Original Contribution

Effect of the *p53* gene on synchronization of cells with hydroxyurea and on the cell phase response to hyperthermia *in vitro*

MASANORI HATASHITA¹, EIICHI KANO^{1*}, SACHIKO HAYASHI¹,
HIDEKI MATSUMOTO¹, TOSHIO OHTSUBO², ZHAO-HUI JIN¹ and HARUMI ITOH³

Departments of ¹Experimental Radiology and Health Physics, ²Otorhinolaryngology, and ³Radiology,
Fukui Medical University, Matsuoka, Fukui 910-1193, Japan.

Abstract : The effects of the *p53* gene on synchronization of the cell phase with hydroxyurea (HU) and on cellular thermosensitivity in each cell phase were investigated using the murine transfectants with either wild-type *p53* (*wtp53*) or mutated *p53* (*mp53*), or those with a *p53*-deficient genotype. The *p53*-deficient and the *wtp53* cells were well synchronized in the early S phase by HU. A much lower quality of synchronization was obtained in the transfectants with *mp53* than either those with a *p53*-deficient or with *wtp53*. Cells in the S phase were the most thermosensitive among all the cell phases irrespective of the *p53* status of the cells. However, among the cells bearing different status of *p53* gene, there were differences in the cellular thermosensitivity in each phase. The cellular thermosensitivity was higher in the order of the *wtp53*, *p53*-deficient and the *mp53* cells in all cell phases. The *p53* gene is therefore at least one of the factors to determine cellular thermosensitivity.

Key Words : thermosensitivity, *p53*, cell cycle, hydroxyurea, cell phase response

Introduction

Since the early 1970s, cellular thermosensitivity has been known to be changeable according to cell cycle progression^{1) 2)}, indicating that the cells in S phase are the most sensitive to hyperthermia. Many cancer-related genes are suggested to contribute to determination of cellular thermosensitivity³⁾. In particular, many studies reported on the relationship between tumor suppressor gene, *p53*, and cellular thermosensitivity⁴⁻⁸⁾. It has been reported that the *wtp53* gene could sensitize cellular thermosensitivity due to the stimulation of heat-induced apoptosis, whereas the *mp53* cells could suppress heat-induced apoptosis in comparison with the cells with *wtp53* or with a *p53*-deficient genotype⁴⁾. However, since almost all the studies on the relationships between the status of *p53* gene and cellular thermosensitivity have been performed with asynchronous cultured cell populations, cell cycle analysis on the contribution of *p53* gene to cellular thermosensitivity has not been enough adequately clarified. A number of procedures have been established to synchronize the mammalian cell growth *in vitro*⁹⁾. Among these procedures, HU has been known to show its potential to synchronize cells in the early S phase¹⁰⁻¹³⁾. This mechanism of synchronization has been believed that HU inhibits ribonucleotide reductase, preferentially

kills cells in the S phase, and inhibits cells to enter the S phase^{14) 15)}. The wtp53 protein accumulated in response to both genotoxic stressors such as γ -rays and ultraviolet and nongenotoxic stressors such as heat shock and changes in metabolism, has been shown to be a key protein acting as a cell cycle checkpoint^{4) 16)}. Thus, it takes a part of signaling pathway by which cells regulate the G₁-S transition following either genotoxic or nongenotoxic insults; in this way, wtp53 functions as a transcriptional factor to control the cell cycle, especially in stressed cells. Recently, it has been considered that a major functional role of wtp53 is an integrator of stress-response signals¹⁷⁾. Therefore, the synchronization mechanism of cells with HU suggests the possibility involved in p53-dependent cell cycle checkpoint for changes in metabolism of cells.

In the present study, the effect of the p53 gene on synchronization of cells with HU into the early S phase and on cellular thermosensitivity in each cell phase was examined, using the murine transfectants with either wtp53 or mp53, or those with a p53-deficient genotype.

Materials and Methods

Cells and Culture medium

The transfectants of MT158-8/neo-1 and -2, MT158/wtp53-0 and -2, and MT158/mp53-0 and -2 (provided by Prof. T. Ohnishi, Nara Medical University, Japan) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), streptomycin (50 μ g/ml) and kanamycin (50 μ g/ml) (DMEM-10). These transfectants were established by electroporation of *neo*, murine wtp53 or mp53 gene respectively into the fibroblasts from the embryonic p53-deficient mice^{4) 18)}.

Treatment with HU

HU was dissolved in the DMEM-10 medium at the final concentration of 4 mM. The adhered cells in 25-cm² flasks were treated with HU by replacement with 6 ml of the DMEM-10 medium containing HU. The surviving cell fractions from HU treatments for graded periods up to 5 h were determined. After the first treatment with HU for 5 h, the cells were incubated at 37°C for graded interval periods followed by the second treatment with HU or 44°C hyperthermia; the final surviving fractions were determined by colony forming unit.

Treatment with caffeine

Caffeine was dissolved in the DMEM-10 medium containing 4 mM HU at the final concentration of 2 mM. After treatment with HU, MT158/mp53-0 cells in 25-cm² flasks were treated with caffeine by replacement with 6 ml of the DMEM-10 medium containing caffeine and HU to release the cells from arrest in the G₂/M border.

Treatment with 44°C hyperthermia

The surviving fractions of cells from hyperthermia at 44°C were determined using the clonogenic assay. The flasks with 6 ml of DMEM-10 medium were transferred into polystyrene rack with their tightened screw tops, which was immersed in a finely temperature-regulated water bath for 30 min (Model EPS-47, Toyo Seisakusho, Tokyo, Japan). The DMEM-10 medium was overlaid on the adhered cells on bottom

surface of flasks at heat. The water bath preset at 44°C was maintained within the error of $\pm 0.05^\circ\text{C}$ as measured with a thermistor (Model D116-1251, Takara Thermistor Instruments, Yokohama, Japan). After the hyperthermia, the DMEM-10 medium was replaced with 6 ml of fresh DMEM-10 medium at 37°C, the cells were then further incubated to allow colony formation of the surviving cells. The surviving fraction estimated from the number of colonies for each heating period was normalized by the plating efficiency of the non-heated control cells.

Cell cycle analysis

Cell phase distribution was assayed by determination of DNA contents, for which the harvested cells were centrifuged (1,500 rpm, 5 min) in sample tube. The supernatant was discarded and 300 μl of ice cold PBS was supplemented in the tube and 700 μl of ethanol at 0°C was further supplemented to fix cells. The thus supplemented tubes were placed stationarily in the crushed ice for 2 h. After the fixation, cells were rinsed with PBS, and incubated for 30 min at room temperature with RNase (1 mg/ml) and propidium iodide (50 $\mu\text{g/ml}$). The stained cell samples were filtered through 40 μm nylon mesh and subjected to flow cytometry (EPICS Cytometer, Beckman Coulter Inc., Fullerton, CA, USA).

Western blot analysis

The cells were suspended in RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.05% SDS), and treated by freezing and thawing three times. The protein concentration of the supernatant was determined using a BIO-RAD protein assay kit (Bio-Rad Labs., Richmond, CA, USA) after centrifugation ($15,000 \times g$, 15 min). Each aliquot of the supernatant was subjected to western blot analysis. After electrophoresis on 10% polyacrylamide gels containing 0.1% SDS, the proteins were transferred electrophoretically onto PVDF membranes. The membranes were then incubated with anti-cdc2 or anti-cyclin B1 monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). For visualization of the bands, horseradish peroxidase-conjugated antimouse IgG antibody (Zymed Labs. Inc., San Francisco, CA, USA) and 4-chloronaphtol as a substrate for horseradish peroxidase were used.

Results

Response to fractionated treatment with HU

When the three transfected MT158 cell lines were treated with first HU, the survival curves regressed until first 2 h in all the cell lines as shown in Fig. 1. When the cells were treated with HU for more than 2 h, the survival curves were nearly level. After the cells were treated with first HU for 5 h, HU was chased and the cells were rinsed with DMEM-10 medium three times and incubated for graded interval periods and further treated with second HU for 2 h. There was no difference in the cell cycle synchronization status between MT158/neo (*p53*-deficient) and MT158/wtp53 cells as shown in Fig. 1. It was observed that approximately 95% of the survival cells from first HU treatment were killed by the second exposure to HU after the interval period of 4 to 5 h. It was suggested that the survival cells from first HU treatment progressed synchronously according to the interval period and that the cells progressed to the S phase during the interval period were selectively killed by the second HU treatment. However, MT158/mp53 cells indicated a different profile of survival curve from the other two cell lines in the survivals from the second HU treatment. The surviving fractions of MT158/mp53 cells from the second HU treatment

Fig. 1. The cell phase response to the treatment with second HU in the transfectants after synchronization by first HU. Left panel shows surviving fractions from the treatment with 4 mM HU during 5 h. Ordinate; log surviving fractions: Abscissa; treatment periods (h) with HU. Right panel shows surviving fractions from the fractionated treatments with 4 mM HU for 5 h and 2 h interposed with graded interval periods at 37°C. Ordinate; same to that in the left panel: Abscissa; periods of interval incubation in hour between the two fractionated HU treatments. Open circles; MT158/neo-2: open triangles; MT158/wtp53-0: open squares; MT158/mp53-0. Bars represent standard error. Symbols without error bar represent standard error within the symbols.

Fig. 2. Analysis on cell phase distribution analysis of the transfectants with flow cytometry was represented by cellular DNA contents. At various graded incubation periods after HU synchronization with 4mM for 5 h, cells were fixed with ethanol, stained with propidium iodide and analyzed with flow cytometer. Three left panels represent the profiles of the flow cytometric analysis of the transfectants synchronized by HU followed by graded periods of interval incubation. 1; MT158/neo-2: 2; MT158/wtp53-0: and 3; MT158/mp53-0. Left panel represents profiles of the flow cytometric analysis of the transfectants immediately after the HU synchronization. 1-a; MT158/neo-2: 1-b; MT158/neo-1: 2-a; MT158/wtp53-0: 2-b; MT158/wtp53-2: 3-a; MT158/mp53-0: 3-b; MT158/mp53-2. 3-c; the profile of the flow cytometric analysis of the MT158/mp53-0 cells after the treatment with 4mM HU and 2 mM caffeine for 1.5 h simultaneously.

Fig. 3. The time course profiles of cyclin B1 in Western blot analysis. The profiles of cyclin B1 of the transfectants interposed with graded periods at 37°C between HU and the fixation for western blot. 1; MT158/neo-2; 2; MT158/wtp53-0; 3; MT158/mp53-0. Horizontal lane represents C; unsynchronized control: 0; immediately after HU synchronization: 4,8 and 12; 4,8 and 12 h after the interposed 37°C interval.

decreased gradually up to 6 h and then increased gradually up to 10 h of the interval period, suggesting a much lower quality of synchronization.

Cell phase distribution analysis after the HU treatment

Cell phase distributions after the HU treatment were analyzed by flow cytometry. There was no difference in the progression of the synchronized cell cycle between MT158/neo and MT158/wtp53 cells (Fig. 2, left). Both cells were synchronized in the early S phase and proceeded to the mid-S phase and to the G₂/M phase with time after HU was chased. Thus synchronized cell population was desynchronized to considerable extent for the interval period of 12 h. However, MT158/mp53 cells were synchronized at not only G₁/S but also G₂/M borders immediately after the treatment with HU. In the profile of cell phase distribution by flow cytometry, no different profile between the mutant clones so far assayed was observed (Fig 2. right).

Western blot analysis

To distinguish whether or not the synchronization to G₂/M phase of MT158/mp53 cells by the treatment with HU is correlated with the accumulation of cell cycle regulators, cdc2 and cyclin B1 proteins, western blot analysis was performed for the three cell lines, MT158/neo-2, MT158/wtp53-0 and MT158/mp53-0. The expression of cyclin B1 was not observed in MT158/neo and MT158/wtp53 cells, but observed in MT158/mp53 cells immediately after the HU treatment (Fig. 3). However, there was no marked difference in time-dependent changes of protein amount of cdc2, irrespective of the cell line (data not shown).

Cell phase response to hyperthermia of synchronized cells

The surviving fractions from 44°C hyperthermia for 30 min during the graded interval periods for cell phase progression after the treatment with HU for 5 h were plotted against the interval periods in Fig. 4, left. Cell phase response to hyperthermia was found obviously in both MT158/neo and MT158/wtp53 cells. The cells thus treated with HU followed by the interval period of 4 h at 37°C showed most remarkable thermosensitivity to hyperthermia at 44°C. Other hand, cell phase response to hyperthermia was not appreciable for MT158/mp53 cells after the treatment with HU for 5 h, as shown in Fig. 4, left. When MT158/mp53 cells were treated with 4 mM HU for 5 h followed by the treatment with 2 mM

Fig. 4. The cell phase response to the hyperthermia at 44°C for 30 min of the three transfectants interposed with 37°C interval periods after HU synchronization. Left panel represent surviving fractions from hyperthermia at 44°C for 30 min during graded interposed interval periods between 4mM HU for 5 h and hyperthermia. Ordinate; log surviving fractions; Abscissa; interposed interval periods (h): Open circles; MT158/neo-2; open triangles; MT158/wtp53-0; and open squares; MT158/mp53-0. Closed symbol on the ordinate represents surviving fractions from the hyperthermia immediately after 4mM HU for 5 h. Light panel represents surviving fractions of MT158/mp53-0 cells from hyperthermia at 44°C for 30 min after 4mM HU for 5 h followed by 2mM caffeine for 1.5 h. The 37°C interval periods were interposed after the caffeine treatment and the hyperthermia. Ordinate; log surviving fractions; Abscissa; interposed interval periods same to that of left panel. Open symbols represent surviving fraction from hyperthermia at 44°C for 30min after the treatments with HU and caffeine. Closed symbol on the ordinate represents surviving fractions immediately after the caffeine treatment following the HU treatment. Bars represent standard error. Symbols without error bar represent standard error within the symbols.

caffeine for 1.5 h, the population of G₂/M phase disappeared and almost all the cells were synchronized in the early S phase (Fig. 2, right). The surviving fractions of MT158/mp53 cells from 44°C hyperthermia for 30 min during the graded interval periods for cell phase progression after the treatment with HU for 5 h followed by the treatment with 2 mM caffeine for 1.5 h were plotted against the interval periods in Fig. 4, right. Cell phase response to hyperthermia was found more clearly rather than that without the caffeine treatment. The synchronized MT158/mp53 cells in the early S phase by the treatment with HU followed by the treatment with caffeine were most sensitive to the hyperthermia 4 h after chasing the caffeine. In addition, the cellular thermosensitivity in the S phase is much higher in the wtp53 cells rather than that in the p53-deficient or the mp53 cells.

Discussion

It was found that (i) not all the mp53 cells could be synchronized in the early S phase by HU as opposed the p53-deficient and the wtp53 cells and that (ii) the cellular thermosensitivity of the wtp53 cells was much higher in the S phase rather than that in the p53-deficient and in the mp53 cells. HU, which is an inhibitor of DNA synthesis due to specific inhibition of ribonucleotide reductase¹⁴⁾, has been reported to synchronize cells in the early S phase¹⁰⁻¹²⁾. When cells undergoing exponential growth are treated with HU, cells in the S phase are killed¹⁵⁾, while the cells in the other phases survive and arrest in the early S phase, where the survived cells are synchronized. In the present study, the p53-deficient and the wtp53 cells were

synchronized in the early S phase by HU. As for the cells with *mp53*, however, flow cytometry assay indicated that most of the cells were synchronized in the early S phase by HU, but a considerable portion of the cell population was accumulated in the G₂/M phase. It has been previously reported that the synchronization of cells in the early S phase by HU was confirmed in Chinese hamster V79 and CHO-K1 cells bearing the *mp53* gene¹⁹⁻²²⁾ and murine L cells bearing the *wtp53* gene^{5) 23)}. Therefore, the present results suggest that a certain function of the *mp53* gene, which is gained by the mutation, may interfere the signal(s) for induction of synchronization in the early S phase, although the synchronization of cells by HU in the early S phase may be substantially p53-independent.

It is well known that the cellular thermosensitivity is varied by cell cycle progression. Cells in the S phase are highly thermosensitive, and cells in the G₁/S border and those in the G₂/M phase are minimally thermosensitive^{1) 2)}. It could be confirmed that the cells in the S phase are the most sensitive to hyperthermia among all the cell phases with the *p53*-deficient and the *wtp53* cells. In the case of the *mp53* cells, however, this tendency was not clearly observed. Not only the cells in the early S phase but also in the G₂/M phase were accumulated by the treatment with HU as described in the present discussion. The treatment with caffeine reduced radiation-induced cell-cycle delay, especially G₂ phase of the cell cycle²⁴⁾. Whether or not caffeine could release the *mp53* cells arrested in the G₂/M phase forward was examined after the treatment with HU. As shown in Fig. 2, right, caffeine could release the G₂/M arrest of the *mp53* cells induced by the treatment with HU. After the treatment with caffeine, the cells in the S phase are more sensitive to hyperthermia among all the cell phases as well as in the *p53*-deficient and the *wtp53* cells. These results demonstrated that cells in the S phase were the most thermosensitive among all the cell phases irrespective of the *p53* status of the cells. However, there were differences in the cellular thermosensitivity in the S phase among the different status of *p53* gene. The cellular thermosensitivity was higher in the order of the *wtp53*, *p53*-deficient and the *mp53* cells irrespective of cell phase. It has been previously reported that the *wtp53* gene contributed to thermosensitization due to enhancement of heat-induced apoptosis⁴⁾. Taking all those into account, we would conclude that the *p53* gene plays significant role to determine cellular thermosensitivity.

Acknowledgments

The adopted transfectants of MT158 were kindly shared by Prof. T. Ohnishi, Nara Medical University, Nara, Japan. The present research activity was supported in part by a grant in aid for "Assessment on radiation application 1997-2000" of province of Fukui.

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ハイドロキシウレアによる細胞周期同調および各細胞周期位相における温熱感受性に及ぼすがん抑制遺伝子p53の影響

畑下昌範¹・加納永一¹・林 幸子¹・松本英樹¹・大坪俊雄²・金 朝暉¹・伊藤春海³

¹福井医科大学医学部放射線基礎医学講座

²福井医科大学医学部耳鼻咽喉科学講座

³福井医科大学医学部放射線医学講座

要 旨：ハイドロキシウレアによる細胞周期の同調および各細胞周期位相における細胞温熱感受性に及ぼすがん抑制遺伝子p53の影響について検討した。p53遺伝子欠損マウス由来線維芽細胞と野生型および変異型p53遺伝子を導入した細胞を用いた。p53遺伝子欠損細胞および野生型p53細胞はハイドロキシウレアによりS期初期に高品位に同調された。一方、変異型p53細胞の同調は低品位であった。S期細胞はp53遺伝子の型に関わらず各細胞周期の中で最も温熱感受性であった。しかし、各細胞間では細胞周期位相に関わらず温熱感受性に差異があり、感受性の高い順に、野生型p53細胞>p53遺伝子欠損細胞>変異型p53細胞であった。p53遺伝子が温熱感受性を規定する因子の一つであることが示された。