Role of Retinoblastoma Tumor Suppressor Protein in DNA Damage Response

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Growth arrest induced by DNA damage in mammalian cells requires the function of the retinoblastoma tumor suppressor protein (RB). RB-deficient cells cannot undergo G1, mid-S or G2 arrest following DNA damage, although they can activate the G2 checkpoint, which is reversible. RB-deficient cells are also hypersensitive to DNA damage-induced apoptosis. Induction of apoptosis in RB wild-type cells is associated with the loss of RB protein through cleavage by caspase. Two substitution mutations in exon 25 of the *Rb* gene have been created in the mouse germline to generate the *Rb-MI* allele that codes for a caspase-resistant RB protein. The RB-MI protein desensitizes cells to apoptosis. Taken together, these results suggest that RB plays a critical role in determining the cell fate following DNA damage. Growth arrest is dependent on RB and apoptosis is activated following RB degradation.

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DNA DAMAGE RESPONSE IN YEAST VERSUS MAMMALIAN CELLS

Genetic analyses in yeast have shown that survival after DNA damage is dependent on two important biological responses: the activation of DNA repair and the activation of cell cycle checkpoints (1). A major cell cycle checkpoint response occurs at the G2/M transition. DNA damage activates a cascade of protein kinases that transmit the damage signal to inhibit the M-phase promoting factor and thus prevent the entry into mitosis (2). DNA repair is more efficient during the G2-phase of the cell cycle, because of the existence of sister chromatids. Thus, the G2 cell cycle checkpoint plays a critical role in protecting the genome and in survival. A unicellular organism must recover from the G2 checkpoint to resume life, following DNA repair. Because the G2 checkpoint mechanism involves protein phosphorylation, it is readily reversible. Hence, the G2 checkpoint in the unicellular organism is not a permanent arrest of the cell (3). When the damage is severe or when the damage cannot be properly repaired, a yeast cell would not recover. Thus, DNA damage can lead to two alternative outcomes in yeast, recovery or no recovery (Fig. 1). Recovery is compromised in yeast by mutations in either DNA repair or cell cycle checkpoints.

It is well established that DNA repair mechanisms are conserved. Recent work has also established that the G2 checkpoint mechanism is also conserved from yeast to humans (2). The activation of DNA repair and of the G2 checkpoint is therefore important in protecting a damaged mammalian cell as well. However, besides repair and checkpoint, DNA damage can lead to two additional outcomes in mammalian cells: these are (a) a permanent growth arrest and (b) programmed cell death, also known as apoptosis (Fig. 1). These additional outcomes are likely to have evolved to protect the organism against a damaged cell. A permanent growth arrest, which can occur from any phase of the cell cycle (see below), will prevent a damaged cell from reproducing itself while maintaining the cytoplasmic function of that cell. Apoptosis will completely eliminate a damaged cell. These alternative biological outcomes in mammalian cells can be influenced by developmental lineage, and may also be affected by the repair capacity. Defects in DNA damage-induced permanent arrest or apoptosis would increase the probability of cell survival as long as the damage is not severe enough to compromise recovery (Fig. 1).

MAMMALIAN CELL SENSITIVITY TO DNA DAMAGE IS DETERMINED BY MANY FACTORS

By convention, sensitivity to DNA damage of mammalian cells is determined by the clonogenic survival assay. This assay measures the ability of damaged cells to recover from the damage and resume proliferation to form colonies. Cells that have lost the ability to recover, owing

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to severe or irreparable DNA damage, will not form colonies in these assays. These are the 'classically sensitive' cells. Cells that have undergone a permanent growth arrest will not form colonies in these assays, either. Thus, permanently arrested cells would be scored as being 'sensitive' to DNA damage. Cells that have undergone apoptosis, of course, are also scored as being 'sensitive' in these assays. Therefore, sensitivity to DNA damage determined by the clonogenic assay can be the result of a number of different causes. The fact that for clonogenic survival a distinction cannot be made between the three different fates, i.e., permanent arrest, inability to recover and apoptosis, might have contributed to some confusion in the study of DNA damage response in mammalian cells.

Similar to yeast, defects in cell cycle checkpoints or DNA repair will sensitize mammalian cells to DNA damage. Unlike yeast cells, however, defects in the permanent growth arrest or in apoptosis can lead to 'survival' in clonogenic assays. Survival resulting from defects in arrest or apoptosis is not the same as 'recovery', because the damage may not have been properly repaired in these surviving cells. Clearly, sensitivity to DNA damage in mammalian cells is determined by many different factors, some of which are not found in yeast.

In this paper, we discuss results that suggest an important role for the retinoblastoma tumor suppressor protein (RB) in determining the biological outcomes following DNA damage in mammalian cells. Our experiments with genetically defined mouse embryo fibroblasts and embryonic stem cells have allowed us to show that RB is required for a damaged cell to undergo permanent growth arrest. We have been able to distinguish between this RB-dependent permanent arrest from the reversible G2 checkpoint that is conserved through evolution. We have also obtained evidence that supports a role for RB in the inhibition of apoptosis. Our results suggest that inactivation of RB should sensitize mammalian cells to DNA damage-induced apoptosis. In light of these results, we propose that the loss of RB in tumor cells must be accompanied by defects in the apoptosis pathways that are inhibited by RB. We will discuss the possibilities that RB itself and the RB-inhibited apoptosis pathway may be a target of therapeutic intervention in sensitizing tumor cells to DNA damage-induced apoptosis.

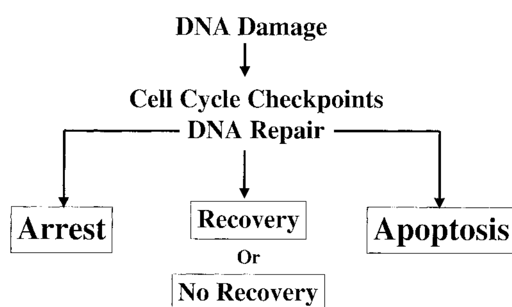

Fig. 1. DNA damage can lead to one of four different outcomes in mammalian cells. The activation of cell cycle checkpoints and DNA repair is a universal response to DNA damage. DNA repair and cell cycle checkpoint mechanisms, particularly the G2 checkpoint, are conversed from yeast to man. In yeast, DNA damage can lead to two outcomes: recovery from the checkpoint and damage to resume proliferation, or a lack of recovery owing to irreparable damage. In mammalian cells, DNA damage can cause two additional outcomes: permanent growth arrest, which can be distinguished from the evolutionarily conserved checkpoints, and apoptosis. See text for discussion of the role of RB in these alternative cell fates following DNA damage.

RESULTS

RB is required for DNA damage to induce long-term arrest

The retinoblastoma tumor suppressor protein is a nuclear phosphoprotein that has a critical function in the regulation of gene expression. The active, unphosphorylated form of RB protein can assemble transcription repression complexes at specific promoters (4). In particular, RB can repress E2F-regulated genes such as cyclin E, cyclin A, Cdc2, and other DNA synthetic enzymes to block cell proliferation (5, 6). RB is inactivated prior to the onset of DNA replication by a series of phosphorylation events that are catalyzed by several different cyclin-dependent protein kinases (7, 8). The inability to phosphorylate RB, owing to inhibition of the cyclin-dependent kinases, would prevent cells from entering into S-phase.

It should be noted that RB-deficient cells, derived from the *Rb*-knockout mouse embryos, are responsive to growth factors. The RB-deficient cells can withdraw from the cell cycle when they are deprived of serum (9, 10). This is because the RB-related p130 and p107 proteins can also repress E2F-regulated genes to arrest cell proliferation in the absence of RB. Thus, RB does not have an essential role in the growth factor response.

It has been established that RB has an essential role in arresting cells in G1 following DNA damage (11–13). The RB-positive cells can stop in G1 after being exposed to ionizing radiation or cisplatin, while the RB-deficient cells do not stop in G1 (11–13). It is well established that p53 is also essential to the G1-arrest response, because p53 activates the expression of p21Cip1 to inhibit the cyclin-dependent kinases. The activation of p53 and p21 is normal in *Rb*-deficient cells, although these cells do not undergo G1-arrest because they lack RB. These results demonstrate that RB is necessary for the p53/p21 pathway to exert a negative effect on G1/S transition. These results also show that p130 and p107, in the absence of RB, cannot arrest cells in G1 following DNA damage.

Knudsen et al. have recently reported that RB also plays a role in causing an S-phase arrest in cells that have encountered a high dose of DNA damaging agents, such as cisplatin and mitomycin C. The RB protein is shown to

become dephosphorylated in S-phase cells following DNA damage (13). Following the dephosphorylation of RB, DNA synthesis is completely blocked even in cells with S-phase DNA content. However, the RB-deficient cells do not become arrested in S-phase (13). Knudsen et al. have prepared a constitutively active RB protein, known as the phosphorylation site mutated (PSM)RB, which cannot be inactivated by the cyclin-dependent protein kinases (14). When this PSM-RB is expressed in S-phase cells, it can block ongoing DNA replication (15). Taken together, these results demonstrate that RB can become activated inside S-phase by DNA damage, and the consequence of RB activation is an S-phase arrest (13).

While RB is required for DNA damage to arrest the cell cycle in G1 or in S-phase, RB is not required for the G2-checkpoint response, that is conserved from yeast to man (Naderi et al., in preparation). The accumulation of primary mouse embryo fibroblasts (MEFs) in G2-phase of the cell cycle could be observed following ionizing radiation. The duration of the G2 arrest is increased with increasing doses of radiation. When MEFs were irradiated with 2 Gy or 4 Gy, we observed a transient stop in G2 with both the $Rb^{+/+}$ and $Rb^{-/-}$ cells. By 24 h after 2 to 4 Gy IR, the cell cycle distribution was similar to that of unirradiated controls, suggesting a complete recovery (Fig. 2). At 8 Gy of IR, however, $Rb^{+/+}$ cells became permanently arrested in G2 (Fig. 2). This permanent arrest has been described previously by others as being dependent on p53 and p21Cip1 and is associated with the transcription repression of the Cdc2 and cyclin B genes (16–19). We have found that the long-term G2 arrest is also dependent on RB, because $Rb^{-/-}$ cells can recover from 8 Gy of irradiation and re-enter S-phase (Naderi et al., in preparation). By cell counting, we could show that the entire population of $Rb^{-/-}$ MEFs could resume the cell cycle, while the $Rb^{+/+}$ MEFs never divided again, following 8 Gy of IR. The resumption of proliferation with 8 Gy-irradiated $Rb^{-/-}$ MEFs did not occur until after 4 to 6 days. After these $Rb^{-/-}$ MEFs recovered from 8 Gy of IR, they still remained responsive to another dose of IR and could activate the G2 checkpoint again. Therefore, the $Rb^{-/-}$ MEFs did not 'adapt' to the G2 checkpoint, but, instead, recovered from the high dose of radiation. These results strongly suggest that MEFs could repair the damage caused by 8 Gy of IR if they are given enough time (4–6 days). The inability of $Rb^{+/+}$ MEFs to recover from 8 Gy of IR is caused by an RB-dependent permanent arrest. These results also allowed us to distinguish between the G2 cell cycle checkpoint, which is reversible and does not depend on RB, and the G2 cell cycle arrest, which is irreversible and requires the function of RB (Fig. 2).

RB-deficient cells are sensitized to apoptosis

In previous studies it has been found that RB-deficient cells exhibit an increased sensitivity to agents that cause

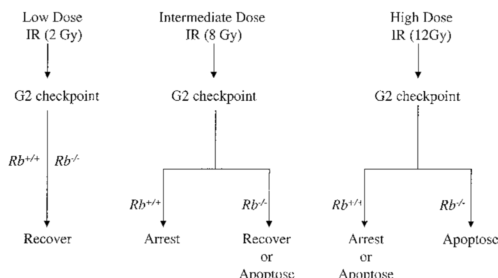

Fig. 2. Contrasting the response of RB-positive and RB-deficient cells to different doses of ionizing radiation.Primary fibroblasts were prepared from the $Rb^{+/+}$ and $Rb^{-/-}$ embryos in the same pregnancy and subjected to the indicated dose of ionizing radiation. The G2 checkpoint response was monitored by bi-variant FACS analysis and by the inhibition of Cdc2 kinase activity, as previously described (13). With 2 Gy of IR, $Rb^{+/+}$ and $Rb^{-/-}$ cells exhibited a transient delay in G2-phase, and resumed proliferation between 24 and 48 h after irradiation. With 8 Gy of IR, both the $Rb^{+/+}$ and $Rb^{-/-}$ cells exhibited a prolonged arrest in G2-phase. The arrest with the $Rb^{+/+}$ cells was permanent, no proliferation being observed for 8 passages (24 days), while the cells maintained viability. The arrest with the $Rb^{-/-}$ cells was reversible after 8 Gy of IR. The $Rb^{-/-}$ cells began to re-enter S-phase at 3 days after 8 Gy of IR, and resumed proliferation at a rate that was similar to the unirradiated $Rb^{-/-}$ cells. With 12 Gy of IR, the $Rb^{+/+}$ cells underwent either a permanent arrest or apoptosis. By contrast, $Rb^{-/-}$ cells only underwent apoptosis. These results show that RB is required for the establishment of a permanent growth arrest. The RB-mediated growth arrest is radiation dose dependent. These results also show that RB-deficient cells are more sensitive to radiation-induced apoptosis. Interestingly, $Rb^{+/+}$ cells lose the RB protein when they undergo apoptosis. The loss of RB is catalyzed by caspase cleavage and proteolysis (see Fig. 3).

DNA damage (20). This is also true with *Rb*-null mouse embryos. Increased apoptosis in the central and peripheral nervous systems was found with mouse embryos that are homozygously mutated for the *Rb* gene (21–23). In the RB-deficient embryos, apoptosis was observed with neurons in the post-mitotic ventricular zone and with the lens epithelial cells in the post-mitotic compartment (21–23). The abnormal apoptosis phenotypes associated with *Rb*-null mutation can be partially corrected by mutating the p53 gene or the E2F-1 gene (24). Embryos with the combined mutations *Rb* and E2F-1, or *Rb* and p53, do not exhibit apoptosis in the developing lens or in the developing central nervous system. However, apoptosis in the peripheral nervous systems was unabated in *Rb*-null embryos irrespective of the p53 or E2F-1 genotypes (24). As a result, mutation of p53 or of E2F-1 cannot rescue the embryonic lethality of RB-deficient mice.

We have extended these previous results by showing that RB-deficient MEFs are sensitized to apoptosis following exposure to ionizing radiation. As summarized in Fig. 2, RB-deficient MEFs do not undergo long-term G2 arrest following 8 Gy of IR, whereas the wild-type MEFs become arrested in G2 at this dose of IR. While the RB-defi-

cient cells re-entered the cell cycle, a fraction of these cells underwent apoptosis, evidenced by the fragmentation of genomic DNA. The increased sensitivity to apoptosis was more evident when RB-deficient cells were exposed to a higher dose of IR, at 12 Gy. At 48 h following 12 Gy of IR, more than 80% of the RB-deficient cells were found to have fragmented their genomic DNA. In comparison, less than 40% of the RB-positive cells underwent apoptosis at this dose of IR. Similar to the results with the RB-deficient mouse embryos, we have found that the mutation of p53 could block the IR-induced apoptosis in RB-deficient MEFs. We have prepared MEFs from embryos that are deficient for both the *Rb* and the p53 gene. These MEFs, deficient for both RB and p53, are resistant to apoptosis. Thus, the loss of RB function can sensitize cells to IR-induced, and p53-dependent apoptosis.

RB is cleaved and degraded in apoptotic cells

Previous studies have shown that the RB protein is cleaved by caspases during apoptosis at a specific C-terminal site (25–28). The caspase-cleavage sequence is present in the RB protein of vertebrate species, including human and mouse. However, this caspase-cleavage sequence is not found in the RB-related p130 and p107 proteins, or in the RB-related proteins of invertebrates and plants. The cleavage of RB generates an unstable form of the RB protein, which is rapidly degraded by other proteolytic enzymes (25, 26). As a result, caspase cleavage leads eventually to the complete loss of the RB protein (Fig. 3). Thus, caspase can convert RB-positive cells into RB-negative cells. The cleavage of RB is observed in a wide variety of cell types under conditions of apoptosis induction (27). These include apoptosis induced by DNA damage, by tumor necrosis factor, and by the withdrawal of survival factors (27). Because RB-deficient cells are sensitized to apoptosis, and because RB-positive cells can be converted into RB-negative cells upon the activation of caspases, these published results suggest that the cleavage and degradation of RB might contribute to the induction of apoptosis.

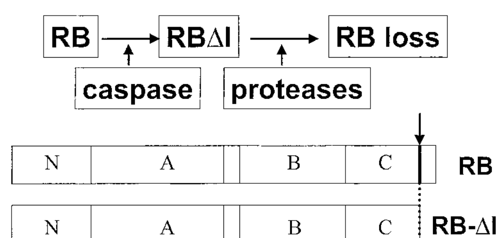

Fig. 3. Cleavage of RB protein by caspase leads to the loss of RB. RB protein contains a cleavage site for the type II caspases at its C-terminal end. Cleavage generates the RB-ΔI protein that is unstable. The RB-ΔI protein is further degraded by other proteases leading to the loss of RB (26). Thus, activation of the caspases can convert RB-positive cells into RB-negative cells.

Cleavage-resistant RB-MI desensitizes cells to apoptosis

The caspase-cleavage site in the RB protein has been mutated to produce a cleavage-resistant RB-MI protein (26). The RB-MI protein is not cleaved by caspase in vitro or in vivo (26). As a result, the RB-MI protein is stable in cells that contain activated caspases. Transient expression of the RB-MI protein in RB-deficient cells can lead to protection from TNF-induced apoptosis. Recently, the RB-MI protein has also been expressed in neurons and shown to protect neurons from apoptosis (28).

The direct demonstration that the cleavage of RB is relevant to physiological apoptosis has come from the construction of a mutant mouse, which expresses the RB-MI protein (Chen & Wang, submitted). Using the gene targeting method in mouse embryo stem cells, we have introduced two point mutations into the germline *Rb* gene and succeeded in transmitting this mutated *Rb-MI* allele through the construction of chimeric mice. We then bred the heterozygous *Rb-MI* mice to generate *Rb-MI* homozygous mutant mice. Interestingly, we observed increased cellularity in the developing brains of the *Rb-MI* embryos when compared to their wild-type littermates (Chen & Wang, submitted). With adult *Rb-MI* mice, close examination also revealed increased cellularity in some regions of the brain. The *Rb-MI* mice do not show increased tumor incidence, thus mutation of the caspase-cleavage site did not inactivate the tumor suppression function of RB. We challenged the *Rb-MI* mice with intraperitoneal injections of bacteria lipopolysaccharides (LPS), which can induce septic shock. At the high dose of 40 mg/kg-body weight, LPS causes the death of wild-type mice between 24 to 48 h after injection. With the *Rb-MI* mutant mice, we observed the initial reactions to the injected LPS and could detect comparable levels of increase in the blood levels of TNF-α and IL-1β as with the wild-type mice. However, these *Rb-MI* mice recovered and survived the dose of LPS that was lethal to the wild-type mice. We measured apoptosis in different tissues collected from LPS-treated mice and found that LPS induced apoptosis in the spleen and the intestinal microvilli. Interestingly, apoptosis in the spleen of *Rb-MI* mice was comparable to that of the wild-type mice. However, apoptosis in the intestinal microvilli was suppressed in the *Rb-MI* mice (Chen & Wang, submitted). These results demonstrate that the RB-MI protein can protect specific cell types from apoptosis either during embryonic development or during an aggravated inflammatory response.

Previous studies have shown that the cleavage and the degradation of RB can be observed in a large variety of cell types following treatment with different types of apoptosis inducers, including DNA damage (27). We therefore examined whether RB-MI can protect against DNA damage-induced apoptosis and found that the thymocytes, and the developing neuroblasts from the *Rb-MI* mice do un-

dergo apoptosis following ionizing radiation. The MEFs from the *Rb-MI* mice can also undergo apoptosis following ionizing radiation. Thus, RB-MI cannot protect thymocytes, the developing neuroblasts and MEFs against IR-induced apoptosis. Interestingly, however, we found that RB-MI ES cells are less sensitive to apoptosis induced by adriamycin (Chen & Wang, submitted). The differential ability of RB-MI to protect against DNA damage-induced apoptosis may be due to the levels of expression of the RB protein in the different cell types. Alternatively, RB-MI may become inactivated by phosphorylation in these cell types and thus unable to its inhibitory effect on the apoptosis response.

DISCUSSION

Two models for the role of RB in apoptosis

Two alternative models have been proposed to explain the sensitivity of *Rb*-deficient cells to apoptosis. These two models are summarized in Fig. 4. The first model, widely known as the 'conflict model', is proposed to explain both the apoptotic function of E2F-1 and the apoptosis of RB-deficient cells. In this model, apoptosis is considered to be an adaptive response to over-stimulation with mitogenic signals and to the simultaneous exposure of cells to mitogenic and anti-mitogenic signals. Because the RB-deficient cells cannot undergo growth arrest, they are thought to be under 'conflict' when presented with anti-mitogenic signals. The essence of this model is based on the idea that growth arrest overrides apoptosis (Fig. 4).

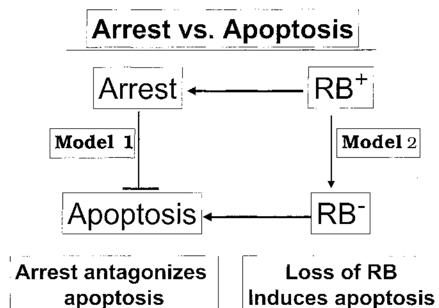

Fig. 4. Two alternative models for the role of RB in growth arrest and apoptosis. The sensitivity of RB-deficient cells to apoptosis can be explained by two different models. In Model 1, RB is proposed to cause growth arrest. When RB is absent, cells cannot activate the growth arrest response, become conflicted and this 'conflict' triggers apoptosis. Model 1 does not invoke a direct role of RB in inhibiting apoptosis and therefore is consistent with the tumor suppression function of RB. In Model 2, RB is proposed to cause growth arrest and actively to inhibit apoptosis. When RB is absent, cells not only cannot undergo growth arrest but are also sensitized to apoptosis. Model 2 appears to be in contradiction with the tumor suppressor function of RB, as inhibitors of apoptosis are tumor promoters. Model 2 is, however, supported by our results that the preservation of RB through the mutation of the RB caspase-cleavage site (RB-MI) can desensitize cells to apoptosis. The RB-MI protein retains its function in growth arrest and tumor suppression. Model 1 would predict that RB-MI and RB should have the same effect on apoptosis. Model 2 would predict that RB-MI should inhibit apoptosis under conditions when RB does not have this effect. Our results with the RB-MI mutant mice are better explained by Model 2.

The second model proposes that RB actively inhibits apoptosis. This model explains the cleavage and degradation of RB during apoptosis. In this model, RB inhibits the functions of pro-apoptotic factors, such as E2F-1. When RB is cleaved and degraded, E2F-1 becomes activated and contributes to the induction of apoptosis. The RB-deficient cells are prone to apoptosis because they lack the RB-dependent inhibitory mechanism. The RB-positive cells undergo apoptosis after RB is degraded through caspases and other proteases. This model would predict that if the cleavage of RB is prevented, cells would be less likely to commit suicide (Fig. 4). This model, therefore, is supported by the phenotype of the *Rb-MI* mice.

RB is a conditional tumor suppressor

Retinoblastoma protein has a well-established function in tumor suppression, because RB inhibits cell proliferation. Mice with heterozygous mutation of the *Rb* gene ($Rb^{+/-}$) develop pituitary tumors with $>90\%$ penetrance (21–23), again supporting a role of RB in tumor suppression. The oldest *Rb-MI* mice in our colony died of old age (>2 years) without increased tumor incidence. Thus, the *Rb-MI* allele retains its tumor suppression function. We have crossed the *Rb-MI* mice with $p53^{-/-}$ mice. Interestingly, we have observed an acceleration of tumor development with mice of the $Rb+/MI/p53^{-/-}$ genotype (D. Cheng, T.-T. Chen & J. Y. J. Wang, unpublished). These results suggest that *Rb-MI* has gained a tumor promotion function, consistent with its ability to inhibit apoptosis.

The role of RB in inhibiting both cell proliferation and cell death may seem contradictory in the context of tumor development. However, these functions of RB may be well suited to the establishment of terminally differentiated cells that are resistant to apoptosis. In fact, RB does play an important role in the development of muscles and neurons, and these are cell types that do not proliferate and are generally more resistant to apoptosis. The anti-apoptotic function of RB appears to be tissue-specific, indicated by the differential protection of intestinal microvilli but not spleen in LPS-treated *Rb-MI* mice. The tumor suppression function of RB also exhibits tissue specificity: RB suppresses the formation of retinoblastoma in human and pituitary tumor in mice. Human retinoblastoma involves only the mutation of the *Rb* gene. Perhaps the loss of RB does not activate apoptosis in human retinoblasts. Alternatively, RB-deficient retinoblasts may suppress apoptosis through epigenetic mechanisms.

In sporadic cancer, the loss of RB is almost always complemented by mutations that inactivate apoptosis. Although the p53 gene is not mutated in retinoblastoma, p53 mutation is usually detected in sporadic cancers that in-

volve the mutation of the *Rb* gene. This is also illustrated by the necessary inactivation of both RB and p53 in transformation induced by viral oncoproteins, e.g., E1A and E1B, E6 and E7 (29). It is interesting to note that the *Rb* gene itself is seldom mutated in sporadic tumors. Rather, there is a selection for genetic defects that increase the phosphorylation of RB in sporadic cancer (30). The inactivation of RB by phosphorylation is associated with cell proliferation but not cell death. This suggests that the RB-suppressed apoptosis pathways may also be inactivated during normal cell cycle progression in parallel with RB phosphorylation.

Because RB can inhibit cell proliferation and apoptosis, we consider it to be a 'conditional' tumor suppressor gene. In order for tumor cells to take advantage of the loss of RB, they must inactivate the apoptosis mechanisms that are inhibited by RB. RB is known to inhibit the function of E2F-1, which has the ability to activate genes that induce apoptosis. Previous studies have linked E2F-1 to the activation of p53, through the transcriptional induction of the p19Arf protein (31–34). Recent reports have linked E2F-1 to the p53-related gene p73, which is directly activated at the transcriptional level by E2F-1 (35). Welch & Wang have shown that RB can also inhibit the function of c-Abl tyrosine kinase (36, 37). Recent reports have linked c-Abl to the stabilization and activation of p73 protein (38–40). It will be of interest to determine whether the E2F-1, c-Abl and/or p73 function is compromised in tumor cells that have mutations in the *Rb* gene.

Therapeutic implications

The increased sensitivity of RB-deficient cells to apoptosis can be explored in cancer therapy. The RB function is inactivated, either by the mutation of the *Rb* gene or by the increased RB phosphorylation, in sporadic cancer (30). The inactivation of RB should sensitize tumor cells to apoptosis, unless the tumor cells have developed compensatory mechanisms to suppress apoptosis. Based on this rationale, cancer cells with inactivated RB should possess anti-apoptotic functions that are not found in normal cells. One of the anti-apoptotic mechanisms of cancer cells is the mutation of the p53 gene. This defect has been explored in a therapeutic strategy that targets p53-mutated cells for destruction. It is possible that other anti-apoptotic functions may also be found in cancer cells that have inactivated RB. Each one of these functions can become a target for therapeutic intervention because they are unlikely to be important in cells that retain wild-type RB.

Considering our results from the opposite angle, the RB-MI protein may be used to protect normal cells from apoptosis during cancer therapy. For example, RB-MI may be selectively expressed in patient bone marrow cells to increase their resistance to cancer therapeutic agents.

Finally, the *Rb* gene itself is seldom mutated in sporadic cancer. The inactivation of RB in a majority of cancer cells is through RB phosphorylation. Because phosphorylation is readily reversible, the RB protein in a majority of cancer cells can be reactivated. Perhaps the reactivation of RB under conditions of physiological stress can protect cancer cells from apoptosis. This might explain why cancer cells choose to inactivate RB by phosphorylation rather than by the direct mutation of the *Rb* gene. It is conceivable that RB-positive tumor cells might escape apoptosis by reactivating RB through dephosphorylation in response to radiation or chemotherapy. Further studies of RB-regulated apoptosis pathways in cancer cells will be required to determine whether the inactivation of RB might have a therapeutic advantage in cancer treatment.

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