Anti-tumor and anti-metastatic effects of intralesional recombinant interferon-alpha and -beta on human malignant melanoma xenografts in nude mice

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Directed by Professor Kee Yang Chung

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ABSTRACT

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Interferon (IFN)-alpha (α) is the most commonly used biologically active cytokine in the treatment for high risk patients with melanoma. Although IFN-α has shown to be clinically effective in the adjuvant setting of high risk for melanoma relapse, the reasons are unknown yet. Malignant melanomas of the skin primarily metastasize to lymph nodes, and the detection of sentinel lymph node metastases serves as an important prognostic marker. Recent evidences strongly revealed that tumor-induced lymphangiogenesis plays an important and active role in the promotion of cancer metastasis to lymph nodes.

The purpose of this study was to evaluate and compare the efficacy of IFN-α2b and IFN-β1a on primary tumor growth and lymph node metastasis and investigate the mechanisms regarding lymph node metastasis by using human melanoma xenograft model. Both IFN-α2b and IFN-β1a showed inhibitory effect on tumor cell proliferation and induced apoptosis. IFN-β1a showed

significantly potent anti-proliferative and apoptotic effect compared to IFN-α2b in xenograft tumors (p<0.05). Both IFN-α2b and IFN-β1a were effective in inhibiting lymph node metastasis compared to the control. Control group showed lymph node metastasis in 5 out of 6 mice compared to 3 out of 6 in IFN-β1a group and 1 out of 6 in IFN-α2b group. Microvessel density decreased in tumors treated with IFN-α2b and IFN-β1a compared to the control, but it was not statistically significant. As for lymphatic vessel density, it significantly decreased only in tumors treated with IFN-α2b (p<0.05). Both IFN-α2b and IFN-β1a decreased *in vitro* and *in vivo* VEGF-C and VEGFR-3 protein expression and secretory VEGF-C level *in vitro*. IFN-α2b showed earlier and sustained effect in decreasing VEGF-C and VEGFR-3 protein expression and superior effect in decreasing secretory VEGF-C level compared to IFN-β1a. In conclusion, IFN-α2b and IFN-β1a both showed anti-tumor and anti-metastatic effect in human melanoma xenograft through different action mechanisms. IFN-β1a showed stronger anti-proliferative and pro-apoptotic effect while IFN-α2b showed stronger anti-metastatic effect through inhibition of lymphangiogenesis.

Key words: malignant melanoma xenograft model, interferon-α2b, interferon-β1a, anti-tumor effect, anti-metastatic effect

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I. INTRODUCTION

Malignant melanoma is the leading cause of death among skin cancers. The incidence of cutaneous melanoma has increased more than tenfold over the last fifty years and its incidence is increasing more rapidly than any other malignancies in Caucasian population.[1]

Appropriate surgical management remains the mainstay of therapy for patients with early-stage, nonmetastatic disease. However, effective therapies for both adjuvant therapy and patients with metastasis are required since the mortality rates rises in advanced stages. Patients with American Joint Committee on Cancer (AJCC) stage IIB-IIC disease or stage III disease have an adverse prognosis, with reported 5-year survival of 24-67%.[2] These patients are regarded as intermediate risk group for metastasis. Therefore, there has been great interest in the use of adjuvant therapies such as immunotherapy, vaccine therapy and chemotherapy in patients with risk of tumor recurrence.

However, at present, there is no well-confirmed therapy that prolongs overall survival in advanced staged melanoma. The only FDA approved substance for this purpose in stage II and III melanoma is IFN-α. However, various trials showed an improved relapse free survival but did not show a benefit in overall survival with high-dose and intermediate-dose IFN-α2b regimen.[3-4] Although IFN-α2b has shown to be clinically effective in the adjuvant setting of high risk for melanoma relapse, the reasons are unknown yet.

Malignant melanomas of the skin primarily metastasize to lymph nodes, and the detection of sentinel lymph node metastases serves as an important prognostic marker. Recent evidences strongly revealed that tumor-induced lymphangiogenesis plays an important and active role in the promotion of cancer metastasis to lymph nodes in various tumors.[5] Although IFNs have shown to have inhibitory effect on tumor angiogenesis,[6-7] little is known about theirs effect in tumor-associated lymphangiogenesis in malignant melanoma.

By using human melanoma xenograft model, we sought to evaluate and compare the therapeutic efficacy of IFN-α2b and IFN-β1a on primary tumor growth and lymph node metastasis and investigate the mechanisms regarding lymph node metastasis.

II. MATERIALS AND METHODS

1. Cell culture, antibodies and reagents

The human melanoma cell lines SK-MEL-2 and SK-MEL-24 were supplied by the ATCC (Rockville, MD, USA). All cell lines were maintained in microvascular endothelial cell (MEM) medium supplemented with 10% FBS, 100 U/ml ampicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Gibco BRL, Gaithersburg, MD, USA) in a 5% CO_2 humidified atmosphere at 37℃. For vascular endothelial cells, human dermal microvascular endothelial cell (HDMEC) (Cambrex, Walkersville, MA, USA) was purchased and used. HDMEC was prepared and treated in a tissue incubator with 0.1% gelatin, using MEM-2 (Cambrex, Walkersville, MA, USA) containing human epidermal growth factor, hydrocortisone acetate, vascular endothelial growth factor (VEGF, human basic fibroblast growth factor (bFGF), gentamycin, amphotericin B, 5% FBS, R^3-insulin-like growth factor-1, and ascorbic acid. They were subcultured serially from passage 5 to passage 25 at 37℃ in CO_2 incubator.

2. Recombinant human interferon-α and -β

Recombinant human interferon-alpha2b (IFN-α2b) was kindly supplied by Donga Research Laboratories, Seoul, Korea. This IFN was 96.21% pure and its concentration was 0.729 mg/mL. Recombinant human interferon-beta1a (IFN-β1a) was kindly supplied by Merck Serono Research Laboratories,

Geneva, Switzerland. This IFN was 99% pure and its specific activity was 2 $x10^8$ units/mg protein. For mouse experiments, IFNs were diluted in 0.9% normal saline for final concentration of $2x10^5$ IU and intralesional injections were performed in cycles of 5 consecutive days per week for 4 weeks.

3. Analysis of cell viability

For cell viability assay using IFN-α2b and IFN-β1a, cells were plated in six-well culture plates and allowed to adhere overnight. IFN-α2b and IFN-β1a were then added the following day at the indicated concentrations. The viability of the cells was determined 72 hr after drug addition by trypan blue exclusion assay at least in duplicates as described previously.[8]

4. Invasion assay using transwell filters

A modified version of the standard transwell filter assay for invasion was conducted. Transwell filters (diameter, 6.5 mm; pore size, 8 μm; BD Biosciences, Bedford, MA, USA) were coated on the lower side with 8 μg/μL Matrigel and placed on a 24-well plate containing medium supplemented with 0.1% bovine serum albumin. Cells were harvested with a cell dissociation solution and suspended in medium with 1% bovine serum albumin. Cells (1×10^5) were added to the upper compartment of a transwell chamber and allowed to migrate for 24 hr at 37℃. For protocols involving drug treatment, IFN-α2b and IFN-β1a were added to the upper compartment. After 48 hr,

nonmigrated cells on the upper side of membrane were removed with a cotton swab, and migrated cells on the bottom surface of the membrane were fixed in 3.7% paraformaldehyde in PBS and stained with crystal violet for 10 min at room temperature (RT). Cell migration was quantified by counting the number of cells in three inserts. The data are expressed as the average number of cells per insert.

5. Tube formation assay

24-well plates were pre-coated with 1:1 mixture of cold Matrigel Basement Membrane (10 mg/ml, BD Biosciences, Bedford, MA, USA) and EGM-2 medium. After 45 min of polymerization at 37˚C, HDMEC grown for 24 hr in EGM-2 were plated at 10^5 cells/well in EGM-2 supplemented or not with IFN-α2b or IFN-β1a at 10^2 IU/mL and 10^4 IU/mL. After 6 and 24 hr, pictures of 5 representative fields were taken for each condition under an inverted microscope at x200 magnification.

6. Western blot analysis of VEGF-C and vascular endothelial growth factor receptor-3 (VEGFR-3)

A total of $5x10^5$ cells of the SK-MEL-24 cell line were plated into on each 100 mm culture dish. After 24 hr, IFN-α2b and IFN-β1a were treated and proteins were extracted as time dependent. The media was discarded and the cells were

washed twice with PBS. Then, 150 µl of lysis buffer (50 mM Tris-Cl (pH 7.4), 150 mM EDTA, 1 mM PMSF) was added. Cells were detached by scrapping, incubated on ice for 30 min, and centrifugation was done at 13,000 rpm for 20 min. Whole cell lysates were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blotting was performed by standard methods using antibodies to VEGF-C (Abcam, Cambridge, UK, 1:1,000), VEGF-R3 (Abcam, Cambridge, UK, 1:250), and GAPDH (Abcam, Cambridge, UK). Detection of proteins was done using peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies and visualized with ECL kit (PerkinElmer, Waltham, MA, USA).

7. Assessment of VEGF-C secretion by SK-MEL-24 cells *in vitro*

SK-MEL-24 cells were suspended in 10 mL of media with or without IFN-α2b and IFN-β1a and incubated in 100 mm culture dishes. Media concentration was performed using centriprep centrifugal filter devices (Millipore, County cork, Ireland). Basal levels of VEGF-C in cell culture supernatants were measured using VEGF-C ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations.[9] The VEGF-C ELISA detects all human VEGF-C forms containing the VEGF homology domain, including full-length VEGF-C and the proteolytically processed mature form.

8. Human melanoma xenograft model

Female, 6- to 8-week-old athymic nude (*nu/nu*) mice were housed in laminar-flow cabinets under specific-pathogen-free conditions. The present study was performed under protocols approved by the Animal Care and Use Committee of the College of Medicine of Yonsei University. Our animal research program is accredited by AAALAC International. Murine model of sentinel lymph node metastasis based on injection of human melanoma cells into the preauricular area of athymic nude mice was made as described previously.[10] Briefly, 1 x 10^7 of SK-MEL-24 human melanoma cells were intradermally injected into the right preauricular area of nude mice. Human melanoma xenograft mice were randomized into three treatment groups (*N*=6 per group) when tumor volume measured between 200~300 mm^3. Group 1 was treated with IFN-α2b (2x10^5 IU), group 2 with IFN-β1a (2x10^5 IU) and group 3 was treated with normal saline as control. Intralesional injections in the tumor and peritumoral area were performed 5 consecutive days per week for 4 weeks. Tumor sizes were determined every 3 days blinded to the treatment conditions by measuring the length and width of the nodules with a caliper and volumes were calculated as previously described.[11] After 4 weeks of treatment, mice were sacrificed and the primary tumor and lymph nodes were removed.

Preceding experiments (data not shown) were the basis for this protocol; in untreated animals, lymph node metastasis was found when the tumor volume was larger than 300 mm^3. Waiting for longer period of time resulted in systemic

metastasis to lung and gastrointestinal tracts, which was difficult to quantify. Therefore, in our experiment, treatment was initiated at day 54 after inoculation.

9. MicroPET imaging

Three mice (one mouse per group) were randomly selected for microPET imaging before and after 4 weeks of treatment. Mice were injected with 7.4 MBq ^{18}F-FDG. Mice were fasted overnight before each ^{18}F-FDG scan.[12] One hour after tracer injection mice were anesthetized with 3% sevofluran (Abbott Scandinavia AB, Solna, Sweden) mixed with 35% O_2 in N_2. A 20 min PET scan was acquired using a MicroPET Focus 120 (Siemens Medical Solutions, Malvern, PA, USA). After data acquisition, PET data were arranged into sinograms and subsequently reconstructed with the maximum a posteriori (MAP) reconstruction algorithm. The pixel size was 0.866 x 0.866 x 0.796 mm and in the center field of view the resolution was 1.4 mm full-width-at-half-maximum. MicroPET images were fused in the Inveon software (Siemens Medical Solutions). Region of interests (ROIs) were drawn manually by qualitative assessment covering the whole tumors and standardized uptake value (SUV) mean and maximum were generated.

10. Immunohistochemistry

Formalin-fixed, paraffin-embedded 4μm tissue sections were deparaffinized with xylene, hydrated with graded ethanol. Endogenous peroxidase activity

were blocked with endogenous enzyme block solution (DAKO, Carpinteria, CA, USA), antigen retrieval was then performed by pressure-cooking for 2 min at full pressure in antigen retrieval buffer (DAKO, Carpinteria, CA, USA).

1) Detection of micrometastasis in lymph nodes

Anti-S100 (rabbit polyclonal anti-S100, DAKO, Carpinteria, CA, USA) antibody was used to detect micrometastasis of human melanoma cells in lymph nodes. Detection of S100 protein expression was achieved with Real™ Envision™ HRP Rabbit/Mouse (DAKO, Carpinteria, CA, USA) detection system. Antibody was applied for 30 min at RT. Then, the slides were covered with HRP conjugated rabbit/mouse link for 30 min at RT and developed with 3, 3'-diaminobenzidine (DAB) working solution. Counter staining was also performed as before.

2) TUNEL assay

The apoptotic cells were detected with TUNEL assay using DermaTACS *in situ* apoptosis detection kit (Trevigen, Inc., Gaithersburg, USA), according to the specifications and instructions recommended by the manufacturer. Briefly, after being washed in PBS, sections were incubated with proteinase K solution for 15 min and immerse slides in 3% H_2O_2 in methanol for 5 min. For detecting apoptotic DNA fragments in cuff-induced vascular remodeling cells, 50 µl of TUNEL reaction mixture (biotinylated TdT dNTP mix , 50×Mn^{2+}, TDT enzyme, 1×TDT labeling buffer) was added to each of them and incubate for 1 hr at 37°C. Then cover sample with 50 µL of Strep-HRP Solution (dilute Strep-HPP

in Blue Streptavidin-diluent, 1:800) for 10 min at RT. Finally, visualized by Blue Label Solution for 10 min and counterstained with Red Counterstain C.

3) Staining for endothelial CD34 and LYVE-1

Blood vessel endothelial cells were highlighted with CD34 (rat polyclonal anti-human CD34, 1:50, Abcam, Cambridge, UK) antibodies and lymphatic endothelial cells were highlighted with lymphatic vessel hyaluronan receptor-1(LYVE-1) (rat polyclonal anti-human LYVE-1, 1:40, Abcam, Cambridge, UK). For immunostaining, slides were then incubated with CD34 and LYVE-1 antibodies for 1 hr at RT. After incubation with secondary antibody (Real^{TM} Envision^{TM} HRP Rabbit/Mouse, DAKO, Carpinteria, CA, USA) for 30 min at RT, the slides were developed with 3, 3'-DAB chromogen and counterstained with hematoxylin.

4) Immunostaining for VEGF-C, VEGFR-3 and Ki-67

VEGF-C (goat polyclonal anti-human VEGF-C, 1:40, Abcam, Cambridge, UK) antibody was used to determine the expression levels of angiogenic factor protein in human melanoma cells. Expression of angiogenic factor receptor was detected by VEGFR-3 antibody (rabbit polyclonal anti-human VEGFR-3, 1:500, Abcam, Cambridge, UK). Proliferation index was determined by the expression of Ki-67 (anti-Ki-67, 1:100, DAKO, Carpinteria, CA, USA). Immunohistochemical staining for VEGF-C was performed by the labeled streptavidin-biotin (LSAB) method using the LSAB kit (DAKO, Carpinteria,

CA, USA). Slides were incubated for 1 hr at RT with VEGF-C antibody and then were sequentially incubated with biotinylated secondary antibody and peroxidase labeled streptavidin for 30 min. After color development with DAB as chromogen, hematoxylin counterstaining was performed. Detection of VEGFR-3 and Ki-67 expression was achieved with Real™ Envision™ HRP Rabbit/Mouse detection system. Antibody was applied for 30 min at RT. Then, the slides were covered with HRP conjugated rabbit/mouse link for 30 min at RT and developed with DAB working solution. Counter staining was also performed as before. Interpretation of VEGF-C and VEGFR-3 protein expression was performed using weighted histoscore method as follows.[13] The tumor cell intensity was scored as 0 (negative), 1 (light brown), 2 (brown), 3 (dark brown). The final score was assessed as below: Score= (0 x percentage of negative cells)+(1 x percentage of light brown-staining cells)+(2 x percentage of brown staining cells)+(3 x percentage of dark brown-staining cells). Then, the final score was divided into 4 groups: negative (score 0), weak positive (score 1-100), moderately positive (score 101-200) and strong positive (score 201-300). The proportion of cells immunopositive for Ki-67 and TUNEL assay was determined by counting the number of positive cells out of 100 cells in three different areas of each tissue section.

5) Quantitative analysis of microvessel and lymphatic vessel density

Blood or lymphatic vessel counts were performed in tissue sections stained by

CD34 or LYVE-1 antibodies. The tissue sections were scanned at low power (x40) to identify the areas with highest microvessel or lymphatic vessel density (hot spots). After the three hot-spots were identified, vessel count was performed on a x400 field. The mean of the vessel counts in three hot-spots of the sections was considered as microvessel density (MVD) or lymphatic vessel density (LVD). Each count of hot-spot was expressed as the mean count of three x400 fields within the hot spot. According to Weidner et al.[14], any positive stained endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels, tumor cells and other connective tissue elements was considered a single, countable microvessel.

11. Statistical analysis

All analyses were performed using SPSS 18 statistical package (SPSS, Inc., Chicago, IL). Quantitative data are represented as the mean of at least three independent experiments. Comparison among the three groups was done by variance analysis (ANOVA for repeated measurements) for results of transwell invasion assay, the overall growth of melanoma and immumohistochemical staining results. Statistical results are presented as mean ± standard deviation (SD) or standard error of the mean (SEM) where appropriate. Differences were regarded as significant when the *p* value was less than 0.05.

III. RESULTS

1. IFN-α2b and IFN-β1a inhibit proliferation of melanoma cells

To investigate whether IFN-α2b and IFN-β1a have an anti-proliferative effect, melanoma cell lines SK-MEL-2 and SK-MEL-24 were treated with various doses of IFN-α2b and IFN-β1a from 1 IU/mL to 10^4 IU/mL. The proliferation assay showed that proliferation was inhibited in SK-MEL-2 (Figure 1A) and SK-MEL-24 cell lines (Figure 1B) in a dose-dependent fashion. IFN-β1a showed superior inhibitory effect in cell proliferation compared to IFN-α2b in both cell lines (p<0.05).

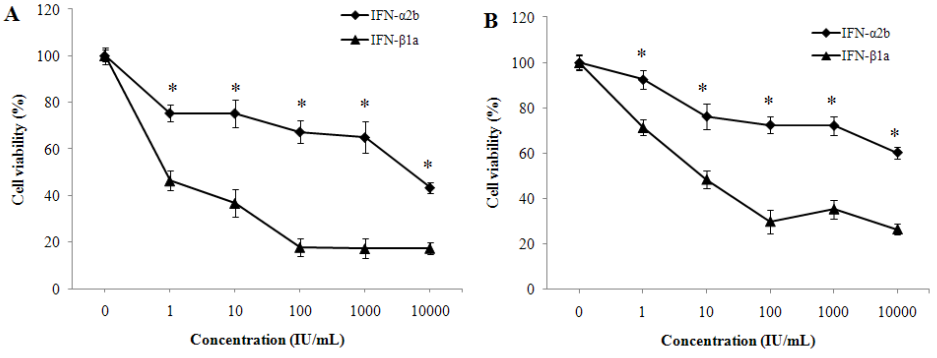

Figure 1. Dose-dependent inhibition of melanoma cell proliferation by IFN-α2b and IFN-β1a in A) SK-MEL-2 and B) SK-MEL-24 melanoma cells. Data are expressed as means ± SD of three such experiments performed in duplicate. Statistical significance was set as p<0.05(*).

2. IFN-α2b and IFN-β1a inhibit melanoma cell invasion

Since the ability of tumor cells to invade tissues is essential to the metastatic process, the effects of IFN-α2b and IFN-β1a on invasion were also examined. Similar to the results in melanoma proliferation assays, both IFN-α2b and IFN-β1a inhibited melanoma cell invasion into Matrigel compared to control in both SK-MEL-2 (Figure 2A) and SK-MEL-24 (Figure 2B) cell lines. However, there was no significant difference between IFN-α2b and IFN-β1a in ability to inhibit melanoma cell invasion in both cell lines. Higher dose (10^4 IU/mL) of IFN-α2b and IFN-β1a significantly inhibited melanoma cell invasion compared to control in both cell lines ($p< 0.05$).

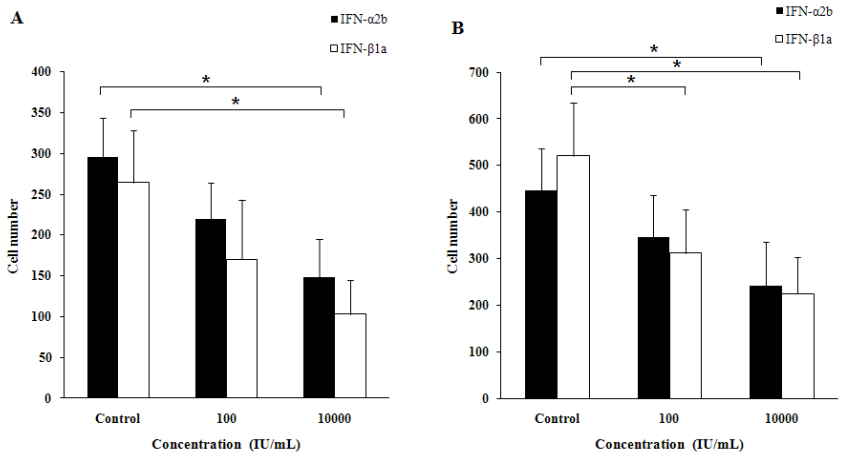

Figure 2. Inhibition of melanoma cell invasion into Matrigel. A) SK-MEL-2 B) SK-MEL-24 melanoma cells were suspended in serum-free medium with or without the indicated concentration of 10^2 and 10^4 IU/mL. Data are expressed as means ± SD of three such experiments performed in duplicate. Statistical significance was set as $p<0.05$(*).

3. IFN-α2b and IFN-β1a inhibit endothelial cell tube formation

To determine whether IFN-α2b and IFN-β1a could affect endothelial cell tube formation, HDMECs were cultured on Matrigel-coated tissue culture plates and then incubated with IFN-α2b and IFN-β1a in doses of 10^2 IU/mL and 10^4 IU/mL. Tube-like structures formed after 16 hr in untreated control wells. In contrast, treatment with IFN-α2b and IFN-β1a resulted in disrupted, poorly connected tube networks in both doses (Figure 3).

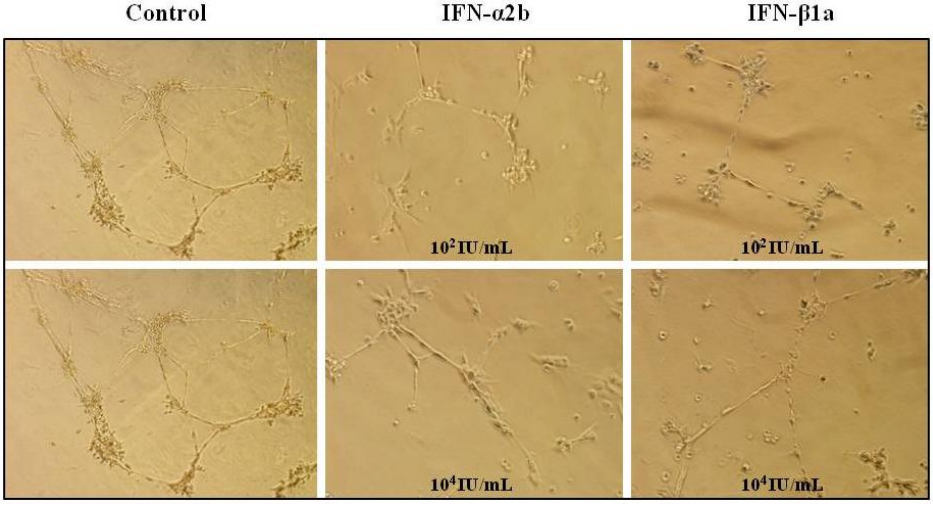

Figure 3. IFN-α2b and IFN-β1a inhibit tube formation by HDMECs. Tube formation by HDMECs was disrupted by IFN-α2b or IFN-β1a at 10^2 IU/mL and 10^4 IU/mL. After 6 and 24 hr, pictures of 5 representative fields were taken for each condition under an inverted microscope at x200 magnification.

4. IFN-α2b and IFN-β1a decrease VEGF-C and VEGFR-3 protein expression and secretion of VEGF-C in SK-MEL-24 melanoma cells

Lysates from SK-MEL-24 cell line were probed for VEGF-C and VEGFR-3, and dominant bands were seen at 21 kDa and 147 kDa. Both IFN-α2b and IFN-β1a decreased VEGF-C and VEGFR-3 protein expression compared to control. IFN-α2b decreased VEGF-C protein expression from 1 hr while IFN-β1a decreased protein expression from 12 hr. IFN-α2b decreased VEGFR-3 protein expression from 1 hr and maintained inhibition until 12 hr. However, decreased VEGFR-3 protein expression at 1 hr recovered at 12 hr with IFN-β1a (Figure 4A). SK-MEL-24 cells were incubated in culture for 24 hr and then treated with IFN-α2b and IFN-β1a at 10^2 IU/mL and VEGF-C secretion was measured by ELISA for further 24 hr. ELISA analysis showed that secretion of VEGF-C was time-dependent, with secretion levels being observed from 12 hr. Treatment of SK-MEL-24 cell line with 10^2 IU/mL IFN-α2b resulted in significant decrease in secretory VEGF-C level at 12 hr and 24 hr compared to control (control: 12 hr: 236.2 ± 5.4 pg/mL/10^7 cells, 24 hr: 260.3 ± 26.4 pg/mL/10^7 cells). IFN-β1a also decreased secretory VEGF-C level at 24 hr.

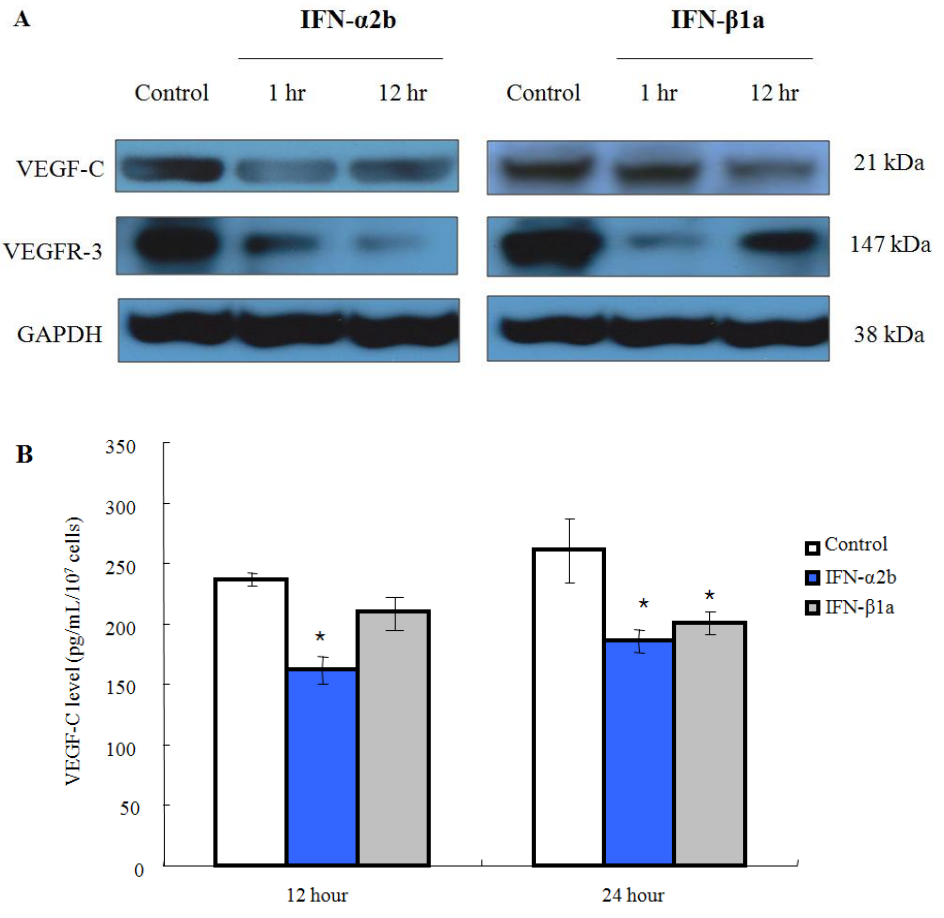

Figure 4. IFN-α2b and IFN-β1a decrease VEGF-C and VEGFR-3 protein expression and secretory VEGF-C in SK-MEL-24 melanoma cells. A) Expression of VEGF-C and VEGFR-3 protein in SK-MEL-24 cell lysates in response to *in vitro* treatment with IFN-α2b and IFN-β1a. B) VEGF-C secretion in response to *in vitro* treatment with IFN-α2b and IFN-β1a. Data are expressed as mean ± SEM of three such experiments performed in duplicate. Statistical significance was set as $p<0.05$(*).

5. IFN-α2b and IFN-β1a inhibit growth of primary human melanoma in nude mice

Therapeutic effects of IFN-α2b and IFN-β1a were evaluated on the growth of primary human melanomas on nude mice. Human SK-MEL-24 melanoma cells were injected intradermally into mice and treatment was initiated when the mean tumor volume measured 235.48 ± 43.8 mm^3 at day 54 after inoculation. Treatment with IFN-α2b and IFN-β1a both reduced tumor volumes compared to tumors of control group treated with normal saline and the extent of reduction was statistically significant from day 65 (p<0.05). However, there was no significant difference between IFN-α2b and IFN-β1a group (Figure 5).

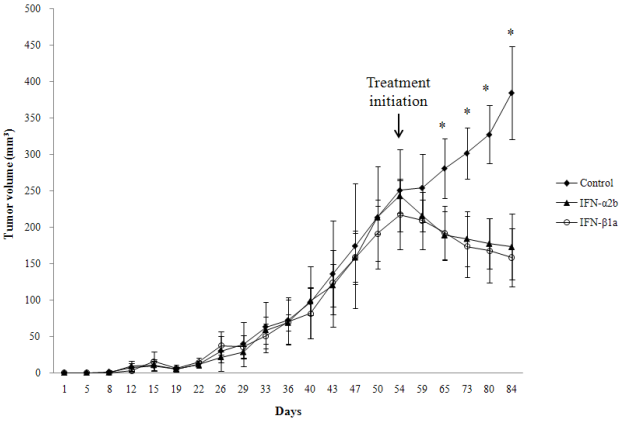

Figure 5. Reduction of primary tumor growth in human melanoma xenograft model formed by human SK-MEL-24 melanoma cells. N=6 animals per group; volumes are expressed as mean ± SD. Statistical significance was set as p<0.05(*).

6. ^{18}F-FDG uptake of human melanoma xenograft tumor decreased by IFN-α2b and IFN-β1a

Baseline tumor uptake of ^{18}F-FDG in the human melanoma xenograft model was relatively high (SUV mean 2.3586), making it easy to differentiate tumor from non-tumor tissues. In the control mouse, uptake of ^{18}F-FDG assessed by SUV mean increased from 1.3404 at baseline to 1.4642 at 4 weeks after normal saline treatment (Figure 6A). Uptake of ^{18}F-FDG assessed by SUV mean decreased from 2.2151 at baseline to 1.1176 at 4 weeks in mouse treated with IFN-α2b (Figure 6B) and from 1.1616 at baseline to 0.6683 at 4 weeks in mouse treated with IFN-β1a (Figure 6C).

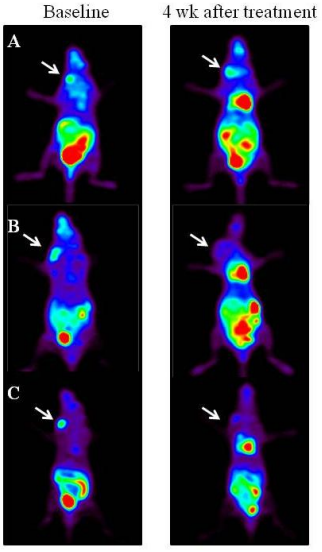

Figure 6. MicroPET images. The six images are representative coronal PET images of three mice scanned with ^{18}F-FDG before and after 4 weeks of treatment. A) Mouse treated with normal saline. B) Mouse treated with IFN-α2b. C) Mouse treated with IFN-β1a .The arrows point towards the tumors.

7. IFN-α2b and IFN-β1a inhibit melanoma metastasis to lymph nodes

Lymph node metastases were detected by immunohistochemistry using an antibody against S100 (Figure 7). The ipsilateral cervical lymph node was invariably the first lymph node (the so-called sentinel lymph node) that showed melanoma metastases in our model. There were no cells staining positive for S100 antibody in normal lymph nodes of nude mice. Sentinel lymph nodes within all treatment groups (*N*=18, total yield 100%) were examined for melanoma metastases. In control group, 5 out of 6 mice showed lymph node metastases. One mouse showed lymph node metastasis in IFN-α2b group and 3 mice showed lymph node metastasis in IFN-β1a group, respectively.

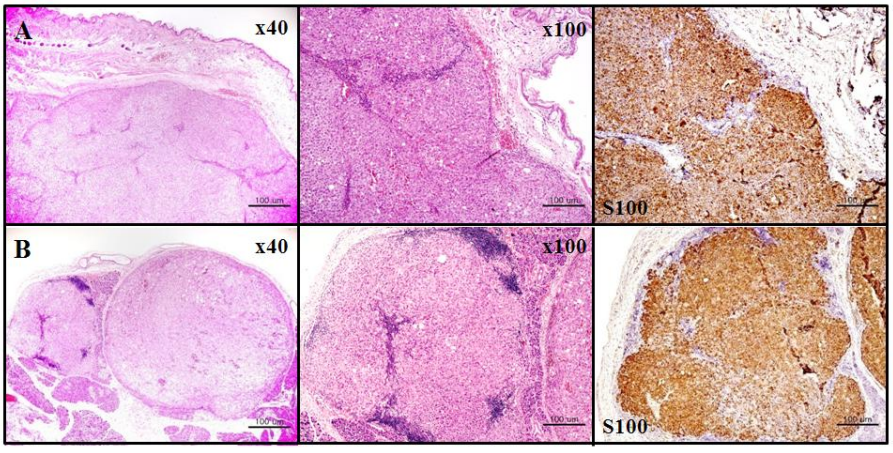

Figure 7. Identification of primary melanoma and lymph node metastasis. A) Primary melanoma and B) lymph node metastases were identified with H&E and S100 immunostaining.

8. IFN-α2b and IFN-β1a inhibit tumor cell proliferation and induce apoptosis in human melanoma xenograft tumors

The effects of IFNs on proliferative activity, angiogenesis and lymphangiogenesis in human melanoma xenograft tumors were evaluated. Both IFN-α2b and IFN-β1a decreased the number of proliferating tumor cells as assessed with Ki-67 immunohistochemistry. IFN-β1a showed significantly potent anti-proliferative effect compared to IFN-α2b (p<0.05) (Figure 8 A & B).

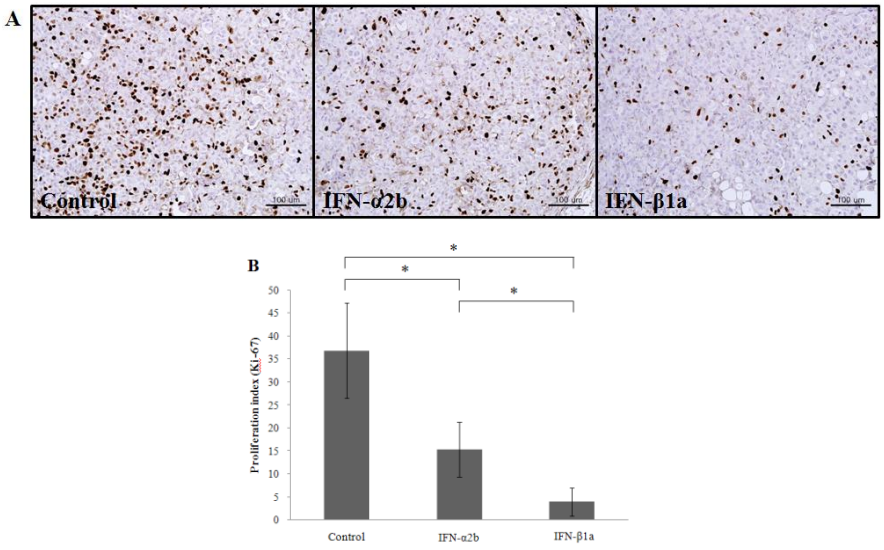

Figure 8. IFN-α2b and IFN-β1a showed anti-proliferative effect in human melanoma xenograft tumors. A) Immunohistochemical staining for Ki-67 in human melanoma xenograft tumors (x100). B) Proliferation index significantly decreased in tumors treated with IFN-α2b and IFN-β1a compared to control tumors. N=6 animals per group; data are expressed as means ± SD. Statistical significance was set as p<0.05(*).

To detect cells undergoing apoptosis after IFN treatment, an *in situ* TUNEL assay was conducted. Both tumors treated with IFN-α2b and IFN-β1a showed significantly increased number of TUNEL-positive cells compared to control (Figure 9A). Tumors treated with IFN-β1a showed significantly increased number of TUNEL-positive cells compared to tumors treated with IFN-α2b (Figure 9B).

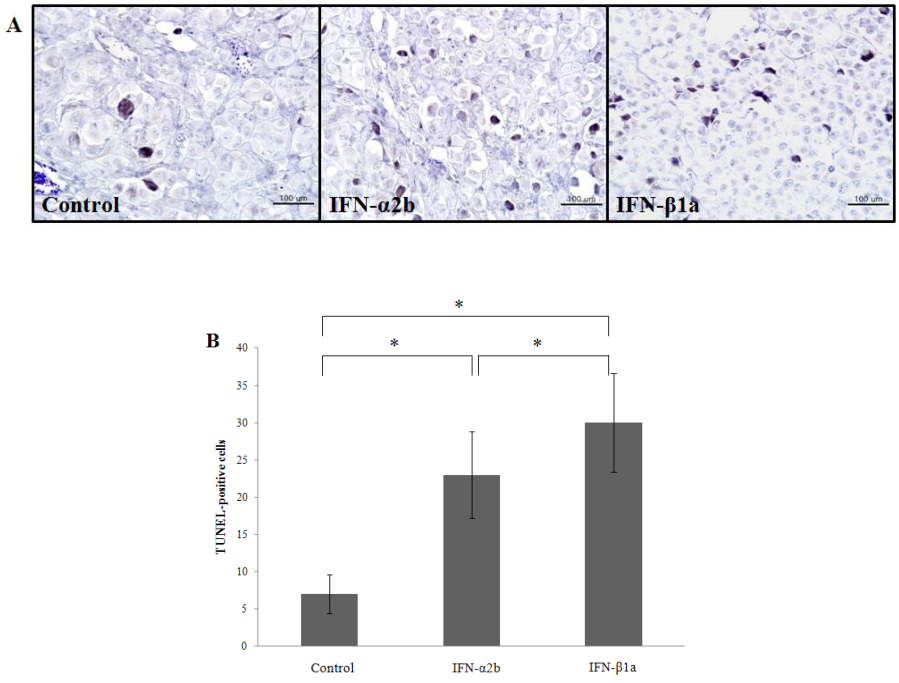

Figure 9. *In situ* detection of apoptotic cells in human melanoma xenograft tumors. A) TUNEL staining in human melanoma xenograft tumors (x100). B) The number of apoptotic cells significantly increased in tumors treated with IFN-α2b and IFN-β1a compared to control. *N*=6 animals per group; data are expressed as means ± SD. Statistical significance was set as $p<0.05$(*).

9. IFN-α2b decreases lymphatic vessel density in human melanoma xenograft tumors

Immunohistochemical staining for CD34 highlighted blood vessels and LYVE-1 highlighted intra- and peritumoral lymphatic vessels in human melanoma xenograft tumor. MVD decreased in tumors treated with IFN-α2b and IFN-β1a, however, it was not statistically significant (Figure 10 A & B).

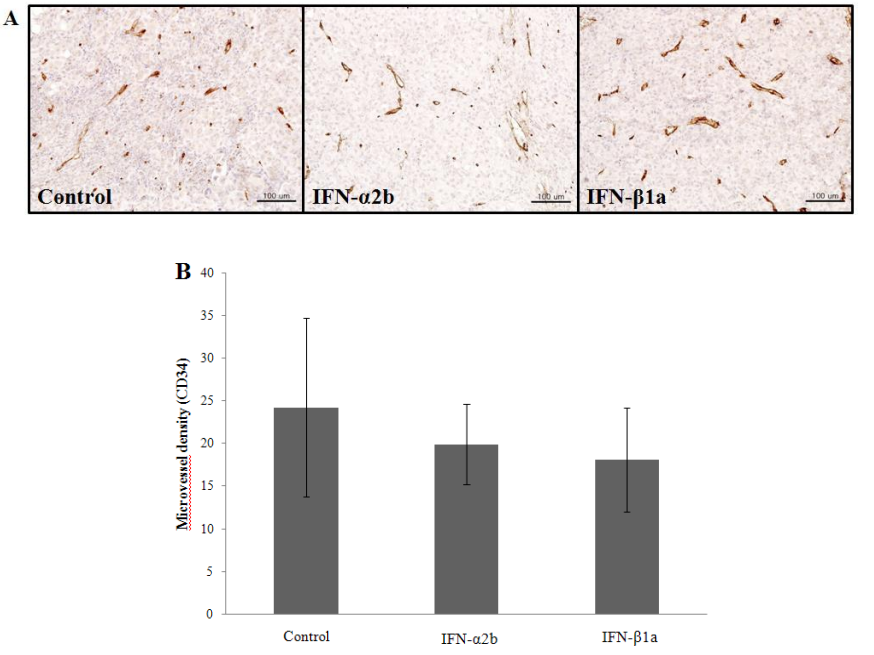

Figure 10. Effect of IFN-α2b and IFN-β1a on MVD in human melanoma xenograft tumors. A) Immunohistochemical staining for CD34 in human melanoma xenograft tumors (x100). B) MVD was decreased in tumors treated with IFN-α2b and IFN-β1a compared to control, but it was not statistically significant. *N*=6 animals per group; data are expressed as means ± SD. Statistical significance was set as $p<0.05$(*).

As for LVD, it significantly decreased only in tumors treated with IFN-α2b (p<0.05) (Figure 11 A & B).

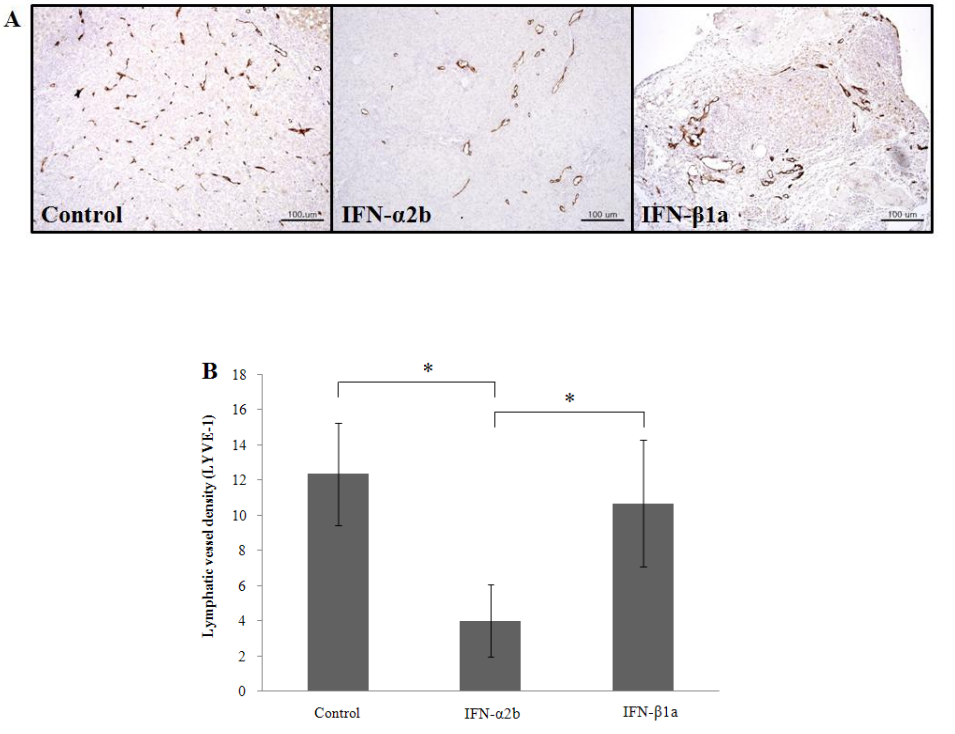

Figure 11. Effect of IFN-α2b and IFN-β1a on LVD in human melanoma xenograft tumors. A) Immunohistochemical staining for LYVE-1 in human melanoma xenograft tumors (x100). B) LVD was significantly decreased in tumors treated with IFN-α2b. Tumors treated with IFN-β1a showed no difference in LVD compared to control. N=6 animals per group; data are expressed as means ± SD. Statistical significance was set as p<0.05(*).

10. IFN-α2b and IFN-β1a decrease expression of lymphangiogenic factors VEGF-C and VEGFR-3 in human melanoma xenograft tumors

Immunohistochemical staining with VEGF-C and its receptor VEGFR-3 were performed to see the expression of lymphangiogenic factors in xenograft tumors. All of the human melanoma xenograft tumors expressed VEGF-C in the tumor cell cytoplasm. Histoscores were significantly higher in the control compared to tumors treated with IFN-α2b and IFN-β1a, however, there was no significant difference between the two groups (Figure 12 A & B).

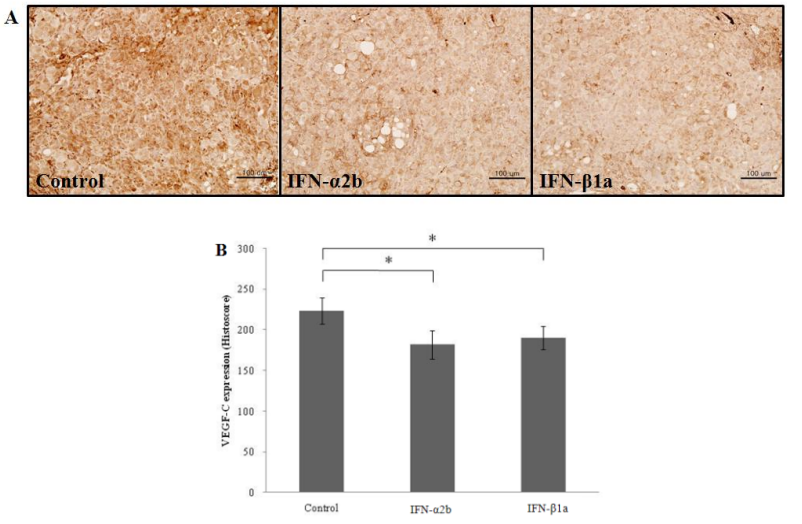

Figure 12. IFN-α2b and IFN-β1a decrease expression of VEGF-C in human melanoma xenograft tumors. A) Immunohistochemical staining for VEGF-C in human melanoma xenograft tumors (x200). B) Expression of VEGF-C decreased in tumors treated with IFN-α2b and IFN-β1a compared to control. *N*=6 animals per group; data are expressed as means ± SD. Statistical significance was set as $p<0.05$(*).

All of the human melanoma xenograft tumors also expressed VEGFR-3 in the tumor cell cytoplasm with membrane-associated staining detected in some tumor cells. Histoscores were significantly higher in the control compared to tumors treated with IFN-α2b and IFN-β1a. However, there was no significant difference between tumors treated with IFN-α2b and IFN-β1a (Figure 13 A & B).

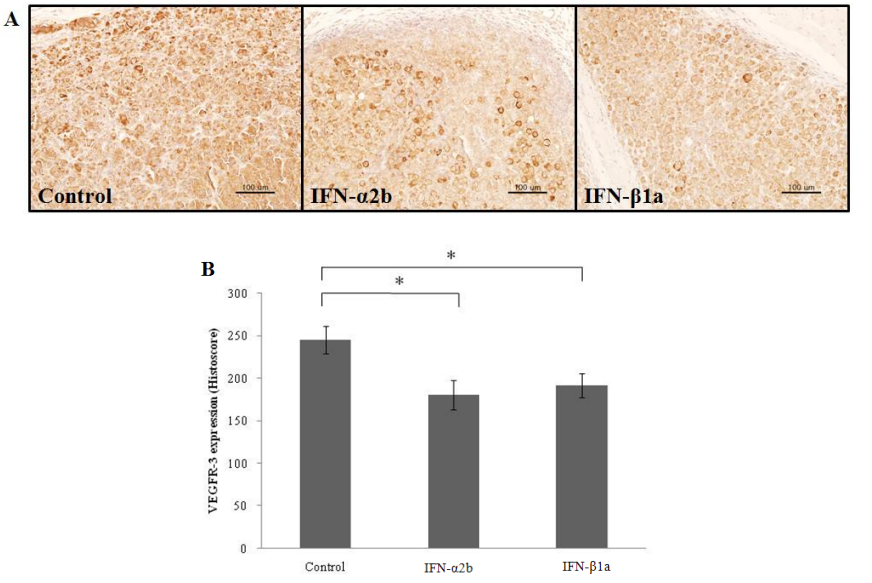

Figure 13. IFN-α2b and IFN-β1a decrease expression of VEGFR-3 in human melanoma xenograft tumors. A) Immunohistochemical staining for VEGFR-3 in human melanoma xenograft tumors (x200). B) Expression of VEGFR-3 decreased in tumors treated with IFN-α2b and IFN-β1a compared to control. *N*=6 animals per group; data are expressed as means ± SD. Statistical significance was set as $p<0.05$(*).

IV. DISCUSSION

IFN-α2b is the only FDA approved adjuvant therapy for malignant melanoma that has undergone rigorous testing in randomized intergroup trials. However, for reasons that are currently unknown, IFN-α2b has only been shown to be clinically effective in the adjuvant setting of high risk for melanoma relapse and not in distant metastatic disease.[15] Various trials showed an improved relapse free survival but did not show a benefit in overall survival with high-dose and intermediate-dose IFN-α2b regimen.[3-4] There are also reports of preliminary studies on the effectiveness of intralesional IFN-β treatment of malignant melanoma.[16-20] In preclinical studies, IFN-β induced higher levels of IFN-stimulated gene products, induced more apoptosis in melanoma cells, and had higher anti-tumor effects against melanoma when compared to IFN-α.[21] However, in a phase II trial of IFN-β, it did not have clinical benefit with overall response rate lower than 10% and median progression free survival of 1.8 months.[22] Despite the fact that IFN-α2b therapy can bring about long-term remissions in malignant melanoma is well established, the mechanisms by which this is achieved is yet unknown.

IFNs represent a complex family of heterogeneous proteins that have been traditionally classified based on the mechanism of their production and the receptor through which they propagate their effects inside the cell. IFN-α and IFN-β are type I IFN and their effects are mediated predominantly via the

IFNαβ receptor. The possible known mechanisms of anti-tumor effect of type I IFN are as follows: direct inhibition of cell proliferation[23-24], induction of apoptosis of tumor cells[25-27], activation of host anti-tumor immunity[28], and inhibitory effect on tumor angiogenesis.[6-7] Although, IFNs are widely known to inhibit angiogenesis[29], little is known about the effect of IFN on lymphangiogenesis.

Therefore, in our study, we compared the anti-tumor and anti-metastatic effects, more importantly anti-lymphangiogenic effect, of IFN-α2b and IFN-β1a on human melanoma xenograft model and investigated the underlying mechanism of IFNs on lymph node metastasis.

IFN-α2b and IFN-β1a both showed inhibitory effects on tumor cell proliferation. The number of proliferating tumor cells as assessed with Ki-67 immunohistochemistry and tumor size decreased in both groups compared to the control group. Also, tumors treated with IFN-α2b and IFN-β1a showed significantly increased number of TUNEL-positive cells compared to the control. MicroPET images also revealed decreased SUV in tumors treated with IFN-α2b and IFN-β1a compared to control. In our study, IFN-β1a group showed significantly potent anti-proliferative and apoptotic effect compared to IFN-α2b group which is consistent with previous studies. IFN-β is known to be more potently anti-proliferative apoptotic in tumor cells *in vitro* compared to IFN-α.[25,30-31] In murine studies, IFN-β has shown anti-tumor effects for transplantable syngeneic melanomas,[32-33] human IFN-β also showed greater

anti-tumor effects than IFN-α in melanoma xenograft model.[34-35]

The induction of angiogenesis, the generation of new capillary vessels from pre-existing vessels, is generally considered as essential to ensure the supply of oxygen and nutrients for malignant tumor growth, invasion and even metastasis.[36] New blood vessel formation is a prominent feature of human cutaneous melanomas, indicating that these tumors have angiogenic activity.[37] Also, multiple proangiogenic factors such as VEGF[38], bFGF[39], interleukin (IL)-8[40] are known to be produced by melanoma cells. IFNs are known to have a potent anti-angiogenic effect which has been clinically translated to treatment of infants with large life-threatening hemangiomas and giant cell tumor of the bone.[41-42] In a previous study, IFN-α inhibited VEGF secretion by melanoma cell lines *in vitro* and showed similar actions in melanoma patients that responded to IFN-α treatment.[43] Our study results revealed that MVD decreased in xenograft tumors treated with IFN-α2b and IFN-β1a compared to control, but it was not statistically significant.

In human malignant melanomas of the skin, lymphatic vessels represent the major route of metastatic dissemination.[44] A direct correlation between increased tumor lymphangiogenesis and increased lymphatic metastasis has been established in animal models[45] and in clinical studies on melanoma patients.[46-47] It has recently been shown that lymphangiogenic growth factors such as VEGF-C and its receptor VEGFR-3, may play a pivotal role in the promotion of metastasis to regional lymph nodes. A stable overexpression of

VEGF-C or VEGF-D by cancer cells potently enhanced tumor-assisted lymphatic vessel growth, leading to an increased incidence of lymph node metastasis.[48-49] Moreover, it has recently been shown that lymphangiogenic growth factors secreted from a primary tumor can induce lymphangiogenic niche in nearby lymph nodes, even before arrival of tumor cells, which may facilitate further metastasis.[50] In melanomas, increased expression of VEGFR-3 has been seen in tumor cells as well as in blood vessels and lymphatics[51]. However, in benign melanocytic lesions, it has been shown to be confined to lymphatic vessels only implicating a role in metastatic spread.[52] Also, Mouawad et al.[53] reported that high levels of VEGFR-3 in melanoma tumor tissue were accompanied by significantly higher pre-treatment serum levels of VEGFR-3 in melanoma patients. In these patients, median serum VEGFR-3 levels were increased in patients with high tumor burden as well as in non-responders compared to responders to treatment. In our experiment, *in vitro* studies revealed that both IFN-α2b and IFN-β1a decreased expression of VEGF-C and VEGFR-3 in melanoma cells. IFN-α2b showed earlier and sustained inhibitory effect compared to IFN-β1a. Treatment with IFN-α2b and IFN-β1a also decreased secretory VEGF-C level with superior effect by INF-α2b. *In vivo* studies revealed that IFN-α2b and IFN-β1a were both effective in inhibiting lymph node metastasis compared to the control group. However, only IFN-α2b showed significant reduction of LVD in primary tumors. *In vivo* VEGF-C and VEGFR-3 expressions were significantly decreased in tumors treated with

IFN-α2b and IFN-β1a with no significant difference between the two groups. Our study results may suggest that IFN inhibits lymph node metastasis partly by blocking lymphangiogenesis through inhibition of VEGF-C/VEGFR-3 signaling system.

Several mechanisms are responsible for the development of metastases: (i) increased tumor growth, (ii) inhibition of apoptosis, (iii) neoangiogenesis of blood and lymph vessels, (iv) direct interactions with the surrounding stroma, and (v) increased concentrations of chemoattractants. Our study results show that IFN acts on melanoma through various mechanisms. IFN shows its effect through inhibition of tumor growth, induction of apoptosis, and inhibition of angiogenesis and lymphangiogenesis. Although IFN-α and IFN-β are both type I IFNs that act through the same receptor, our results indicate that their action mechanism and effect may differ on melanoma cells.

This study has limitations that the therapeutic effect of IFN-α2b and IFN-β1a in melanoma cells was demonstrated *in vitro* and the lymph node metastasis experiments were conducted in immunocompromised mice. In melanoma patients, activation of the immune system by IFNs may also make an important contribution to their overall anti-tumor and anti-metastatic effects.

V. CONCLUSION

In conclusion, both IFN-α2b and IFN-β1a showed efficacy in inhibiting primary tumor growth and lymph node metastases in human melanoma xenograft model. However, IFN-α2b and IFN-β1a showed different mechanism regarding anti-tumor and anti-metastatic effects on malignant melanoma. IFN-β1a showed higher anti-proliferative and apoptotic effect while IFN-α2b showed superior inhibitory effect in inhibiting lymph node metastasis by inhibition of lymphangiogenesis compared to IFN-β1a. Further studies regarding the molecular mechanisms that control the interaction among IFNs, melanoma cells and blood vessel/lymphatic endothelium need to be clarified to define the mechanism of IFNs on melanoma.

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ABSTRACT (IN KOREAN)

재조합 Interferon-α 및 -β의 병변내 주사가 nude mice에 이식한 인체 악성 흑색종에 미치는 항암 및 항전이 효과

<지도교수 정기양>

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노 미 령

인터페론 알파는 고위험 재발성 악성 흑색종 환자에서 보조적인 치료법으로 유일하게 FDA 허가된 약제로서, 악성 흑색종 환자의 relapse free survival 를 증대시키며, 고위험 재발성의 악성 흑색종 환자의 보조적인 치료법으로 인정되고 있다. 그러나 보조적인 치료로 임상적으로는 효과를 보이지만, 그 작용 기전에 대해서는 완전히 밝혀지지 않고 있다. 악성 흑색종은 초기에 림프절 전이를 많이 일으키는 종양으로, 전초 림프절 전이의 여부가 중요한 예후 인자로 알려져 있다. 최근 연구에 의하면 종양 유도 림프관생성(tumor-associated lymphangiogenesis)이 림프절로의 전이를 유도하는데 중요한 역할을 한다고 보고되었다. 본 실험에서는 재조합 인터페론 알파와 베타의 병변내 주사가 nude mice 에 이식한 인체 악성 흑색종에 미치는 항암 및 항전이 효과를 연구하고자 한다.

연구 결과 인터페론 알파와 베타 모두 종양 세포 성장 억제와 apoptosis 유도 효과를 보였다. *In vitro*상, 인터페론 알파와 베타는 악성 흑색종 세포주의 성장을 농도에 비례하여 억제하였으며, 인터페론 베타가 알파에 비해 세포 성장 억제 효과가 더 컸다.

Nude mice에 이식한 악성 흑색종 원발 종양에 대한 Ki-67 면역염색 및 TUNEL assay결과, 대조군에 비교하여 인터페론 알파와 베타 치료군에서 proliferating tumor cell의 수가 감소하고 TUNEL 양성 세포의 수가 증가하였다. 종양 세포 성장 억제 및 apoptosis 유도 효과의 경우, 인터페론 베타 치료군에서 인터페론 알파 치료군에 비해 통계학적으로 유의하게 높게 나타났다 (p<0.05). 인터페론 알파와 베타 모두 *in vitro*에서 human dermal microvascular endothelial cell (HDMEC)의 tube formation 형성을 억제하였다. Nude mice에 이식한 악성 흑색종 원발 종양의 microvessel density (MVD) 및 lymphatic vessel density (LVD) 측정 결과, MVD는 대조군에 비해 치료군에서 감소하였으나 통계학적으로 유의하지 않았으며, LVD는 인터페론 알파 치료 군에서 유의하게 감소하였다 (p<0.05). Nude mice에 이식한 악성 흑색종 모델에서 림프절 전이 정도는 인터페론 알파 및 베타 치료군에서 대조군에 비해 적게 관찰되었다. 각 군당 여섯 마리 중, 인터페론 알파 치료군에서는 전이가 1마리, 인터페론 베타 치료군에서는 3마리에서 관찰된 반면, 대조군에서는 5마리에서 림프절 전이 소견이 관찰되었다. Lymphangiogenic factor인 VEGF-C 및 VEGFR-3의 발현은 *in vitro*와 *in vivo*에서 모두 인터페론 알파와 베타 치료에 의해 감소하였다. *In vitro*에서 인터페론 알파의 경우, 베타에 비해 VEGF-C 및 VEGFR-3의 발현 억제 양상이 짧은 시간 안에 나타났고, 오래 지속되는 효과를 보였고, secretory VEGF-C를 감소시키는 효과가 통계학적으로 유의하게 크게 나타났다. 결론적으로 nude mice에 이식한 악성 흑색종 모델에서 인터페론 알파와 베타 치료는 대조군에 비해 원발 종양 성장 및 림프절 전이를 억제하는데 효과를 보였으며, 이는 각각 다른 기전을 통함을 보여주었다.

핵심되는 말: 악성흑색종 xenograft model, 인터페론-α2b, 인터페론-β1a, 림프절 전이, 림프관생성