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Effect of Hyperthermia on the Cell Cycle and Cyclin B Expression in Human Glioblastoma Cell Line

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Abstract Cell kinetic effects of hyperthermia on glioblastoma cells were investigated using flow cytometry. Pulse-labeling with 5-bromodeoxyuridine (BUdR) and chasing of the labeled cells showed a temporary accumulation of the labeled cells in the G_2M phase and a reduction of DNA synthesis.

Cyclin B protein rises rapidly in G_2 phase and falls at the end of mitosis in normal cycling cells. Cyclin B protein binds to $p34^{cdc2}$, resulting in histone kinase activity which is necessary for the initiation of mitosis. The amount of $p34^{cdc2}$ is constant throughout the cell cycle. The level of cyclin B was measured by an anti-cyclin B antibody and flow cytometry in order to investigate the cause of the G_2 accumulation induced by hyperthermia. The low level of cyclin B after hyperthermia, in comparison with that of normal cycling cells, persisted for more than 3 hours. These results indicate that the temporary accumulation of cells in the G_2M phase after hyperthermia may be caused at least in part by an insufficient level of cyclin B.

Key Words: Hyperthermia, Glioblastoma, Cell kinetics, Bromodeoxyuridine, Cyclin B

Introduction

The cell kinetic effects of hyperthermia have not been studied in detail. However, flow cytometric analysis of human glioblastoma cells after heat treatment has revealed a temporary accumulation of cells in the G_2M phase[1)22)] and pulse-labeling with BUdR analysis of human pancreatic adenocarcinoma cells also showed a reduced DNA synthesis rate and a temporary accumulation of cells in the G_2M phase[13)]. As shown by labeling with BUdR, the effect is most marked in S phase cells[21)] and chase of the labeled cells provides more additional information[15)16)].

Recent investigations on the mechanisms of cell cycle progression have demonstrated that several proteins, initially isolated from yeast cells but later identified in higher eukaryotic species as well, appear to play a direct role in cell cycle control[5)6)9)11)14)18)20)]. Among these, $p34^{cdc2}$ and cyclin B are particularly important to bring cells into mitosis. However, the amount of $p34^{cdc2}$ is constant throughout the cell cycle. For the entry into mitosis, it is required that the $p34^{cdc2}$ is dephosphorylated and bound to cyclin B. The cyclin B is synthesized mainly in G_2 phase and destroyed abruptly in M phase (Fig. 1). Therefore, we directed our attention to the change in abundance of cyclin B after hyperthermia and analyzed kinetics of heat-treated cells using the technique of BUdR pulse labeling and a monoclonal antibody against cyclin B and $p34^{cdc2}$, particularly at G_2M phase.

Materials and Methods

Cell Culture

Human glioblastoma A172 cells, obtained from the Japanese Cancer Research

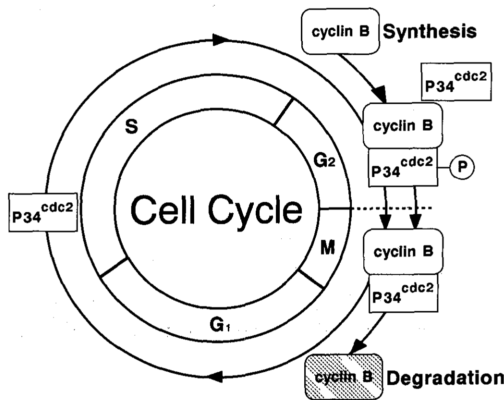

Fig. 1. The cycle of association between p34cdc2 and cyclin B involves phosphorylations, dephosphorylations, and proteolytic degradation. The level of cyclin B rises during G_2 phase, and then this protein combines with p34cdc2. Secondly, dephosphorylations of p34cdc2 are required to generate mitosis-promoting activity. Cyclin B is precipitously destroyed at the end of M phase.

Resources Bank, were maintained in Petri dishes with Dulbecco's modified Eagle medium (DMEM, Gibco Co.) supplemented with 10% fetal bovine serum (FBS, Gibco Co.) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. In this experimental condition, doubling time of cells was about 24 hours. This cell line was used at the exponential growth phase after subculture in completely fresh medium. All experiments were performed with a starting cell density of about 5×10^5 cells per 100-mm tissue culture dish. For each experiment, approximately 10^7 cells were harvested by trypsinization.

Pulse-labeling with BUdR and Chasing of Labeled Cells

For determination of the relative DNA synthesis rates and periods of the cell cycle, the cells were labeled with 15μM BUdR (Sigma Chemical Co.) at 37°C. Twenty minutes later, the pulse-labeled cells were washed twice with warm PBS and recultured in the BUdR-free standard medium to monitor their passage through the cell cycle. The cells were harvested using 0.02% trypsin solution at intervals of 6 hours for 24 hours, and then fixed in 70% cold ethanol. This method was established by Dolbeare and Sasaki, including BUdR/DNA double staining[4)23)].

Heat treatment

Cells were exposed for 60 minutes in a humidified incubator of 95% air and 5% CO_2 at 43°C. In this atmosphere, 10 minutes was needed to elevate medium temperature at 43°C.

Staining Procedure

1) *BUdR/DNA double staining*

The ethanol-fixed cells were rinsed with cold PBS and incubated in 4N HCl at room temperature for 20 minutes. The cells were washed twice with borax-borate buffer and subsequently once with PBS and suspended in 0.5ml PBS containing 0.5% Tween 20 (Sigma Chemical Co.), 0.5% bovine serum albumin, and 10μl of monoclonal anti-BUdR antibody (Becton-Dickinson Co.) at room temperature for 1 hour. After washing twice in PBS, the cells were exposed to FITC-conjugated goat anti-mouse IgG antibody (CALTAG Co.) diluted 1:50 with PBS containing 0.5% Tween 20 and BSA at room temperature for 30 minutes. After rinsing twice in PBS, the cells stained with FITC were resuspended in PBS containing 0.1% RNase (Sigma Chemical Co.). The staining of DNA was performed in 10μg/ml propidium iodide (PI, Calbiochem-Behring Co.).

2) *Cyclin B1 and p34cdc2/DNA double staining*

Non-BUdR-treated cells with or without heat treatment were also harvested for staining of cyclin B1 and p34cdc2. These cells were washed twice in PBS and fixed overnight in 50% vol/vol methanol/acetone. The fixed cells were washed twice in PBS and incubated with monoclonal anti-cyclin B1 (Oncogene Science Co.) or anti-p34cdc2 antibody (Pharmingen Co.), diluted in PBS containing 3% BSA, for 1 hour at room temperature. After 3 washes in PBS, the secondary antibody, FITC-conjugated goat anti-mouse IgG (CALTAG Co.), was applied for 1

hour at room temperature. After 3 washes in PBS, DNA staining was performed in the same way as for BUdR/DNA double staining.

Flow cytometry

Flow cytometric measurements were made using a Becton Dickinson FACScan Analyzer. Data were acquired and analyzed on a CellFIT, the final result being a cytogram of green (FITC) vs. red (DNA) fluorescence. For calculation of the percentage and the level of FITC-labeled cells, the threshold was set to exclude nonspecific fluorescence of the control cells. The labeling index in G_2M phase were calculated as a percentage of FITC-positive cells in G_2M phase. The level of FITC in G_2M phase were calculated as the mean content of only positively stained cells in G_2M phase.

Results

BUdR pulse labeling

Cells in S phase were marked with BUdR by pulse-labeling at the start of the experiment and, were able to progress their cell cycle. At the start of the experiment, the BUdR/DNA distribution of unheated cells showed a horseshoe pattern (Fig. 2). The rate of DNA synthesis increased in early S phase, reached a maximum in mid S phase, and dropped sharply again in the late S phase. The labeled unheated cell cohort passed through the late S, G_2, M, G_1, and S phases in order. In the cycle of the labeled cell cohort, the intensity of FITC per cell decreased to half because of the dilution of label with cell division in the preceding mitosis (Fig. 2: top panel).

In contrast to the control, the labeled 43°C heated cell cohort shifted to G_2 phase slowly at the first stage of the experiment and accumulated at G_2M phase (Fig. 2: bottom panel). This accumulation was gradually released by 24 hours after hyperthermia. The percentage of BUdR-positive heated at 43°C cells in G_2M phase remained higher than that of unheated cells since 12 hours after

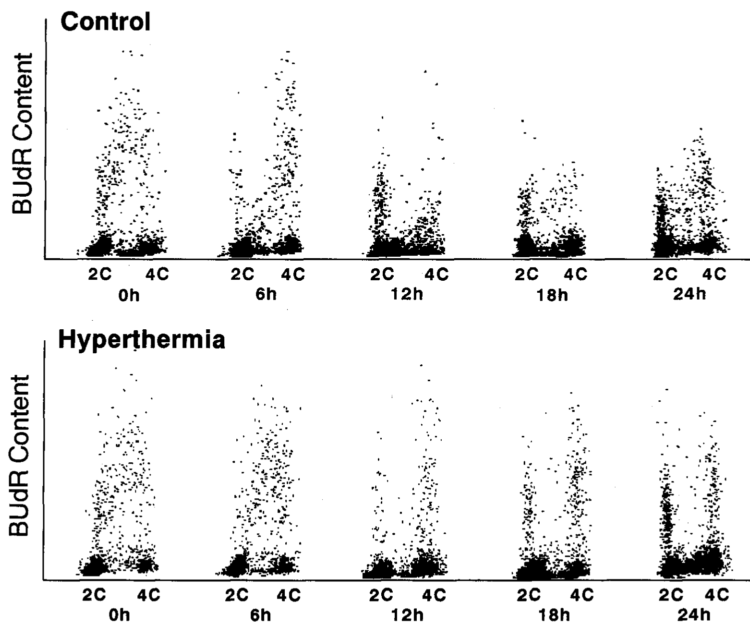

Fig. 2. The change of BUdR/DNA dot-plot distribution of A-172 cells pulse-labeled for 20 minutes with BUdR.
Top panel: Control cells in log phase growth. The labeled unheated cell cohort traversed the cell cycle in order. In the cycle of the labeled cell cohort, the intensity of FITC per cell decreased to half.
Bottom panel: Cells heated at 43°C. The labeled 43°C heated cell cohort shifted to G_2 phase slowly at the first stage of the experiment and accumulated at G_2M phase.

heat treatment, which rose and fell with the course of the cell cycle (Fig. 3). In the study using BUdR pulse-labeling after hyperthermia, the total amount of BUdR incorporated into the heated cells in S phase also decreased until 6 hours after heat treatment.

Hyperthermia and the level of Cyclin B

Cyclin B content versus DNA distribution showed a bootlike pattern, as the level of cyclin B was low in G_1 and S phases and increased rapidly in the G_2 and M phases. Heat treatment of asynchronous glioblastoma cell line A172 (43°C for 1 hour) caused significant inhibition of cyclin B expression (Fig. 4). The level of cyclin B in G_2M phase was lower at 3 hours than immediately after hyperthermia. The level of cyclin B in G_2M phase recovered by 6 hours after treatment (Fig. 5). The labeling index of cyclin B took the similar course of the cyclin B level (Fig. 5).

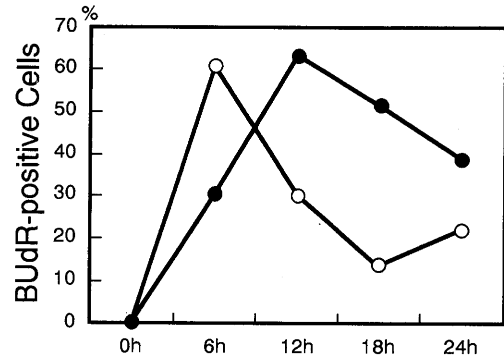

Fig. 3. Percentage of BUdR-positive cells in G_2M phases against time. Open circles: control; closed circles: 43°C heated cells. Abscissa: Time (hour), Ordinate: % of BUdR-positive Cells.

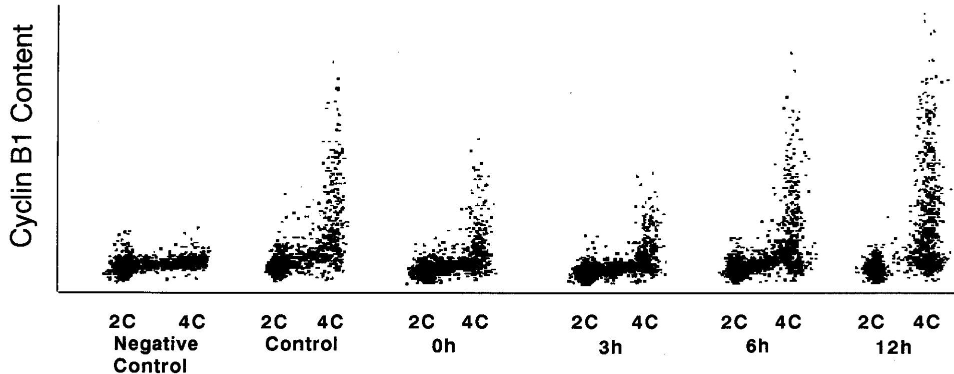

Fig. 4. The change of cyclin B/DNA dot-plot distribution against time after hyperthermia.

Hyperthermia and the level of $p34^{cdc2}$

$p34^{cdc2}$ content versus DNA distribution showed a gradual increase of $p34^{cdc2}$ in line with the cell cycle from G_1 phase to G_2 in accordance with cell size (Fig. 6). The level of $p34^{cdc2}$ in G_2 phase was about double that in G_1 phase. Throughout 24 hours after heat treatment, the content and labeling index of $p34^{cdc2}$ was unchanged (Fig. 7).

Discussion

The U87MG cell line (derived from human glioblastoma) is sensitive to mild hyperthermia[1)], but there is little information about its effect on cell kinetics[22)].

In a number of human tumors, approximately the same effect is achieved by halving a set exposure time for each additional degree of temperature elevation above 42°C. Above 44°C, the thermal sensitivity of normal cells rapidly approaches that of neoplastic cells, it is necessary either to restrict the field of thermal irradiation to abnormal tissues only or to use temperatures within the range 42-43°C[21)]. In this way, the therapeutic index of hyperthermia is quite narrow. The present study showed that hyperthermia at 43°C for

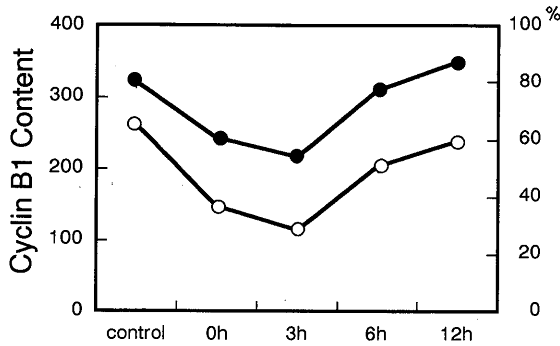

Fig. 5. Contents and percentages of cyclin B after hyperthermia. Open circles: percentages of cyclin B1-positive cells in G_2 M phases; closed circles: cyclin B1 content. Mean channel fluorescence values for the cyclin B1 of cells in G_2M phases for distinct durations after hyperthermia. Only positively stained cells were included in the analysis.
Abscissa: Time (hour), Ordinate: Cyclin B1 Content or % of Cyclin Bl-positive Cells

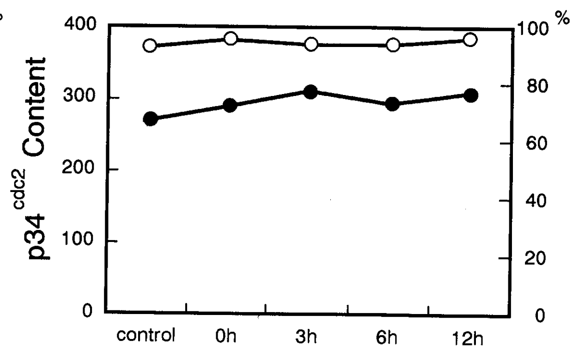

Fig. 7. Contents and percentages of $p34^{cdc2}$ after hyperthermia. Open circles: percentages of $p34^{cdc2}$ positive cells in G_2M phases; closed circles: $p34^{cdc2}$ content. Mean channel fluorescence values for the $p34^{cdc2}$ of cells in G_2M phases for distinct durations after hyperthermia. Only positively stained cells were included in the analysis.
Abscissa: Time (hour), Ordinate: $p34^{cdc2}$ Content or % of $p34^{cdc2}$-positive Cells

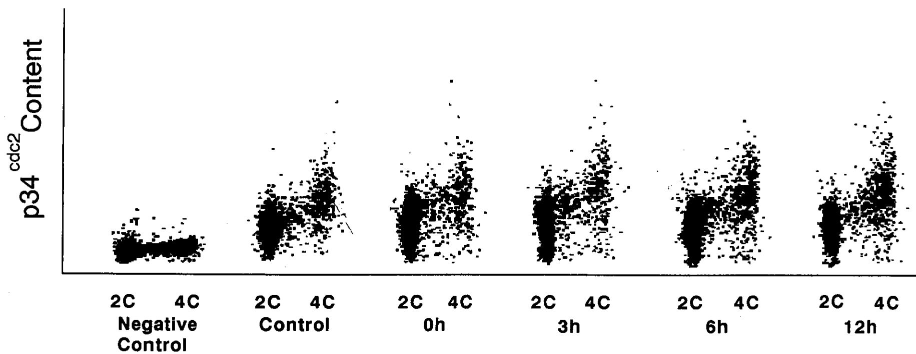

Fig. 6. The change of $p34^{cdc2}$/DNA dot-plot distribution against time after hyperthermia.

50 minutes resulted in no loss of cells and influenced the DNA histogram obtained by flow cytometry.

Monoclonal anti-BUdR antibodies have been used to analyze cells pulse-labeled with BUdR both in vivo or in vitro[4)15)23)]. However, labeling with BUdR before hyperthermia is crucial, because pulse-labeling after heat treatment gave limited information only about the low rate of DNA synthesis in our experiment (data not shown).

In previous studies of cell kinetics after hyperthermia using flow cytometry, block in the S phase and G_2M phase was revealed by DNA histogram analysis[3)8)24)]. Pulse-labeling of cells with BUdR allowed us to follow up cells heat-treated in S phase and it was found that they reached G_2 phase by 12 hours after treatment, where they experienced a further block. The accumulation in G_2M phase revealed on the DNA histogram is attributed to this G_2 block.

The entry of cells from G_2 phase into M phase is induced by an activity called

maturation-promoting factor, which has been identified as a complex between p34cdc2 and cyclin B. Levels of cyclin B mRNA increase abruptly at the S/G_2 boundary. In humans, two types of cyclin B have been cloned, and designated cyclins B1 and B2; these have been unknown to behave in the same way in the cell[19]. In this investigation, anti-cyclin B1 antibody was used. Cyclin B accumulates around the nucleus throughout S and G_2 phases. As a human cell enters mitosis, after chromatin has begun to condense but before nuclear lamina breakdown, a large proportion of cyclin B enters the nucleus[19]. Cyclin B is destroyed very rapidly at the end of metaphase[6][11]. Thus cyclin B appears to be indispensable for mitosis and its level fluctuates throughout the cell cycle.

Cyclin B was analyzed quantitatively after hyperthermia using anti-cyclin B1 monoclonal antibody and flow cytometry. This method requires no isotopes and no electrophoresis. Furthermore, cyclin B/DNA double staining makes it possible to correlate the level of cyclin B with the cell cycle without synchronization. Anti-cyclin B1 antibodies stain the cytoplasm and/or nuclei more strongly in methanol/acetone-fixed cells than in ethanol-fixed cells[19]. Therefore, we used methanol/acetone fixation for staining of cyclin B1 and p34cdc2.

The low level of cyclin B after hyperthermia in comparison with that of normally cycling cells, persisted for more than 3 hours. The level of cyclin B in G_2M phase was lower at 3 hours than immediately after hyperthermia, because of cyclin B synthesized before hyperthermia. The level of p34cdc2 in G_2M phase, which was about twice as high as that in G_1 phase, in accordance with cell size, was unchanged after hyperthermia. G_2 block due to reduction of cyclin B synthesis produced accumulation at G_2M on the DNA histogram obtained by flow cytometry. This G_2 block was released after recovery of cyclin B synthesis.

Lock and Ross examined p34cdc2 kinase activity in G_2-enriched Chinese hamster ovary cells after exposure to etoposide and in an asynchronous culture after exposure to γ-irradiation[10]. In both the etoposide- and the radiation-treated cells, a reduction in the activity of p34cdc2 kinase was observed. Muschel et al. investigated the effect of cyclin B mRNA and protein level induced by irradiation in synchronized HeLa cells[12]. Their experiments revealed two effects on cyclin B regulation which might contribute to the division delay. Irradiation of the cells in S phase produced a delay in the accumulation of cyclin B mRNA. On the other hand, irradiation of the cells in G_2 phase, when level of mRNA was increasing, markedly lowered the level of cyclin B protein, despite the high level of the mRNA. These results suggest that the absence of cyclin B may have contributed to the reduction of p34cdc2 kinase activity in both the drug- and radiation-treated cells.

Our data showed a temporary reduction in the percentage and content of cyclin B-positive cells in G_2M phase. Our findings suggest that hyperthermia induces a division delay, at least in part, by producing insufficient levels of cyclin B, similar to the effect of drug and radiation treatment.

The rate of polypeptide synthesis is inhibited in Ehrlich cells incubated at 43°C compared with those incubated at 37°C[17]. Hyperthermia has been shown to reduce the incorporation of amino acids into proteins in in vitro studies[2]. This reduction of amino acid uptake is attributed to a suppressed demand for amino acids due to inhibition of protein synthesis. The inhibition of protein synthesis caused by heat treatment is observed to be reversible[7]. The temporary reduction of cyclin B content after hyperthermia may be ascribed to inhibition of protein synthesis. However, our data do not indicate which stage of cyclin B synthesis is damaged by hyperthermia. Further studies will be required in order to clarify the effect of hyperthermia on cell kinetics in detail.

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