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Original article

Radiosensitivity of prostate cancer cells is enhanced by EGFR inhibitor C225☆

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Abstract

Purpose: To determine the direct effects of the epidermal growth factor receptor (EGFR) inhibitor C225 on the radiosensitivity of human prostate cancer cells.

Experimental design: Human prostate cancer DU145 cells were irradiated with ^{60}Co (1.953 Gy/min) at various doses in the presence or absence of C225. The cellular proliferation and cell-survival rate were evaluated by MTT and colony-forming assays after irradiation. The cell-cycle distribution, cell apoptosis, and MAPK expression were investigated using FCM. The expression of Cyclin D1, CDK2, CDK4, and Survivin were determined by RT-PCR.

Results: The RBE in the C225 group compared with that in the control group was 1.39. Cells treated with C225 and irradiated at 4 Gy predominantly exhibited G_0/G_1 phase arrest and significant decrease in the fraction of cells in the S phase in comparison with those in the control cells, respectively. An evidently higher apoptosis rate on irradiation at 4 Gy was observed in C225-treated cells compared with that in the control cells. Decreased cell proliferation and increased cell death were further supported by the down-regulation of cyclin D1, CDK2, CDK4, and survivin in C225-treated DU145 cells, as determined by RT-PCR. Furthermore, C225 significantly inhibited the phosphorylation of P38-MAPK in DU145 cells.

Conclusions: The EGFR inhibitor C225 increased the radiosensitivity of DU145 cells through antiproliferative effect, inhibition of clonal growth, G_0/G_1 phase arrest, apoptosis induction, and inhibition of EGFR-signaling pathways by the down-regulation of MAPK activation.

Keywords: EGFR inhibitor; Prostate cancer cells; Cell proliferation; Apoptosis

1. Introduction

Growth factors control cellular proliferation and differentiation and are important for the initiation and maintenance of neoplastic transformation. Transforming growth factor-α (TGF-α) and epidermal growth factor (EGF) and its specific receptors, the epidermal growth factor receptors (EGFRs), have been implicated in the development and progression of the majority of human epithelial cancers, including prostate cancer [1–3]. TGF-α and EGF and/or EGFR are expressed at high levels in prostate cancers, which are generally associated with advanced disease and poor prognosis [4,5]. EGFR activation is not only critical for cell proliferation but EGFR-mediated signals also contribute to other processes that are crucial to cancer progression, including angiogenesis, metastatic spread, and the inhibition of apoptosis [6,7]. The high expression of EGFR is also associated with resistance to cytotoxic drugs or ionizing radiation, as determined in several preclinical models.

EGFR activation may prevent apoptosis induced by radiation in cancer cells. This may be clinically relevant because it could represent a mechanism via which cancer cells escape radiation-induced cell death. A large body of experimental and clinical work supports the view that the EGFR is a relevant target for cancer therapy. Different pharmacological and biological approaches have been developed for blocking EGFR activation and/or function in cancer cells.

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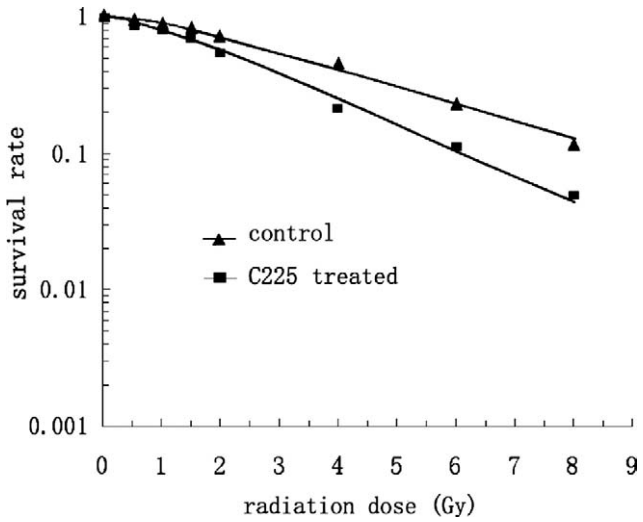

Fig. 1. Dose-survival curves of DU145 and C225-treated DU145 cells after irradiation.

Intensity-modulated radiation therapy for early-stage prostate cancer has produced similar therapeutic effect and fewer complications than those produced by radical surgery [8–10]; however, further improvements in the therapy of prostate cancer are required. It was reported that prostate cancer cells exhibited decreased requirement for exogenous growth factors compared with that exhibited by normal cells. This is attributable in part to the ability of prostate cancer cells to overexpress growth factors and/or their specific cell membrane receptors, resulting in the autonomous activation of autocrine and paracrine growth pathways. Thus, blocking these receptors by monoclonal antibodies (mAbs) or other agents may significantly impact clinical prostate cancer therapy. Several anti-EGFR blocking mAbs were recently developed. C225 (cetuximab), a chimeric human-mouse IgG1 mAb [11–13], has been clinically used for the therapy of human head and neck cancers and colon cancers [14,15]. In the present study, we directly evaluated the potential antagonistic effects of ionizing radiation and C225 by detecting cell proliferation, cell cycle distribution, and apoptotic activity in human prostate cancer cells (DU145), which express both TGF-α and EGFR. Our data showed that C225 significantly enhanced cell death induced by ionizing radiation. Our present study may have potential impacts on the clinical applications of combining C225 with irradiation therapy in patients with cancers.

2. Materials and methods

2.1. Cell lines and cell culture

The DU-145 prostate carcinoma cell line was kindly provided by the Urinary Surgery Department of the First Affiliated Hospital of Peking University. It was maintained in RPMI1640 supplemented with 20 mM HEPES (pH 7.4), 100 IU/ml penicillin, 100 mg/ml streptomycin, 4 mM glutamine, and 10% heat-inactivated fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Hangzhou, China) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. C225-treated DU145 cells were obtained by culturing cells with 100 nmol/l C225 (EGFR inhibitor; Boehringer Co., Ingelheim, Germany) for 24 h before irradiation. The concentration of cetuximab (100 nmol/l) in vitro was comparable to in vivo dose of cetuximab (3 to 8 mg/kg) in prostate cancer and NSCLC xenografts [16,17]. Phospho-P38 mitogen-activated protein kinase (MAPK) mAb (Alexa Fluor) was provided by Cell Signaling Technology (Boston, MA).

2.2. Cell-growth analysis by methyl thiazolyl tetrazolium (MTT) assay

Cell proliferation was determined by assessing the mitochondrial reduction of MTT. Cells from the control and C225-pretreated groups were exposed to different radiation dosages (0, 2, 4, 6, and 8 Gy) according to previous reports [18–20]. Cells were plated at 1×10^3 cells/well in 96-well plates containing 200 μl growth medium and allowed to attach for 24 h. The medium was removed, and the C225-treated cells were quiesced for 2 days in a medium supple-

Table 1
Growth-inhibitory effects of radiation and C225 treatment on DU145 cell line (%, $\bar{X} \pm s$)

	Radiation dose (Gy)				
	0	2	4	6	8
	Growth inhibitory rate				
Control	0	57 ± 4.6	68 ± 3.4	70 ± 4.0	73 ± 3.3
C225 group	43 ± 3.0[a]	67 ± 3.8[b]	72 ± 5.8[c]	79 ± 3.7[d]	88 ± 4.2[e]

Compared with control group t = −25.4.
[a] $P < 0.01$; t = −3.1.
[b] $P < 0.05$; t = −1.1.
[c] $P < 0.05$; t = −3.0.
[d] $P < 0.05$; t = −4.7.
[e] $P < 0.05$.

Table 2
Cell-cycle distribution of DU145 cells treated with radiation and C225 (%, $\bar{X} \pm s$)

Group	Radiation dose (Gy)	G_0/G_1	S	G_2/M
Control	0	53.13 ± 2.60	37.44 ± 2.15	9.43 ± 2.05
	2	55.60 ± 3.52	39.34 ± 3.24	5.05 ± 2.04
	4	44.37 ± 3.12	40.03 ± 2.05	15.60 ± 2.08
	6	27.80 ± 3.02	37.84 ± 2.26	34.36 ± 2.35
	8	27.82 ± 2.84	34.77 ± 2.96	37.41 ± 3.06
C225 group	0	59.55 ± 3.62	34.69 ± 3.62	5.76 ± 1.86
	2	59.41 ± 3.65	33.82 ± 4.06	6.77 ± 2.10
	4	65.95 ± 3.60[a]	32.05 ± 2.24[d]	20.00 ± 1.02[g]
	6	50.25 ± 3.82[b]	29.06 ± 3.05[e]	20.69 ± 2.42[h]
	8	37.88 ± 2.51[c]	29.42 ± 2.58[f]	32.70 ± 3.16

Compared to the control group irradiated with the same dose t = −7.92.
[a] $P < 0.01$; t = −8.64.
[b] $P < 0.01$; t = −4.90.
[c] $P < 0.01$; t = 4.33.
[d] $P < 0.05$; t = 3.90.
[e] $P < 0.05$; t = 3.08.
[f] $P < 0.05$; t = −2.97.
[g] $P < 0.05$; t = 6.91.
[h] $P < 0.01$.

mented with 100 nmol/l C225. The medium was changed on day 2 of the 4-day experiment. At harvest, the medium was removed from the appropriate wells, replaced with 50 μl MTT solution (2.5 mg MTT/ml), and incubated for 4 h at 37°C. After incubation, the MTT solution was carefully aspirated and replaced with 150 μl DMSO. Cell growth was analyzed on a plate reader by using SoftMax program (Molecular Devices Corp., Menlo Park, CA). Experiments were performed in quadruplicate and repeated at least 3 times. Inhibition ratio (%) = (1 − A_{190} of testing group/A_{190} of the 0-Gy-treated (control) group) × 100.

2.3. Ionizing radiation treatment

Exponentially growing DU145 cells in a tissue-culture flask (25 cm^2) were irradiated with ^{60}Co γ rays, and the absorbed dose rate was 1.953 Gy/min. Radiation was performed in the Radiation Department of Peking University.

2.4. Clonogenic assay

Cells of the control and C225 groups were exposed to different radiation dosages (0, 0.5, 1.0, 1.5, 2, 4, 6, and 8 Gy). After incubation for 14 d, the cells were fixed with methanol and stained with Giemsa. Colonies containing more than 50 cells were counted. The plating efficiency (PE) and survival fraction (SF) were calculated as follows: PE = (colony number/inoculating cell number) × 100%. SF = PE (tested group)/PE (0-Gy group) × 100%. A dose-survival curve was obtained for each experiment and used for calculating several survival parameters. Parallel samples were set at each radiation dosage. The cell-survival curve was plotted with Origin (Northampton, MA) ver. 7.5 software, using the equation: $SF = 1 - (1 - e\text{-}D/D0)^N$. The multi-target,

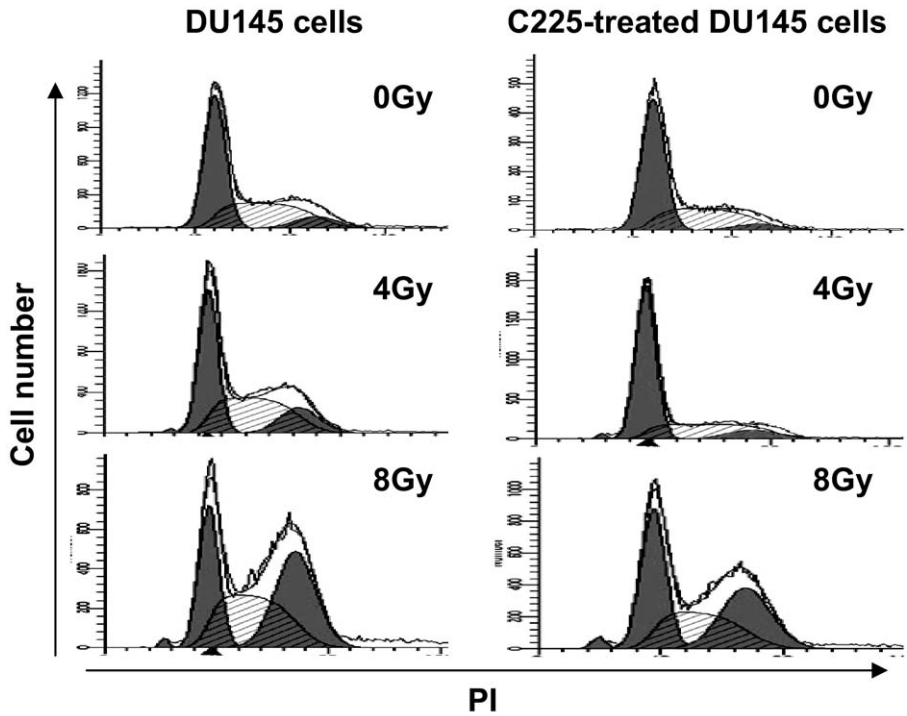

Fig. 2. Effect of radiation and C225 on the cell cycle in DU145 cells. In C225-treated DU145 cells, a sharp decrease in the fraction of cells in the S phase is observed, and the G1 arrest rate is higher at every radiation dose included in the study.

Table 3
Effect of radiation and C225 on early apoptosis in DU145 cells (%, $\bar{X} \pm s$)

Radiation dose (Gy)	Early apoptosis rate	
	DU145 cells	C225-treated DU145 cells
0	0.92 ± 0.23	1.60 ± 0.26
2	1.30 ± 0.28	1.74 ± 0.22
4	3.56 ± 0.32	8.14 ± 0.42[a]
6	1.90 ± 0.25	4.09 ± 0.24[b]
8	1.34 ± 0.20	1.36 ± 0.18

Compared to the control group irradiated with the same dose t = −14.55.
[a] $P < 0.01$ and t = −12.26.
[b] $P < 0.01$.

single-hit model was applied to calculate the cellular radiosensitivity (mean lethal dose, D_0), the capacity for sublethal damage repair (quasithreshold dose, Dq), and the extrapolation number (N). The values for alpha and beta were determined from best-fit curves according to the linear-quadratic model, and these values were used to calculate the SF after irradiation with a dose of 2 Gy (SF_2) and the relative biological effect (RBE).

2.5. Cell cycle and apoptosis analysis by flow cytometry (FCM)

Cells from the control and C225 groups were exposed to different radiation dosages (0, 2, 4, 6, and 8 Gy). Cells were harvested at 24 hours after irradiation for cell cycle analyses by FCM. Cells for apoptosis analysis were harvested at 48 hours after irradiation. Each test was performed 3 times. Cells used for cell-cycle testing were stained with propidium iodide. Cells used for apoptosis tests were stained with propidium iodide (PI) and annexin V and analyzed by fluorescence-activated cell sorting (FACS) using Coulter EPICS and ModFit software (Verity Software House, Topsham, MN).

2.6. mRNA extraction and reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared using Tri-Pure isolation reagent. cDNA was produced from 2.5 μg total RNA by using reverse transcriptase in a reaction volume of 20 μl. PCR analyses were carried out in 20 μl reaction solution by using Qiagen Taq Polymerase Kit (Shanghai, P.R. China), which contained 1 μl cDNA, 50 μM deoxynucleotide triphosphates, and 0.1 μM of both sense and antisense primers. Amplification

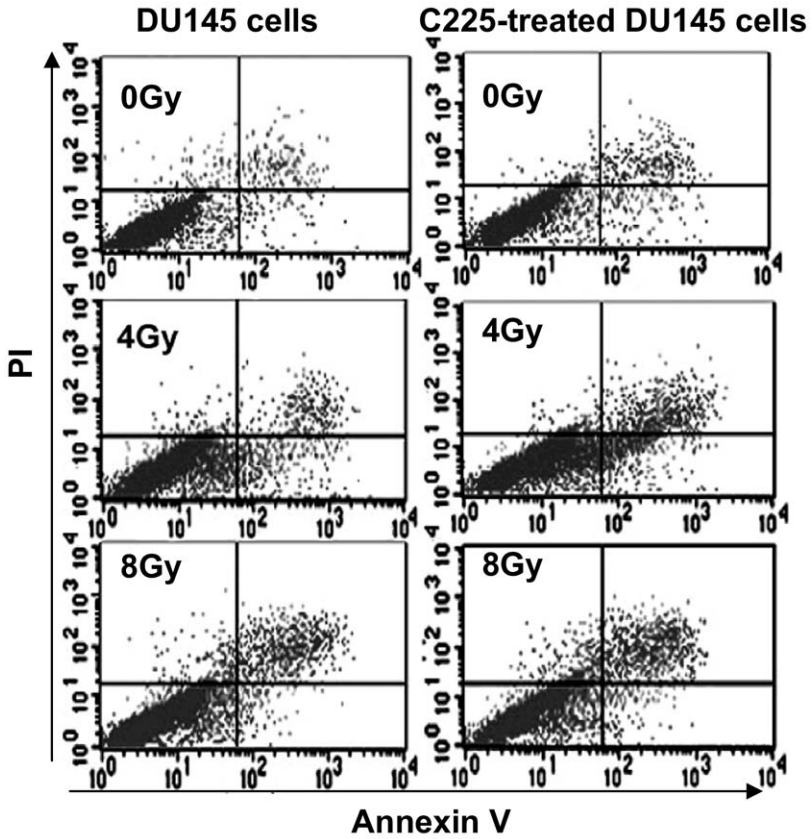

Fig. 3. C225 treatment significantly enhanced radiation-induced cell death in DU145 cells. The early apoptosis rate of C225-treated DU145 cells was significantly higher than that of the control group cells at the radiation dosage of 4 Gy. Higher late apoptosis rate of the C225-treated DU145 cells was observed at every radiation dose included in this study.

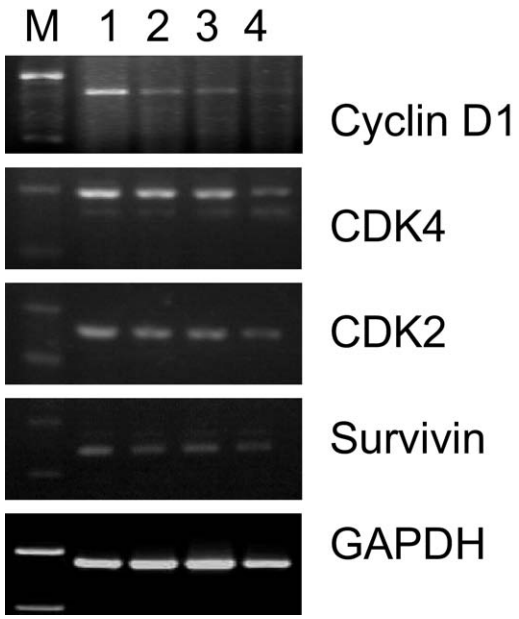

Fig. 4. Expressions of cyclin D1, CDK2, CDK4, and survivin mRNAs in DU145 cells subjected to irradiation and/or C225 treatment, respectively. M, marker; 1, control DU145 cells; 2, C225-treated DU145 cells (0 Gy); 3, 4-Gy irradiated DU145 cells; and 4, 4-Gy irradiated, C225-treated DU145 cells.

was performed as follows: samples were heated at 94°C for 5 min, followed by 30 cycles at 94°C for 30 seconds, 55°C to 60°C for 40 seconds, and 72°C for 30 seconds. After a final extension at 72°C for 7 minutes, the samples were analyzed using agarose gel electrophoresis.

2.7. Phospho-P38-MAPK quantification by FCM

Control and C225-treated DU145 cells for phospho-P38-MAPK quantification by FCM were harvested at 24 hours after 4-Gy irradiation. Each test was performed 3 times. Cells used for apoptosis tests were stained with P38-MAPK mAb (Alexa Fluor) and analyzed by FACScan using Coulter EPICS and ModFit software.

2.8. Statistical analysis

All data are presented as mean ± SD. The *t*-test was used to compare the means of different groups. *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Survival curve and RBE of C225-treated DU145 cells after irradiation

The cloning efficiency of DU145 was between 60% and 90%. The survival curve of control and C225-treated DU145 cells after irradiation is shown in Fig. 1. The radiobiological parameters of C225-treated DU145 cells were $D_0 = 2.145$, Dq = 0.647, N = 1.352, and $SF_2 = 0.49$, while those of control DU145 cells were $D_0 = 2.832$, Dq = 1.428′N = 1.656, and $SF_2 = 0.68$. In the present study, RBE = SF_2 (control)/SF_2 (C225 group) = 0.68/0.49 = 1.39. The enhanced RBE in C225-treated cells indicated that treatment with C225 significantly improved the biological effect of irradiation.

3.2. Effects of C225 on DU145 cell growth

In our preliminary study, C225 produced dose-dependent inhibition on DU145 cell growth (data not shown). In this study, 100 nmol/l C225, which produced approximately 50% inhibition of DU145 cell growth, was selected to perform the following experiments. Using MTT assays, we evaluated the growth-inhibitory effects of radiation and C225 on DU145 cells. As shown in Table 1, although both C225 and irradiation individually produced significant inhibition of DU145 cell growth, the inhibition produced by their combination was significantly greater than that produced by either treatment alone ($P < 0.05$). To further determine the effects of C225 on the proliferation of DU145 cells, we detected the cell-cycle distribution of DU145 cell by using FCM. The results are shown in Table 2 and Fig. 2. The percentage of C225-treated cells in the S-phase was significantly lower than that of control cells after irradiation. Moreover, the rate of G1 arrest was consistently higher among the C225-treated cells than among untreated cells after irradiation.

3.3. Significantly enhanced cell death in C225-treated DU145 cells after irradiation

Cell death was detected by FCM in the present study. As shown in Table 3 and Fig. 3, the early apoptosis rate among

Table 4
Expression of cyclin D1, DK2, CDK4, and survivin in DU145 cells (%, $\bar{X} \pm s$)

Gene expression	Radiation dose (0 Gy)		Radiation dose (4 Gy)	
	Control	C225 treatment	Control	C225 treatment
CyclinD1	0.92 ± 0.12	0.46 ± 0.08[a]	0.48 ± 0.10	0.31 ± 0.06[b]
CDK2	0.89 ± 0.20	0.59 ± 0.06[c]	0.65 ± 0.09	0.42 ± 0.04[d]
CDK4	0.96 ± 0.18	0.70 ± 0.07[e]	0.72 ± 0.09	0.45 ± 0.04[f]
Survivin	0.69 ± 0.15	0.56 ± 0.06[g]	0.52 ± 0.07	0.43 ± 0.05[h]

Compared to the control group irradiated with the same dose t = −8.32.
[a] $P < 0.01$; t = −8.52.
[b] $P < 0.01$; t = −5.62.
[c] $P < 0.01$; t = 4.48.
[d] $P < 0.05$; t = 4.22.
[e] $P < 0.05$; t = 3.66.
[f] $P < 0.05$; t = −3.05.
[g] $P < 0.05$; t = 5.96.
[h] $P < 0.01$.

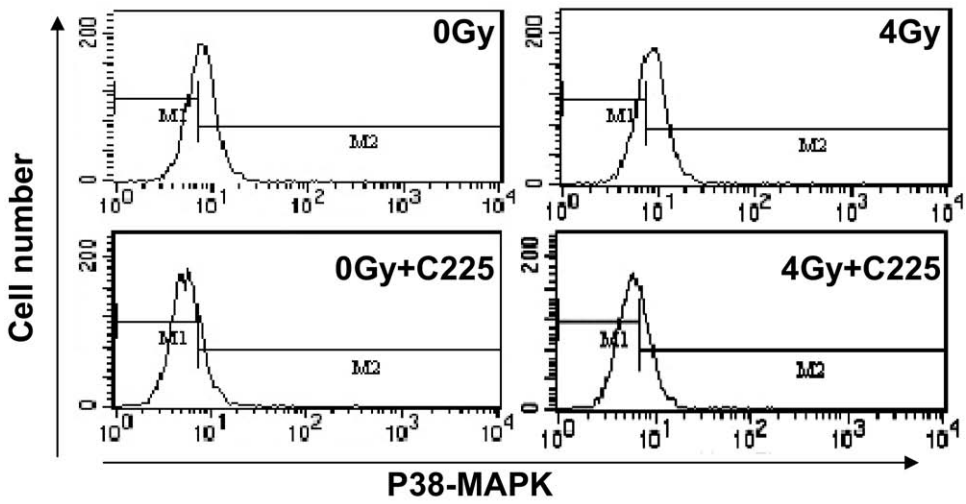

Fig. 5. Phosphorylation level of P38-MAPK in DU145 cells after irradiation and/or C225 treatment. C225 significantly inhibits MAPK phosphorylation in both the control and radiated DU145 cells.

C225-treated DU145 cells was significantly higher than that among control DU145 cells after 4-Gy irradiation. The percentage of C225-treated DU145 cells that underwent late apoptosis was remarkably higher than that of untreated DU145 cells exposed to different irradiation doses.

3.4. Expression of phospho-P38-MAPK, cyclin D1, CDK2, CDK4, and survivin in DU145 cells after irradiation and/or C225 treatment

It is known that cyclin D1, DK2, CDK4, and survivin are some of the important genes related to cell proliferation and survival [21–24]. We therefore detected their expressions by using RT-PCR. As shown in Fig. 4 and Table 4, the expressions of these genes were lower in C225-treated DU145 cells than in control DU145 cells. The lowest expressions were observed in DU145 cells treated with both 4-Gy irradiation and C225. P38-MAPK is one of the major factors in the EGFR/ras/raf/MAPK proliferative pathway [25–28] and is activated by phosphorylation. We therefore measured the phosphorylation level of p38-MAPK by FCM. As shown in Fig. 5 and Table 5, C225 significantly inhibited the phosphorylation of p38-MAPK in DU145 cells, regardless of whether or not these cells were irradiated.

Table 5
Expressions of P38-MAPK in DU145 cells ($\bar{X} \pm s$)

	Flurescence intensity		
	M1% (gated)	M2% (gated)	Mean (all)
0 Gy	40.59 ± 2.84	60.21 ± 3.06	7.92 ± 0.42
0 Gy + C225	82.64 ± 3.85*	18.03 ± 2.01*	5.26 ± 0.32*
4 Gy	34.89 ± 2.94	66.84 ± 3.25	9.37 ± 0.46
4 Gy + C225	74.34 ± 3.97*	26.42 ± 2.74*	6.41 ± 0.36*

Statistically significant difference was observed between the control and C225 groups with regard to M1, M2, and mean fluorescence intensity (*$P < 0.05$).

4. Discussion

In mammalian cells, many components of the biologic response to ionizing radiation are mediated via signal transduction, cell-cycle regulation, and DNA-repair pathways. Recent studies have shown that EGF can act not only as a growth factor and promote cell proliferation but also function as a survival factor and suppress apoptosis when cells are challenged with various death signals.

It has been reported that prostate cancer cells frequently exhibit high expression of EGFR as well as of its ligands EGF and TGF α [29–31]. EGFR inhibitors, such as C225, were shown to inhibit the growth of some EGFR-expressing cell lines, including DU145. The mechanism of growth inhibition seemed to involve the inhibition of EGF-induced downstream signaling pathways. In the present study, we confirmed using MTT and clonogenic assays that C225 significantly inhibited DU145 cell growth. Importantly, irradiation in combination with C225 produced significant synergistic inhibition of DU145 cell growth. The dose-survival curve of C225-treated DU145 cells exhibited a narrow shoulder (indicating decrease in D_0 and N) and a greater slope rate (indicating decrease in Dq). The decreased SF_2 value of the C225-treated DU145 cells indicated the radiosensitization effect of C225 on DU145 cells. Consistent with this observation, the RBE of C225-treated DU145 cells was 1.39, which is comparable to that of ^{125}I seeds brachytherapy for prostate cancer (1.2–1.4) [32–34].

The mechanisms by which C225 and other EGFR inhibitors augment tumor response to radiation are multiple, especially in an in vivo model. These involve both direct

and indirect interaction with tumor cells, increase in radiation-induced apoptosis or necrosis, the inhibition of cellular repair after radiation damage, the accumulation of cells in radiosensitive phases of the cell cycle, the blocking of accelerated repopulation during radiotherapy, and the induction of tumor reoxygenation. C225 inhibits tumor neovascularization, resulting in tumor-growth inhibition and indirectly increasing tumor radio-response. In addition, the C225 antibody may itself be cytotoxic, contributing mainly to the induction of apoptosis, and it may inhibit the proliferation of tumor cells, both of which reduce the total tumor cell mass, making tumors more amenable to control by radiotherapy.

The cell-cycle phase is one of the determinative factors of cell radiosensitivity [35–37]. Radiation combined with C225 treatment of DU145 cells induced significant G0/G1 arrest, a sharp decrease in the fraction of cells in the S phase, and the accumulation of cells in the G2/M phase, as revealed by FCM. Cyclin D1 is a G1 cyclin whose long-term overexpression along with CDK2 and CDK4 is believed to be oncogenic due to the abrogation of G1-checkpoint controls [38]. Using RT-PCR, we showed that cyclin D1, CDK2, and CDK4 expression were down-regulated at the transcriptional level in DU145 cells after C225 treatment or irradiation. The synergetic cyclin D1, CDK2, and CDK4 down-regulation produced by radiation and C225 treatment may be responsible for their synergetic effect on G1 arrest in DU145 cells.

Survivin is a member of the apoptosis-suppressor family and is overexpressed in many malignancies, including prostate cancer, but not in terminally differentiated normal cells [39,40]. Survivin expression has been reported to be important for the regulation of cell division and apoptosis. Irradiation along with C225 treatment induced markedly less survivin expression, as shown by RT-PCR as well as significant apoptosis compared with that induced by either treatment alone, indicating that the synergetic down-regulation of survivin expression by radiation and C225 nay contribute to the synergetic effects to induce cell death in DU145 cells.

Cellular apoptosis represents another process that may influence the ultimate response of a particular tumor to ionizing radiation. EGFR activation reduces the apoptosis rate and the radiosensitivity of tumor cells [41]. Oleg and Graham reported that EGFR signals significantly down-regulated bcl-2 (apoptosis-suppressor gene) expression and up-regulated the expression of apoptosis-trigger gene (Bax) [42]. Wang et al. found that EGFR inhibitors lead to apoptosis in many tumor cells. Moreover, the mechanism of apoptosis induction was related to the activation of the MAPK and Akt genes, which are downstream of the EGFR gene [43]. Treatment with ionizing radiation can activate the EGFR/ras/raf/MAPK proliferative pathway through the release of TGF and the activation of the EGFR tyrosine kinase. EGFR activation may be a cell-survival response that blocks apoptotic signals in cancer cells that have undergone cytotoxic damage. Mounting evidence suggests that when EGFR-ERK signaling is inhibited, radiation-induced cell apoptosis increases. In the present study, C255 increased the rate of DU145 cell apoptosis after exposure to different doses of irradiation. Importantly, C255 treatment significantly inhibited the radiation-induced EGFR-ERK signaling pathway activation in DU145 cells as evidenced by the reduced the phosphorylation of p38-MAPK molecules by C225.

In summary, EGFR inhibitors, such as C225, significantly enhanced radiosensitization possibly by inhibiting the MAPK pathway. This provides a rationale for combining C225 and radiotherapy in the treatment of human epithelial cancers that express EGFR. Radiotherapy combined with C225 might improve therapeutic effect and reduce the total radiation dose in a clinical setting, which is of potential clinical interest.

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