Enhanced antitumor effect of dendritic cell-based immunotherapy after internal radiotherapy against B16 melanoma

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Enhanced antitumor effect of dendritic cell-based immunotherapy after internal radiotherapy against B16 melanoma

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감사의 글

2000년 봄의 문턱을 넘어 난 새로운 학문의 길에 첫 발을 내 디뎠다. 미래에 대한 기대감과 불안감을 동시에 지닌 출발, 하지만 6년의 세월이 지난 지금 불안감보다는 자신감과 기대감으로 충만해 있는 나를 바라본다. 6년의 세월을 한결같이 지도해주시고 본 논문이 나오기까지 물심양면으로 도와주신 이 민걸 선생님께 깊은 감사를 드립니다. 또한 바쁘신 가운데 논문 지도 및 심사를 해주신 이 종태 선생님, 서 창옥 선생님, 이 종두 선생님, 그리고 임 종석 선생님께도 깊은 감사를 드립니다. 짧은 기간이나마 부산이라는 새로운 환경에서 생활할 수 있게 도와주시고, 연구함에 있어 연구자의 길을 일깨워주신 박 영민 선생님께도 깊은 감사를 드립니다. 처음 낯선 환경과 실험에 적응하도록 도와주신 장 남수 선생님과 계 진철 선생님, 동물실험에 많은 도움을 주신 김 형관 선생님, 교통사고 후 많은 도움을 주신 이 승준 선생님, 졸업 후 미래에 대해 많이 조언을 해준 김 형근 선생님, 송 경섭 박사, 이 승범 박사에게도 감사드립니다. 또한 실험실에서 밤 늦게까지 일하며 실험을 도와준 허 민규 선생, 박 기청 선생, 부산의 정 영일 선생, 이 준식 선생, 이 창민 선생, 그리고 부산서 서울까지 올라와 실험을 도와주고 있는 제 정환 선생에게도 감사드립니다. 지금은 미국에 있지만 논문에 많은 도움을 준 조 영훈 선생님에게도 감사의 마음을 전합니다. 좁은 지면에 도움을 주신 모든 분들을 일일이 열거하면서 감사의 마음을 전하지는 못하지만, 이 모든 것이 결코 저 혼자의 힘만으로 된 것이 아니었음을 알고 있습니다. 도움을 주신 모든 분들께 감사드립니다.

오늘이 있기까지 끝없는 보살핌과 도움을 주신 양가 부모님, 어려움 속에서도 끝까지 지켜봐 준 동생내외와 미형이 그리고 이해와 사랑으로 참고 견딘 아내와 아들 윤성이에게 고마움과 사랑의 마음을 전하며 오늘의 작은 열매에 자만하지 않고 계속 정진하면서 살아가도록 하겠습니다.

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LIST OF ABBREVIATIONS

Ho : holmium

DCs : dendritic cells

LNs : lymph nodes

CM : complete medium

Ab : antibody

RT : room temperature

GABA : Φ-labeled anti-biotin Abs

ABSTRACT

Enhanced antitumor effect of dendritic cell-based immunotherapy after internal radiotherapy against B16 melanoma

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Internal radiotherapy involving the intratumoral injection of the beta emitting radionuclide, Holmium(Ho)-166, into B16 melanoma resulted in a reduction in size and growth rate; however, complete remission was not always achieved. Therefore, additional dendritic cells (DCs) therapy was investigated to determine whether it could improve therapeutic results. Malignant melanoma was induced in mice by inoculating B16F10 cell line subcutaneously. Fifty-four mice were divided into 4 groups: 1) non-treated (n=11, group I), 2) treated with Ho-166 (n=16, group II), 3) treated with

immature DCs (n=8, group III), and 4) treated with immature DCs after Ho-166 injection (n=19, group IV). Changes in tumor size, survival rates, and immunologic profiles were observed. Nineteen days after Ho-166 or PBS injection, mean tumor sizes in the four groups were $6044\pm3469mm^3$, $1658\pm2092mm^3$, $3871\pm2604mm^3$, and $444\pm729mm^3$, respectively. We observed a significant decrease in tumor size ($p<0.05$) and an increase in survival in group IV. When the B16F10 cell line was reinjected into the contralateral backs of survivors, much slower growth was observed in group IV ($p<0.05$). Both tumor-specific CTL and natural killer cell activities and the infiltration of inflammatory cells into tumor tissues were found to be elevated in group IV. In addition, strong immune responses as determined by *in vitro* T cell proliferation, ELISA and ELISPOT assay were induced in group IV. Our results suggest that a combination of internal radiotherapy using Ho-166 and immature DCs could be used either to treat unresectable melanoma or as an adjuvant therapy after surgery.

Key words : dendritic cell, melanoma, immunotherapy, radiotherapy, holmium

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I. INTRODUCTION

Malignant melanoma is a potentially lethal cancer that arises from melanocytes present in the skin, mucosa, or the epithelial surfaces of the eyes and ears, and its incidence has increased substantially over the past two decades[1, 2]. Although primary tumor excision can sometimes achieve compete remission, most melanomas are beyond surgical margins when diagnosed, and are usually resistant to chemotherapy and radiotherapy[3]. Consequently, many other therapeutic modalities are being investigated, and one of them is dendritic cell-based immunotherapy[4, 5].

Dendritic cells (DCs) are one of the most potent antigen-presenting cells[6]. DCs residing in the skin or mucosa are in their immature state and have great phagocytic capacity. After antigen uptake, DCs undergo maturation, i.e., downregulate antigen uptake and increase their antigen processing abilities, and migrate to the regional lymph nodes (LNs) where they present antigen to naïve T cells, which generate potent host immunity to the antigen[7]. It has been reported that efficient antitumor immunity is generated by immature DCs because DCs phagocytose both necrotic and apoptotic tumor cells and mature *in vivo*[8]. The abilities of DCs to phagocytose tumor cells *in vivo* and migrate to regional LNs 24 hours after injection have also been demonstrated. These findings suggest that DCs can be used efficiently in cancer immunotherapy. Such DC-based immunotherapies have been tried in mice and humans, and positive results have been obtained for many tumors, especially for malignant melanoma, B cell lymphoma, colorectal cancer, and prostate cancer[9-12]. However, some problems remain to be solved. First, no clear consensus has been reached concerning the antigen most capable of stimulating an antitumor immune response, or the method of preparing such antigens[13]. Investigators have used many antigens, e.g., crude tumor lysates[14, 15], apoptotic tumor cells[16, 17], and several known tumor peptide antigens such as melanoma antigen (MAGE) or tyrosinase-related protein 2 (TRP-2)[18], or tumor DNA/RNA[19-21], but results are conflicting with regard to which stimulate host immune response most efficiently. Second, obtaining the quantities of tumor tissues required for use as autologous tumor antigens is not always possible.

And third, pulsing DCs with tumor antigen *in vitro* differs from the physiologic alternative *in vivo*, due to the presence of cytokines and danger signals. Moreover, the fact that the immune systems of cancer patients are down regulated by many factors secreted by tumor cells or host cells in response to cancer tissues is of utmost importance. DCs and T cells are commonly detected in tumors, but they are immature and have been functionally inactivated by inhibitory cytokines, like IL-10 and vascular endothelial growth factor, which are secreted by tumor cells, and thus they cannot generate adequate antitumor immunity[22-26].

Therefore, investigators have tried to isolate DC precursors and generate functional DCs *in vitro*, and to re-administer them as an adoptive immunotherapy to recover the immunosuppressive effects of tumor cells and thus generate proper anti-tumor immunity[27, 28]. Other investigators have tried the new physiologic method of *in vivo* antigen preparation and pulsing, in which immature DCs are injected directly into tumor tissues to capture tumor antigens *in vivo*, and then migrate to the regional LNs and generate antitumor immune responses[29]. However, tumor cells secret many inhibitory cytokines, and DCs delivered to tumor tissues express low surface molecules, such as CD80, CD86, and MHC class II, so that they cannot play a proper role[30-32]. Thus, we tried to find a way of enhancing DCs survival and of reducing the immunosuppressive effects of tumors.

The administration of radiation *in vitro* and *in vivo* has been utilized to create an inflammatory milieu, via the induction of apoptosis and necrosis,

and via the upregulation of immunomodulatory surface molecule expression and secretary molecules in tumor cells. Experimental data indicates that the radiation-induced microenvironment enables DC-mediated antigen-specific immune responses[33]. Although the radiation sensitivity of melanoma cells is generally low[34-36], it has been proposed that higher fractional and total radiation doses would increase melanoma radiation response[37-39]. As an alternative to external radiotherapy, the direct intratumoral injection of unsealed high-energy beta-emitting radionuclides has emerged as a new technique for the local control of tumors[40, 41]. Recently, we reported that a significant reduction in tumor size and tumor growth inhibition was achieved by direct intratumoral injection of the high-energy beta-emitter Holmium (Ho)-166 into a malignant melanoma animal model. However, this technique did not achieve complete remission[42].

In this study therefore, we combined Ho-166 and intratumoral adoptive DC-based immunotherapy. We injected DCs directly into tumor tissues after Ho-166 injection, in the belief that a local intratumoral injection of Ho-166 would lead to a release of tumor antigen and reduce tumor size, and that unpulsed-DCs injected into a tumor would uptake tumor antigen, migrate to regional LNs, and stimulate T lymphocytes to increase immune responses and survival rate of mice.

II. MATERIALS AND METHODS

Mice

C57BL/6 female mice (6-10 weeks old) were purchased from Daehan Biolink (Seoul, Korea) and housed in pathogen-free units at the Yonsei Medical Research Center.

Media and Cytokines

Complete medium (CM) consisting of RPMI 1640 (Gibco, BRL, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Gibco), 100IU/㎖ penicillin (Gibco), 100 ㎍/㎖ streptomycin (Gibco), 0.1mM nonessential amino acids (Sigma, Saint Louis, MO), 1mM sodium pyruvate (Sigma), 10mM HEPES (Sigma), and 50μM 2-mercaptoethanol (Sigma). DMEM (Gibco) and RPMI 1640 were used to culture hybridoma cell lines. Recombinant mouse GM-CSF and IL-4 were purchased from Endogen (Woburn, MA) and used to generate DCs.

Preparations of Bone Marrow-derived DCs

Bone marrow was obtained from the tibias and femurs of C57BL/6 mice (6-8 weeks old) and depleted of erythrocytes using commercial lysis buffer (Sigma). Bone marrow cells were then treated with a mixture of antibodies (Abs) [anti-CD4 (GK1.5, TIB-207), anti-CD8 (53-6.72, TIB-105), anti-B220 (RA3-3A1/6.1, TIB-146), anti-I-$A^{b,d,q}$ & I-$E^{d,k}$ (M5/114.15.2, TIB-120), anti-

erythrocytes, neutrophils & B cells (J11d.2, TIB-183); all were obtained from the ATCC] for 30min at 4℃, and then treated with rabbit complement (Cedarlane, Ontario, Canada) for 1h at 37℃. Cells were then layered onto lympholyte M (density: 1.0875±0.0010g/㎖, Cedarlane) gradients, centrifuged, and low-density interfaces were collected. Cells were washed 3 times with CM, and then incubated in CM supplemented with 10ng/㎖ GM-CSF and 10ng/㎖ IL-4 in 24 well plates at 7-10×10^5/well. On day 2 of culture, floating cells were gently removed and fresh medium containing GM-CSF and IL-4 was replaced. On day 4, floating cells were harvested and fresh medium containing GM-CSF and IL-4 was replaced. These harvested cells were then plated in new 24 well plates in CM supplemented with 10ng/㎖ GM-CSF and 10ng/㎖ IL-4 at 5×10^5/㎖. On day 6, non-adherent and loosely adherent proliferating DCs were collected and counted for injection.

Flow Cytometry

To identify surface molecules expressed on DCs, they were harvested on day 5(D5), 6(D6) and 7(D7). Cells (6-10×10^5) were washed twice with 0.4% BSA/PBS and stained for 30min at 4℃ with monoclonal Abs against CD11c (Pharmingen, San Diego, CA), CD80 (Pharmingen), CD86 (Pharmingen) and MHC class II (I-$A^{b,d,q}$ & I-$E^{d,k}$). Hamster IgG (Pharmingen) and rat IgG2a (Pharmingen) isotype control were used. After 2 washes with 0.4% BSA/PBS, secondary staining was performed using FITC-conjugated $F(ab')_2$ goat anti-rat Ig's (Biosource, Camarillo, CA) and FITC-conjugated anti-hamster IgG

(Pharmingen). After 30min, cells were washed twice with 0.4% BSA/PBS and resuspended in 400 μℓ of 0.4% BSA/PBS. Propidium iodide (Sigma) was added to exclude dead cells from the analysis. Flow cytometric analysis was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA).

Experimental Design

A malignant melanoma model was made by inoculating 2.5×10^5 B16F10 cells in 100 μℓ PBS subcutaneously into C57BL/6 mice (6-10 weeks old). Ten to fourteen days later when tumors were about 1.0cm, 54 mice were divided into 4 groups: 1) non-treated control (11 mice, Group I), 2) treated with Ho-166 (16 mice, Group II), 3) treated with immature DCs (D6) (8 mice, Group III), and 4) treated with immature DCs (D6) from 7 days after Ho-166 injection (19 mice, Group IV).

Intratumoral Injection of Ho-166 and DCs

The concentration of Ho-166 was fixed at 1mCi/㎖ (determined by preliminary experiments) and 300 μℓ Ho-166 was injected directly into the melanoma. Seven days after Ho-166 injection, DCs (D6) were injected at 1.5×10^6 per mouse in 50 μℓ of PBS and given another two injections at 2-3 day intervals.

Of the possible beta-emitters, Ho-166 was chosen for internal radiotherapy because 94% of its emission is in the form of 1.76- and 1.86-MeV beta rays, which have a relatively short half-life, 26.9h, and because their maximum soft

tissue penetration range is 8.7 mm (average 2.1 mm), thus they cause less damage to surrounding normal tissue. Although the radiation dose to the tumor center exceeded 885 Gy/mCi, doses applied to the surrounding tissues at 1, 2, 3 and 4 mm from the tumor center were approximately 128, 40, 13 and 3.6 Gy/mCi, respectively[42].

Measurement of Tumor Size and Survival Rate.

After injecting Ho-166 and DCs, tumor long and short axes were measured with calipers (Mitutoyo, Japan) at 2-3 day intervals, and tumor volumes were calculated using the formula: $V=1/2\times A^2\times B$, where A is the length of the short axis and B that of the long axis. Surviving mice were counted until all group I mice succumbed. Survival rates were recorded as percentage survivals.

In Vivo Protection Assay

To identify specific immunity against B16F10 melanoma, mice surviving after treatment with Ho-166, DCs, or Ho-166+DCs were re-injected into the contralateral side of the backs with 2.5×10^5 B16F10 cells in 100 $\mu\ell$ PBS.

T cell Proliferation Assay

Splenocytes were obtained from each experimental group and commercial erythrocyte lysis buffer was added. After 5min, the reaction was stopped by adding CM, and cells were washed twice with CM. Triplicate samples of 1.5×10^5 cells in CM were seeded in 96 well round-bottom plates (Corning,

NY). The splenocytes (responder; R) were stimulated with B16F10 cells (stimulator; S) treated with mitomycin c and irradiation (30,000 rad) at a S:R ratio of 1:50. Splenocytes from untreated mice were used as negative control. The cells were incubated at 37℃ for 96h, then 1μCi/well [^{3}H] thymidine was added for another 16h. Cells were harvested onto glass-fiber filters using a Harvester 96 (Tomtec, Hamden, CT), and [^{3}H] thymidine incorporation (CPM) was counted on a scintillation counter (Wallac, Truku, Filand).

IFN-γ ELISA

To measure IFN-γ secreted by splenocytes, splenocytes were stimulated with B16F10 cells and prepared as described above. Quantities of secreted IFN-γ were measured by ELISA using an OptEIA set (Pharmingen) as described by the manufacturer. Briefly, anti-mouse IFN-γ capturing Abs in 0.1M pH 9.5 sodium carbonate (100 ㎕/well), were plated in the wells of 96 well plates (Corning) at 100 ㎕ per well and incubated overnight at 4℃. After washing, blocking solution (10% FBS/PBS) was plated at 200 ㎕ per well and incubated for 1h at room temperature (RT). After washing, culture supernatants were plated at 100 ㎕ per well and incubated for 2h at RT. After washing, a mixture of biotinylated anti-mouse IFN-γ Ab and streptavidin-HRP reagent were plated at 100 ㎕ per well and incubated for 1h at RT. After washing, substrate solution (100 ㎍/ml tetramethyl benzidine and 0.003% H_2O_2) was plated at 100 ㎕ per well and incubated for 30min at RT in the

dark, followed by 4 N HCl. OD_{450} was measured on an ELISA Reader (Molecular Devices, Sunnyvale, CA, USA).

IFN-γ ELISPOT Assay

Erythrocyte-depleted splenocytes were obtained as described above. ELISPOT was performed according to the protocol supplied by U-CyTech (Utrecht, Netherlands). 96 well PVDF plates were coated with anti-mouse IFN-γ and incubated overnight at 4℃. After washing, plates were blocked with 1% BSA/PBS buffer for 1h at 37℃. After removing the buffer, splenocytes were plated in wells at 3.3×10^5/100 μℓ and B16F10 cells treated mitomycin c and irradiation (30,000 rad) were added at 1×10^4/100 μℓ. After 24h of incubation at 37℃, cells were removed, washed, and biotinylated detector Ab was plated at 100 μℓ/well and incubated for 1h at 37℃. After washing, 50 μℓ of Φ-labeled anti-biotin Abs (GABA) was added to each well, and plates were incubated for 1h at 37℃. After washing, Activator I/II was plated at 30 μℓ/well and developed for 25min at RT in the dark. After developing, plates were washed with running water, and dried. Spot-forming colonies were counted using an ELISPOT reader system (AID, Germany). To identify $CD8^+$ T cells, $CD4^+$ T cells were depleted using anti-CD4 Ab (H129.19).

Cytotoxic Assays of CTLs and Natural Killer (NK) Cells

Erythrocytes-depleted splenocytes were obtained as described above. Splenocytes (2×10^7) from each experimental group were restimulated with 1% paraformaldehyde-fixed B16F10 cells (5×10^5) in the presence of rhIL-2 (10 U/㎖, Endogen). Five days later, the restimulated cells were used as effectors against B16F10 and YAC-1 cells in ^{51}Cr release assays. Briefly, target cells (1×10^6 of each) were labeled with 200μCi $Na_2{}^{51}CrO_4$ for 4h, and after washing twice, these effector and target cells were plated in 96 well round-bottomed plates at a proper E:T ratio. After culturing for 6h, supernatant (100 ㎕) was obtained and radioactivity was measured using a gamma counter (Packard, Downers Grove, Illinois). Percentage specific lysis was calculated using the following formula: % specific lysis = 100 × (experiment release – spontaneous release) / (maximal release-spontaneous release).

Histological Examination

Tumors of C57BL/6 mice were removed 2 days after the 3rd DC injection and fixed in 10% neutral buffered formalin. Tissues were then dehydrated in a graded ethanol series and embedded in paraplast, and tissue blocks were sectioned in 3-4 ㎛. Sections were stained with hematoxylin and eosin, and examined under an optical microscope (Olympus, Tokyo, Japan).

Statistical Analysis

Statistical analysis was performed using SPSS (SPSS Inc., Chicago). ANOVA with the Sheffé method for multiple comparisons was applied to compare tumor sizes in the main experiment and in the protection assay. The Kruskal-Wallis test, a nonparametric equivalent to ANOVA, was used to compare T cell proliferation, ELISA, ELISPOT, and cytotoxicity CTL and NK cell assay results. *P* values <0.05 were considered statistically significant.

III. RESULTS

Changes in DC Surface Molecule Expression. Erythrocyte-, lymphocyte-, and neutrophil-depleted bone marrow cells of C57BL/6 mice were incubated with rmGM-CSF and rmIL-4, and the surface expressions of CD11c, CD40, CD80, CD86, and MHC class II molecules were examined with respect to days cultured. On FACS analysis, high expressions of costimulatory and MHC class II molecules and moderate expression of CD11c were identified, indicating that the DCs had been generated from bone marrow cells with rmGM-CSF and rmIL-4 (**Fig. 1**). The expressions of CD40, CD86, and MHC class II molecules were much increased on culture day 7 versus day 6. In our experiments, immature DCs in day 6 were used to uptake the tumor antigens released by Ho-166 treatment.

Changes in Tumor Sizes and Mice Survival. Preliminary experiments were performed to determine the optimal concentration and injection schedules of Ho-166 and DCs to increase tumor destruction and antitumoral immune response while minimizing the effects of Ho-166 on DCs. After repeated experiments, the optimal Ho-166 concentration was found to be 1 mCi/㎖, and DCs were injected initially 1 weak after Ho-166 injection (data not shown).

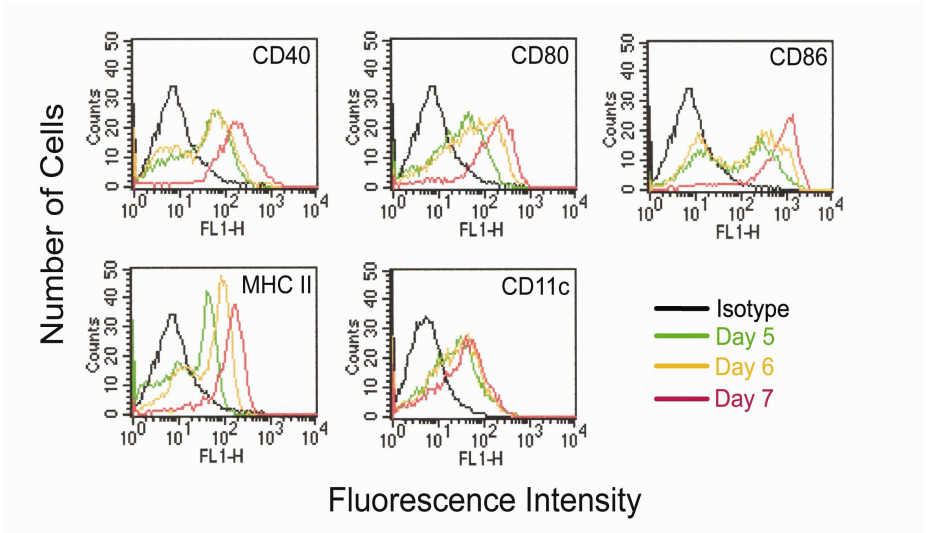

Figure 1. Flow cytometric analysis of mouse bone marrow-derived DCs. Bone marrow cells were cultured with rmGM-CSF and rmIL-4. On days 5, 6, and 7, the surface phenotypes of bone marrow-derived DCs were examined by flow cytometry using CD40, CD80, CD86, MHC class II and CD11c mAb. Similar results were obtained from three experiments. The data shown are from one representative experiment. In this study, bone marrow-derived DCs on day 6 were used.

Three hundred $\mu\ell$ of Ho-166 were injected when the long axes of tumors were about 1.0cm (day 0), and tumor sizes were then measured at 2-3 days intervals (**Fig. 2**). Three cycles of DCs were injected in total, starting 1 weak after Ho-166 injection at 2-3 days intervals. Reduced tumor growth was observed in the Ho-166-treated groups (groups II and IV) after day 11 versus the non-treated controls. This growth inhibitory effect reduced after day 14, but tumor growth rates were slower in the Ho-166-treated groups (group II) than in the non-treated group. On the other hand, the growth inhibitory effects of additional DCs after Ho-166 (group IV) continued after day 14, and tumor sizes were much smaller in this group. On day 19 after Ho-166 injection, the mean tumor sizes of Groups I, II, III, and IV were 6044±3469 mm^3, 1658±2092 mm^3 (2 complete remissions), 3871±2604mm^3, and 444±729 mm^3 (12 complete remissions) (*$p<0.05$ versus other 3 groups*), respectively.

Survival rates are expressed as percentage survivals (**Fig. 3**). The survival rates of non-treated (group I) and DC-treated (group III) mice were much lower after day 20 and all died before day 31 and day 40, respectively. When measured on day 60, survival rates were 19% (3/16) in the Ho-166-treated (group II), and 53% (10/19) in the Ho-166+DC-treated group (group IV). These results indicate that additional antitumoral immunity was generated by DC administration.

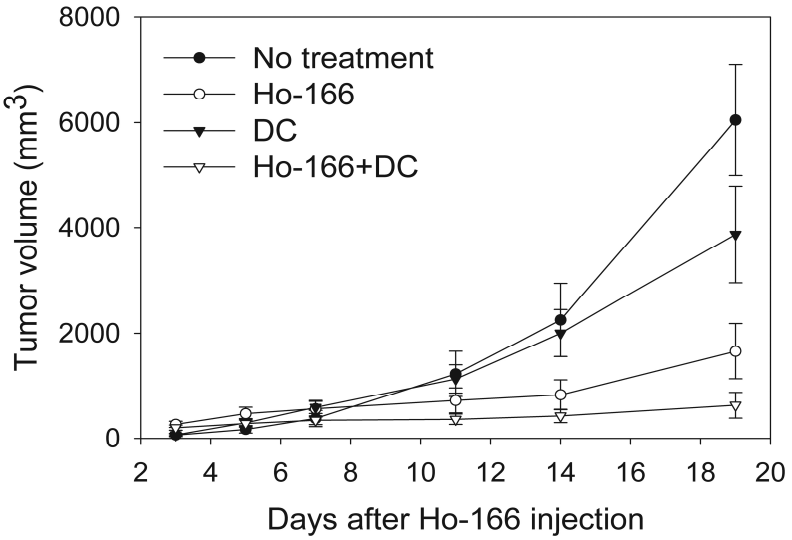

Figure 2. Tumor volume changes after various treatments. Melanomas were generated by subcutaneously injecting B16F10 melanoma cells (2.5×10^5 cells) suspended in 100 $\mu\ell$ of normal saline into the backs of C57BL/6 mice. These mice were then treated with Ho-166 when tumor sizes reached approximately 1.0 cm in maximal diameter (10-14 days after inoculation). Seven days after Ho-166 injection, DCs were injected intratumorally. Mean tumor volumes were much reduced in the Ho-166+DC-treated group ($p<0.05$ *versus* groups treated with Ho-166 or DCs) on day 19 after inoculation. The data shown are the mean values and their standard errors.

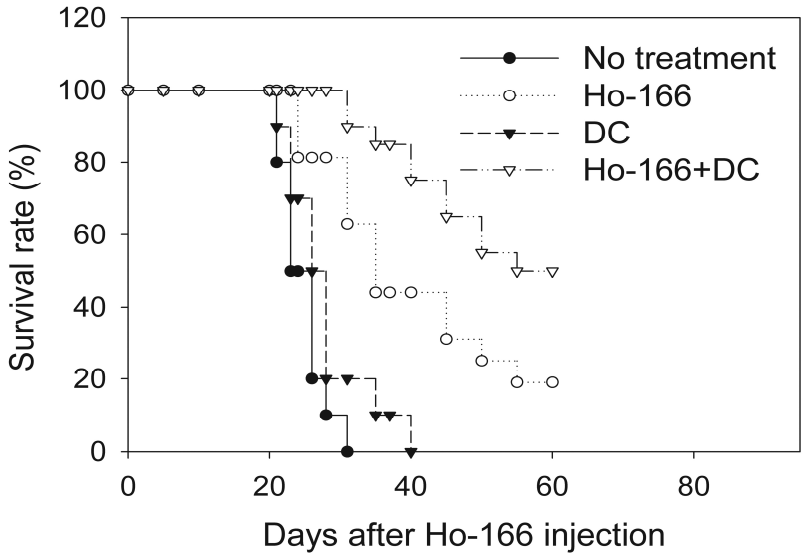

Figure 3. Survival rates of mice with melanoma in each treatment group. Survival rates are presented as number of animals surviving (%). Non-treated mice lived for up to 31 days. However, 3 of 16 (19%) mice in the Ho-166-treated group and 10 of 19 (53%) mice in the Ho-166+DC-treated group remained alive on day 60.

Generation of Tumor-specific Immunity In Vivo (In Vivo Protection Assay). Additional B16F10 melanoma cells (2.5×10^5) were also injected into the contralateral sides of surviving mice on day 60 to determine whether tumor-specific immunity was generated or not. Growth rates of newly developed tumor were determined (**Fig. 4**). As all mice in the non-treated and DC-treated groups succumbed before day 40, healthy mice were used as negative controls and no protection assay could be performed on the DC-treated group. Though newly developed tumors grew rapidly in negative control mice 14 days after the protection assay started, and slowly in Ho-166-treated mice 11 days after the protection assay started, tumors growth in the Ho-166+DC-treated group was much retarded (*$P<0.05$ versus other 2 groups*). When measured 17 days after the protection assay started, the mean sizes of tumors in non-treated, Ho-166-treated, and Ho-166+DC-treated mice were $2148\pm561mm^3$, $966\pm808mm^3$ and $192\pm266mm^3$ (no tumor in 2 mice), respectively (n=5 mice per group).

Generation of Tumor-specific Immunity In Vitro. T cell proliferation, IFN-γ ELISA, and ELISPOT assay were performed with splenocytes from the non-treated, Ho-166-treated, DC-treated and Ho-166+DC-treated groups to confirm whether melanoma-specific immunity was generated. Erythrocyte-depleted splenocytes (R) were plated in the wells of 96 well round-bottom plates (1.5×10^5/well) and stimulated with B16F10 melanoma cells (S) treated with mitomycin c and irradiation (30,000 rad) at a

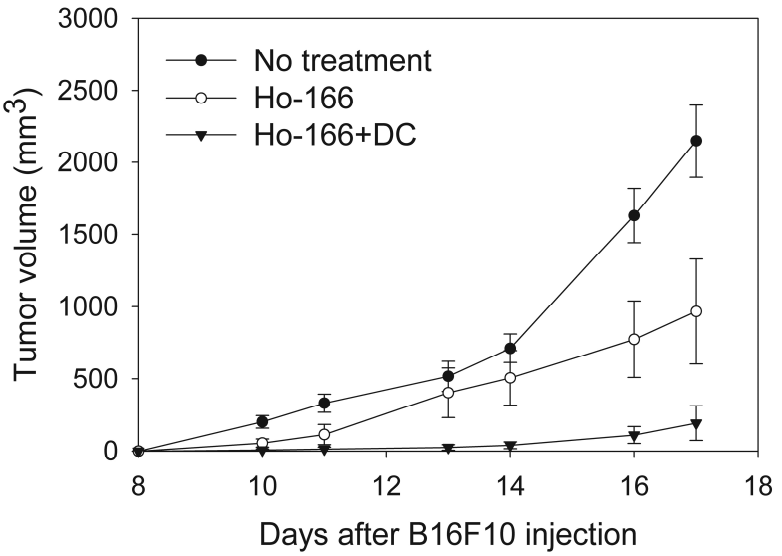

Figure 4. Tumor volume changes during in vivo protection assays. B16F10 melanoma cells (2.5×10^5 cells) were re-injected into the contralateral dorsal sides of mice that had survived initial experiment. Seventeen days after re-injection, the mean sizes and standard errors of tumors in the non−treated group, the Ho-166 group, and the Ho-166+DC group were $2148\pm561mm^3$, $966\pm808mm^3$ and $192\pm266mm^3$ (*P<0.05 versus other 2 groups*), respectively (n=5 mice per group).

S:R ratio of 1:50. T cell proliferation was then determined.

According to the T cell proliferation assay, the mean CPM values of the non-treated, Ho-166-treated, DC-treated and Ho-166+DC-treated groups were 246±25, 1991±804, 2592±1280, and 7449±53, respectively ($P<0.05$). Thus splenocyte proliferation was much higher in Ho-166+DC-treated group (**Fig. 5*A***).

IFN-γ ELISA was also performed with the supernatants of splenocytes stimulated with irradiated B16F10 melanoma cells. The determined IFN-γ concentrations were 306±44pg/㎖, 1859±21 pg/㎖, and 2705±50pg/㎖ in the Ho-166-treated, DC-treated, and the Ho-166+DC-treated groups, respectively ($P<0.05$). IFN-γ was not detected in the non-treated group. Thus the amounts of IFN-γ secreted by the DC- and Ho-166+DC-treated groups were much higher than in the Ho-166-treated group (**Fig. 5*B***).

Finally, ELISPOT assays were performed to identify melanoma-specific T cells in each experimental group. Splenocytes were stimulated with irradiated B16F10 melanoma cells for 24 hours, and analyzed according to the protocol of U-CyTech. IFN-γ-secreting cells were much higher in the Ho-166+DC-treated group than in the Ho-166-treated or DC-treated groups. After depleting $CD4^+$ T cells with anti-CD4 Ab to analyze $CD8^+$ T cells, IFN-γ-secreting cells remained higher in the Ho-166+DC-treated group (**Fig. *5C***). These findings indicate that anti-melanoma immunity was significantly enhanced by Ho-166 and DC combination therapy.

Cytotoxic Assays of CTL and NK Cells. To verify the induction of systemic and specific antitumor immunity, we determined the CTL activities of splenocytes in each experimental group. Splenocytes were restimulated with irradiated B16F10 for 5 days, and subjected to ^{51}Cr-release assays to determine antitumor CTL and NK cell activities. The measured cytolysis were 0.84% (non-treated), 1.76% (Ho-166-treated), 4.46% (DC-treated), and 10.28% (Ho-166+DC-treated), respectively ($P<0.05$), at an E:T ratio of 100:1 (data not shown). CTL activity against B16F10 cells was much higher in the Ho-166+DC-treated group, as was NK cell activity against YAC-1 cells (13.23% in the non-treated group, 13.24% in the Ho-166-treated group, 24.86% in the DC-treated group, and 29.38% in the Ho-166+DC-treated group ($P<0.05$) at an E:T ratio of 100:1 (data not shown). These results suggest that CTL and NK cell activities induced in the Ho-166+DC-treated group are B16F10 melanoma-specific.

Histopathologic Observations of B16 Melanoma Injected with Ho-166 and DCs. As shown in **Fig. 6**, many mononuclear inflammatory cells were observed to have infiltrated tumor tissues in mice treated with Ho-166+DC, whereas no inflammatory cell infiltration was observed in the tumor tissues of mice in the other groups.

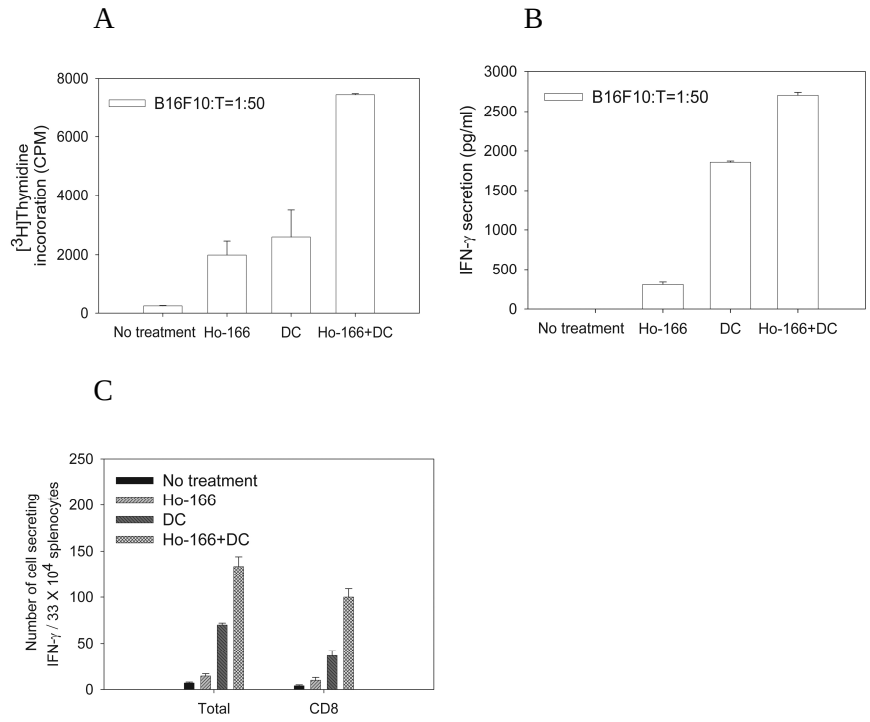

Figure 5. Erythrocyte-depleted splenocytes (R) were obtained from each group and stimulated with irradiated B16F10 cells (S) at a S:R ratio of 1:50. ***A.*** Results of T cell proliferation assays. After stimulating for 4 days, the levels of proliferation induced were measured by thymidine incorporation for another 16h of culture. Treatment with Ho-166+DCs stimulated higher levels of tumor-specific proliferation by splenocytes. Data presented are the mean values of three repeated experiments with standard error bars. ***B.*** IFN-γ secretions measured by ELISA. The cytokine contents of splenocyte supernatants after 4 days of stimulation were determined by ELISA. Data presented are the mean values of three repeated experiments with standard error bars. ***C.*** IFN-γ-secreting cells measured by ELISPOT assay. To identify $CD8^+$ T cells, $CD4^+$ T cells were depleted with anti-CD4 Ab (H129.19). Data

presented are the mean values of spots per 33×10^4 responders with standard error bars for three repeated experiments conducted in duplicate.

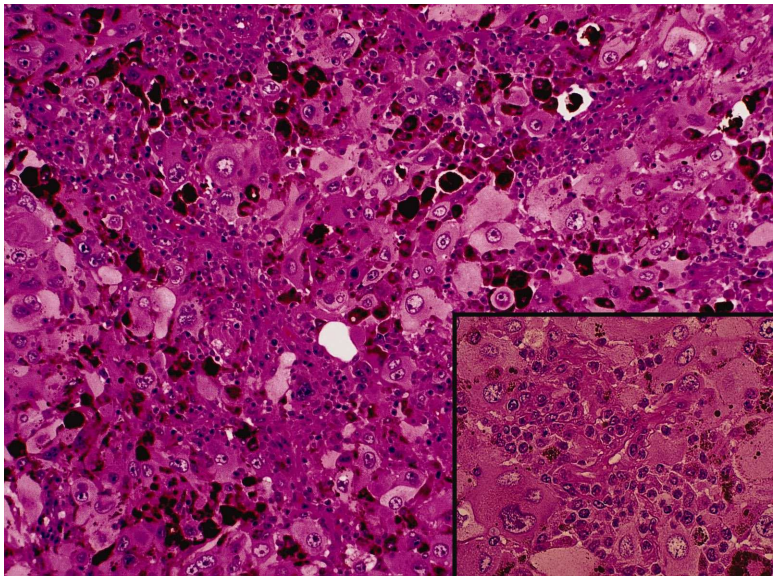

Figure 6. Histological findings of B16F10 melanoma from mice injected intratumorally with Ho-166+DCs. Two days after the final DC injection, resected melanomas were examined histologically. Many mononuclear inflammatory cells were found to have infiltrated necrotic melanoma cells in this group alone. Haematoxylin & Eosin stain, ×100; inset, ×400.

IV. DISCUSSION

In this study, we injected unstimulated immature DCs into established tumors 1 week after Ho-166 injection. The half-life of Ho-166 is relatively short (26.9h), and the interval of 1 week between Ho-166 and DCs injection was determined by preliminary experimentation to prevent residual radiation damaging the DCs. Our results show that the Ho-166+DC combination greatly retarded tumor growth and increased survival rates versus Ho-166 or DC treatment alone. We also demonstrated that tumor-specific immune responses *in vitro* involved T cell proliferation, IFN-γ release (ELISA and ELISPOT assay), and CTL and NK cell activities. Ho-166+DC treatment generated much higher T cell proliferation and IFN-γ release in response to tumor cells. CTL and NK cell activities were also increased in Ho-166+DC-treated mice. Furthermore, it was found histologically that Ho-166+DC treatment induced inflammatory cell infiltration into tumors and tumor cell necrosis.

Tumor growth inhibitory effects were also demonstrated by the Ho-166 or DC treatment alone versus the negative control. However, these effects were much weaker at inducing anti-tumor immune responses, and the effect of Ho-166 treatment differed little from that of the control. Thus though internal radiotherapy was able to reduce tumor volume with localized necrosis and growth arrest, it failed to induce tumor-specific immune responses under the conditions used, which suggests that radiation therapy, whether internal or

external, might be insufficient to induce complete remission from melanoma. Likewise, DC treatment failed to generate potent tumor-specific immunity. As described earlier, tumor cells secret many cytokines that down-regulate host immunity. Therefore, DCs require an adjuvant therapy capable of destroying tumor cells and eliminating immune suppressing molecules. Furthermore, as the danger signal model describes, inflammation should be created around the inoculated DCs.

Internal Ho-166 radiotherapy is known to induce central tumor cell necrosis and peripheral growth arrest[42]. These changes generate inflammation and promote the secretion of many danger signals by host and tumor cells. Internal radiotherapy can also destroy the architecture of melanoma tissues, and residual necrotic and growth-arrested tumor cells may provide an antigenic source for inoculated DCs. As expected, Ho-166+DC treatment was found to induce a more potent immune response than DC treatment, thus demonstrating that radiation therapy well complements DC treatment. These relations have also been demonstrated by experiments conducted by others[43, 44].

The identities of the specific tumor antigens involved in our experiments and also in normal immune responses to tumors remain unknown. However, tumor necrosis and growth arrest seem to be required for tumor antigen recognition by immune cells. DCs would uptake some antigens released from tumor cells with the help of Ho-166, and migrate to regional LNs and stimulate naïve T cells to become effective cytotoxic T cells. At this

juncture our results appear to support these initial assumptions. In fact, it has been reported that radiation enhances the antitumor therapeutic effects of DCs. Nikitina and Gabrilovich showed enhanced therapeutic results by treating DCs intravenously and subcutaneously after local external gamma-irradiation had been applied to tumors[43]. A substantial proportion of mice in the combined treatment group completely rejected their tumors (80% for MethA sarcoma and 40% for C3 tumor), but these effects were not observed in the other groups treated with individual modalities. IFN-γ production by splenocytes in response to tumor-specific peptides as determined by ELISPOT assay and tumor peptide-specific $CD8^+$ T cell counts, were significantly increased in the combined treatment group[43].

Teitz-Tennenbaum et al. also demonstrated that radiotherapy potentiates the therapeutic efficacy of intratumoral DC administration[44]. These workers used external local γ-irradiation followed by intratumoral DC injection, and in addition to a local tumor suppressive effect, found that systemic antitumor immunity could be induced by the adoptive transfer of splenocytes of treated mice to other mice with pulmonary metastasis. Moreover, these effects were not observed in mice treated with the individual modalities.

In terms of the underlying mechanisms which enhance the therapeutic results, γ-irradiation was proposed to increase apoptotic tumor cell numbers, which serve as an antigenic source for DC activation, and which in turn generate potent systemic antitumor immunity[43]. However, Teitz-

Tennenbaum et al. failed to find apoptotic or necrotic tumor cells after irradiation, and suggested that γ-irradiation inhibited tumor cell proliferation and modified them to become more immunogenic and thus enhanced DC immune response. Although the total irradiated dose were similar in both studies, Nikitina and Gabrilovich administered the total radiation dose over 2-3 weeks with 2-3 days intervals and observed apoptosis, whereas Teitz-Tennenbaum et al. administered similar dose for 5 consecutive days without inducing apoptosis or necrosis[44].

The biologic effects of radiation on skin are well-known. Primarily, radiation can induce DNA damage and programmed cell death (apoptosis), but it can also damage endothelial cells, and induce partial necrosis and inflammatory cell infiltration[33, 45]. However, it appears that therapeutic results and the immune responses generated are dependent on the radiation protocol used and the total radiation dose delivered, and thus the mechanisms of radiation-mediated immune modulation require clarification. Above two protocols and ours increased the efficacy of DC vaccines, although their effects on tumor cells were different, i.e., apoptosis, growth arrest, and central necrosis with peripheral growth arrest, respectively. Although the exact tumor antigens involved are unknown and the methods of antigen preparation have not been optimized, all three of these *in vivo* antigen preparation models are similar to physiologic reactions to tumors. Major research effort is required to progress this technology, and we are of the opinion that the mode of applying ionizing radiation can be further optimized in terms of maximizing tumor-

specific cellular immunity.

In the present study, we chose to administer a high-energy β-emitter intratumorally to generate antigens *in vivo*, because γ-irradiation applied by an external source damages normal peri-tumoral tissues[43, 44]. Thus we view the main advantage of Ho-166 as its local effect, because its maximum soft tissue penetration range is 8.7 mm (average 2.1 mm), and it can induce central necrosis, peripheral growth arrest, and theoretically cause the release of inflammatory mediators in a restricted area[42]. Ho-166 can also be used for deep-seated tumors without exposing normal skin and surrounding tissues to harmful radiation levels.

NK cell activity was also found to be elevated in our experimental model. Recently, experimental findings support the notion that NK cells are key players in tumor immunotherapy[46]. Although we performed no experiments after removing all $CD8^+$ cytotoxic T cells to examine the roles of NK cells in isolation, it is evident that NK cells participated in antitumor immune responses.

In summary, in this study, we used a modality based on Ho-166 for *in vivo* antigen preparation, which enhanced the efficiency of DC vaccine and generated a potent antitumor immune response. Our results show reduced tumor growth, increased survival, and the generation of tumor-specific immunity. These findings suggest that *in vivo* prepared tumor antigens can be used to efficiently generate effective antitumor immunity. Despite our lack of knowledge concerning the natures of the tumor antigens that evoked immune

responses, the described method of preparing tumor antigens could be used in patients in whom sufficient autologous tumor tissues are difficult to obtain, or be used as an alternative adoptive immunotherapy using antigen-loaded DCs.

V. CONCLUSION

In this study, we used Ho-166, high-energy β-emitter, as a method of in vivo antigen preparation, and let the immature DCs phagocytize the antigens and generate potent antitumor immune response.

1. **A combination of Ho-166 and immature DCs decreased tumor size and increased survival rates significantly.**
2. **Ho-166+DC treatment generated much higher T cell proliferation and IFN-γ release (ELISA and ELISPOT assay) in response to tumor cells.**
3. **CTL and NK cell activities were increased in Ho-166+DC-treatment mice.**
4. **Ho-166+DC treatment induced inflammatory cell infiltration into tumor and tumor cell necrosis.**
5. **Although Ho-166 treatment alone decreased tumor size and increased survival rates, it did not induce tumor-specific immune responses.**

Taken together, a combination of internal radiotherapy using Ho-166 and immature DCs could be used either to treat unresectable melanoma or as an adjuvant therapy after surgery.

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ABSTRACT (in Korean)

마우스 악성흑색종에서 Holmium-166 과 수지상세포를 이용한 병용 치료 효과

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이 태 형

베타선을 방출하는 Holmium (Ho)-166 의 종양내 주사 후 흑색종의 크기와 생장율은 감소하였으나 완전관해는 일어나지 않았다. 따라서 우리는 치료 효과를 증가시키기 위하여 미성숙 수지상세포를 이용한 면역치료를 병용하였다. 악성흑색종은 B16F10 세포주를 마우스의 피하에 주사하여 유발하였다. 악성흑색종이 유발된 54 마리의 마우스를 4 군으로 나누었다: 1) 치료하지 않은 군, 2) Ho-166 으로 치료한 군, 3) 미성숙 수지상세포로 치료한 군, 4) Ho-166 주사 후 미성숙 수지상세포로 치료한 군. Ho-166 또는 생리식염수 주사 19 일 후 4 군의 종양 크기는 각각 6044±3469mm^3, 1658±2092mm^3,

3871±2604mm^3, 그리고 444±729mm^3 이었다. Ho-166 과 수지상세포를 병용치료한 군에서 종양의 크기(p<0.05) 가 현저히 줄어들고 생존율이 증가함을 관찰하였다. 치료 후 각 군에서 생존한 마우스의 항종양 효과를 확인하기 위하여 악성흑색종 세포주를 다시 반대쪽 등 피하에 주사한 결과 Ho-166 과 수지상세포를 병용치료한 군이 다른 군에 비하여 종양의 생장 속도가 느림을 확인하였다(p<0.05). Ho-166 과 수지상세포를 병용치료한 군에서 종양특이적 세포용해성 T 림프구(CTL) 및 자연살해세포(NK cell) 활성화 그리고 염증세포의 종양 조직내 침윤이 관찰되었다. 또한 Ho-166 과 수지상세포를 병용치료한 군에서 악성흑색종에 대한 강한 면역반응이 유도됨을 T 세포 증식, ELISA 그리고 ELISPOT 분석을 통하여 확인하였다. 이러한 결과들을 토대로, 수술로 완전히 제거할 수 없거나 수술후의 보완치료로 Ho-166 의 종양내 주사와 미성숙 수지상세포를 이용한 병용치료가 흑색종의 치료에 이용될 수 있을 것으로 사료된다.

핵심되는말 : 수지상세포, 흑색종, 면역치료, 방사선치료, 홀미늄