Neutron Radiation Enhances Cisplatin Cytotoxicity Independently of Apoptosis in Human Head and Neck Carcinoma Cells

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ABSTRACT

Recent advances in combined modality treatment of locally advanced head and neck cancer have improved local and regional disease control and survival with better functional outcome. However, the local and regional failure rate after radiation therapy is still high for tumors that respond poorly to cisplatin-based neoadjuvant chemotherapy. This clinical observation suggests a common biological mechanism for resistance to cisplatin and photon irradiation. In this report, we investigated the molecular basis underlying cisplatin resistance in head and neck squamous carcinoma (HNSCC) cells and asked if fast neutron radiation enhances cisplatin cytotoxicity in cisplatin-resistant cells. We found that cisplatin sensitivity correlates with caspase induction, a cysteine protease family known to initiate the apoptotic cell death pathway, suggesting that apoptosis may be a critical determinant for cisplatin cytotoxicity. Neutron radiation effectively enhanced cisplatin cytotoxicity in HNSCCs including cisplatin-resistant cells, whereas photon radiation had little effect on cisplatin cytotoxicity. Interestingly, neutron-enhanced cisplatin cytotoxicity was associated neither with apoptosis nor with cell cycle regulation, as determined by caspase activity assay, annexin V staining, and flow cytometric analysis. Taken together, the present study provides a molecular insight into cisplatin resistance and may also provide a basis for more effective multimodality protocols involving neutron radiation for patients with locally advanced head and neck cancer.

INTRODUCTION

HNSCC constitutes ~5% of all new cancers diagnosed annually in the United States (1). Frequently, they are locally advanced and inoperable because of the extent of primary lesions or metastatic lymph nodes in the neck. Recent advances in management with a multidisciplinary approach including radiotherapy and chemotherapy resulted in improved local and regional disease control with better functional outcomes (2, 3). Cisplatin is among the most widely used and most effective chemotherapeutic agents for HNSCC patients. However, locally advanced head and neck tumors with poor response to neoadjuvant cisplatin chemotherapy also respond poorly to subsequent photon radiation treatment (4, 5). This clinical observation suggests the possibility of a common biological mechanism for resistance to cisplatin and photon irradiation treatments. Failure of photon radiation therapy is thought to involve tumor hypoxia and genetic changes causing intrinsic radioresistance. Compared with photons, neutrons have a higher linear energy transfer and generate more dense ionization, resulting in a greater number of free radicals and causing more double-stranded DNA breakage than photons. Neutrons differ from photons in the mode of their interactions with tissue. Whereas photons interact with the orbital electrons of the atoms of the absorbing material, neutrons interact with the nuclei of atoms of the absorbing material (6). At present, neutron radiation therapy for head and neck squamous cancer has been limited, especially in combination with chemotherapy.

In the present study, we investigated the molecular basis underlying cisplatin resistance in human HNSCC and whether neutron irradiation enhances cisplatin-induced cytotoxicity more effectively than photon irradiation. We also examined whether cytotoxicity is associated with apoptosis sensitivity and/or cell cycle arrest. For this study, we used five HNSCC cell lines derived from patients with well-documented clinical histories.

MATERIALS AND METHODS

SRB Assay. Cells in 96-well plates were washed with PBS, fixed with 10% ice-cold trichloroacetic acid at 4°C for 1 h, then washed with water five times, and dried at room temperature. The cellular proteins in each well were stained with 100 µl of 0.4% SRB in 1% acetic acid at room temperature for 20 min and then washed with 1% acetic acid four times and dried at 37°C for another 30 min. To dissolve the SRB bound to cellular protein, 200 µl of 10 mM Tris were added to each well and incubated at room temperature with mechanical agitation until the color became homogeneous. SRB bound to protein was quantified by the absorbance of SRB at 570 nm. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; SRB, sulforhodamine B; RBE, relative biological effectiveness.
measured by absorbance at a 550-nm wavelength using a Benchmark Micro-Plate Reader (Bio-Rad, Hercules, CA).

Caspase Activity Assay. Cells were collected at 0, 36, and 48 h after treatment with 5 μM cisplatin and then lysed in 50 mM Tris buffer (pH 7.5) containing 0.03% Nonidet and 1 mM DTT. Nuclei were removed by low-speed centrifugation (800 × g for 5 min), and the cytosol fraction was incubated with 40 μM DEVD-amc, 10 mM HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM DTT in a total volume of 200 μl for 60 min at 37°C. Fluoromethylcoumarin fluorescence, released by caspase activity, was measured using 360-nm excitation. A CCD device (Instaspec IV; Oriel, Stratford, CT) fitted with a monochromator was used to measure the fluorescence emission spectrum. The intensity at the optimum (~450 nm) was measured. DEVDase activity was normalized per mg of protein determined by BCA protein assay kit (Pierce).

Photon Irradiation. A Co-60 beam was used to irradiate the cells at a single dose of 2, 4, 6, or 12 Gy. The flasks containing cells were irradiated in a Lucite phantom at a dose rate of 85–100 cGy/min. Neutron Irradiation. The Wayne State d(48.5) + Be fast neutron beam was used to irradiate the cells at a dose of 0.67, 1.34, 2, or 4 Gy. The flasks containing cells were placed in a tissue-equivalent plastic phantom (TEP-A150) at the isocenter of the machine. The calibration of the experiment set-up was performed according to the international protocol for neutron dosimetry (7). The cells were irradiated at a dose rate of 20–30 cGy/min.

Cell Culture and Clonogenic Cell Survival Assay. Establishment of human HNSCC lines was described previously (8, 9). Cells were cultured in three parts DMEM/F-12 with 1 part F-12 nutrient medium supplemented with 10% fetal bovine serum, 30 μg/ml bovine pituitary extract, 0.1 ng/ml human epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 0.5 μg/ml fungizone in a 95% air and 5% CO₂ incubator at 37°C. One day prior to the experiment, cells were plated into T-25 flasks. After irradiation, cells were trypsinized and counted, and appropriate dilutions were made. The appropriate number of cells was plated into two replicate plates. After 1–3 weeks, colonies were stained and counted.
Detection of Apoptotic Cells by Annexin V Staining.

Cells were washed with ice-cold PBS, trypsinized, and resuspended in 1× binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂] at a concentration of 1 × 10⁶ cells/ml. One hundred µl of the cell solution were mixed with 5 µl of annexin V FITC (PharMingen) and 10 µl of propidium iodide stock solution (50 µg/ml in PBS) by gentle vortexing, followed by 15 min incubation at room temperature in the dark. Four hundred µl of 1× buffer were added to each sample and analyzed at the Imaging Flow Cytometry Core Facility within.

Fig. 3 Neutron enhances cisplatin-induced cytotoxicity in HNSCC. Confluent cells were treated with 1 µM cisplatin 2 h prior to photon or neutron radiation and cultured for an additional 48 h in the presence of 1 µM cisplatin. Cell survival was determined by SRB assay. A, cell survival after different treatments was normalized to untreated cells. B, cell survival after cisplatin treatment alone was arbitrarily given as 100%, and cell survival after cisplatin treatment in combination with photon or neutron radiation was normalized to that after cisplatin treatment alone in each cell line. All experiments were performed in triplicate; bars, SD.
1 h. Apoptotic cells were detected by annexin V FITC staining, and necrotic cells were detected by propidium iodide staining.

**Determination of Cell Cycle Distribution.** Cells were trypsinized, washed with PBS, and fixed with 70% ethanol. The fixed cells were spun down and resuspended in Hoechst staining solution at a concentration of 1 × 10⁶ cells/ml and incubated for 3 min at room temperature. The Hoechst staining solution consisted of 3 mg/ml Hoechst 33258 (Sigma Chemical Co., St. Louis, MO) in Tris buffer (2 mm MgCl₂, 0.1% Triton X-100, 154 mm NaCl, and 100 mm Tris, pH 7.5). The percentage of cells in each cell cycle phase was determined at the Imaging Flow Cytometry Core Facility at our institute.

**RESULTS**

**Cisplatin-induced Cytotoxicity Varies in Human HNSCC Cell Lines.** To investigate the molecular basis for resistance to cisplatin in human HNSCC, we tested cisplatin-induced cytotoxicity on HNSCC lines (HN6, HN12, HN13, HN17, and HN30) derived from head and neck cancer patients (8, 9). Anatomical locations of tumors from which cell lines were generated, the clinical stages of patients (T₁-T₄), and the degrees of cancer cell metastasis to lymph node (N₀–N₃) are listed in Table 1. The cell numbers after cisplatin treatment were measured by SRB staining, which stains for cellular proteins (10). Cisplatin-induced cytotoxicity in a dose-dependent manner in these cells as shown in Fig. 1. Forty-eight h of treatment with 1 μM cisplatin induced significant cytotoxicity in HN6, HN13, and HN17 cells, whereas it had little effect on HN12 and HN30 cells. More than 95% of HN12 and HN30 cells remained viable after 48 h of 5 μM cisplatin treatment. In contrast, approximately 55–65% of HN6 and HN13 cells survived after the same treatment (Fig. 1). Whereas HN17 cells showed intermediate sensitivity after 48 h of 5 μM cisplatin treatment (Fig. 1), 72 h of treatment with 5 μM cisplatin resulted in cytotoxicity at a comparable level among HN6, HN13, and HN17 cells (data not shown). Treatment with 10 μM cisplatin for 48 h further induced cytotoxicity in HN6, HN13, and especially in HN17 cells but significantly less in HN12 and HN30 cells. Taken together, we concluded that HN12 and HN30 are relatively resistant to cisplatin compared with HN6, HN13, and HN17 cells.

**Cisplatin Inducibility of Caspase Activity Correlates with Its Cytotoxicity.** Although the molecular mechanisms underlying resistance to chemotherapy agents are unclear at present, recent studies suggest that apoptosis plays a critical role for determining chemosensitivity (11, 12). Activation of caspases is known to be a hallmark of apoptosis induction. Caspases are a group of cysteine proteases that cleave substrates after aspartic acid residues and initiate the apoptotic cell death process (13, 14). We asked whether cisplatin sensitivity correlates with caspase activity in HNSCC. Because caspase-3 and caspase-7 are the two most common caspases activated in the apoptotic cell death process and their recognition sequences are the tetrapeptide DEVD (reviewed in Refs. 15 and 16), we measured caspase activity using the fluorogenic substrate Ac-DEVD-amc. As shown in Fig. 2, DEVDase activity increased over time in cisplatin-sensitive HN6, HN13, and HN17 cells after 5 μM cisplatin treatment. In contrast, there was no detectable increase in DEVDase activity in cisplatin-resistant HN12 and HN30 cells after the same cisplatin treatment. This showed that caspase induction is associated with cisplatin-induced cytotoxicity in HNSCC.

**Neutron Radiation Enhances Cisplatin-induced Cytotoxicity More Effectively than Photon Radiation.** We next asked whether neutron radiation enhances cisplatin-induced cytotoxicity more effectively than photon radiation, especially in cisplatin-resistant cells. We compared neutron and photon radiation efficacy in HNSCC. Clonogenic cell survival was examined 1–3 weeks after irradiation (as described in “Materials and Methods”), showing more effective HNSCC killing by neutron irradiation (Table 2). The RBEs of neutrons relative to photons for 10% tumor growth inhibition (90% cell survival) was 4.2 in HN12, 2.8 in HN17, and 3.1 in HN30, RBE for 50% growth inhibition was 3.4 in HN12, 2.7 in HN17, and 2.5 in HN30, and RBE for 90% inhibition was 2.7 in HN12, 2.4 in HN17, and 2.3 in HN30 (Table 2). Because the RBE of neutrons relative to photons is ~3, we compared the combined effect of cisplatin and 2 Gy photon irradiation with cisplatin and 0.67 Gy neutron irradiation, and 6 Gy photon irradiation with 2 Gy neutron irradiation. To determine cisplatin-induced cytotoxicity in combination with photon or neutron irradiation, cells were treated with 1 μM cisplatin 2 h prior to irradiation, and cell survival was determined 48 h after irradiation. As shown in Fig. 3A, cisplatin treatment followed by neutron irradiation was far more effective to induce short-term cytotoxicity in HNSCC cells than cisplatin treatment followed by photon irradiation. To compare the effects of photon and neutron irradiation on cisplatin-induced cytotoxicity, combined cytotoxicity was plotted after normalizing to cisplatin-induced cytotoxicity in the respective cell line as shown in Fig. 3B (cell survival after 48 h of 1 μM cisplatin treatment alone shown as 100%). Photon irradiation failed to significantly enhance cisplatin-induced short-term cytotoxicity in all lines tested. Interestingly, combined treatment with photon irradiation at 2 Gy enhanced cell survival >60% in HN6 cells compared with 1 μM cisplatin treatment alone in HN6, the most sensitive line to 1 μM cisplatin treatment (see Fig. 1). In contrast to photon irradiation, neutron effectively enhanced cisplatin-induced cytotoxicity in all HNSCC lines tested.

**Neutron Irradiation Enhances Cisplatin-induced Cytotoxicity Independently of Apoptosis or Cell Cycle Regulation.** We next examined whether neutron-enhanced cisplatin cytotoxicity resulted from further activation of caspases, resulting in apoptotic cell death. DEVDase activity was measured using the fluorogenic substrate Ac-DEVD-amc in five HNSCC lines after cisplatin treatment, neutron irradiation, or combined

<table>
<thead>
<tr>
<th>Treatments</th>
<th>% apoptotic cells</th>
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<tbody>
<tr>
<td>Control</td>
<td>2.45</td>
</tr>
<tr>
<td>1 μM cisplatin</td>
<td>13.20</td>
</tr>
<tr>
<td>6 photon gray (Gy)</td>
<td>5.13</td>
</tr>
<tr>
<td>1 μM cisplatin + 6 (Gy)</td>
<td>3.76</td>
</tr>
<tr>
<td>2 neutron gray (N Gy)</td>
<td>4.13</td>
</tr>
<tr>
<td>1 μM cisplatin + 2 (N Gy)</td>
<td>3.27</td>
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treatment of cisplatin and neutron irradiation. Caspase activity was insignificant after neutron irradiation. Caspase activity after combined treatment was significantly lower than cisplatin-induced caspase activity (data not shown). This suggests that neutron-enhanced cytotoxicity was not associated with apoptosis induction. To confirm this, we examined the appearance of phosphatidylserine on the cell surface, recognized as a universal feature of apoptosis (17–19). Apoptotic cells, identified as those binding to annexin V, were readily detected after cisplatin treatment (Table 3) in cisplatin-sensitive HN13 cells. In cisplatin-resistant HN30 cells, only 5% of cells were apoptotic after the same treatment, further substantiating the conclusion that apoptosis is critical for HNSCC sensitivity to cisplatin. Interestingly, combined treatment with either neutron or photon irradiation significantly reduced cisplatin-induced apoptosis in HN13 and had little effect on HN30 apoptosis. Both caspase assay and annexin V staining indicate that neutron enhancement of cisplatin-induced cell killing is independent of apoptosis induction.

Although apoptosis can be induced at any point of the cell cycle, apoptosis sensitivity seems to differ depending on cell cycle points. Increasing evidence suggests that photon irradiation induces cell cycle arrest at G1-S or G2-M, and the ability to induce cell cycle arrest after photon irradiation may be a critical determinant for radiation sensitivity. However, the relationship between neutron and cell cycle regulation is not understood. We next investigated whether differences between photon- and neutron-induced cytotoxicity in combination with cisplatin resulted from differences in cell cycle regulation. To this end, we analyzed cell cycle distribution after a single treatment of cisplatin, photon or neutron irradiation, or combined treatment of cisplatin with radiation. As shown in Fig. 4, neutron irradiation alone or together with cisplatin treatment resulted in cell cycle arrest, similar to photon irradiation. This suggests that the ability of neutron, but not photon, irradiation to effectively enhance cisplatin cytotoxicity is not directly associated with cell cycle regulation.

**DISCUSSION**

Recent advances in combined modality treatment of locally advanced HNSCC have improved local and regional disease control with better functional outcome. However, local and regional failure rate after radiation therapy is still high for tumors that poorly respond to cisplatin-based neoadjuvant chemotherapy (2, 3). Recent in vitro studies showed that pre-exposure of human HNSCC cells to photon radiation increased resistance to subsequent cisplatin treatment (20). Similarly, cisplatin-resistant tumors also respond poorly to subsequent photon radiation treatment (4, 5). At present, the molecular basis underlying cisplatin and photon radiation resistance is largely unknown, and the design of more efficacious multimodality protocols is in demand.

Neutron-induced cytotoxicity appears to be less dependent on tissue oxygenation or genetic background of the cells, and neutron-induced DNA damage is less repairable than photon radiation (6). To overcome resistance to photon therapy, neutron radiation treatments have been attempted since the 1960s (21–23). Several Phase III randomized studies have been completed in HNSCC. Six used low energy/or laboratory-based neutron
generators of marginal capability, and the seventh used state-of-the-art equipment (24–28). Direct comparisons between these trials were difficult because of the diversity of treatment equipment, total radiation doses, and patient populations. A recent Phase III study used hospital-based cyclotrons in a prospective collaborative international randomized trial (29). A total of 178 patients participated in the study that directly compared state-of-the-art fast neutron radiation therapy with photon and electron radiation therapy. The complete response rate in the neutron-treated group of patients was 70% versus 52% in the low linear energy transfer-treated group. Although neutron therapy improved the initial response rate, it failed to improve permanent local-regional tumor control and increased the incidence of late normal tissue cytotoxicity. The strategy for neutron therapy was based on limited clinical experience. Time schedule and fractionation scheme modification or combined modality therapy with chemotherapy may be needed to improve tumor cell killing with reduced normal tissue cytotoxicity. The present study demonstrated that moderate neutron dose enhancement cisplatin cytotoxicity in all HNSCC tested, including cisplatin-resistant cell lines. This may provide a basis to revisit the use of neutron radiation in patients with locally advanced HNSCC and to establish more effective multimodality protocols.

In summary, the present study clearly suggests that cisplatin-induced cytotoxicity correlates with apoptosis sensitivity and caspase induction, providing an insight into the molecular basis for cisplatin resistance in HNSCC cells. In agreement with previous studies, neutron enhancement of cisplatin cytotoxicity was associated neither with cell cycle phase nor apoptosis. By understanding the molecular mechanism of neutron cytotoxicity in these cell lines, novel approaches to overcoming cisplatin resistance may be elucidated.

REFERENCES
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