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Response of Metastatic Cancer Cells to Thermal Changes in vitro

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배양온도 변화에 대한 전이성 암세포의 반응

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ABSTRACT

Alteration of temperature is one of cancer therapies. In general, severe hyperthermia (around 43°C) and hypothermia (around 18°C) trigger apoptosis through mitochondria, though the specific mechanism is still unknown. CC-t6 and GB-d1 cell lines, which were originally derived from human cholangiocarcinoma and gall bladder cancer, were established from a metastatic lymph node. To investigate the mechanism of metastatic cancer cell response to thermal stresses, hyperthermia (37°C → 43°C) and hypothermia (37°C → 17.4°C) were designed. Thermal stresses did not induce apoptosis but necrotic cell death. Any alterations of caspase-3, -9, cytochrome c, Bax, and Bcl-2 were not found in both hyperthermia and hypothermia exposed cells using western blot analysis. In the transmission electron microscopy, typical necrotic, but not apoptotic, changes were observed. These results suggest that temperature changes induce cell death through necrotic pathway in metastatic cancer in vitro, and it can be one of effective anticancer methods.

Keywords : Thermal stress, Necrosis, Apoptosis, Anti-cancer immunity, Metastatic cancer

INTRODUCTION

According to World Health Organization (WHO), cancer is a leading cause of death worldwide - from 58 million deaths in 2005, cancer accounts for 7.6 million (or 13%) of all deaths. To solve the problem, manifold cancer-killing methods were designed. In particular, to

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conquer the cancer, many scientists have developed various kinds of anticancer drugs. Wide ranges of anticancer agents, including chemotherapeutic agents, antibody, hormones, and various biologicals, induce apoptosis in malignant cells (Arends et al., 1991; Mesner et al., 1997; Houshmand & Zlotnik, 2003), and the mitochondrial pathway plays a predominant role in drug-induced apoptosis in the cell types examined to date (Kaufmann & Earnshaw, 2000). However, because a number of molecular processes conduct cancer at the same time (Hornberg et al., 2006), anticancer agents are well-known that various side effects are induced. Thus, diverse research and treatment methods have been developing for cancer therapy.

Besides the anticancer agents, alteration of temperature is one of the promising approaches in cancer therapy. The oldest report of thermal therapy was found in the Egyptian Edwin Smith surgical papyrus, dated around 3,000 BC. Hippocrates (460~370 BC) believed that any diseases could be cured with artificial control of body temperature, and the method he described in his aphorisms, i.e. hot irons, concerns higher temperatures, used in cauterization. In the 19th and 20th centuries, fever therapy has been used as a method to increase temperatures, while other investigators started to apply radiofrequency techniques. A worldwide interest in hyperthermia was initiated by the first international congress on hyperthermic oncology in Washington in 1975 (van der Zee, 2002; Ito et al., 2006). However, the effect and response of thermal stresses to metastatic cancer cell are not well studied.

Hyperthermia, one of the well-known thermal stresses, is recognized as teratogen in vivo and toxic agents in vitro (Edwards et al., 1995; Hildebrandt et al., 2002; Mirkes, 2002; Edwards et al., 2003). Conventional hyperthermia are designed to around 42~44°C. In general hyperthermia is known to trigger an apoptotic signal that converges on the mitochondria in susceptible cells to induce the release of cytochrome c in vivo (Mirkes, 2002). The specific mechanism of cell death by hyperthermia is still unknown (Lepock, 2003). Elevated temperatures can kill great numbers of tumor cells, and in principle, hyperthermia can influence all kinds of tumor cells (van der Zee, 2002; Ito et al., 2006). The other thermal stress, hypothermia may damage cells through induction of apoptosis if the exposure period of hypothermia is long enough (Shibano et al., 2002; Aslam et al., 2006). However, the mechanism of cell death by hypothermia is also still unclear.

Thermal stresses are closely related with heat shock proteins (HSPs). The expression of HSPs is a common response to a variety of adverse conditions particularly thermal change (Theodorakis, 1999), thus HSPs are families of highly conservative stress proteins, 'molecular chaperones' (Ellis & van der Vies, 1991; Lindquist & Craig, 1998). HSPs, which are typically overexpressed after various stressful conditions, are integral part of response to environmental changes (Christians et al., 2003). The genes for HSPs can be upregulated in response to cellular trauma, resulting in enhanced cell survival and protection (Fairfield et al., 2004). These induction of heat shock genes due to stresses is heat shock response (Thayer & Mirkes, 1997; Buckley et al., 2001). The central role of HSPs is to act as chaperone transiently associated to other polypeptides and modulate cell proliferation, antigen presentation, and cytoskeleton organization (Christians et al., 2003). Moreover, HSPs are closely related with cancer. HSPs expressed in increased amounts in many cancers owing to the de-repression of their genes during malignant progression. At these high levels, HSP family members play an essential, facilitating role in cancer by permitting autonomous growth through the accumulation of overexpressed and mutated oncogenes and by inhibiting the death of tumor cells (Calderwood et al., 2006).

However, despite the development of applicable researches using thermal stresses, the basic knowledge of metastatic cancer cell response to thermal stresses is at a standstill. Do metastatic cancer cells die by thermal stresses? What is the main death pathway of metastatic

cancer cells by thermal stresses? What molecules are participated in the response? How their morphology should be changed? What kind of thermal stresses kill them effectively? What is the correlation between thermal stresses and HSPs in metastatic cancer cells?

To solve these questions and to investigate the mechanism of metastatic cancer cell response to thermal change, hyperthermia and hypothermia were introduced. To identify the main death pathway, immunoblotting using apoptosis related antibody were executed. In addition, to confirm the morphological changes, metastatic cancer cells were observed after thermal stresses by transmission electron microscopy (TEM).

MATERIALS AND METHODS

Cell culture

CC-t6 and GB-d1 cell lines (kindly provided by Dr. Hideo Shimura, Fukuoka University of Medicine, Japan), which were originally derived from human cholangiocarcinoma and gallbladder cancer, were established from a metastatic lymph nodes of a patient. Cells underwent up to 6~7 passages from the original cell line were used in this experiment. Cells were maintained in T-75 or T-25 flasks or 35-mm dishes in Dulbecco's modified Eagle's medium (Sigma M2278), supplement with 10% heat-inactivated fetal bovine serum (Sigma F2442) and 100 U/mL penicillin G (Sigma P7764), 100 mg/mL streptomycin (Sigma S1277), 20 mM HEPES (Amresco 1291B68), 2 mM L-glutamine (Sigma G-8540), 1% insulin-tranferrin-sodium selenite media supplement (Sigma I-1884), 1% MEM vitamin solution (Sigma M6895), and 1% MEM non-essential amino acid solution (Sigma M7145) at 37°C in a 100% humidified atmosphere containing 5% carbon dioxide (CO_2).

Thermal stresses

CC-t6 and GB-d1 cells were counted 2×10^6 and subcultured to T-75 flasks and incubated in normal culture conditions. 6hr or 17hr after seeding, the cells were exposed to hyperthermia (37°C → 43°C) and hypothermia (37°C → 17.4°C). Hyperthermia was induced by pouring 43°C heating normal culture media before using and kept the temperature in 43°C incubator for 30 min. Hypothermia was induced by the incubating temperature maintained at 4°C for 10 min and the temperature of media decreased from 37°C to 17.4°C. Lastly, these two thermal stresses, both hyperthermia and hypothermia, were immediately conducted in succession by same methods. The temperature of the media altered from 37°C to 43°C and to 18.6°C. Severe hyperthermia is well-studied around 43°C, and severe hypothermia is well-studied around 20°C, thus this experiment focused on 43°C and 18°C. All groups recovered in 37°C normal culture incubator. The recovery times of each group were similar at 30 min. Control group had no thermal stress. Sample acquisitions executed at 1 hr (early response) or 12 hr (late response) after experiment when the total lifetimes of all cells are the same as 18 hr.

Western blotting

Thermal stresses exposed CC-t6 and GB-d1 cell were prepared, and proteins were extracted by using lysis buffer (PRO-PREP protein extraction kit 17081 iNtRON biotechnology). The protein concentrations were determined according to Bradford's method. Additionally, for concentration of dead and damaged cells, supernatants of each experiment were collected by floating just after 1 min of mildly shakings the culture dishes. And the proteins of supernatants were also extracted by same methods.

Equal amounts of protein were subjected to SDS PAGE and electrophoretically were transferred to nitrocellulose membranes. Proteins were analyzed by immunoblotting using 1:30,000 dilution of anti-HSP70 antibody (Stressgen SPA-812), 1:2,000 dilution of anti-HSP27 antibody (Cell Signaling G31), 1:1,000 dilution of anti-

Bcl-2 antibody (Cell Signaling 2872), 1 : 1,000 dilution of anti-Bax antibody (Santa Cruz sc-7480), 1 : 1,000 dilution of anti-cytochrome c antibody (BD sciences 556 433), 1 : 1,000 dilution of anti-caspase-9 antibody (Santa Cruz sc-7885), 1 : 3,000 dilution of anti-β-actin antibody (abcam ab6276), and 1 : 2,000 dilution of anti-cleaved caspase-3 antibody (Cell Signaling Asp175). Using image analyzer, Gel-Doc 2000 (Bio-RAD), the results of western blot were photographed and densitometrically analyzed.

Transmission electron microscopy

After thermal stresses 12 hr later, each sample was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4 for 2hr and washed three times for 30 min in 0.1 M PB. They were postfixed with 0.002 M OsO_4 dissolved in 0.1 M PB for 1h and dehydrated in ascending gradual series (50～100%) of ethanol and infiltrated with propylene oxide. Specimens were embedded by EMbed 812 kit (Electron Microscopy Sciences 14120). After pure fresh resin embedding and polymerization at 60°C electron microscope oven (TD-500 Dosaka) for 72 hr, 1 μm thick section were initially cut and stained with toluidine blue for light microscope. 70 nm thin section were double stained with 3% uranyl acetate and lead citrate for contrast staining. These sections were cut by Reichert-Jung E ultramicrotome (Leica Microsystems). All of the thin sections were observed by transmission electron microscopy (H-7600s, Hitachi) at the acceleration voltage of 80 kV.

RESULTS

Cell death mechanism

To confirm the cell death mechanism induced by thermal stresses, apoptotic proteins were investigated by immunoblotting. Apoptosis related proteins-caspase-3, -9, cytochrome c, Bcl-2, Bax- are important staple components of apoptosis and these proteins showed no meaningful changes during thermal stresses (Fig. 1 A～E). After each thermal stress was exposed, dead and damaged cells floated and these supernatants were collected after 1min of mildly shakings of culture dishes and were analyzed by immunoblotting (Fig. 1 F, G). CC-t6 cells had little HSP70 and HSP27 at normal culture conditions. However, after thermal stresses, significantly increasing amount of HSP 70 and HSP27 were detected in the dead and damaged cells. Thermal stresses upregulated the expression of HSPs, which indicated that thermal stresses induced necrotic cell death. In contrast, GB-d1 cells, which originally expressed high levels of HSP70 and HSP27 at normal culture conditions, were not significantly affected by thermal stresses. The results only showed the expressions of HSP70 and HSP 27 either slightly increased or decreased. This result suggested that no obvious changes in expression of HSP 70 and HSP27 indicated necrotic cell death.

Morphological analysis

Using transmission electron microscopic techniques, morphological changes were observed (Fig. 2). Hyperthermia induced severe cell damage. Hypothermia and hyperthermia immediately followed by hypothermia also induced cell damage. Compared with controls, all thermal stresses induced cell death in each cell line. The most of damaged cells clearly showed typical necrotic changes: mitochondrial swellings, cell membrane ruptures, cytoplasmic vacuolizations, irregular nuclear contour, and secondary lysosomes. On the other hand, typical apoptotic morphological changes were not observed at all. Interestingly, cells that suffered hypothermia showed high frequencies of mitotic figures. In TEM survey, the results were similar irrespective of exposure time and cell type.

DISCUSSION

Necrosis is the main cell death pathway

Most cancer treatment using anti-cancer drugs in-

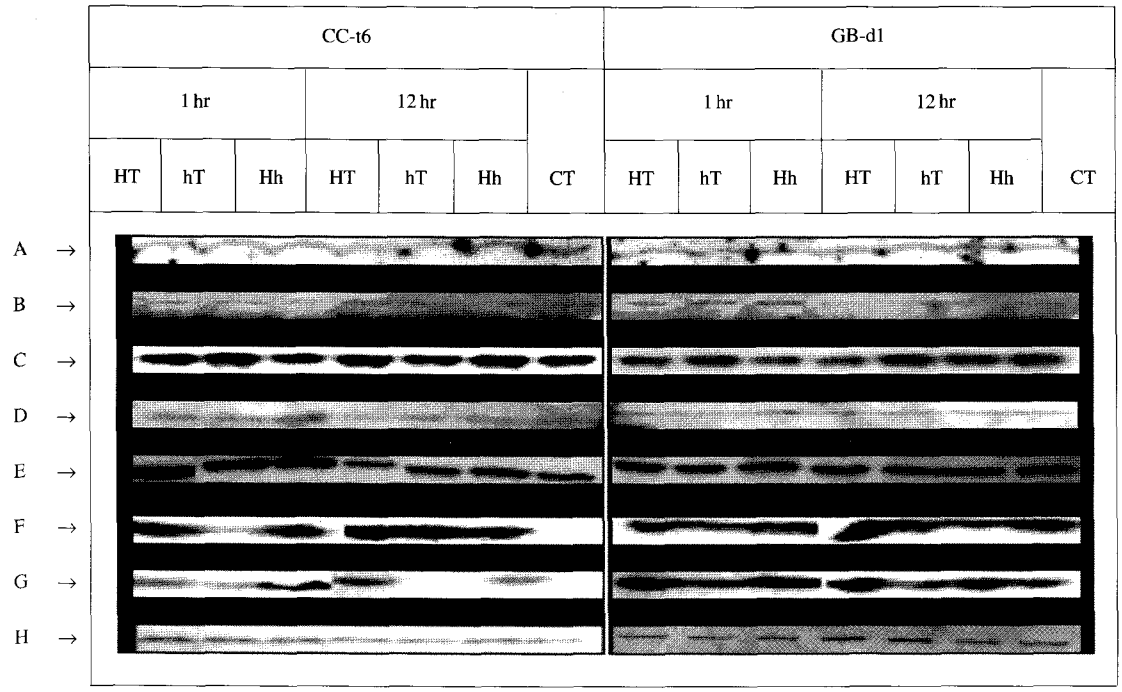

Fig. 1. Effect of thermal stresses on apoptosis regulatory proteins in CC-t6 and GB-d1 cells by western blotting. Each thermal stresses induced cell lysate (A ~ F) and supernatant cell lysate (G, H) was electrophoretically separated on polyacrylamide gel and immunoblotted with antibodies against target proteins. A: cleaved caspase-3, B: caspase-9, C: cytochrome c, D: Bax, E: Bcl-2, F: HSP70, G: HSP27, H: β-actin.

duced apoptotic cell death. Apoptosis in response to cancer therapy proceeds through activation of the core apoptotic machinery including the receptor and the mitochondrial signaling pathway (Kaufmann & Earnshaw, 2000). And the majority of cytotoxic drugs initiates cell death by triggering the cytochrome-c/Apaf-1/caspase-9-dependent pathway through the mitochondria (Debatin, 2004). Thermal stresses also induce apoptosis in general.

However, thermal stresses induced necrotic cell death in our experiments, regardless of exposure time, cell type, thermal stresses. Expressions of apoptotic related proteins-caspase-3,-9, cytochrome c, Bcl-2, Bax-were not changed. In the survey of TEM, mitochondrial swellings, cell membrane ruptures, cytoplasmic vacuolizations, irregular nuclear contour, and secondary lysosomes were observed, and these were the characteristic morphology of necrosis. Whereas apoptotic morphology, such as, chromatin condensations, nuclear fragmentations, cytoplasm shrinkages and apoptotic body formations did not observed.

Recently, Basu et al. (2000) has reported that necrotic, but not apoptotic, cell death leads to release of HSPs. However, they only researched HSPs depleted cells in normal states. Thus, our experiments were designed two cell line of metastatic cancer that have or do not have HSPs in normal conditions. CC-t6 cells did not have any HSP70 and HSP27 in non-stressed states. However, thermal stresses induced the increase of HSP-70 and HSP27 in dead and damaged cells; particularly hyperthermia most significantly induced. This increase indicated that thermal stresses induced necrotic cell death. In contrast, GB-d1 cells have abundantly HSP70 and HSP27 in control. After thermal stresses, the amo-

unts of HSPs were slightly fluctuated. Hyperthermia and hyperthermia immediately followed by hypothermia induced small degrees of increasing of HSP70 and HSP 27. This increases indicated that necrotic cell death. Hypothermia induced small degrees of decreasing of HSP70 and HSP27. However, there were no disappearances of HSP70 and HSP27. Therefore, compare with another our results; these situations indicated necrotic cell death.

Necrosis is a regulated cellular response to stress, and another type of programmed cell death (Bursch, 2001; Leist & Jaattela, 2001; Proskuryakov, 2003; Yuan et al., 2003; Borst & Rotterberg, 2004; Zong et al., 2004). Necrosis strongly produces a danger signal to immune system to repair or kill these regions. Necrotic cell death engages the innate and adaptive components of the immune system through the release of HSP-peptide complexes. Generation of necrotic cell death via HSPs expression induces antitumor immunity (Srivastava, 2003). Moreover, increasing HSPs with cell death is not only a marker of necrosis but also a specific signal for the immune system (Basu et al., 2000; Proskuryakov et al., 2003). HSPs protect cancer from a wide range of apoptotic and necrotic stimuli, and are overexpressed in a wide range of human cancers, and are implicated in tumor cell proliferation, survival, differentiation, invasion, metastasis, death, and recognition by the immune system (Nylandsted et al., 2000; Proskuryakov et al., 2003; Ciacca & Calderwood, 2005; Cladeerwood et al., 2006). Increased expression of HSP occurs through derepression by p53, which is mutated with 45% of cancers, and are thought positive regulation by oncogenic signaling pathways (Agoff et al., 1993; Tsutsumi-Ishii et al., 1995; Madden et al., 1997; Ghioni et al., 2002; Calderwood et al., 2006). The increase of HSPs in tumors presents an opportunity for cancer immunotherapy through the innate ability of many HSPs to function as biological adjuvants and to chaperone tumor antigens (Calderwood et al., 2005). HSPs interact with HSP receptors on antigen-presenting cells (APCs) and activate through the NF-κB pathway (Srivastava et al., 1994; Binder et al., 2000). When HSPs are increased in cancer cells and such cells are destroyed through a necrotic mechanism, cells at distant sites are destroyed by a specific anti-tumor immune response that is strictly dependent on increased release of HSPs from the primary cells (Calderwood, 2005). Thus, necrotic cell death with high HSPs stimulates the patient's own immune responses against tumor (Masse et al., 2004).

Hyperthermia is the most effective thermal stress against metastatic cancer cells

Hyperthermia has a toxic effect on many cells and this phenomenon has been applied many cancers. In fact, hyperthermia can influence and kill all kinds of tumor cells although the mechanisms of cell death is not clear (Edwards et al., 1995; Hilderbrandt et al., 2002; Mirkes, 2002; van der Zee, 2002; Edwards et al., 2003; Lepock, 2003; Ito et al., 2006). Our results also showed the possibility of hyperthermia to kill metastatic cancer cells. The most toxic damages were induced by hyperthermia in MTT assay, trypan blue staining, and flow cytometric analysis using PI and Annexin V doubling staining (data not shown). Hyperthermia decreased the cell viability of metastatic cancer cell by half and this damage continuously influenced on 12 hr group (data not shown). In the morphological analysis using TEM, hyperthermia also induced the most severe insult than other thermal stresses did. A major technical problem with hyperthermia is the difficulty of heating the local tumor region to the intended temperature without damaging normal tissue. However, tumors can be subjected to tumor-specific local hyperthermia (like intracellular hyperthermia systems using magnetite nanoparticles in alternating magnetic field) or other means of radiation that may be deemed or determined to be capable of enhancing the expression of HSPs (Srivastava, 2003; Ito et al., 2006). Thus, cell therapy using hyperthermia through necrotic cell death is a powerful me-

thod in metastatic cancer cells.

Meanwhile, hypothermia caused damages to cells similar pattern like hyperthermia in the beginning, however these damages did not continuously influence. After 12 hr of hypothermia hypothermia exposed cells showed higher frequencies of mitotic figures. Low temperature cultivation of mammalian cells results in prolonged generation time and maintenance of cell viability for longer periods, reduced glucose and glutamine consumption, suppressed release of waste products, delayed apoptosis, reduced protease activity, and improved tolerance against shear stress (Al-Fageeh et al., 2006). Moreover, hypothermia is employed in a variety of medical applications, including the preservation of organs before transplantation because of these protective function (Russotti et al., 1996).

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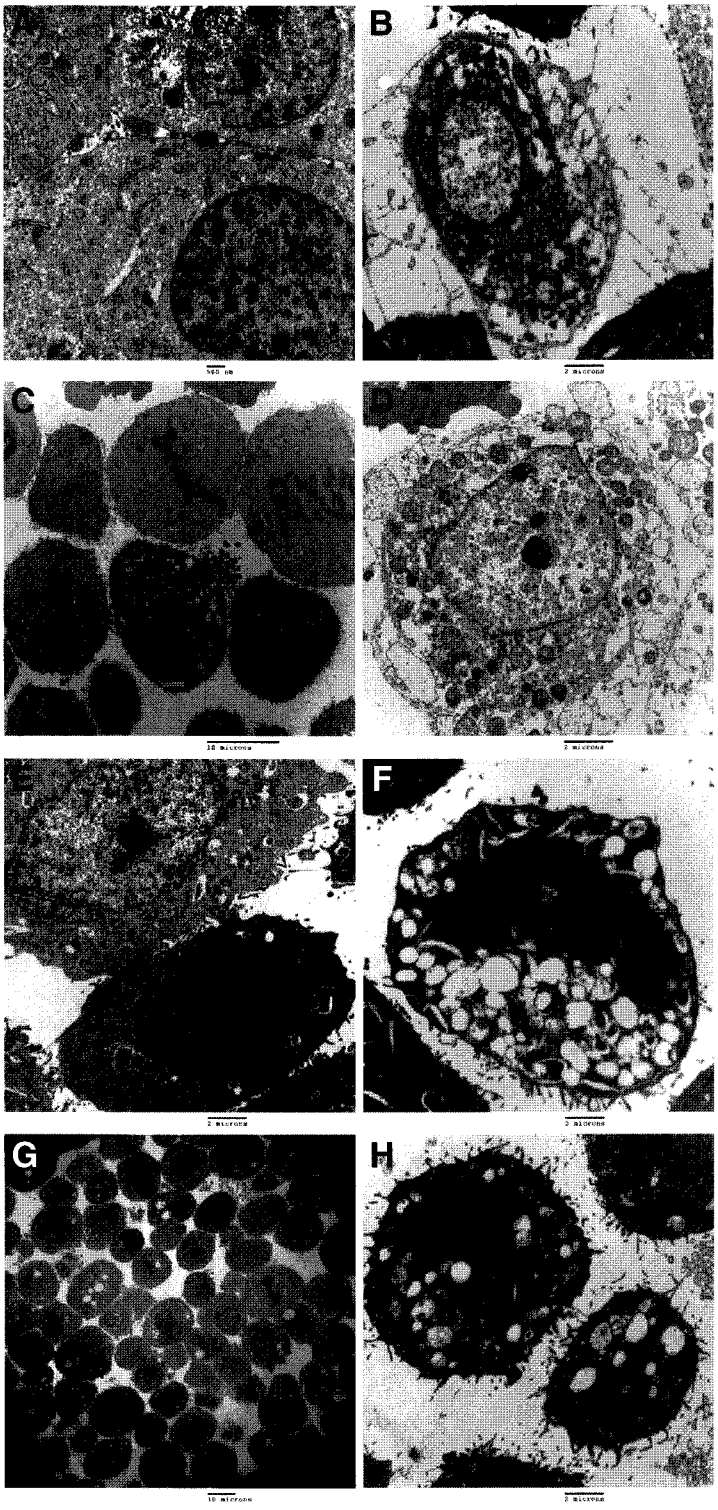
<국문초록>

암세포가 있는 장소의 온도를 변화시키는 것은 하나의 암 치료 방법이 될 수 있다. 명확한 기전은 아직 잘 밝혀져 있지 않지만, 고온은 미토콘드리아로 신호를 전달해서 cytochrome c를 분비시키는 세포자멸사로의 길로 유도하는 것으로 알려져 있다. 저온은 30°C 미만에서 세포자멸사를 유도하지만 심하지 않은 저온에서는(35～33°C 혹은 31～29°C) 오히려 세포자멸사를 막는 것으로 알려져 있다. CC-t6와 GB-d1세포 주는 림프절로 전이된 사람의

담관암과 담낭암에서 확립한 것으로, 이와 같은 전이성 암세포가 온도 변화에 어떻게 반응을 하는지를 연구하기 위해 고온노출(37 → 43°C)과 저온노출 (37 → 17.4°C)을 시행하였다. 세포의 종류나 온도 변화를 통한 스트레스의 방법과 관계없이 죽는 세포가 관찰되었으며, 고온노출이 가장 심한 영향을 주었다. 이런 죽어가는 세포는 세포자멸사가 아닌 세포괴사의 경로를 거치고 있었다. 투과전자현미경을 이용한 관찰에서 세포자멸사적인 모습은 보이지 않았고, caspase-3, -9, cytochrome c, Bax 같은 세포자멸사와 관련된 단백질의 변화도 관찰되지 않았고, 열충격단백질 70과 27도 증가하였다. 결국 CC-t6와 GB-d1 세포는 온도변화를 통한 스트레스를 주었을 경우 세포괴사로 죽음을 알 수 있었다. 온도변화를 통한 스트레스는 열충격단백질의 증가와 함께 세포괴사를 일으켰다. GB-d1과 CC-t6 세포에서 고온은 가장 심각하게 세포괴사를 일으켰으며, 저온은 초기에는 세포괴사를 유발하였으나 12시간 경과후에는 세포분열이 더욱 활발하게 일어나 세포의 생명력을 연장시켜주었다. 결국 이 실험에서는 전이성 암세포를 제거하는 방법으로는 고온이 가장 효과적이며 유용함을 알 수 있었다.

FIGURE LEGENDS

Fig. 2. Morphological observations of CC-t6 and GB-d1 cells using transmission electron microscopy. Electron micrographs of CC-t6 (A~D) and GB-d1 (E~H) cells were acquired after thermal stresses. A and E are morphology of control each cell line respectively. Mitochondrial swellings, membrane ruptures, vacuolizations, irregular nuclear contour, and secondary lysosomes were found in hyperthermia-exposed cells (B, F). Mitotic figures plentifully observed in hypothermia-exposed cells (C, G). Hyperthermia followed by hypothermia treated cells showed similar morphological characteristics of hyperthermia-exposed cells (D, H). A: CC-t6 CT, B: CC-t6 HT, C: CC-t6 hT, D: CC-t6 Hh, E: GB-d1 CT, F: GB-d1 HT, G: GB-d1 hT, H: GB-d1 Hh.


A
B
C
D
E
F
G
H