Enhancement of radiosensitivity by a unique novel NF-κB inhibitor, DHMEQ, in prostate cancer

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BACKGROUND: Inducible activation of nuclear factor (NF)-κB is one of the principal mechanisms through which resistant prostate cancer cells are protected from radiotherapy. We hypothesised that inactivation of inducible NF-κB with a novel NF-κB inhibitor, DHMEQ, would increase the therapeutic effects of radiotherapy.

METHODS: PC-3 and LNCaP cells were exposed to irradiation and/or DHMEQ. Cell viability, cell cycle analysis, western blotting assay, and NF-κB activity were measured. The antitumour effect of irradiation combined with DHMEQ in vivo was also assessed.

RESULTS: The combination of DHMEQ with irradiation resulted in cell growth inhibition and G2/M arrest relative to treatment with irradiation alone. Inducible NF-κB activity by irradiation was inhibited by DHMEQ treatment. The expression of p53 and p21 in LNCaP, and of 14-3-3σ in PC-3 cells, was increased in the combination treatment. In the in vivo study, 64 days after the start of treatment, tumour size was 85.1%, 77.1%, and 64.7% smaller in the combination treatment group than that of the untreated control, DHMEQ-treated alone, and irradiation alone groups, respectively.

CONCLUSION: Blockade of NF-κB activity induced by radiation with DHMEQ could overcome radio-resistant responses and may become a new therapeutic modality for treating prostate cancer.

Keywords: prostate cancer; radiation; nuclear factor-κB

Radiation therapy, such as external beam radiotherapy (EBRT) or brachytherapy, is one of the standard options for organ-confined or locally advanced prostate cancer. The therapeutic efficacies of radical prostatectomy, high dose of EBRT, and brachytherapy are similar (Kupelian et al, 2004), being approximately 75–80% for stage T1–T2 prostate cancer. Doses used in EBRT or brachytherapy are highly associated with therapeutic efficacy; however, the incidences of related side effects increase as the dose of radiation increases (Pollack et al, 2002). Therefore, a modality for improving the therapeutic efficacy of radiotherapy for locally confined or advanced prostate cancer is warranted both to enhance radiation-induced cytotoxicity and to reduce related side effects.

With respect to the technical aspects of radiation therapy, three-dimensional conformal radiation therapy and intensity-modulated radiotherapy for external therapy have been implemented (Zelefsky et al, 2001; Hanks et al, 2002). Meanwhile, the therapeutic mean, including manipulating the radio-adaptive response of prostate cancer cells, could be another tool for improving the therapeutic effects of radiotherapy and minimising related side effects. Several factors associated with radio-resistance, such as the expression of antiapoptotic proteins (Chin et al, 2005) and regulation of the cell cycle (Takagi et al, 1998) in cancer cells, have been reported. However, the mechanism of the radio-adaptive response has not yet been adequately elucidated.

It has been reported that in radio-adaptive resistance, the prosurvival network was initiated by nuclear factor (NF)-κB. Activated NF-κB signal produces various genes associated with antiapoptotic proteins and modulation of cell cycle regulation, which contribute to increased cell survival and cell radio-resistance. Among the transcription factors, NF-κB is one of the most decisive regulators of irradiation-induced gene expression.

Activation of NF-κB has also been reported to reduce the therapeutic effect of radiotherapy in cancer cells (Starenki et al, 2004). It has been reported that ionising radiation activates DNA binding of NF-κB (Brach et al, 1991; Sun et al, 2007). Furthermore, blocking NF-κB activation increases the apoptotic response and decreases the growth and clonogenic survival of several human cancer cells (Tang et al, 2001; Chen et al, 2002). In prostate cancer that includes the hormone-insensitive prostate tumour cell, DU145, PC-3 lines, and the hormone responsive LNCaP cell line, radiation exposure was found to lead to an increase in NF-κB activity (Palayoor et al, 1999; Wen et al, 2003; Raffoul et al, 2006; Sun et al, 2007). Inhibition of NF-κB by a dominant-negative IκB mutant enhanced apoptosis in DU145 cells (Flynn et al, 2003). Thus, these results strongly suggest that NF-κB plays a key role in radio-adaptive resistance under ionising radiation in prostate cancer.

We have previously synthesised a dehydroxymethyl derivative of epoxyquinomicin, DHMEQ, from a natural product and it is a novel and potent NF-κB inhibitor (Matsumoto et al, 2000). The mechanism by which DHMEQ inhibits activation of NF-κB is...
unique because DHMEQ inhibits NF-κB translocation from the cytoplasm to the nucleus (Ariga et al., 2002). Recently, it was shown to covalently bind to the specific cysteine of NF-κB components (Yamamoto et al., 2008). The antitumour effect of DHMEQ has been proven in various types of cancer, including breast cancer (Matsumoto et al., 2005), pancreatic cancer (Takatsuna and Umezawa, 2004), thyroid cancer (Starenki et al., 2004), multiple myeloma (Watanabe et al., 2005), and leukaemia (Ohsugi et al., 2005). In our laboratory, we have investigated the therapeutic effect of DHMEQ on hormone-insensitive prostate cancer cells (Kikuchi et al., 2003; Kuroda et al., 2005). We speculated that the NF-κB inhibitor would enhance the therapeutic effect of radiation in prostate cancer cells. Therefore, in this study, we examined whether DHMEQ had the potential to inhibit NF-κB activity induced by radiation and to enhance cytotoxicity in an *in vitro* study and animal model.

**MATERIALS AND METHODS**

**Cell lines and chemicals**

LNCaP and PC-3 prostate cancer cells (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 μg ml⁻¹ streptomycin, and 100 IU ml⁻¹ penicillin. Racemic DHMEQ was synthesised as described previously (Matsumoto et al., 2000), dissolved in dimethyl sulfoxide (DMSO), and subsequently diluted in culture medium to a final concentration of less than 0.1%.

**Irradiation**

Cells and tumours were irradiated using a MBR 1520 R (Hitachi, Tokyo, Japan) radiation source operating at 150 kV and 20 mA X-rays with 0.5 mm Al and 0.1 mm Cu filters at a radiation dose rate of 1.74 Gy min⁻¹. In the *in vivo* study, irradiation was locally confined to the tumour by shielding the rest of the body with a lead block.

**WST-1 assay**

Cells were seeded in 96-well plates and incubated overnight at 37 °C under 5% CO₂ in a humidified incubator. Cells were treated with various concentrations of DHMEQ for 24 h. Cells treated with the same concentrations of DMSO served as controls. After 24 h of incubation, cytotoxicity was determined by WST-1 reagent in accordance with the manufacturer’s instructions (Roche, Indianapolis, IN, USA). The quantity of formazan dye was measured using a microplate reader at 450 nm.

**Clonogenic survival assay**

LNCaP and PC-3 cells were plated at a density of 2000 to 10 000 cells per well and 100 to 500 cells per well into 6-well plates, respectively. Treatments with various doses of DHMEQ and irradiation were performed. After 14 days incubation, they were washed and stained with crystal violet, and colonies containing >50 cells were counted as clonogenic survivors. Survival fractions were obtained according to the standard protocol by comparing cell counts with the plating efficiency of untreated controls.

**Flow cytometric analysis of cell cycle phase distribution**

The vehicle control, 5.0 μg ml⁻¹ of DHMEQ alone, 4 Gy of irradiation alone, and their combination treatments were analysed by flow cytometric analysis of cells labelled with BrdU assay in LNCaP and PC-3 cells. After incubation for 48 h, cells were labelled with BrdU assay using BrdU kits (Cell Signaling Technology, Beverly, MA, USA) according to the manufacturer’s protocol. Anti-BrdU was detected by flow cytometry (Beckman Coulter, Fullerton, CA, USA).

**Electrophoresis mobility shift assay**

NE-Per Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA) were used to prepare nuclear extracts. The binding reaction mixture contained nuclear extract (5 μg of protein), 2 μg poly (dl-dC), and 10 000 c.p.m. ³²P-labelled probe in binding buffer (75 mM NaCl, 1.5 mM EDTA, 1.5 mM DTT, 7.5% glycerol, 1.5% NP-40, 15 mM Tris-HCl, pH 7.0). By using the recombinant protein, we incubated the reaction mixture containing 20 μM recombinant protein and 5% DMSO with or without DHMEQ in PBS at 4°C. To determine the DNA binding activity of recombinant protein, we used 20 ng of recombinant protein from the reaction mixture for the electrophoresis mobility shift assay (EMSA). Samples were incubated for 20 min at room temperature in this mixture. DNA/protein complexes were separated from free DNA on a 4% native polyacrylamide gel in 0.25 mM TBE buffer. The following sequences were used as a C/EBPβ probe (Promega, Madison, WI, USA): 5'-TGCAGATTGGCCAATCTGCA-3' and 5'-TGCAGATTGGCCAATCTGCA-3'. These oligonucleotides were labelled with [γ-³²P]-ATP (3000 Ci mmol⁻¹; GE Healthcare, Little Chalfont, Buckinghamshire, UK) using T4 polynucleotide kinase (Takara, Ohtsu, Japan) and purified by passage through a Nickel column (GE Healthcare).

**Western blot analysis**

The expressions of p53, p21, and 14-3-3σ were determined by western blot analysis. Samples containing equal amounts of 20 μg protein were subjected to electrophoresis on a sodium dodecyl sulphate–polyacrylamide gel and transferred to a nitrocellulose filter. Filters were blocked with Tris-buffered saline containing 5% nonfat milk powder at 4°C overnight and then incubated for 1 h with an anti-p53 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), an anti-p21 Waf1/Cip1 mouse monoclonal antibody (Cell Signaling Technology), an anti-14-3-3σ goat polyclonal antibody (Santa Cruz Biotechnology), or an anti-β-actin mouse antibody (Sigma, St Louis, MO, USA). Filters were then incubated for 1 h with an anti-mouse secondary antibody (Dako A/S, Glostrup, Denmark), an anti-goat secondary antibody (Dako A/S), or an anti-rabbit secondary antibody (Dako A/S), and reactivity was detected using an enhanced chemiluminescence system (Amersham Life Science, Little Chalfont, Buckinghamshire, UK).

**In vivo treatment**

All procedures involving animals and their care in this study were approved by the Animal Care Committee of Keio University in accordance with institutional and Japanese government guidelines for animal experiments. The antitumour effect of irradiation combined with DHMEQ was evaluated in an animal model. Male BALB/c-nu/nu mice were obtained from Sankyo Lab Service (Tokyo, Japan). PC-3 cells (5 × 10⁶) were implanted subcutaneously into the flank of each nude mouse. When an animal in DHMEQ-treated groups developed a palpable tumour, it was given a once daily intraperitoneal injection of DHMEQ at a concentration of 4 mg kg⁻¹ in 0.5 ml of PBS for 14 consecutive days. As a control, 0.5 ml of vehicle alone was administered. In irradiation groups, irradiation consisted of 8 Gy in 2 fractions on the 1st and 8th days. Tumour volume (V) was calculated using the formula $V = \frac{A \times B^2}{2}$, where $A$ is the greatest diameter and $B$ is the diameter at a perpendicular to $A$. Tumour volume was monitored every 4 days.
Statistical analysis
All data are expressed as the mean ± s.e. from three or more independent experiments. Differences between groups were examined for significance with ANOVA and/or the Student’s t-test where appropriate. A P < 0.05 indicated a significant difference.

RESULTS
DHMEQ enhanced the growth inhibition of PC-3 cells by irradiation
The cell growth rate in LNCaP and PC-3 cells at various doses of 24-h exposure of DHMEQ was evaluated (Figure 1). Significant cell growth inhibitory effects were observed at a 10 μg ml⁻¹ or higher dose of DHMEQ and 20 μg ml⁻¹ or higher dose of DHMEQ in PC-3 and LNCaP cells, respectively. The IC₅₀ values were 18.2 and 33.5 μg ml⁻¹ in PC-3 and LNCaP cells, respectively.

The combination of irradiation with various doses of DHMEQ treatment was then evaluated by clonogenic survival assay (Figures 2A and B). As shown in Figure 2A, at a radiation dose of 4 Gy, the inhibitory effect on colony formation in LNCap cells was significantly higher after treatment with the 2.5 μg ml⁻¹ or higher dose of DHMEQ than that with the vehicle control. At a radiation dose of 4 Gy, the inhibitory effect of colony formation in PC-3 cells was significantly higher after treatment with the 5 μg ml⁻¹ or higher dose of DHMEQ than that with the vehicle control (Figure 2B).

DHMEQ inhibited NF-κB activation induced by irradiation
In the experiment of EMSA assay, the DNA activity of NF-κB was induced 4 h after irradiation in LNCaP cells and this increase was inhibited by 5.0 μg ml⁻¹ of DHMEQ treatment (Figure 3A). In PC-3 cells, the DNA activity of NF-κB was completely inhibited by 6-h exposure of 5.0 μg ml⁻¹ of DHMEQ treatment (Figure 3B). The DNA binding activity of NF-κB was increased after irradiation (especially 0.5, 1, and 2 h after irradiation) and this increase was inhibited by 5.0 μg ml⁻¹ of DHMEQ treatment. These data confirm that DHMEQ could inhibit the activation of NF-κB induced by irradiation.

Cell cycle analysis
The percentage of G2/M arrest in LNCaP cells treated with 4 Gy of irradiation (38.0 ± 1.4%) was higher than that of non-treated cells (26.7 ± 4.2%) and of 5.0 μg ml⁻¹ of DHMEQ treatment alone (24.8 ± 1.8%; P < 0.05 in each, Table 1). Furthermore, the percentage of G2/M arrest in LNCaP cells treated with the combination treatment of DHMEQ and 4 Gy of irradiation (44.8 ± 2.3%) was higher than that of irradiation alone (P < 0.05).

In PC-3 cells, the percentage of G2/M arrest in the combination treatment of DHMEQ with irradiation group (56.1 ± 2.1%) was significantly higher than that in the non-treated control (30.8 ± 3.6%), DHMEQ treatment alone (42.5 ± 3.3%), and irradiation alone (44.6 ± 2.4%) groups (P < 0.05, in each).

Analysis of cell cycle–related protein expression, p53, p21, and 14-3-3σ
In LNCaP cells, the expression of p53 and p21 were enhanced in cells treated with the combination of 5.0 μg ml⁻¹ of DHMEQ and 4 Gy of irradiation. In PC-3 cells, no p53 expression was observed and their expressions did not change after DHMEQ and/or irradiation treatment. On the contrary,
Table 1  Cell cycle analysis after combination treatment with 5 \( \mu \)g ml\(^{-1}\) of DHMEQ and 4 Gy of irradiation in LNCaP and PC-3 cells

| Cell line | Treatments | LNCaP | PC-3 |
|-----------|------------|-------|------|
| % G1      | Untreated  | 58.8 ± 2.4 | 55.2 ± 4.2 |
|           | DHMEQ      | 63.9 ± 3.4 | 46.2 ± 3.8 |
|           | Irradiation| 54.4 ± 2.3 | 42.9 ± 1.7 |
|           | DHMEQ + irradiation | 46.9 ± 3.9 | 35.5 ± 2.2 |
| % S       | Untreated  | 14.5 ± 3.0 | 14.0 ± 5.1 |
|           | DHMEQ      | 11.3 ± 1.8 | 11.3 ± 1.0 |
|           | Irradiation| 7.6 ± 2.3  | 10.5 ± 1.1 |
|           | DHMEQ + irradiation | 8.3 ± 2.6  | 8.4 ± 5.1 |
| % G2/M    | Untreated  | 26.7 ± 4.2 | 30.8 ± 3.6 |
|           | DHMEQ      | 24.8 ± 1.8 | 42.5 ± 3.3 |
|           | Irradiation| 38.0 ± 1.4 | 46.6 ± 2.4 |
|           | DHMEQ + irradiation | 44.8 ± 2.5 | 56.1 ± 2.1 |

The expression of 14-3-3-\(\sigma\) increased after 4 Gy of irradiation. The increase in 14-3-3-\(\sigma\) expression after irradiation was enhanced in cells treated with the combination of 5.0 \( \mu \)g ml\(^{-1}\) of DHMEQ and 4 Gy of irradiation.

The antitumour effect of DHMRQ and irradiation treatment in vivo

Mean tumour volume in mice treated with irradiation and DHMEQ (66.8 ± 9.3 mm\(^3\)) was significantly lower than that in untreated controls (215.7 ± 27.3 mm\(^3\)). DHMEQ treated alone (200.9 ± 29.5 mm\(^3\)), and irradiation alone (105.2 ± 13.4 mm\(^3\)) 36 days after the start of treatment \((P<0.05\) for each; Figure 5). No body weight loss or skin reactions were observed in the combination treatment or other groups. Sixty-four days after the start of treatment, tumour size was 85.1%, 77.1%, and 64.7% smaller in the combination treatment group than that of the untreated control, DHMEQ treated alone, and irradiation alone groups, respectively. In another set of experiments, we evaluated the toxic and side effects of DHMEQ itself in mice. No abnormal data, including liver and renal function, were demonstrated in mice treated with any dose of DHMEQ up to 16 mg kg\(^{-1}\) (data not shown). We also analysed the histological appearance of major organs, including the heart, lung, liver, kidney, bladder, and prostate. Haematoxylin and eosin staining did not reveal significant differences in histological appearance and damage between control and DHMEQ-treated mice.
DISCUSSION

We first investigated the cytotoxic effect of DHMEQ treatment in both LNCaP and PC-3 cells. No significant cytotoxic effects of DHMEQ itself were observed at doses of up to 5.0 μg ml⁻¹ in either LNCaP or PC-3 cells. At a radiation dose of 4 Gy, a significant inhibitory effect of colony formation in LNCaP and PC-3 cells was observed after treatment with the 2.5 μg ml⁻¹ or higher dose and 3 μg ml⁻¹ or higher dose of DHMEQ, respectively. Next, we evaluated if the combined treatment of DHMEQ with irradiation could affect NF-κB binding activity. NF-κB binding activity was induced by radiation alone in both PC-3 and LNCaP cells. DHMEQ also inhibited NF-κB activation induced by irradiation, as confirmed by EMSA assay. These results indicate that DHMEQ could inhibit NF-κB activation induced by irradiation, thereby reducing the progression of prostate cancer cell lines.

We then evaluated cell cycle analysis, which showed that G2/M arrest was enhanced by the combined treatment of irradiation with DHMEQ in both LNCaP and PC-3 cells. This result showed that the combination of irradiation with DHMEQ induced strong G2/M arrest in prostate cancer cells.

According to western blot results, the appearance of p53 increased after irradiation and p53 expression was enhanced by the combination treatment of DHMEQ with irradiation in LNCaP cells. Previous reports showed that p53 is also responsible for cell cycle arrest upon DNA damage and is a key regulator of apoptosis (Voussen, 2002). Similar results were observed in p21 expression cycle arrest upon DNA damage and is a key regulator of apoptosis.

CONCLUSIONS

DHMEQ inhibits NF-κB binding activity induced by irradiation and causes an increase in the inhibitory effect on colony formation and in the enhancement of cell cycle arrest in prostate cancer cells. Blockade of NF-κB function induced by radiation with DHMEQ could overcome radio-resistant responses and may become a new therapeutic modality for treating organ-confined and locally advanced prostate cancer.

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DHMEQ is a unique and potent NF-κB inhibitor that works at the level of nuclear translocation of NF-κB (Ariga et al, 2002). It has recently been demonstrated that DHMEQ can inhibit constitutive NF-κB activity consisting of p50/p65, although its effect is less potent against the p50 homodimer. This may explain why DHMEQ can effectively induce apoptosis in cancer cells with constitutive NF-κB activity consisting of p50/p65, but does not affect normal-resting lymphocytes whose NF-κB activity consists mainly of the p50 homodimer.

CONCLUSIONS

DHMEQ inhibits NF-κB binding activity induced by irradiation and causes an increase in the inhibitory effect on colony formation and in the enhancement of cell cycle arrest in prostate cancer cells. Blockade of NF-κB function induced by radiation with DHMEQ could overcome radio-resistant responses and may become a new therapeutic modality for treating organ-confined and locally advanced prostate cancer.
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