Biochimica et Biophysica Acta 1842 (2014) 2656–2663

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Inhibition of soluble adenylyl cyclase increases the radiosensitivity of prostate cancer cells ☆

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ARTICLE INFO

Article history:
Received 8 May 2014
Received in revised form 15 September 2014
Accepted 16 September 2014
Available online 23 September 2014

Keywords:
Prostate cancer
Radioresistance
cAMP
EPAC
ERK1/2

ABSTRACT

Pharmacological modulation of tumor radiosensitivity is a promising strategy for enhancing the outcome of radiotherapy. cAMP signaling plays an essential role in modulating the proliferation and apoptosis of different cell types, including cancer cells. Until now, the regulation of this pathway was restricted to the transmembrane class of adenylyl cyclases. In the present study, the role of an alternative source of cAMP, the intracellular localized soluble adenylyl cyclase (sAC), in the radiosensitivity of prostate cancer cells was investigated. Pharmacological inhibition of sAC activity led to marked suppression of proliferation, lactate dehydrogenase release, and induction of apoptosis. The combination of ionizing radiation with partial suppression of sAC activity (~50%) immediately after irradiation synergistically inhibited proliferation and induced apoptosis. Overexpression of sAC in normal prostate epithelial PNT2 cells increased the cAMP content and accelerated cell proliferation under control conditions. The effects of radiation were significantly reduced in transformed PNT2 cells compared with control cells. Analysis of the underlying cellular mechanisms of sAC-induced radioresistance revealed the sAC-dependent activation of B-Raf/ERK1/2 signaling. In agreement with this finding, inhibition of ERK1/2 in prostate cancer cells enhanced the cytotoxic effect of irradiation. In conclusion, the present study suggests that sAC-dependent signaling plays an important role in the radioresistance of prostate cancer cells. This article is part of a Special Issue entitled: The role of soluble adenylyl cyclase in health and disease.

1. Introduction

Prostate cancer remains the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in men [1]. The current standard of care consists of prostatectomy and radiation therapy, which may often be supplemented with hormonal therapies. However, nearly 30% of patients experienced relapse at the irradiation sites [2]. The resistance of prostate cancer to radiotherapy has prompted a search for new methods of treatment, such as a combination of radiotherapy with the targeting of certain molecular mechanisms responsible for the radioresistance of prostate cancer.

In particular, many anti-tumor strategies focus on suppressing the cell cycle or inducing apoptosis. Within several signaling pathways, cAMP-dependent signaling plays a substantial role in controlling cell proliferation and apoptosis. However, the specific effects of cAMP signaling on proliferation and apoptosis are controversial. For example, the elevation of cellular cAMP content by stimulating G protein-responsive transmembrane adenylyl cyclases (tmAC) or by treatment with cAMP analogs has been shown to either induce or suppress proliferation in different cell types [3–6]. Similarly, cAMP signaling has conflicting effects on apoptosis [7–10]. This discrepancy may be due to differences in cell types or experimental models. Alternatively, the lack of specificity of methods to affect the cAMP signaling, e.g. forskolin, cAMP analogs or PKA inhibitors, used in majority of studies, may also be a cause for this discrepancy. Indeed, overproduction of cAMP by tmAC due to treatment with forskolin unlikely will lead to selective activation of plasmalemma-localized targets.

Traditionally, cAMP signaling was thought to rely solely on the production of cAMP at the plasma membrane by tmAC. However, work by many groups, including our own, has changed this view. tmAC can continue to signal within the cell following internalization, thereby defining endocytic cAMP microdomains [11,12]. Furthermore, a second source of cAMP, type 10 soluble adenylyl cyclase (sAC), has been identified in mammalian cells [13]. sAC, unlike tmAC, possesses no transmembrane domains and is localized throughout the cell in the cytosol, nucleus, mitochondria, and centriole [14]. Several studies demonstrated the unique activation of sAC by bicarbonate and bivalent

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http://dx.doi.org/10.1016/j.bbadis.2014.09.008
0925-4439/

cations as well as the role of sAC in the function of various cells and organs, such as sperm and neutrophils as well as the brain, kidney, eye, and pancreas [15]. Furthermore, recent studies have demonstrated that sAC regulates oxidative phosphorylation in mitochondria [16] and activates the mitochondrial pathway of apoptosis [17].

The role of the sAC-dependent cAMP pool in proliferation control was unknown. Our recent study demonstrates that sAC promotes prostate cancer cell proliferation in an EPAC-dependent manner [18]. Other studies have also suggested that nuclear sAC expression is associated with the transition of keratinocytes and melanocytes from benign cells into cancers, such as squamous cell carcinoma of the skin and melanoma [19,20]. A recent report by Onodera et al. [21] also demonstrated the involvement of sAC/EPAC signaling in the proliferation of breast cancer cells. Altogether, these observations suggest that sAC plays a role in proliferation of some cancer cells and may contribute to the tumor malignancy. We therefore hypothesized that the inhibition of sAC could be effective for potentiation of the radiation-induced cytotoxicity. To test this hypothesis, we utilized human prostate carcinoma cells and investigated the anti-proliferative, cytotoxic and pro-apoptotic effects of a combined treatment including sAC inhibition and irradiation.

2. Material and methods

2.1. Cell culture

Androgen-sensitive LNCaP (ATCC-Nr. CRL-1740D) and androgen-insensitive PC3 (ATCC-Nr. CRL-1435D) human prostate carcinoma cell lines were purchased from the American Type Culture Collection, and the normal human prostate epithelial cell line PNT2 was purchased from Sigma-Aldrich (Cat. Nr. 95012613). Cells were expanded and frozen in aliquots within 4 weeks of purchase. For the experiments in this study, the cells were thawed and cultured for no more than 3 additional passages. The LNCaP and PC3 cells were cultured in Dulbecco's modified Eagle's medium that was supplemented with 5% fetal calf serum, 100 U/mL penicillin, and 100 μg/μL streptomycin. PNT2 cells were cultured in RPMI-1640 medium that was supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/μL streptomycin. The cells were seeded in culture medium containing 2% fetal calf serum (LNCaP and PC3) or 5% fetal calf serum (PNT2) 12–18 h prior to each experiment.

2.2. Experimental protocols

U0126 (Cell Signaling), KH7 (Cayman) or its inactive analog, KH7.15 (kindly provided by Dr. J. Buck, Cornell University, NY), was applied to the cells throughout the duration of the experiment as indicated. At the end of the experiments, the floating cells were collected and combined with the attached cells for further analyses.

2.3. Radiation treatment

The cells were irradiated with 250 keV X-rays (16 mA) produced by a Müller RT250 (Philips Industrial X-Ray) at a dose rate of 1.5 Gy/min. The cells were returned to an incubator after the irradiation procedure and maintained at 37 °C and 5% CO_2 until further use.

2.4. sAC overexpression

The cloning of the untargeted expression of sAC, i.e., predominantly in the cytosol, was performed as previously described [22]. Briefly, the human influenza hemagglutinin (HA)-tagged rat sAC gene constructs were generated by PCR, digested with AgeI and NotI, and cloned into the eukaryotic expression vector pTurbo-G418 (Evrogen-Axxora). The plasmids encoding sAC or control vector (donated by Dr. Giovanni Manfredi, Weill Cornell Medical College, NY) were transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) and then incubated for 24 h. sAC expression in the transfected cells was analyzed using western blotting and antibodies against sAC.

2.5. Western blotting

The cells were lysed in Laemmli buffer containing 2% SDS, 10% glycerol, 0.1% 2-mercaptoethanol, 0.002% bromophenol blue, and 0.0625 mol/L Tris–HCl. The protein concentrations were determined using the Pierce 660 nm Protein Assay Reagent combined with the Ionic Detergent Compatibility Reagent (Thermo Scientific). Equal amounts of total proteins were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The utilized primary antibodies were as follows: sAC (clone R21, kindly provided by Dr. J. Buck, Cornell University, NY), actin (Chemicon International), cleaved caspase-3, phos-B-Raf, B-Raf, phos-ERK, and ERK1/2 (Cell Signaling). After incubation with peroxidase-linked and horseradish peroxidase-labeled secondary antibodies, specific bands were visualized by chemiluminescence using the ECLPlus Kit (Amersham Pharmacia). Equivalent sample loading (10–30 μg/well) was confirmed by stripping the membranes with Restore Western Blot Stripping Buffer (Pierce) prior to incubating the membranes with antibodies against actin.

2.6. Rap1 activity assay

The level of activated Rap1 was determined using a pull-down assay kit (Jena Bioscience, Jena, Germany), which is based on glutathione S-transferase fusion to the Ras-binding domain of RalGDS, following the manufacturer's instructions. The amounts of GTP-bound Rap1 and total Rap1 were determined by western blotting followed by staining with an anti-Rap1A antibody.

2.7. Analysis of cell number

After staining with 4% trypan blue (Gibco), trypan blue-negative cells were counted using a Neubauer hemocytometer (depth: 0.1 mm).

2.8. Cellular cAMP analysis

Analysis of the total cellular cAMP content was performed using the cAMP (direct) enzyme immunoassay kit (Assay Designs). The preparation of cell extracts and cAMP measurements were performed following the manufacturer's protocol. The absorbance measured at 405 nm was used to calculate the concentration of cAMP by applying a calibration curve.

2.9. FACS-based subG1-population analysis

The cells were fixed with 70% alcohol, stained with propidium iodide and treated with RNase (BD Biosciences, Heidelberg, Germany), which was followed by DNA content analysis using a FACSCalibur flow cytometer (BD Biosciences).

2.10. Analysis of lactate dehydrogenase in the culture medium

Lactate dehydrogenase (LDH) activity in the cell culture medium was used as an indicator of necrosis and was determined using the Cytotoxicity Detection Kit (Roche Applied Science). After each experiment, the culture medium was centrifuged at 500 ×*g* for 5 min at 4 °C, and the supernatant was subjected to LDH analysis using an ELISA reader.

2.11. Statistical analysis

The data are presented as the mean ± SEM. Comparisons of the means of the groups were performed using one-way analysis of variance followed by the post hoc Bonferroni test. $P < 0.05$ was considered statistically significant.