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Si RNA inhibition of GRP58 associated with decrease in mitomycin C-induced DNA cross-linking and cytotoxicity

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Abstract

The anti-cancer drug mitomycin C is metabolically activated to bind and cross-link DNA. The cross-linking contributes significantly to the cytotoxicity. The complex chemical structure of mitomycin C allows its metabolism by several known (cytosolic NAD(P)H:quinone oxidoreductase and microsomal NADPH:cytochrome P450 reductase) and unknown enzymes. The identification of new enzymes/proteins that metabolize mitomycin C and like drugs is an area of significant research interest since these studies have direct implications in drug development and clinical usage. In the present studies, we have investigated a role of cytosolic glucose regulatory protein GRP58 in mitomycin C-induced DNA cross-linking and cytotoxicity. The control and GRP58 siRNA were transfected in human colon carcinoma HCT116 cells in culture. The transfection of GRP58 siRNA but not control siRNA significantly inhibited GRP58 in human colon carcinoma HCT116 cells. The inhibition of GRP58 led to decrease in mitomycin C-induced DNA cross-linking and cytotoxicity. These results establish a role of GRP58 in mitomycin C-induced DNA cross-linking and cytotoxicity. Site-directed mutagenesis of cysteines to serines in thioredoxin domains of GRP58 and cross-linking assays revealed that both N- and C-terminal thioredoxin domains are required for GRP58-mediated mitomycin C-induced DNA cross-linking. These results suggest that GRP58 might be an important target enzyme for further studies on mitomycin C and similar drug therapy.

Keywords: GRP58; Mitomycin C; DNA cross-linking; Cytotoxicity

1. Introduction

The bioreductive chemotherapy is a successful treatment for certain types of cancer. It is based on activation of drugs by specific proteins and the tumor types that are rich in those proteins (1). Bioreductive drugs development is also based on differences in oxygen content and cellular pH between normal and tumor tissues [1,2]. Mitomycin C is a prototypical bioreductive alkylating anti-tumor agent that is effective against several tumor tissues including colon, breast, lung, head and neck [3]. Mitomycin C is metabolically activated to cause DNA adducts formation and DNA cross-linking that leads to cytotoxicity and cell death [4,5]. Cytosolic NAD(P)H:quinone oxidoreductase 1 (NQO1) and microsomal NADPH:cytochrome P450 reductase (P450 reductase), cytochrome b5 reductase,

Abbreviations: GRP58, 58 kDa glucose regulatory protein also known as ERp57 or ERp60; MMC, mitomycin C; HCT116, human colon carcinoma cell; siRNA, small interfering RNA; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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xanthine oxidoreductase and dehydrogenase, have been shown to catalyze mitomycin C activation leading to DNA cross-linking and cytotoxicity [6–12]. There is continued interest to explore and identify additional cytosolic and microsomal proteins that selectively activate mitomycin C and other bioreductive drugs in tumors.

Glucose regulatory protein GRP58, also known as ERp57 or ERp60 is localized in endoplasmic reticulum, cytosol and nucleus [13–15]. In the endoplasmic reticulum, GRP58 specifically interacts with glycoproteins such as calnexin and calreticulin, playing an important role as a molecular chaperone of glycoprotein biosynthesis and assembly of newly synthesized MHC class I molecules, a process that also involves the chaperones calnexin and calreticulin [13]. In the nucleus, GRP58 has been indicated as a component of the subset of nuclear matrix proteins that are responsible for DNA attachment to the nuclear matrix and for the formation of DNA loops due to GRP58 binding to DNA [14,15]. In the cytosol, GRP58 has been identified as a chaperone for the signal transducer and activator of transcription (STAT) signaling in which STAT3 protein function in the cytoplasm as complexes with novel accessory scaffolding protein [16]. The biological role of GRP58 except its chaperone function remains unknown. Recently, cytosolic GRP58 is suggested to mediate mitomycin C-induced DNA cross-linking [17]. However, further experiments are needed to establish a role of GRP58 in mitomycin C-induced DNA cross-linking and also to determine the role of GRP58 in mitomycin C-induced cytotoxicity. In addition, the studies are required to investigate the role of thioredoxin domains at N- and C-terminus of GRP58 protein in mitomycin C-induced DNA cross-linking.

In the present report, we used GRP58 siRNA to study the role of GRP58 in mitomycin C-induced DNA cross-linking and cytotoxicity. We also investigated the role of thioredoxin domains of GRP58 in mitomycin C-induced DNA cross-linking. The GRP58 siRNA significantly inhibited GRP58 in human colon carcinoma HCT116 cells. The inhibition of GRP58 led to a decrease in mitomycin C-induced DNA cross-linking and decreased cytotoxicity. Site-directed mutagenesis of cysteines to serines in thioredoxin domains resulted in complete loss of GRP58 to mediate mitomycin C-induced DNA cross-linking. These studies establish a role of GRP58 in mitomycin C-induced DNA cross-linking and cell death. The studies also demonstrated requirement of both N- and C-terminal thioredoxin domains of GRP58 in mitomycin C-induced DNA cross-linking.

2. Materials and methods

2.1. Materials

The human colon carcinoma HT116 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cell culture reagents were obtained from Gibco-BRL (Gaithersburg, MD, USA). Mitomycin C (MMC) was purchased from Sigma–Aldrich Chemical Company (MO, USA). Polyclonal antibodies against purified full length GRP58 protein (SPA-585) was purchased from StressGen Biotechnologies Corp. (Victoria, Canada). All other reagents used in the experiments were of highest purity available commercially. The pre-designed control and GRP58 siRNA was obtained from Ambion, Austin, TX (catalogue #4510, 4511).

2.2. Cell culture, transfection and western analysis

HCT116 cells were grown in McCoy's 5a medium (modified) with 1.5 mM L-glutamine adjusted to contain 2.2 g/l sodium bicarbonate supplemented with 10% fetal bovine serum in incubator containing 95% air and 5% carbon dioxide. The cells were grown in monolayer and transfected with control or GRP58 siRNA by siRNA transfection kit and procedure from Ambion, Austin, TX. Si RNA concentration used was 30 nM as recommended by Ambion protocol and double concentration of 60 nM. The cells were washed with ice cold PBS and scraped, homogenized and subcellular fractionated to prepare cytosolic fraction by procedures as previously described [18].

2.3. Mitomycin C-induced DNA cross-linking assay

Mitomycin C-induced DNA cross-linking assays were performed by procedure as described [7,11,12]. Briefly, the two strands of the twenty-three base pairs of oligonucleotides containing mitomycin C binding site, were synthesized and used for the cross-linking experiments. The nucleotide sequence of the 23 base pair oligonucleotide was: sense strand 5′-CTA CAT CGT GTC ATG CAC AGG AT-3′ and anti-sense strand 5′-A GAT CCT GTG CAT GAC ACG ATG T-3′. The complementary strands were mixed in equal amounts and annealed by heating to 70 °C for 15 min and slowly cooling to room temperature. The 3′-end of the top strand was selectively labeled with DNA polymerase I large (Klenow) fragment (Promega, Madison WI) and [α-^{32}P]dCTP (NEN, Boston, MA). The labeled oligonucleotides were purified on a 15% non-denaturing poly-