Role of p53 in Growth Suppression by Bromodeoxyuridine in Human Gastric Cancer Cell Lines

Guang-Zhi Zhan¹ ², Hiroyuki Sugihara¹, Dun-Fa Peng¹, Zhi-Qiang Ling¹, Xiao-Hong Yao¹, Kazusada Yoshitake² and Takanori Hattori ¹

¹First Department of Pathology and ²Department of Oral and Maxillofacial Surgery, Shiga University of Medical Science, Ohtsu 520-2192

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Bromodeoxyuridine (BrdU) is known to cause base mispairs and DNA strand breaks during DNA synthesis, culminating in growth suppression. To know whether P53 gene plays any role in the BrdU-induced growth suppression, we continuously gave BrdU (20 μM) to the synchronized culture of human gastric cancer cell lines, MKN-45 (P53-wild type) and MKN-28 (P53-mutant), shortly after release from the G1/S block by hydroxyurea. In comparison with the control culture, the growth of MKN-28 was not suppressed until 48 hr of BrdU exposure, while that of MKN-45 was already suppressed at the 24 hr point. Continuous exposure to BrdU caused a S-phase delay and G2 arrest of around 6 hr each in MKN-45 and a delay only of the second S phase in MKN-28 in comparison with synchronized control cultures of matched cell cycle phase. BrdU-exposed MKN-45 cells showed a significantly higher incidence of apoptosis after the cells passed through the first G2 phase and the second S/G2 phases, but MKN-28 did not. It thus appears that the delays of S/G2 phases and an increased incidence of apoptosis in the first cell cycle are p53-dependent. The growth suppression in the second S/G2 phase or later observed in both of the cell lines may be p53-independent.

Key words: Bromodeoxyuridine, Cell cycle, Apoptosis, p53, Gastric cancer cell line

I. Introduction

Bromodeoxyuridine (BrdU), a synthetic analogue of thymidine, is incorporated into DNA during the S phase of cell cycle, and is widely used to label DNA in studies of cellular proliferation [8, 30, 34, 36]. BrdU is also known to cause increased sensitivity to ionizing radiation [29] and to have various biological effects including toxicity, mutagenicity, teratogenicity and inhibition of differentiation [7]. Though its toxicity might exert an influence on the results of cell kinetic analysis, the concrete effects of BrdU on cellular viability and cell cycle parameters have not fully been studied.

BrdU is known to have ambiguous pairing properties due to the presence of the bromine atom; a shift from a keto form (that makes a base pair with adenine) to an enol form (that does the same with guanine) occurs with a certain probability [18]. When this shift occurs in BrdU incorporated in DNA, it may lead to incorporation of an inappropriate base during the replication of BrdU-incorporated DNA strand [13] or a reparative process by base excision repair system [15], resulting in AT-GC transition. BrdU is also known to cause DNA strand breaks during DNA synthesis through disturbance of cellular nucleotide pools particularly in a deoxyuridine-less state [20, 26, 35].

Recently, BrdU has been noted to induce apoptosis [14, 34]. The above-mentioned mispairs and strand breaks elicited by BrdU are thought to be responsible, at least in part, for BrdU-induced apoptosis. But it is still unknown whether this induction of apoptosis is related to P53 gene expression, which plays important roles in cell cycle control, DNA repair and regulation of apoptosis [1, 17, 19, 24]. It is known that p53 mediates apoptosis in response to various stimuli including DNA damage, withdrawal of growth factors and expression of certain genes such as MYC in certain cell types [4, 12, 23]. Apoptosis is also known to be induced in a p53-independent manner by various stimuli, such as antineoplastic agents [16, 25, 31], virus oncogenes [27], and X-irradiation [10].

BrdU was also demonstrated to induce growth suppression in a certain cell line [14]. The present study was conducted to determine to what extent the BrdU-induced...
growth suppression is ascribed to apoptosis and which of the cell cycle phase is responsible for the apoptosis and cell cycle arrest using a synchronized culture system. To know whether p53 takes any part in the growth suppression by BrdU, we compared the effects of BrdU on growth, cell cycle progression and the occurrence of apoptosis between P53-wild type and P53-mutated human gastric cancer cell lines.

II. Materials and Methods

Cell lines and chemicals

Two human gastric cancer cell lines, MKN-45 (P53-wild type) and MKN-28 (P53-mutant) [2, 21] were grown in RPMI 1640 medium with 2 mM L-glutamine (Nacalai Tesque, Inc., Kyoto, Japan), 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL Life Technologies, Inc., Rockville, USA) and 5% antibiotic-antimycotic (Gibco-BRL). Cell culture dishes of 6 cm in diameter were incubated in 5% CO2 and 95% air at 37°C. The cells were subcultured every third day. The initial cell density of culture was adjusted to 5×10^4 cells/ml using a hemocytometer. BrdU (Sigma Chemical Co., Ltd., St. Louis, USA) was dissolved in distilled water immediately before use and added to the medium at a final concentration of 20 μM. Control cultures were treated with distilled water at a portion of 0.1% in culture medium. All cultures with BrdU treatment were carefully protected from light of short wavelength by aluminum foil wrappings.

Growth rate and viability

MKN-45 and MKN-28 cells were distributed into 6 cm dishes at a concentration of 5×10^4 cells/ml 24 hr after subculture, and were divided into two groups: control and BrdU-treated (at the final concentration were 20 μM and 200 μM) cultures. Cells were harvested at the following time points: 24 hr, 48 hr, 72 hr, 96 hr, 120 hr and 144 hr after addition of BrdU. The cell density (cell number/ml) of each sample was determined with a hemocytometer. Cell viability was determined as a percentage of the cells positive for trypan blue (Sigma) dye exclusion test.

Cell kinetic analysis

Dishes of exponentially growing cells were prepared and treated with BrdU for 1 hr, 8 hr, 16 hr, or 24 hr. The cells were harvested at the same time point, 48 hr after the subculture. All the samples were fixed in methanol/acidic acid (3:1) and stored in -20°C until use. The BrdU-labeled cells were detected using an immunoperoxidase method with streptavidin-biotin kit (Nichirei, Tokyo, Japan) and an anti-BrdU antibody (Dako A/S, Glostrup, Denmark, 1:50). Mean BrdU labeling indices (LIs) were calculated from LIs of 3 dishes for each time point. The maximum cell cycle time (Tc) and the maximum S phase time (Ts) were estimated from the labeling index curve. Tc was determined by extrapolating the time required for the labeling index to gain from 0% to 100%. Ts was determined by the following equation: Ts=Tc×LI.

Synchronization and continuous exposure to BrdU

Cells were subcultured into 75 cm² culture flasks with 30 ml medium at a density of 5×10^4/ml. 24 hr after subculture, the cells were treated with hydroxyurea (HU, Nacalai Tesque) at a final concentration of 3 mM for 24 hr. Then, the cells were cultured in HU-free medium for 15 hr and treated again with HU at the same final concentration for 24 hr. Thereby, the cells were synchronized at the G1/S boundary. The synchronized cells were distributed into 6 cm culture dishes (5×10^4/ml cells in 5 ml medium) after change of the medium to an HU-free one and treatment with trypsin (Gibco BRL). The dishes were divided into two groups: control ones and BrdU (20 μM BrdU)-treated ones with the period of treatment ranging from 6 hr to 51 hr at intervals of 3 hr. The cell harvest was fixed in methanol/acetic acid (3:1) and stored at -20°C. The experiments were repeated in triplicate.

DNA cytofluorometry

The cells smeared on glass slides were stained with 50 ng/ml 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) in antifade solution (10 mM Tris/10 mM EDTA/100 mM NaCl/10 mM 2-mercaptoethanol, pH 7.4) for 30 min at room temperature and were mounted with a staining solution [9]. The DNA contents of 300 nuclei were measured on each smear slide with a fluorescence cytophotometer (Nikon P-100, Nikon, Tokyo, Japan), simultaneously classifying the cell types into interphase, mitosis, apoptosis and the others. Apoptotic cells/bodies were identified by the morphologic criteria [33]. The nuclear DNA content was expressed in frequency histograms.

TdT-mediated dUTP-biotin nick end labeling (TUNEL)

TUNEL was done after the method of Gavriel et al. [6] after microwave treatment [28] for a short time as described previously [34].

Data processing and statistical analysis

DNA histogram analysis and statistical data analysis were carried out by Statview software. Differences of average apoptotic indices of matched cell cycle phase between the control and BrdU-treated groups were assessed by Student’s t-test. A difference was considered to be significant when the P value was smaller than 0.05.

III. Results

Effect of BrdU on growth curve and viability test

Until 48 hr of 20 μM BrdU treatment, MKN-28 grew at almost the same rate as the control. Thereafter, growth of MKN-28 was suppressed. The growth of MKN-45 appeared to be suppressed from the beginning of the BrdU exposure (Fig. 1). There was no significant difference in
cell viability between the control and BrdU-treated cells until 120 hr of 20 μM BrdU treatment in both MKN-45 and MKN-28 (Fig. 2). When the cells were exposed to a higher concentration of BrdU (200 μM), marked growth suppression and remarkable increase of non-viable cells were observed in both cell lines.

**Cell kinetic analysis by continuous BrdU labeling**

Up to 24 hr of exposure to BrdU, the percentage of BrdU-positive nuclei increased time-dependently. After exposure to BrdU for 1 hr, 8 hr, 16 hr and 24 hr, the average BrdU LI of MKN-45 were 37.9%, 77.9%, 94.0%, 95.5%, and those of MKN-28 were 44.8%, 71.3%, 89.8%, 94.4%, respectively. Plotting the period of labeling, X (hr) and the LI, Y (%), as the abscissa and the ordinate, respectively, we determined, regression curves: Y=3.681X + 39.086 (R²=0.928) for MKN-45 and Y=2.986X + 43.761 (R²=0.98) for MKN-28, hence R² is the coefficient of determination. From these curves, the maximum Tc/Ts was estimated to be 27 hr/10 hr in MKN-45 and 33.5 hr/14.5 hr in MKN-28.

Taking the effect of BrdU on the growth described above into consideration, Tc of MKN-45 may be overestimated because of BrdU-induced delay of cell cycle in this cell line, while there may be little problem in the estimation of Tc in MKN-28.

**Effect of HU on cell cycle and apoptosis in BrdU-free cultures**

Just after release from the arrest by HU, the syn-
Fig. 3. Sequential DNA histograms of MKN-45 after release of cell cycle arrest by HU. A: DNA histograms of viable cells in control synchronized cultures without BrdU. B: DNA histograms of viable cells in BrdU-treated cultures. C: DNA histograms of apoptotic cells of control (upper) and BrdU-treated (lower) groups.
Fig. 4. Sequential DNA histograms of MKN-28 after release of cell cycle arrest by HU. A: DNA histograms of viable cells in control synchronized cultures without BrdU. B: DNA histograms of viable cells in BrdU-treated cultures. C: DNA histograms of apoptotic cells of control (upper) and BrdU-treated (lower) groups.
chronized cells (0 hr) showed a high G1 peak and depleted S/G2 phase cells with a lower G1 peak of polyploid population in both cell lines (Figs. 3A, 4A). They entered S phase and then G2 phase without overt arrest. The G2 phase was defined as the time point with the high G2 peak and decreased S phase fraction. From sequential changes of mitotic index (data not shown) and the pattern of DNA histograms, the G2/M phase was inferred to correspond to 9–12 hr point in both MKN-45 and MKN-28. The G1/S boundary was defined as the time point after which the S phase fraction increased. Both cell lines seemed to pass the G1/S boundary just before the second S phase at 21–24 hr point, corresponding to the mean cell cycle time, which was smaller than the maximum cell cycle time estimated above. Both cell lines reached the G2 phase for the second time at the 30–33 hr point. The second G1/S boundary was noted at 42–48 hr point (Table 1). No overt G2 arrest was observed thereafter.

The DNA histogram of the second but not the first S/G2 phase was accompanied by a higher G1 peak; a small part of the cells seemed to be arrested at the G1 phase for about 12 hr. We also observed a small number (up to around 5%) of apoptosis of which the DNA content often exceeded the value of the G1 peak (S/G2 apoptosis, Fig. 5A). We tentatively define pre-G1 apoptosis as the one whose DNA contents were smaller than the G1 peak value (Fig. 5B). This pre-G1 apoptosis includes G1 apoptosis as well as degraded S/G2 apoptosis. The pre-G1 apoptosis and the S/G2 one were positively stained by the TUNEL method (Fig. 5C, D).

Effect of continuous exposure to BrdU on cell cycle and apoptosis

As HU itself induced some apoptosis in the control experiment, we describe the difference in incidence of apoptosis of matched cell cycle phase between the BrdU-treated and control cells (Figs. 3, 4). Mitotic index of MKN-28 cells abruptly increased at 12 hr after removal of HU, while it took about 18 hr for MKN-45 cells to present the G2 peak and increase of mitotic index, which appeared 6 hr later than those of the control, indicating the presence of the S-phase delay. This delay of S phase progression was accompanied by an increase of S/G2 apoptosis in the S phase in comparison with control cells (Fig. 3B, C and Table 1), though this increase was not statistically significant. In MKN-28, neither S-phase delay nor significantly increased incidence of S/G2 apoptosis was observed in the first cell cycle (Fig. 4B, Table 1).

After 30 hr of synchronization, high G2 peak remained in MKN-45, indicating a G2 arrest (Fig. 3B).

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**Fig. 5.** Morphology of apoptosis detected at 48 hr point of BrdU exposure in MKN-45. A: G2/S apoptosis. DAPI. B: Pre-G1 apoptosis. DAPI. C: TUNEL stain of G2/S apoptosis. D: TUNEL stain of pre-G1 apoptosis. Arrows indicate apoptosis.
Table 1. Sequential changes in incidence of apoptosis in synchronized cultures of MKN-45 and MKN-28.

<table>
<thead>
<tr>
<th>Cell cycle phase</th>
<th>S</th>
<th>G2</th>
<th>G2-G1</th>
<th>G1/S</th>
<th>Second S</th>
<th>Second G2</th>
</tr>
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<tr>
<td><strong>MKN-45</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>BrdU-treated time after HU removal (hr)</td>
<td>6</td>
<td>12-21</td>
<td>21-30</td>
<td>30-39</td>
<td>36-42</td>
<td>42-48</td>
</tr>
<tr>
<td>Incidence of apoptosis (%)</td>
<td>5.51 ± 2.03</td>
<td>8.67 ± 2.70</td>
<td>6.40 ± 1.13</td>
<td>6.61 ± 1.96</td>
<td>8.32 ± 0.99</td>
<td>6.37 ± 0.04</td>
</tr>
<tr>
<td>Time after HU removal (hr)</td>
<td>6</td>
<td>9-12</td>
<td>15-18</td>
<td>24</td>
<td>27</td>
<td>30-33</td>
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<tr>
<td>Apoptosis of control cells (%)</td>
<td>2.78 ± 1.13</td>
<td>2.68 ± 1.24</td>
<td>3.13 ± 0.64</td>
<td>4.81 ± 0.47</td>
<td>4.27 ± 1.53</td>
<td>3.79 ± 0.83</td>
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<td>P value</td>
<td>0.353</td>
<td>0.03</td>
<td>0.007</td>
<td>0.231</td>
<td>0.025</td>
<td>0.009</td>
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<tr>
<td><strong>MKN-28</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>BrdU-treated time after HU removal (hr)</td>
<td>6</td>
<td>9</td>
<td>15</td>
<td>21-24</td>
<td>33-39</td>
<td>39-42</td>
</tr>
<tr>
<td>Incidence of apoptosis (%)</td>
<td>2.35 ± 0.49</td>
<td>4.82 ± 2.20</td>
<td>4.68 ± 1.75</td>
<td>5.20 ± 1.75</td>
<td>10.69 ± 4.32</td>
<td>10.31 ± 4.16</td>
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<tr>
<td>Time after HU removal (hr)</td>
<td>6</td>
<td>9</td>
<td>15</td>
<td>21</td>
<td>27-33</td>
<td>33</td>
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<tr>
<td>Apoptosis of control cells (%)</td>
<td>1.46 ± 0.70</td>
<td>3.12 ± 0.145</td>
<td>5.84 ± 0.48</td>
<td>6.04 ± 1.04</td>
<td>7.32 ± 2.80</td>
<td>6.75 ± 1.19</td>
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<tr>
<td>P value</td>
<td>0.304</td>
<td>0.292</td>
<td>0.301</td>
<td>0.38</td>
<td>0.212</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Undertable legend: * mean ± standard deviation (SD).

This arrest caused an additional cell cycle delay of 6 hr. At 30-39 hr point, about 12 hr later than the control culture, BrdU-treated MKN-45 cells seemed to reach the G1/S boundary, while MKN-28 did at 21-24 hr of synchronization. At the G1/S boundary, BrdU-treated MKN-45 cells but not MKN-28 cells showed an increasing number of pre-G1 apoptosis (Fig. 3C), though this increase was not statistically significant compared with non-BrdU control cells (Table 1).

MKN-45 cells reached the second G2 phase at 42-48 hr of synchronization, which was about 18 hr later than the control culture, due to the G2 arrest and repeated S phase delay (Fig. 3B). MKN-28 cells reached the middle point of the second S phase at 33-39 hr point and second G2 phase at 39-42 hr point, which was 6-9 hr later than the cells without BrdU treatment (Fig. 4B). From DNA histograms, this delay did not reflect G1/S arrest but represents prolonged second S phase. Both cell lines showed increased S/G2 apoptosis (Figs. 3C, 4C and Table 1), though this increase was not statistically significant in MKN-28.

IV. Discussion

In comparison with the control culture, the growth of MKN-28 was not suppressed until 48 hr of BrdU exposure, while that of MKN-45 was already suppressed at 24 hr point. The viability test indicates that the growth suppression could not be explained by cell death. Accordingly, as a result of a provisional experiment in a non-synchronized condition, only a small increase in the incidence of apoptosis was observed in BrdU-treated cells of either cell line.

It is well known that p53 plays a key role in induction of cell cycle arrest and apoptosis in the G1/S and G2/M checkpoints [1, 17, 19, 24]. The DNA strand breaks elicited by nucleotide pool imbalance due to BrdU during S phase is thought to be checked up at the G2/M checkpoint, while base mispairs induced by DNA-incorporated BrdU is at the G1/S checkpoint. Because the mispairs and DNA strand breaks occur at a certain lower incidence, the apoptosis analyzed in this study is so infrequent that it would not be detected as DNA ladder on gel electrophoresis.

To determine which phase in cell cycle is responsible for the BrdU-induced apoptosis or cell cycle delay, a synchronized culture system with HU [22] was used in this study. HU is known to cause cell cycle arrest at earliest S phase just after the cells pass through the G1/S boundary. On the basis of the determined Tc and Ts values, we adjusted the protocol of HU treatment, so that the cells were successfully synchronized. As HU itself induced some apoptosis as reported previously [3], we had to carefully assess the difference in incidence of apoptosis between the BrdU-treated and non-treated cells after matching the cell cycle phase (Table 1). The present study demonstrated that up to 48 hr of BrdU exposure, cell cycle delay in S phase and G2 phase rather than induction of apoptosis may be the major cause of growth suppression in MKN-45. The cell cycle time of MKN 45, which was shorter than that of MKN-28 without HU or BrdU treatment, became about 12 hr greater than that of MKN-28 after HU and BrdU treatments.

In the present study, continuous exposure to BrdU caused S-phase delay of about 6 hr, a G2 arrest of a further 6 hr and induction of S/G2 apoptosis in P53-wild type MKN-45. In P53-mutant MKN-28, S-phase delay was observed only in the second cell cycle, and no significant increase of apoptosis was observed throughout the observed period of synchronization (up to 51 hr after release from the arrest by HU). When the cells passed the G1/S boundary, the incidence of pre-G1 apoptosis tended to increase in MKN-45 but not in MKN-28. Our results suggest that the S-phase delay of the first cell cycle and apoptosis in the
S/G2 phase and possibly those at the G1/S checkpoint are p53-dependent. To confirm this possibility we have to check the expression of the downstream genes of p53, such as WAF1/CIP1 [5], and the genes taking part in induction of p53-independent apoptosis, such as MYC [11, 37].

In the second S/G2 phases of MKN-45, we observed not only S phase delay as in the first cell cycle but also a significantly greater number of apoptosis than that in the synchronized control cells of matched cell cycle phase, while MKN-28 cells showed S phase delay only in the second S/G2 phase. This difference between the first and the second S/G2 phases can be explained as follows. In the first S/G2 phase, apoptosis may be elicited by the presence of single strand breaks in p53-dependent manner as mentioned above. In the second S/G2 phases, BrdU-incorporated DNA replicate in the presence of BrdU and imbalance of nucleotide precursor pools. In this context, BrdU may elicit strand breaks of DNA that induce apoptosis or mitotic death in a p53-independent manner. In MKN-28 as well as MKN-45, DNA synthesis was thus partially disturbed and apoptosis was induced in the second S phase. This circumstance may continue after the second G2/M phases causing growth suppression in both cell lines.

The cell line in which apoptosis was first reported to be induced by BrdU was HL-60 [14], which is known to have large deletion in P53 gene and amplified MYC gene [32]. Interestingly, the growth curve of HL-60 reported showed the time sequence of growth suppression very similar to that of MKN-28, indicating that the apoptosis commonly observed in these cell lines were not only p53-independent but also inducible in the second S phase or later after BrdU exposure. Accordingly, using HL-60, it was shown that the decision to undergo p53-independent apoptosis induced by X-irradiation was made during the arrest at G2 checkpoint [10].

In our preliminary data, BrdU induced not only p53-dependent and -independent apoptosis but also non-apoptotic cell death after continuous exposure for more than 72 hr. BrdU-induced cell death is an interesting system in that various types of cell death can be induced by the same agent and may be useful for disclosing relationships among various kinds of cell death.

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