Mutually Exclusive Cyclin-Dependent Kinase 4/Cyclin D1 and Cyclin-Dependent Kinase 6/Cyclin D2 Pairing Inactivates Retinoblastoma Protein and Promotes Cell Cycle Dysregulation in Multiple Myeloma

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Abstract

Multiple myeloma, the second most common hematopoietic cancer, ultimately becomes refractory to treatment when self-renewing multiple myeloma cells begin unrestrained proliferation by unknown mechanisms. Here, we show that one, but not more than one, of the three early G1 cyclins is elevated in each case of multiple myeloma. Cyclin D1 or D3 expression does not vary in the clinical course, but that alone is insufficient to promote cell cycle progression unless cyclin-dependent kinase 4 (cdk4) is also elevated, in the absence of cdk6, to phosphorylate the retinoblastoma protein (Rb). By contrast, cyclin D2 and cdk6 are coordinately increased, thereby overriding the inhibition by cdk inhibitors p18INK4c and p27Kip1 and phosphorylating Rb in conjunction with the existing cdk4. Thus, cyclin D1 pairs exclusively with cdk4 and cdk6 pairs only with cyclin D2, although cyclin D2 can also pair with cdk4 in multiple myeloma cells. The basis for this novel and specific cdk/D cyclin pairing lies in differential transcriptional activation. In addition, cyclin D1 or cyclin D3—expressing multiple myeloma cells are uniformly distributed in the bone marrow, whereas cdk6-specific phosphorylation of Rb occurs in discrete foci of bone marrow multiple myeloma cells before proliferation early in the clinical course and is then heightened with proliferation and disease progression. Mutually exclusive cdk4/cyclin D1 and cdk6/cyclin D2 pairing, therefore, is likely to be a critical determinant for cell cycle reentry and progression and may play a pivotal role in the expansion of self-renewing multiple myeloma cells. (Cancer Res 2005; 65(24): 11345-53)

Introduction

Control of cell cycle reentry and progression from G1 to S phase regulates physiologic responses, and loss of this control can be critical for oncogenesis (1). G1 to S cell cycle progression is modulated by the balance between positive cell cycle regulators [cyclins and cyclin-dependent kinases (cdk)] on one hand and cdk inhibitors (CKI) on the other (ref. 2; Fig. I.A). D cyclins are essential for development beyond the early embryonic stage and for the hematopoietic lineage, including B lymphocytes (3). Cyclin D2, in particular, is the major D cyclin expressed in mature B cells, the precursors of antibody secreting plasma cells. It is required for cell cycle activation in response to physiologic signals, such as antigen (4, 5), that lead to coordinated elevation of cyclin D2 and cdk4, and then cdk6 (6). Inhibition of cdk6 by the early G1 CKI p18INK4c (7, 8) is specifically required for G1 cell cycle arrest and terminal differentiation of antigen-activated B cells to plasma cells (9, 10). Thus, although neither cdk4 nor cdk6 is required for cell cycle progression or viability in mice (11), specific D cyclins, cdk4/6, and CKIs are required for B-cell physiologic functions, implying that perturbation of this balance is likely to underlie oncogenesis in the B lineage.

Multiple myeloma represents a clinically defined collection of plasma cell neoplasms in which plasmacytoid cells are arrested at varying stages of B-cell terminal differentiation (12). Unlike normal plasma cells, multiple myeloma cells retain their self-renewing potential. Although impaired apoptosis accounts for accumulation of multiple myeloma cells in the bone marrow during the stable phase of the disease (13), cell cycle dysregulation underlies unrestrained proliferation of self-renewing multiple myeloma cells in aggressive multiple myelomas and during relapse from treatment.

Overexpression of cyclin D1 or D3, but not D2, is frequently associated with chromosomal translocations (14, 15), although cyclin D1 overexpression has also been attributed to a gene dosage effect in the absence of t(11:14) translocation (16). Gene expression profile analysis suggests that cyclin D1, D2, or D3 RNA is elevated in most multiple myeloma cases (15, 17, 18). In addition, the p18INK4c gene is deleted in some multiple myeloma cell lines (19), and the p16INK4a and p15INK4b genes are inactivated by methylation in multiple myeloma cells (20, 21). These observations suggest that gain of D cyclin or loss of CKI function might promote cell cycle reentry and progression in multiple myeloma pathogenesis. However, there has been no functional evidence to support this concept. Moreover, elevation of cyclin D1 RNA has been paradoxically linked to a more favorable prognosis in multiple myeloma (22–24). The mechanism that underlies cell cycle dysregulation in multiple myeloma, therefore, remains undefined.

We have addressed the mechanism by which positive and negative G1 cell cycle regulators control cell cycle progression exclusively in primary bone marrow multiple myeloma (BMMM) cells in the context of disease progression. We showed that only one of the three D cyclins is expressed in BMMM cells of each patient. By functional analysis of cdk4/6-specific Rb phosphorylation in vivo at the single cell level by immunohistochemistry in combination with immunoblotting and quantitative real-time reverse transcription-PCR (RT-PCR) analysis of purified BMMM
controls G1 cell cycle progression in BMMM cells, which underlies a mechanism by which specific cdk/cyclin D pairing critically associated with advanced disease stage. Our findings suggest the expansion of self-renewing multiple myeloma cells in myeloma pathogenesis and progression.

Figure 1. Phosphorylation of Rb by cdk4/6 correlates with myeloma progression. A, phosphorylation of Rb by cyclin D and cdk4/6 in early G1, and cyclin E/cdk2 in late G1, leads to the release of E2F and S-phase entry. The INK4 family of CKIs (p16, p15, p18, and p19) inhibits cdk4/6 and the Cip/Kip family of CKIs (p21, p27, and p57) inhibits cyclin E/cdk 2. B, immunohistochemical analysis of simultaneous expression of CD138 (red) and Rb, phosphorylated Rb (pS780 and pS807/811), or Ki67 (all brown) in bone marrow core biopsy sections from a normal volunteer (1239), or an untreated multiple myeloma patient (PT) in a 29-month interval (247-1 and 247-2). The nuclei were counterstained with hematoxylin (blue). Pretreatment with calyculin A (CA) or cdk4 and cdk6 phosphatase (CIP) are as indicated. Sections from a primary retinoblastoma (Retina) tumor were used as a negative control for Rb. C, frequencies of CD138+ bone marrow cells expressing pS780, MCM7 or MCM2, or Ki67 in untreated stage I (329) and stage III (327), and treated stage III (246) and stage IIII (243) patients were determined by immunohistochemistry. BrdUrd uptake ex vivo was determined in purified CD138+ BMMM cells. The error bars represent triplicate analysis. The results are representative of 5 independent analyses.

cells, we found that cyclin D1 or D3 expression in BMMM cells rarely leads to G1 cell cycle progression. By contrast, cdk4/cyclin D2– or cdk6/cyclin D2–specific phosphorylation of Rb is accompanied by DNA replication and cell proliferation and is preferentially associated with advanced disease stage. Our findings suggest a mechanism by which specific cdk/cyclin D pairing critically controls G1 cell cycle progression in BMMM cells, which underlies the expansion of self-renewing multiple myeloma cells in myeloma pathogenesis and progression.

Immunohistochemistry. Immunohistochemistry was done on 4-μm sections of paraffin-embedded bone marrow tissues using a TechMate500 BioTek automated immunostainer and reagents (Ventana Medical Systems, Inc., Tucson, AZ) according to manufacturer’s specifications. CD138+ plasma cells were detected using an anti-CD138 mouse monoclonal antibody (mAb; Serotec, Oxford, England) and a red chromogen. The nuclei were visualized by counterstaining with hematoxylin (blue). Simultaneous expression of other proteins was detected with mAbs to Ki67 (Zymed, San Francisco, CA); cyclin D1, MCM2, and MCM7 (Lab Vision, Fremont, CA); cyclin D3 (Novocastra, Newcastle Upon Tyne, England); Rb and cdk6 (Cell Signaling, Beverly, MA); and polyclonal rabbit antibodies to phospho-Ser780 and phospho-Ser780 of human Rb (Cell Signaling). Tissue sections were pretreated with calyculin A (Cell Signaling) to prevent dephosphorylation during or after antigen retrieval, or with cdk4 specific phosphatase (Cell Signaling) to verify phosphorylation. As a control for immunoglobulin synthesis, serial sections were stained with rabbit antibodies to IgM, IgG, IgA, Igk, and Igl (DakoCytohm, Carpinteria, CA). The frequency of CD138+ cells expressing a specific protein was scored by counting 150 cells in triplicate, in three areas each time. As positive controls, parallel analyses were done on tissue sections containing centroblasts (Rb, phospho-Rb, p18, and Ki67), mantle zone B cells (p27), suprabasal suprabasal epithelial cells (cyclin D3), and mantle cell lymphoma with t(11;14) (cyclin D1). Correlations between D1 and D3 with Ki67 were assessed by the t test and P value using SPSS for Windows (release 11.0, SPSS, Inc., Chicago, IL). P < 0.05 was considered statistically significant.

Results

Phosphorylation of retinoblastoma protein by cyclin-dependent kinase 4/6 increases with cell proliferation during multiple myeloma progression. Phosphorylation of Rb by cdk4...
or cdk6 in cooperation with one of the D cyclins promotes early G1 cell cycle progression and the release of E2F transcription factors that are necessary for S-phase entry (refs. 26–28; Fig. 1A). Normal and malignant plasma cells express a common surface marker CD138, a proteoglycan not present on B-lineage cells until the plasma cell stage (29). Because cyclins and cdks are subject to complex regulation at the transcriptional and posttranscriptional levels (2), we investigated their expression and roles in cell cycle dysregulation in CD138+ myeloma cells by a combination of approaches. We assayed cdk4/6-specific phosphorylation of Rb on Ser780 (pS780) and Ser807/811 (pS807/811) in vivo in bone marrow CD138+ plasma cells (BMMC) by double immunohistochemistry of formalin-fixed bone marrow sections. In parallel, we quantified the expression of G1 cell cycle regulators at the protein and RNA levels by immunoblotting and real-time RT-PCR and determined their interactions by immunoprecipitation/immunoblotting in purified CD138+ BMMC cells.

No pS780 or pS807/811 was detected in normal, G1-arrested BMMCs (Fig. 1B, 1239). Nonetheless, pS780 and pS807/811 were similarly absent in bone marrow CD138+ multiple myeloma cells at the time of initial multiple myeloma diagnosis but seemed to increase prominently with disease progression without therapy, as shown in one representative multiple myeloma case (Fig. 1B, 247-1 and 247-2). The increase in p53-Rb was accompanied by cell proliferation based on the expression of the proliferation antigen Ki67 (Fig. 1B). The phosphorylation signals were confirmed because they did not vary by protection with a phosphatase inhibitor calcineurin A and were completely eliminated by calf intestine phosphatase (Fig. 1B). Thus, cdk4/6-specific phosphorylation of Rb increases with multiple myeloma progression in the absence of therapy.

The frequencies of multiple myeloma cells expressing pS780 in early (stage I; 329) and advanced multiple myeloma (stage III; 327, 243, and 246; ref. 25) correlated tightly with those expressing mini-chromosome maintenance protein 7 (MCM7) and MCM2, two components of the replication origin recognition complex, in addition to Ki67 (Fig. 1C). This linear relationship among Rb phosphorylation, DNA replication, and cell proliferation in vivo was verified by the uptake of BrdUrd in 3 hours ex vivo in freshly purified BMMC cells (Fig. 1C). Thus, cdk4/6 phosphorylation of Rb increases with DNA replication and cell proliferation during multiple myeloma progression.

**Expression of cyclin D1 and cyclin D3 is mutually exclusive and insufficient to promote G1 progression.** To determine which of the D cyclins cooperates with cdk4/6 in phosphorylating Rb in multiple myeloma cells in vivo, we analyzed serial sections of bone marrow biopsies from 251 additional multiple myeloma patients, along with 56 symptomatic monoclonal gammopathy of undetermined significance (MGUS) patients and 64 normal individuals (Fig. 2; Table 1). MGUS is a clinically benign condition that is thought to be a precursor to multiple myeloma. Cyclic D1 was not detected in BMPCs of any normal individuals; however, it was present in bone marrow CD138+ cells in 20% of the MGUS patients and 25% of the multiple myeloma patients, regardless of disease stage or treatment history (Fig. 2A). These results are consistent with the notion that cyclin D1 overexpression occurs early in multiple myeloma pathogenesis (32). The expression of cyclin D3 was less frequent (9%) and mainly in treated multiple myeloma patients (Fig. 2A). In most cyclin D1– or cyclin D3–positive cases, expression was detected in >90% of CD138+ multiple myeloma cells (Fig. 2A and C).

Importantantly, only cyclin D1 or D3, but not both, was expressed in each case of multiple myeloma, and this was not accompanied by cdk4/6 phosphorylation of Rb (pS807/811) or Ki67 expression (Fig. 2B). The lack of correlation between cyclin D1 and Ki67 expression (P = 0.878), or between cyclin D3 and Ki67 expression (P = 0.527), was verified by statistical analysis of all 251 multiple myeloma patients (Fig. 2C).

We then asked whether cyclin D1 expression varies during multiple myeloma progression by sequential immunohistochemistry analysis of bone marrow biopsies (5-26 biopsies per patient) from 21 additional multiple myeloma patients for an average of 72 months (31-166 months; Fig. 2D). In most cases, cyclin D1 expression did not vary significantly in individual patients during the clinical course. In four of five cyclin D1–expressing cases, the frequency of D1 expression among BMMC cells was maintained at 2% (U), 41% (V), 75% (G), and 92% (S). The only exception was the reduction of D1 expression from 10% to 0% of multiple myeloma cells in one case (Q). These results corroborate our observation that cyclin D1 is expressed in ~25% of multiple myeloma patients in the separate study of 251 patients (Fig. 2A and C). On this basis, we conclude that the expression of cyclin D1 and D3 is mutually exclusive and insufficient to promote G1 progression.
Table 1. Patient clinical data: distribution of patients in data set

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NOTE: Total numbers of patients analyzed in each diagnosis group in this study. Detailed analyses presented in the figures are summarized in Table 2.

exclusive in multiple myeloma cases, and that cyclin D1 expression is maintained throughout the clinical course but insufficient to promote G1 cell cycle progression.

Retinoblastoma protein is phosphorylated by either cyclin-dependent kinase 4/cyclin D1 or cyclin-dependent kinase 4/cyclin D2 or cyclin-dependent kinase 6/cyclin D2 in each case of myeloma. The lack of correlation between cyclin D1 or D3 expression, even at high levels, and Rb phosphorylation (Fig. 2) suggests that cdk4 or cdk6 is limiting and that cyclin D2 expression might underlie cell cycle dysregulation in multiple myeloma. To address this possibility, we investigated the timing and the mechanism of Rb phosphorylation further in early untreated multiple myeloma cases that had limited proliferation (Ki67 was expressed in <0.1% of multiple myeloma cells; n = 30), by immunohistochemistry along with immunoblotting and quantitative RT-PCR analyses of freshly isolated BM MM cells (Fig. 3; Table 2).

Rb was not phosphorylated in normal BMPCs despite the presence of cdk4, apparently due to the lack of D cyclins (Fig. 3B, 13). It was phosphorylated by cdk4/cyclin D1 in multiple myeloma cells, when cdk4 was elevated in the absence of cdk6, cyclin D2, or cyclin D3 (Fig. 3A-B, 336). Thus, enhanced expression of cyclin D1 in multiple myeloma can, although infrequently, lead to cdk4-specific phosphorylation of Rb when cdk4, but not cdk6, is correspondingly increased. However, in multiple myeloma cells from other early-stage multiple myeloma patients where neither cyclin D1 nor cyclin D3 was expressed, Rb was phosphorylated by cyclin D2 in cooperation with cdk6 or cdk4 following coordinated elevation of cyclin D2 and cdk6 (Fig. 3B, 346). Thus, cyclin D1 pairs exclusively with cdk4, whereas cdk6 pairs exclusively with cyclin D2, although cdk4 can also pair with cyclin D2, Rb, therefore, is phosphorylated early in the clinical course by either cyclin D1/cdk4 or cyclin D2/cdk6 or cyclin D2/cdk6 but not both.

Correspondingly, the mRNAs encoding cyclin D2 and cdk6 were increased based on quantitative real-time RT-PCR analysis (Fig. 3C). Likewise, cyclin D1 and cdk4 mRNAs were coordinately increased, and this was accompanied by prominent decreases in cyclin D2 and cdk6 mRNAs early in the clinical course. The basis for the striking and mutually exclusive cdk4/cyclin D1 and cdk6/cyclin D2 partnerships, therefore, lies in coordinated activation of specific D cyclin and cdk4 or cdk6 genes.

Phosphorylation of retinoblastoma protein by cyclin-dependent kinase 6/cyclin D2 in discrete bone marrow foci. Sequential immunohistochemistry analysis of cdk6 expression in bone marrow biopsies early in the clinical course further revealed the emergence of discrete bone marrow foci in which multiple myeloma cells simultaneously expressed high levels of cdk6, pS780, and Rb before cell proliferation, as indicated by the absence of Ki67 (Fig. 3D, compare 137-2 with 137-1). Cyclin D2, although not detectable by the immunohistochemistry method, cooperates with cdk6 in phosphorylating Rb in these cells, because it is the obligatory catalytic partner of cdk6 in the absence of cyclin D1 or D3 (Fig. 3B). Phosphorylation of Rb by cdk6/cyclin D2 in discrete focal bone lesions early in the clinical course is in sharp contrast to expression of cyclin D1 or D3, which is uniform in >90% multiple myeloma cells, at lower frequency in scattered multiple myeloma cells, or not at all (Fig. 2A-B). Collectively, these findings suggest that the cdk6/cyclin D2 pairing is focal and likely to be coordinately regulated during multiple myeloma progression.

Phosphorylation of retinoblastoma protein by cyclin-dependent kinase 4/cyclin D2 or cyclin-dependent kinase 6/cyclin D2 increases with multiple myeloma progression. This led us to ask which of the cdk/cyclin pairs phosphorylates Rb during multiple myeloma progression and relapse. In the presymptomatic MGUS stage, both pS780 and pS807/811 were below the level of immunohistochemistry detection in CD138+ cells.

**Figure 3.** Selective phosphorylation of Rb by cdk4/cyclin D1, cdk4/cyclin D2 or cdk6/cyclin D2 in untreated early stage multiple myeloma. **A,** immunohistochemical analysis of simultaneous expression of CD138 (red) and cdk4/6-specific phosphorylated Rb (pS780), Rb, Ki67, cyclin D1, cyclin D3, CD20 (all brown) in serial sections of bone marrow core biopsies from stage I multiple myeloma patients (PT). **B,** immunoblot analysis of purified CD138+ BMPC from a normal volunteer (13) and multiple myeloma patients 336 and 346 (A). Bottom, ratios of pS780 to total Rb and the multiple myeloma stage of each patient. C, relative RNA levels from the same cells were determined by real-time RT-PCR analysis. **D,** immunohistochemical analyses of an untreated stage I multiple myeloma patient, initially (137-1) and 14 months later (137-2), which show discrete foci of multiple myeloma cells with strong expression of cdk6, Rb, and pS780 but not cyclin D1, cyclin D3, or Ki67. Represent nine independent analyses.
(376), although pS780 was appreciable by immunoblotting (Fig. 4A-B, 16). The frequency of multiple myeloma cells expressing pS780 and pS807/811 was extremely low in stage I (329) but increased in stage III in the absence of therapy (327) to levels approaching that of relapsed stage III multiple myeloma (Fig. 4A, 243). The increase in Rb phosphorylation was confirmed by immunoblotting (Fig. 4B) and seemed unrelated to cytogenetic abnormalities (Table 2; data not shown).

Cyclin D2 and cdk6 were copiously expressed in stage III (327) multiple myeloma cells and further amplified in relapsed multiple myeloma cells (243), concomitant with enhanced Rb phosphorylation (Fig. 4B). cdk4 levels also increased but much more modestly. Because neither cyclin D1 nor D3 was expressed in myeloma (Fig. 4F), 310), neither cyclin D2 nor D3 was expressed in multiple myeloma cells in which Rb was phosphorylated by cdk4/cyclin D1 (Fig. 5A, 336). However, the increase in p27Kip1 (Fig. 5B) did not match the more profound elevation of cyclin D2 and cdk6 (Fig. 4B). Phosphorylation of Rb by cdk4 or cdk6 in multiple myeloma cells is due, at least in part, to differential gene regulation that leads to an imbalance between cdk/cyclin D pairs and G1 CKIs.

Table 2. Patient clinical data: patients presented in figures

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NOTE: Figures listed in the first column present primary data for each specimen. Stage is according to the Durie-Salmon staging (25). Translocation t(11;14) is associated with cyclin D1 overexpression (14).

*p, absence of heavy chains.

Abbreviations: Pt no., patient number (arbitrary identifier assigned to database); Dx, diagnosis; Ig, immunoglobulin, Tx, treatment; N, no; Y, yes; N, negative; NL, normal; ND, not done; FISH, fluorescence in situ hybridization, reported as % positive cells.

differentiation (9, 10), was not increased in most early and late multiple myeloma or insufficient to inhibit cdk4/6 despite its prominent increase in a stage III relapsed multiple myeloma (Fig. 5A-B, 243). The p27Kip1 (34, 35) protein was modestly elevated, apparently due to increased p27Kip1 RNA, in early multiple myeloma cells in which Rb was phosphorylated by cdk4/cyclin D1 (Fig. 5A, 336). However, the increase in p27Kip1 (Fig. 5B) did not match the more profound elevation of cyclin D2 and cdk6 (Fig. 4B). Phosphorylation of Rb by cdk4 or cdk6 in multiple myeloma cells is due, at least in part, to differential gene regulation that leads to an imbalance between cdk/cyclin D pairs and G1 CKIs. p27Kip1, but not p21Cip1, is expressed in G1-arrested normal plasma cells (10). The correlation between cdk4/6-specific Rb phosphorylation and DNA replication in advanced multiple myeloma stages (Fig. 1C) further suggests that coordinated elevation of cyclin D2 and cdk4/6 may also lead to inactivation of late G1 CKIs by the formation of ternary complexes, thereby promoting late G1 progression. We therefore tested this possibility by determining the association of p27Kip1 with cyclin D2 and cdk6. Corroborating the fact that Rb was phosphorylated by cdk4/cyclin D2 or cdk6/cyclin D2, cyclin D2 associates with both cdk6 and cdk4, as determined by reciprocal immunoblotting analysis of cyclin D2 and cdk6 immune complexes in relapsed multiple myeloma (Fig. 5C). In addition, p27Kip1 was present in both cyclin D2 and cdk6 immune complexes (Fig. 5C). Coordinated activation of cyclin D2 and cdk6, therefore, seem to not only promote cdk4/6-specific phosphorylation of Rb and early G1 progression but also
Multiple myeloma cell lines are invariably established from end-stage multiple myeloma when self-renewing multiple myeloma cells proliferate without constraint. Together, these results reinforce that Rb is phosphorylated by selective cdk/D cyclin and suggest that inactivation of Rb by cdk4/cyclin D2 or cdk6/cyclin D2 is preferentially associated with advanced multiple myeloma (Fig. 6C). The association between increases in Rb and cell cycle progression in multiple myeloma cells is reminiscent of elevation of Rb protein levels upon cell cycle reentry in response to physiologic in primary B cells (6). In addition, Rb is subject to caspase cleavage (36), suggesting that the increase in Rb expression may reflect progressive loss of apoptotic control in multiple myeloma progression. To address this possibility, we showed that inhibition of apoptosis of primary BMMMs cells ex vivo by the pan-caspase inhibitor Z-VAD-FMK led to a modest increase in Rb level and phosphorylation of Rb (Fig. 6B). This result is consistent with the notion that Rb is a substrate for caspase cleavage in primary BMMMs cells, and its up-regulation may implicate decreased apoptosis of BMMMs cells. However, although high levels of cdk4/6-specific phosphorylation correlate with high Rb expression, low or lack of cdk4/6-specific phosphorylation is not due to low Rb expression (Fig. 6D, compare 393 with 373 and 383). In addition, the ratios of cdk4/6-specific phosphorylation of Rb to total Rb increased in advanced multiple myeloma (Fig. 4B), confirming heightened inactivation of Rb by specific cdk4/cyclin D1 or cdk6/cyclin D2 pairing in the course of disease progression.

Discussion

Phosphorylation of retinoblastoma protein by cyclin-dependent kinase 4/cyclin D1, cyclin-dependent kinase 4/cyclin D2 or cyclin-dependent kinase 6/cyclin D2 is critical for cell cycle dysregulation in myeloma. In this study, we have shown, for the first time, that Rb is phosphorylated by mutually exclusive pairing of cdk4 with cyclin D1 and cdk4/6 with cyclin D2 in multiple myeloma, and that this exclusive pairing seems to critically control cell cycle dysregulation in multiple myeloma (Fig. 6C). The basis for the mutually exclusive pairing between cdk4/6 and D cyclins lies in the restricted expression of one and}

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only one D cyclin in each case of multiple myeloma and the differential transcriptional regulation of cdk4, cdk6, and cyclin D2 genes. We showed that overexpression of cyclin D1 or D3 rarely promotes Rb phosphorylation or G1 cell cycle progression (Fig. 2), apparently due to limiting cdk4 (Fig. 3). cdk6 and cyclin D2 are coordinately and prominently increased in the clinical course, indicating the association of p27Kip1 with cdk6 and cyclin D2 in the expansion of self-renewing multiple myeloma cells.

Inactivation of retinoblastoma protein by cyclin-dependent kinase 4 in cooperation with cyclin D1. Translocation of the cyclin D1 gene to the immunoglobulin heavy chain locus t(11:14) and overexpression of cyclin D1 RNA have been implicated in cell cycle dysregulation in multiple myeloma (14, 15, 17, 18). Paradoxically, cyclin D1 overexpression has also been linked to a more favorable prognosis in multiple myeloma (22–24), calling into question the precise role of cyclin D1 overexpression in multiple myeloma. We showed by sequential analysis of bone marrow biopsies (5-26) that cyclin D1 expression did not vary during the clinical course of 21 patients (Fig. 2D) or correlate with cell proliferation in a study of an additional 251 patients (Fig. 2C), because it alone was insufficient to inactivate Rb and promote G1 progression (Fig. 2B-C). Phosphorylation of Rb by the exclusive cyclin D1/cdk4 pair is in the absence of cdk6, cyclin D2, or cyclin D3 in early (Fig. 3A-B) and advanced (Fig. 6A) multiple myeloma further suggests that the level of cdk4 expression is rate limiting in Rb phosphorylation and G1 progression in cyclin D1 overexpressing multiple myeloma. This result is in line with the requirement for cdk4 in transformation of primary mouse cells (37). Together, our findings also raise an important question concerning the basis for the striking absence of cdk6, cyclin D2, and cyclin D3 in cyclin D1–expressing multiple myeloma cells, which awaits future exploration.

The cyclin-dependent kinase 6/cyclin D2 partnership preferentially promotes multiple myeloma progression. cdk6 and cyclin D2 are undetectable at the RNA and protein levels in normal bone marrow plasma cells, although cdk4 is present at a low level (Fig. 3). cdk6 and cyclin D2 are coexpressed in ~37% of multiple myeloma cases (15 of 40, including those shown in Table 2) and 90% of multiple myeloma cell lines characterized (Fig. 6) based on complementary immunoblotting, quantitative RT-PCR, and immunohistochemistry analyses. Together with the mutually exclusive expression of cyclins D1, D2, and D3 in all multiple myeloma cases (Figs. 3-6) and the lack of association between cyclin D1 expression and cell proliferation (Fig. 2), these findings suggest that the more favorable clinical outcome in cyclin D1–expressing multiple myeloma may in part due to the absence of the cdk6 and cyclin D2 expression.

Contrasting the modest changes in cdk4 levels, cdk6 expression is detectable in discrete bone marrow foci of myeloma cells along with Rb phosphorylation (Fig. 3D) and is prominently increased in advanced multiple myeloma along with cyclin D2 expression (Figs. 4 and 6). Coordinated elevation of cdk6 and cyclin D2 could have two functional consequences: phosphorylating Rb in conjunction with cdk4 by overriding early G1 CKI, such as p18INK4c, and accelerating G1-S cell cycle progression by sequestering the late G1 CKI p27kip1. Both require that in aggregate, cdk6 and cdk4 be in molar excess of the early G1 CKIs.

Early G1 progression in multiple myeloma is due, at least in part, to insufficient p18INK4c, which does not increase above the level seen in normal BMPCs, except in relapsed multiple myeloma cells expressing extremely high levels of cyclin D2 (Figs. 4-6). p27kip1 RNA and protein are modestly elevated along with the prominent increases in cyclin D1 and cdk4 in early multiple myeloma (Figs. 3 and 5). Whether differential elevation of p27kip1 inhibits late G1 progression in cyclin D1–expressing multiple myeloma cells is presently unclear. However, p27kip1 coprecipitated with cdk6 and cyclin D2 (Fig. 5), confirming the cdk6/cyclin D2 partnership and indicating the association of p27kip1 with cdk6 and cyclin D2 (Fig. 5C). These findings further raise an interesting possibility that p27kip1 may, in turn, also stabilize cyclin D2 and cdk6 as observed in the formation of cyclin D1/cdk4/p27kip1 complexes in primary mouse embryonic fibroblasts (33). Collectively, our results suggest that coordinated elevation of cdk6 and cyclin D2 leads to tandem

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loss of early and late G1 cell cycle control in myeloma cells, the former by cdk4/6-specific phosphorylation of Rb and the latter by sequestration of p27Kip1 by cdk6 and cyclin D2. Cell cycle dysregulation in multiple myeloma, therefore, is due to selective gain of cdk and D cyclin function as well as insufficient G1 CKIs.

Molecular myeloma subtypes defined by the expression of early G1 cell cycle regulators. The partnership between cdk4/6 and D cyclins is determined largely by selective RNA activation (Figs. 3–4). On this basis, we propose that there are two molecular subtypes of self-sustaining multiple myeloma cells that are distinguishable by selective activation of cdk4/6 and D cyclins early in multiple myeloma pathogenesis and during disease progression. Deciphering the genetic alterations and the epigenetic signals that cooperate in generating and differentially maintaining these two multiple myeloma subtypes poses an important challenge for the immediate future.

Overexpression of cyclin D1, but not D2, is frequently but not always associated with a t(11;14) chromosomal translocation or trisomy 11 (14–16). In this context, t(11;14) has been shown to correlate with expression of CD20 (38). Consistent with this finding, we detected an association between overexpression of cyclin D1, but not cyclin D2 or D3, with t(11;14) by fluorescence in situ hybridization (Table 2) and strong CD20 expression on all cyclin D1–expressing multiple myeloma cells (Fig. 2B; data not shown). CD20 is normally present on the surface of B cells but not end-stage plasma cells. Whether the association between CD20 and cyclin D1 expression in multiple myeloma cells reflects a specific timing of transformation and differentiation arrest during B-cell terminal differentiation is an intriguing possibility that remains to be followed.

Cyclin D2 has been shown to be trans-activated by the transcription factor c-Maf, which is elevated in 50% of multiple myeloma cases based on microarray analysis, and seems to promote pathogenic interactions between multiple myeloma cells and bone stroma (39). In preliminary studies, we found a positive correlation among cdk6, cyclin D2, and c-Maf mRNA levels in primary BMMM cells. In the bone marrow microenvironments, cdk4/6 and D cyclins are likely to be coordinately regulated by multiple signals. CD40L represents one such signal, as it is aberrantly expressed in both multiple myeloma cells and bone marrow stromal cells, and through autocrine and paracrine signaling, CD40L enhances cyclin D2, cdk6, and MCM7 expression. The functional cell cycle immunohistochemical assay presented here offers an opportunity for longitudinal analysis of G1 cell cycle dysregulation in vivo in archived bone marrow specimens in the context of multiple myeloma progression. Functional and mechanistic analyses of cell cycle dysregulation in primary bone marrow myeloma cells, although challenging, are possible despite the exceedingly low cell numbers. Elucidating the mechanisms by which cdks and D cyclins are regulated in the bone microenvironment to control G1 cell cycle progression may advance our understanding of cell cycle dysregulation in multiple myeloma progression and guide treatment selection.

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References


Mutually Exclusive Cyclin-Dependent Kinase 4/Cyclin D1 and Cyclin-Dependent Kinase 6/Cyclin D2 Pairing Inactivates Retinoblastoma Protein and Promotes Cell Cycle Dysregulation in Multiple Myeloma

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