Retinoblastoma Pathway Defects Show Differential Ability to Activate the Constitutive DNA Damage Response in Human Tumorigenesis

Frederic Tort,1 Jirina Bartkova,1 Maxwell Sehested,2 Torben Ørntoft,2 Jiri Lukas,1 and Jiri Bartek1

1Institute of Cancer Biology and Centre for Genotoxic Stress Research, Danish Cancer Society; 2Department of Pathology, University Hospital, Copenhagen, Denmark; and 3Department of Clinical Biochemistry, Aarhus University Hospital, Skjebby, Aarhus N, Denmark

Abstract
Loss of G1-S control and aberrations of the p16INK4a-cyclin D1/cyclin-dependent kinase (CDK) 4(6)-pRb-E2F-cyclin E/CDK2 pathway are common in human cancer. Previous studies showed that oncogene-induced aberrant proliferation, such as on cyclin E overexpression, causes DNA damage and checkpoint activation. Here, we show that, in a series of human colorectal adenomas, those with deregulation of cyclin D1 and/or p16INK4a showed little evidence of constitutive DNA damage response (DDR), contrary to cyclin E-overexpressing higher-grade cases. These observations were consistent with diverse cell culture models with differential defects of retinoblastoma pathway components, as overexpression of cyclin D1 or lack of p16INK4a, either alone or combined, did not elicit detectable DDR. In contrast, inactivation of pRb, the key component of the pathway, activated the DDR in cultured human or mouse cells, analogous to elevated cyclin E. These results highlight differential effect of diverse oncogenic events on driving the 'cancer cell cycles' and their ability to deregulate the replication-driving CDK2 kinase and to alarm the DDR as a potential anticancer barrier in accordance with their hierarchical positions along the retinoblastoma pathway. Our data provide new insights into oncogene-evoked DDR in human tumorigenesis, with potential implications for individualized management of tumors with elevated cyclin D1 versus cyclin E, due to their distinct clinical variables and biological behavior. (Cancer Res 2006; 66(21): 10258-63)

Introduction
Cell cycle is a tightly controlled process that ensures proper duplication and distribution of the genetic material to the next generation of cells. Transitions through the cell cycle phases are orchestrated by the cyclin-dependent kinases (CDK), bound to their distinct activatory subunits, the cyclins. In mid-late G1, the CDK4/6-cyclin D complexes phosphorylate and inactivate the retinoblastoma protein (pRb), thereby promoting progression through the restriction point and the release of the E2F transcription factors, which in turn induce the synthesis of cyclin E and other factors required for DNA replication. The activity of cyclin D-CDK4(6) is negatively regulated by members of the INK4 family of CDK inhibitors, including p16INK4a, a tumor suppressor that prevents the phosphorylation-mediated inactivation of pRb (1).

Another layer of protection that ensures genomic integrity and guards against genetic diseases, such as cancer, is provided by the DNA damage response (DDR) pathways, a signaling network that leads to cell cycle arrest, DNA repair, or death of cells exposed to genotoxic insults (2). Aberrations of both G1-S transition and DDR are among hallmarks of cancer. Frequent defects in tumors include overexpression of proto-oncogenic D-type cyclins and cyclin E and inactivation of p16INK4a, pRb, and p53 tumor suppressors, the latter a key effector of the DDR network (2–4). Recent data showed that oncogene-induced aberrant proliferation can cause DNA damage and trigger DDR, and constitutive DDR activation occurs in early human lesions, likely acting as a barrier against progression to malignancy (5, 6). However, not all premalignant lesions show DDR activation. We hypothesize that this may reflect either inactivation of the DDR network in subsets of lesions or distinct capacity of diverse oncogenic events to evoke DNA damage and hence activate the DDR barrier. Here, we test the latter hypothesis and determine the ability of the deregulation of several components along the p16INK4a-cyclinD1-pRb-E2F-cyclinE pathway to trigger DDR, both in cell culture models and by analysis of human colorectal adenomas.

Materials and Methods

Antibodies. For immunofluorescence, Western blotting, immunoprecipitation, and immunohistochemistry, we used antibodies against cyclin D1 (DCS-6 and DCS-11), p16INK4a (DCS-30 and 16P04), Mcm7 (DCS-141), and Cdk7 (MO-1; NeoMarkers, Fremont, CA; refs. 5, 7, 8); pRb (BP Pharmingen, San Diego, CA); cyclin E (HE12 and E172), p21, Cdk2, and human papillomavirus (HPV)-E7 (Santa Cruz Biotechnology, Santa Cruz, CA); phosphorylated specific Ser139-H2AX (Upstate, Charlottesville, VA); and Ser(10) and Ser(11)-Chk1 (Cell Signaling, Danvers, MA).

Immunohistochemistry and clinical specimens. Paraffin blocks of formalin-fixed specimens of human colorectal adenomas (27 grade 1-2 lesions and 43 grade 3 lesions) were from the University Hospitals of Copenhagen and Aarhus. Sensitive immunoperoxidase staining of tissue sections was done as described (5) and the results scored as indicated in legend of Fig. 4.

Cell culture and biochemistry. U-2-OS osteosarcoma cell line (and derived clones), BJ cells, and breast cancer cell lines (see Fig. 2D) were grown in DMEM supplemented with 10% FCS and antibiotics. U-2-OS-derived cells with tetracycline-repressible expression of cyclin D1 and E7 (provided by B. Norrild, University of Copenhagen, Copenhagen, Denmark) genes were generated and characterized using previously described plasmids and methods (5). Human primary p16INK4a-deficient fibroblasts were kindly provided by Gordon Peters (Cancer Research UK, London, United Kingdom). Cyclin D1–expressing retroviral vectors and infection of human diploid fibroblasts were done as described (9). Cells were synchronized by nocodazole treatment (12 hours) and released. From late mitosis to mid G1, cells were harvested at the indicated time points and analyzed as described in the figures. When indicated, cells were treated with ionizing radiation using an X-ray generator (HF160; Pantak, East Haven, CT; 150 kV, 15 mA, and dose rate of 2.18 Gy min-1). Protein extraction, immunoblotting, and kinase assays were done as described (7).

Immunofluorescence. Cells grown on glass coverslips were fixed in 4% formaldehyde (10 minutes, room temperature) and permeabilized with
0.2% Triton X-100 (5 minutes) before application of the primary antibodies specified in the figure legends. Images were captured and processed using a Zeiss (Göttingen, Germany) Axioplan 2 microscope.

**Results and Discussion**

To analyze which defects along the p16<sup> Ink4a </sup>-cyclin D1/CDK4(6)-pRb-cyclin E/CDK2 pathway trigger DDR, we used U-2-OS-derived cell lines with tetracycline-repressible overexpression of cyclin D1 or cyclin E (U-2-OS-CycD1 and U-2-OS-CycE, respectively) or pRb inactivation (U-2-OS-E7). U-2-OS cells are checkpoint proficient and wild-type for p53 and pRb, but deficient for the Ink4 locus, providing a p16<sup> Ink4a </sup>-null genetic background.

Immunofluorescence and immunoblotting analysis of U-2-OS-CycD1 cells showed induction of cyclin D1 protein (homogeneously in 90-95% cells) to a level even higher than in breast cancer cell lines with known cyclin D1 overexpression due to gene amplification or enhanced mRNA expression (Fig. 1A). Consistent with its rate-limiting role for G1 progression (8, 10), induction of cyclin D1 accelerated the S-phase onset in U-2-OS-CycD1 cells (Supplementary Fig. S1).

To test whether cyclin D1 overexpression can cause DNA damage similarly to oncogenes, such as cyclin E, Cdc25A, or E2F1 (5), we examined several markers of activated DDR, including Ser<sup>15 </sup>-phosphorylated p53, Ser<sup>317 </sup>-phosphorylated Chk1, and γH2AX, in U-2-OS-CycD1 cells. Immunoblotting and immunofluorescence analyses showed that, in contrast to isogenic cells overexpressing cyclin E, cells overexpressing cyclin D1 had no significant increase in any of these markers. The slight increase in Ser<sup>15 </sup>-phosphorylated p53 was not reflected by elevated p21, a p53-regulated effector of cell cycle arrest in response to DNA damage (Fig. 1B and C). Control, irradiated cells showed strong p53 and Chk1 phosphorylation, excluding the possibility that the absence of DDR activation after cyclin D1 overexpression reflects an inability of these cells to respond to genotoxic insults (Supplementary Fig. S2).

The U-2-OS cells are deficient in the Ink4 tumor suppressor locus, a frequent target in human tumors, which encodes p16<sup> Ink4a </sup>and p14<sup> ARF </sup>. Whereas p16<sup> Ink4a </sup>arrests cells in G1 through cyclin D/CDK4(6) inhibition, p14<sup> ARF </sup>interferes with mdm2-mediated degradation of p53 (11). Consistent with the data obtained with U-2-OS cells, we found no evidence of DNA damage and constitutive checkpoint activation in normal (BJ strain) or primary p16<sup> Ink4a </sup>-deficient human fibroblasts, either mock-infected or overexpressing cyclin D1 on retroviral infection. In contrast, fibroblasts infected with vectors encoding cyclin E showed strong DDR activation, as documented by histone H2AX phosphorylation (Fig. 2A and B). We concluded that neither the lack of p16<sup> Ink4a </sup>nor...
nor the combined defect of p16\(^{Ink4a}\) and p14\(^{ARF}\) is sufficient to cause DDR activation. Taken together with the data in U-2-OS cells, even the concomitant absence of p16\(^{Ink4a}\) and overexpression of cyclin D1 did not evoke a significant DDR.

Given that the cyclin E/CDK2 kinase is a major driving force of DNA replication, that the deregulated cyclin E causes 'replication stress' (5), and that cyclins D1 and E operate in the same pathway, yet differ in their ability to induce DNA damage and checkpoint activation (this study), we wondered whether such intriguing biological differences could be attributable to differential effects of cyclins D1 and E on CDK2 kinase activity. To this end, biochemical analyses of U-2-OS-derived clones showed that overexpression of cyclin E, but not D1, resulted in an increase of CDK2 kinase activity in exponentially growing cells (Fig. 3A). An even more striking difference was observed in synchronized mitotic cells, when cyclin E overexpression, but not that of cyclin D1, caused a pronounced increase of CDK2 kinase activity in exponentially growing cells (Fig. 3A). An even more striking difference was observed in synchronized mitotic cells, when cyclin E overexpression, but not that of cyclin D1, caused a pronounced increase of CDK2 kinase activity in exponentially growing cells (Fig. 3A).

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Thus, our data, together with previously reported observations, implicate the abnormally enhanced and/or unscheduled CDK2 activity in the replicational stress and DDR activation induced by certain oncogenes, such as cyclin E. These experiments also offer a plausible explanation for the differences seen in cyclin D1–overexpressing and cyclin E–overexpressing cells despite these proteins function in a linear pathway during G1 and G1-S transition.

Cyclin D1 and p16\(^{Ink4a}\), respectively, represent the main positive and negative regulators of CDK4(6) activity to neutralize pRb and promote progression through the G1 restriction point and S-phase entry. The fact that E2F1, cyclin E, and Cdc25A operate downstream of pRB in the same regulatory pathway raises the question whether pRb inactivation by itself could induce DNA damage and the subsequent checkpoint activation. To assess the potential of pRb inactivation to activate the DDR, we have used U-2-OS cells with regulatable expression of the HPV (type 16) E7, an oncogene involved in the pathogenesis of several types of human carcinomas, through its binding to, and functional interference with, pRb. E7-mediated inactivation of pRb generated DNA damage and checkpoint activation, as seen by increasing amounts of phosphorylated H2AX and p53 (Fig. 1B and C). These results corroborate a recent report that pRb down-regulation by small interfering RNA induces DNA double-strand breaks and γH2AX foci formation (13). Consistent with the human cell model, early
passage embryonic fibroblasts from \textit{pRb} knockout mice, but not from their normal littermates, showed a strong constitutive activation of DDR markers analogous to that seen in U-2-OS cells overexpressing E7 (Fig. 2C).

We further extended the above results using a panel of human breast cancer cell lines with different defects in the p16\textsuperscript{Ink4a}–cyclinD1–pRb–cyclin E cascade. As shown in Fig. 2D, cell lines overexpressing cyclin D1 (MCF7 and ZR-75) had no accumulation of activated DDR proteins, such as phosphorylated forms of p53 and Chk2. In contrast, pRb-deficient cell lines (MDA-468 and BT-549) and cyclin E-overexpressing (MDA-157) cell lines showed pronounced constitutive DDR activation. Similarly to U-2-OS clones, such constitutive DDR in breast cancer cell lines seems to be independent of p16\textsuperscript{Ink4a} aberrations, suggesting that presence or absence of this tumor suppressor may not account for this phenomenon. Interestingly, the three breast cancer cell lines with constitutive DDR have been reported to harbor p53 inactivation. These data support previous observations, in which constitutive DDR has been linked to cancer cell lines with mutant p53 (14).

Aberrations of p53 are among the most common genetic alterations in human cancer, frequently associated with tumor progression and poor prognosis of the patients (15–18). Interestingly, previous studies in breast cancer clinical specimens revealed a correlation between overexpressed cyclin E, but not D1, with the presence of p53 mutations, higher histologic grade, and poor survival of the patients (19, 20). Our cell culture experiments suggest a possible explanation for the different behavior of cyclin E-overexpressing tumors versus cyclin D1–overexpressing tumors, reflecting the different abilities of these oncogenes to induce DNA damage and checkpoint activation, including p53. Activated DDR and p53 would pose a barrier to growth and hence be expected to be more commonly inactivated in progressing tumors among lesions harboring elevated cyclin E rather than cyclin D1.

To complement the cell culture experiments with analysis of clinical specimens, we examined the expression of cyclin D1, p16\textsuperscript{Ink4a}, and the DDR markers γH2AX and phosphorylated Chk2 in a large series of human colorectal adenomas. Whereas normal colon tissue shows barely detectable cyclin D1 and no DDR activation, despite similar rates of proliferation compared with adenomas (as judged from the Ki67 proliferation marker), subsets of adenomas showed clear activation of DDR, yet this did not correlate with either elevated cyclin D1 or lack of p16\textsuperscript{Ink4a} (Fig. 4A and B). Interestingly, the degree of constitutive DDR activation was higher among the grade 3 lesions, which harbor higher risk of

\begin{figure}[h]
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\caption{Cyclin E– and cyclin D1–associated kinase activity in U-2-OS–inducible clones. \textit{A,} in vitro kinase assay showing the increase in the CDK2 activity of U-2-OS cells overexpressing cyclin E but not cyclin D1, \textit{RU,} relative units. \textit{B,} CDK2 kinase activity of U-2-OS-CycE and U-2-OS-CycD1 cells induced (–\textit{Tet}) or not (+\textit{Tet}) to express the transgenes was analyzed from late mitosis to mid G1 in a time course experiment after nocodazole (\textit{Noc}) release. \textit{C,} a time course of cyclin E– and cyclin D1–associated kinase activities in U-2-OS-CycE–inducible and U-2-OS-CycD1–inducible cell lines, respectively, using histone H1 and glutathione S-transferase (GST)–pRb as substrates.}
\end{figure}
progression into carcinomas compared with grade 1 and 2 adenomas. In neither category, however, did the DDR correlate with cyclin D1/p16\textsuperscript{ink4a} status, and especially among the lower-grade adenomas, there were subsets of cases with enhanced cyclin D1 and/or lack of p16\textsuperscript{ink4a}, which showed very little or no DDR activation. This result contrasts with good correlation between DDR activation and elevated cyclin E in grade 3 colorectal adenomas (5) and strongly supports our cell culture data on differential effects of distinct aberrations in the retinoblastoma pathway.

In conclusion, our results provide evidence of distinct roles for particular oncogene and tumor suppressor defects within the retinoblastoma pathway, corresponding to hierarchical positions of

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**Figure 4.** Immunohistochemical analysis of DDR markers compared with cyclin D1 and p16\textsuperscript{ink4a} deregulation in human colorectal adenomas. A, immunoperoxidase staining of normal colon mucosa and two colorectal adenomas with antibodies against the indicated markers. Note the very low level of cyclin D1 and lack of γH2AX in normal epithelium and the lack of correlation between DDR activation (γH2AX) and elevation of cyclin D1 and lack of p16\textsuperscript{ink4a}. B, graphical summary of the data, separately for lower-grade (1-2) and high-grade (3) adenomas. The degree of γH2AX was scored as described (5), whereas p16\textsuperscript{ink4a} was regarded as negative-low (up to 5% positive cells) and medium-high (6-60% positive cells) and cyclin D1 as negative (0-2% positive cells), medium (3-7% positive cells), or high (8% or more positive cells).
our data suggest why some premalignant lesions lack the hallmarks of DDR activation and indicate that at least some oncogenic events may help clonal expansion of the early lesions without activation of the DDR barrier (5, 6). Given the role of DDR in response to radiotherapy and chemotherapy, assessment of these pathways may help select individualized treatment strategies in the future.

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