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Intratumoral Administration of Dendritic Cells Combined with Hyperthermia Induces Both Local and Systemic Antitumor Effect in Murine Tumor Models

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Purpose: We examined whether intratumoral (i.t.) administration of dendritic cells (DCs) into a treated tumor could induce local and systemic antitumor effects in a mouse tumor model.

Methods and Materials: C57BL/6 mice were inoculated s.c. in the right and left thighs with MCA-102 fibrosarcoma cells on day 0 and on day 7, respectively. On day 7, the tumors (usually 6 mm in diameter) on the right thigh were heated by immersing the tumor-bearing leg in a circulating water bath at 43°C for 30 min; thereafter, the immature DCs were i.t administered to the right thigh tumors. This immunization procedure was repeated on days 7, 14 and 21. The tumors in both the right and left thighs were measured every 7 days and the average sizes were determined by applying the following formula, tumor size=0.5×(length+width). Cytotoxicity assay was done to determine tumor-specific cytotoxic T-lymphocyte activity.

Results: Hyperthermia induced apoptosis and heat shock proteins (HSPs) in tumor occurred maximally after 6 hr. For the local treated tumor, hyperthermia (HT) alone inhibited tumor growth compared with the untreated tumors ($p<0.05$), and furthermore, the i.t. administered DCs combined with hyperthermia (HT+DCs) additively inhibited tumor growth compared with HT alone ($p<0.05$). On the distant untreated tumor, HT alone significantly inhibited tumor growth ($p<0.05$), and also HT+DCs potently inhibited tumor growth ($p<0.001$); however, compared with HT alone, the difference was not statistically significant. In addition, HT+DCs induced strong cytotoxicity of the splenocytes against tumor cells compared to DCs or HT alone.

Conclusion: HT+DCs induced apoptosis and increased the expression of HSPs, and so this induced a potent local and systemic antitumor response in tumor-bearing mice. This regimen may be beneficial for the treatment of human cancers.

Key Words: Dendritic cell, Heat shock protein, Hyperthermia

Introduction

It is now well established that dendritic cells (DCs) primed with tumor antigens have the capacity to elicit antitumor immune responses *in vitro* as well as mediate effective tumor regression *in vivo*.[1] They have unique pathways for the phagocytosis, processing and presentation of antigens derived from destructed cells.[2~4] Immature DCs capture antigens *in situ* and undergo maturation; thereafter matured DCs migrate to lymphoid organs to interact/activate naive T cells.[1]

Hyperthermia (HT), which is currently applied in the treatment of some human cancers, is known to induce apoptotic and necrotic death of tumor cells and expression of heat shock proteins (HSPs).[5] One study compared the manner of cell death induced by hyperthermia; at 42°C, apoptotic cell death was induced; at 44°C, necrotic cell death was induced.[6] Although it may differ between cell types which temperature induces apoptosis or necrosis, necrotic cell death occurs at higher temperature.

Heat shock proteins, whose syntheses are induced by a

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variety of stresses, including heat stress, are able to; chaperone antigenic peptides; interact with DCs through a receptor; stimulate DCs to secrete inflammatory cytokines; and mediate maturation of DCs as danger signals.[5]

It has been reported that apoptotic bodies are engulfed by DCs, followed by processing and presentation of tumor antigens by MHC class I.[3] In addition, HSPs released from necrotic tumors chaperone antigens; HSP-antigen complexes bind to the surface of DCs.[5] In fact, appropriate processing by APCs may occur in both apoptosis and necrosis.

Therefore, we hypothesized that DCs would phagocyte, process and present the antigens derived from apoptotic or necrotic cells destructed with HT, would be enhanced by HSPs released by HT, and thereafter would generate tumor-specific immune response. Based on this hypothesis, we examined whether intratumoral administration of dendritic cells combined with hyperthermia (HT+DCs) could induce systemic, as well as local, antitumor effect in murine tumor models.

Materials and Methods

1. Cells and animals

MCA-102 fibrosarcoma cells; EL-4 lymphoma cells, which originated from a C57BL/6 mouse; and CT-26 colon adenocarcinoma cells, which originated from a Balb/C mouse, were maintained by *in vitro* culture in complete medium (CM). CM consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Bethesda, MD), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37°C in an atmosphere containing 5% CO_2 and 95% air. Female wild-type C57BL/6 mice, 6~8 weeks of age, were purchased from Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea) and housed at the Animal Maintenance Facility of Pusan Medical Research Center. The mice were used for experiments at 7~9 weeks of age.

2. Generation of bone marrow-derived DCs

Bone marrow-derived DCs were generated as previously described,[7] with minor modifications. Briefly, bone marrow cells from flushed marrow cavities of femurs and tibias of naive syngeneic mice were depleted of erythrocytes by incubating in 0.9% ammonium chloride for 3 min at 37°C. The cells were washed in PBS and cultured in CM with 20 ng/ml recombinant mouse granulocyte macrophage colony stimulating factor (GM-CSF) and 10 ng/ml recombinant mouse interleukin (IL)-4 (Preprotech, Rocky Hill, NJ) at 5×10^5 cells/ml. On day 3 in culture, the media and floating cells were removed and centrifuged for 5 min at 500 g. The cells were resuspended in CM with 10 ng/ml GM-CSF and 20 ng/ml IL-4, and replaced in the original dishes. On day 6, nonadherent cells were harvested by gentle pipetting and used as immature DCs. The DC preparation, analyzed with a Coulter Epics XL cytometer (Beckman Coulter, Miami, FL), showed expression of CD11c, MHC class II and CD86, typically 50~60%, 60~70% and 20~40%, respectively (data not shown).

3. Hyperthermia

Tumors were heated by immersing the tumor bearing leg in a circulating water bath (Model MCB-3011D, Mono-Tech Eng. Co., Daejeon, Korea). The water bath was covered with a Lucite plate with holes allowing immersion of the legs. The tumor-bearing leg was pulled down using a sinker, and the tumor was immersed at least 5 mm below the water surface. The temperature of the water bath was controlled at 43±0.01°C for 30 min. Mice were anesthetized with intraperitoneal injection of pentobarbital sodium at a dose of 60 mg/kg before HT. The mice were air-cooled using a fan during hyperthermic treatment to avoid whole body hyperthermia.

4. Treatment protocols

C57BL/6 mice were inoculated s.c. in the right and left thigh with MCA-102 fibrosarcoma cells (1×10^6 and 1×10^4, respectively) on day 0. Immature DCs (1×10^6) were intratumorally (i.t.) administered to right thigh tumors on days 7, 14, and 21. Before DCs injections, hyperthermia was given as described above. Control groups of mice received either no treatment (i.t. PBS), i.t. DCs alone, or HT plus i.t. PBS. Tumor diameter was measured every 7 days and the average size was determined by applying the following formula, tumor size=0.5×(length+width) where length and width are measured in millimeters using vernier calipers. Data are reported as the average tumor size±SE of five mice/group.

5. TUNEL assay

C57BL/6 mice bearing subcutaneous MCA-102 tumors were treated with HT as described above. Tumors were harvested 2, 6, 12, 24, 48, and 72 h after hyperthermia. Control tumors received no treatment. Paraffin-embedded tissues were prepared for both tumors. Slides were deparaffinized and stained using TUNEL method with the In Situ Cell Death Detection Kit (Boehringer-Mannheim, Mannheim, Germany), according to the manufacturer's instructions.

6. Immunohistochemistry

Slides were also deparaffinized for immunohistochemistry of HSP 70 expression and incubated in a humidified chamber with mouse monoclonal HSP 70 (sc-24; 1 : 100; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. After a complete wash in PBS, the slides were treated with goat anti-mouse antibody (1 : 100) for 30 min at 37°C. After a complete wash in PBS, the slides were treated with 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Aldrich, St. Louis, Mo, USA) for 5 min, and then counterstained with hematoxylin for 1 min.

7. Cytotoxicity

Splenocytes (3×10^7/5 ml) from treated or control mice were restimulated by coculture with mitomycin C (10 μg/ml for 20 min)-treated target cells (3×10^6) for 5 days. After restimulation, target cells (2×10^4/well) were cultured with restimulated splenocytes at various ratios in 96-well, round-bottomed plates (200 μl/well) for 6 hr at 37°C. After centrifugation for 10 min at 250 g, 100 μl of supernatant from triplicate cultures were collected and LDH released from target cells was measured in vitro using the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI), according to the manufacturer's protocol. Briefly, cell-free supernatants were incubated in a separate 96-well plate with LDH substrate for 30 min before measuring absorbance using a microplate reader (Quant; Bio-Tek Instruments, Winooski, VT) at 490 nm. Percent cytotoxicity was calculated according to the following formula: $\%\text{Cytotoxicity}=([E-St-Se]/[M-St])\times100$ (with E being the LDH release by effector-target coculture, St the spontaneous release by target cells, Se the spontaneous release by effector cells and M the maximal release by target cells). Spontaneous release of effector and target cells was controlled by separate incubation of the respective populations. Maximal LDH release was measured after lysis of target cells with 1% Triton-X 100.

8. Statistics

For statistical analysis, Kruskal-Wallis and Friedman test with Dunn's multiple comparisons were used to compare the cytotoxicity and *in vivo* tumor growth in individual group, respectively.

Results

1. Induction of apoptosis and HSP expression in heat treated tumor

First, we determined the optimal time of i.t. DC injection after HT, sufficient to induce apoptosis and hsp expression in tumor tissue. The site of established MCA-102 fibrosarcoma was heated at 43°C for 30 min and harvested 0, 2, 4, 6, 8, 12, 24 hr later. In *In situ* TUNEL staining of MCA-102 tumor sections, apoptosis was observed 2 hr and peaked 6 hr after HT (Fig. 1). At peak time, approximately 20~35% of tumor cells had undergone apoptotis. In immunohistochemistry, HSP

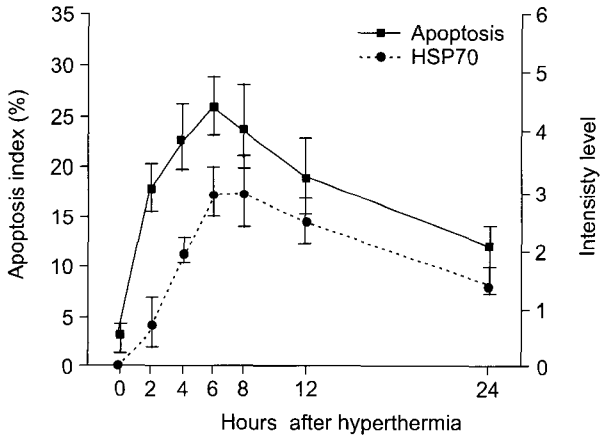

Fig. 1. Induction of apoptosis and HSP 70 expression in tumor by hyperthermia. MCA-102 fibrosarcoma cells (1×10^6) were inoculated into the right thigh of syngeneic C57BL/6 mice. When the tumors reached approximately 6 mm diameter (usually on day 7), they were heated in water bath at 43±0.01°C for 30 min. After the indicated time, the tumor masses were removed. DNA fragmentation was determined with fluorescence microscopy after TUNEL reaction and HSP 70 expression with light microscopy after immunohistochemistry.

70 expression also was detected 2 hr and peaked 6 hr after HT. According to these data, i.t. DCs injection was performed at 1 hour after HT in subsequent experiments.

2. I.t. DCs administration combined with hyperthermia enhance the local antitumor effect of hyperthermia

We examined whether i.t. DCs injection into HT tumors could augment the local antitumor effect of HT. I.t. DCs injection into untreated tumors didn't cause significant inhibition of tumor growth (Fig. 2A). Hyperthermia alone inhibited tumor growth by 31.5% on day 31 after tumor inoculation compaired with untreated tumors ($p<0.05$). I.t. DCs injection combined with HT resulted in an additive inhibition of tumor growth. This inhibition reached 68.2% on day 31 ($p<0.05$ vs all other group).

3. I.t. DCs administration combined with hyperthermia enhance the systemic antitumor immunity of hyperthermia

Next, we examined whether i.t. DCs injection into HT tumors could mediate systemic antitumor immunity on a distant tumor. Hyperthermia alone (HT+PBS) caused a significant inhibition of tumor growth at the distant untreated site (Fig. 2B, $p<0.05$ vs. PBS group). I.t. DCs injection into HT tumors (HT+DCs) induced a potent inhibition of tumor growth at the distant site ($p<0.001$ vs. PBS group). But we didn't able to show a statistical significant difference of HT+DCs group compaired with HT+PBS ($p>0.05$).

4. Induction of tumor-specific cytotoxic T-lymphocyte (CTL) activity by i.t. DCs injection into HT tumor

Apoptotic cells, which could be engulfed and processed by DCs, would be good sources of antigen. Through induction of apoptosis, most current antitumor therapies including chemotherapeutic agents, ionizing radiation and hyperthermia are known to kill tumor cells.[8~10] So, we examined if direct injection of DCs into HT tumor would give rise to tumor-specific CTL activity (Fig. 3). Inoculation of MCA-102 fibrosarcoma cells, which are poorly immunogenic, did not stimulate antitumor cytotoxicity. Each single procedure, which included DCs and HT alone, stimulatd antitumor cytotoxicity weakly ($p<0.05$, all observations of group DCs or HT compared to control group). In contrast, HT+DCs induced strong antitumor cytotoxicity against poorly immunogenic tumor cells ($p<0.05$, all observations of group HT+DCs compared to group DCs or HT). To demonstrate the specificity of antitumor immunity induced by HT+DCs, cytotoxicity was an-

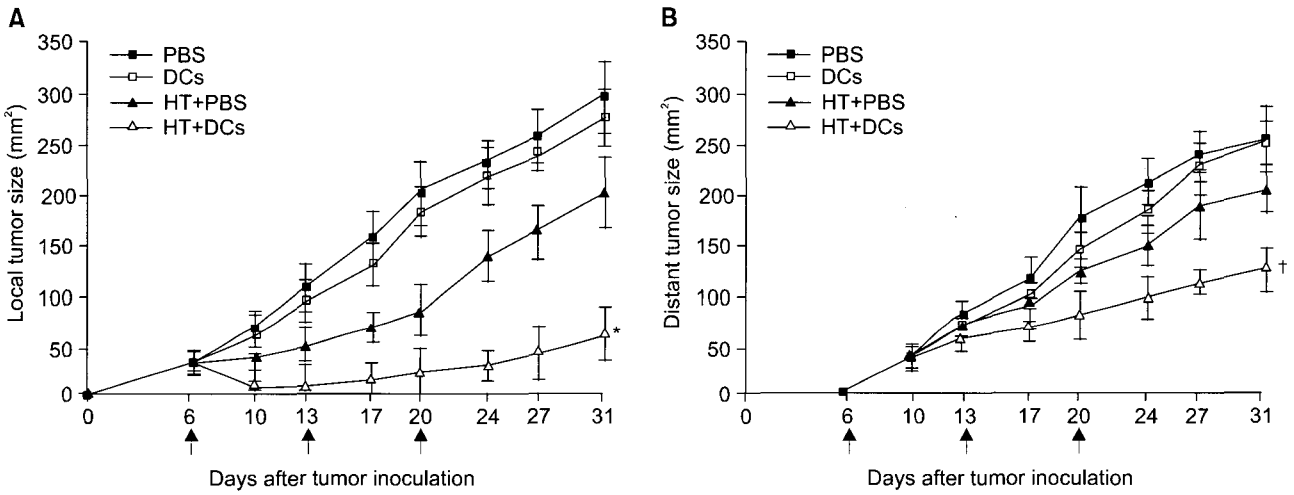

Fig. 2. Induction of antitumor effect against a local treated tumor (A) and a distant untreated tumor (B) by i.t. injection of DCs into heat-treated tumor. A solid primary tumor was produced on the right thigh of a C57BL/6 mouse by injecting s.c. 1×10^6 viable MCA-102 fibrosarcoma cells (black arrows in A and B). On day 7, a solid distant tumor was established on the left thigh by injecting MCA-102 cells (5×10^4). After the tumors on the right thigh had grown to approximately 6 mm diameter (usually on day 7), half of each group of mice were heat treated (HT) at $43\pm0.01^{\circ}C$ for 30 min and the other half received i.t. injection of DCs (1×10^6 in 100 μl of PBS) after HT to the local tumor. These immunization processes were performed 3 times at 7 days intervals (black arrows). Data are average tumor area±SE of 5 mice/group. A: *$p<0.001$ vs. PBS and $p<0.05$ vs. DCs or HT, B: †$p<0.001$ vs. PBS; $p<0.05$ vs. DCs; $p>0.05$ vs. HT+PBS.

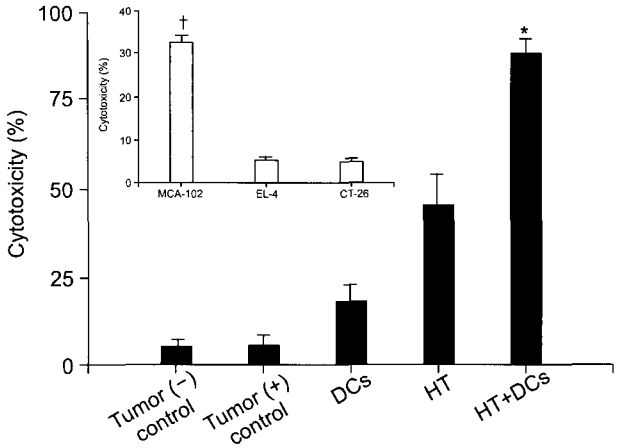

Fig. 3. Enhancement of antitumor immunity by i.t. injection of DCs into heat treated (HT) tumor. Solid tumors were produced on the right thigh of C57BL/6 mice by injecting s.c. 1×10^6 viable MCA-102 fibrosarcoma cells. After tumors grew to 6 mm diameter, mice were untreated [Tumor (+) Control], HT only or injected with DCs (1×10^6) into tumor unheated (DCs) or heated (HT+DCs) at $43\pm0.01^{\circ}C$ for 30 min. These immunization processes were performed 3 times at 7 days intervals. Cytotoxicity of splenocytes isolated from each group 1 week after last immunization was determined against tumor target cells (E : T ratios=20 : 1). Tumor-free mice were included [Tumor (−) Control]. Data are reported as the average cytotoxicity±SE of 5 mice/group. To demonstrate the specificity of antitumor immunity induced by injection of DCs into HT tumor, cytotoxicity was analyzed against EL-4 and CT-26 cells, which are MHC-matched and mismatched tumor cell lines, respectively (inset). In this case, mice (n=5) were immunized once, and the E:T ratio was 20 : 1. *$p<0.01$ vs. Tumor (−) Control or Tumor (+) Control, †$p<0.05$ vs. EL-4 or CT-26.

alyzed against EL-4 and CT-26, which are MHC-matched and mismatched tumor cell lines, respectively (Fig. 2, inset). Since cytotoxicity against EL-4 and CT-26 cells was not observed, our results indicated that DCs injection into tumor apoptosed by hyperthermia leads to induction of tumor-specific CTL activity.

Discussion

This study in murine tumor models demonstrates that intratumoral administration of DCs combined with HT induces systemic antitumor immunity on untreated tumors on the contralateral thigh, as well as induces local antitumor effect on heat treated tumors. In our study, for the local treated tumor, hyperthermia alone inhibits tumor growth; furthermore, i.t. DCs administration combined with hyperthermia results in an additive inhibition (Fig. 2A). And, for the distant untreated tumor, hyperthermia alone causes a significant inhibition of tumor growth; i.t. DCs administration combined with hyperthermia also induces an additive inhibition (Fig. 2B). In our cytotoxicity assay, i.t. DCs administration combined with hyperthermia leads to induction of tumor-specific CTL activity (Fig. 3).

An earlier report showed that i.t. DC administation after hyperthermia using magnetic nanoparticles induced antitumor immunity.[11] Although the heating method is different from ours, these findings are consistent with our result showing that i.t. DCs combined with hyperthermia stimulated systemic antitumor immunity.

Recent reports have shown the importance of HSPs in immune reactions, including HSP 70, HSP 90, and glucose-regulated protein 96.[12] Okamoto et al.[13] demonstrated that tumor-derived cellular lysate enhanced tumor-specific CTL responses when combind with hyperthermia and reported that a fraction enriched in HSP 70 appeared to be involved. HSP-mediated antitumor immunity has been reported in human melanoma and HSP-peptide complexes purified from human melanoma cells induced a vaccine-like effect.[14]

It is unknown how i.t. DCs administration combined with hyperthermia induces antitumor immunity. Tanaka et al.[11] demonstrated 2 mechanisms of antitumor immunity in hyperthermia using magnetic nanoparticles: augmentation of antigenicity by increasing MHC class I expression on cell surface via HSP 70 expression and necrotic cell killing via HSP 70 release, which caused *in situ* vaccination.

Recently, several groups, including ours,[15] described induction of antitumor response after intratumoral DCs administration with high spontaneous rates of apoptosis[16] or after the combination of i.t. DCs administration with apoptosis-inducing therapy.[17,18] These experiments established proof-of-concept that the combination of the intratumoral placement of *ex vivo* generated autologous DCs in concert with apoptosis-inducing therapy (chemotherapy or radiation) might lead to a generation of local and systemic antitumor immunity.

Hyperthermia is currently used in the treatment of some human cancers.[19] Recent evidence indicates that besides exerting direct tumor necrosis, hyperthermia also exhibits various immunomodulatory effects.[11] Inflammatory responses are triggered within heated tissues. These, in turn, discharge danger/alarm signals that recruit DCs to sites of inflam-

mation.[20] At the site, DCs acquire antigens, undergo maturation, and then migrate to the draining lymph node, where they present processed antigens to T cells.[21] Thus hyperthermia, as danger signal, engenders antigen specific cellular immunity. As our data (Fig. 3), animal studies suggest hyperthermia can mediate modulation of tumor-specific immunity.[11] In addition, heated tumor cells have been shown to serve effectively as a source of tumor associated antigens elicit specific T-cell responses *in vitro* when processed and presented by DCs.[22]

The data presented in this report indicates that i.t. DCs administration combined with HT mediates potent inhibition of local tumors. In addition, the combined treatment induces a systemic antitumor immunity. Thus, this approach may be beneficial in the treatment of patients with advanced metastatic disease as well as in the neoadjuvant setting before resection of tumors known to have a high recurrence rate.

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국문초록

온열 요법 후 종양 내 주입한 수지상 세포의 국소 및 원격 항종양 효과

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목 적: 마우스 대퇴부에 심은 종양에 43도의 열을 가한 뒤 수지상 세포를 종양내에 주입하여 국소 및 원격 항종양 효과를 관찰하였다.

대상 및 방법: 마우스 우측 대퇴부에 MCA-102 fibrosarcoma를 피하로 주입하여 종양을 만들었고, 7일째는 반대편 대퇴부에도 주입하여 종양을 만들었다. 종양 세포를 접종한 지 7일째 약 6 mm가 된 종양에 43도의 열을 30분간 가하고 골수 유래 미성숙 동종 수지상 세포를 종양내에 직접 주입하고, 이를 1주 간격으로 2회 더 시행했다. 3~4일 간격으로 양측 대퇴부의 종양의 크기를 측정하여 국소 및 원격 항종양 효과를 평가하였다. 또한 종양 특이적 면역 반응을 평가하기 위해 마우스의 비장 세포를 분리하여 cytotoxicity를 측정하였다.

결 과: 온열치료는 apoptosis를 유도하고 heat shock protein 발현을 증가시켰고, 6시간경에 최고치를 보였다. 치료한 국소 종양에 있어서 온열 요법 단독만으로 종양의 성장을 억제했지만, 온열 요법 후 수지상세포를 주입했을 때는 그 성장 억제 효과는 온열요법 단독의 효과보다 높게 나왔다($p<0.05$). 한편 치료하지 않은 원격종양에 있어서도 온열 요법 단독으로 원격 종양의 성장 억제 효과를 보였으며 온열 요법 후 수지상 세포를 주입했을 때도 원격 종양의 성장 억제 효과가 온열 요법 단독보다 높았으나, 그 억제 정도가 온열 요법 단독과 비교하여 통계적인 차이는 없었다($p>0.05$). Cytotoxicity 검사에서 온열요법과 수지상 세포 복합 치료군에서 가장 높은 세포살해능을 보였고($p<0.05$), 이러한 면역 반응은 종양 특이적이었다.

결 론: 온열요법으로 apoptosis와 heat shock protein을 유도하고 종양내에 동종의 미성숙 수지상 세포를 주입하여 국소 및 원격 종양에 높은 항종양 효과를 유도할 수 있었다. 이러한 새로운 치료법은 암치료에 응용될 수 있을 것이다.

핵심용어: 수지상 세포, 온열 요법, Heat shock protein