Biochemical and Biophysical Research Communications 469 (2016) 363–369

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibition of cyclic AMP response element-directed transcription by decoy oligonucleotides enhances tumor-specific radiosensitivity

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ARTICLE INFO

Article history:
Received 20 November 2015
Accepted 26 November 2015
Available online 2 December 2015

Keywords:
Cyclic AMP response element
γ-Irradiation
p38 MAPK
Radiosensitization
Decoy

ABSTRACT

The radiation stress induces cytotoxic responses of cell death as well as cytoprotective responses of cell survival. Understanding exact cellular mechanism and signal transduction pathways is important in improving cancer radiotherapy. Increasing evidence suggests that cyclic AMP response element binding protein (CREB)/activating transcription factor (ATF) family proteins act as a survival factor and a signaling molecule in response to stress. We postulated that CREB inhibition via CRE decoy oligonucleotide increases tumor cell sensitization to γ-irradiation-induced cytotoxic stress. In the present study, we demonstrate that CREB phosphorylation and CREB DNA-protein complex formation increased in time- and radiation dose-dependent manners, while there was no significant change in total protein level of CREB. In addition, CREB was phosphorylated in response to γ-irradiation through p38 MAPK pathway. Further investigation revealed that CREB blockade by decoy oligonucleotides functionally inhibited transactivation of CREB, and significantly increased radiosensitivity of multiple human cancer cell lines including TP53- and/or RB-mutated cells with minimal effects on normal cells. We also demonstrate that tumor cells ectopically expressing dominant negative mutant CREB (KCREB) and the cells treated with p38 MAPK inhibitors were more sensitive to γ-irradiation than wild type parental cells or control-treated cells. Taken together, we conclude that CREB protects tumor cells from γ-irradiation, and combination of CREB inhibition plus ionizing radiation will be a promising radiotherapeutic approach.
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1. Introduction

Radiotherapy is one of the major treatment modalities for a wide range of human tumors. However, the exact cellular and molecular events following exposure to ionizing radiation (IR) are yet to be clearly understood. IR produces a series of responses including cell cycle arrest and apoptosis [1]. IR also induces activation of several key cytoprotective molecules including c-FOS, c-JUN, EGR1, TP53 and cyclic AMP response element (CRE)-binding protein (CREB) [2]. In particular, cytoprotective responses to IR frequently involve signal transduction pathways of MAPK, leading to CREB-mediated transcriptional activation [3]. The 43 kDa CREB, which binds to the consensus motif (5′-TGACGTCA-3′), is ubiquitously expressed and is a pleiotropic activator that participates in the induction of a wide variety of cellular genes. Increasing evidence support that CREB is involved in cell growth, differentiation, and tumor cell survival to various genotoxic agents. Previously, we demonstrated that synthetic CRE palindromic decoy oligonucleotide can penetrate cells, bind sequence-specific DNA-binding proteins, and interfere with the CRE-directed transcription *in vivo*, which has proven that the CRE decoy oligonucleotides are harmless for normal cells but are potent inhibitors for cancer cell growth [4,5]. Increasing evidence suggests that CREB/ATF family protein may act as a survival factor to radiation stress [6], while expression of dominant negative mutant CREB (KCREB) reduces resistance to UV-radiation of human melanoma cells [7]. In addition, CREB was activated by ionizing radiation in Jurkat T cells [8]. Taken together,

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http://dx.doi.org/10.1016/j.bbrc.2015.11.122
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we postulated that CREB is phosphorylated in response to γ-irradiation through MAPK pathway, playing a role in cytoprotective responses to IR. We also tested whether CRE decoy oligonucleotide increases tumor-specific radiosensitivity.

2. Materials and methods

2.1. Cells and γ-Irradiation

Human cancer cells (AGS, HeLa, MCF-7, DU-145, SaOs-2 and HepG2) were grown in RPMI-1640 media supplemented with 10% fetal bovine serum and penicillin/streptomycin in a humidified 5% CO_2 incubator at 37 °C [9]. For IR treatment, cells were irradiated in a ^{137}Cs Source chamber irradiator (Biointernational Co.)

2.2. Oligonucleotides and transfection

CRE-decoy and control oligonucleotides used in the present studies were phosphorothioate oligonucleotides. Their sequences are as follows: 24-mer CRE decoy, 5′-TGACGTCA TGACGTCA TGACGTCA-3′ and 24-mer nonsense sequence control, 5′-CTAGCTAG CTAGCTAG CTAGCTAG-3’. Cationic liposome (DOTAP) was used to transfect oligonucleotides to cells.

2.3. Nuclear extraction and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared and EMSA was performed as previously described [4]. Briefly, nuclear extracts were pre-incubated with poly(dI-dC)•poly(dI-dC), dithiothreitol and reaction buffer for 30 min at 4 °C. ^{32}P-labeled oligonucleotide was then added, and reaction mixtures were incubated for 10 min at 37 °C. The reaction mixture was then separated on a 5% nondenaturing polyacrylamide gel at 4 °C, 180 V for 1.5 h. To prepare probes, oligonucleotide (5′-AGAGATGCCTGACGTCAGAGAGCTAG-3′) was labeled with [γ-^{32}P] ATP using 5′-end labeling by T4 polynucleotide kinase.

2.4. Soft agar colony formation assay

Cells (10^4 cells/35 mm dish) were mixed with 1% aqueous agar and plated in 35 mm dish. After attaining sufficient growth, iodonitrotetrazolium violet was added to stain the cell colonies. Experiments were performed in triplicate and repeated twice. The results in Figs. 2–4 are shown in a linear-logarithmic plot relating γ-irradiation exposure to survival. To compare the efficacy of oligonucleotides on radiosensitization, we employed a method described in Choi EJ et al. [10]. Briefly, sensitization enhancement ratio (SER) was calculated as the ratio of the isoeffective dose at survival fraction 0.5 in the absence of each inhibitor to that in the presence of each inhibitor. Therefore, SER 1.0 means no radiosensitization effect, and the higher numbers (SER > 1.0) means the stronger radiosensitization effect.

2.5. Production of stable transfectants

Cells (1×10^5 cells/60 mm dish) were transfected with 6 μg of KCREB plasmid, a dominant negative binding mutant form of CREB, using DOTAP. Stably transfected cells were selected by growing cells in the presence of G418 (400 μg/ml) [11,12]. The G418-resistant colonies were isolated after 2 weeks of selection and mutant CREB overexpression was identified by Western blot analysis using anti-CREB antibody.

2.6. Transient transcription assay of somatostatin-chloramphenicol acetyltransferase fusion gene

Cells in serum-free medium were transfected with 2 μg of somatostatin-chloramphenicol acetyltransferase (CAT) fusion gene plasmid, 1 μg of pSV-β-Gal plasmid and 4 μg of CRE decoy or control oligonucleotide using DOTAP. After 24 h, the medium was changed with fresh medium containing 10% FBS, and the cells were harvested at 48–72 h, and then assayed for CAT activity. Lysates (50–100 μg of protein) were incubated with 0.4 μCi of [^{14}C]-chloramphenicol, 0.53 mM acetyl-CoA, and 250 mM Tris, pH 7.8, for 90 min at 37 °C. Under these conditions, CAT activity was linear with time. Reaction products were analyzed by thin layer chromatography (TLC), and the plate was autoradiographed. The transfection efficiency was normalized by β-galactosidase activity. The activity of β-galactosidase was determined using luminescent β-galactosidase detection kit and luminometer.

2.7. Western blotting analyses

Nuclear proteins (20–40 μg) were separated on 12.5% SDS-polyacrylamide gel electrophoresis, and separated proteins were transferred onto nitrocellulose membrane using semidry blotting [13,14]. Anti-CREB and anti-phospho CREB antibodies were used as primary antibodies at a 1:1000 dilution.

3. Results

3.1. γ-Irradiation induces CREB phosphorylation, CREB-DNA binding and CRE-directed transcriptional activity

Western blot analyses of nuclear extracts showed that CREB was rapidly phosphorylated in response to γ-irradiation (Fig. 1**A**). Increased levels of CREB phosphorylation were observed 20 min after 10 Gy γ-irradiation and maintained up to 6–24 h depending on the cell type. In addition, when cancer cells were treated with increasing doses of γ–ray and harvested in 2 h, CREB phosphorylation was gradually increased from 2 to 10 Gy in all three cancer cell lines (Fig. 1B). Collectively, CREB phosphorylation increased in time- and dose-dependent manners. In addition, *in vitro* CRE DNA-protein binding of nuclear extracts of the γ-irradiated cells clearly increased. (Fig. 1C). Transcription of somatostatin-CAT fusion gene reporter was also increased in a dose-dependent manner (Fig. 1D).

It has been previously reported that the CRE-palindromic oligonucleotide can penetrate cells, compete with CRE enhancers for binding transcription factors, and specifically interfere with CRE- and AP-1-directed transcription [4]. As shown in Fig. 1F, the shift bands disappeared in adding 25-fold molar excess of unlabeled cold oligonucleotide containing a copy of CRE but not AP-1 or Oct-1, indicating sequence specificity (Lanes 1–3). In the presence of anti-CREB antibody (Lane 4), the loss of lower shift band was accompanied by the appearance of supershift band, indicating CREB-DNA binding. Lanes 5–7 in Fig. 1F confirms that CRE decoy oligonucleotide specifically interferes *in vivo* with the formation of CREB-DNA complex including CREB as compared to saline or control oligonucleotide.

To confirm CRE decoy oligonucleotide inhibits not only DNA binding of CREB but also transcriptional activity, cells were transfected with somatostatin-CAT fusion gene in the presence of CRE decoy oligonucleotide or control oligonucleotide (Fig. 1E). CRE decoy oligonucleotide markedly decreased CAT activity, as compared to saline or control oligonucleotide treatment.

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