Cyclin D1 Overexpression Enhances Radiation-induced Apoptosis and Radiosensitivity in a Breast Tumor Cell Line

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ABSTRACT

Overexpression of cyclin D1, a G1 cell cycle regulator, is often found in many different tumor types, such as breast carcinoma and squamous cell carcinoma of the head and neck. The overexpression of this protein is, in several cases, associated with a poor prognosis. In this study, the effect of cyclin D1 on radiosensitivity was investigated in a breast tumor cell line, MCF7, containing a cyclin D1 gene construct under the control of a tetracycline-sensitive regulator. MCF7 cells cultured without tetracycline resulted in a 6-fold increase in the cyclin D1 protein.

Cyclin D1-overexpressing MCF7 cells were more sensitive to ionizing radiation than the nonoverexpressing counterparts. The cyclin D1-overexpressing cells also exhibited a higher induction of apoptosis. Treatment with a dose of 5 Gy resulted in a rapid increase of p53 and p21 in the cyclin D1-overexpressing cells. Nonoverexpressing cells showed a more transient expression of these proteins after ionizing radiation. A pronounced G2-M block was observed in both cell lines. The cyclin D1-overexpressing cells were, however, released earlier from the block than the control cells.

These data suggest that overexpression of cyclin D1 alters sensitivity toward ionizing radiation by modulating G1 radiation-induced G2-M transition.

INTRODUCTION

Overexpression of cyclin D1 is often found in many different tumor types, e.g., breast carcinoma and squamous cell carcinoma of the head and neck. Amplification of the 11q13 region, where the cyclin D1 gene is located, is found in ~20–40% of human breast, ovarian, and squamous cell carcinomas (1, 2). Overexpression of this protein or amplification at the 11q13 region is associated with a poor prognosis or recurrence in several cases (2–6). These studies indicate the clinical significance of cyclin D1-overexpressing tumors.

Cyclin D1, their catalytic counterparts, the Cdks (3) and the inhibitors of the Cdks, the Ckis, regulate the progression through the G1 phase of the cell cycle. This suggests that alterations in the expression of these cell cycle regulators may be of critical importance in determining the sensitivity of tumors toward cytostatic drugs and radiation.

Cyclin D1:Cdk4 regulates transition through the early G1 phase of the cell cycle by phosphorylation of pRb (7), which results in the release of transcription factor E2F from pRb. Free E2F mediates transcription of E2F-dependent genes, including DNA polymerase, thymidine kinase, and dihydroflorote reductase. Expression of cyclin D1 is sensitive to growth factors (8) and to adhesion of cells onto extracellular matrix components (9). Cyclin D1:Cdk4 kinase activity is specifically inhibited by the Cki p16 (10), whereas the Cki p21 binds to all of the cyclin Cdks. p21 is expressed in a p53-dependent and -independent manner (11–13) and is increased by cyclin D1 overexpression (14, 15). At low concentrations, p21 promotes the assembly of an active cyclin D:Cdk4 complex, whereas it inhibits its activity at higher concentrations (16). Cki p27 also binds to all of the Cdk but inhibits cyclin D:Cdk4 activity much less efficiently than cyclin A:Cdk2 kinase activity (17). Cki p21 in particular, mediates either a cell cycle arrest or apoptosis upon treatment of cells with either ionizing radiation or genotoxic agents (11).

Cyclin D1, on the one hand, binds to Ckis p21 and p27 (16, 17) and, on the other hand, induces expression of these Ckis (14, 18, 19). To study these complex interactions, we examined the effect of overexpression of cyclin D1 on apoptosis induced by radiation, which involves induction of p53 and p21 (20). We studied the effect of ionizing radiation in MCF7-cl3 cells in which exogenous cyclin D1 expression was under the influence of a tetracycline-sensitive promoter (21). These MCF7 cells lack p16 and contain wt p53 (22). Our results indicate that overexpression of cyclin D1 reduces clonogenic survival of cells upon radiation by apoptosis. This occurs via an accelerated induction of p53 and p21, leading to a faster progression through the G2-M phase of the cell cycle and, finally, apoptosis.

MATERIALS AND METHODS

Cell Culture and Induction of Overexpression of Cyclin D1. Human epithelial MCF7 breast tumor cells and the cyclin D1-transfected MCF7-cl3 cells were cultured in DMEM supplemented with 10% FCS, 2 mm glutamine, 100 units of penicillin, and 100 μg/ml streptomycin. The cyclin D1-transfected MCF7-cl3 cells contained the puHD15–1, the pSV2neo-tetracycline transactivator, and the tet-cyclin D1 as well as thymidine-kinase-hygromycin plasmid as described previously (21). These cells were cultured in the presence of 10 μg/ml tetracycline to suppress expression of ectopic cyclin D1, whereas tetracycline was washed off when overexpression of cyclin D1 was wanted.

Clonogenic Survival Assays. Cells were plated at different densities and exposed to various doses of ionizing radiation using a 137Cs irradiation unit with a dose rate of ~1 Gy/min. After 10 days of incubation at 37°C, cells were stained with a solution of crystal violet. The number of colonies per dish was counted, and the surviving fractions were calculated as the ratio of plating efficiencies for treated and untreated cells. The plating efficiency is defined as the colony number divided by the number of cells plated.

Apoptosis Assays. Cells growing on coverslips in six-well Falcon plates were exposed to indicated doses of ionizing radiation and cultured thereafter for the indicated periods of time. The cells were irradiated using 137Cs irradiation unit with a dose rate of ~1 Gy/min. Cells were fixed in methanol (~20°C) for 20 min, briefly immersed in cold acetone, and stained with 0.1 μg/ml 4,6 diamidine-2-phenylindole-dihydrochloride and 200 μg/ml 1,4 diazobicyclo[2,2,2]octane (Merck) in glycercol. The percentage of apoptotic cells was determined microscopically as cells with visible micromet. The percentage of apoptotic cells was determined in three independent experiments, and in each experiment, 300 cells were scored for each time/dose point.

Cell Cycle Progression. S-phase cells were labeled with 1 μM BrdUrd by incubating the cells for 10 min at 37°C immediately after irradiation. Cells were harvested at several time points after irradiation by trypsinization of the cells, followed by resuspension of the cells in 1 ml PBS and fixation in 5 ml of 70% cold ethanol (~4°C). Anti-BrdUrd staining was performed as previously described by Begg and Hofland (23). Briefly, nuclei were isolated by pepsin digestion, and -independent manner (11–13) and is increased by cyclin D1 overexpression (14, 15). At low concentrations, p21 promotes the assembly of an active cyclin D:Cdk4 complex, whereas it inhibits its activity at higher concentrations (16). Cki p27 also binds to all of the Cdk but inhibits cyclin D:Cdk4 activity much less efficiently than cyclin A:Cdk2 kinase activity (17). Cki p21 in particular, mediates either a cell cycle arrest or apoptosis upon treatment of cells with either ionizing radiation or genotoxic agents (11).

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RESULTS

Clonogenic Cell Survival and Induction of Apoptosis after Ionizing Radiation. Ionizing radiation can induce \( G_1 \) as well as \( G_2-M \) delays, dependent upon the status of p53 (20, 26, 27). In addition, cyclin D1 might have a significant effect on the progression through the cell cycle and potentially modulate these blocks, leading to a different cell survival. To study the final effect of ionizing radiation, a clonogenic survival assay was performed. MCF7 cells overexpressing cyclin D1 were more radiosensitive than nonoverexpressing cells (Fig. 1). Cell survival parameters of these survival curves are shown in Table 1. The enhanced sensitivity as derived from the cell survival parameters ranged from a factor 1.3 (\( SF_2 \)) to 1.4 (\( D_{10} \)).

To elucidate whether the clonogenic cell death was associated with the induction of apoptosis, changes in nuclear morphology were examined. The apoptotic process is characterized by nuclear deformation, blebbing, and activation of endogenous nuclease, which leads to degradation of nuclear DNA (28). All but the latter phenomena of apoptosis are observed when apoptosis is induced in MCF7 human breast cancer cells by transforming growth factor-\( \beta \), etoposide, or deprivation of growth factors (29, 30). After a radiation dose of 5 Gy, a time-dependent increase of apoptosis was observed for MCF7 cells both overexpressing cyclin D1 and in wt cells. Induction of apoptosis was more pronounced, however, in the cyclin D1-overexpressing cells (Fig. 2). A dose-dependent increase in apoptotic cells at 24 h after radiation was observed for both cell lines (Fig. 3); again, more apoptotic cells were observed in the cyclin D1-overexpressing cells.

When control MCF7-cl6 cells containing only the pSV2-tetracycline transactivator (21) were cultured in the presence or absence of tetracycline, no difference in clonogenic survival and apoptosis was observed after \( \gamma \)-radiation (data not shown).

Cell Cycle Progression after Ionizing Radiation. To determine to what extent the cell cycle was affected by radiation and the role of cyclin D1, cell cycle progression was examined after a pulse label

Table 1 Radiation survival parameters for MCF7-cl3 cells overexpressing cyclin D1 (+tetr) and control cells (+tet) from linear-quadratic fitted dose-response curves\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>( SF_2 )</th>
<th>( D_{10} ) (Gy)</th>
<th>( \alpha/\beta ) ratio (Gy)</th>
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<tbody>
<tr>
<td>+ tet</td>
<td>0.55</td>
<td>4.7</td>
<td>3.3</td>
</tr>
<tr>
<td>- tet</td>
<td>0.40</td>
<td>3.7</td>
<td>2.2</td>
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\(* SF_2 \), surviving fraction at 2 Gy; \( D_{10} \), dose to give \( SF \) of 10%; \( \alpha/\beta \), ratio of linear and quadratic coefficients.

\(2^\dagger\) Data points, means of three independent experiments; bars, SD.

\(4^\dagger\) Radiation survival parameters for MCF7-cl3 cells overexpressing cyclin D1 (+tetr) and control cells (+tet) from linear-quadratic fitted dose-response curves.*
with BrdUrd. In both cell lines, a clear G2-M block could be observed (Fig. 4) because no cells labeled with BrdUrd were observed in G1 at 8 h after radiation. Twelve h after radiation a small population of cyclin D1-overexpressing cells labeled with BrdUrd was observed in G1, indicating the exit from G2-M into the next cycle. This was only the case, however, for cyclin D1-overexpressing cells, as was confirmed by quantifying the fraction of labeled cells in G1 (Fig. 5A). Sixteen h after radiation, 25% of the cells overexpressing cyclin D1 were released from G2-M. For the nonoverexpressing cells, only 7% was able to progress into the next cell cycle. In both the control and the cyclin D1-overexpressing cells, a G1-S block was observed upon radiation. Release from the G1-S block was, however, not significantly different for control and cyclin D1-overexpressing cells, as determined on the basis of the fraction of unlabeled S-phase cells (Fig. 5B). The progression of nonradiated cells from G1 into S was not affected by overexpression of cyclin D1, as was determined by unlabeled cells entering S phase (Fig. 5B).

Effect of Cyclin D1 on Expression of Regulators of the Cell Cycle and of Apoptosis after Ionizing Radiation. Expression of exogenous cyclin D1 from the cyclin D1-tet-cDNA plasmid in MCF7 cells was prevented by the addition of 10 μg/ml tetracycline to the medium and is maximal in the absence of tetracycline (21). Using this tetracycline-sensitive expression vector system (31), we obtained a medium and is maximal in the absence of tetracycline (21). Using this.

In nontreated cells, a basal level of Cki p21 protein was evident both in cyclin D1-overexpressing and in control cells. A further increase in the p21 protein level was observed 2–4 h after radiation in the cyclin D1-overexpressing cells, and a second rise was observed 24 h after radiation. In control cells, only a minor increase in p21 protein levels was observed 4 h after radiation, and no increase was seen at 24 h (Fig. 6A).

Cki p27 expression was elevated in cyclin D1-overexpressing cells as compared with control cells but did not vary upon exposure of cells to radiation (Fig. 6A). No variation in expression of other regulators of apoptosis, bax, bcl-2, and bcl-xS, was observed after radiation (Fig. 6B) in cyclin D1-overexpressing cells, whereas a decrease was observed for bcl-2 and bax after ionizing radiation in control MCF7-cl3 cells. Bcl-xS expression in MCF7-cl3 cells was absent, as was also observed by others (32).

Effect of Cyclin D1 on Cdk2 and Cdk4 and on Cyclin B-associated Kinase Activity after Ionizing Radiation. To examine whether cyclin D1 affected radiation-induced G2-M arrest and/or apoptosis by altering cyclin:Cdk activities, we determined Cdk4, Cdk2, and cyclin B1-associated kinase activity in MCF7-cl3 at 4, 10, 25, and 49 h after 5 Gy of radiation. This dose resulted in a rapid decline of Cdk2 and cyclin B-associated kinase activity within 4 h in cyclin D1-overexpressing as well as in control cells (Fig. 7A). Thereafter, Cdk2-associated kinase activity was recovered in cyclin D1-overexpressing cells, but less in control cells by 10 h after radiation, whereas also cyclin B-associated kinase activity increased more significantly 16 h after ionizing radiation in cyclin D1 overexpressing cells than in control cells. Cdk4 activity did not change significantly after radiation.

To further investigate the difference in kinase activities after radiation between cyclin D1-overexpressing and control cells, we studied the association between cyclin:Cdk complexes and their inhibitors, Cks p21 and p27. The cellular concentration of Cki p27 was already elevated in nontreated, cyclin D1-overexpressing cells (r = 0), and more p27 was associated with Cdk4 in these cells than in control cells (Fig. 7B). The total cellular concentration of p27 did not change upon radiation. Relatively low levels of p27 became associated with Cdk2, which did not alter after radiation. The association between cyclin B and p27 could not be determined in these studies, because of comigration of IgG bands with p27.

Total levels of Cki p21 protein clearly increased upon radiation, with a more pronounced effect in cyclin D1-overexpressing than in control cells (Figs. 6A and 7B). As a result, p21 became increasingly associated after radiation not only with Cdk4 but also with Cdk2 and cyclin B in cyclin D1-overexpressing cells (Fig. 7B). The radiation-induced p21 appeared to associate in first instance with cyclin B and Cdk2 and subsequently with Cdk4.

The decline in Cdk2 and cyclin B-associated kinase activity after radiation was associated with an increased binding of p21 to these kinases. Sixteen h after radiation, p21 appears to bind to Cdk4 more efficiently in cyclin D1-overexpressing cells than in control cells, which might be responsible for a more enhanced recovery of Cdk2 and cyclin B-associated kinase activity. This enhanced recovery of kinase activities coincided with an accelerated exit from the G2-M block.

DISCUSSION

Here, we demonstrated that a 6-fold overexpression of cyclin D1 enhances apoptosis and reduces cell survival after ionizing radiation. We used, in these experiments, a clone of MCF7 cells in which the expression of exogenous cyclin D1 can be varied by altering the concentration of tetracycline added to the culture medium. By using...
this tetracycline-sensitive expression system, we avoid the variability of results by the use of different cell lines or clones of cells. The amounts of tetracycline used in our experiments did not affect any of the apoptotic features examined in this study.

Excessive overexpression of cyclin D1 that results from transient transfection may induce apoptosis on its own (19, 33), whereas a moderate overexpression of cyclin D1, as is observed in stable transfectants, results in an accelerated transition through the G1 phase of the cell cycle in human fibroblasts (34, 35) and leads to apoptosis only when cells are deprived of serum (36). The maximal level of cyclin D1 protein in the induced MCF7 cells used in this study is ~6-fold over the basal level present in noninduced MCF7 cells. This level of cyclin D1 expression is also observed in most of the breast cancers showing overexpression of cyclin D1 as determined by immunohistochemistry (37, 38), and thus, the system used here mimics the clinical situation.

The enhanced apoptosis after radiation in cyclin D1-overexpressing cells is most likely caused by the sustained higher levels of p53 and p21 after the initial induction at 1 h after radiation (Fig. 6A). The initial level of p53 is slightly higher in cyclin D1-overexpressing cells than in control cells. Upon radiation, the higher levels of p53 subsequently induce expression of p21. These proteins are, therefore, the most likely candidates for the increased apoptosis in cyclin D1-overexpressing cells because bax, bcl-2, and bcl-xL levels remained unaffected by radiation. The increase in p53 and p21 levels in MCF7 cells upon radiation was transient, as reported previously (39), but p53 and p21 levels remained higher after radiation in cyclin D1-overexpressing cells than in control cells. These elevated levels of p53 and

Fig. 4. Flow cytometric analysis of MCF7-cl3 cells control cells (+tet) and MCF7 cells overexpressing cyclin D1 (-tet) after a dose of 5 Gy. Cells were pulse-labeled with BrdUrd and analyzed 0, 8, 12, 16, 20, 24, 30, and 36 h after radiation.
In the case of ionizing radiation, cyclin D1 overexpression accelerates p21, overexpression of cyclin D1 enhances the p53-mediated events. However, upon exposure to conditions that induce p53 and/or p27 and p21, which does not affect transition through the cell cycle by itself, leads to increased levels of Ckis due to the absence of wt p53. Hain et al. (39) showed induction of p53 and p21 protein upon ionizing radiation, indicating that their results are most likely due to increased levels of cyclin D1 and p21 (40), where induction of p21 by p53 may lead to an arrest required for impairment of DNA damage. Cyclin D1-overexpressing cells, however, exit faster from this radiation induced G2-M arrest than do control cells (Fig. 5A). The elevated cyclin B-associated kinase activity at 16 h after radiation in these cells is indicative of this (Fig. 7A) and may be due to an increased capturing of p21 by Cdk4 complexes (Fig. 7B) because more cyclin D1:Cdk4 complexes are present at that time point in cyclin D1 overexpressing cells than in control cells (Fig. 6). The rapidly radiation-induced p21 apparently inhibits at first (4 h after radiation) cyclin D1-associated kinase activity and is in cyclin D1 overexpressing cells “neutralized” by being captured in cyclin D1:Cdk4 complexes.

Residual DNA damage in cells following ionizing radiation manifests as micronuclei, which we considered to be features of apoptosis. We observed an increased appearance of these micronuclei in cyclin D1 overexpressing cells. The results of this study and of those of others (14, 19) indicate that overexpression of cyclin D1 by itself leads to increased levels of Ckis p27 and p21, which does not affect transition through the cell cycle when MCF7 cells are cultured under optimal growth conditions (19, 21). However, upon exposure to conditions that induce p53 and/or p21, overexpression of cyclin D1 enhances the p53-mediated events. In the case of ionizing radiation, cyclin D1 overexpression accelerates exit from the G2-M arrest with the subsequent formation of micronuclei. Cyclin D1-associated overexpression of p53 in this way facilitates radiation-induced death of MCF7 cells, as does acute overexpression of p53 via transient transfection of exogenous p53 into various cancer cell lines (43).

Epperly et al. (44) did not find any radiosensitization after overexpressing cyclin D1 in the hematopoietic mouse cell line 32Ccl3, which, however, did not show any induction of p53 and p21 protein. These studies indicate that discrepancies may exist between cell lines in the induction of p53 levels, leading to a G1-S or G2-M block, as was also recently demonstrated by Nagasawa et al. (42).

In MCF7 cells overexpressing cyclin D1, increased apoptosis was observed after radiation. This apoptosis was not related to a decline of the apoptosis-promoting protein bcl-2 (45, 46) or induction of the apoptosis promoting protein bax (47). Expression of these proteins was not affected by radiation in cells overexpressing cyclin D1. The enhanced induction of apoptosis after ionizing radiation might, therefore, be related an induction of p53 and p21 by cyclin D1. The higher levels of cyclin D1 and p21 lead to a redistribution of p21 upon radiation. Transition through G2-M is likely to be influenced by levels of p53 and p21 (40), where induction of p21 by p53 may induce an arrest required for impairment of DNA damage. Cyclin D1-overexpressing cells, however, exit faster from this radiation induced G2-M arrest than do control cells (Fig. 5A). The elevated cyclin B-associated kinase activity at 16 h after radiation in these cells is indicative of this (Fig. 7A) and may be due to an increased capturing of p21 by Cdk4 complexes (Fig. 7B) because more cyclin D1:Cdk4 complexes are present at that time point in cyclin D1 overexpressing cells than in control cells (Fig. 6). The rapidly radiation-induced p21 apparently inhibits at first (4 h after radiation) cyclin D1-associated kinase activity and is in cyclin D1 overexpressing cells “neutralized” by being captured in cyclin D1:Cdk4 complexes.

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D1 overexpressing cells after release from the radiation-induced G2-M arrest.

Enhanced exit from G2-M after radiation by overexpression of cyclin D1 may result in entry into the next cell cycle, whereas not all of the radiation-induced DNA damage is completely repaired. This may then lead to reinduction of apoptosis and formation of micronuclei. These findings are consistent with the second wave of p53 and of the radiation-induced DNA damage is completely repaired. This result, ultimately, in clonogenic cell death (Fig. 2).

This finding may be of clinical importance, and it also suggests that patients with breast cancer with a wt p53 and pRb and with an overexpression of cyclin D1, may benefit more from treatment with γ-radiation than do patients with breast cancer without overexpression of cyclin D1.

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