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Hypoxia and retinoic acid-inducible NDRG1 expression is responsible for doxorubicin and retinoic acid resistance in hepatocellular carcinoma cells

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ABSTRACT

Hypoxia may activate survival signals in cancer cells. Moreover, hypoxic cells are less sensitive than normoxic cells to doxorubicin cytotoxicity, a potent activator of the p53 tumor suppressor gene. N-myc downstream-regulated gene-1 (NDRG1) is a hypoxia- and retinoic acid-inducible protein, and has been previously implicated in carcinogenesis. As this protein is also a downstream target of p53 and hepatocellular carcinoma (HCC) cells frequently evidence resistance to retinoic acid (RA) cytotoxicity, we attempted to determine whether the suppression of NDRG1 expression may sensitize HCC cells to doxorubicin and/or RA cytotoxicity. HCC cells expressed NDRG1 protein, and the expression of this protein was hypoxia- and RA-inducible. Doxorubicin treatment induced HCC cell cytotoxicity via the activation of mitochondrial apoptotic signals, including caspase-9 activation. Hypoxic HCC cells are less sensitive to doxorubicin-induced apoptosis. The suppression of NDRG1 expression either by siRNA or flavopiridol sensitized hypoxic HCC cells to doxorubicin cytotoxicity, and this was attributed to more profound augmentation of JNK and caspase-9 activation. The suppression of NDRG1 expression also sensitized RA-resistant HCC cells to RA-induced apoptosis, and this sensitization was more apparent in hypoxic HCC cells than in normoxic cells. Glutaredoxin2 expression was down-regulated in NDRG1-suppressed HCC cells. These results show that hypoxia- and RA-inducible NDRG1 expression is responsible for doxorubicin and RA resistance in HCC cells. Thus, the selective interruption of NDRG1 signaling may prove to be therapeutically useful in HCCs, particularly in the advanced infiltrative type of tumors exposed to hypoxic environments.

1. Introduction

Hepatocellular carcinoma (HCC) development is a major cause of mortality in patients with chronic liver disease. HCC is generally considered to be a hypervascular tumor [1] and therefore, transarterial chemoembolization (TACE) is employed as a standard treatment in unresectable HCCs on the basis of hypervascularity. However, advanced infiltrative HCCs seldom evidence hypervascularity and are usually refractory to TACE [2,3], although they grow more aggressively than mass-forming hypervascular HCCs. Moreover, the surviving cancer cells in HCC nodules treated with TACE, which confers a profound hypoxic insult, occasionally grow more rapidly than those in neighboring

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nodules [4]. Therefore, hypoxia is a strong stimulus for the generation of signals that enable HCC cells to survive and proliferate under hypoxic conditions [5].

Doxorubicin, a potent activator of p53, is commonly utilized for HCC chemotherapy, delivered via the intra-hepatic artery [6]. However, its efficacy is rather low, and its systemic injection has not thus far provided any significant and substantial benefits in terms of tumor regression and overall survival [7]. Moreover, hypoxic tumor cells generally show chemoresistance [8] and therefore, the surviving HCC cells following TACE or HCC cells of infiltrative-type are expected to be highly resistant to doxorubicin.

Retinoic acid (RA), a metabolite of vitamin A, exhibited anti-tumor activity against a variety of cancers, and is currently utilized as a therapeutic or chemo-preventative agent in the treatment of several human cancers, including acute promyelocytic leukemia [9]. With regard to HCCs, RA significantly reduced the incidence of second primary HCCs and exhibited survival benefits in patients who underwent resection or local ablation of HCCs [10,11]. Moreover, a phase II trial of a synthetic RA combined with TACE versus TACE alone in advanced HCC patients is currently underway. However, HCC cells occasionally show resistance to RA cytotoxicity [12].

N-myc downstream-regulated gene-1 (NDRG1) is a hypoxia- and RA-inducible protein, and has previously been implicated in carcinogenesis [13]. Doxorubicin is a potent p53 activator, and this NDRG1 protein is also a downstream target of p53. As hypoxic tumor cells are generally chemoresistant [8] and HCC cells frequently evidence RA resistance [12], we hypothesized that NDRG1 expression is responsible for doxorubicin and RA resistance in HCC cells, particularly under hypoxic conditions. In order to evaluate this hypothesis, we formulated the following questions: (i) Does hypoxia or RA increase NDRG1 expression in HCC cells? (ii) Are hypoxic HCC cells resistant to doxorubicin cytotoxicity? (iii) Does the suppression of NDRG1 expression sensitize HCC cells to doxorubicin and/or RA cytotoxicity, and finally (iv) What is the underlying mechanism of cell death? Collectively, these results show that hypoxia- and RA-inducible NDRG1 expression is responsible for doxorubicin and RA resistance in HCC cells. Thus, the selective interruption of NDRG1 signaling may prove therapeutically useful in HCCs, particularly in advanced infiltrative-type tumors that are exposed to a hypoxic environment.

2. Materials and methods

2.1. Cell line and culture

Human HCC cell lines were used in this study: Huh-7 cells (derived from a well-differentiated HCC [14]) and RA-resistant SNU-761 cells (derived from a poorly-differentiated HCC [15]). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 100,000 U/l of penicillin and 100 mg/l of streptomycin. In all of the experiments conducted in this study, the cells were serum-starved overnight in order to avoid the confounding variable of serum-induced signaling. According to the experimental design, the cells were subsequently incubated under standard culture conditions (20% O_2 and 5% CO_2, at 37 °C) or hypoxic culture conditions (1% O_2, 5% CO_2 and 94% N_2, at 37 °C). All-trans retinoic acid (RA) was purchased from MP Biomedicals (Solon, OH, USA). Doxorubicin and flavopiridol were obtained from Sigma–Aldrich (St. Luis, MO, USA).

2.2. Immunoblot analysis

The cells were lysed for 20 min on ice with lysis buffer (50 mM Tris–HCl, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 μl/ml aprotinin, leupetin, pepstatin; 1 mM Na3VO4; 1 mM NaF) and centrifuged for 10 min at 14,000*g* at 4 °C. Samples were resolved via 10% or 12% SDS–PAGE, transferred to nitrocellulose membranes, and blotted using appropriate primary antibodies with peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA, USA). Bound antibodies were visualized with a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, USA) and exposed to film (X-Omat; Kodak, Hannover, Germany). The primary antibodies utilized were mouse anti-phospho-c-Jun N-terminal kinase (anti-phospho-JNK), rabbit anti-phospho-p38, rabbit anti-phospho-p42/44 and rabbit anti-phospho-eIF2α purchased from Cell Signaling Technology (Beverly, MA, USA); rabbit anti-caspase-8 and rabbit anti-caspase-9 from Pharmingen (San Diego, CA, USA); goat anti-actin from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and anti-glutaredoxin-2 from Abcam (Cambridge, MA, USA). Images were detected by an analyzer (LAS-1000, Fuji Photo Film, Tokyo, Japan), and then densitometric analysis was performed using Image Gauge Software (Fuji Photo Film, Tokyo, Japan). The arbitrary units were calculated by densitometric scanning of the intensity of NDRG1 relative to actin intensity, taking that of 0 h as 1.

2.3. cDNA microarray analysis

In order to compare the relative gene expression profiles in Huh-7 cells with selectively suppressed NDRG1 expression, total RNA from Huh-7 cells transfected with NDRG1-specific or control siRNA was extracted and purified with a Micro-FastTrack 2.0 kit (Invitrogen, Carlsbad, CA, USA). Biotin-labeled complementary RNA was synthesized in accordance with the protocols recommended by Affymetrix (Affymetrix, Santa Clara, CA, USA). Complementary RNA samples were hybridized to GeneChip Human Genome U133A (Affymetrix), which contains 54,000 probe sets and 47,400 transcripts from 339,000 genes. Hybridization signals were scanned according to the protocol from Affymetrix, and data were analyzed with GeneChip Operating Software (Affymetrix).

2.4. Apoptosis

Apoptosis was evaluated by assessing the nuclear changes of apoptosis (i.e. chromatin condensation and nuclear fragmentation) using the binding dye 4′,6-diamidino-