Holmium complex를 이용한 치료 경험

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Experience with Holmium complexes

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Ho-166 is a beta-emitting radionuclide and physical characteristics are suitable for internal radiotherapy especially in malignant tumors. However, radiation biology of internal radiotherapy using beta-emitter has not been fully investigated. In this study, radiation response and radiobiology of internal therapy are investigated using radioresistant tumor, malignant melanoma.

The incidence of malignant melanoma has been increasing substantially over the past two decades (1). Excision of the primary lesion, lymph node metastases and distant metastases is the only therapeutic strategy that leads to long-term disease free survival. The data from the National Cancer Data Base indicate that additional adjuvant therapy has been applied in about 3 % of all cases because radiotherapy and/or chemotherapy were not proven to be highly successful and there was little evidence to suggest a benefit for any adjuvant therapy (2-4).

Regarding the use of external radiotherapy, melanoma has been considered as a radioresistant tumor because of having a large shoulder on their radiation survival curve (5-6), therefore, it had a limited role as a primary therapeutic modality. However, recent studies demonstrated that the radiation sensitivity of melanoma cells varies within the same individual, and varies between tumors (7). In addition, treatment method such as dose per fraction and total dose would affect on the radiosensitivity (8). The purpose of this study was to evaluate therapeutic effect of high dose internal radiotherapy as an alternative to external radiation by intra-tumoral injection of beta-emitting radionuclide, Ho-166, in an animal model with B16 melanoma and a patient with subcutaneous metastasis of malignant melanoma.

Beta rays have some advantages over gamma or X-rays since maximum soft tissue penetration range of beta rays is much shorter than gamma rays, therefore, higher radiation dose can be delivered to the tumor tissue without adverse radiation effect on the underlying normal tissue. Ho-166 is an ideal radionuclide for internal radiotherapy because it emits 94 % of 1.76 and 1.86 MeV beta rays and also emits small proportion of 81 KeV gamma rays suitable for gamma camera imaging. The physical half-life is 26.9 hour and maximum soft tissue penetration range is 8.7 mm (average 2.1 mm) (9).

In terms of radiation biology, previous studies

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have demonstrated that growth arrest and apoptosis induced by activation of p53 and downstream effector genes play an important role in radiation induced cell death mechanisms (10-12), however, tumor responses to high dose continuous irradiation using beta-emitting radionuclides have not been extensively evaluated. In this study, morphological and biological effects of this method were evaluated by analysis of p53 tumor suppressor and p53 downstream effector genes using immunohistochemistry, TUNEL staining, flow cytometry and RT-PCR whether these genes and related protein products play an important role in cell death mechanisms.

Materials and Methods

1. Animal experiments

B16 melanoma cells cells were cultured at 37℃ in RPMI 1640 media (Gibco laboratories, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (Gibco laboratories, NY) 2 mL L-glutamine (Gibco laboratories, NY), 100 IU/ml penicilline and 100 g/ml streptomycin (Gibco laboratories, NY). 5×10^5 cells suspended in 100 ㎕ of normal saline were inoculated subcutaneously into the back of 8- week-old C57BL/6 mice and the mice were grown in a specific pathogen free condition with standard diet and water ad libitum. The length of the short and long axes of the tumors was measured with a caliper (Mitutoyo, Japan) at 2 days interval until all normal control and 50 % of experimental group died. The tumor volume was calculated by the formula: $V= 1/2\times A^2\times B$ where A is the length of the long axis and B is the length of the short axis. When tumors were grown to be approximately 1 cm of their long axes, tumor volume was approximately $500mm^3$. Total 92 mice were divided into 4 groups and 0.3 ml normal (group 1, n=25), 1 mCi of carrier free Ho-166 (group 2, n=15), 5 mCi (group 3, n=27) in 0.3 ml of saline or 5 mCi in 0.5 ml of saline (group 4, n=25) were injected intra-tumorally to evaluated survival rate according to the injection volume and radioactivity. The radionuclide, Ho-166, was prepared by bombarding Ho-165 in a nuclear reactor (HANARO center, Taejon, Korea). The amount of the radioactivity to be injected was determined by computer simulation using the formula provided by Prestwich et al (15): Ho-166 injected into the tumor is assumed to be distributed uniformly inside the 1 cm diameter tumor, radiation dose to the surrounding normal tissue 1,2,3 and 4mm apart from the tumor surface is approximately 120, 36, 10 and 3 Gy/mCi respectively. The survival rate of the mice after injection of the radionuclide was evaluated until mice died.

Additionally, 30 mice were prepared with the same method as previously described for morphological and biological analysis of the radiation effect. Five mice each were sacrificed by cervical dislocation before injection, 1st, 2nd, 3rd, 6th and 14th days post injection of 5 mCi of Ho-166 in 0.3 ml of saline. Gamma camera images were obtained with dual-headed gamma camera (ADAC Laboratory, Milpitas, USA) before sacrifice, which showed sustained retention of injected radionuclide without leakage. After gamma camera imaging, the tumor tissues were removed and sent for flow cytometry (n=2 each) or fixed in 10 % formaldehyde and embedded in paraffin for hematoxylin-eosin (H&E) staining, immunohistochemical analysis, the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining and reverse transcription-polymerase chain reaction (RT-PCR). Macro-autoradiography was

performed to evaluate the distribution pattern of the injected Ho-166, which showed relatively even distribution of the injected radionuclide inside the tumor, however, peripheral margin was devoid of the radioactivity.

For visual side-by-side comparison, melanoma cells were inoculated into the bilateral back of 10 additional mice, and 5 mCi of Ho-166 in 0.3 ml of saline was injected in one side and normal saline in the other side of the tumors. Histological evaluation with H&E staining were performed 2 weeks after injection. This study was undertaken based on the Guidelines and Regulations for the Use and Care of Animals in our institute.

2. Immunohistochemistry

Hematoxylin-eosin staining of 5-m thick sections was performed and then sections were deparaffinized and rehydrated for immunohistochemistry. Antibodies used for immunohistochemistry were polyclonal anti-p53 (Santa Cruz, Santa Cruz, CA), monoclonal anti-p21 (BD PharMingen, San Diego, CA), polyclonal anti-Bax (BD PharMingen, San Diego, CA), monoclonal anti-cyclin D1 (BD PharMingen, San Diego, CA), and monoclonal anti- proliferating cell nuclear antigen (PCNA) (Dako,Glostrup, Denmark) antibodies. Dilution factors of the antibodies were 1:20, 1:50, 1:500, 1:50 and 1:100, respectively. Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 10 min. Antigen retrieval was performed by microwave pretreatment using 0.01M citric acid buffer (pH 6.0) in a pressure cooker for 15 minutes. After cooling, immunostaining was performed by an avidin-biotin-peroxidase complex method using a commercially available kit (Vector® M.O.M.™ Immunodetection Kit, Vector Laboratories, Burlingame, CA) for monoclonal antibodies and by an EnVisionTM kit (Dako) for polyclonal antibodies according to the manufacturers instructions. After blocking procedures, sections were incubated at 4℃ overnight with either mouse monoclonal or rabbit polyclonal antibodies. Diaminobezidine was used as a chromogen except for the BAX immunostaining for which 3-amino-9-ethylcarbazole was used as a chromogen. The nuclei were lightly counterstained with hematoxylin.

3. TUNEL stain

Apoptosis was assessed by the TUNEL method using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Intergen, Purchase, NY, USA) according to the manufacturers instructions with some modifications. Sections were deparaffinized in xylene and rehydrated in a graded concentration of ethanol and then in distilled water. They were then treated with proteinase K (20μg/ml) for 15 minutes at 37℃ and washed in distilled water. After applying equilibrium buffer, the sections were incubated with digoxigenin-dNTP and TdT at 37℃ for 60 minutes. After being washed and endogenous alkaline phosphatase was inhibited, sections were incubated with alkaline phosphatase conjugated rabbit F(ab) anti-digoxigenin (Dako, Glostrup, Denmark) at 1:20 for 30 minutes at room temperature. They were washed, and the signal was detected by incubation with 5-bromo-4-chloro-3-indolyl-phosphatase/Nitroblue tetrazolium for 20 minutes before being counterstained nuclear fast red.

4. Flow cytometry

Flow cytometric analysis using annexin V

B16 melanoma cells were collected by centifugation at 1,000×g for 10 minutes. Then the cells were washed once in cold PBS buffer. The sediment of the B16 melanoma cells was

resuspended with 100μl of optimal concentration of annexin V-FITC and propidium iodide (Trevigen, Inc. Gaithersburg, MD, USA) and incubated in the dark for 15 minutes at room temperature. Then the cells were analyzed with FACScan (Becton Dickinson, Mountain View, CA, USA)

DNA labelling with propidium iodide (PI) and flow cytometric analysis

The 200×g centrifuged cell pellet(2×10^6 B16 melanoma cells) was fixed in 2 ml cold 70% ethanol at 4 C for 30-60 min. The cells were then centrifuged, washed in 1 ml PBS and resuspended in 1 ml of staining solution including 50 μg/ml of PI and 50μg/ml of RNAse.

The mixed cells were incubated in the dark at room temperature for 60 min.

Then the cells were analyzed with FACScan (Becton Dickinson, Mountain View, CA, USA).

5. RT-PCR

Tumor tissues within the peripheral margin containing viable cells were removed from the previously prepared paraffin blocks and total RNA isolation was performed according to the manufacturer's instructions using paraffin block RNA lsolation kit (Ambion Inc., Austin, Texas, USA). To synthesize cDNA, 3μg of total RNA was mixed with 100ng random hexamer (Phamacia, Uppsala, Sweden), boiled at 70℃ for 10min, and then quickly chilled on ice. Three μl of 5X first strand buffer, 1.5μl of 0.1M DTT, 2 μl of 10mM dNTPs and 200units murine molony leukemia virus-reverse transcriptase (MMLV-RT) (Gibco BRL, Grand Island, NY, USA) were added into the reaction mixture and incubated at 42℃ for 2hrs. The reaction mixture was boiled at 95℃ for 5min, quickly chilled on ice, and added 20 μl distilled water. To determine that the equal amount of RNA was used, the expression level of beta-actin mRNA was also examined. PCR reaction was performed in 50 μl vol containing 4 μl cDNA, 4 μl of 2.5 mM dNTPs, 1 μl for 10 pmol each up-stream primers, 1 μl of 10 pmol each down-stream primers, 5 μl of 10X PCR buffer, 30.5 μl distilled water and 2.5units of Taq polymerase (Solgent, Korea). After initial 3 min incubation at 94℃, PCR reaction was performed by the following conditions: denaturation at 94℃ for 1min, annealing at 60℃ (p21; 62°C) for 1min, and polymerization at 72℃ for 1min for 40cycles. The primers used were:

mouse p53 forward:
5-CAAGTGAAGCCCTCCGAGTG-3,
reverse :
5-GGCAGCGTCTCACGACCTCC-3,
bax forward:
5-CAGCTCTGAGCAGATCATGAAGACA-3,
reverse :
5-GCCCATCTTCTTCCAGATGGTGAGC-3,
p21 forward:
5-AAGACCATGTGGACCTGTCA-3,
reverse :
5-GGCTTCCTCTTGGAGAAGAT-3,
beta-actin forward :
5-CGTGGGCCGCCCTAGGCACCA-3,
reverse:
5-TTGGCCTTAGGGTTAGGGGGG 3.

Results

Tumor volumes and survival rates following intra-tumoral injection of Ho-166.

Nine to ten days after inoculation of melanoma cells, mean tumor volume reached 492.5± - 631.9 ± mm^3. Control group showed rapid growing of tumor and the mean tumor volume reached

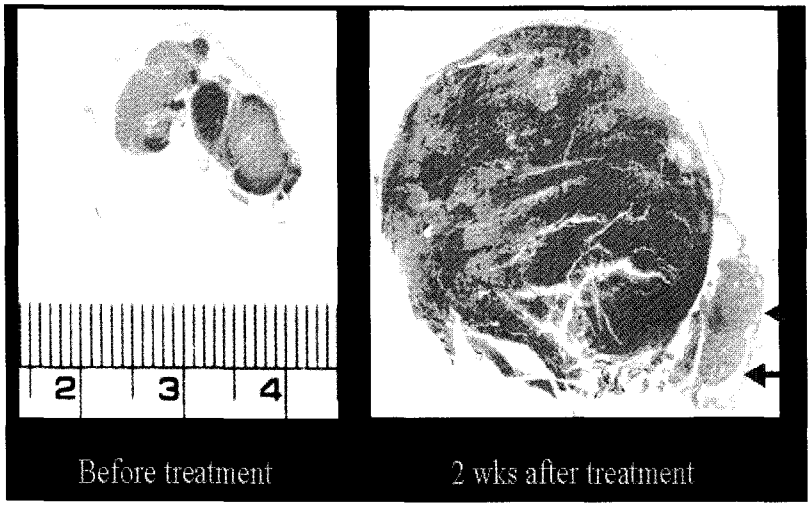

Fig 1. Ho-166 was injected in left side tumor. Two weeks after injection, left tumor has been decreased in size (arrows) with necrosis, but right side tumor grew rapidly.

approximately 30 times of original volume and none of control group was alive until 16th day post injection of normal saline. Morphologically, the tumors of control group were initially round shape with pseudocapsule but progressively enlarged with lobulated contour. The median survival of the control group was 9 days after injection. On the other hand, mean tumor volumes of the treated groups showed gradual decrease and 66.7~74.1 % of treated subjects were alive when all control subjects died. Histological examination demonstrated wide central necrosis that was matched with the area of Ho-166 deposition. Viable tumor cells distributed along the peripheral margin, however, those cells became progressively swollen and vacuolization and giant formation with increased melanin pigmentation were seen without apoptotic features such as cellular shrinkage with nuclear condensation and fragmentation. The median survival was 29 days in group 2, 33 days in group 3 and 33 days in group 4. The median survival of treated group was longer than control group and survival rate was also higher in treated group ($p<0.001$). The survival rate of group 3 was higher than that of group 2 and 4 but there was no statistical significance. Skin ulceration was developed in 4 (group 4) and 1 (group 3) mice.

Gross and histological evaluation with H&E staining 2 weeks after treatment in mice with melanomas in bilateral back showed large tumor mass in non-treated side but necrosis with growth delay in treated side (Fig 1).

Expression of p53, p21, bax, PCNA and cyclin-D1 proteins following intra-tumoral injection of Ho-166.

Control group showed strong positive reaction of PCNA and cyclin D1 within the nuclei of viable cells, however, expression of p53, p21 and bax protein was not observed . Twenty-four hours after treatment, cellular swelling and nuclear enlargement occurred. PCNA was positive as strong as in control group but cyclin D1 was markedly reduced. p53 was strongly positive in

most viable cells, a positive reaction of p21 could be seen but not as abundant as p53 ,and faint positive reaction of bax within cytoplasm occurred. Two days after treatment, swelling of cytoplasm and enlargement of nuclei were more prominent. Expression of PCNA and p53 persisted without remarkable change but cyclin D1 was not detected and the expression of p21 and bax increased. Three days after, PCNA was persistently positive but p53 was almost negative and the reaction of p21was slightly diminished. Cyclin D1 reaction reappeared and bax expression showed persistently strong positive. Until 2 weeks after treatment, expression of PCNA, cyclin D1, p21 and bax was seen although the intensity of positive reaction was decreased. Cytoplasmic swelling, vacuolization, enlargement of nuclei and disruption of cell membrane progressed .

Analysis of p53, p21 and bax mRNA with RT-PCR.

Expression pattern of mRNAs of p53, p21 and bax was closely correlated with that of immunohistochemistry . Expression of bax mRNA was increased until 2 weeks after treatment, but p53 and p21 were increased until 72 hours after treatment and then decreased

TUNEL stain and flow cytometry for the evaluation of apoptosis

TUNEL positive nuclei could be seen from 2 days until 2 weeks after treatment despite morphological features of the positive cells were compatible with radiation induced necrosis. Flow cytometry also did not demonstrate any apoptotic features compared to the control.

Discussion

Despite the high incidence of distant metastasis, locoregional control remains an important goal in the management of melanoma. Traditionally conventional external radiation therapy had a limited role in the local control. High-dose-per-fraction (HDPF) strategy has been proposed to overcome the reparative capacity of the tumor cells with sublethal damage (13-14) and a recent study demonstrated the adjuvant postoperative HDPF regimen was effective in reducing local recurrence in patients at high risk of locoregional failure (15), nevertheless, HDPF scheme is considered to be only as good as conventional fractionated regimens at the present time and inherent variability in the radiation sensitivity of malignant melanoma is the dominant factor in determining radiation response until recently (16).

In the radiation biological aspect, delivering very intense large dose confined to the tumor tissue can be more effective than conventional or HDPF unless underling or overlying normal structures are spared. Indeed, stereotactic radiosurgery of cerebral metastatic melanoma with 166cGy in a single session in addition to the whole-brain irradiation showed a marked improvement of neurological deficit (17), which suggests total dose (very-intense-dose) is also an important determinant in the radiation sensitivity of melanoma. However, conventional external radiotherapy has an inherent limitation to treat with an extremely large dose due to its long penetration ranges of high-voltage X-rays or high-energy gamma rays, which lead to inevitable radiation hazard to the normal tissues. On the other hand, penetration ranges of the high-energy α -rays or α -particles are much shorter than X or gamma rays, therefore, a much greater fraction of energy is deposited into the tumor cells without damage to the surrounding normal tissues if large amount of radiotracers can be specifically delivered to the tumors. For an effective targeted

therapy, various types of radioimmunoconjugates and methods were developed using anti-melanoma monoclonal antibodies and boron compound labeled with high-energy β or α emitting radionuclides (18-21). Radionuclide has also been attempted for targeted gene therapy using sodium/iodide symporter gene and radioiodine in melnoma and hepatoma (22-23). These targeted therapy methods were proposed to deliver a very intense large tumor radiation dose based on the physical characteristics of β or α rays. Although the results of the radioimmunotherapy or boron captured neutron therapy appeared to be promising but survival benefit is still under investigation.

In this study, direct intra-tumoral injection of high-energy β -emitting radionuclide, Ho-166, was attempted in animal model. With this method, radiation dose to the central portion of tumor tissue calculated by computer simulation was approximately 800 Gy/mCi and doses to surrounding normal tissue 1,2,3 and 4mm apart from the tumor surface were approximately 120, 36, 10 and 3 Gy/mCi respectively, however, actual tumor dose was more than 800 Gy/mCi since administered radionuclide was not homogenously distributed but peripheral rim was devoid of radionuclide as seen on autoradiography. Despite the intense radiation dose, surrounding normal tissue injury was minimal and central tumor necrosis with prolonged survival and growth arrest was observed.

Pathological and molecular analysis demonstrated high dose internal radiotherapy produces tumor necrosis and growth arrest of tumor cells but apoptosis plays a minimal role, which is different from that of external radiotherapy.

Future Prospective

Ho-166 has many physical characteristics for internal radiotherapy, therefore, it can be used in local control of malignant tumors such as hepatoma, skin cancers, recurrent breast cancer, prostate cancer.

In our hospital, Ho-166 is incorporated in polyurethane membrane and covered over a metallic stent and animal experiments are being conducted for palliative treatment of malignant esophageal stricture and biliary obstruction.

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