Modulating ICBP90 to Suppress Human Ribonucleotide Reductase M2 Induction Restores Sensitivity to Hydroxyurea Cytotoxicity

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Abstract. Background: Ribonucleotide reductase (RR) inhibition by hydroxyurea (HU) causes deoxyribonucleotide (dNTP) depletion, which activates the replication checkpoint, a part of the S-phase checkpoint that responds to DNA damage by inhibiting late origin firing. It also transactivates RR and other genes involved in DNA replication and repair. ICBP90 (overexpressed in breast cancer) is a novel Rb-associating transactivator for the human topoisomerase IIα gene and responds to DNA damage-induced checkpoint signaling. Materials and Methods: ICBP90 expression was monitored by Western blot. Promoter activity was detected via the luciferase assay and gene silencing via siRNA. Cell death was monitored by the MTT assay. Results: dNTP depletion by HU induced ICBP90, ICBP90 transactivated RR’s M2 subunit gene, and ICBP90 induction was necessary for HU-induced M2 accumulation. Blocking the M2 accumulation via anti-ICBP90 siRNA caused greater sensitivity in HU-resistant human cancer. Conclusion: A transcriptional intervention strategy is presented through which HU-resistant cancers may be eradicated without dose escalation.

The prototypic antimetabolite drug hydroxyurea (HU) has been used to treat chronic myelogenous leukemia, head and neck carcinoma and sarcoma, and other types of human cancer as a radio-sensitizer, but recurrent cancers eventually diminish its therapeutic role (1). HU’s anticancer property derives primarily from its ability to inhibit ribonucleotide reductase (RR), which catalyzes the conversion of ribonucleotides to their corresponding deoxyribose form essential for DNA synthesis (2). The human RR is composed of M1 and M2 subunits. Their transcription heightens during the S-phase; yet, the half-life of the M2 subunit is considerably shorter than that of M1, making the availability of M2 the prime determinant of RR activity. In eukaryotes, dNTP (deoxynucleoside triphosphate) depletion by HU yields aberrant replication forks, which activate the replication checkpoint (3). The replication checkpoint is a component of the S-phase checkpoint that responds to DNA damage by executing multiple functions including the inhibition of late origin firing, stabilization of replication forks and prevention of M-phase entry (4). Depletion of dNTPs (as well as DNA damage) activates yet another function, i.e., transactivation of multiple genes involved in DNA replication and repair (3). Consistent with this, we previously showed that HU induced the transactivation of the M2 gene in human head and neck cancer cells (5). ICBP90 (expressed in high-grade breast carcinoma) is a recently identified Rb-associating transcription factor for the human topoisomerase IIα gene, and inactivation of its murine homolog NP95 rendered murine cells more sensitive to HU cytotoxicity (6). In this report, we investigated whether the above response to dNTP depletion could be modulated to restore sensitivity in a HU-resistant human cancer. By suppressing the ICBP90 induction necessary for the dNTP depletion-induced RR M2 accumulation, resistance to HU cytotoxicity could be reversed. Thus, a treatment paradigm, based on modulating the HU-induced lytic path, is presented through which HU-resistant cancers may be eradicated without requiring dose escalation.

Materials and Methods

Reagents. KB human head and neck cells were maintained in RPMI medium supplemented with 10% FBS and pen-strep. KB-HURs cells were maintained in identical medium containing 1 mM HU. HU and nucleotides were purchased from Sigma (St. Louis, MO, USA).
Plasmids. The construction of pSG5-ICBP90 was previously described (7). The parental plasmid pSG5 was reconstructed from pSG5-ICBP90 by cleaving pSG5-ICBP90 with EcoR1 endonuclease (to remove the ICBP90 cDNA insert) and re-ligating. The plasmid phRRM2-Lucif has been described (8).

Western blot. Western blot analysis was performed using anti-human ICBP90, anti-human β-tubulin monoclonal antibody (BD Pharmingen, San Diego, CA, USA) or anti-human RR M2 subunit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as the primary antibody, followed by alkaline phosphatase-conjugated anti-mouse IgG antibody as the secondary antibody. The resulting blot was visualized through the enhanced chemiluminescence method (8).

Promoter assay. The lysate of cells that had been transfected with reporter plasmids were analyzed for luciferase activity using a luciferase assay kit (8). The signal was measured using a luminometer. The activity was standardized by adjusted protein concentration.

siRNA interference assay. The construction of the anti-ICBP90 siRNA-expressing plasmid was performed as described (9). For this, previously published anti-ICBP90 siRNA sequences were utilized (e.g. 5’-AATGAGTACGTCGATGCTCGG-3’ and 5’-AACGACTGTGGATCAGCCTGC-3’ representing ICBP90 nucleotides 412-432 and 799-819, respectively) (10). In each case, the annealed oligonucleotide duplex encoding the siRNA was subcloned into the expression vector psiRNA-hH1zeo (InvivoGen, San Diego, CA, USA) to express under the control of the RNA polymerase III-dependent H1 RNA promoter. Cells were transfected with the indicated plasmid at equimolar concentration via electroporation.

Cytotoxicity. The MTT cytotoxicity assay was performed by incubating with MTT and monitoring the MTT-formazan formed via the enhanced chemiluminescence method (8).

Results

dNTP depletion by HU induces ICBP90 in human head and neck cancer cells. Multiple observations, including the fact that ICBP90’s murine homolog NP95 modulates sensitivity to HU cytotoxicity, that ICBP90 physically interacts with Rb involved in the checkpoint response to dNTP depletion, and that ICBP90 represents a component of the DNA damage checkpoint indicated that ICBP90 may function in the cellular response to dNTP depletion (6, 10, 11). As several checkpoint proteins undergo induction following a stimulus, the expression status of ICBP90 following dNTP depletion was monitored.

Western blot analysis of electrophoretically separated KB human head and neck cancer cell lysate, using a commercially available anti-human ICBP90 antibody, detected a discrete band (Figure 1A) migrating at a position consistent with its molecular mass of 89 kDa (7). Its authenticity was confirmed when the band intensified following the transfection with an ICBP90-expressing plasmid (Figure 2A). KB cells were persistently treated with HU at a sublethal dose of 0.1 mM (IC50 value for KB is ~0.3 mM), which is capable of inhibiting intracellular RR. It led to a significant increase in ICBP90 (Figure 1A). The expression level of the endogenous protein β-tubulin was unaffected, indicating that the increase in ICBP90 observed was specific (Figure 1A).

To confirm that the ICBP90 induction observed was specifically due to dNTP depletion, the classic dNTP pre-accumulation experiment was performed. ‘Pre-accumulation’ refers to the prior replenishing of a certain nucleoside, which attenuates dNTP depletion caused by subsequent HU treatment. For instance, pre-treatment with dA (deoxyadenosine) attenuated the decline in the dATP (deoxyadenosine triphosphate) and dGTP (deoxyguanosine triphosphate) pools in HU-treated V79/dC hamster lung fibroblasts (12). HU treatment initially precipitated >80% decline in the dATP and dCTP (deoxyguanosine triphosphate) pools and >18% decline in the dGTP and dTTP pools in KB cells (5). To counter this decline, the KB cells were pre-incubated with dA, dC (deoxycytidine) or dG (deoxyguanosine). Subsequent treatment with HU resulted in a significantly lower induction of ICBP90 in cells pre-incubated with dA or dG (Figure 1B; compare the ratio of band intensity before vs. after HU treatment for each sample), indicating that the ICBP90 induction was specifically in response to dNTP depletion.

ICBP90 transactivates the human ribonucleotide reductase M2 subunit gene in vivo. Multiple data indicate that ICBP90 may regulate the human RR M2 gene. These include the findings that ICBP90 transactivates the human topoisomerase IIα gene (which is also involved in DNA replication and repair) (7), that ICBP90 expression occurs in late G1 preceding the strong M2 expression during the S-phase, that both genes are highly expressed in, amongst others, bone marrow and thymus, that ICBP90 binds to an inverted CCAAT motif (7) and multiple such motifs (albeit inverted) are present in the human M2 promoter, both genes being overexpressed in breast cancer (13).

To investigate this point, KB cells, which contain little endogenously expressed ICBP90, were transiently transfected with the pSG5-ICBP90-expressing human ICBP90 gene controlled by the SV40 early promoter (7). Western blot analysis of the resulting lysate showed an intense ICBP90 band when compared to the mock-transfected or parental pSG5-transfected plasmid, indicative of ICBP90 transgene expression (Figure 2A). The expression level of the endogenous protein β-tubulin remained unaffected (Figure 2A).

Next, KB cells were co-transfected with pSG5-ICBP90 and phRRM2-Lucif expressing the firefly luciferase gene
driven by the human M2 promoter. The construction of pH7RM2-Lucif was previously described (8). Ectopic expression of ICBP90 led to a significantly greater M2 promoter activity (Figure 2B), suggesting that ICBP90 transactivates the M2 gene in vivo.

ICBP90 induction is necessary for dNTP depletion-induced accumulation of the human ribonucleotide reductase M2 subunit protein. As mentioned, dNTP depletion causes activation of the replication checkpoint, which executes multiple functions including the transactivation of RR and other genes involved in DNA repair and replication. Consistent with this, we previously observed that HU treatment, which initially reduced the intracellular concentrations of dATP and dCTP, triggered an increase (>450%) in the M2 transcript by 12 hpt (hours post treatment) in KB cells (5). The M2 protein continued to increase thereafter, and the RR activity rose accordingly (5). Hence, HU treatment induced accumulation of both ICBP90 (Figure 1) and M2 at the protein level. This correlation, along with the fact that ICBP90 transactivates the M2 gene in vivo (Figure 2), inspired further investigation into whether the dNTP depletion-induced accumulation of the M2 protein requires ICBP90 induction.

To block ICBP90 induction, previously published siRNA sequences that efficiently degrade ICBP90 mRNA were utilized (10). To achieve stable expression, the siRNA sequences were expressed as short hairpin RNA (forms a RNA duplex upon expression in vivo) using a mammalian

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**Figure 1.** dNTP depletion by HU induced ICBP90 in human head and neck cancer cells. A. HU induced ICBP90. KB cells were incubated with the indicated agent for 24 h and lysed. An equivalent amount of the lysate was separated by 10% SDS-PAGE and subjected to Western blot analysis using anti-human ICBP90 (left panel) or anti-human β-tubulin (right panel) as the primary antibody. Lanes 1 and 3, none; lanes 2 and 4, HU (0.1 mM). Markers (in kilodaltons) shown. [Inset shows IC_{50} values (± standard deviation) of the cell lines.] B. ICBP90 induction by HU was in response to dNTP depletion. KB cells were pre-incubated with dA (100 μM), dC (20 μM), or dG (100 μM) for 1 h, incubated with HU (0.1 mM) for 24 h, and analyzed as described in (A). Pre-incubation with dA (lanes 1 and 2); with dC (lanes 3 and 4); with dG (lanes 5 and 6). Lanes 1, 3 and 5, untreated; lanes 2, 4 and 6, HU-treated. [Inset shows the intracellular dNTP level (± standard deviation) following HU (0.1 mM) treatment in KB cells.]
expression vector. The utility of this system in generating a functional siRNA was previously demonstrated. When KB cells were transiently transfected with the anti-ICBP90 siRNA-expressing plasmid psiRNA-hH1zeo-ICBP90 and subsequently treated with HU, little ICBP90 induction occurred (though ICBP90 expression was not completely prevented). ICBP90 induction by HU occurred unperturbed in KB cells transfected with the parental vector psiRNA-hH1zeo, indicating that the suppression of ICBP90 induction observed above was specifically due to the anti-ICBP90 siRNA (Figure 3, left panel).

The effect of suppressing ICBP90 induction on the dNTP depletion-induced accumulation of the M2 protein was examined next. KB cells were transiently transfected with the anti-ICBP90 siRNA-expressing plasmid psiRNA-hH1zeo-ICBP90 and subsequently treated with HU, little ICBP90 induction occurred (though ICBP90 expression was not completely prevented). ICBP90 induction by HU occurred unperturbed in KB cells transfected with the parental vector psiRNA-hH1zeo, indicating that the suppression of ICBP90 induction observed above was specifically due to the anti-ICBP90 siRNA (Figure 3, left panel).

The possibility of the decrease in M2 protein accumulation resulting from the degradation of M2 mRNA via anti-ICBP90 siRNA appears highly unlikely as little homology exists between the two sequences for cross-hybridization. Taken together, the results suggested that ICBP90 induction is necessary for HU-induced M2 accumulation.

Suppressing dNTP depletion-induced M2 protein accumulation via anti-ICBP90 siRNA reverses resistance to HU cytotoxicity, The RR activity induced by dNTP depletion may have a neutralizing effect on the therapeutic efficacy of HU. The corollary of the above is that, by suppressing this response, a greater sensitivity to HU cytotoxicity may be attained. We previously reported that the increase in RR activity following HU treatment was due to M2 protein accumulation resulting from an increased transcription of the M2 gene (5). In the preceding section, it was shown that ICBP90 induction was
necessary for this process. Here, whether this response via anti-ICBP90 siRNA could restore sensitivity to a HU-resistant cancer was investigated.

KB-HURs is a derivative of the KB cell line exhibiting ~15-fold resistance to HU cytotoxicity due to its M2 gene amplification. To determine whether the ICBP90 response to dNTP depletion was preserved, KB-HURs cells were treated with HU at a sublethal dose of 1 mM (IC50 value for KB-HURs is ~4.5 mM), incubated for 24 h, and lysed. The resulting lysate was separated by 10% SDS-PAGE and subjected to Western blot analysis using an anti-human ICBP90 monoclonal antibody or anti-human M2 antibody as the primary antibody (left panel). The lysate corresponding to the equivalent cell number was loaded. Lanes 1 and 2, psiRNA-hH1zeo; lanes 3 and 4, psiRNA-hH1zeo-ICBP90. Lanes 1 and 3, untreated; lanes 2 and 4, HU treated. Coomassie blue staining of identical samples electrophoresed in parallel is shown (right panel). Data shown reflect the results of multiple independent experiments utilizing distinct anti-ICBP90 siRNAs.

Finally, the effect of suppressing the dNTP depletion-induced M2 protein accumulation on the resistance to HU cytotoxicity was examined. KB-HURs cells were transiently transfected with the anti-ICBP90 siRNA-expressing plasmid and then treated with HU after allowing time for siRNA expression. HU was more cytotoxic in the psiRNA-hH1zeo-ICBP90-transfected KB-HURs cells than in the mock- or null-vector-transfected KB-HURs cells. The difference was most pronounced at HU concentrations approaching the IC50 value of KB-HURs (Figure 4B). Thus, by suppressing dNTP depletion-induced M2 protein accumulation through anti-ICBP90 siRNA, resistance to HU cytotoxicity could be reversed.
Discussion

Following the recent delineation of the pathway underlying the S-phase checkpoint, a study was undertaken to examine the potential of exploiting it to develop a therapeutic strategy against HU-resistant cancer. In particular, the component of the S-phase checkpoint responding to dNTP depletion, i.e., the replication checkpoint, was focused on. The checkpoint responds by executing multiple functions, one of which is to transactivate certain genes involved in DNA replication and repair. Consistent with this, HU treatment induces the RR M2 gene in human cancer cells. Here, it was shown that ICBP90 induction was necessary for dNTP depletion-induced M2 accumulation. Furthermore, by suppressing dNTP depletion-induced M2 protein accumulation via anti-ICBP90 siRNA, greater sensitivity was attained in a HU-resistant cancer, indicating that the above strategy could be used to reverse resistance to HU cytotoxicity. It also provided a mechanistic insight into a similar observation made previously after inactivating the murine homolog NP95 in mouse embryonic stem (ES) cells (6). The greater sensitivity to HU cytotoxicity (>80% cell death) attained in that case was probably due to the complete block in NP95 expression achieved via gene knock out (plus the fact that the ES cells were not HU-resistant).

The lytic path induced by HU entails RR inhibition, which leads to dNTP depletion, resulting in the arrest of DNA replication, as evidenced by the appearance of stalled replication forks. The dNTP depletion-induced transactivation of the M2 gene is a response that affects the activity of RR, an upstream event in the HU-induced lytic path. As such, interfering with this response may alter the extent of lethality induced by HU. Our finding that attenuating this response via anti-ICBP90 siRNA enhanced HU-induced lethality is consistent with this view and implicates ICBP90 in the HU-induced lytic path. Increasingly, checkpoints are being implicated in the lytic paths induced by chemotherapeutic drugs and further understanding of their role may allow better manipulation of the parameters influencing their potency.

Increases in side effects pre-empt the efforts to eliminate recurrent cancers via dose escalation. The strategy outlined here provides a novel perspective on how to resolve this...
dilemma. A synergistic therapy employing dual drugs (one that inhibits ICBP90 and the other being HU or other types of RR-inhibiting drugs) could be envisioned to counter HU resistance. The easily degradable nature of anti-ICBP90 siRNA delimits its usefulness in clinics and may require the development of an alternative with greater stability.

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References


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