Restoration of FGF Receptor Type 2 Enhances Radiosensitivity of Hormone-refractory Human Prostate Carcinoma PC-3 Cells

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Abstract. *Background: This study was undertaken to investigate the radiosensitizing effects of fibroblast growth factor receptor 2IIIb (FGFR2IIIb) in androgen-independent human prostate carcinoma PC-3 cells devoid of normally resident epithelial cell FGFR2IIIb. Materials and Methods: A clonal line of PC-3 cells expressing FGFR2IIIb was established by stable transfection. Clonogenic cell survival, apoptosis and cell cycle distribution with and without gamma-irradiation were then compared between FGFR2IIIb-expressing PC-3 cells and control cells mock-transfected with vector alone. Results: Gamma-irradiation resulted in an increase of clonogenic cell death concurrent with enhanced apoptosis and cell cycle arrest in the G2/M-phase in both transfected and untransfected cells. A quantitative analysis of all three parameters indicated that cells expressing FGFR2IIIb were significantly more sensitive to irradiation than control cells. Conclusion: These results indicate that restoration of FGFR2IIIb to PC-3 cells enhances their sensitivity to irradiation through acceleration of apoptosis and cell cycle arrest.*

Prostate cancer is initially androgen-dependent and sensitive to androgen ablation therapy. However, it eventually acquires the ability to grow independently of androgen and thus becomes resistant to hormonal therapy (1). For this so-called

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hormone-refractory prostate cancer (HRPC), currently available methods such as chemotherapy and radiotherapy are only modestly effective. It is therefore very important to elucidate the mechanisms of malignant progression from androgen dependence to independence and to develop a novel therapeutic strategy for inducing apoptosis and cell death preferentially in HRPC cells.

Fibroblast growth factor (FGF) signaling has been shown to be a local mediator of stromal–epithelial dialogue and homeostasis in both animal and human normal prostate and prostate tumors (2-8). The IIIb isoform of FGF receptor 2 (FGFR2IIIb), a splice variant of FGFR2 tyrosine kinase, is the resident isoform of the FGFR family expressed in prostate epithelial cells and, in partnership with epithelial cell heparan sulfate, is specific for FGF7 (also known as keratinocyte growth factor, KGF) and FGF10, whose expression is limited to stromal cells (2, 3, 6, 9, 10). FGF7 and FGF10 have been proposed as candidate andromedins, which mediate the effect of androgen on stromal cells and epithelial cells (2, 3). Concomitant loss of FGFR2IIIb and FGFR2 kinase is a common correlate of loss of androgen responsiveness and response of epithelial cells to stromal signaling during their progression to malignancy in prostate tumor cell lines and animal models (6-8, 11-13). In the same models, restoration of FGFR2IIIb to tumor cells has been reported to restore responsiveness to the stroma and dramatically reduce tumorigenicity *in vivo* (14, 15). Loss of FGFR2 has also been confirmed in human prostate tumors and loss of FGFR2IIIb in particular is associated with tumor androgen independence (7, 16). In addition, restoration of FGFR2IIIb by transfection in a prototypic hormone-refractory human prostate cancer cell line PC-3 has been shown to suppress cell growth and tumorigenicity concurrent with increased cell differentiation and apoptosis (16). Similar results have also been obtained in human models of salivary and bladder cancer (17, 18).

Given this background and observations suggesting that the FGF system affects radiosensitivity by controlling apoptosis or the cell cycle in various kinds of cells (19, 20), we hypothesized that FGFR2IIIb might improve the response of HRPC to radiotherapy.

Materials and Methods

Cell culture and transfection. Cloned PC-3 cells were maintained in OPTI-MEM1 medium (Life Technologies, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Life Technologies) and 100 mg/ml kanamycin (Life Technologies). Preparation of FGFR2IIIb cDNA and transfection to PC-3 were performed as described elsewhere (16). Briefly, wild-type, full-length FGFR2IIIb cDNA (15) was cloned into the expression vector IRES2 neo (Clontech Laboratories, Inc., Palo Alto, CA, USA; IRES2 FGFR2IIIb). PC-3 cells were transfected with IRES2 FGFR2IIIb, or the IRES2 empty control vector, by electroporation using the Gene Pulser II apparatus (Bio-Rad, Hercules, CA, USA). The cells were then incubated for 14 days in selection medium comprising OPTI-MEM1 medium containing the antibiotic G418 sulfate (200 μg/ml; Promega, Maddison, WI, USA). Colonies of cells emerging from the selection medium were isolated and selected according to their level of FGFR2IIIb expression, which was determined by immunoblot analysis. Among them, a clonal PC-3 cell line expressing FGFR2IIIb at the highest level was chosen for analysis and designated PC-3 R2IIIb. Control cell cultures transfected with the empty vector were designated PC-3 neo.

Clonogenic cell survival. Cells grown to subconfluency in 90-mm dishes were exposed to 0, 4 or 8 Gy irradiation from a ^{60}Co gamma-ray source (Shimazu Seisakusyo, Tokyo, Japan). Forty-eight hours later, the cells were retrieved, plated into 90-mm dishes at 10^3 cells/dish and cultured in OPTI-MEM1 medium supplemented with 5% heat-inactivated FBS at 37°C under a 5% CO_2 atmosphere. After 14 days of culture, the cells were fixed and stained with crystal violet and colonies containing more than 50 cells were counted. The surviving fraction was normalized to that of untreated cells.

Assay of apoptosis. An APOPercentage™ kit (Biocolor Ltd., Belfast, N. Ireland, UK) was used for detection of apoptotic cells in accordance with the manufacturer's protocol. Cells at 2×10^3 and 5×10^3 cells/well for PC-3 neo and PC-3 R2IIIb, respectively, were plated in 96-well plates in OPTI-MEM1 medium supplemented with 5% FBS and subjected to 0, 4 or 8 Gy of gamma-ray irradiation. After 48 h of culture, the cells were exposed to fresh OPTI-MEM1 medium containing APOPercentage™ Dye Label for 1 h. After two washes with phosphate-buffered saline (PBS), the numbers of positively stained red to purple apoptotic cells were counted using an inverted microscope.

Radiation-induced cell apoptosis was also confirmed by dual-colored (propidium iodide, PI, and Annexin V) flow cytometry. Cells were grown to subconfluency in 90-mm dishes and exposed to 0 or 8 Gy of gamma-irradiation. After 48 h of incubation, 1×10^5 cells were prepared and stained with an antibody against Annexin-V conjugated to FITC and with PI using an Apoalert Kit (Clontech Laboratories, Inc.) in accordance with the manufacturer's instructions. Cells that were viable (Annexin-V−/PI−), apoptotic (Annexin-V+/PI−), or showing residual damage (Annexin-V+/PI+,

Annexin-V−/P+) were estimated by fluorescence-activated cell sorting (FACS) using a FACS Calibur (Beckton-Dickinson, San Jose, CA, USA).

Cell cycle analysis by flow cytometry. Cell cycle phase distribution after irradiation was assessed using flow cytometry. Cells grown to subconfluency in 90-mm dishes were exposed to 0 or 8 Gy gamma-irradiation and incubated for 48 h. They were then washed with ice-cold PBS, fixed with ice-cold 70% ethanol, and incubated at –20°C overnight. The cells were centrifuged, pellet-washed twice with ice-cold PBS, and resuspended in the staining solution containing PI and RNase. Cell cycle phase distribution was analyzed using a FACS Calibur.

Statistical analysis. Each experiment was performed at least three times. Results are expressed as the mean value±standard deviation (SD). Statistical analysis was performed using repeated measures ANOVA and Wilcoxon test.

Results

Increased radiosensitivity in PC-3 expressing FGFR2IIIb. PC-3 cells expressing high levels of FGFR2IIIb (PC-3 R2IIIb cells) and control cells (PC-3 neo) were prepared as described in Materials and Methods. To test the effect of FGFR2IIIb and irradiation on PC-3 cell population dynamics, clonogenic cell survival was assessed. Figure 1 shows the effect of PC-3 R2IIIb and PC-3 neo after exposure to 4 and 8 Gy of radiation. The mean survival fraction without irradiation was 0.54 for PC-3 neo and 0.25 for PC-3 R2IIIb ($p < 0.05$, Wilcoxon) (data not shown). These differences were normalized to the indicated percentage survival curves. Although cell survival exhibited a radiation dose-dependent decrease in both cell types, the survival fraction of the PC FGFR2IIIb population decreased to 40% and 8% that of the PC-3 neo cells at 4 and 8 Gy, respectively ($p < 0.01$, repeated measures ANOVA). This indicated that restoration of FGFR2IIIb to PC-3 cells dramatically increased their radiosensitivity.

FGFR2IIIb-dependent increase in radiosensitivity is accompanied by increased apoptosis. Based on the results of the clonogenic cell survival assay and our previous observations that the growth suppression of PC-3 cells by restoration of FGFR2IIIb resulted in increased cellular apoptosis (16), we compared the rate of apoptosis in PC-3 R2IIIb to that in PC-3 neo cells upon irradiation.

As expected, the APOPercentage™ assay indicated that the percentage of apoptotic cells in both populations of PC-3 R2IIIb and PC-3 neo cells increased proportionally to the dose of radiation (Figure 2A, B). As reported earlier, PC-3 R2IIIb cells exhibited a significantly higher rate of apoptosis than PC-3 neo cells in the unirradiated state ($p < 0.05$, Wilcoxon). Similar to cell population survival rates, the rate of apoptosis increased proportionally to the dose of radiation in both cell populations.
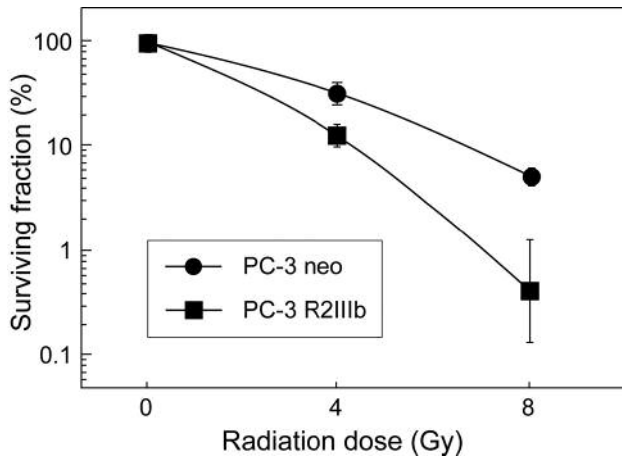
Figure 1. *Clonogenic cell survival after gamma-ray irradiation. Clonal PC-3 cells transfected with FGFR2IIIb cDNA (PC-3 R2IIIb) and those transfected with a control vector (PC-3 neo) were exposed to 4 or 8 Gy of gamma-ray radiation and cultured for 14 days. The resulting colonies were stained and counted (>50 cells). The number of colonies at each dose point was normalized to that of the corresponding unirradiated control. Resulting survival curves were fitted using a linear quadratic model. Each point on the graph represents a mean value±SD of three independent experiments, each plated in triplicate. The significance of differences between the two cell types was determined by repeated measures ANOVA ($p < 0.01$).*

Most notably, at 8 Gy the rate of apoptosis in the PC-3 R2IIIb population was nearly 3-fold that of mock-transfected PC-3 neo cells ($p < 0.05$ for all measurement points, Wilcoxon). The rates of increase in numbers of apoptotic cells resulting from irradiation were significantly higher for PC-3 R2IIIb than for PC-3 neo ($p < 0.01$, repeated measures ANOVA).

The Annexin-V/PI apoptotic assay confirmed that the percentages of apoptotic cells (Annexin-V$^+$/PI$^-$) increased with 8 Gy of radiation in both cell types (Figure 2C). PC-3 R2IIIb cells exhibited a higher rate of apoptosis than did PC-3 neo cells when unirradiated, although the difference did not reach significance (11.7±1.4% *vs.* 9.1±0.7% for PC-3 R2IIIb and PC-3 neo). At 8 Gy, the rate of apoptosis in the PC-3 R2IIIb population was 1.5-fold higher than that of PC-3 neo cells (20.7±3.8% *vs.* 13.7±0.4% for PC-3 R2IIIb and PC-3 neo, $p < 0.05$, Wilcoxon). The rates of increase in numbers of apoptotic cells resulting from irradiation were significantly higher for PC-3 R2IIIb than for PC-3 neo ($p < 0.01$, Wilcoxon).

Cell cycle arrest in G2/M correlates with increased radiosensitivity. Finally, the effect of FGFR2IIIb on irradiation-induced cell cycle distribution was assessed by FACS analysis. Parallel to the increased rates of apoptosis, PC-3 cells with restored FGFR2IIIb exhibited an increased proportion of cells in the G2/M stage relative to control cell populations, independent of irradiation (Figure 3). Although less dramatic than clonogenic population survival rates and

the coincident rates of apoptosis, PC-3 R2IIIb cells exhibited 1.4-fold more cells in the G2/M-stage at 8 Gy relative to the 1.7-fold increase in control cell populations devoid of FGFR2IIIb ($p < 0.05$, Wilcoxon).

Discussion

PC-3 is a representative hormone-refractory human prostate cancer (HRPC) cell line, highly tumorigenic and metastatic in nude mice, and resistant to normal rates of apoptosis (21-23). We previously confirmed that the expression of normally resident epithelial cell FGFR2IIIb is lost in PC-3 tumor cells and that restoration of FGFR2IIIb dramatically promoted apoptosis and suppressed overall population growth rates and tumoriginicity of these cells (16). Here, we report results of a preclinical study that shows that restoration of FGFR2IIIb sensitizes PC-3 cells to irradiation, a commonly used treatment in refractory prostate cancer.

Clonogenic PC-3 cells with restored FGFR2IIIb exhibited a significantly reduced rate of survival compared to control mock-transfected cells that is further reduced relative to control cells upon exposure to ionizing radiation. This was coincident with a significant increase of apoptosis and cell cycle arrest in G2/M phase. These results indicate that even though HRPC cells have lost FGFR2 expression, functional downstream reception mechanisms are intact and these signals are capable of sensitizing the HRPC cells to irradiation through enhanced apoptosis.

Radiotherapy remains a viable treatment for HRPC, but its efficacy for restriction of HRPC is modest and generally only palliative for symptoms such as bone pain (1). In addition, recent phase II clinical studies using single agent therapy aimed at specific molecular targets such as hyperactive epidermal growth factor receptor (EGFR) tyrosine kinase in HPRC are disappointing (24-26). For these reasons, attention has turned to a combined therapeutic strategy for HRPC in many clinical trails (27). Our results indicate that the combination of radiotherapy and reactivation of FGFR2 kinase may be a promising treatment strategy for HRPC.

In animal models, forced overexpression of FGFR1 in epithelial cells expressing normal levels of FGFR2IIIb overrides homeostasis and accelerates tumorigenicity (14, 28). In contrast, restoration of FGFR2IIIb appears to induce apoptosis and overrides the tumor-promoting effect of ectopic FGFR1 in malignant cells (14-16). Restored FGFR2 kinase may act as an apoptosis inducer through a fundamentally different downstream signaling pathway than FGFR1 (16). Alternatively, "dominant-negative" heterodimerization by forced coexpression of both FGFR2 and FGFR1 kinases may also contribute to a dampening effect of one over the other (16).

The mechanisms underlying the combined apoptosis-promoting and radiosensitizing effect of FGFR2IIIb when
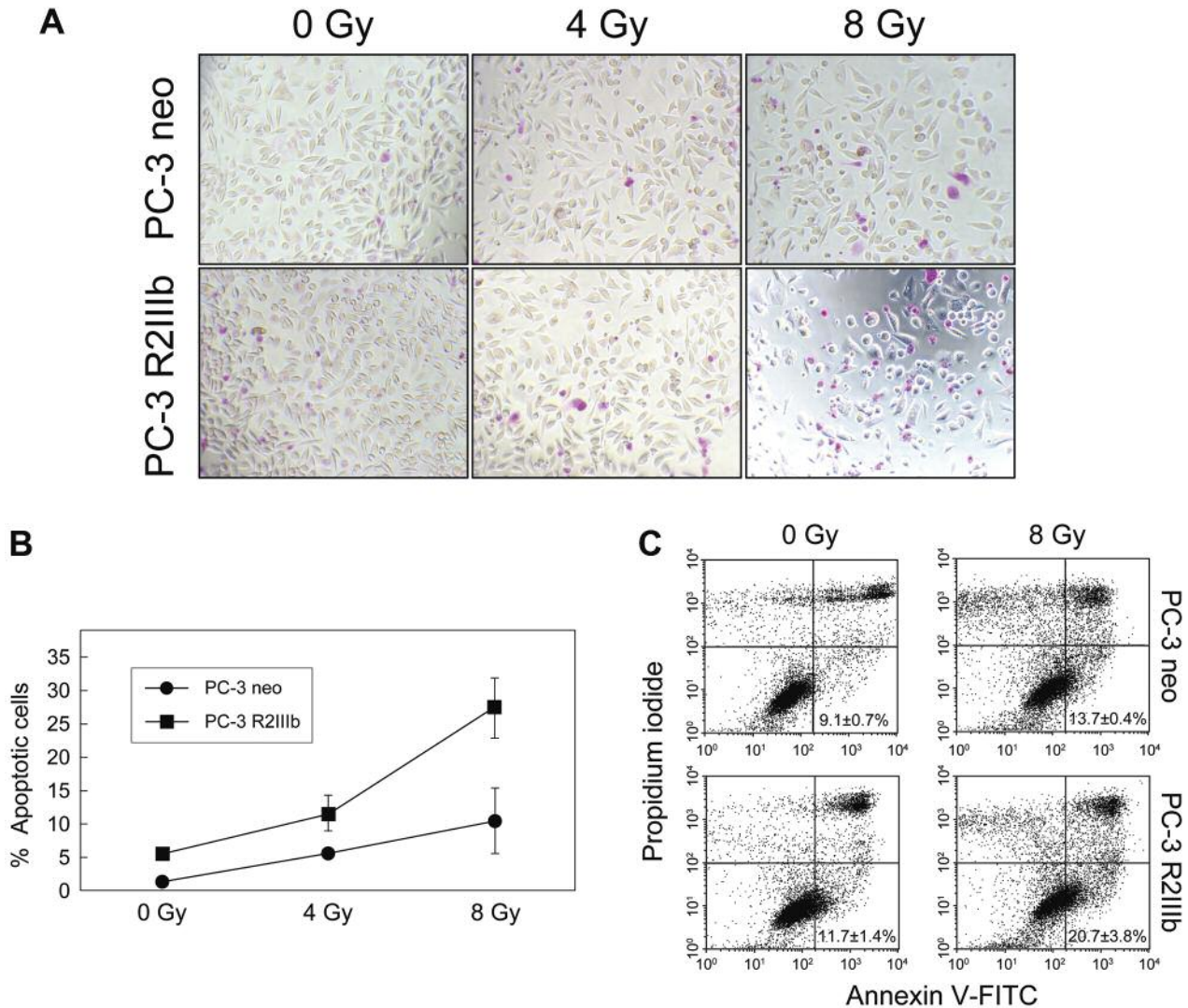
Figure 2. *Effect of FGFR2IIIb on radiation-induced apoptosis in PC-3 cells. Cells undergoing apoptosis were determined by APOPercentage™ staining (A) as described in Materials and Methods. The purple stain indicates apoptotic cells. Images are representative examples from triplicate wells. The percentage of positively stained cells was estimated by direct counting of at least 300 cells in each of triplicate wells (B). Each point on the graph represents the mean±SD of three independent experiments, each plated in triplicate. The P-value between PC-3 R2IIIb and PC-3 neo for the rate of increase in apoptotic cells induced by irradiation was <0.01 (repeated measures ANOVA). Apoptosis was also analyzed by Annexin-V/propidium iodide staining as described in Materials and Methods. (C). Apoptotic cells (Annexin-V+/PI−) were detected in the lower right quadrant. A representative example from three independent experiments is shown. The figures indicated in the lower right quadrant are percentages of apoptotic cells.*

restored to malignant HRPC cells remain to be established. Recent studies have shown that down-regulation of antiapoptotic factors such as Bcl-2 confers sensitization of PC-3 cells to both radio- and chemotherapy (29, 30). It is conceivable that the FGFR2 kinase inactivates such antiapoptotic factors in PC-3 cells. Further studies are needed to clarify how restored FGFR2IIIb promotes apoptosis in HRPC and its enhancement of radiosensitity in order to establish clinical application of combined FGFR2IIIb restoration therapy with irradiation.

Acknowledgements

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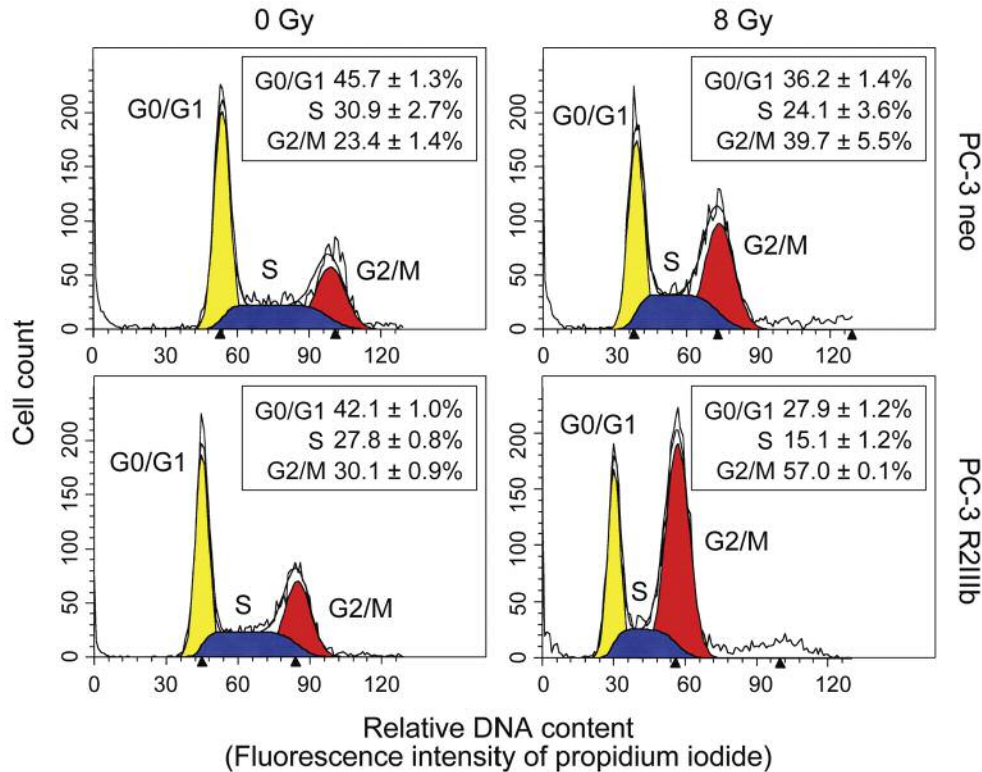
Figure 3. *Effect of FGFR2IIIb on radiation-induced cell cycle distribution in PC-3 cells. The DNA histogram after 8 Gy or 0 Gy (as a control) of radiation was measured by flow cytometry. The red areas indicate the cell population in G2/M phase. The data (graphs) are representative examples for triplicate tests. The cell cycle arrest in G2/M phase was marked for PC-3 R2IIIb compared with PC-3 neo in both unirradiated conditions, and after exposure to 8 Gy of radiation (p<0.05 for both).*

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