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Involvement of mitogen-activated protein kinases and p21^{Waf1} in hydroxyurea-induced G1 arrest and senescence of McA-RH7777 rat hepatoma cell line

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Abbreviations; cdk, cyclin dependent kinase; ERK, extracellular signal-associated kinase; HU, hydroxyurea; JNK, c-Jun NH_2-terminal kinase; MAP kinase, mitogen-activated protein kinase; p38, p38 MAP kinase; SA, senescence associated; TBS-T, Tris-buffered saline containing Tween 20

Abstract

Hydroxyurea is commonly used to treat hematologic disorders and some type of solid tumors, but the mechanism for its therapeutic effect is not clearly known. In this study, we examined the effect of hydroxyurea on rat hepatoma McA-RH7777 cells, specifically, on the role of mitogen-activated protein (MAP) kinase signal transduction pathways and p21^{Waf1}, p27^{Kip1} and p53. Rat hepatoma McA-RH7777 cells treated with hydroxyurea for 7 days, caused the inhibition of cell growth in a dose-dependent manner. But, this growth inhibition was not caused by necrosis or apoptosis but instead was associated with cell senescence-like change as evidenced by senescence associated-β-galactosidase staining, and cells arrest at G1 phase of cell cycle. Phosphorylation of MAP kinases, such as ERK, JNK, and p38, was found to be decreased after treatment of cells with hydroxyurea. But, the expression of p21^{Waf1} was increased, while p27^{Kip1} and p53 were not detected in hydroxyurea treated rat hepatoma cells. Hydroxyurea treatment induced G1 arrest and a senescence-like changes in rat hepatoma McA-RH7777 cells may be the likely results of signal disruption of MAP kinases (ERK, JNK, and p38 MAP kinase) and p21^{Waf1} over-expression.

Keywords: hydroxyurea; MAP kinases; p21^{Waf1}; rat hepatoma cells

Introduction

Hydroxyurea (HU) is a free radical quencher that inhibits a cellular enzyme, ribonucleoside diphosphate reductase and, in so doing, reduces the levels of deoxyribonucleotides (Krakoff *et al.,* 1968). HU has been used over the last 30 years for the treatment of human diseases such as chronic myelogenous leukemia, myeloproliferative syndromes and more recently sickle cell anemia (Alter and Gilbert, 1985; Charache *et al.,* 1995). In addition, refractory solid tumors such as pancreas carcinoma, cholangiocarcinoma, hepatocellular carcinoma, renal cell carcinoma, etc. were tried with HU alone or combined with known chemotherapeutic regimens (Walder *et al.,* 1996). HU has been known to produce least cytotoxity, to arrest at S phase, reversibly and to inhibit DNA synthesis through the induction of DNA damage. However, in spite of its well known biological and clinical importance, its mechanisms of action remain unknown.

Recently, a family of serine-threonine protein kinases that is structurally similar yet functionally distinct has been identified. These mitogen-activated protein (MAP) kinases fall into 4 distinct groups: the extracellular signal-related kinases (ERKs) (Seger and Krebs, 1995), the c-Jun N-terminal kinases (JNKs) (Minden and Karin, 1997), p38 MAP kinase (p38) (Ichijo, 1999), and Erk5/BMK1 (Zhou *et al.,* 1995). The MAP kinase signaling cascade has been shown to regulate a wide variety of cellular events such as cell proliferation, differentiation and development (Se-

ger and Krebs, 1995; Lewis *et al.,* 1998; Kim *et al.,* 2003; Seo *et al.,* 2004). Therefore the molecules could be a potential target of HU action. To learn more about the signaling pathways used by HU and the molecules which are involved in transducing signaling from HU, we investigated whether cell cycle regulators in MAP kinase signaling pathways, $p21^{Waf1}$, $p27^{Kip1}$ and p53 can play roles in HU effects toward rat hepatoma cell line McA-RH7777.

Materials and Methods

Cell culture and trypan blue exclusion

The rat hepatoma cell line McA-RH7777 was obtained from American Type Culture Collection (Philadelphia, PA) and maintained in DMEM (Life technologies Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Life technologies Inc., Grand Island, NY), 100 units/ml penicillin and streptomycin (Life technologies Inc., Grand Island, NY) in a humidified 5% CO_2 atmosphere at 37°C. For experiments, cells were seeded at 5×10^5 cells/10 ml in 10 cm dishes (Nunc., Naperville) and cultured for 7 days in the presence or absence of HU (Sigma, St. Louis, MO) with the concentrations of 200 μM and 400 μM.

Cells were harvested and mixed with 0.4% trypan blue dye (Sigma Chemical Co., St. Louis, MO), and the mixture was allowed to sit briefly at RT. Cells were counted in hemocytometer chamber under light microscope and scored viable cells.

Flow cytometric analysis

For cell cycle analysis, McA-RH7777 cells were seeded and treated with HU at concentration of 0, 200, and 400 μM, respectively. After 4 days, cells were harvested and processed for propidium iodide staining of the nuclei. The cellular DNA contents were analyzed by the EPICS XL Flow Cytometer (Beckman Coulter). The ranges for G1, S, and G2-M phase cells were established based upon their corresponding DNA contents of the histograms. Results were analyzed and expressed as percentages of the total gated cells.

SA-β-galactosidase staining

HU-untreated or -treated McA-RH7777 cells were stained as previously described (Dimri *et al.,* 1995). Cells were washed in PBS, fixed for 3-5 min in 2% formaldehyde, washed, and incubated at 37°C with the fresh senescence-associated β-galactosidase (SA-β-galactosidase) stain solution: 1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) per ml in 40 mM citric acid/sodium phosphate, pH 6.0 with 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$. Slides were counterstained with hematoxylin, rinsed with distilled water, and mounted onto microscope slides using mounting media.

Western blot analysis

To determine whether HU-treated McA-RH7777 cells express increased MAP kinases-dephosphorylated forms and phosphorylated forms, and cdk inhibitors, we carried out Western blot analysis for ERK1/2, p-ERK, p38, pp38, JNK1/2 and p-JNK, and $p21^{Waf1}$, $p27^{Kip1}$ and p53 with the respective specific antibodies (Santa Cruz, CA). McA-RH7777 cells treated with or without HU were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.2 mM Na vanadate, 1% SDS, 1 mM PMSF, 1 ug/ml aprotinin, 1mg/ml leupeptin) by extensive vortexing. Samples were centrifuged at 13,000 *g* for 10 min to remove cell debris. Total protein content was determined using BioRad Protein Assay (Bio-Rad Laboratories, Hercules). Protein samples were separated by SDS-polyacrylamide gel electrophoresis (12%) and transferred from gels on nitrocellulose membranes (Scheicher and Schuell, Keene), and then blocked in TBS-T buffer (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.05% Tween 20) containing 5% non-fat dried skim milk for 1 h. The blots were incubated with the respective primary antibodies (1 mg/ml) for 1 h, and washed three times in TBS-T buffer, and followed by incubation for 1 h with peroxidase-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch, West Grove) in TBS-T buffer plus 5% nonfat dried milk. Blots were developed using enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Results and Discussion

Liver cancer, especially hepatocellular carcinoma is one of malignant cells with high incidence and mortality rate. The prognosis for liver cancer is poor with current chemotherapeutic agents, for the most part, ineffective. *In vitro* rat hepatoma cell lines showed differential sensitivities to bleomycin, adriamycin, HU and other chemotherapeutic agents (Barranco *et al.,* 1978). Although HU chemotheraphy in hepatoma-bearing rats and HU effect on Novikoff rat hepatoma cell lines have been investigated (Plagemann & Erbe, 1974; Cameron & Rogers, 1977), detailed manifestations and mechanisms involving cell cycle regulators or MAP kinases have not been studied.

It has been known that HU inhibits the growth of

human diploid fibroblasts and K562 human erythroleukemia cells (Park *et al.,* 2000) and induces senescence-like changes. We investigated whether HU induced similar effects on rat hepatoma McA-RH7777 cells. To assess the effect of HU on rat hepatoma cells, McA-RH7777 cells were treated with 200 μM and 400 μM of HU for up to 7 days. Cells were harvested and performed trypan blue exclusion. Viable cells were counted in hemocytometer chamber and graphed the viable cell proportion. As shown in Figure 1, the growth of McA-RH7777 cells was retarded by treatment of HU compared with HU untreated cells. In case of high dose treatment (400 μM), McA-RH7777 cells were more inhibited of growth than low dose treatment (200 μM). To examine whether HU could induce replicative senescence in the culture, McA-RH7777 cells were treated with 200 μM and 400 μM concentration of HU for 4 days, then cell cycle was analyzed by FACS. G1 phase of cell cycle was increased with HU treatment, but G2/M and S phase were decreased by HU treatment. In case of 400 μM HU treatment, McA-RH7777 cells arrested about 54.2% at G1 phase, while G2/M phase cells reached 0% (Figure 2). This result indicated that HU in rat hepatoma cells induced cell cycle arrest at G1 phage resulting inhibition of growth.

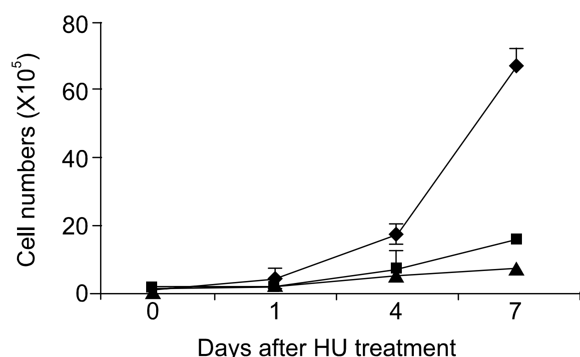

Figure 1. Hydroxyurea-induced cell growth inhibition of McA-RH7777 cells. Cell numbers/ml were determined after incubating McA-RH7777 cells up to 7 days in the presence of 0 to 400 μM of HU. Closed diamond symbol (◆) represents untreated control cells, closed square (■) HU -treated cells at a concentration of 200 μM, closed triangle (▲) HU-treated cells at a concentration of 400 μM.

In an attempt to further understand the mechanism of HU action, a possible association of apoptotic path with the HU-induced growth retardation was examined. Apoptotic state of cells was analyzed by acridine orange and ethidium bromide staining, DNA fragmentation and qualitative and quantitative immunoassay with anti-histone-biotin and anti-DNA POD. However, no evidences of apoptosis by HU was found (data not shown).

Induction of cellular senescence quantified by determining senescence-associated β-galactosidase activity showed a typical HU-induced senescent cells: cytoplasmic blue dots representing the SA-β-galactosidase activity were observed in HU-induced cells, but not in the HU-untreated McA-RH7777 cells (Figure 3). As shown in Figure 3, these cells also appeared to be enlarged, a typical senescent-like morphology (Stanulis-Praeger, 1987). These results suggested that HU induced senescence-like change in hepatoma McA-RH7777 cells. A typical characteristics of senescence cells were found in the HU treated K562 erythroleukemia cells where the changes in morphological phenotypes, such as loss of proliferative capacity, enlarged cell size, and positive SA-β-galactosidase staining (Park *et al.,* 2000).

The mechanism of induction for senescence-like changes of K562 cells by HU has not been clearly understood yet, although its inhibitory activity to ribonucleotide reductase has been suggested (Krakoff *et al.,* 1968). The inhibition of ribonucleotide reductase causes a lack of deoxynucleotides required for DNA synthesis, which in turn results in DNA single strand breaks (Mata *et al.,* 1989). A cellular senescence could be triggered also by several DNA damaging agents, with a cell cycle arrest at G1 or

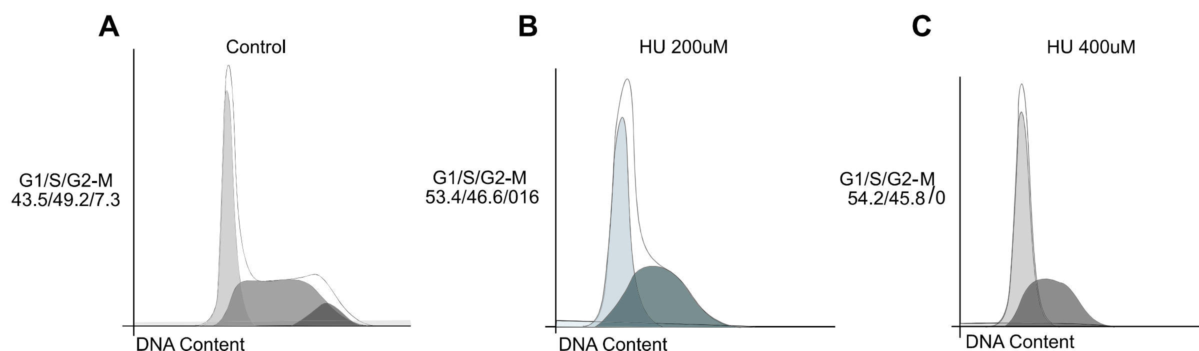

Figure 2. Flow cytometric analysis. McA-RH7777 cells treated with HU for 4 days, show increased distribution of G1/S fraction. (A) untreated control cells; (B) HU-treated cells at a concentration of 200 μM; (C) HU-treated cells at a concentration of 400 μM.

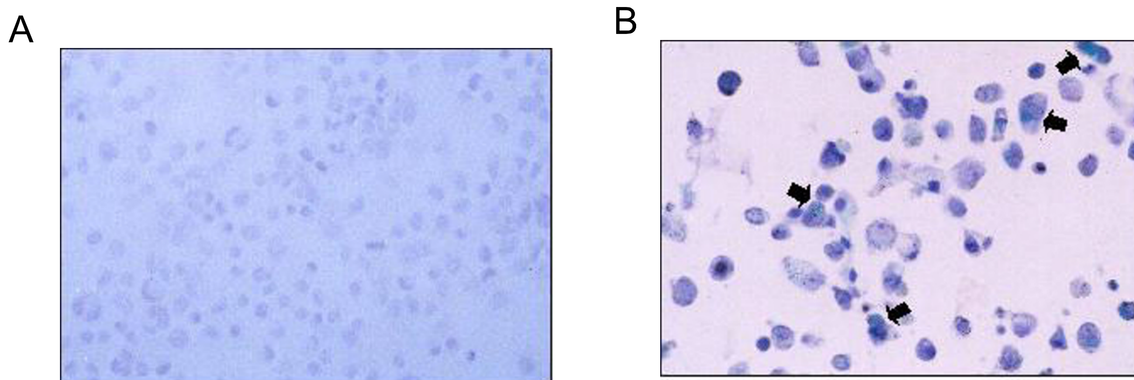

Figure 3. HU-induced SA-β-galactosidase activity. McA-RH7777 cells were treated with HU for 4 days, stained with X-gal, observed under light microscope. (A) untreated cells; (B) HU-treated cells (Arrows indicate cytoplasmic blue reaction.).

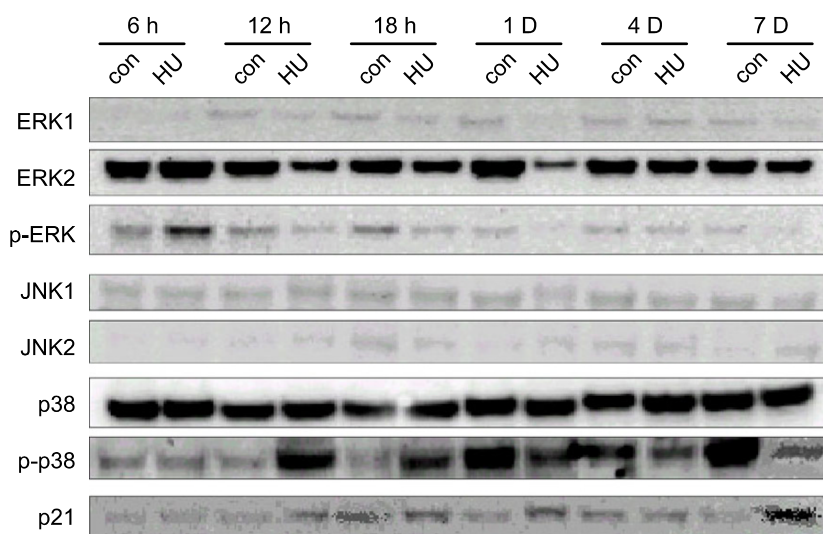

Figure 4. Western blot of MAP kinases, $p21^{Waf1}$ during HU-treatment in McA-RH7777 cells up to 7 days. Expression of ERK1/2, JNK 1/2, p38 are relatively consistant, but p-ERK, p-p38 decrease during HU treatment. Expression of p21 increases during HU-treatment.

G2 phase (Kastan *et al.,* 1991). Lesions in DNA decrease the transcription of many genes, not only for cell proliferation but also for cellular metabolism, which might result in cellular senescence. For example, DNA damage induced by UV irradiation caused cellular senescence with the increase of tumor suppressor p53 protein (Kastan *et al.,* 1991), in association with transcription of several genes, such as gadd 45 and $p21^{Waf1}$, that arrest the cell cycle progression at the G1/S interphase (el-Deiry *et al.,* 1993; Harper *et al.,* 1993). But HU-induced senescence seems to be a phenomenon not necessarily dependent on the p53 pathway, since K562 human erythroleukemia cell, already known that senescence induced by HU treatment, is p53-null.

To understand the cellular changes associated with HU-induced senescence, we investigated the possible involvement of the MAP kinases, such as ERK1/2, JNK, p38 and cell cycle inhibitors, such as 21^{Waf1} and $p27^{Kip1}$ in McA-RH7777 cells. Dephosphorylated and phosphospecific antibodies against the ERK1/2, JNK1/2, and p38 MAP kinases were utilized for the assessment of activities of MAP kinase signaling molecules. As shown in Figure 4, the levels of $p21^{Waf1}$ were highly elevated by HU treatment, while ERK1/2, JNK, and p38 levels were not changed by HU treatment. Although the individual MAP kinases, such as ERK1/2, JNK, and p38, were expressed constantly, but appeared to be dephosphorylated after HU treatment. Therefore, our results showed that HU induced dephophorylation of ERK1/2, JNK, and p38, and up-regulated $p21^{Waf1}$ levels in rat hepatoma McA-RH7777 cells. To elucidate whether the $p21^{Waf1}$ induction is accompanied by p53 in the senescent cells, we also examined the levels of p53 and $p27^{Kip1}$. However, we did not detect p53 and $p27^{Kip1}$ in McA-RH7777 cells with or without treatment of HU (data not shown).

The induction of senescence was closely related to cell growth arrest presumably *via* induction of cell cycle inhibitors. In the case of hydrogen peroxide-induced aging, the induction of p53 and p21^{Waf1} has been reported, while another senescence inducer, sodium butyrate, showed an increase of p21^{Waf1} in a p53-independent manner (Xiao *et al.,* 1997). Also, the senescence induced by a treatment with 5 ₵-aza-2 ₵ -deoxycytidine showed an increase of p16^{INK4a} (Vogt *et al.,* 1998). These data suggested that for senescence induction, any of the cdk inhibitors might play an important role, independently from each other. Analysis of the cdk inhibitors, such as p21^{Waf1}, and p27^{Kip1}, in HU-treated rat hepatoma McA-RH7777 cells showed that HU elevated level of p21^{Waf1}. The accumulation of p21^{Waf1} could cause the cells to arrest at G1 stage, which could result in cellular senescence. Expression of p21^{Waf1} provided protection against the cytotoxic effect of radiation and doxorubicin but not of taxol (Wang *et al.,* 1999b). These results tend to demonstrate that the impact of the cell cycle regulators on susceptibility to cytotoxic agents may depend on the type of drug and DNA damage. Therefore, it can be suggested that HU mediates its effects mainly through p21^{Waf1} in rat hepatoma McA-RH7777 cells.

In conclusion, HU induces cell growth inhibition, G1 arrest and senescence, not apoptosis in rat hepatoma McA-RH7777 cells. It is suggested that decreased activities of ERK, JNK and p38 MAP kinases, and up-regulatory expression of p21^{Waf1} independent of p53 might be important factors, although further investigations for the specific inhibitors of MAP kinases and identification of substrates for the p21^{Waf1} will be required.

Acknowledgement

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