Free Radical Biology and Medicine 53 (2012) 1500–1513

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

Radiosensitization by 2-deoxy-D-glucose and 6-aminonicotinamide involves activation of redox sensitive ASK1-JNK/p38MAPK signaling in head and neck cancer cells

Pradeep Kumar Sharma [a,b], Bilikere Srinivasa Dwarakanath [a], Rajeev Varshney [a,c,*]

[a] Institute of Nuclear Medicine and Allied Sciences, DRDO, Brig. S K Mazumdar Road, Delhi-110 054, India
[b] University School of Biotechnology, Guru Gobind Singh Indraprastha University, sector-16 C, Dwarka, New Delhi-110075, India
[c] 533, O/o SA to RM, Secretary Defence R&D & DG DRDO, DRDO Bhawan, Ministry of Defence, Government of India, Rajaji Marg, New Delhi-110 105, India

ARTICLE INFO

Available online 21 July 2012

Keywords:
2-deoxy-D-glucose
6-aminonicotinamide
Oxidative stress
ASK1
Redox alterations

ABSTRACT

Our previous studies on simultaneous inhibition of glycolysis by 2-deoxy-D-glucose (2-DG) and pentose phosphate activity by 6-aminonicotinamide (6-AN) have been shown to induce oxidative stress mediated selective radiosensitization in wide range of human malignant cells. However, the mechanism of radiosensitization induced by this combination (2-DG+6-AN) is not completely understood. Since activation of apoptotic signal regulating kinase (ASK1) and subsequent apoptosis are implicated in oxidative stress response, the role of ASK1 activation in radiosensitization by this combination was investigated in the present study. Our results demonstrated that redox alterations induced by this combination activated ASK1 and subsequent apoptosis during radiosensitization of head and neck carcinoma cells (KB). In addition, mRNA and protein expression of thioredoxin and thioredoxin reductase decreased significantly under similar treatment conditions. Further, the downstream targets such as JNK and p38MAPK were also activated by this combination, and their pharmacological inhibition by SP600125 and SB201291 respectively resulted in suppression of 2-DG+6-AN mediated apoptosis in irradiated KB cells. Interestingly, the activation of ASK1 was mediated by hydrogen peroxide rather than superoxide anions as PEG-catalase but not PEG-SOD suppressed its activation. Our observations clearly suggest that redox alterations by inhibition of glucose metabolism serves as a molecular switch that activate ASK1-JNK/p38MAPK signaling in malignant cells during radiosensitization by 2-DG+6-AN. The present study emphasizes the importance of redox alterations in determining radiosensitivity of tumor cells that may greatly influence the outcome of radiation therapy.

Introduction

Evasion from apoptosis is one of the hallmarks of cancer phenotype that has been suggested as one of the factors responsible for their resistance towards variety of treatments including radiotherapy and pharmacological drugs [1]. Cancer cells often exhibit an elevated level of reactive oxygen species (ROS) than their normal counterparts and therefore, maintenance of redox homeostasis has been suggested as an important mechanism of their survival [2]. Moreover, enhanced aerobic glycolysis (the Warburg effect) and pentose phosphate pathway (PPP) activity have been shown to protect cancer cells from oxidative damage by maintaining their redox homeostasis via producing more pyruvate and NADPH respectively [3–5]. Further, many lines of evidences have clearly shown that either glucose deprivation or inhibition of glucose metabolism resulted in oxidative stress-mediated activation of apoptotic signaling and subsequent cytotoxicity in malignant cells [6–11]. Therefore, it has been hypothesized that inhibition of glucose metabolism may shift the cellular redox homeostasis in favor of increasing intracellular ROS leading to oxidative stress-mediated activation of apoptotic signaling and render cancer cells more susceptible to radiation-induced cytotoxicity.

Although, it is known that 2-deoxy-D-glucose (2-DG) inhibits both glycolysis and PPP, however, a great degree of heterogeneity in 2-DG-induced radiosensitization has been observed in variety

Abbreviations: 2-DG, 2-deoxy-D-Glucose; 6-AN, 6-aminonicotinamide; ROS, reactive oxygen species; PPP, pentose phosphate pathway; Trx, thioredoxin; ASK1, Apoptosis signal-regulating kinase 1; JNK, c-Jun N-terminal kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MAPKK, mitogen-activated protein kinase kinase; DCFDA, 2'7'dichlorofluorescein diacetate; DHE, Dihydroethidium; PBS, phosphate buffered saline; NAC, N-acetyl cysteine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Redox sensor red CC-1, 2, 3, 4, 5, 6-pentafluorotetramethyldihydrorosamine)

* Corresponding author at: 533, O/o SA to RM, Secretary Defence R&D & DG DRDO, DRDO Bhawan, Ministry of Defence, Government of India, Rajaji Marg, New Delhi-110 105, India. Fax: +91 01123792628.
E-mail addresses: rvarshney@rediffmail.com, rvarshney@hqr.drdo.in (R. Varshney).

0891-5849/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.freeradbiomed.2012.07.001

of human malignant cell lines [10]. Also it has been reported that 2-deoxy-D-glucose-6-phosphate (phosphorylated product of 2-DG by Hexokinase) thus formed is then oxidized to 2-deoxygluconate-6-phosphate by glucose 6-phosphate dehydrogenase (G6PD), leading to the regeneration of one molecule of NADPH [12]. Hence, inhibition of glycolysis and PPP by 2-DG alone did not inhibit NADPH regeneration maximally [13]. Therefore, inhibition of PPP activity by 6-aminonicotinamide (6-AN; inhibitor of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) along with inhibition of glycolysis (by 2-DG) was suggested to have an advantage in radiosensitization of tumor cells due to severe depletion of both NADPH and ribose-5-phosphate moieties which are required for the maintenance of redox homeostasis and biosynthesis of nucleotides respectively, besides minimizing the heterogeneous response of 2-DG-induced radiosensitization in variety of malignant cell lines.

Indeed, our previous studies have clearly shown that simultaneous inhibition of glycolysis by 2-deoxy-D-glucose (2-DG) and pentose phosphate activity by 6-aminonicotinamide (6-AN) enhanced oxidative stress-mediated radiation damage selectively in variety of human malignant cells [14–18]. Abrogation of GSH-mediated antioxidant defense besides non-coordination in the expression of antioxidant enzymes led to oxidative stress mediated apoptosis in irradiated malignant cells via mitochondrial dysfunction following treatment with their combination (i.e., 2-DG+6-AN) [14–18]. Moreover, alterations in cellular redox homeostasis due to inhibition of glucose metabolism has been suggested as a molecular switch that may serve as a promising therapeutic target to potentiate radiation damage selectively in malignant cells. However, the mechanisms underlying and downstream signaling events involved in 2-DG+6-AN-induced selective radiosensitization of malignant cells are not completely understood.

It is increasingly becoming clear that ROS-mediated redox alterations play important roles in the regulation and control of apoptotic signaling via activation of apoptosis signal-regulating kinase 1 (ASK1). Various cellular redox regulating systems, such as the glutathione (GSH), thioredoxin (Trx), and pyridine nucleotide redox couples ($NADP^+$/NADPH), are being considered as central in redox regulation, cell signaling and ASK1-mediated apoptosis (as reviewed in ref. [19]). ASK1 is a redox sensitive member of mitogen-activated protein kinase kinase kinase (MAPKKK) family that induces apoptotic cell death when activated by exposure to various cytotoxic stresses including reactive oxygen species (ROS) [20–22]. The activated ASK1 activates two different subgroups of MAP kinase kinases (MAPKK), SEK1 (or MKK4) and MKK3/MAPKK6 (or MKK6), which subsequently activates c-Jun N-terminal kinase (JNK) and p38 MAPK pathways respectively, and induces apoptosis in cells through the mitochondria-dependent caspase activation [23,24]. In addition, activated ASK1 is required for sustained activation of JNK and p38MAPK as ASK1 deficient mouse embryonic fibroblasts were resistant to hydrogen peroxides (H_2O_2) or tumor necrosis factor (TNF-α)-mediated apoptosis [22]. Several lines of evidence have suggested an association between activated ASK1and apoptosis under various stress conditions including H_2O_2 and TNF-α treatment [23,25,26]. Consistent to these observations, a catalytically inactive form of ASK1 has been shown to inhibit TNF-α-induced apoptosis [22,23,27]. Therefore, oxidative stress has been suggested as one of the most potent activators of ASK.

Thioredoxin (Trx), a redox regulatory protein, is a physiological inhibitor of ASK1 activity and also acts as an antioxidant [28], a transcription factor regulator [29], and an anti-apoptotic molecule [30]. The reduced Trx remains bound to ASK1 in redox dependent manner to form a complex to inhibit the activity of ASK1 and subsequent induction of apoptosis via activation of ASK1-JNK/p38 pathway [21]. In fact, dissociation of Trx from ASK1 and activation of downstream ASK1-p38MAPK/JNK-MAP signaling has been shown to promote apoptosis during oxidative conditions possibly due to oxidative modifications in Trx which prevented the formation of Trx-ASK complex to inhibit the ASK1 activity [23]. These observations suggest that redox state of a cell could be an important determinant of either formation or dissociation of ASK1-Trx complex and subsequent apoptosis in response to apoptotic stimuli such as oxidative stress. Since, a strong correlation has been suggested between ROS and ASK1-mediated apoptosis, the present study was, carried out to investigate the possible involvement of ASK1-mediated signaling events in the induction of apoptosis as one of the mechanisms responsible for radiosensitization induced by this combination (2-DG+6-AN) in malignant head and neck squamous carcinoma cells (KB). Results indeed demonstrated that simultaneous inhibition of glycolysis and PPP activity results in ROS-mediated activation of ASK1-JNK/p38MAPK signaling and subsequent apoptosis due to severe redox alteration during radiosensitization of KB cells by 2-DG+6-AN.

Materials and methods

Cell culture

KB {Plating Efficiency (PE)=0.82} cells derived from human head and neck squamous carcinoma were maintained as monolayer cultures at 37 °C in a humidified CO_2 incubator (5% CO_2, 95% air). KB cells were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), HEPES (10 mM) and antibiotics (30 μg ml^{-1} penicillin G, 50 μg ml^{-1}streptomycin and 2 μg ml^{-1} nystatin). KB cells were routinely sub-cultured (twice a week) using 0.05% trypsin in 0.02% EDTA and reseeded in fresh medium. All experiments were carried out in exponentially growing cells. High glucose DMEM and FBS were purchased from SIGMA chemicals (SIGMA, USA).

Experimental procedure and irradiation

Monolayer KB cells were grown for 24 h (exponential growth phase) before treatment in high glucose DMEM containing 10% fetal bovine serum (FBS). All treatments were carried out under liquid holding conditions using Hanks balanced salts solution (HBSS) containing 5 mM glucose. 6-aminonicotinamide (6-AN; 5 μM) and 2-deoxy-D-Glucose (2-DG; 5 mM) were added simultaneously just prior to irradiation (2 Gy) [16]. The drug concentrations were optimized previously for effective radiosensitization response. After 4 h of incubation, drugs were removed by washing and replaced with fresh growth medium. A ^{60}Co γ-radiation source (Gamma Cell, Atomic Energy of Canada Ltd (AECL) Chalk River, Ontario, Canada) was used for irradiation at a dose-rate of 0.8 Gy/min.

Detection of reactive oxygen species and superoxide radicals

Reactive oxygen species (ROS) generation was detected using two dyes namely 2' 7' dichlorofluorescein diacetate (DCFDA) and dihydroethidium (DHE) as described earlier [17]. Detection of superoxide anions was performed by using Mitosox red probe (Molecular probes). Briefly, KB cells (0.1×10^6 cells) were plated in 35 mm Petri dishes and treated as mentioned in experimental procedure. After 24 h following treatment, cells were incubated with Mitosox red (5 μM) at 37 °C for 15 min in the dark. Finally, cells were washed with cold PBS, trypsinized and resuspended in