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(Original article)

Cell cycle of tsAF8 after heating

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Abstract

Thermotolerance in tsAF8 cells develops during incubation at 34℃ after heating at 45℃, while it is suppressed by the following incubation at a non-permissive temperature of 39.7℃ after the same heating. The incubation temperature after heating may affect the cell cycle and consequently thermotolerance. In the present study, a relationship between the thermotolerance and the cell cycle of tsAF8 was investigated. The cell cycle fractions and DNA synthesis were measured by flow cytometry using double staining with propidium iodide and bromodeoxyuridine. When the tsAF8 cells were heated at 45℃ for 20 min, and thereafter incubated at 34℃, bromodeoxyuridine uptake in the S phase cells (DNA synthesis) was recovered to 65.1% 6 h after the heating, and the cells showed gradual accumulation in the G_2/M phase. When the cells were incubated at 39.7℃ after heating at 45℃ for 20 min, then showed inhibition of thermotolerance development, the DNA synthesis was recovered to 15.1% temporarily 6 h after the heating, but it became 0% after 12 h, and the cells did not remarkably accumulate in any phases of the cell cycle. This inhibition of DNA synthesis at 39.7℃ was considered to be the result of cell survival decreasing by a step-down heating. However, the relationship between the thermotolerance and the cell cycle was not found out in tsAF8 cells, because the cells did not accumulate in any phases of the cell cycle under the inhibitory condition of thermotolerance.

Keywords : thermotolerance, hyperthermia, tsAF8, cell cycle

Introduction

Development of thermotolerance is one of the most critical problems in clinical hyperthermic treatment of cancer. Although the sequential mechanism of thermotolerance development is not fully understood, heat shock proteins (HSPs) induced by heating relate to the induction of thermotolerance[1-3]. The HSPs induction may involve in intracellular metabolism through protein kinase C (PKC), because the thermotolerance development is suppressed by PKC inhibitors[4-8].

Heat sensitivity differs among the phases of the cell cycle[9]. The S phase cells are the most sensitive to heating, while the G_1 phase cells are resistant to it[9]. With heating of the growing cells, progression of the cell cycle is remarkably inhibited[10,11]. When asynchronous Chinese hamster ovary (CHO) cells were treated at 42℃ for more than 12

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h, a heat-induced delay of the G_2 phase cells occurred[10, 11]. When CHO cells were heated at 45℃ for 15 min and then incubated at 37℃, the cells were delayed in the S and G_2 phase for 24 h[10, 11].

tsAF8 cells, temperature sensitive mutants of Syrian hamster BHK21 cells, grow at 34℃, but they are arrested in the G_1 phase, and the activity of RNA polymerase II decreases in the cells at the non-permissive temperature of 39.5 - 40.6℃[12-16]. The above phenomena are not observed in the wild-type BHK21 cells at 34 - 40.6℃[12-16]. We also previously reported that thermotolerance in the tsAF8 cells developed during incubation at 34℃ after heating at 45℃ for 20 min, while it was suppressed completely by the following incubation at 39.7℃ after the same heating[14]. The cell cycle of tsAF8 might be disturbed by the heating and incubation temperature after the heating, and consequently affect the thermotolerance. Therefore, in the present study we investigated a relationship between the thermotolerance and the tsAF8 cell cycle to clarify the above phenomena.

Materials and Methods

1. Cell culture

Temperature sensitive mutant tsAF8 cells derived from the Syrian hamster cell line BHK21 were used in the present study. They were cultured in Dulbecco's modified Eagle medium (DMEM) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 100 unit/*mℓ* of penicillin (Meiji Seika Co. Ltd., Tokyo, Japan), 100 μg/*mℓ* of streptomycin (Meiji Seika), and 10% bovine calf serum (HyClone, Laboratories Inc., Utah, USA) in a incubator with 5% CO^2 and 95% air at 34℃. Experiments were carried out with exponentially growing cells.

2. Heat treatment.

Twenty-four hours before each experiment, 6×10^5 cells were plated in 25 cm^2 Falcon plastic flasks, and heating was performed by totally immersing in a circulating water bath. Temperature was controlled within ± 0.05℃.

3. Analysis of the cell cycle and DNA synthesis.

To investigate the cell cycle and DNA synthesis, after heating at 45℃ for 20 min, tsAF8 cells were incubated at 34℃ or 39.7℃ for 4 to 12 h, then they were incubated with 30 μg/*mℓ* bromodeoxyuridine (BrdU) at 34℃ for 45 min. BrdU is taken up in the S phase cells, because its chemical structure is similar to the thymidine DNA composition. The cells were trypsinized and fixed in 70% ethanol for 10 min at -20℃ in a 15 *mℓ* test tube. The cells were rinsed twice with cold PBS (2% FCS, 0.1% NaN_3). After the addition of normal goat serum to the cells and mixing, the cells were incubated with 0.75 μg/*mℓ* monoclonal antibody to BrdU (Medical & Biological Laboratories Co, Ltd., Nagoya, Japan) for 30 min at room temperature. The cells were incubated with fluorescein-conjugated anti-mouse Ig (Immunotech, Marseille, France) diluted 1:40 in PBS for 30 min at room temperature. The cells were incubated with 100 μg/*mℓ* propidium iodide in PBS for 30 min at room temperature. The cells were rinsed well when drugs were changed. The intensity of intracellular fluorescences from BrdU and propidium iodide were measured by flow cytometry using an EPICS PROFILE II (Coulter Corporation Hialeah, FL, USA). The wave length of excitation light was 488 nm, and the intensity of fluorescence from 575 to 625 nm of the emission light was measured in 20000 cells.

Results

1. Effects of heating on Cell cycle of tsAF8

Fig. 1 shows the cell cycle fractions of the cells which were heated at 45℃ for 20 min, and then incubated at 34℃ for up to 12 h. The G_1 phase cells decreased from 31.3% to 18.6% after 12 h. The S phase cells decreased from 60.5% to 51.6%, and the G_2/M phase cells increased from 8.2% to 29.8% at 12 h after heating. The cells accumulated in the G_2/M phase under these conditions, that is, heating induced the G_2-block.

Fig. 2 shows the cell cycle fractions of the cells which were heated at 45℃ for 20 min, and incubated at 39.7℃ for up to 12 h. The S phase cells decreased slightly from 60.5% to 55.5%, and the G_2/M phase cells increased slightly from 8.2% to 13.1% at 12 h after heating. Ratio of the G_1 phase

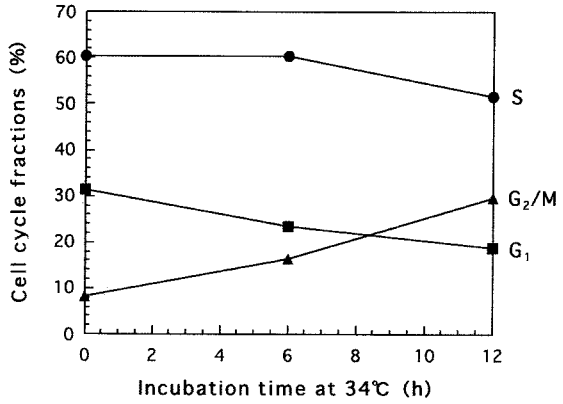

Fig. 1 Changes in cell cycle fractions of tsAF8 cells incubated at 34℃ after heating at 45℃ for 20 min. The tsAF8 cells were incubated at 34℃ for 6 and 12 h after heating, and cell cycle fractions were analyzed by flow cytometry.

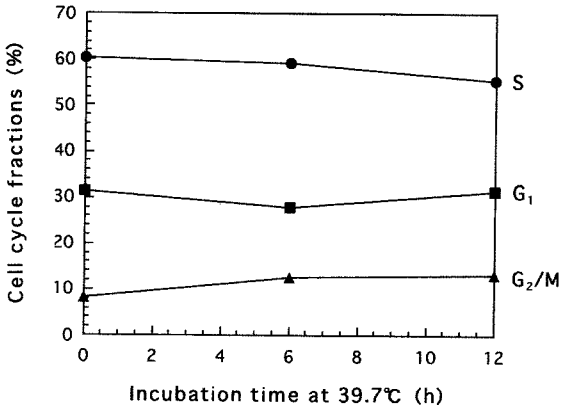

Fig. 2 Changes in cell cycle fractions of tsAF8 cells incubated at 39.7℃ after heating at 45℃ for 20 min. The tsAF8 cells were incubated at 39.7℃ for 6 and 12 h after heating at 45℃ for 20 min, and fractions of cell cycle were analyzed by flow cytometry.

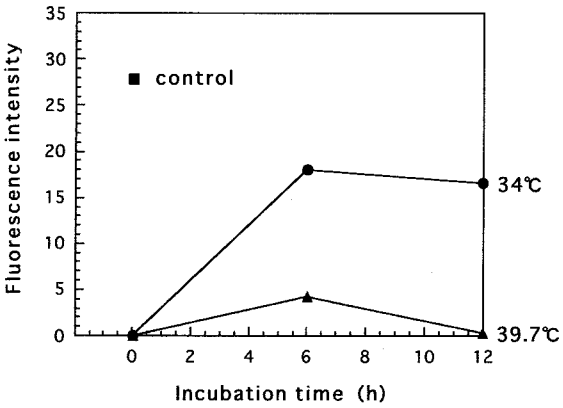

Fig. 3 Changes in BrdU uptake in S phase cells after heating at 45℃ for 20 min. After heating at 45℃ for 20 min, tsAF8 cells were divided into two groups, and incubated at 34℃ or 39.7℃. BrdU of 30 μg/mℓ was added to the medium at 0, 6, and 12 h after the heating, and then cells were incubated at 34℃ for 45 min. Cells were stained with monoclonal antibody to BrdU. Then the intensity of the intracellular fluorescence from BrdU was measured by flow cytometry. A vertical axis shows the relative intensity of fluorescences of the S phase cells.

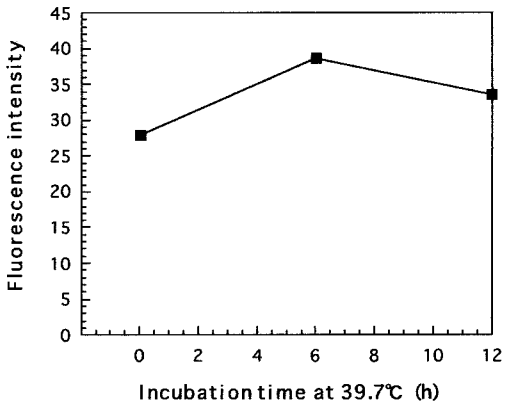

Fig. 4 Mean fluorescence from BrdU in the S phase cells incubated at 39.7℃ without heating. tsAF8 cells were incubated at 39.7℃ without heating, and they were incubated with BrdU at 34℃ for 45 min, 0, 6 and 12 h after the incubation at 39.7℃. Then the intensity of the intracellular fluorescence from BrdU was measured by flow cytometry. The vertical axis shows the relative intensity of fluorescences of the S phase cells.

cells did not change for 12 h. The progression of the cell cycle was almost restrained under these conditions.

2. Effects of heating on DNA synthesis of tsAF8

Fig. 3 shows the mean value of fluorescence intensity from BrdU in the S phase cells after heating at 45℃ for 20 min. Intensity of the fluorescence indicates the uptake amount of BrdU. The horizontal axis is a incubation time at 34℃ or 39.7℃after the heating. The control in Fig. 3 shows the intensity of fluorescence from unheating cells. The fluorescence intensity was completely inhibited immediately after the heating, and then being incubated at 34℃ after the heat-

ing, the fluorescence intensity recovered to 65.1 % of that of the control cells, and thereafter it was kept at the same level until 12 h. In the case of incubation at 39.7℃ after the heating, the fluorescence intensity temporarily recovered to 15.1% of that of the control cells after 6 h, but it became 0% after 12 h. However, the fluorescence intensity of the cells incubated at 39.7℃ without heating showed no change compared to that of incubation at 34℃ (Fig. 4).

Discussion

Read et al. reported that thermotolerance was dependent on the phase of the cells in the cell cycle, and that thermotolerance did not develop in synchronized S phase CHO cells[16, 17]. In the present study, we investigated the relationship between the thermotolerance and the tsAF8 cell cycle to clarify whether the cell cycle relates to the thermotolerance development in the tsAF8 cells. The cells heated at 45℃ for 20 min were arrested in the G_2/M phase during the following incubation at 34℃. However, in the case of tsAF8 cells incubated at the non-permissive temperature of 39.7℃ after the same heating, they did not remarkably accumulate in any phases of the cell cycle.

DNA and protein synthesis are inhibited by heating[18]. To investigate the DNA synthesis rate of S phase cells, BrdU uptake of the cells was measured under the above conditions by flow cytometry. As a result, BrdU uptake of the cells at 34℃ after heating at 45℃ for 20 min was completely inhibited immediately after the heating, and then recovered to 65.1% at 6 h after the heating, and thereafter it was maintained at the same level until 12 h. While the BrdU uptake of the cells incubated at 39.7℃ after the same heating recovered to 15.1% temporarily, but it was inhibited completely at 12 h after the heating. Surviving fractions of tsAF8 cells not heated at 45℃ did not decease during the incubation at 39.7℃, but in the case of the cells incubated at 39.7℃ after the heating at 45℃, they gradually decreased, and became about 4% after 12 h[14]. This decreasing were considered to be caused by a phenomenon called step-down heating[19], and the inhibition of DNA synthesis at 39.7℃ after heating was considered to be the result of the cell survival decreasing. However, the relationship between the thermotolerance and the cell cycle was not found out in tsAF8 cells, because the cells did not accumulate in any phases of the cell cycle under the inhibitory condition of thermotolerance. tsAF8 cells may become inactive or be dying probably in any phases of the cell cycle under these conditions. Further studies about the metabolism from a different point of view, at the molecular levels are necessary to clarify the mechanisms of thermotolerance development.

Conclusion

In the present study, the relationship between the thermotolerance and the cell cycle of tsAF8 was investigated. However, it was not found out in tsAF8 cells.

Acknowledgement

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加温後のtsAF8細胞の細胞周期

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抄　　録

tsAF8 細胞は45℃の加温後34℃で培養すると温熱耐性が速やかに発現するが，加温後，制限温度である39.7℃で培養すると温熱耐性の発現が抑制される。加温後の培養温度が細胞周期に影響し，その結果として温熱耐性発現に影響を与えている可能性があることから，今回，Propidium Iodide (PI) とbromodeoxyuridine (BrdU) で tsAF8 細胞を二重染色し，フローサイトメトリーによって温熱耐性と細胞周期の関係の有無について調べた。tsAF8 細胞を45℃20分の加温後34℃で培養すると，6時間後にはG_1期の細胞が減少し，12時間後にはG_2/M期への蓄積が見られた。しかし，加温後39.7℃で培養した場合には細胞周期の進行がほとんど見られなかった。BrdU の取込みは，加温せずに39.7℃で培養した場合には活発に行われ，また，45℃20分加温後34℃で培養した場合には，6時間後には BrdU の取り込みは65.1%まで回復した。しかし，温熱耐性発現の抑制が観察される45℃20分加温後39.7℃で培養した場合には，BrdU の取込み量は6時間後に一時的に15.1%に回復するが，12時間後には取込み量はゼロとなった。BrdU の取り込みが阻害されたのは step-down heating の現象による細胞生存率の減少が原因だと考えられたが，温熱耐性発現の抑制が観察される条件下では細胞周期の特定の時期への集積がなかったことから，温熱耐性と細胞周期との関係は tsAF8 細胞においては見い出されなかった。

キーワード：温熱耐性，ハイパーサーミア，tsAF8，細胞周期

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