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Regulation of Retinoblastoma Protein (Rb) by p21 Is Critical for Adaptation to Massive Small Bowel Resection

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Abstract

Background—Adaptation following massive intestinal loss is characterized by increased villus height and crypt depth. Previously, we demonstrated that p21-null mice do not adapt after small bowel resection (SBR). As retinoblastoma protein (Rb) levels are elevated in p21-null crypt cells, we first sought to determine whether Rb is required for normal adaptation. Next, we tested whether Rb expression is responsible for blocked adaptation in p21-nulls.

Methods—Genetically manipulated mice and wild-type (WT) littermates underwent either 50% SBR or sham operation. The intestine was harvested at 3, 7, or 28 days later and intestinal adaptation was evaluated. Enterocytes were isolated and protein levels evaluated by Western blot and quantified by optical density.

Results—Rb-null mice demonstrated increased villus height, crypt depth, and proliferative rate at baseline, but there was no further increase following SBR. Deletion of one Rb allele lowered Rb expression and restored resection-induced adaptation responses in p21-null mice.

Conclusion—Rb is specifically required for resection-induced adaptation. Restoration of adaptation in p21-null mice by lowering Rb expression suggests a crucial mechanistic role for Rb in the regulation of intestinal adaptation by p21.

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Keywords

Cyclin-dependent kinase inhibitor; Enterocyte proliferation; Adaptation; Apoptosis; p21; Retinoblastoma protein

Introduction

Short gut syndrome (SGS) results from massive intestinal loss which limits absorptive and digestive capacity. As a consequence, patients with SGS frequently require parenteral nutrition in order to meet caloric needs.¹ Adaptive responses in the remnant bowel are critical for ultimate autonomy from parenteral nutrition. Normal adaptation is characterized structurally by increases in villus height and crypt depth resulting from enhanced rates of enterocyte proliferation.^{2,3} However, the mechanisms for the induction of proliferation following massive small bowel resection (SBR) remain unknown.

The p21^{waf1/cip1} protein (p21) is a potent cyclin-dependent kinase inhibitor (CDKI). This CDKI binds to and inhibits the activity of cyclin-dependent kinase (CDK)2 or CDK4 complexes, thus regulating cell cycle progression at G₁.^{4,5} Although p21 is usually considered to be a cell cycle inhibitor, prior studies from our laboratory have established that p21 expression is paradoxically required for resection-induced adaptation. Using p21-null mice, we demonstrated that resection-induced proliferation or adaptive growth did not occur.⁶

The actual mechanism for how p21 is required for adaptive increases in enterocyte proliferation is presently unknown. We found that p21 deficiency did not significantly affect the expression of other cell cycle regulatory proteins within the intestine.⁷ Further, we revealed distinct effects of p21 versus the related cip/kip family member p27 deficiency on enterocyte differentiation and migration.⁶ Mice with deficiency of p27 demonstrated normal adaptation following SBR; therefore, p21 seems to be specifically required. Finally, we found that p21 deficiency did not affect intestinal stem cell populations.⁸

In order to delineate a mechanism for how p21 regulates the intestinal adaptation response, we sought to evaluate other proteins that may interact with p21. Retinoblastoma protein (Rb) is a prototype tumor suppressor, a well-established cell cycle regulator,^{9–11} and cellular levels may be affected by p21. Rb is expressed in virtually all tissues and controls cell cycle progression via interactions with the E2F family of transcription factors.^{12,13} Despite the longstanding knowledge of the role for Rb in cell cycle regulation, the relevance of Rb activity to normal enterocyte turnover has only recently been implicated in mice with intestine-specific deletion of Rb resulting in mucosal hyperplasia.^{14,15} These observations provide the first direct evidence for the regulation of intestinal epithelial homeostasis by Rb.

The most prominent mechanism for governing cellular levels of Rb protein involves degradation via the proteasome.¹⁶ The p21 protein has been shown to be both a positive and negative regulator of Rb by influencing either Rb phosphorylation or degradation, respectively.¹⁷ These conflicting studies illustrate the current gaps in our knowledge regarding p21 and Rb stability. These findings also outline a potential mechanism for p21/Rb interactions as a means to govern adaptation. The experiments in this report seek to test the hypothesis that p21 is required for intestinal adaptation following massive SBR via a mechanism regulating Rb protein stability.

Materials and Methods

Animals

The protocol for this study was approved by the Washington University Institutional Animal Care and Use Committee (protocol # 20090190). Homozygous breeding pairs for p21^{waf1/cip1}-null mice, (developed on a C57/B16 background) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice containing Rb alleles in which exon 19 is flanked by loxP sites (*Rb(flox/flox)*),¹⁹ p107-null, and p130-null mice were the generous gifts of Dr. Eric Knudsen (Thomas Jefferson University, Philadelphia, PA, USA). Intestine-specific Rb knockout (KO) mice were generated by crossing *villin-Cre* mice with *Rb flox/flox* mice as previously described.¹⁵ Male and female mice aged 8 to 13 weeks were used in this study with a weight range of 18 to 25 g. Mice were kept on a 12-h light–dark schedule and were housed in a standard facility and allowed to acclimate to their environment for at least 7 days.

Experimental Design

Genetically manipulated mice and their wild-type littermates were randomly assigned to either 50% proximal SBR or sham operation (transaction and re-anastomosis alone). Mice were harvested on postoperative days 3, 7, or 28. Parameters of adaptation (villus height, crypt depth, and proliferation rate) were compared between wild-type littermates (sham vs. SBR) and null mice (sham vs. SBR) as well as between the SBR groups to determine if the deletion of a particular gene resulted in different adaptation responses.

Operative Procedure

Specific details of this procedure have been described previously.¹⁸ Briefly, 50% proximal SBR was performed by transecting the small bowel 12 cm proximal to the cecum and at the ligament of Trietz and then removing the intervening proximal small intestine. An end-to-end, single-layered, interrupted anastomosis using a 9–0 monofilament suture was used to restore intestinal continuity. A sham operation was performed by complete transaction of the bowel 12 cm proximal to the cecum and then re-anastomosing the cut ends together without removing any intestine. All mice were placed on a preoperative liquid diet (Micro-stabilized Rodent Liquid Diet LD101, Purina Mills, St. Louis, MO, USA) 1 day before their operation. After operation, the animals received water only for the first 24 h, followed by the same liquid diet until sacrifice. Animals that died, appeared ill (unkempt fur, lethargy), or had signs of intestinal obstruction at the time of sacrifice were excluded from further analyses.

Small Bowel Harvest and Enterocyte Isolation

At the time of harvest, mice were first anesthetized with a subcutaneous injection of ketamine, xylazine, and acepromazine (4:1:1 proportion). The abdominal cavity was then opened, the intestinal anastomosis identified, and the remaining distal bowel excised from the mesentery and cecum. The mice were sacrificed via cervical dislocation following removal of the intestine. After enterectomy, the intestine was immediately flushed with ice-cold phosphate-buffered saline. The first centimeter of the segment distal to the anastomosis was discarded, the next 2 cm was fixed for histology in 10% neutral-buffered formalin, and the remaining intestine was cut open and transferred into tubes containing 5 mL of ice-cold PBS with protease inhibitors (0.2 mmol/L phenylmethanesulfonyl fluoride, 5 µg/mL of aprotinin, 1 mmol/L benzamidine, 1 mmol/L sodium orthovanadate, and 2 µmol/L cantharidin (Gibbstown, NJ, USA). Crypts and villi were separated from the resected intestine using our previously described technique of enterocyte isolation involving calcium chelation and mechanical dissociation.¹⁵

Histology

All histologic measurements were performed by one investigator who was blinded with regard to mouse strain and operative procedure. Five-micrometer-thick longitudinal sections of paraffin-embedded tissue sections were mounted on glass slides and used for morphology. Hematoxylin-and-eosin-stained sections were used to measure villus height and crypt depth with a video-assisted computer program (Metamorph, UIC; Dowington, PA, USA). At least 20 well-oriented crypts and villi were counted per slide. Crypts were counted only if the crypt-villus junctions on both sides of the crypt were intact and if Paneth cells were present at the base of the crypt. Villi were counted only if the central lymphatic channel extended from the villus base to the tip and if the mucosal surface was in continuity with an intact crypt.

Crypt Cell Proliferation

At 90 min before sacrifice, the mice received an intraperitoneal injection of 5-bromo-deoxyuridine (BrdU; 0.1 mL/10 g body weight; Zymed Laboratories Inc, San Francisco, CA, USA). Incorporation of BrdU into proliferating crypt cells was detected in paraffin-embedded tissue sections by immunohistochemistry using a biotinylated monoclonal antibody system with streptavidin-peroxidase as a signal generator. The staining methods were generated by the Digestive Disease Research Core Center (Washington University School of Medicine, St. Louis, MO, USA). The number of cells staining positive (incorporating BrdU) per crypt were counted along with the total number of cells per crypt. A proliferative rate was calculated from the ratio of these measurements. Twenty well-oriented crypts were counted for each mouse by blinded scoring.

Crypt Cell Apoptosis

Apoptotic indices were calculated from H&E-stained slides. Apoptotic bodies were determined by the presence of pyknotic nuclei, condensed chromatin, and nuclear fragmentation. An apoptotic index was defined as the number of apoptotic bodies per 100 crypts. One hundred well-oriented crypts were counted for each mouse by blinded scoring.

Western Blotting

Frozen isolated crypt samples were thawed, reconstituted with Tris buffer, and sonicated for 10 s. The samples were then lysed with sodium dodecyl sulfate sample buffer (50 mM Tris-HCL, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, and 5% mercaptoethanol). The lysate was then heated for 5 min at 100°C and the protein concentration was determined by using the RC DC kit (Bio-Rad; Hercules, CA, USA) following the manufacturer's protocol. Proteins in equal amounts were analyzed by Western blot assay. The following antibodies were used: total Rb (5541436, BD Pharmingen), phosphorylated Rb on Ser807/811 (9308, Cell Signaling), p107 (SC-318, Santa Cruz Biotechnology), p130 (SC-137, Santa Cruz Biotechnology), and p21 (556431, BD Pharmingen). Protein levels were quantified by optical density using Image-J software.

Statistics

All results are presented as a mean $\pm$ SE of measure. Statistical differences were determined by using SigmaStat software (SPSS, Chicago, IL, USA). Statistical significance was established at $p<0.05$.

Results

p21 Deficiency Results in Elevated Levels of Rb Protein

We have previously demonstrated that p21^{cip1/waf1} is required for the stimulation of enterocyte proliferation following massive SBR in the mouse. However, these findings are contrary to common beliefs about the general role of p21 as a negative controller of the cell cycle. To understand the unique role of p21 in regulating small intestinal adaptation, we first investigated the role of p21 on the phosphorylation status of Rb protein. Both hyper- (inactive) and hypo-phosphorylated (active) Rb were elevated in the crypts of p21-null mice (Fig. 1). Further, lower baseline rates of enterocyte proliferation were observed in the crypts of unperturbed p21-null mice as compared to WT (data not shown).

Rb Deletion Results in Impaired Morphologic Intestinal Adaptation

Because p21 null mice do not adapt to massive SBR and have elevated Rb expression, we sought to determine the effect of Rb deletion on adaptation following SBR. Specifically, if Rb deletion is known to result in hyperplastic and hyperproliferative mucosa, it is possible that deletion of Rb in the context of SBR may result in a magnified adaptation response. We therefore performed SBR procedures on WT and Rb null animals, and morphological adaptation was measured at 3, 7, and 28 days after resection. As shown in Fig. 2a, b, adaptation occurred in WT mice at all time points after SBR. However, in the Rb-null animals with, at baseline, taller villi and deeper crypts, no further growth was observed in either parameter. Similarly, increased proliferation occurred only in the WT mice at all time points. There was no further increase in the rate of proliferation in the Rb-null mice subjected to SBR (Fig. 2c). Consistent with prior observations, rates of apoptosis were increased in WT mice following SBR at 3 days, while Rb-null mice had no change in this parameter²⁰ (Fig. 2d).

Lack of Resection-Induced Mucosal Growth is Specific to Rb

Rb is a member of a larger family of cell cycle regulatory pocket proteins that includes p107 and p130.¹³ We have previously demonstrated that the deletion of Rb, but not p107 or p130, results in a hyperplastic mucosa.¹⁵ We therefore sought to determine the effect of deletion of these other pocket proteins on resection-induced adaptation. To test this, we subjected p130 and p107-null mice to SBR. As shown in Fig. 3a, b, both crypt depth and villus height increased in p107-null and p130-null mice following intestinal resection. We therefore conclude that the effects on resection-induced adaptation appear to be specific to Rb.

Attenuated Rb Expression Rescues Adaptation in p21-Null Mice

Thus far, we have established that absent Rb expression results in augmented mucosal growth at baseline, which cannot be further enhanced by SBR. Further, we have demonstrated increased expression of Rb in p21-null mice in which adaptation is prevented. We therefore hypothesized that elevated Rb expression is the mechanism for why p21 deficiency prevents adaptation. Specifically, we sought to determine whether attenuation of Rb expression could rescue adaptive responses in the background of p21 deficiency. To reduce Rb protein levels in p21-null mice, one *Rb* allele was deleted by breeding *villin Cre Rb (flox/flox)* mice with p21-null mice, resulting in offspring genotyped as *Villin Cre (+); Rb (+/flox); p21 (-/-)*; these mice will be referred to as p21^{-/-}-Rb het mice). Levels of Rb protein were markedly attenuated in the p21^{-/-}-Rb het strain when compared to C57BL/6 J (WT) and p21 null strains (Fig. 4). Optical density values of Rb in p21^{-/-}-Rb het strain, C57BL/6 J, and p21-null were 0.20±0.02, 0.34±0.05, and 0.87±0.03, respectively.

Indeed by reducing Rb protein levels in the p21-null background to near-wild-type levels, the adaptive response to SBR was restored. Mice that underwent SBR had significantly

increased crypt depth, villus height, and rates of enterocyte proliferation when compared to sham-operated animals. However, rates of apoptosis were not significantly different comparing sham to SBR (Fig. 5a–d).

Discussion

In the present study, we attempted to determine a mechanism for how p21 expression is required for the regulation of resection-induced intestinal adaptation. Deletion of Rb results in a hyperplastic phenotype with no further adaptive changes following SBR. This seems to be specific to Rb as deletion of other members of its pocket protein family, p107 and p130, results in normal adaptive responses. We have identified that Rb protein levels are significantly elevated in the context of p21 deletion. Knocking down Rb expression in the context of p21 deficiency restores the adaptive capacity of p21-null mice. Taken together, these results suggest that in adapting enterocytes, p21 functions to regulate Rb protein levels, thereby providing a plausible mechanism for p21 as a required protein for resection-induced intestinal adaptation.

Rb deletion produces a unique hyperplastic intestinal phenotype not seen with deletion of other members of its pocket protein family. Rb has been previously shown to be required for normal epithelial cell homeostasis.^{14,15} The hyperplasia seen with Rb deletion is associated with defects in epithelial cell terminal differentiation as well as increased epithelial cell proliferation in mutant animals. At the molecular level, perturbation of the Rb pathway results in increased expression of transcription factors, such as Math1, Cdx1, and Cdx2, which regulate the proliferation and differentiation of the intestinal epithelium.¹⁴ Additionally, Rb, but not p107 or p130, has been demonstrated to be required for the maintenance of the post-mitotic epithelial cells in quiescence so that absorptive enterocytes can complete differentiation.¹⁵

It seems counterintuitive that the deletion of a cell cycle inhibitor (Rb) would result in no adaptive response to SBR. Because Rb deletion results in a hyperplastic mucosa that already resembles the adaptive growth seen after SBR, perhaps there is a plateau or a maximal level of enterocyte proliferation that the added proliferative stimulus of SBR cannot further enhance. Alternatively, because deletion of Rb results in a marked proliferative stimulation to enterocytes, the alteration of multiple counter-regulatory molecular pathways could mask the contribution of Rb to adaptation. However, deletion of other important transcription factors, such as p27⁷ or the other pocket proteins p107 and p130, does not result in abrogated adaptation to intestinal resection.

The proliferative response to SBR is seen as early as 12–24 h after resection, although it is maximally increased at day 3.¹⁸ Because adaptation is maintained over time by increased rates of proliferation and Rb-null mice have baseline elevated proliferative rates, we evaluated adaptation in the Rb-null animals over a range of postoperative time intervals from early (3 days) to late (28 days). However, there was no difference in adaptation between the early vs. middle vs. late time points, and the higher levels of baseline proliferation did not translate into further increases in villus height or crypt depth following massive SBR.

Apoptosis is also a critical hallmark of the crypt enterocyte response to intestinal resection.²⁰ After SBR, the intrinsic pathway of apoptosis is initiated via the p38 - dependent activation of Bax in intestinal epithelial cells.²¹ New data suggest that Rb is directly phosphorylated by p38 independent of the cell cycle, leading to the degradation of Rb, release of E2F1, and cell death.²² Although Rb-null mice have similar baseline rates of apoptosis as observed in WT mice, Rb deficiency prevented the usual increase in apoptosis

following SBR. This observation suggests that in addition to Bax, resection-associated apoptosis may also require Rb, perhaps via a mechanism of p38⁻-induced Rb phosphorylation. Further, since Rb-nulls had similar rates of apoptosis at baseline, the contribution of apoptosis does not appear to represent a significant factor in the hyperplastic phenotype of these mice. Finally, in the Rb-null animals where the normal increase in apoptotic rate following SBR is prevented, exaggerated adaptation does not occur.

The function of p21 as a cell cycle inhibitor vs. cell cycle inducer seems to be situation and tissue dependent. While the predominant literature supports the concept of p21 involvement in cell cycle arrest, such as in the blood and brain,^{24, 25} increased expression of this CDKI has been reported in other models in association with induction of proliferation. Following partial hepatectomy, p21 expression was found to be elevated in the regenerating liver.²³ Increased expression of p21 in the cytosol of vascular smooth muscle cells was associated with increased cell cycle progression.²⁴

There is increasing evidence in the literature for the regulation of Rb by p21 and that their dual oncogenic/tumor suppressor functions are intimately associated. Through the inhibition of CDK2/4 complexes and other transcription factors, p21 affects cell survival, morphology, and gene expression.²⁶ Phosphorylation by CDK2 and CDK4/6 inactivates Rb in proliferating cells; therefore, the induction of p21 results in Rb dephosphorylation, activation, and G1 arrest.¹⁶ However, p21 has also been reported to be associated with the proteasomal degradation of Rb.²⁷ The observations that p21 can both activate Rb transcription through dephosphorylation and then deactivate it through degradation suggest a powerful negative feedback regulation between these two proteins.¹⁶

Our observation that Rb protein levels are elevated in the context of p21 deletion fits the concept of regulation of Rb by p21. When p21 is absent, Rb is not degraded and thus accumulates in the cell. This accumulation of Rb inhibits resection-induced proliferation and explains the lack of adaptation seen in the p21-null mouse following SBR. Additionally, reducing Rb levels to near-normal in the context of p21 deletion resulted in restoration of the proliferative response to SBR. This highlights not only the important regulatory relationship between p21 and Rb but also that the presence of Rb is critical for adaptation.

Conclusion

As Rb is a transcription factor for the E2F gene family, future directions for research will not only focus on controls of Rb expression and decay but will also need to investigate the downstream contributions of Rb-regulated proteins on adaptation. Finally, because Rb activity is governed by phosphorylation on multiple sites with varied cellular effects, other factors which affect Rb function independent of absolute Rb levels must be explored. Through these considerations, it is possible that Rb represents a novel therapeutic target as a means to amplify adaptation responses, thereby helping patients with SGS achieve autonomy from parenteral nutrition.

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Fig. 1.

p21 deficiency is associated with increased expression of total and phosphorylated Rb. Western blot of total Rb, phosphorylated Rb (p-Rb), p107, and p21^{waf1/cip1} expression in isolated crypt enterocytes from wild-type (*WT*) or p21-null (*P21*^{-/-}) mice. Actin was used as the loading control

Fig. 2.

Rb is indispensable for SBR-induced adaptation. WT ($n=37$) and Rb-deficient mice ($n=45$) underwent Sham operation (bowel transaction with reanastomosis alone) or 50% proximal small bowel resection and were sacrificed at 3, 7, or 28 days later. Structural adaptation was measured in crypts (a) and villi (b). Proliferative rate was calculated by the ratio of BrdU-positive cells to total crypt cells (c). Apoptotic rate was calculated by counting apoptotic bodies/100 crypts on H&E-stained slides; only postoperative day 3 data are shown here (d). * $p < 0.05$ (Student's *t* test)

Fig. 3.

Deletion of related Rb pocket protein family members p107 and p130 does not affect the magnitude of intestinal adaptation. Crypt depth (a) and villus height (b) in wild-type background strain (*WT*) ($n=14$), p107-null ($n=16$), and p130-null ($n=16$) mice at 7 days following either Sham operation (bowel transaction with reanastomosis alone) or 50% proximal small bowel resection were measured and compared. * $p<0.05$ (Student's *t* test)

Fig. 4.

Deletion of a single Rb allele results in decreased Rb levels in p21-null mice. Western blot for Rb protein in p21-null with Rb haploinsufficient mice (*p21*^{-/-} *Rb* *het*), C57BL/6 J (wild-type) mice, and p21-null mice

Fig. 5.

Attenuated Rb expression restores adaptation in p21-null mice. Haploinsufficient Rb/p21-null ($p21^{-/-}$ Rb het) mice ($n=14$) underwent either Sham operation (bowel transection with reanastomosis alone) or 50% proximal small bowel resection and were sacrificed at 7 days later. Crypt depth (a), villus height (b), rates of apoptosis (c), and proliferation (d) were measured. Rates of apoptosis were calculated by counting the number of apoptotic bodies/100 crypts on H&E-stained slides while rates of proliferation were calculated by the ratio of BrdU-positive cells to the total number of crypt cells. * $p < 0.001$, Student's t -test