite of adaptation is currently unknown. Adapted cells could then continue dividing (Figure 4, Adaptation I,), senesce (Adaptation II), or execute a death pathway (Adaptation II or III). Tao et al. (2005) now provide suggestive evidence for Adaptation III, in which cells escape to G1 despite continued mitotic checkpoint signaling, and the inappropriate presence of elevated levels of checkpoint inhibitors provokes apoptotic death in interphase. A final possibility is execution of a death pathway directly from mitosis.

The recent work by Tao and colleagues (2005) brings mitotic arrest and its linkage to cell death to the fore. The authors report the discovery of KSPIA, a novel small-molecule inhibitor of KSP (also known as Eg5), a member of the kinesin family of microtubule-dependent motors. KSP activity is required for spindle pole separation, and inhibition of its ATP-dependent motor activity results in monopolar spindles with unattached kinetochores (Figure 3C). Other KSP inhibitors (Kapoor et al., 2000; Sakowicz et al., 2004) have previously been reported, and one (ispinesib, produced by Cytokinetics) is currently being evaluated in Phase I and II clinical trials. Theoretically, KSP inhibitors have an advantage over the taxanes and vinca alkaloids in that KSP apparently functions only in mitosis, so this class of anti mitotic drug may reduce the unwanted side effects of microtubule disruption, especially in neurons.

Although the effect of KSP inhibitors on microtubules is different from the taxanes and vinca alkaloids, all three classes of drugs lead to chronic activation of the mitotic checkpoint. Tao et al. (2005) present some of the first evidence indicating that cell death following such long-term arrest is mediated by caspase-dependent death accompanied by activation of Bax, cleavage of PARP, and activation of the "executioner" caspase-3. Since treatment with the pancaspase inhibitor zVAD efficiently prevents KSPIA-induced cell death, these results offer strong support for the hypothesis that apoptotic cell death is induced by KSP inhibitors.

Tao and colleagues also offer suggestive evidence that cells treated with taxol or KSPIA exit to G1 before undergoing apoptosis (Figure 4, Adaptation III). They find that cells that can sustain a long-term (>48 hr) arrest in mitosis (e.g., HT29 colorectal adenocarcinoma cells) are less susceptible to KSPIA and taxol-mediated killing than are cells that more rapidly adapt into G1

after less than 24 hr in either drug (e.g., HCT116 colorectal carcinoma cells). Moreover, cell death is induced in a substantial number (about 25%) of the KSPIA-resistant HT29 cells by treating mitotically arrested cells with the Cdk1 inhibitor purvalanol, which presumably drives the cells out of mitosis. This lends support to the idea that cells arrested in mitosis must undergo adaptation in order to execute apoptosis. Observation of adaptation and/or apoptotic activation is now needed in live cells treated with antimitotic drugs to provide a direct test of this hypothesis.

Based on their indirect evidence, Tao and colleagues propose that it is the presence of high levels of checkpoint complexes during G1 that triggers apoptosis in cells that have undergone adaptation (Figure 4, Adaptation III). This suggestion is an attractive idea, but one that now remains to be put to an experimental test. Is the mechanism of adaptation through overriding an activated checkpoint signal, or through attenuation of its signaling? A third possibility that is not mutually exclusive with either of the others is that that long-term checkpoint activation may result in the production and accumulation of a currently unidentified proapoptotic complex, which may or may not include checkpoint proteins themselves. Further studies are now eagerly anticipated to identify the molecular links between the mitotic checkpoint and the apoptotic cascade.

In the meantime, another question of clinical significance is whether chromosomally unstable cells are more or less sensitive to antimitotic drug induced cell death, since chromosomal instability is such a common characteristic of tumor cells. The limited data now available are contradictory on this point, and the evidence reported by Tao et al. is no exception. As introduced above, some cells that can sustain prolonged checkpoint signaling (HT29 cells) are resistant to both taxol and KSPIA-mediated killing. However, cells with a weakened checkpoint (either by expression in HeLa cells of a dominant fragment of Bub1 or heterozygous deletion of Mad2 in HCT 116 cells) are also less sensitive than normal controls to KSPIA-induced cell death, even though in the presence of the drug they exit mitosis more quickly than those controls. This latter example also raises the possibility that there is a minimal window of time during which cells must remain arrested in mitosis in order to effectively activate the apoptotic machinery. With the data now available, no unifying mechanistic conclusion is yet possible, and further efforts will be required to determine whether chromosomally unstable cancers with a weakened mitotic checkpoint are more or less suitable for treatment with antimitotic drugs.

Similarly, the link between the mitotic checkpoint and the apoptotic machinery remains unclear. What proteins initiate the signal transduction cascade leading to cell death? Do checkpoint proteins participate directly? How long must the cells be delayed in mitosis in order to trigger apoptosis? What determines which cells will activate an apoptotic pathway in response to antimitotic treatment? Moreover, in an example of the "glass half full or half empty" conundrum, even in the best of circumstances reported by Tao et al., approximately 50% of cells escaped apoptosis after treatment with KSPIA. Such cells would be predicted to survive chemotherapeutic treatment and repopulate the tumor in between drug doses, and could therefore be argued to be the most problematic. Perhaps by dissecting the molecular linkage between mitotic arrest and cell death, it may be possible to manipulate insensitive cells in order to produce a more desirable outcome.

What is clear, especially with the new evidence from Tao et al., is that there are connections between the mitotic checkpoint, aneuploidy, adaptation, and cancer. In all cases, weakened mitotic checkpoint signaling promises to be dangerous, both in driving tumorigenesis and, possibly, by promoting resistance to chemotherapy. New insights that focus on the molecular links between mitotic checkpoint activation and the apoptotic machinery are now needed, if we are to rationally improve the therapeutic targeting of mitosis.

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