Genotoxic Stress Induces Expression of E2F4, Leading to Its Association with p130 in Prostate Carcinoma Cells

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Abstract

The retinoblastoma (pRb), p107, and p130 pocket proteins bind to the E2F transcription factors to control gene expression. E2F4 protein levels increased and accumulated in the nuclei of prostate carcinoma cells subjected to ionizing radiation (IR). The IR-induced increase of E2F4 levels led to an increase in E2F4 binding to p130 but had no effect on E2F4/p107 or E2F5/p130 complexes. The increase in E2F4/p130 association after IR was observed in prostate carcinoma cells regardless of their sensitivity to androgens, but not in breast carcinoma cells. E2F4/p130 complex formation was dependent on dissociation of p130 from cyclin-dependent kinase 2 and p130 dephosphorylation. Disruption of E2F4 through small interfering RNA prevented p130/E2F4 complex formation and sensitized cells to IR-induced apoptosis, leading to caspase-3 activation, cleavage of its substrate, poly(ADP-ribose) polymerase, and nuclear condensation. The E2F4/p130 pocket protein complex emerges as a new target of radiation in prostate carcinoma cells.

Introduction

Prostate cancer is the most widely diagnosed cancer and the second cause of cancer death in North American men, affecting one third of all men >50 years old (1). Surgery and radiation therapy are considered the best treatments for differentiated, clinically localized cancers (2). We have studied the changes that occur during the G1-S-phase transition in differentiated, clinically localized cancers (2). Previous studies have revealed a critical role for the retinoblastoma susceptibility gene product, pRb, in the cell growth suppressive function after treatment with genotoxic agents (11, 12). pRb inactivation sensitizes cells to genotoxic stress; however, the role of related pocket proteins and their E2F partners in the response to genotoxic stress is not clear. To further assess the role of these proteins in the genotoxic stress response, we examined the effects of IR on E2F and partner pocket protein expression levels. Our data indicate that formation of the E2F4/p130 complex is specifically regulated by IR in prostate but not in breast cancer cells. Moreover, our data also suggest that the interaction between CDK2 and p130 is also affected by IR, which may be a result of the increased levels of E2F4 associated with p130, leading to a decrease in p130 phosphorylation and decreased cell viability. Small interfering RNA (siRNA)-mediated down-regulation of E2F4 prevents p130/E2F4 complex formation and sensitizes cells to IR-induced apoptosis.

Materials and Methods

Cell Culture and Treatments. LNCaP prostate and MCF-7 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA). C4-2, the androgen-hypersensitive derivative of LNCaP (13), was a gift from Dr. W. Heston (Cleveland Clinic). All cells were grown in monolayer culture in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA; Ref. 14). Cells were either left untreated or subjected to 10 Gy of γ-irradiation (15).

Western Blot Analysis. Cells were collected at the indicated times after IR. Protein samples in SDS-containing lysis buffer were loaded on 6–10% acrylamide gels (30 μg/lane). After electrophoresis, proteins were transferred onto a nitrocellulose membrane and blocked with 5% nonfat milk. The membrane was incubated for 1.5 h at room temperature with primary antibodies for p107 (sc-317; 1:100), p130 (sc-317; 1:100), E2F4 (sc-866; 1:200), and caspase-3 p20 (sc-1748; 1:500) from Santa Cruz Biotechnology and poly-ADP-ribose) polymerase (1:500) from Biomol, followed by a 1-h incubation with secondary horseradish peroxidase-conjugated antibodies (Amersham Biosciences). β-Actin (1:500; Sigma Chemical Co., St. Louis, MO) was used as a loading control. The membrane was incubated in Lumiglo Chemiluminescent Substrate (Kirkegaard and Perry Laboratories) and visualized by exposure to X-ray film (Eastman Kodak). All other chemicals, unless otherwise specified, were obtained from Sigma Chemical Co.

Immunocytochemistry. Control and treated C4-2 cells grown on coverslips in 6-well plates were fixed using a 3.7% formaldehyde/PBS solution.
After three washes with 1× PBS for 5 min each, coverslips were incubated to block nonspecific binding with 2% goat serum, 0.3% Triton X-100, and 1× PBS for 10 min, followed by anti-E2F4 antibody (1:400) for 1 h. After three additional washes with PBS for 5 min, the cell monolayers were incubated with an Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:1000; Molecular Probes, Eugene, OR) for 45 min. The coverslips were washed three times, mounted with Vectashield containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and observed under confocal microscopy (Leica TCS-S2P, Heidelberg, Germany).

**Immunoprecipitation Analysis.** Control and irradiated cells were lysed as described above, except that 0.1% NP40 was substituted. p130, E2F4, E2F5 (sc-999), and CDK2 (sc-6248) primary antibodies (0.2 μg/μl each; Santa Cruz Biotechnology) were used for immunoprecipitation as well as 15 μg of protein A/G-agarose (Oncogene) beads. The beads were washed in lysis buffer and boiled in 30 μl of Laemmli buffer; the entire sample was loaded on a SDS-polyacrylamide gel and processed by Western blotting. The membranes were immunoblotted with anti-p130, anti-CDK2, and anti-E2F4 primary antibodies and visualized as described above, except that the IgG heavy chain was used as a loading control.

**siRNA Transfection.** Oligonucleotides corresponding to the U15641 e2f4 NCBI sequence (16) at positions 904–924, 74–94, and 651–671 (all followed by 5′-CCC-TGT-CTC-3′ for annealing to the T7 promoter sequence) were used to generate sie2f4-1, -2, and -3, respectively. siRNAs were generated using the Silencer siRNA Construction Kit (Ambion) as described previously (14). C4-2 cells were plated into a 24-well plate. At 24 h after transfection with Oligofectamine (Invitrogen) and 100–200 nm siRNA, cells were subjected to irradiation. They were then collected, lysed, and analyzed for protein expression by immunoblotting. Cell death was determined by the Hoechst 33258 staining (14). After IR, C4-2 cells (attached and in suspension) were pooled and washed with serum-free RPMI 1640. An aliquot of the resuspended cells was incubated with Hoechst 33258 (10 μg/ml) for 1 min before being examined and scored under UV fluorescence microscopy (~200–300 cells/experimental sample). Each experiment, with the exception of the reagent-only control, was performed on six replicates. The sigapdh (glyceraldehyde-3-phosphate dehydrogenase) was generated in the same fashion as the sie2f4 siRNAs, with sense and antisense strands provided by Ambion.

**Results and Discussion**

**Radiation Induces Changes in E2F4 and p130 Expression.** Examination of the IR response in prostate carcinoma C4-2 cells revealed that the expression level of E2F4 protein was elevated during a 16–24-h period (Fig. 1A). The levels of the phosphorylated form of p130 (p-p130), however, decreased in a time-dependent manner, whereas the unphosphorylated form of p130 was elevated. These changes were specific for p130 because at the same time, the levels of p107 did not increase and, in fact, slightly decreased beginning at 8 h after radiation treatment. These data suggest that both E2F4 expression and p130 phosphorylation are regulated by genotoxic stress. The localization of E2F4 before and after IR was examined next in C4-2 cells that were subjected to immunocytochemical staining at 24 h post-IR (Fig. 1B). E2F4 was present diffusely in both the cytoplasm and nuclei of the control (unirradiated) cells. In contrast, E2F4 was localized predominantly to the nuclei after IR. Because E2F4, which can form an active complex with p130, is considered to be a repressing E2F, our results indicate the possible role of E2F4/p130 in mediating the radiation-induced cell growth suppression and changes in gene expression.

**IR Induces E2F4/p130 Complex Formation.** Examination of E2F4/p130 complex formation in irradiated cells revealed that the level of p130 in E2F4 immunoprecipitates increased starting at 8 h after irradiation, consistent with the hypothesis that E2F4 and p130 form a complex after IR. The level of p130 in E2F4 immunoprecipitates increased starting at 8 h after IR treatment.
and continued up through 24 h postirradiation (Fig. 2A). At the same time, the E2F5 association with p130 remained relatively unaffected, as did the association between E2F4 and p107 (data not shown). The amount of E2F4 immunoprecipitated also increased (Fig. 2C), which parallels the IR-induced increase of E2F4 protein levels (Fig. 1A). These data suggest that IR causes increased levels and nuclear localization of E2F4 as well as p130 dephosphorylation, all of which contribute to E2F4/p130 complex formation. To further determine the generality of E2F4/p130 complex formation after IR, we examined the specificity of this response in several other tumor cell lines (Fig. 2B). C4-2 cells are androgen hypersensitive (17), whereas the parental LNCaP cells are androgen dependent. As in C4-2 cells, the IR-induced formation of the E2F4/p130 complex was also dramatically elevated after irradiation of LNCaP cells. In contrast, no such response was observed after treatment of MCF-7 breast carcinoma cells. The reciprocal immunoprecipitation, which examined the levels of E2F4 in p130 immunoprecipitates, gave very similar results (Fig. 2D). These data indicate that the E2F4/p130 association after IR may be a prostate tumor cell type-specific response to IR, which is independent of the androgen sensitivity of these cells.

It has been reported that the p130/E2F4 repressor complex binds to the Cyclin E/CDK2 complex (7, 8, 10, 18). This interaction has been suggested to facilitate the phosphorylation of p130 and the subsequent release of E2F4, thus leading to the activation of E2F-responsive genes and inhibition of growth suppression (19). Examining the levels of p130 in CDK2 immunoprecipitates indicated a stable p130/CDK2 complex before irradiation, an association that persisted up to at least 8 h after treatment (Fig. 3A). However, at 16 h after IR, this association was dramatically decreased because very little p130 remained in complex with CDK2. Decreased levels of p130 association with CDK2 were observed up to at least 72 h (data not shown). The levels of CDK2 immunoprecipitated did not change (Fig. 3B). These observations, in conjunction with the decrease of phosphorylated p130 protein expression (Fig. 1A), suggest that IR causes a decrease in p130 phosphorylation due to the increased association between p130 and E2F4.

**E2F4 Down-Regulation Inhibits IR-Induced E2F4/p130 Complex Formation and Sensitizes C4-2 Cells to IR.** To directly examine the role of E2F4 in the IR response, we used siRNAs that were targeted against the e2f4 gene to inhibit protein expression. Of the three siRNAs targeted to e2f4 that we have screened in C4-2 cells, sie2f4-1 was the most effective. After transient transfection, the E2F4
protein expression level was substantially down-regulated by si\textit{e2f4}-1 to reach \(-30\%\) of what was found in untreated cells (Fig. 4A). We then used si\textit{e2f4}-1 to determine the effect of E2F4 down-regulation on IR-induced E2F4/p130 complex formation (Fig. 4B). In control cells, we confirmed the increase in E2F4 association with p130, observed above, at the 24 h time point. However, in cells that were transfected with si\textit{e2f4}-1, this association was totally inhibited. This suggests that si\textit{e2f4}-1 is effective in down-regulating E2F4 expression and, as a result, the E2F4 protein that is found in association with p130 after IR. These data also indicate that there is a pool of E2F4 protein that does not form a complex with p130; however, the role of this free form of E2F4 has not been determined.

By inactivating E2F4 and E2F5 in mice, it was reported that at least one E2F4 or E2F5 functional allele was required for live birth and that these mice displayed major defects in the G1-phase control of the cell cycle (20). To investigate the role of E2F4 expression on cell survival, we examined the activation of the caspase-mediated pathway, which is known to be critical for apoptosis in C4-2 cells (14). After IR, the si\textit{e2f4}-transfected cells (E4) exhibited decreased levels of the pro form of caspase-3, indicative of its proteolytic cleavage and activation (Fig. 4C). Moreover, there was a detectable p85 proteolytic cleavage product of poly(ADP-ribose) polymerase, a well-known caspase-3 substrate (14). In contrast, these changes were not apparent in cells treated with reagent only or transfected with \textit{sigapdh}. Levels of E2F4 were decreased by siRNA in control and irradiated cells; in the absence of siRNA, E2F4 levels were increased after IR. Finally, we determined the effect of E2F4 inactivation on cell survival (Fig. 4D). Examination of nuclear condensation, a hallmark of apoptosis, revealed that the untransfected cells and those transfected with \textit{sigapdh} exhibited similar cell viability after IR. In contrast, C4-2 cells transfected with si\textit{e2f4} displayed a significant increase in nuclear condensation, which was further increased after IR, indicating that E2F4 inactivation renders C4-2 cells sensitive to IR-induced DNA damage.

In summary, we have shown that IR controls prostate tumor cell growth by increasing the expression of the E2F4 transcription factor in the nucleus and promoting its association with the p130 pocket protein. Our data indicate that the inactivation of E2F4 by siRNA prevents formation of the p130/E2F4 transcription complex and sensitizes cells to IR-induced apoptosis. Because E2F4 is overexpressed in prostate tumor epithelial cells (21), it may provide a suitable target for effective radiation therapy. Additional studies will be needed to examine the specific contribution of critical genes to the radiation-resistant phenotype of C4-2 cells, whose expression is modulated after E2F4 inactivation.

Acknowledgments

We thank Dr. W. Heston (Cleveland Clinic) for the C4-2 cells and Dr. J. Drazba, A. Vasanji, and D. Leontiev (Imaging Core Facility) for fluorescence and confocal microscopy. We also thank Dr. J. Hissong for assistance with statistical analyses and M. Crosby and Dr. S. Ray for critical reading of the manuscript.

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