In vivo Significance of the G2 Restriction Point

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Abstract

Loss of activity of the retinoblastoma pathway is a common event in human cancer. Mouse models have revealed that tumorigenesis by loss of Rb was accelerated by concomitant loss of the cell cycle inhibitor p27KIP1. This has been attributed to reduced apoptosis and weakening of the G1 checkpoint. However, the role of p27KIP1 in a recently identified G2 restriction point may offer an alternative explanation for this synergy. Here, we have investigated the significance of the G2 restriction point in Rb-deficient pituitaries. We show that Rb loss in the pituitary gland activated the G2 restriction point, as evidenced by the appearance of cyclin B1–p27KIP1 complexes. Somewhat unexpectedly, these complexes remained present in Rb-deficient tumors. These results indicate that the G2 restriction point does operate in vivo. However, in the pituitary gland, this mechanism seems to retard rather than to prevent tumor growth. [Cancer Res 2007;67(19):9244–7]

Introduction

The retinoblastoma gene family, which is composed of Rb, p107 and p130, plays an essential role in regulating the cell cycle and is commonly affected in human cancer (1). Its gene products, the so-called pocket proteins, inhibit E2F transcription factors, whose activity stimulates DNA synthesis and cell cycle progression (2–5). Several forms of stress, such as DNA damage, high cell density, loss of anchorage or mitogenic signaling, or cell differentiation, result in arrest of cells in the G1 phase of the cell cycle. This G1 arrest is completely abrogated by ablation of the three pocket proteins in arrest of cells in the G1 phase of the cell cycle. This G1 arrest is completely abrogated by ablation of the three pocket proteins.

Monoallelic mutation of Rb in humans strongly predisposes to retinoblastoma, a tumor of the developing retina (9, 10). In mice, however, Rb hemizygosity led to tumors originating from the intermediate lobe of the pituitary gland with an average latency of 40 to 50 weeks. These tumors consistently showed loss of the wild-type Rb allele, thus emphasizing the critical role of Rb in pituitary tumor suppression (11, 12). Furthermore, in chimeric mice composed of wild-type and Rb−/− cells or in mice engineered to inactivate Rb exclusively in the anterior and intermediate lobe of the pituitary gland, the latency time of tumor development was strongly reduced (12–14). Interestingly, concomitant loss of p27KIP1 in Rb heterozygous animals accelerated tumor development to 20 weeks, and the tumors were more aggressive. Although the synergistic effect of p27KIP1 and Rb loss has often been explained by progressive loss of G1 control, the observed synergy may also suggest p27KIP1 and pRb to function in different pathways, which both need to be disrupted or attenuated for pituitary tumor growth. Indeed, we and others have recently identified a role for p27KIP1 in G2 control that may provide an alternative explanation for the observed synergy (8, 15–17).

In this study, we show that loss of p27KIP1 accelerated tumorigenesis in Rb-deficient pituitaries. In the presence of p27KIP1, loss of Rb resulted in an increase of cells arrested or delayed in the G2 phase of the cell cycle and accumulation of cyclin B1–p27KIP1 complexes. However, by comparing the levels of cyclin B1–p27KIP1 complexes in tumors in mice of different ages, we did not find selection for loss of this interaction during tumor development. Therefore, p27KIP1 may decelerate rather than block the growth of Rb-deficient pituitary gland tumors.

Materials and Methods

Mouse strains. Rbp+/− mice (18) were bred with Oluc-Cre mice (19) to obtain Oluc-Cre Rbp+/− mice. In addition, Oluc-Cre Rbp−/− mice were bred with p27KIP1−/− mice (20) to obtain Oluc-Cre Rbp−/−p27KIP1−/− mice. All mice were in an FVB:129Ola mixed background.

Histologic analysis. Pituitary glands were removed immediately after euthanasia and fixed in 4% formaldehyde (Sigma) in PBS (Invitrogen-Life Technologies) for at least 24 h. For histologic analysis, formaldehyde-fixed pituitary tissues were embedded in paraffin, cut into 5–μm sections, and stained with H&E.

Fluorescence-activated cell sorting analysis. Pituitary glands were removed immediately after euthanasia and homogenized to single-cell suspensions through mechanical disaggregation. Single-cell suspensions were fixed in 70% ethanol in PBS and stained for DNA content by propidium iodide. Cell cycle distributions were analyzed by fluorescence-activated cell sorting using “Cell Quest” software (BD Biosciences) and “FACS Express” software (De Novo Software).

Immunoprecipitations, immunoblots, and antibodies. Pituitary glands were removed immediately after euthanasia and homogenized to single-cell suspensions through mechanical disaggregation. Subsequently, cells were lysed for 30 min in ELB [150 mmol/L NaCl, 50 mmol/L HEPES (pH 7.5), 5 mmol/L EDTA, 0.1% NP40] containing protease inhibitors (Complete; Roche) and phosphatase inhibitors (5 mmol/L NaF, 0.5 mmol/L sodium vanadate, and 20 mmol/L β-glycerophosphate). Protein concentrations were determined using the Bradford assay (Bio-Rad). For immunoprecipitations, 50 μg of protein was incubated with 0.5 μg of immobilized antibody overnight at 4°C while rotating. The antibodies used were mouse anti-p27KIP1 (BD Transduction Laboratories), tubulin-α (Sigma), rabbit polyclonal cyclin E (M20), mouse monoclonal CDK1 (17), rabbit polyclonal CDK4 (C22), rabbit polyclonal cyclin B1 (H33) and cyclin B1 (GNS1; Santa Cruz). Secondary antibodies used were horseradish peroxidase (HRP)–conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit (Dako).

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Results

Loss of \( p_{27}^{\text{KIP1}} \) accelerates pituitary tumorigenesis. Both \( p_{27}^{\text{KIP1}-/-} \) mice (20, 21) and \( Rb \) heterozygous mice (11, 12, 14, 22) developed tumors originating from the intermediate lobe of the pituitary gland. Furthermore, \( p_{27}^{\text{KIP1}} \) loss accelerated pituitary tumorigenesis in \( Rb \) heterozygous mice (23). We wondered whether this acceleration was (partly) caused by alleviation of the \( G_2 \) restriction point. To address this issue, we examined pituitary glands from \( p_{27}^{\text{KIP1}} \)-proficient and \( p_{27}^{\text{KIP1}} \)-deficient mice at 4 weeks of age (Table 1), although histologic analysis revealed that the intermediate lobe of \( Rb \)-deficient pituitaries was much enlarged at 8 weeks and showed higher cell density (Fig. 1, compare \( B \) and \( A \)). Concomitant loss of \( p_{27}^{\text{KIP1}} \) dramatically changed this phenotype, causing hyperplasia in the intermediate lobe within 3 weeks (Fig. 1C) and full-blown pituitary gland tumors at 4 weeks of age (Fig. 1D; Table 1). Thus, tumorigenesis in the pituitary gland, as a consequence of \( Rb \) loss, is delayed by activity of \( p_{27}^{\text{KIP1}} \).

\( Rb \)-deficient pituitary glands show an increased \( G_2 \) population. Next, we examined whether the delay of pituitary tumorigenesis by \( p_{27}^{\text{KIP1}} \) is caused by arrest of \( Rb \)-deficient pituitary cells in \( G_2 \). Therefore, we homogenized \( Rb \)-proficient and \( Rb \)-deficient pituitaries and determined DNA content by flow cytometry. Figure 2A shows that the vast majority (~83%) of wild-type pituitary residues in the \( G_1 \) phase of the cell cycle. A similar profile was seen in \( p_{27}^{\text{KIP1}} \)-deficient pituitaries (Fig. 1C). Loss of \( Rb \) alone resulted in an 8% decrease of \( G_1 \) cells and an increase of \( G_2 \) cells from 12% to 21% (Fig. 2, compare \( A \) and \( B \)). Concomitant loss of \( Rb \) decreased the number of \( S \)-phase cells was increased up to 7% (Fig. 2, compare \( A \) and \( B \)), indicative of a partial \( G_2 \) arrest. The number of \( S \)-phase cells was unaffected (4% in wild-type and 3% in \( Rb^{-/-} \) pituitaries).

In contrast, in \( Rb/p_{27}^{\text{KIP1}} \)-deficient tumors, which arose at 4 weeks, the number of \( S \)-phase cells was increased up to 7%, whereas the number of \( G_2 \) cells was decreased (Fig. 2, compare \( A \) and \( B \)). These data suggest that \( Rb \) deficiency leads to increased \( G_2 \) arrest in the pituitary gland, which is alleviated upon concomitant loss of \( p_{27}^{\text{KIP1}} \).

\( Rb \) deficiency in pituitary glands leads to accumulation of cell cycle proteins. \( pRb \) is a well-established suppressor of E2F activity (1). Consequently, loss of \( Rb \) leads to activation of E2F and increased transcription of E2F target genes in vitro (24). We wondered whether \( Rb \) loss in the pituitary gland similarly affected the expression of E2F target genes. Therefore, we compared the protein levels of the E2F targets cyclin B1 and cyclin E in \( Rb \)-proficient and \( Rb \)-deficient pituitaries harvested at 4 and 8 weeks of age. Figure 3A shows that both cyclin B1 (top) and cyclin E (second row) levels were strongly elevated in \( Rb \)-deficient pituitaries. Interestingly, cyclin accumulation coincided with increased \( p_{27}^{\text{KIP1}} \) levels (third row). Because we observed an increased number of \( G_2 \) cells in \( Rb \)-deficient pituitaries, and \( p_{27}^{\text{KIP1}} \) was shown to prevent \( G_2 \) progression through interaction with cyclin B1 (8, 15–17), we immunoprecipitated cyclin B1 from \( Rb \)-proficient and \( Rb \)-deficient pituitary glands harvested at 4 weeks of age, the stage at which we observed partial \( G_2 \) arrest. Consistent with the presence of

Table 1. \( p_{27}^{\text{KIP1}} \) loss accelerates pituitary tumorigenesis by loss of \( Rb \)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (wk)</th>
<th>Tumors</th>
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<tr>
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<tr>
<td>( Rb^{f/f} ); Cre</td>
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<td>10</td>
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<tr>
<td>( Rb^{f/f} ); ( p_{27}^{\text{KIP1}} ); Cre−</td>
<td>3</td>
<td>0/1</td>
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<td></td>
<td>4</td>
<td>0/1</td>
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<tr>
<td>( Rb^{f/f} ); ( p_{27}^{\text{KIP1}} ); Cre−</td>
<td>3 (enlarged)</td>
<td>1/1</td>
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Figure 1. Loss of \( p_{27}^{\text{KIP1}} \) accelerates pituitary gland tumorigenesis. Histologic slides showing control pituitary tissue at 6 wk (A), premalignant pituitary tissue of an \( \text{Oloc-Cre} \) \( Rb^{f/f} \) mouse at 8 wk (B), premalignant pituitary tissue of an \( \text{Oloc-Cre} \) \( Rb^{f/f}; p_{27}^{\text{KIP1}} \) mouse at 3 wk (C), and an intermediate lobe pituitary tumor in a \( \text{Oloc-Cre} \) \( Rb^{f/f} p_{27}^{\text{KIP1}} \) mouse at 4 wk (D). Magnification, 10 \times \text{left} \) and 40 \times \text{right}.
G2-arrested cells, we detected an interaction between cyclin B1 and p27KIP1 in Rb-deficient pituitaries (Fig. 3B, top). Additionally, we detected p27KIP1-CDK2 complexes (Fig. 3B, bottom), which can be indicative for G1 (25), but also G2, arrest (8, 15–17). These results suggest that Rb deficiency in the pituitary gland stimulates cell cycle progression by induction of the E2F targets cyclin B1 and E, which is counteracted by accumulation of the CKI p27KIP1, resulting in G1 and G2 cell cycle arrest or delay.

Cyclin B1–p27KIP1 interaction persists in tumors. Because p27KIP1 loss accelerated pituitary tumorigenesis, we wondered whether p27KIP1-mediated inhibition of cyclin B1 was attenuated or even abrogated during tumor progression. To address this issue, we first determined the levels of p27KIP1 and CDK1, the latter as a measure for E2F activity. Figure 3C (middle) shows that loss of Rb led to increased levels of CDK1, both in normal pituitary glands (3 weeks) and in full-blown tumors (10 weeks). However, also p27KIP1 was increased in Rb-deficient pituitary glands at ages ranging from 3 to 10 weeks (Fig. 3C, top). Furthermore, when we immunoprecipitated cyclin B1 from Rb-deficient pituitaries, we found that the cyclin B1–p27KIP1 interaction, rather than disappearing, persisted or became even increasingly evident upon aging (Fig. 3D, compare 3, 5, 8, and 10 weeks). These data indicate that Rb loss resulted in E2F activation and stimulation of the cell cycle, but also in elevated p27KIP1 levels and cyclin B1–p27KIP1 interaction.

Discussion

Our findings confirm that loss of p27KIP1 strongly synergizes with loss of Rb in tumorous outgrowth of the pituitary gland. Rb/p27KIP1-deficient tumors arose earlier and grew more aggressively than Rb-deficient tumors. We have investigated here whether tumor development by loss of Rb alone was delayed by p27KIP1-mediated G2 arrest by a mechanism that we have previously described in vitro (8). Taken together, our findings indicate that the G2 restriction point also operates in vivo: upon loss of Rb in the pituitary gland, the number of cells in G2 increased, and this was accompanied by the appearance of cyclin B1–p27KIP1 complexes.
We have previously shown that this interaction inhibits cyclin B1–dependent kinase activity (8). The cyclin B1–p27\(^{KIP1}\) interaction was induced upon loss of Rb, but strikingly, was not lost during tumor progression. We envisage that in Rb-deficient pituitary tumors, p27\(^{KIP1}\) contributed to slower tumor growth by interacting with cyclin B1 and that within the time frame tumor development and behavior was followed, no selection occurred for loss of this interaction. We do realize that the contribution of the cyclin B1–p27\(^{KIP1}\) interaction to slower tumor growth was only modest. Our earlier in vitro experiments have shown that the G2 restriction point is particularly important in TKO cells that are devoid of all three pocket proteins. In the presence of one or two of the pocket proteins, the major mechanism of cell cycle control operated in G1. The G2 arrest was less prominent. Thus, the presence of p107 and p130 and the interaction of p27\(^{KIP1}\) and CDK2 (Fig. 3B) likely explain the modest level of G2 cells in Rb-deficient pituitary glands. Furthermore, it should be noted that activation of the G2 restriction point in pocket protein-deficient cells in vitro only occurred in response to mitogen deprivation. It is therefore possible that inhibition of CDK2 and CDK1 by p27\(^{KIP1}\) was confined to a subset of tumor cells that were deprived of mitogens and that only these cells were arrested in G1 or G2. Alternatively, p27\(^{KIP1}\) may have inhibited CDK activity in the majority of tumor cells but rather than imposing cell cycle arrest, this only caused a slower progression through the cell cycle. Either mechanism may explain that loss of p27\(^{KIP1}\) strongly synergizes with loss of Rb in tumorigenesis and that loss of p27\(^{KIP1}\) expression in human cancers is associated with poor prognosis (26, 27).

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