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Independent regulation of P53 stabilisation and activation after Rb deletion in primary epithelial cells

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Abstract. We have previously reported that deletion of the retinoblastoma gene Rb leads to rapid but transient p53 stabilisation. We investigated here the pathways involved. We show that upon Rb-deletion dysregulated E2F activates p19ARF expression that localises in the nucleoli. There it interacts with MDM2, leading to P53 stabilisation. At the same time, ATR is activated, activating CHK1 that may phosphorylate P53 but also contribute to inhibition of MnSOD expression leading to accumulation of ROS (reactive oxygen species) and subsequent DNA injury, which in turn maintains ATR/CHK1 activated. However, from 72 h after Rb deletion, NPM interacts with P19ARF and concomitantly the interaction between p19ARF and MDM2 decreases leading to a return to P53 degradation. This occurs despite the persistence of the DNA damage response pathways. We therefore observe in primary cells not subjected to exogenous gene expression or exogenous DNA damaging treatment, activation of 2 concomitant pathways of activation of P53 that are dealt with in independent manner: an oncogenic pathway with rapid activation of ARF which is ‘switched off’ downstream of p19ARF activation after 72 h of induction and a DNA damage response pathway keeping a low level of transcriptionally active P53 sufficient to deal with a physiological elevation of oxidative DNA injury. A possible connection between the two pathways is discussed.

Introduction

The retinoblastoma protein pRb is a critical regulator of cellular proliferation. It forms a complex with E2F transcription factors and inhibits their transcriptional activity. Upon phosphorylation by cyclin D-cdk4/6 in early G1 and cyclin E-cdk2 in late G1, pRb dissociates from the complex allowing E2F to activate expression of genes required for S phase. pRb is a tumor suppressor, and decreased expression is common in cancer.

Using a Cre/Lox system to specifically delete the Rb gene from adult mouse hepatocytes by infection with an adenovirus expressing the Cre recombinase (1,2), we are studying the early effects of the loss of Rb. We have previously shown in vitro that Rb deletion from primary liver cells quickly leads to dysregulated proliferation, with the concomitant appearance of multinucleated cells, cells with nuclei of abnormal morphology or cells showing abnormal mitosis reminiscent of abnormalities observed after γ-irradiation (1). Interestingly, we have also reported that in response to Rb deletion, P53 is rapidly stabilised and activated (2). However, this stabilisation is only transient and resolves within 4 days in culture.

P53 regulates signalling pathways controlling cell proliferation, apoptosis and DNA repair. Cellular P53 levels are normally low due to rapid degradation by the proteosome, promoted by the ubiquitin ligase and P33 partner MDM2. In response to various stimuli, the interaction of P53 with MDM2 is reduced through mechanisms ranging from post-translational modification of either protein to protein/protein interaction preventing the formation of MDM2/P53 complex (reviewed in ref. 3), thus leading to P53 stabilisation and accumulation.

In response to DNA injury, for example, various pathways are activated depending on the nature of the DNA damage and the cell type. However, all activate specific kinases that are responsible for the phosphorylation of P53 at key residues including Ser18 (Ser15 in human) (reviewed in refs. 3-6). This phosphorylation is thought to disrupt the interaction with MDM2, contribute to nuclear retention and to activate P53. The same kinases also phosphorylate other target proteins important for the regulation of cellular responses to DNA damage, which can be used as markers of DNA injury. One such marker is H2Ax, a Histone2A variant that rapidly undergoes phosphorylation on Ser139 (γH2Ax) in response to DNA damage, particularly double strand breaks (7-9).

P53 can also be stabilised in response to aberrant activation of oncogenes (10), including E2F1, in which case instability arises from activation of the ARF pathway (p14ARF in human, p19ARF in mouse). ARF is a nucleolar protein which can sequester MDM2 (11-13), preventing its association with P53 and restricting its E3 ligase activity (14,15). In this instance P53 is stabilised without requiring phosphorylation.

One of the critical effects of Rb deletion is to increase E2F activity (2,16). It has been reported that overexpression

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of E2F activates p19ARF (17). With this in mind, we asked what pathway(s) activated after Rb deletion from otherwise genetically normal primary epithelial cells might account for the rapid but transient stabilisation of P53.

Materials and methods

Hepatocyte isolation, culture and adenovirus infection. Mouse primary hepatocytes (male, 6-12 weeks old), were isolated by a two-step retrograde liver perfusion (18) of Rb-floxed mice (19) and plated onto fibronectin-coated plastic in serum-free medium supplemented as previously described (1).

Wild-type and Rb⁻/⁻ isogenic hepatocytes were obtained by infection of the hepatocytes in vitro with an adenovirus expressing Cre-recombinase under the human CMV promoter (Ad-Cre) or an adenovirus control (Ad-D70) (Virapur, USA) a multiplicity of infection of 10 (2,20). Both adenoviruses are replication-deficient (E1 deleted) serotype 5. Infection with the adenovirus expressing Cre-recombinase leads to deletion of the Rb gene within 16-24 h while cells infected by the adenovirus control are phenotypically wild-type.

Immunoblotting. Proteins were prepared from snap-frozen cell pellets using the appropriate lysis buffer. H2A.X, 10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl. All other cell pellets using the appropriate lysis buffer. H2A.X, 10 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween-20.

Proteins were separated on SDS-PAGE and detected by immunoblotting. Detection of P19 ARF required an avidin-biotin amplification Kit (574596 Calbiochem), diluted in 5% Marvel TBST or TBST (Bio-Rad) to capture images and Versadoc Imaging for densitometric analysis. Proteins were visualized by ECL-plus using a versadoc4000 (Bio-Rad) with an adenovirus

ROS and DNA damage quantification

8-oxo-dG. DNA was extracted using sodium iodide method (40) according to the manufacturer's recommendation (DNA Extractor WB-Kit, Wako Pure Chemical Industries, Japan). Briefly cells were lysed in a non-ionic surfactant, polyoxyethylene oxyphenyl ether buffer and spun at 10,000 g for 20 sec at 4˚C. The pellet was suspended in a SDS containing protease solution for 1 h at 37˚C. After addition of NAI the DNA was precipitated by addition of 0.5 ml of isopropanol alcohol and resuspended in water. The levels of 8-oxo-dG were measured using an ELISA kit according to the manufacturer's instructions (JaICA, Japan) in control and Rb⁻/⁻ DNA or 8-oxo-dG standards.

AP site. Apurinic and apyrimidinic sites (AP sites) in hepatocyte DNA were detected using a DNA damage quantification kit according to the manufacturer's recommendation (K253-25 Biovision, UK). Briefly, the extracted DNA is incubated with a probe reacting with the aldehyde groups in the AP sites in the DNA, leading to the tagging with biotin residues which are detected with a colorimetric assay.

Reactive oxygen species (ROS). Measurement of ROS was done using 5-(&-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). CM-H2DCFDA is a diacetate ester that enters passively into cells where it is cleaved by intracellular esterases leading to its retention in the cells. Reaction with intracellular ROS (hydrogen peroxide, hydroxyl radical, peroxy radical and peroxynitrite anion) leads to emission of green fluorescence. The cells were treated with 10 μM CM-H2DCFDA and fluorescence quantified over one hour using a fluorimeter (Fluoroskan, Thermo Life Sciences) at 485 nm and 510 nm with readings taken every 3 min. This was normalised according to the amount of protein in each sample.
Superoxide anion. Dihydroethidium (DHE) was used to measure the amount of superoxide anion in the hepatocytes. Upon reaction with ROS DHE is oxidised to ethidium that intercalates into the DNA and fluorescence red. Cells were incubated with DHE for 4 h, at 37˚C. Red fluorescence (567 nm) was quantified as above.

Statistical analyses. Statistical analyses were performed using Minitab 13.1 software. Effects of time and genotypes were evaluated by univariate analysis of variance (ANOVA). Differences were taken to be significant when p<0.05. Satisfactory homogeneity of variances was determined with Bartlett’s test. Where a significant difference between means was identified, we performed pairwise comparison of means to the control using a Bonferroni correction for multiple comparisons.

Results

We have previously shown that after Rb deletion p53 is rapidly stabilised and that P21 expression is induced in a p53-dependent manner (2): 72 h after infection of primary RB-loxed hepatocytes with an adenovirus expressing the Cre recombinase, 70-80% of the cells show strong p53 and p21 nuclear positivity (2) (Fig. 1).

However, we also observed that the nuclear p53 stabilisation is only transient with the proportion of cells showing nuclear positivity decreasing sharply 80-96 h after infection (Fig. 1A). In contrast, P21 remains high and this activation is P53-dependent (Fig. 1B).

After Rb deletion, a DNA damage pathway is activated. A hallmark of P53 activation by DNA damage is phosphorylation of Ser18 (Ser15 in human). This phosphorylation was detected in Rb-/- cells only from 48 h after Rb deletion (Fig. 2). Further evidence to support activation of DNA damage pathways was the detection of increasing levels of γH2AX (phosphorylated at Ser139), a recognised marker of DNA damage, in Rb-/- cells: 96 h after Rb deletion 98-99% of Rb-/- cells were positive for γH2AX (Fig. 2). Both P53 and γH2AX are components of two DNA damage pathways that are driven by the DNA damage kinases ATR and ATM via checkpoint kinases 1 and 2 (Chk1 and Chk2) respectively. However, neither changes in ATM expression, nor phosphorylation of Chk2 on thr68 (target site for ATM) were detected (data not shown), suggesting that the ATM pathway is not involved here. By contrast, from 72 h after plating, ATR was found to localise in the nucleoli of 50-80% of Rb-/- hepatocytes (Fig. 3), suggesting activation of the ATM pathway is not involved here. By contrast, from 72 h after plating, ATR was found to localise in the nucleoli of 50-80% of Rb-/- hepatocytes (Fig. 3), suggesting activation of the protein (22). Phosphorylation of Chk1 kinase on Ser317, a site targeted by ATR was also detected in Rb-/- cells from 72 h, and could be inhibited by the addition of caffeine [at a concentration of 2 mM, sufficient to inhibit the activity of ATM and ATR but not DNA-PK (23)] (Fig. 3B and C). Caffeine similarly inhibited the phosphorylation of P53 at
superoxide anion turnover (27). We hypothetised that in reduction of MnSOD (26) a mitochondrial enzyme catalysing were repeated with consistent results. (C) Immunofluorescence for or absence (-) of caffeine. ß-actin is shown as loading control. The experiments with the activation of Chk1 (Fig. 3B) in control (wt) and Rb-/-. 

First, we quantified ROS using two different compounds (Fig. 5A and B). From 48 h in culture ROS levels were consistently greater in Rb\(^{-}\) cells compared with wild-type control (Fig. 5A) (Student’s t-test p=0.03621). The same trend was observed using DHE which detected higher levels of superoxide anion in Rb\(^{-}\) hepatocytes compared to wild-type controls (Fig. 5B) (asterisk denotes a significant difference using a two-tailed t-test).

We then quantified 2 types of DNA injury known to arise from ROS: 8-oxo-dG produced through the oxidation of guanine and secondly, AP sites formed via hydrolysis of the N-glycosyl bond. From 72 h in culture, the level of 8-oxo-dG rose and was greater in Rb\(^{-}\) compared with control hepatocytes (Fig. 5C) (asterisk denotes a significant difference using a two-tailed t-test). This increase was also observed for AP sites, although statistical significance was not achieved (Fig. 5D, p=0.1773) which could be expected as levels of endogenously produced ROS levels are low and changes small.

Our results therefore support the hypothesis that in response to Rb deletion, MnSOD expression is inhibited leading to an increase in endogenous ROS with consequences for DNA damage and activation of P53. However, while the DNA damage pathway is still active 96 h after plating (H2AX activated, P53 phosphorylated on Ser18, P53-dependent activation of P21 expression, increased ROS and DNA damage); the number of hepatocytes showing P53 stabilisation decreased sharply (Figs. 1 and 6) to ~10%. This suggests that the activation of the DNA damage pathway by a low level of endogenous DNA injury, including phosphorylation of P53 on Ser18 is not sufficient to maintain high levels of P53 stabilisation. Indeed some Rb\(^{-}\) hepatocytes positive for γH2AX were negative for p53 (data not shown). We investigated whether p19ARF could be involved.

After Rb deletion, P19ARF is activated and contributes to P53 stabilisation. E2F overexpression has been shown to induce P19ARF expression, and interaction of ARF with MDM2 stabilises P53. We therefore investigated whether P19ARF was activated in response to Rb deletion.

P19ARF expression was higher in Rb\(^{-}\) cells compared with control (Fig. 7). It was detected from 72 h in culture by RT PCR (Fig. 7A) and from 48 h by Western blotting (Fig. 7B) in Rb\(^{-}\) cells. By contrast, there was no expression detectable in control cells at early time-points and only weak expression was observed 96 h after plating. Real-time PCR confirmed an increase of ~10-fold of p19ARF expression in Rb\(^{-}\) cells (Fig. 7C), and this was reduced when the cells were treated with E2F antisense (Fig. 7D).

Currently there is not one unifying model able to accurately describe the regulation of p53 by ARF in the different systems.
studied. It is clear that ARF stabilises P53 through binding to MDM2, thus preventing the formation of MDM2/p53 complexes and targeting of P53 for degradation but the regulation of ARF itself seems to be different in different systems. ARF localisation and its interaction with the nucleolar protein NPM/B23 are nevertheless central to the problem and were studied next.

There were no significant differences between MDM2 protein levels detected in control and Rb−/− cells (data not shown). P19ARF immunoprecipitation pulled down MDM2 in Rb−/− cells only (Fig. 8) confirming an interaction between these proteins 48 and 72 h after plating. The interaction was greatly decreased 96 h after plating, in agreement with the decreased P53 nuclear staining observed at that time (Fig. 6). According to some models, ARF localisation is thought to affect its ability to stabilise p53. P19 ARF was detected in Rb−/− cells only (Fig. 9A) and localised in the nucleoli at all time-points as confirmed by co-staining with nucleolin (Fig. 9B). Quantification showed that the number of Rb−/− cells positive for P19ARF rose sharply, following the increased of P53 positive cells but remained high after 72 h when the number of P53 positive cells decreases (Fig. 9C). Thus the interaction between P19ARF with MDM2 did correlate closely with P53 stabilisation but was not regulated by a change in the localisation of P19ARF.

Nucleophosmin (NPM), a nucleolar phosphoprotein, can compete with MDM2 for interaction with P19ARF (28). We investigated if NPM was involved in the changes of interaction between MDM2 and P19ARF. Wild-type and Rb−/− hepatocytes showed similar NPM expression patterns (Fig. 10A), but immunoprecipitation with P19ARF antibodies pulled down
NPM in Rb-/- only, showing an interaction between the two proteins from 72 h after plating (Fig. 10B).

Discussion

Oncogenic stimuli leading to P53 stabilisation are complex as they can potentially trigger multiple apparently unrelated pathways. An example is shown here using primary epithelial cells, where a physiological increase in E2F activity is obtained in response to acute Rb deletion. On one hand, increased E2F activity activates the expression of ARF which interacts
with MDM2 and contributes to P53 stabilisation. On the other hand, E2F leads to decreased MnSOD expression, accumulation of reactive oxygen species and DNA damage, triggering a DNA injury response that can activate P53 through post-translational modifications including phosphorylation of Ser18. While the DNA damage response is sustained, the stabilisation of P53 resolves within 96 h showing that the two pathways are regulated independently.

**Rb deletion leads to DNA injury.** Based on our data and others we suggest the following model: upon Rb deletion, E2F activation leads to Arf induction, ATM/Chk1 activation which can inhibit relA (NF-κB) (22). This relA inhibition leads to decreased MnSOD (SOD2) expression resulting in accumulation of endogenous ROS leading to DNA injury and activation of DNA damage pathways: activation of ATR/chk1, γH2AX and phosphorylation of P53. Interestingly, others have also observed activation of γH2AX in response to Rb deletion (29,30). However, these studies clearly suggest that γH2AX is not associated with ROS induced DNA damage (30) but rather with DSB induced at the replication fork (29). Although in some cases activation of ATM/chk2 or ATR/chk1 are clearly associated with a specific type of DNA injury, often the situation is more complex with simultaneous activation and/or interconnection of the two pathways. Pickering and Kowalik found that γH2AX induction in response to Rb deletion was ATM-independent supporting our result (30). By contrast in response to TWIST depletion, which activates ARF, γH2AX increases together with activation of both chk1 and chk2 kinases (31). This suggests that activation of γH2AX in response to Rb deletion and/or ARF over-expression may result from a combination of DNA damage pathways that may be cell type specific. In summary, the increased ROS and γH2AX activation clearly shows that Rb loss leads to DNA injury and P53 stabilisation. In fact this could be contributing to the early activation of DNA damage checkpoints observed in early human carcinogenesis (32).
Arf-dependent regulation of P53 stabilisation. Interestingly, we observed a dynamic regulation of P53 with rapid but transient stabilisation, then return to low protein levels, which was correlated with ARF-MDM2 interaction. The currently favoured model of regulation of P53 by ARF suggests that in the nucleoli, ARF is sequestered by NPM and unable to interact with MDM2. In that model, in response to high levels of DNA injury, ARF relocates to the nucleoplasm where it binds to MDM2, leading to P53 stabilisation (reviewed in refs. 33,34).

In the present system, where gene expressions are endogenous, we confirm that ARF interaction with MDM2 dictates the level of P53 protein and that interaction between ARF and MDM2 is abolished by NPM binding to ARF. However this change in interaction occurs without dissociation of the nucleoli or relocalisation of ARF. Brady et al (28) have recently demonstrated that ARF/NPM and ARF/MDM2 are independent protein complexes. Interestingly, they also demonstrated a binding preference of ARF for MDM2 over NPM suggesting that an active regulation must take place for the binding to change from MDM2 to NPM. NPM has been shown to be induced by exogenous DNA damage (35,36) and a difference in the stoichiometry between MDM2 and NPM could be sufficient to affect the binding partner (28,34), even in absence of damage to the nucleoli structure. However, we did not observe a change in NPM protein level suggesting that another mechanism is involved for the observed regulation of ARF binding to MDM2. We have observed in Rb−/+ cells homodimeric forms of P19ARF at the time of decreased interaction with MDM2 (data not shown). ROS have been shown to induce homodimerisation of the human form of P19ARF (P14ARF) which leads to a decreased affinity for MDM2 (37). Based on this work in human cells and our own work, we postulate that in response to Rb deletion, increased endogenous ROS may promote homodimerisation of P19ARF at late time-points leading to a decrease affinity for MDM2. NPM can bind to ARF leading to a return to MDM2-dependent degradation of P53. Feed-back loops and interaction of two pathways. P53 is critical in the regulation of many cellular responses and (the) many ways by which P53 activity can be regulated are now clearly established. However, as discussed by Harris and Levine, the regulatory mechanisms have often been studied in established, frequently transformed cell lines, which are likely to bear various mutations, using overexpression vectors, mutant proteins and/or high levels of DNA damage (38). The many feed-back loops that exist are likely to provide a different picture in a physiological system.

Using primary epithelial cells, in which Rb is deleted acutely, we have shown how deletion of Rb can trigger 2 pathways of activation of P53 which may be interconnected but are regulated independently: P53 remains transcriptionally active at times when NPM interacts with ARF and the overall P53 protein level has returned to baseline. This suggests that P53 stabilisation and activation are independent in this setting as previously suggested by others (39). ARF has been reported to promote some post-translational modifications liable to activate P53 such as acetylation (40,41) or ATM dependent phosphorylation on Ser15 (42), however, these modifications can also be activated by DNA damage pathways whose activation was not ruled out in those studies. By contrast P53 can be stabilised without phosphorylation on Ser15 (43) supporting our hypothesis that arf overexpression stabilises P53 but activation itself results from another pathway, activation of ATR/Chk1 pathway by ROS-induced DNA damage. This regulation may provide a mechanism where the cells are able to sense the level of stress and prevent P53 stabilisation, possibly until the stress is such that elimination of the cells is necessary (44). The role of P53 in controlling apoptosis needs indeed to be tightly controlled to allow its activity in regulating other critical cellular pathways such as proliferation and differentiation.

This is supported by work suggesting that interaction between NPM and ARF has a regulatory role by promoting the accumulation of inactive ARF until it is required, and thus could set a threshold for P53 response (45,46). In the presence of high level of DNA injury, such as in studies involving UV-induced DNA damage (47) NPM can repress P53 activation until the levels of damage are such that DNA repair pathways or apoptosis should be activated (46,47). In this case the high level of DNA damage promotes a redistribution of ARF; NPM/ARF complexes disappear from the nucleoli and ARF complexes with MDM2 in the nuclei (47) although this may be due to damage to the nucleoli itself rather than to the DNA (48). A recent study further suggests that redistribution of ARF may be limited to DNA damage that induces the JNK pathway (49). In the absence of JNK induction, as observed after ionising radiation (49), ARF could be activated without relocalisation as observed in the present study.

In conclusion, the present data from primary cells suggests that the mdm2/p53/NPM/ARF pathway provides a pathophysiological sensor mechanism allowing inhibition of proliferation by low levels of active P53, perhaps giving the opportunity for DNA repair to occur, while apoptosis is inhibited. In normal cells, a low level of ROS and DNA damage activates sufficient P53 to induce P21 and lead to cell cycle arrest. However, the overall amount of P53 protein would be kept low as NPM interacts with ARF and P53 is targeted to degradation by MDM2. Additional signal, such as high levels of damage could activate ARF and stabilisation of P53 would allow induction of apoptosis. This mechanism allows to maintain P53 below the threshold necessary for cell death (44).

Acknowledgments

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