

Hydroxyurea Induces p53 Accumulation and Apoptosis in Human Cervical Carcinoma Cells

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Abstract: Cervical carcinoma is one type of cancer with a high mortality rate in women. In this research, we investigated the cytotoxic, antiproliferative and apoptosis-inducing effects of hydroxyurea on a human cervical carcinoma cell line, HeLa. We found that hydroxyurea has cytotoxic and antiproliferative effects on HeLa cells in a dose-dependent manner. We also found that hydroxyurea can increase cellular levels of p53 and p21, and induce DNA fragmentation in a dose-dependent manner. We concluded that the observed antiproliferative, cytotoxic and apoptosis inducing activities of hydroxyurea might be mediated by the induction of p53 and its transcriptional target, p21.

Key Words: Hydroxyurea, p53, p21, Cervical Carcinoma, DNA fragmentation, Apoptosis

p53 Birikimi ve Apoptosisin İnsan Servikal Karsinoma Hücrelerinde Hidroksiüre Tarafından İndüklenmesi

Özet: Servikal karsinoma kadınlarda yüksek ölüm oranı gösteren bir kanser tipidir. Bu araştırmada, hidroksiüre'nin insan servikal karsinoma hücre dizisi, HeLa, üzerindeki sitotoksik, antiproliferatif ve apoptosis indukleyici etkileri araştırılmıştır. Hidroksiüre'nin doz bağımlı olarak sitotoksik ve antiproliferatif etki gösterdiği saptanmıştır. Ayrıca, konsantrasyona bağlı olarak hidroksiüre'nin hücre içi p53 ve p21 seviyelerini artırdığı ve DNA fragmentasyonunu induklediği saptanmıştır. Sonuç olarak hidroksiüre'nin antiproliferatif, sitotoksik ve apoptosisi indukleyici etkilerinin p53 ve onun transkripsiyonel hedefi, p21'in indüksiyonu aracılığı ile bağlantılı olabileceği tartışılmıştır.

Anahtar Sözcükler: Hidroksiüre, p53, p21, Servikal Karsinoma, DNA fragmentasyonu, Apoptosis.

Introduction

p53 was identified nearly two decades ago by virtue of its ability to bind to the simian virus 40 and adenovirus oncoproteins; later, its cDNA was cloned (1,2). p53 is a multifunctional protein that is involved in a variety of biological processes such as growth arrest, apoptosis, differentiation and senescence. Although the function of p53 in normal development remains unclear, its predominant role is probably the regulation of cell proliferation through the induction of growth arrest or apoptosis in response to stress signals, such as DNA damage and hypoxia (3,4). p53 is a transcription factor that activates the transcription of a large number of cellular genes including p21^{WAF1} (hereafter referred to as p21), bax, cyclin G, and mdm2 (5-7). The induction of the cyclin-dependent kinase inhibitor p21 is one of the

principal effectors of growth arrest (8-10). In addition to the growth inhibitory effect of p21, bax induces apoptosis by interacting with Bcl2 family members, which may modify the mitochondrial ionic potential (11).

Cervical carcinoma is one of the most common cancers in women. High-risk human papillomaviruses (HPV) such as HPV18 play an important role in the development of essentially all cases of cervical carcinoma. Mutations in the p53 tumor suppressor gene are the most common specific genetic changes in human tumors; however, in cervical carcinomas p53 mutations are uncommon and most cervical carcinomas and carcinoma cell lines, like HeLa, harbor wild-type p53 (12). However, the E6 protein of HPV binds to p53 and targets it for accelerated ubiquitin-mediated degradation. Therefore, levels of p53 are very low in cells expressing E6. As a

result of this, expression of E6 proteins in cultured cells disturbs cell cycle checkpoint control and results in increased rates of mutations and genetic instability; therefore, mutations accumulate over time and carcinoma develops years to decades after initial HPV infection (13,14).

Hydroxyurea was first synthesized in Germany in 1869 by Dressler and Stein (15). Hydroxyurea is a potent inhibitor of ribonucleotide reductase (RR), and treatment of cells with this compound leads to rapid depletion of deoxyribonucleotide pools, which results in reversible cell cycle arrest at late G1/early S-phase (16). When first given to animals by Rosenthal et al. (17), hydroxyurea caused leukopenia, macrocythemia, anemia and death, with a megaloblastic profile similar to that of pernicious anemia. In humans, hydroxyurea was found to be effective in the treatment of chronic myelogenous leukemia, cervical carcinoma and pediatric cancers (18-20). Hydroxyurea kills the cells by inhibiting DNA synthesis and inducing apoptosis. Here, we show that hydroxyurea inhibited the proliferation of HeLa cells and increased DNA fragmentation, probably through the induction of p53 and p21.

Materials and Methods

Cells and cells culture: The cervical carcinoma cell line, HeLa, was obtained from Dr. Milton W. Taylor, Indiana University, Bloomington Indiana USA. This cell line was maintained in DMEM supplemented with 10% FCS at 37°C in humidified 5% CO₂ atmosphere. Cells maintained at the exponential growth phase were harvested for the experiments.

Materials, cytokines, antibodies and reagent: Tissue culture plates were purchased from Falcon (Lincoln Park, NJ). DMEM and other cell culture reagents were purchased from Sigma. PAGE reagents were purchased from Bio-Rad. Hydroxyurea was purchased from Sigma. The antihuman monoclonal antibodies to p53, p21 and horse-radish peroxidase-coupled anti-mouse secondary Abs were purchased from Santa Cruz (Santa Cruz, CA). Nitrocellulose sheets (Hybond-C Ext) were purchased from Amersham (Aylesbury, United Kingdom).

Cytotoxic assay: HeLa cells were grown until they completely covered the bottom of a 96-well plate. Cells were treated with various doses of hydroxyurea (100 ng/ml, 25 ng/ml, 6.25 ng/ml, 1.560 ng/ml). Treated

cells were incubated for 72 h at 37°C in humidified 5% CO₂ atmosphere. Following incubation, floating cells were removed and attached cells were stained with crystal violet (8% in methanol) and dye absorbed by live cells was extracted with sodium citrate (0.1 M) in 50% ethanol. Absorbance was read at 600 nm.

Antiproliferative assay: HeLa cells growing at the exponential growth phase were treated with hydroxyurea (100 ng/ml, 25 ng/ml, 6.25 ng/ml, 1.560 ng/ml) for 72 h at 37°C, 5% CO₂ in DMEM medium. At the end of the incubation period, cells were trypsinized and counted after trypan blue staining. The growth inhibition assay was expressed as the percentage increase in the number of treated cells relative to the increase in the number of untreated control cells.

Immunoblotting for p53, p21 and GAPDH: Cells were lysed by incubation in 50 mM HEPES (pH 7.0) containing 100 mM NaCl, 1.2% Triton X-100, 10% glycerol, 1.5 mM MgCl₂, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM EGTA, 1 mM DTT, 1mM phenylmethylsulfonylfluoride, 0.15 unit/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin. Lysates were clarified by centrifugation (12,000 x g for 1 min), and equal amounts of proteins were subjected to SDS-PAGE on 10% polyacrylamide gels, then electrophoretically transferred onto a nitrocellulose sheet overnight at 40 V, and analyzed by immunoblotting with anti-p53, p21 and GAPDH.

DNA Fragmentation: HeLa cells growing at the exponential growth phase were treated with hydroxyurea (100 ng/ml, 25 ng/ml, 6.25 ng/ml, 1.560 ng/ml), for 72 h at 37°C 5% CO₂ in DMEM medium. At the end of the incubation period, adherent cells were collected after trypsin treatment and combined with cells collected from the medium. Cells were washed with PBS and then incubated overnight at 37 °C in lysis buffer (200 mM Tris/HCL, pH: 8.0, 100 mM EDTA, 50 µg/ml proteinaseK, 1% SDS). DNA was extracted with equal volumes of phenol chloroform/3-methylbutan-1-ol (24:1 v/v). Then 50 µg/ml RnaseA was added and the results was incubated for 3 h at 37°C. DNA was precipitated with sodium acetate (0.3 M pH: 5), and 2.5 vol. of ice-cold 100% ethanol and pelleted by centrifugation (12,000 x g 30 min). The pellet was resuspended in TE buffer (10 mM Tris-HCL pH: 8.0, 1 mM EDTA) and equal amounts of DNA samples were electrophoresed on 1.5% agarose gel in TAE buffer

(Tris-acetate 40 mM, EDTA 1 mM pH: 8.0). The gel was stained with 1 µg/ml ethidium bromide for 30 min, and photographed under UV illumination.

Statistical analysis: Statistical significance analysis was done using the SPSS program (paired-samples t test).

Results

Hydroxyurea induces accumulation of p53 and p21

Since the stability of p53 protein has been shown to be altered after genotoxic damage or stress, the effect of hydroxyurea on p53 accumulation was examined in apoptotically responsive HeLa cells by Western blot analysis. As shown in Figure 1 (top), p53 expression increased after 2 h of hydroxyurea treatment and levels of p53 remained high up to 16 h. Since the cyclin-dependent protein kinase inhibitory protein, p21, has been shown to be transcriptionally regulated by p53, we wanted to determine whether p53 accumulation in hydroxyurea-treated HeLa cells also induced changes in

p21 levels. The p53 blot was stripped off and labeled with p21-directed monoclonal antibody. As shown in Figure 1 (middle), hydroxyurea treatment increased the p21 level indicating that accumulated p53 was transcriptionally active.

The antiproliferative effect of hydroxyurea is stronger than its cytotoxic effect on HeLa cells

Hydroxyurea shows cytotoxic and antiproliferative effects on HeLa cells in a dose-dependent manner. At a 100 ng/ml concentration of hydroxyurea, HeLa cells showed a 20% death rate relative to the control cells. This effect was 14% at the 25 ng/ml and 9% at 6.25 ng/ml concentration of hydroxyurea and the lowest concentration of hydroxyurea killed only 4% of cells (Figure 2).

After determining the cytotoxic effect, we wanted to determine the antiproliferative effect of hydroxyurea on HeLa cells. When HeLa cells were treated with 100 ng/ml of hydroxyurea, they showed only 11% growth relative to untreated cells. Even at a 25 ng/ml concentration of hydroxyurea, growth of HeLa cells was only 12% relative to control cells. The antiproliferative effect of

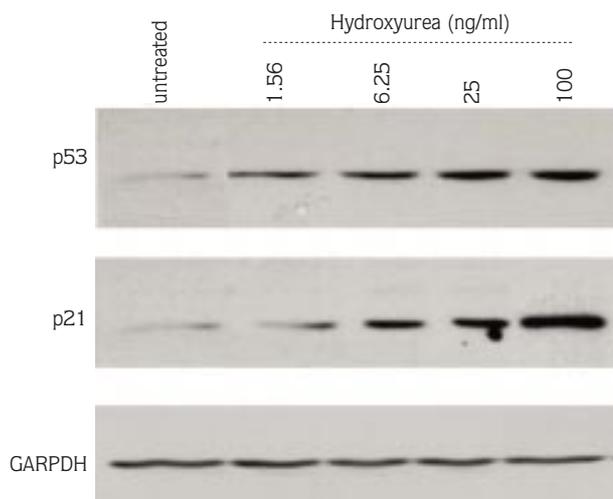


Figure 1. Hydroxyurea induces accumulation of p53 and p21.

HeLa cells were seeded at a density of 1×10^6 cells in 100 mm tissue culture dishes. After 24 h, the cells were washed with PBS, fresh growth media (DMEM) was added and cells were incubated with 100 ng/ml hydroxyurea for various amounts of time. Cell lysates were fractionated on 10% polyacrylamide gels and Western blots were probed with an antibody directed against p53 (top), stripped and reprobed with p21 (middle), and with GAPDH for even loading (bottom).

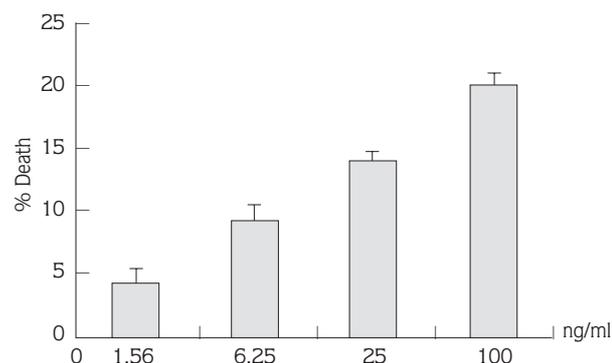


Figure 2. Cytotoxic effects of hydroxyurea are concentration dependent.

Confluent cultures of HeLa cells were treated with various doses of hydroxyurea and incubated for 72 h at 37°C in humidified 5% CO₂ atmosphere. Following incubation, floating cells were removed and attached cells were stained with crystal violet (8% in methanol) and dye absorbed by live cells was extracted with sodium citrate (0.1 M) in 50% ethanol. Absorbance was read at 600 nm. OD values of hydroxyurea-treated samples were divided by that of untreated control cells to determine the rate of cell death.

hydroxyurea began to decrease at lower doses. At 6.25 and 1.5 ng/ml concentrations, HeLa cells showed 72% and 94% growth rates relative to control cells, respectively (Figure 3). Based on these results, it is clear that the antiproliferative effect of hydroxyurea on proliferating cells is much greater than its cytotoxic effects on non-proliferating HeLa cells.

DNA fragmentation effects of hydroxyurea are concentration dependent

After showing the cytotoxic and antiproliferative effects of hydroxyurea, we wanted to determine whether it induces DNA fragmentation in HeLa cells. Hydroxyurea's effects on DNA fragmentation are visible even at its lowest concentration and this effect increased with increasing amounts of hydroxyurea. The amount of intact DNA decreases with increased hydroxyurea concentration and at the highest concentration of hydroxyurea, 100 ng/ml, almost all genomic DNA was degraded (Figure 4).

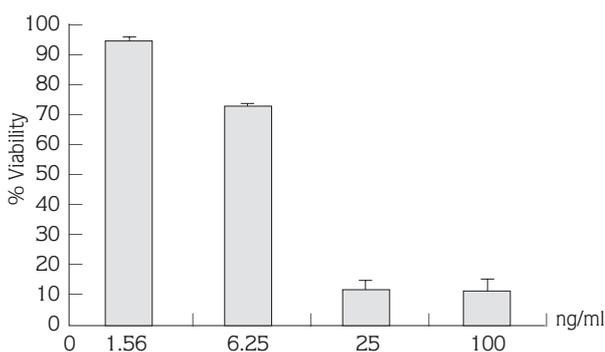


Figure 3. Antiproliferative effects of hydroxyurea are concentration dependent.

HeLa cells growing at the exponential growth phase were harvested by trypsinization and were carried to a 24-well plate (1×10^5 cells/well), then treated with hydroxyurea (100 ng/ml, 25 ng/ml, 6.25 ng/ml, 1.560 ng/ml) and incubated for 72 h at 37°C, 5% CO₂ in DMEM medium. At the end of the incubation period, the live cells were trypsinized and counted after trypan blue staining. The growth inhibition assays are expressed as the percentage increase in the number of cells present in the hydroxyurea-treated cultures relative to the increase in the number of untreated control cells.

Discussion

In over 90% of cervical carcinoma cancer and cancer-derived cell lines, the p53 tumor suppressor pathway is disrupted by the human papillomavirus, HPV (12). The HPV E6 protein promotes accelerated ubiquitin-mediated degradation of p53. HeLa cells express E6 protein from integrated HPV18 DNA and this causes an aberrant checkpoint control in these cells. Recently, it has been

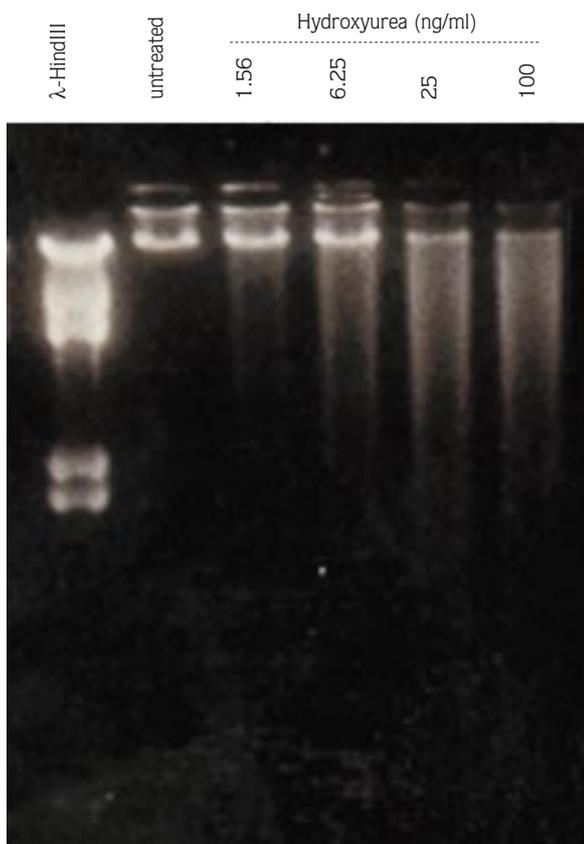


Figure 4. The effects of hydroxyurea on DNA fragmentation.

HeLa cells growing at the exponential growth phase were harvested by trypsinization. Cells were carried to plates (1×10^5 cells /well), then treated with hydroxyurea (100 ng/ml, 25 ng/ml, 6.25 ng/ml, 1.560 ng/ml) and incubated for 72 h at 37°C, 5% CO₂ in DMEM medium. At the end of the incubation period we collected the DNA samples from hydroxyurea treated cells and equal amounts of DNA samples were electrophoresed on 1.5% agarose gel in TAE buffer. The gel was stained with 1 mg/ml ethidium bromide for 30 min, and then photographed under UV illumination.

shown that E2 protein of bovine papillomavirus can repress the transcription of E6 from integrated HPV 18 DNA. The resulting decay in the E6 protein reduced the amount of p53 targeted to the ubiquitin degradation system, leading to increased levels of p53 and p21 in HeLa cells (13,14). Mitogen-dependent progression through the first gap phase (G1) and initiation of DNA synthesis (S) during the mammalian cell cycle are cooperatively regulated by several classes of cyclin-dependent kinases (CDKs), whose activities are in turn constrained by CDK inhibitors (CKIs). p21 is one of the CKIs and plays an important role by acting as a potent inhibitor of cyclin E- and A-dependent cdk2, and it was reported that cdk2 kinase activity is indispensable for G1/S transition (9,10,21).

Different stress signals, such as DNA damage, hypoxia, heat shock and oncogene activation, can induce stabilization and accumulation of wild-type p53. Treatment of cells with hydroxyurea leads to rapid depletion of deoxyribonucleotide pools and results in the arrest of cells at the G1/S boundary or very early in the S phase (16,22). In this investigation, we found that treatment of HeLa cells with hydroxyurea increases the accumulation of p53 and its transcriptional target, p21. Even though we did not look at the cellular level of E6 protein after hydroxyurea treatment, it is possible that down-regulation of HPV E6 by hydroxyurea is one of the key factors increasing the accumulation and function of p53. Cell cycle arrest caused by hydroxyurea may slow down the general transcription machinery, and as a result this, the cellular level of E6 may go down. The decrease in the overall level of E6 may be much faster than that of p53, because hydroxyurea treatment may accelerate the phosphorylation of p53 and thereby increases its half-life, leading to the accumulation of p53. It is also possible that hydroxyurea increases the proteosomal degradation of E6 in these cells.

Recently, Gottifredi et al. showed that treatment of RKO cells with hydroxyurea increases the accumulation of p53 by an unknown mechanism. They claimed that accumulated p53 did not have transcriptional activity, because p53 target genes, e.g. p21 and hdm2, were not induced after hydroxyurea treatment; however, they also showed that transcription of another p53 target gene, PIG3, did increase after hydroxyurea treatment. They also said that the impairment of p53 transcriptional activity was not the result of a general shutdown of RNA

synthesis, since hydroxyurea treatment led to a significant increase in the expression of c-fos and cyclin E mRNAs (23).

What we present in our study somewhat contradicts the findings of Gottifredi et al. because, after hydroxyurea treatment, we did see an increase in p53 and its transcriptional target, p21. Since p53 requires some coactivators, like CBP/p300, and it is not known whether p53 activates transcription of all of its target genes by using identical transcriptional complex, it is very possible that transcription of each p53 target gene may require the formation of a different transcriptional complex depending on the availability of coactivators in different cells.

In this investigation we also found that hydroxyurea exhibits antiproliferative and cytotoxic effects on HeLa cells in a dose-dependent manner. The response of HeLa cells to hydroxyurea differs depending on the growth phase of cells. When non-confluent (proliferating) cultures of HeLa cells were treated with a 100 ng/ml concentration of hydroxyurea, we found a very high level of antiproliferative effect ($p < 0.001$). However, when confluent (non-proliferating) HeLa cells were treated with the same dose of hydroxyurea, we observed only 20% cell death. These are expected observations, because hydroxyurea mainly affects DNA synthesis, thereby interfering with the growth of proliferating cells. In addition, it is clear that hydroxyurea has direct cytotoxic effects on non-proliferating HeLa cells; however, this effect could be mediated by a mechanism independent of DNA synthesis. Yeo et al. (24) found that hydroxyurea inhibits the growth of human diploid fibroblasts and hydroxyurea can induce p53 and p21 levels in human diploid fibroblast cells. Our results are in good agreement with these observations.

Hydroxyurea is currently used as a therapeutic agent in patients with numerous human malignancies (24-30). Although promising results with hydroxyurea have been documented both in experimental animals and in human tumors, the therapeutic efficacy of hydroxyurea is still limited. Furthermore, the mechanism of action of hydroxyurea is not completely understood. It will be very interesting to see whether hydroxyurea has an effect on the transcription, translation, stabilization and degradation of E6 of HPV 18 in cervical carcinoma cell lines.

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