The effect of imiquimod on matrix metalloproteinases and tissue inhibitors of metalloproteinase in malignant melanoma cell invasion

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This certifies that the Doctoral Dissertation
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Written by Jin Young Jung

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ABSTRACT

The effect of imiquimod on matrix metalloproteinases and tissue inhibitors of metalloproteinase in malignant melanoma cell invasion

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(Directed by Professor Kee Yang Chung)

Imiquimod is an immune response modulator for treatment of superficial skin cancers and viral warts. Numerous reports have also been published regarding the use of imiquimod for treatment of melanoma in situ and metastatic melanoma. Cutaneous melanomas are notorious for their tendency to invade and metastasize. Essential steps in this process are degradation of basement membranes and remodeling of the extracellular matrix (ECM) by proteolytic enzymes, including matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs).

This study evaluated the anti-invasive effect of imiquimod in human malignant melanoma cell lines, SK-MEL-2 and SK-MEL-24, *in vitro*. And this study also investigated changes in expression of MMP-2, -9, and membrane type 1 matrix metalloproteinase (MT1-MMP), the key enzymes known to degrade surrounding ECM components during cancer invasion and metastasis,

and their inhibitors, TIMP-1 and -2, under the influence of imiquimod.

Imiquimod treatment resulted in decreased *in vitro* viability of melanoma cells in a concentration-dependent manner. And imiquimod also showed concentration-dependent suppression of invasion in both melanoma cell lines. Both melanoma cell lines showed a concentration-dependent decrease in MMP-2 and MT1-MMP protein levels and a concentration-dependent increase in TIMP-1 and -2 protein levels by imiquimod. However, expression of MMP-9 protein level increased in SK-MEL-2 but decreased in SK-MEL-24 according to the increase in imiquimod concentration. In real-time quantitative RT-PCR, imiquimod was shown to induce similar changes in expression of MMPs and TIMPs mRNA levels to protein levels. These results suggest that imiquimod may have an anti-invasive effect on human melanoma cells via regulation of MMPs and TIMPs.

Key words: imiquimod, invasion, matrix metalloproteinase, malignant melanoma, tissue inhibitor of metalloproteinase

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I. INTRODUCTION

Imiquimod is an immune response modulator approved by the Food and Drug Administration for treatment of actinic keratoses, superficial basal cell carcinomas, and genital and perianal warts. Imiquimod has also been used for treatment of melanoma in situ and metastatic melanoma in patients with confounding morbidities who are not considered surgical candidates, with extensive disease or disease in areas that are not amenable to surgery.[1-5]

Findings from extensive studies conducted in past years have indicated that imiquimod acts in a dual manner. On one hand, imiquimod indirectly activates the innate as well as the adaptive immune system through binding to cell surface receptors, such as Toll-like receptors (TLR) 7 and 8, thereby inducing activation of transcription factors, such as nuclear factor NF-κB, resulting in secretion of pro-inflammatory cytokines, predominantly interferon (IFN)-α, tumor necrosis factor (TNF)-α, and interleukin (IL)-12.[6,7] On the other hand,

imiquimod directly induces *in vitro* and *in vivo* pro-apoptotic activities in a rather tumor selective manner,[6,8,9] requiring activation of the 'work horses' of apoptosis, members of the caspase family of proteases.

Melanoma is a well known tumor that tends to metastasize rather than grow locally. Invasion and metastasis of tumor cells is a complex and multiplex process. During this process, degradation of basement membranes and remodeling of the extracellular matrix (ECM) by proteolytic enzymes, such as matrix metalloproteinases (MMPs), which are regulated by their tissue inhibitors (TIMPs), are essential steps. In melanoma progression, different proteolytic enzyme systems, including members of the plasminogen activator system[10,11] and of the MMP family, play important roles. MMPs, particularly MMP-2 and MMP-9, are key enzymes known to degrade surrounding ECM components during cancer invasion and metastasis. Melanoma cells express a number of MMPs and TIMPs.[12]

There has been only one case report investigating expression of molecules involved in metastasis of melanoma after treatment with imiquimod.[13] Before and after treatment, a skin metastatic lesion was biopsied and expression of the following molecules was investigated using a real-time reverse transcription–polymerase chain reaction (RT-PCR). Expression of TIMP-1, KiSS-1, and MMP-1 was up-regulated and that of MMP-2 was not modified. MMP-9 expression showed a dramatic decrease. This report suggests that imiquimod could down-regulate metastasis and invasion.[13]

The aim of this study was to evaluate the anti-invasive effect of imiquimod, *in vitro* against human malignant melanoma cell lines. And this study also investigated changes in expression of MMP-2, -9, and membrane type 1 matrix metalloproteinase (MT1-MMP), which are key enzymes known to degrade surrounding ECM components during cancer invasion and metastasis, and their inhibitors, TIMP-1 and -2, under the influence of imiquimod.

II. MATERIALS AND METHODS

1. Cell culture

Melanoma cell lines, SK-MEL-2 and SK-MEL-24, and HT1080 cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to the vendor's guidelines. SK-MEL-2 and SK-MEL-24 were routinely maintained in Eagle's minimal essential medium (EMEM; Lonza, Basel, Switzerland) containing 10% fetal bovine serum (FBS; Lonza) and supplemented with 100 units/ml penicillin, and 100 mg/ml. HT1080 cells were routinely maintained in RPMI-1640 (Lonza) containing 10% FBS and supplemented with 100 units/m1 penicillin and 100 mg/m1 streptomycin.

2. Cell viability assay

Melanoma cell lines, SK-MEL-2 and SK-MEL-24, were harvested in the exponential phase and seeded in a 96-well flat bottom tissue culture plate at a concentration of 1 x 10^4 cells/100 μl/well. Cells were allowed to grow and stabilize for 24 h. Subsequently, the cells were treated with different concentrations (5~200 μg/ml) of imiquimod (InvivoGen, San Diego, CA, USA) prepared in complete medium or cultured with different incubation times (6 h~3 d). Each treatment was performed in three well replicates. After incubation, 10 μl of WST-1 reagent EZ-CyTox (Daeil lab, Seoul, Korea) was added to each well, followed by incubation for 4 h at 37℃. Optical density was measured in

an enzyme linked immunosorbant assay (ELISA) plate reader (Molecular Devices, Spectra Max 190 with Soft max Pro, Sunnyvale, CA, USA) at 450 nm with a reference wavelength of 690 nm. Cell viability was plotted as a percentage of untreated control. Results are expressed as mean ± SEM and are representative of three independent experiments. Inhibitory concentration 50 (IC_{50}) was determined from the dose effect curve as the drug concentration that decreased cell viability to 50%.

3. Invasion assay using transwell filters

A modified version of the standard transwell filter assay for invasion was performed. Transwell filters (diameter, 6.5 mm; pore size, 8 μm; Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, 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 (BSA; USB, Cleveland, OH, USA). Melanoma cell lines, SK-MEL-2 and SK-MEL-24, were harvested with a cell dissociation solution (Sigma-Aldrich, St Louis, MO, USA) and suspended in medium with 1% BSA. Cancer cells (1×10^5) were added to the upper compartment of a transwell chamber and treated with 0, 10, and 30 μg/ml imiquimod to the upper compartment for 24 h at 37℃. After 24 h, non-migrated cells on the upper side of the membrane were removed with a cotton swab, and migrated cells on the bottom surface of the membrane were fixed in 3.7% paraformaldehyde in phosphate buffered saline (PBS; Lonza) and stained with

crystal violet for 10 m at room temperature. Cell migration was quantified by counting the number of cells in three inserts. Data were expressed as the average number of cells per insert.

4. Gelatin zymography

Melanoma cell lines, SK-MEL-2 and SK-MEL-24, and HT1080 cell line were cultured in serum-free media for 24 h and the conditioned media was concentrated using VIVASPIN 20 (Sartorius Stedim Biotech GmbH, Goettingen, Germany) per the manufacturer's instructions. An equal volume of sample buffer was added to the concentrated media before loading on a 10% SDS-PAGE gel impregnated with 0.1% gelatin. After electrophoresis, the gel was rinsed with renaturing buffer for 1 h, and incubated in developing buffer overnight at 37˚C. After incubation, the gel was stained with 0.05% Coomassie brilliant blue R-250 (Amresco, Cleveland, OH, USA) and de-stained. MMP-2 and MMP-9 were detected as transparent bands. The HT1080 cell line was used as a positive control.

5. Western blot analysis

To evaluate expression of MT1-MMP, whole-cell protein extracts were prepared from the melanoma cell lines, SK-MEL-2 and SK-MEL-24, and HT1080 cells. Various concentrations of imiquimod (0, 5, 10, and 30 μg/ml) were applied for 24 h. Protein content was determined by the BCA protein assay

method. Total protein of 40 μg per sample was fractionated by 10% SDS-PAGE gel and transferred onto nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). After blocking with 5% skimmed milk, the membranes were incubated with polyclonal antibody against MT1-MMP (1:500 diluation; Abcam Inc., Cambridge, UK) and monoclonal antibody against actin (1:4000 dilution; Santa cruz biotechnology, Santa cruz, CA, USA) at 4˚C overnight. After washing with Tris-buffered saline with Tween 20 (TBST), they were incubated with peroxidase conjugated goat anti-rabbit IgG antibody (1:2000 dilution; Cell signaling, Beverly, MA, USA) and goat anti-mouse IgG antibody (1:2000 dilution; Cell signaling). To evaluate expression of TIMP-1 and -2, cell culture supernatants were collected from melanoma cell lines, SK-MEL-2 and SK-MEL-24, and HT1080 cell line. Samples of conditioned medium (7 ml) were precipitated with 10% TCA at -20˚C overnight. Following centrifugation at 4300 rpm for 20 m at 4˚C, the pellet was washed twice with ice-cold acetone, dried at 37˚C, and dissolved in 100 μl of 2x SDS sample buffer. Twenty μl per sample was fractionated by 10% SDS-PAGE gel and transferred onto nitrocellulose membranes (GE Healthcare). After blocking with 5% skimmed milk, membranes were incubated with monoclonal antibodies against TIMP-1 (1:500 diluation; Abcam Inc.) and TIMP-2 (1:500 diluation; Abcam Inc.) at 4˚C overnight. After washing with TBST, they were incubated with peroxidase conjugated goat anti-mouse IgG antibody (1:2000 dilution; Cell signaling). Chemiluminescence reagents (ECL Plus Western Blotting Detection Reagents;

GE Healthcare) were used for visualization of the labeled protein bands. The HT1080 cell line was used as a positive control.

6. Real-time quantitative PCR

Melanoma cell lines, SK-MEL-2 and SK-MEL-24, and HT1080 cell line were treated with various concentrations of imiquimod (0, 5, 10, and 30 μg/ml) for 24 h. Total cellular RNA was purified from cancer cells using Trizol® reagent (Gibco BRL, Life Techonologies, Carlsbad, CA, USA). After evaluation by gel electrophoresis, the RNA of each sample was stored at −80℃ until use. The total RNA was converted to single-stranded cDNA using a High Capacity kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The resulting cDNA was stored at −20℃. Measurements of mRNA levels of MMP-2, -9, MT1-MMP, TIMP-1, and -2 were based on SYBR® Green PCR Master Mix (Applied Biosystems) using the 7500 Real Time PCR System (Applied Biosystems). Primers for genes MMP-2, -9, MT1-MMP, TIMP-1, and -2 were designed using Primer Express® Software v3.0 (Applied Biosystems). Sense and antisense primers for MMP-2 were 5'-GCAGACATCGTCATCCAGTTTG-3' and 5'- CCGTCCTTCCCGTCGAA-3', respectively. Sense and antisense primers for MMP-9 were 5'-GATGACGATGAGCTATGGACCTT-3' and 5'-TCGGCGTTCCCATACTT CAC-3', respectively. Other primers and probes were synthesized by assay on demand products (Applied Biosystems): MT1-MMP (HS 00237119), TIMP-1

(HS 00171557), TIMP-2 (HS 00234278), and GAPDH (HS 99999905). RT-PCR was performed in triplicate 20 μl reaction volumes consisting of 10 μl 2x TaqMan® Gene Expression Master Mix (Applied Biosystems), and 1 μl 20x Gene Expression Assay Mix and 9 μl cDNA sample diluted in RNAse-free water. Two-step PCR cycling was carried out as follows: 50℃ for 2 m and 95 ℃ for 10 m (1 cycle), 95 ℃ for 15 s, and 60 ℃ for 1 m (50 cycles). At the end of PCR, baseline and threshold values were established using ABI 7500 software v 2. 0. 1 (Applied Biosystems) and the *C*t values were exported to Microsoft Excel (Microsoft Corp., Redmond, WA, USA) for analysis. Relative expression of mRNA was calculated using a relative quantification method, which determines the relative quantification of a target gene in comparison with a reference gene.

7. Statistical analysis

SPSS Graduate Pack 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Quantitative data are represented as the mean of at least three independent experiments. Statistical analysis was performed using the Kruskal-Wallis test followed by the Wilcoxon signed-rank test. Differences were regarded as significant when the *P* values were < 0.05.

III. RESULTS

1. *In vitro* cell viability

A cell viability assay was performed in order to determine the appropriate concentration and incubation time for treatment of melanoma cells with imiquimod. Melanoma cell lines, SK-MEL-2 and SK-MEL-24 cells, were treated with imiquimod for 3 d at a concentration ranging from 5-200 μg/ml. Melanoma cells showed concentration-dependent and incubation time-dependent inhibition in cell viability. Figure 1A shows the concentration-dependent decrease in viability of SK-MEL-2 cells over 24 h of incubation time and the IC_{50} value calculated from the dose effect curve was found to be 56.32 μg/ml. The viability of SK-MEL-24 cells also decreased according to increases in concentration over 24 h of incubation time (Figure 1B). The IC_{50} value of SK-MEL-24 cells was obtained from the dose effect curve as 62.36 μg/ml.

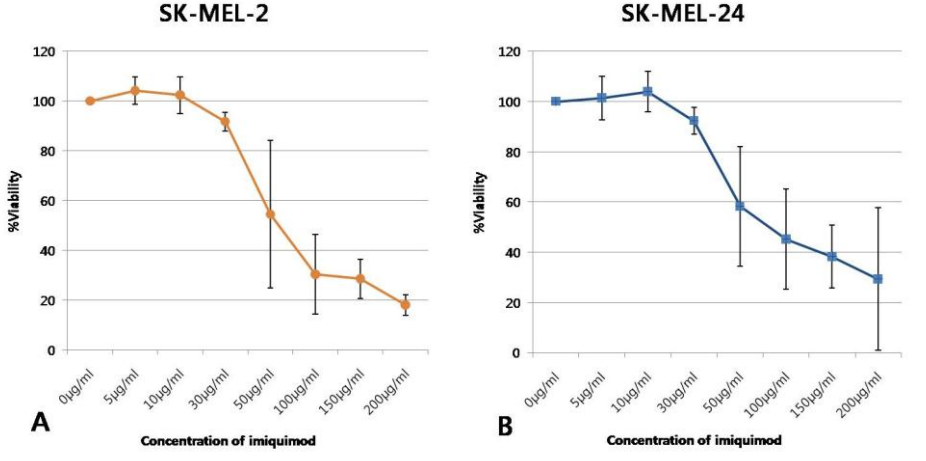

Figure 1. *In vitro* cell viability of melanoma cell lines, SK-MEL-2 (Figure 1A) and SK-MEL-24 cells (Figure 1B), under imiquimod. The dose effect curve showed concentration-dependent inhibition in the percent viability of cells after imiquimod treatment (IC_{50} values: 56.32 μg/ml for SK-MEL-2, 62.36 μg/ml for SK-MEL-24). Values indicate the mean and standard deviation (SD). Results are representative of three independent experiments.

2. Imiquimod suppressed invasion of human melanoma cells

To evaluate the anti-invasive effect of imiquimod according to concentrations of imiquimod in the two melanoma cell lines, SK-MEL-2 and SK-MEL-24 cells, a modified version of the standard transwell filter assay for invasion was performed. Representative micrographs were taken from the lower surface of the transwell filter. As shown in Figures 2A-C and 2E-G, the migratory activity and invasion potential of the two melanoma cell lines were significantly suppressed by increasing concentrations of imiquimod over 24 h of incubation. Figures 2D and 2H show the percentage of the relative cell count after invasion assay. Numbers of invaded melanoma cells showed a significant decrease according to concentrations of imiquimod. Invaded cells were, respectively, 31.57% and 55.96% lower in SK-MEL-2 cells treated with 10 μg/ml and 30 μg/ml imiquimod than in non-treated SK-MEL-2 cells (Figure 2D). Invasion of SK-MEL-24 cells showed a significant decrease, to 43.87% and 67.02%, respectively, after treatment with 10 μg/ml and 30 μg/ml imiquimod, as compared with non-treated SK-MEL-24 cells (Figure 2H).

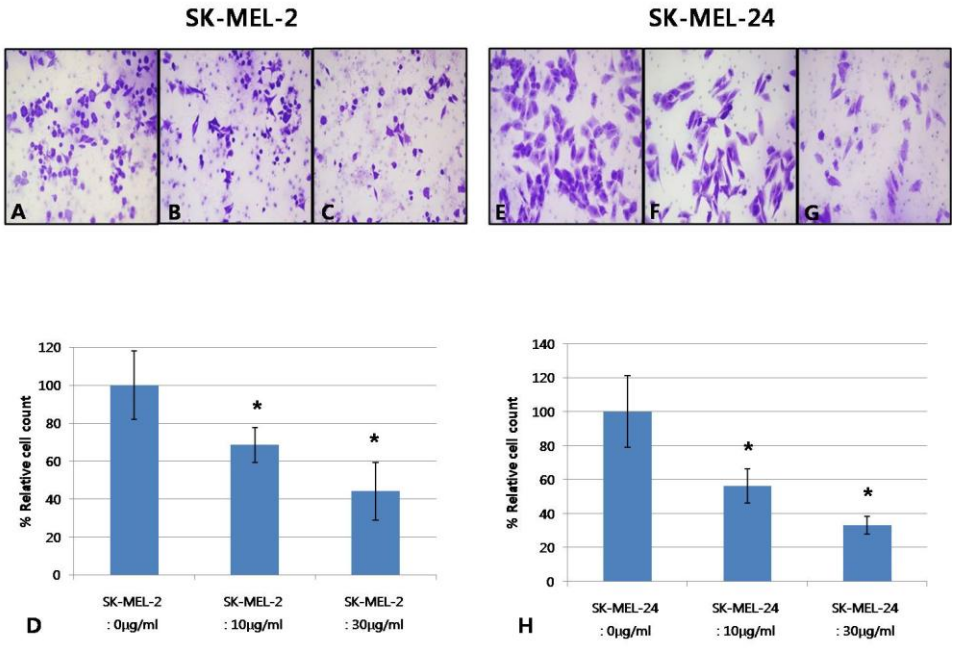

Figure 2. Invasion assay in SK-MEL-2 and SK-MEL-24 cell lines under imiquimod. Suppression of SK-MEL-2 melanoma cell migration and invasion depending on concentration of imiqumod ((A) 0 μg/ml, (B) 10 μg/ml, (C) 30 μg/ml imiquimod, Coomassie stain x 200). (D) The percentage of invasive SK-MLE-2 melanoma cells showed a significant decrease as the concentration of imiquimod increased, compared with untreated cells (*P < 0.05). Suppression of SK-MEL-24 melanoma cell migration and invasion depending on concentration of imiqumod ((E) 0 μg/ml, (F) 10 μg/ml, (G) 30 μg/ml imiquimod, Coomassie stain x 200). (H) The percentage of invasive SK-MLE-24 melanoma cells showed a significant decrease as the concentration of imiquimod increased, compared with untreated cells (*P < 0.05). Results are representative of three independent experiments.

3. Imiquimod induced changes of MMP-2 and -9 in human melanoma cell lines

Melanoma cells express a number of MMPs and TIMPs.[12] MMPs, particularly MMP-2 and MMP-9, are key enzymes in degradation of surrounding ECM components during cancer invasion and metastasis. Gelatin zymography and real-time quantitative RT-PCR were performed for determination of where the function of imiquimod in control of invasion of melanoma cells is dependent on MMP expression and activity.

In gelatin zymography, only the inactive form of MMP-2 was detected in the SK-MEL-2 cell line (Figure 3A), while both the inactive and active forms of MMP-2 were observed in the SK-MEL-24 cell line (Figure 3B). Expression of MMP-2 decreased according to increases in imiquimod concentrations in both cell lines (Figure 3A & 3B). As opposed to MMP-2, which showed similar patterns in both melanoma cell lines, expression of MMP-9 was different between the SK-MEL-2 and SK-MLE-24 cell lines. Inactive and active forms of MMP-9 were observed in SK-MEL-2 cell lines (Figure 3C) and only its inactive form was observed in SK-MEL-24 cell lines (Figure 3D). SK-MEL-2 cells showed a concentration-dependent increase in MMP-9 expression (Figure 3C). However, expression of MMP-9 decreased according to increases in imiquimod level in SK-MEL-24 cell lines (Figure 3D).

In real-time quantitative RT-PCR, expression and change of MMP-2 and -9 mRNA showed a similar pattern with that of MMP-2 and -9 proteins in both cell

lines. In SK-MEL-2 cells, MMP-2 mRNA showed a significant concentration-dependent decrease at all concentrations of imiquimod, as compared with controls (*P < 0.05; Figure 3E). In SK-MEL-24 cells, mRNA expression of MMP-2 also showed imiquimod concentration-dependent decreases. MMP-2 mRNA of SK-MEL-24 cells showed significantly decreased expression at 10 μg/ml and 30 μg/ml imiquimod, as compared with untreated cells (*P < 0.05; Figure 3F) . MMP-9 mRNA expression in SK-MEL-2 cells revealed an increasing tendency according to concentration of imiquimod, but without statistical significance (Figure 3G). In SK-MEL-24 cells, mRNA expression of MMP-9 revealed significantly decreased expression at 30 μg/ml imiquimod, compared with untreated cells (*P < 0.05; Figure 3H).

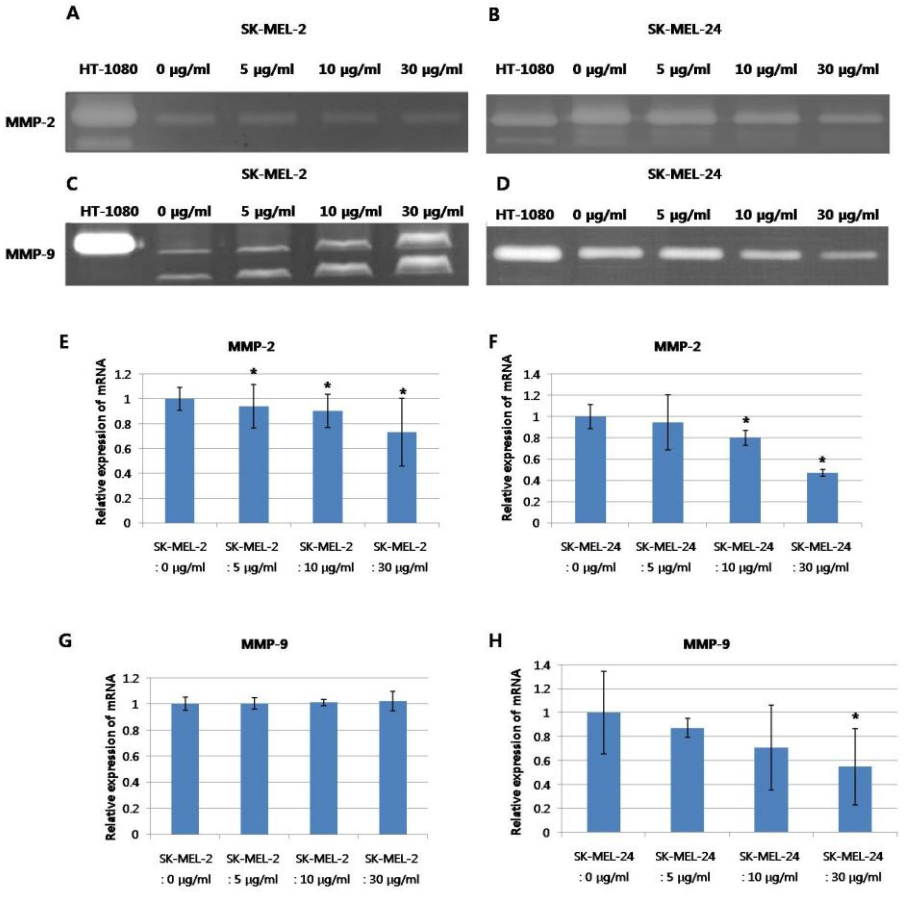

Figure 3. Expression of MMP-2 and -9 protein and mRNA in SK-MEL-2 and SK-MEL-24 cell lines under imiquimod. Gelatin zymography of conditioned media of SK-MEL-2 and SK-MEL-24 cells treated with 0~30 μg/ml of imiquimod. Whole proteins of conditioned media from untreated cells and cells treated with imiquimod for 24 h were collected and concentrated. (A) Expression of MMP-2 decreased according to the increase in imiquimod concentration in SK-MEL-2 cells. (B) Expression of MMP-2 decreased according to increases in imiquimod concentration in SK-MEL-24 cells. (C)

MMP-9 expression in SK-MEL-2 cells showed an increase, depending on the concentrations of imiquimod. (D) However, expression of MMP-9 showed a decrease in SK-MEL-24 cells, depending on the concentrations of imiquimod. Conditioned media of the HT1080 cell line was used as a positive control. Real-time quantitative PCR in SK-MEL-2 and SK-MEL-24 cell lines under imiquimod. Relative mRNA expression of MMP-2 in SK-MEL-2 (E) and SK-MLE-24 (F) cells and MMP-9 in SK-MEL-2 (G) and SK-MLE-24 (H) cells, depending on concentrations of imiquimod (5 μg/ml, 10 μg/ml, 30 μg/ml) in comparison with controls (0 μg/ml). Statistically significant differences were marked "*" ($P < 0.05$ vs control).

4. Expression of MT1-MMP decreased with imiquimod treatment in human melanoma cell lines

Among MMPs, MT1-MMP is a membrane-bound MMP detected on the surface of invasive tumor cells. MT1-MMP is related to cancer invasion by its involvement in pro-MMP-2 activation and degradation of a number of ECM components. To investigate expression and change of MT1-MMP in two melanoma cell lines, Western blot analysis was performed on the whole-cell protein extracts of melanoma cells treated with imiquimod (0~30 μg/ml). Both inactive and active MT1-MMP proteins were detected in both melanoma cell lines. And MT1-MMP proteins were seen to be reversely proportionate to the concentration of imiquimod in both cell lines (Figure 4A & 4B).

MT1-MMP mRNA in SK-MEL-2 cells revealed a significant concentration-dependent decrease at all concentrations of imiquimod (*$P < 0.05$; Figure 4C). In SK-MEL-24 cells, mRNA expression of MT1-MMP showed imiquimod concentration-dependent decreases, with a statistically significant decrease at 30 μg/ml imiquimod, as compared with untreated cells (*$P < 0.05$; Figure 4D).

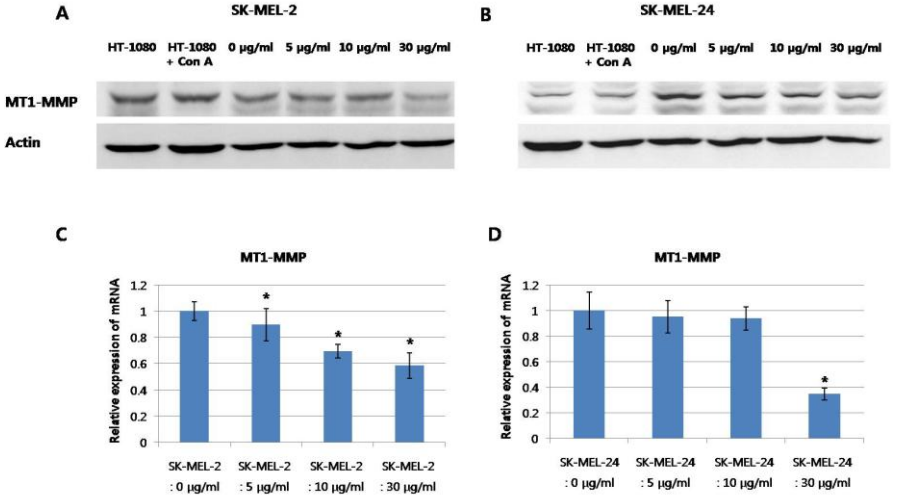

Figure 4. Expression of MT1-MMP protein and mRNA in SK-MEL-2 and SK-MEL-24 cell lines under imiquimod. Expression of MT1-MMP proteins in SK-MEL-2 (A) and SK-MEL-24 (B) cell lines showed a decrease, depending on the concentrations of imiquimod. Melanoma cells were treated with 0~30 μg/ml of imiquimod for 24 h and whole proteins were extracted from cell lysate and immunoblotted with an anti-MT1-MMP antibody. Real-time quantitative PCR was performed in SK-MEL-2 and SK-MEL-24 cell lines under imiquimod. Relative mRNA expression of MT1-MMP in SK-MEL-2 (C) and SK-MEL-24

(D) cell lines, depending on concentrations of imiquimod (5 μg/ml, 10 μg/ml, 30 μg/ml) in comparison with controls (0 μg/ml). Statistically significant differences were marked "*" ($P < 0.05$ vs control).

5. Imiquimod induced expression of TIMP-1 and -2 in human melanoma cells

Expression and change of TIMP-1 and -2 were evaluated according to the concentrations of imiquimod in two melanoma cell lines. Whole proteins from conditioned media of two melanoma cell lines treated with 0~30 μg/ml of imiquimod were collected and concentrated. TIMP-1 and -2 proteins were detected in all concentrations in both melanoma cell lines. Expression of TIMP-1 protein showed a tendency to increase according to increases in imiquimod in both cell lines (Figure 5A & 5B). TIMP-2 protein also expressed concentration-dependent increasing patterns in both cell lines (Figure 5C & 5D).

In real-time quantitative RT-PCR, expression and change of TIMP-1 and -2 mRNA showed a similar pattern with that of TIMP-1 and -2 proteins in both cell lines. mRNA expression of TIMP-1 in SK-MEL-2 cells revealed an increasing tendency according to concentration of imiquimod, but without statistical significance (Figure 5E). In SK-MEL-24 cells, mRNA expression of TIMP-1 also showed an imiquimod concentration-dependent increase with statistically significant differences at 30 μg/ml of imiquimod, as compared with untreated

cells ($P < 0.05$; Figure 5F). TIMP-2 mRNA were expressed with an increasing tendency according to concentration of imiquimod in SK-MEL-2 cells (Figure 5G). However, no statistical significance was observed at all concentrations of imiquimod. mRNA expression of TIMP-2 in SK-MEL-24 cells showed a significant concentration-dependent increase at all concentrations of imiquimod ($P < 0.05$; Figure 5H).

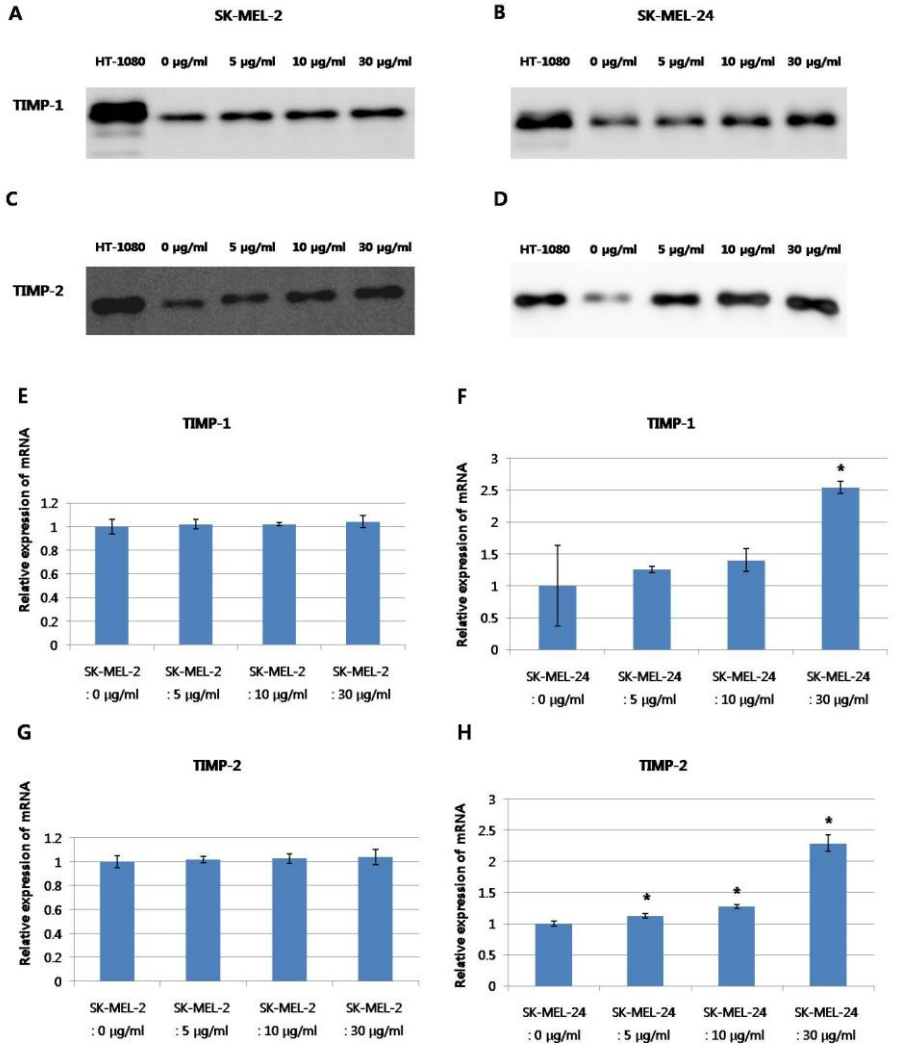

Figure 5. Expression of TIMP-1 and -2 protein and mRNA in SK-MEL-2 and SK-MEL-24 cell lines under imiquimod. Melanoma cells were treated with 0~30 μg/ml of imiquimod for 24 h and whole proteins from conditioned media were extracted and concentrated. The proteins were immunoblotted with anti-TIMP-1 and anti-TIMP-2 antibody. Expression of TIMP-1 protein showed an increasing pattern depending on the concentrations of imiquimod in SK-MEL-2 (A) and SK-MEL-24 cells (B). Expression of TIMP-2 protein also showed an increasing pattern depending on concentrations of imiquimod in SK-MEL-2 (C) and SK-MEL-24 cells (D). Real-time quantitative PCR in SK-MEL-2 and SK-MEL-24 cell lines under imiquimod. Relative mRNA expression of TIMP-1 in SK-MEL-2 (E) and SK-MLE-24 (F) cells and TIMP-2 in SK-MEL-2 (G) and SK-MLE-24 (H) cells, depending on concentrations of imiquimod (5 μg/ml, 10 μg/ml, and 30 μg/ml) in comparison with controls (0 μg/ml). Statistically significant differences were marked "*" ($P < 0.05$ vs control).

IV. DISCUSSION

Previous studies of imiquimod focused mainly on the antitumor effect of imiquimod through induction of profound cellular tumor-directed immune responses by inflammatory cells[6,7] or direct proapoptotic activity against tumor cells.[6,8,9] This study definitely showed that imiquimod suppressed invasion of melanoma cells in a concentration-dependent manner.

Cancer invasion and metastasis occurs through degradation of stromal connective tissue and basement membrane components.[14,15] In order to invade and metastasize, tumor cells adhere to and degrade ECM via secretion and activation of multiple proteolytic enzymes, particularly MMPs.[16] MMPs can be classified into six groups on the basis of their structure, substrate specificity, and cellular localizations. These consist of collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and others.[17-19]

Activity of MMPs is strictly controlled at the level of transcription, activation of precursor zymogens, interaction with ECM components, and inhibition by endogenous inhibitors in physiologic processes.[20-22] Increased activity of MMPs is related to tumor growth, invasion, and angiogenesis.[23-25] TIMPs are specific inhibitors that regulate the local activities of MMPs[20,26,27] and include TIMP-1,2,3, and 4.[23,28,29] Concentrations of TIMP in tissues are much higher than those of MMP, thereby controlling the proteolytic activity of MMPs.[23,30] Various proteolytic enzymes are involved in the process of melanoma

progression. Melanoma cells show expression of MMPs, MMP-1, -2, -9, -13, and MT1-MMP, and TIMPs, TIMP-1, 2, and -3.[12,17]

Among MMPs, MMP-2 and its inhibitor, TIMP-2, have been found to control type IV collagen, which is the main component of the basement membrane; therefore, MMP-2 is an important enzyme known to degrade surrounding ECM components during cancer invasion and metastasis.[31,32] MMP-2 is a gelatinase that dissolves denatured collagens, gelatins. MMP-2 also has collagenolytic activity on type I, II, and III collagens. A relationship between MMP-2 expression and prognosis of melanoma has been reported. An increase in MMP-2 expression was found to show correlation with hematogenous metastasis of melanoma.[17,33,34] In this study, expression of MMP-2 showed imiquimod concentration-dependent decreases in both melanoma cell lines. The results may be associated with the suppressive effect of imiquimod in melanoma invasion and metastasis.

MMP-9 is also one of the gelatinases and has the ability to degrade type IV collagen, a major component of the basement membrane. MMP-9 plays a key role in the early stages of tumor invasion and metastasis that is inhibited by TIMP-1.[35] Imbalance in production of MMP-9 and TIMP-1 relates to control of extracellular matrix degradation, thereby affecting tumor invasion and metastasis.[36] The role of MMP-9 in melanoma progression is controversial. Expression of MMP-9 in primary melanoma was observed in the horizontal growth phase, not in the vertical growth phase.[17,37] On the other hand,

expression of MMP-9 was observed, not in a melanoma cell line derived from early primary melanoma, but in a melanoma cell line derived from advanced primary melanoma.[38] Tumor cells from spontaneous lymph node and pulmonary metastases expressed mostly MMP-9, whereas those from experimental metastases did not express MMP-9.[39] In this study, expression of MMP-9 in the two melanoma cell lines changed conversely under imiquimod. Expression of MMP-9 increased in the SK-MEL-2 cell line and decreased in the SK-MEL-24 cell line according to the increase in imiquimod concentration. This differing tendency in the two cell lines may be related to the origin of the melanoma cell line. The SK-MEL-2 cell line was derived from skin metastasis of melanoma and the SK-MEL-24 cell line was derived from lymph node metastasis of melanoma. Selected tumor cells in different environments may have different properties for invasion and metastasis to different organs; therefore, metastasis to various organs may occur by different mechanisms. These characteristics of each cell could be associated with the response to imiquimod. Further study will be needed in order to find molecules involved in control of MMP-9 expression in melanoma cell lines. Expression of TIMP-1, a natural inhibitor of MMP-9, showed an imiquimod-concentration-dependent increase in both cell lines. In this study, suppressed invasion of the two melanoma cell lines was independent of changes in MMP-9 expression. From these findings, this study suggests that increases in TIMP-1 by imiquimod might play a more important role in control of melanoma cell invasion than changes in MMP-9.

MT1-MMP is a membrane-bound MMP that plays a dual role in degradation of ECM in the tumor invasion process.[40,41] MT1-MMP can activate proMMP-2 and degrade a number of ECM components, including fibronectin, vitronectin, and collagens.[42-45] In this role, MT1-MMP promotes tumor invasion and metastasis. In primary and metastatic melanoma, expression of MMP-2 and MT1-MMP showed a definite increase. MMP-2 and MT1-MMP co-expressing melanoma cells were often located at the front of the melanoma invasion process. Melanoma cells expressing MMP-2 also showed co-expression of MT1-MMP and TIMP-2.[17,46] Among TIMP members, TIMP-2 was unique, bridging the interaction between MMP-2 and MT1-MMP. At low TIMP-2 concentrations, proMMP-2 can effectively be activated by MT1-MMP that is free of TIMP-2. Under conditions of high TIMP-2 concentrations, most MT1-MMP forms a complex with TIMP-2, and these complexes function as receptors for proMMP-2. The ternary complex of MT1-MMP/TIMP-2/proMMP-2 interacts with adjacent free-MT1-MMP and proMMP-2 can be activated. Through this process, activation of proMMP-2 is somewhat decreased.[47,48] In this study, MMP-2 and MT1-MMP showed an imiquimod-concentration-dependent decrease and TIMP-2 showed an imiquimod-concentration-dependent increase. These changes have a synergistic effect on suppression of melanoma invasion through MMPs and TIMPs.

During melanoma progression, over-expression of TIMP-1, -2, and -3 was observed.[49] Over-expression of TIMP-1 and -2 has the effect of reducing

experimental metastasis of melanoma.[50,51] TIMPs were also reported to have apoptosis-inducing properties.[52] In this human melanoma cell line, expression of TIMP-1 and -2 showed an increase, depending on imiquimod. Increases in TIMP-1 and -2 can directly suppress the activity of MMPs in melanoma, thereby inhibiting melanoma invasion and metastasis.

Only a single study[13] has been conducted for evaluation of *in vivo* changes of MMPs and TIMPs after application of imiquimod on cutaneous metastastic melanoma from a patient through real-time quantitative RT-PCR. Results of the study showed up-regulation of TIMP-1 and down-regulation of MMP-9 under imiquimod. MMP-2 was not modified under imiquimod.[13] However, this study demonstrated TIMP-1 elevation and MMP-2 suppression under imiquimod in two melanoma cell lines. Under imiquimod, MMP-9 was increased in the SK-MEL-2 melanoma cell line and decreased in the SK-MEL-24 cell line. The possible reason for these differences between i*n vivo* and i*n vitro* study results was that expression of MMPs and TIMPs by infiltrated inflammatory cells was included in an in vivo study, in addition to melanoma cells, and the author had only investigated changes in MMPs and TIMPs at transcription levels. Findings from a previous study[13] did not conclude that the change of MMP and TIMP mRNA expression was exclusively associated with melanoma cells or other infiltrating cells which were abundant in lesions after imiquimod treatment on metastatic melanoma. However, the results of this study showing that imiquimod itself affected expression of MMPs and TIMPs in melanoma cells

at protein levels and mRNA levels appear to be significant. Several types of inflammatory cells, including mast cells[53] or B cells[54] can produce MMPs. The effect of imiquimod should be studied regarding induction or suppression of MMPs or TIMPs on various inflammatory cells. In addition, in order to further investigate the anti-invasive and anti-metastatic effects of imiquimod, *in vivo* studies using a human or mouse model will be needed.

In the future, cancer drugs that control MMP and TIMP activity will be further developed for clinical use. These results suggest that imiquimod may be used to suppress cancer progression, such as cancer invasion and metastasis.

V. CONCLUSION

Imiquimod induced concentration-dependent suppression of invasion in human malignant melanoma cell lines. And imiquimod also led to concentration-dependent changes of MMPs and TIMPs. These results suggest that imiquimod may have an anti-invasive effect on human melanoma cells via regulation of balances between MMPs and TIMPs.

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ABSTRACT(IN KOREAN)

악성흑색종 세포의 침습에 관여하는 matrix metalloproteinases와 tissue inhibitors of metalloproteinase에 대한 imiquimod의 영향

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정 진 영

Imiquimod는 표재성 피부암과 바이러스성 사마귀에 사용되는 면역반응조절제로 수술적 치료가 어려운 표피내 흑색종이나 전이 흑색종의 치료에 사용된 다수의 보고가 있다. 피부 악성 흑색종은 침습과 전이를 잘 하는 경향이 있어 예후가 좋지 않다. 이러한 악성 흑색종의 침습과 전이 과정에서 matrix metalloproteinases (MMPs)와 같은 단백분해효소와 이것의 억제제인 tissue inhibitors of metalloproteinases (TIMPs)에 의한 기저막의 분해와 세포외 기질의 재구성은 필수과정이다.

본 연구는 SK-MEL-2와 SK-MEL-24라는 2개의 인간 악성 흑색종 세포주에서 imiquimod에 의한 악성 흑색종 세포의 침습 억제효과를 조사하였다. 또한, imiquimod에 의한 암의 침윤과 전이 과정에서 일어나는 주변 세포외 기질의 분해에 관여하는 주된 효소인 MMP-2, -9, membrane type 1 matrix metalloproteinase (MT1-MMP)와 그것의 억제제인 TIMP-1, 2 발현의 변화를

조사하였다.

Imiquimod의 농도가 증가함에 따라 *in vitro*상에서 악성 흑색종 세포의 생존력이 감소하였다. 또한, 두 악성 흑색종 세포주에서 침습검사상 imiquimod에 의해 악성 흑색종의 침습이 억제되었다. 두 세포주 모두에서 imiquimod 농도가 증가함에 따라 단백질 수준에서 MMP-2와 MT1-MMP의 발현은 감소하였고, TIMP-1과 TIMP-2의 발현은 증가하였다. 그러나, MMP-9의 단백질 발현은 imiquimod 농도가 증가함에 따라 SK-MEL-2 세포주에서는 증가하였고, SK-MEL-24 세포주에서는 감소하였다. 실시간 역전사중합효소연쇄반응에서 imiquimod에 의한 MMPs와 TIMPs의 mRNA수준에서 변화는 imiquimod에 의한 단백질 수준에서의 변화와 유사한 양상을 보였다.

이러한 결과들은 imiquimod가 MMP와 TIMP의 조절을 통해 인간 악성 흑색종 세포의 침습을 억제하는 항침습 효과를 보여주는 것으로 생각된다.

핵심되는 말 : imiquimod, 침습, matrix metalloproteinase, 악성 흑색종, tissue inhibitor of metalloproteinase