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Research Paper

S-nitrosoglutathione-mediated STAT3 regulation in efficacy of radiotherapy and cisplatin therapy in head and neck squamous cell carcinoma ☆

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ABSTRACT

S-nitrosoglutathione (GSNO) is an endogenous nitric oxide (NO) carrier that plays a critical role in redox based NO signaling. Recent studies have reported that GSNO regulates the activities of STAT3 and NF-κB via S-nitrosylation dependent mechanisms. Since STAT3 and NF-κB are key transcription factors involved in tumor progression, chemoresistance, and metastasis of head and neck cancer, we investigated the effect of GSNO in cell culture and mouse xenograft models of head and neck squamous cell carcinoma (HNSCC). For the cell culture studies, three HNSCC cell lines were tested (SCC1, SCC14a and SCC22a). All three cell lines had constitutively activated (phosphorylated) STAT3 (Tyr^{705}). GSNO treatment of these cell lines reversibly decreased the STAT3 phosphorylation in a concentration dependent manner. GSNO treatment also decreased the basal and cytokine-stimulated activation of NF-κB in SCC14a cells and reduced the basal low degree of nitrotyrosine by inhibition of inducible NO synthase (iNOS) expression. The reduced STAT3/NF-κB activity by GSNO treatment was correlated with the decreased cell proliferation and increased apoptosis of HNSCC cells. In HNSCC mouse xenograft model, the tumor growth was reduced by systemic treatment with GSNO and was further reduced when the treatment was combined with radiation and cisplatin. Accordingly, GSNO treatment also resulted in decreased levels of phosphorylated STAT3. In summary, these studies demonstrate that GSNO treatment blocks the NF-κB and STAT3 pathways which are responsible for cell survival, proliferation and that GSNO mediated mechanisms complement cispaltin and radiation therapy, and thus could potentiate the therapeutic effect in HNSCC.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer and one of the leading causes of cancer deaths worldwide. Recent advances in the field of HNSCC include development of free flaps for reconstruction after surgery or intensity modulated radiation therapy for targeted radiotherapy [1,2]. However, the prognosis for these patients has not improved significantly. Therefore, better agents are needed to improve therapeutic outcomes.

Aberrant or constitutive activation of STAT3 and NF-κB has been detected in many human malignancies including HNSCC [3,4,6]. STAT3 and NF-κB play key roles in the regulation of immune/inflammatory responses, but growing evidence also supports a major role in oncogenesis [3,7]. Molecular targeting of STAT3 by various methods, such as interfering in dimerization and siRNA approaches, has been shown to inhibit tumor growth in preclinical models of human cancer [8,9]. However, these are not considered drug candidates because of uncertain feasibility of these approaches in clinical practice. NF-κB has been implicated in cancer progression by activation genes that stimulate cancer cell proliferation and survival, angiogenesis, and metastases, and thus

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is considered an interesting therapeutic target for treatment of cancer [4]. However, presently there is no effective treatment strategy for managing the aberrant NF-κB activity in cancers.

While studying the inflammatory pathways in cell culture and animal models of multiple sclerosis (experimental autoimmune encephalomyelitis) and stroke (middle cerebral artery occlusion), our laboratory reported that NF-κB activation under inflammatory conditions is inhibited by S-nitrosylated glutathione (GSNO) treatment [10,11]. Since NF-κB has been also reported to be regulated by glutathionylation [12,13], these studies document the roles of the cellular GSNO as well as redox potential in regulation of NF-κB pathways. In addition, we have also recently reported that STAT3 activation induced by IL-6 is inhibited by endogenously generated nitric oxide (NO) from inducible NO synthase (iNOS) or exogenously supplemented GSNO via S-nitrosylation of Cysteine 259 residue of STAT3 molecule, documenting that STAT3 S-nitrosylation is a physiological regulation [14]. We have also reported that inhibition of STAT3 by GSNO is implicated in TH17 immunomodulation under EAE conditions [15] and anti-proliferation of microglia and cancer cells [16,17]. GSNO is an endogenous NO carrier which plays a crucial role in redox based nitrosylation of protein thiols (S-nitrosylation) in health and disease [18] and is now recognized as an important cell signaling mechanism [19]. Although NF-κB and STAT3 have been implicated in tumorigenesis, metastasis, and chemoresistance [7,20], the biological function of NO and GSNO in regulation of NF-κB and STAT3 in cancer cells and their associated cell signaling pathways leading to cancer progression are not understood well at present.

NO, synthesized by nitric oxide synthases (NOS), is shown to have both pro- and anti-tumor activity [21–28]. These multifactorial effects may be manifested via a range of chemical modifications caused by different properties of the source of NO levels and its metabolites (NO_2, NO^+, and $NO^\bullet$) in nitration, nitrosation or nitrosylation of target molecules as well as the redox environment of the tumor. However, the observed chemosensitization of tumor by NO in doxorubicin resistance by nitroglycerine [27,28], efficacies of NO-nonsteroidal anti-inflammatory drugs [29] as well as direct efficacy of NO compounds in epithelial to messenchymal transition in cancer cells [25], and inhibition of cell proliferation or tumor growth by GSNO treatment in multiple myeloma cell lines [16] and ovarian cancer xenografted mouse models [17] suggests that NO compounds may be considered as potential therapeutic agents for blocking the metastasis cascade and for treatment of patients with refractory cancer. Understanding the different actions of NO and its metabolites in cancer at the molecular level can provide insights in devising potential strategies for cancer treatment.

Based on the fact that STAT3 and NF-κB are involved in tumor growth, angiogenesis and invasion and that GSNO, an endogenous nitrosylating agent, can inhibit activation of STAT3 [15] and NF-κB [10,30], we investigated the efficacy of GSNO in head and neck cancer using HNSCC cells in vitro and in vivo xenograft models. The studies described in this manuscript document that GSNO treatment inhibits cell survival and proliferation in in vitro cultures of HNSCC cells and also inhibits tumor growth in the xenograft animal model. Moreover, GSNO mediated mechanisms against tumor growth complement ionizing radiation and cisplatin treatments to provide greater efficacy. This study for the first time, documents that GSNO mediated inactivation of STAT3 signaling pathway may prove to be of therapeutic potential in the treatment of head and neck cancer.

2. Materials and methods

2.1. Cell cultures

HNSCC cell lines UM-SCC-1 (retromolar trigone/floor of the mouth; SCC1), UM-SCC-14A (SCC of anterior floor of the mouth; SCC14a), and UM-SCC-22A (SCC of hypopharynx; SCC22a) were obtained from Dr. Thomas Carey (Department of Otolaryngology/Head and Neck Surgery, University of Michigan) [31]. These cells lines were cultured in Dulbecco's Modified Eagle's Medium/Ham's F-12 (50/50) (Mediatech Inc., Manassas, VA) with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/mL streptomycin. The cells were grown at 37 °C under 5% CO_2/95% air.

2.2. Cell survival, cell proliferation, and cell cycle assay

The cells in 96-well culture plates in serum free medium were treated with different doses of drugs/radiation. The fractions of the surviving cells were measured by colorimetric assay using WST-8 tetrazolium salt (Cayman Chemical, Ann Arbor, MI). The amount of formazan dye generated from WST-8 by the activity of dehydrogenases in cells was measured by SpectraMax 190 ELISA reader (Molecular Devices, Sunnyvale, CA) at 450 nm. For cell proliferation assay, the cells were treated with 5-bromo-2′-deoxyuridine (BrdU; 10 μM) for 4 h and BrdU incorporated into DNA was measured by colormetric BrdU assay kit (Cell Signaling Technology, Danvers, MA) as per manufacturer's instruction. Cell cycle analysis was performed by flow cytometry following the staining of cells with propidium iodide. Briefly, the ethanol fixed cells were treated with RNase solution and then stained with propidium iodide (50 μg/ml). The samples were analyzed by FACSCalibur™ flow cytometer with Cell QuestTM software (BD Biosciences, San Jose, CA). Expression of cell survival and cell cycle regulators was measured by Western blot analysis using antibodies specific to Bcl-xL, cIAP, and c-Myc (Cell Signaling Technology).

2.3. Assay for STAT3 activation

The activities of STAT3 were analyzed as Western analysis for phosphorylated (Tyr705) STAT3 (pSTAT3) and total STAT3 with specific antibodies (Cell Signaling Technologies, Danvers, MA). For immunofluorescent staining of pSTAT3/STAT3, SCC14a cells cultured on chamber slides (LabTek, Nunc, Inc., Naperville, IL) were fixed in cold methanol and incubated with primary antibodies against phospho- or pan-STAT3 and then secondary antibody (goat anti-Rabbit IgG conjugated with DyLight 488 or 594) (Jackson Immunoresearch Laboratories Inc. West Grove, PA). 4′,6-diamidino-2-phenylindole (DAPI) was used for nuclei counter-stain. Immunofluorescence images were captured by fluorescent microscope equipped with Olympus digital camera (Olympus BX-60, Goleta, CA) and quantified by using Image-Pro 6.2 (Media Cybernetics, Silver Springs, MD). Identical illumination, microscope, and camera settings were used to obtain images for quantification.

2.4. Assay for NF-κB activation and HIF-1α accumulation

Nuclear and cytoplasmic extracts from SCC14a cells treated with GSNO and/or cytokines were prepared using a previously published method [32]. The cytoplasmic and nuclear levels of p65 NF-κB and HIF-1α were analyzed by Western analysis using specific antibodies (Cell Signaling Technologies). H3 histone and β-actin were used for internal loading control for nuclear and cytoplasmic proteins. The nuclear protein extracts were also used for the gel-shift assay for detection of NF-κB DNA binding activities. The gel-shift assay for NF-κB DNA binding activity was performed as described previously [32].