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DNA ligase IV is a potential molecular target in Nimustine (ACNU) sensitivity

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Abstract

Nimustine [1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea; ACNU] is a chloroethylating agent which is used in chemotherapy for glioblastomas. It has been reported that ACNU induces several kinds of DNA lesions such as alkylating modification on base and double-strand breaks (DSBs). This work described here was to clarify repair pathways for ACNU-induced DSBs. Cultured mouse embryonic fibroblasts used here are deficient in DNA DSB-repair genes involved in homologous recombination (HR) repair (*X-ray repair cross-complementing group 2* (*XRCC2*) and *radiation sensitive mutant 54* (*Rad54*)), and in non-homologous end joining (NHEJ) repair (*DNA Ligase IV* (*Lig4*)). We examined the cell survival after drug treatment by colony forming assay. The most effective molecular target which correlated with cellular sensitivity to ACNU was *Lig4*. The results of histone H2AX phosphorylated at serine 139 (γ H2AX) with flow cytometry suggest that *Lig4* can generate cellular resistance to ACNU by repairing DSBs induced by it. In addition, it was found that *Lig4* small interference RNA (siRNA) efficiently enhanced sensitivity to ACNU in human glioblastoma A172 cells. These findings suggest that down regulation of *Lig4* might provide a useful tool to increase cell lethality towards ACNU chemotherapy.

Keywords

ACNU; DSB; NHEJ; HR; *Lig4*

Introduction

Alkylating drugs are the oldest class of anti-cancer drugs which are still commonly in use, and they remain important in the treatment of several types of cancers. Nimustine (ACNU) is a chloroethylating agent which has been used either alone or in combination with other agents for the treatment of brain tumors (1). Following cellular exposure to ACNU, a chloroethyl group is transferred to the *O*⁶-position on guanine (G) residues in DNA, and this *O*⁶-chloroethylG is repaired by the activity of *O*⁶-methylG-DNA methyltransferase

(MGMT) (2). Elevated levels of MGMT have been blamed for much of the observed resistance to chloroethylating agents (3), but it has also been suggested that repair of *O*⁶-chloroethylG by MGMT is not the sole mechanism responsible for resistance to chloroethylating agents (4). About 5% of all solid tumors completely lack MGMT and the frequency of tumors lacking MGMT is 17–30% for gliomas (5). Therefore, if only MGMT, is targeted, improvements in drug efficacy are likely to be limited. A new target to improve chemotherapy with chloroethylating agents is now being sought.

DNA double-strand breaks (DSBs) have been reported to be induced indirectly through the repair processes for the ACNU-induced DNA lesions (6). Since DSBs are likely to be the final event leading to cell death, it would thus be expected that cells defective in DSB repair would be more sensitive to the chloroethylating agent. DSBs are repaired through the homologous recombination (HR) and non-homologous end joining (NHEJ) pathways (7). In human cells, the proteins involved in HR pathway include members of the MRN complex (meiotic recombination 11 (MRE11)/radiation sensitive mutant 50 (Rad50)/Nijmegen breakage syndrome 1 (NBS1)), Rad51, the Rad51 paralogs (Rad51B, Rad51C, Rad51D), X-ray repair cross-complementing group 2 (XRCC2) and XRCC3, Rad54, and Rad54B (7). Proteins involved in the NHEJ pathway include Ku70/80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), DNA Ligase IV (Lig4), and XRCC4 (7).

The work described here was designed to lead to a better understanding of details involved in the DSB-repair pathways which contribute to ACNU sensitivity. The activity of specific components of HR pathway (XRCC2 and Rad54) and NHEJ pathway (Lig4) leading to the repair of ACNU-induced DNA lesions were assessed using clonogenic survival assays. A panel of *p53* tumor suppressor gene knockout mouse embryonic fibroblast cell lines (MEFs) was used which contained cells which were defective in specific components in the repair pathways (XRCC2, Rad54 and Lig4).

Next, to test whether the resulting observations were applicable to gliomas, targeted repair pathways were down regulated using small interference RNA (siRNA), and the sensitivity of human glioblastoma A172 cells to ACNU was measured. To determine the contribution of DSB repair, the expression of γ H2AX was monitored at different times following treatment with ACNU in cell nuclei with comparison between DSB-repair deficient cell lines and the parental cell lines. Hopefully, understanding of the DNA repair mechanism which was identified as contributing to ACNU resistance in this study will lead to the development of new tools or methods which can be utilized to improve drug efficacy.

Materials and Methods

Cell culture

The cell lines used in these studies were the MEF cell lines *XRCC2*^{-/-}*p53*^{-/-} (*XRCC2*^{-/-}); *XRCC2*^{+/+}*p53*^{-/-} (*XRCC2*^{+/+}) (8); *Rad54*^{-/-}*Lig4*^{+/+}*p53*^{-/-} (*Rad54*^{-/-}); *Rad54*^{+/+}*Lig4*^{-/-}*p53*^{-/-} (*Lig4*^{-/-}); *Rad54*^{-/-}*Lig4*^{-/-}*p53*^{-/-} (*Rad54*^{-/-}*Lig4*^{-/-}); and *Rad54*^{+/+}*Lig4*^{+/+}*p53*^{-/-} (*Rad54*^{+/+}*Lig4*^{+/+}) (9). Human glioblastoma A172 cells were purchased from the American Type Culture Collection of Cell Cultures (Manassas, VA). Cells were cultured as previously described (10).

Drug treatments

ACNU (Sigma Aldrich, Saint Louis, MO) was dissolved at a stock concentration of 10 mM in sterile H₂O. ACNU stock solutions were stored at -20°C until used. Cells were treated with medium containing ACNU at various concentrations for 3 h and then rinsed twice with PBS.

Cell survival

Cell survival was measured using a standard clonogenic survival assay as previously described (10). The sensitivity of each cell line was assessed from its D_{50} value, *i.e.* from the ACNU dose which reduced cell survival to 50%. In order to accurately compare ACNU sensitivities in the repair defective cell lines, the relative D_{50} values were normalized using the D_{50} value of the corresponding proficient cell lines.

Flow cytometry

To determine whether DSBs are formed in response to ACNU, and how many DSBs are formed, the overall levels of histone H2AX phosphorylated at serine 139 (γ H2AX) were measured with flow cytometry as previously described (10).

RNA interference

Human *Lig4* siRNA or non-specific negative control siRNA was transfected against human glioblastoma A172 cells as previously described (11). The cells were then trypsinized for colony forming assays.

Statistical analysis

Statistical analysis was performed using the Student's *t* test.

Results and Discussion

The role of DSB-repair genes for ACNU sensitivity

In this study, we focused on DSBs in ACNU chemotherapy. Cellular responses to ACNU were examined using *XRCC2* defective cells (Fig. 1A), *Rad54* and/or *Lig4* defective cells (Fig. 1B). All repair defective cells were more sensitive to ACNU than the corresponding proficient cells (Figs. 1A and B). Also, *Rad54*^{-/-}*Lig4*^{-/-} cells were more sensitive to ACNU than *Rad54*^{-/-} cells or *Lig4*^{-/-} cells (Fig. 1B). The relative D_{50} values listed sequentially in the order in which they increase (reflecting decreasing sensitivities to ACNU) are: *Rad54*^{-/-}*Lig4*^{-/-} cells < *Lig4*^{-/-} cells < *Rad54*^{-/-} cells < *XRCC2*^{-/-} cells (Fig. 1C). The data provide the first evidence that both HR and NHEJ play a critical role in the repair of ACNU-mediated DNA lesions, and observations of the relative D_{50} values support this idea.

The parental cells from which the *Rad54*^{-/-} and *Lig4*^{-/-} cells were derived had the same genetic background, as the defective cells, but the relative D_{50} values of the *Rad54*^{-/-} cells and the *Lig4*^{-/-} cells were 31% and 25% of the parental cells, respectively (Fig. 1C). Among cells defective in a single repair gene, *Lig4*^{-/-} cells showed highest sensitivity to ACNU (Fig. 1C). In addition, the sensitivity of *Rad54*^{-/-}*Lig4*^{-/-} cells to ACNU was higher than that of cells defective in either single gene, which indicates that the HR and NHEJ double knockout cells displayed an additive effect (Figs. 1B and C). These results clearly show the importance of both HR and NHEJ in the repair of ACNU-induced DNA lesions and differ from the result using temozolomide (TMZ) that NHEJ mainly plays a role in the repair of TMZ-induced DNA lesions compared with HR (11). This difference may be explained because *O*⁶-methylG, produced by TMZ, would allow progression of DNA replication fork, on the other hand, ACNU-induced *O*⁶-chloroethylguanine, which prevents progression of DNA replication fork (13). Results for *XRCC2* (Fig. 1A) are in agreement with previous studies which revealed that *XRCC2* knockout MEFs showed a hypersensitivity to other DNA interstrand cross-linking (ICL) agents, such as fotemustine, cisplatin and mitomycin C (14). Although it was reported that in DT40 cells which appear to possess significantly higher HR repair efficiency than any mammalian cell line, NHEJ plays

only a minor role in ICL repair (15), the data here support the idea that not only HR, but also NHEJ both play a major role in protecting cells against ACNU-induced DNA lesions.

Contribution of Lig4 in repair for ACNU-induced DSBs

In proficient cells, γ H2AX levels did not vary much from their initial levels at 12 h and 24 h after ACNU treatment. However, in *Lig4*^{-/-} cells, γ H2AX levels increased more than 4-fold at 12 h and 6-fold at 24 h after ACNU treatment when compared to their initial levels. At 12 h and 24 h after treatment with ACNU, there were significant differences in γ H2AX levels between the proficient and deficient *Lig4* cell lines (Fig. 2). The detection and quantification of γ H2AX are useful tools to monitor the induction of DNA damage response signaling pathways, because many of the early components in the DNA damage response pathway co-localize with γ H2AX at sites of DSBs (7). The data presented here suggests that Lig4 can generate cellular resistance to ACNU exposure by repairing lesions which trigger the activation of DNA damage response cascades. The data indicate that in *Lig4*^{-/-} cells, more DSBs could be produced from ICL processing and left unrepaired, whereas in the corresponding proficient cells, DSBs could be repaired. This data is consistent with the enhanced sensitivity of *Lig4*^{-/-} cells to ACNU when compared with the corresponding proficient cells (Fig. 1C). The formation of DSBs after ICL induction is a possible consequence of stalled replication forks during S phase (16).

Effect of Lig4 siRNA on ACNU sensitivity in A172 glioblastoma cells

To test whether this result was pertinent to chemotherapy used against glioblastomas, *Lig4* expression was silenced using siRNA in A172 glioblastoma cells which exhibit very low levels of MGMT activity (12), and clonogenic survival assays were then performed. *Lig4* silencing caused a 32% reduction in colony formation when compared to cells transfected with negative control siRNA. In addition, after ACNU treatment, *Lig4* silencing caused a 72% reduction in colony formation when compared to cells transfected with negative control siRNA. In A172 glioblastoma cells *Lig4* silencing increased cellular sensitivity to ACNU approximately 2.2 times (Fig. 3). It was demonstrated that the depression of *Lig4* can enhance the sensitivity of glioblastomas to ACNU.

DSB-repair pathways for ACNU-induced DNA lesions are summarized in Fig. 4. Following cellular exposure to ACNU, *O*⁶-G is chloroethylated and transformed into *O*⁶-chloroethylG. If this *O*⁶-chloroethylG is not repaired by MGMT, this adduct is unstable, and undergoes an intramolecular rearrangement leading to an intermediary *N*¹-*O*⁶-ethanoG. The *N*¹-*O*⁶-ethanoG adduct may react with cytosine in the complementary strand to yield a highly toxic DNA-DNA cross-link between position 1 in the guanine and position 3 in the cytosine (1-(3-cytosinyl)-2-(1-guanosinyl)-ethane) (17). The mechanism involved in the repair of this type of DNA ICL seems to involve a combination of FA proteins and NER factors (18). However, the details of this repair pathway remain to be elucidated. The XRCC2 protein and Rad54 protein play a role in HR via its interaction with Rad51 (19,20). In the NHEJ pathway, after DSB formation, the Ku70/80 heterodimer binds to the damaged DNA ends. This facilitates the recruitment of the DNA-PKcs to the DSB. The sequential binding of these proteins activates the phosphorylation function of the DNA-PKcs which then phosphorylates itself, the Ku heterodimer, and other proteins involved in cell cycle regulation (7). It has been suggested that Ku70/80 might also function as an alignment factor which binds DSB ends, and can thus provide ready access for, and greatly stimulate the functioning of the Lig4-XRCC4 complex. This can increase the efficiency and accuracy of NHEJ (7). The Lig4-XRCC4 complex then rejoins the juxtaposed DNA ends. In conclusion, both HR and NHEJ play an important role in the repair of ACNU-mediated DNA lesions. However, among the single repair gene defective cells, the *D*₅₀ value of the *Lig4*^{-/-} cells

was the smallest, so it appears that Lig4 could provide a new molecular target for ACNU chemotherapy.

In conclusion, it is proposed that *Lig4* contributes significantly towards the repair of ACNU-induced DSBs, and that modulating *Lig4* activity could enhance sensitivity to chemotherapeutic agents.

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Figure 1. Contribution of HR or NHEJ to DSB repair and cellular survival following ACNU treatment. **A**, XRCC2^{+/+} cells (open circles) and XRCC2^{-/-} cells (closed circles). **B**, Rad54^{+/+}Lig4^{+/+} cells (open circles), Rad54^{-/-} cells (closed triangles), Rad54^{-/-}Lig4^{-/-} cells (closed squares), and Lig4^{-/-} cells (closed circles). Each point represents the mean of at least three independent experiments; bars indicate the SD. **C**, relative D_{50} value (% compared to proficient cells).

Figure 2. Phosphorylation of H2AX following treatment with medium containing 60 μ M ACNU for 3 h in proficient cells (open columns) or in *Lig4*^{-/-} cells (closed columns) at the indicated time points. Columns show the mean of at least three independent experiments; bars indicate the SD. *, the difference is statistically significant ($P < 0.05$).

Figure 3. Effect of *Lig4* siRNA on ACNU sensitivity in glioblastoma A172 cells. Negative control siRNA (open columns); *Lig4* siRNA (closed columns). Columns show the mean of at least three independent experiments; the bars indicate the SD. **, the difference is statistically significant ($P < 0.01$).

Figure 4.
Hypothesized pathways for the repair of ACNU-induced DNA lesions.