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피부암 세포에서 IL-6/soluble IL-6 Receptor Signaling의 기능연구

조선대학교 대학원

의 학 과

Wagley Yadav

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지도교수 오 재 옥

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의학과

Wagley Yadav

Wagley Yadav의 박사학위논문을 인준함

위원장 **조선대학교** **교수** _____(인)

위원 조선대학교 교수 _____(인)

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위원 조선대학교 교수 _____(인)

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조선대학교 대학원

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ABSTRACT

Functional Studies of IL-6/soluble IL-6 Receptor Signaling in Melanoma

By: Wagley Yadav

Advisor: Prof. Oh Jae-Wook Ph.D.

Department of Medicine,

Graduate School of Chosun University

Interleukin-6 (IL-6) is a cytokine which is involved in the regulation of inflammatory and immunologic responses. IL-6-mediated signaling is initiated by its specific binding to the cognate receptor (IL-6Rα), and then this complex binds to gp130 to occur biological activities. The IL-6Rα form exists as either a membrane-bound or a soluble form. The soluble form of the IL-6Ṛ (sIL-6R) is generated by shedding of the membrane-bound forms or by mRNA alternative splicing. sIL-6R can also form a complex with IL-6, resulting in activation of signal transduction.

The highly metastatic F10.9 melanoma cells, derived from a spontaneous B16 melanoma of C57B1/6 mice, have been shown to be insensitive to IL-6; however, this unresponsiveness can be restored by IL-6, in conjunction with sIL-6R. It has been shown that co-treatment of IL-6 with sIL-6R resulted in growth inhibition and morphological change of F10.9 melanoma cells through p21 accumulation. These findings suggest IL-6/sIL-6R treatment may affect the cytotoxic responsiveness of melanoma cells to other toxic stimuli. IL6RIL6 is a highly active fusion protein of the soluble form of IL-6R and IL-6; this fusion protein has been shown to be highly active in gp130 positive cells. In chapter 1 of these studies, we investigated whether the IL-6/sIL-6R system potentiates the cytotoxic responsiveness of TNF-α in TNF-α-resistant B16/F10.9 melanoma cells. We found that IL6RIL6 sensitizes TNF-α-resistant B16/F10.9 melanoma cells to TNF-α-mediated apoptosis; this effect was associated

with both the upregulation of TNF-R55 expression and the reduction of Bcl-2 expression in response to IL6RIL6. These results suggest that the IL-6/sIL-6R/gp130 system, which sensitizes TNF-α-resistant melanoma cells to TNF-α-induced apoptosis, may provide a new target and for immunotherapy.

Nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) is an important bioactive material and signaling molecule that mediates a variety of biological actions such as vasodilatation, neurotransmission, and host defense. In chapter 2, we explored whether various cytokines can modulate the expression of the *iNOS* gene in melanoma cells. We found that IL6RIL6 plus TNF-α potently induced iNOS gene expression. Gel shift and reporter gene analyses revealed that IL6RIL6 selectively activated AP-1; while TNF-α increased the activities of NF-κB. Stimulation of cells with IL6RIL6 plus TNF-α resulted in the activation of mitogen-activated protein kinases (MAPK) such as c-Jun N-terminal kinase (JNK), ERK1/2 and p38. In addition, we found that JNK and p38 MAPK are involved in the activation of c-Jun and c-Fos, components of AP-1, in response to IL6RIL6 plus TNF-α. These results suggest that IL-6/sIL-6R/gp130 complex signaling has an unexpected positive effect on *iNOS* gene expression through JNK/p38 MAPK mediated-AP-1 activation in melanoma cells.

Astrocytes are the resident glial cells in the central nervous system (CNS) that support brain capillary endothelium, and are in intimate contact with brain endothelial cells via perivascular end-feet. Exposure of astrocytes to inflammatory cytokines, such as TNF-α, IL-lβ, and IFN-γ, induce the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1). In chapter 3 of the present studies, we examined the effect of human plasma high density lipoproteins (HDL) and other lipoproteins on VCAM-1 expression in astroglioma cells. The exposure of astroglioma cells to the major plasma lipoprotein fractions (VLDL, LDL and HDL) had no effect on the VCAM-1 expression. However, TNF-α-induced VCAM-l was inhibited by HDL in a dose-dependent manner, but not by VLDL or LDL. The inhibitory effect of HDL on TNF-α-induced VCAM-l was reversed by the inclusion of apo A-I antibody, the major apolipoprotein of HDL, demonstrating the specificity of

this response. Reconstituted HDL (discoidal complex of apo HDL and DMPC), but not apo HDL or DMPC, was effective in suppressing the VCAM-1 expression. RNase protection assay (RPA) revealed that TNF-α-induced VCAM-l mRNA expression was markedly inhibited by HDL (500 μg cholesterol/ml). These results indicate that HDL-like particles in the CNS may function as an immunosuppressive molecule in pathologic conditions of CNS.

CHAPTER 1

Introduction

1-1. INTERLEUKIN-6

Interleukin-6 (IL-6), first identified in 1981, is known to be produced by T cells, B cells and monocytes as well as non-lymphoid cells such as fibroblasts, keratinocytes, endothelial cells, mesangial cells, and several tumor cells (for review, see Ref (1)). It acts as a pro- or anti-inflammatory cytokine, and is capable of inducing target genes involved in differentiation, proliferation, and apoptosis and in acute-phase immune responses of the organism (2). IL-6 belongs to the Interleukin-6-type cytokine family that also consists of Interleukin-11 (IL-11), Leukemia Inhibitory Factor (LIF), Oncostatin M (OSM), Ciliary NeuroTrophic Factor (CNTF), Cardiotrophin-1 (CT-1), Cardiotrophin-Like Cytokine (CLC), and Neuropoietin (NPN) (2, 3). Recently, IL-27 and IL-31 have also been included in the family (3).

IL-6, a pleiotrophic cytokine, elicits a broad range of biological activities. In activated B-cells, IL-6 augments immunoglobulin synthesis (4-6). It induces growth of T cells (6, 7), and acts as a growth factor for renal mesangial cells, epidermal keratinocytes (8), and various types of tumor cells such as multiple myeloma, plasmacytoma, and renal carcinoma (9, 10). On the contrary, an anti-clonogenic effect of IL-6 has been observed in human breast cancer cells (11, 12). Also, the growth of early stage melanoma and the tumor-metastasis in melanoma-bearing mice are inhibited by IL-6 (13). By augmenting the expression of IL-2 receptor and the production of IL-2, IL-6 supports the differentiation of cytotoxic T cells (14). IL-6 has also shown to induce differentiation of macrophages, megakaryocytes, and osteoclasts (8). In acute-phase reactions, IL-6 stimulates hepatocytes to produce acute-phase proteins such as C-reactive protein (CRP), fibrinogen, α_1-antitrypsin, and serum amyloid A (15, 16). Altered levels of IL-6 have been documented in a variety of human diseases including multiple myeloma, Castleman's disease, Crohn's disease, mesangial glomerulonephritis, osteoporosis, cardiac ischemia, cachexia, rheumatoid arthritis (RA), sepsis, and AIDS (for review, see Ref (17)). Increased IL-6 level have also been associated with a number of skin and mucous membrane pathologies, such as psoriasis, lupus erythematosus and Sjorgren's syndrome (18).

1-2. INTERLEUKIN-6 TRANS-SIGNALING

The membrane-bound glycoprotein receptor complex mediating the biological activities of the IL-6 is the non-signaling alpha-receptors (IL-6Rα, where R refers to receptor) and the signal transducer gp130 (2, 3). A naturally occurring soluble form of the IL-6R (sIL-6R) is generated by two mechanisms; extracellular domain shedding of the membrane protein and translation from an alternatively spliced mRNA (19). The sIL-6R together with IL-6 is functional on the cells that express gp130, a process named trans-signaling. The cells which are unresponsive to IL-6 due to the lack of membrane-bound IL-6R become responsive by the presence of sIL-6R/IL-6 complex. For instance, embryonic stem cells, early hematopoietic progenitor cells, colon cancer cells in transgenic mice, many neural cells, smooth muscle cells, and endothelial cells can be responsive to IL-6 in the presence of sIL-6R (for review, see Ref (20)). The sIL-6R is found in serum, urine, synovial fluid and cerebrospinal fluid (CSF) (21, 22), and augmented serum levels have been documented in a variety of diseases (23-25). In addition to the sIL-6R, soluble gp130 (sgp130) is also found in human serum (26). It has been demonstrated that sIL-6R potentiates the antagonistic activity of sgp130 (20).

To study the IL-6 trans-signaling mechanism, IL6RIL6, a fusion protein consisting of the sIL-6R and IL-6 has been designed (27, 28). This chimeric protein has been found to be highly active with gp130 positive cells, comparing to that of IL-6/sIL-6R complex (28-30).

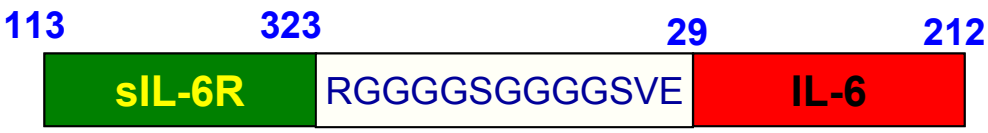

Fig. 1-1. Structure of sIL-6R and IL-6 fusion protein, IL6RIL6.

Fig 1-2A

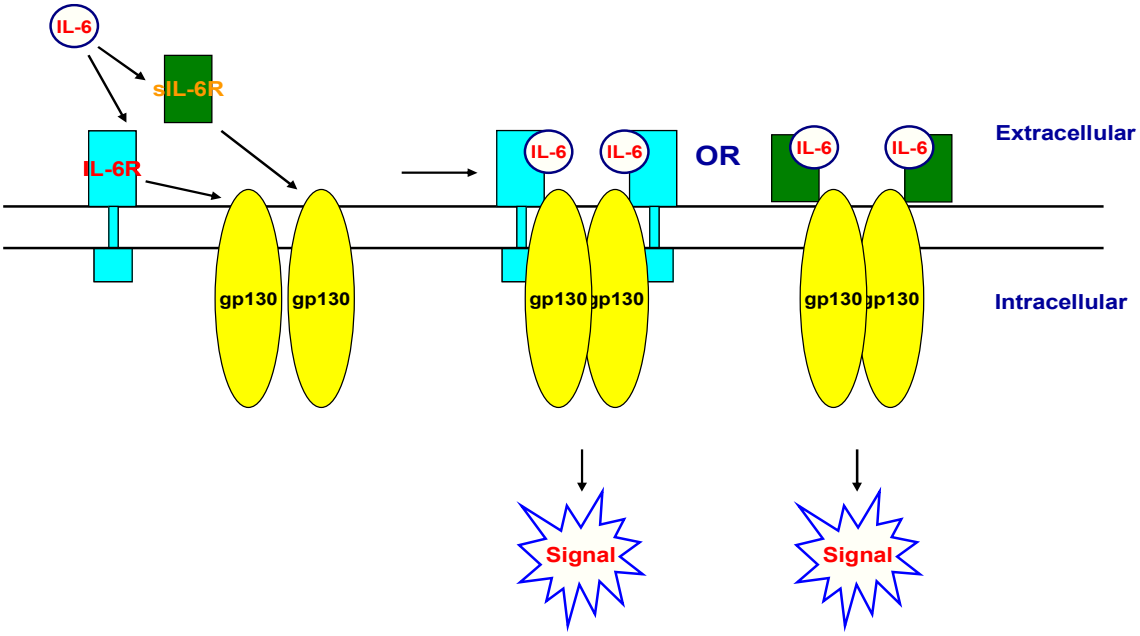

Fig 1-2B

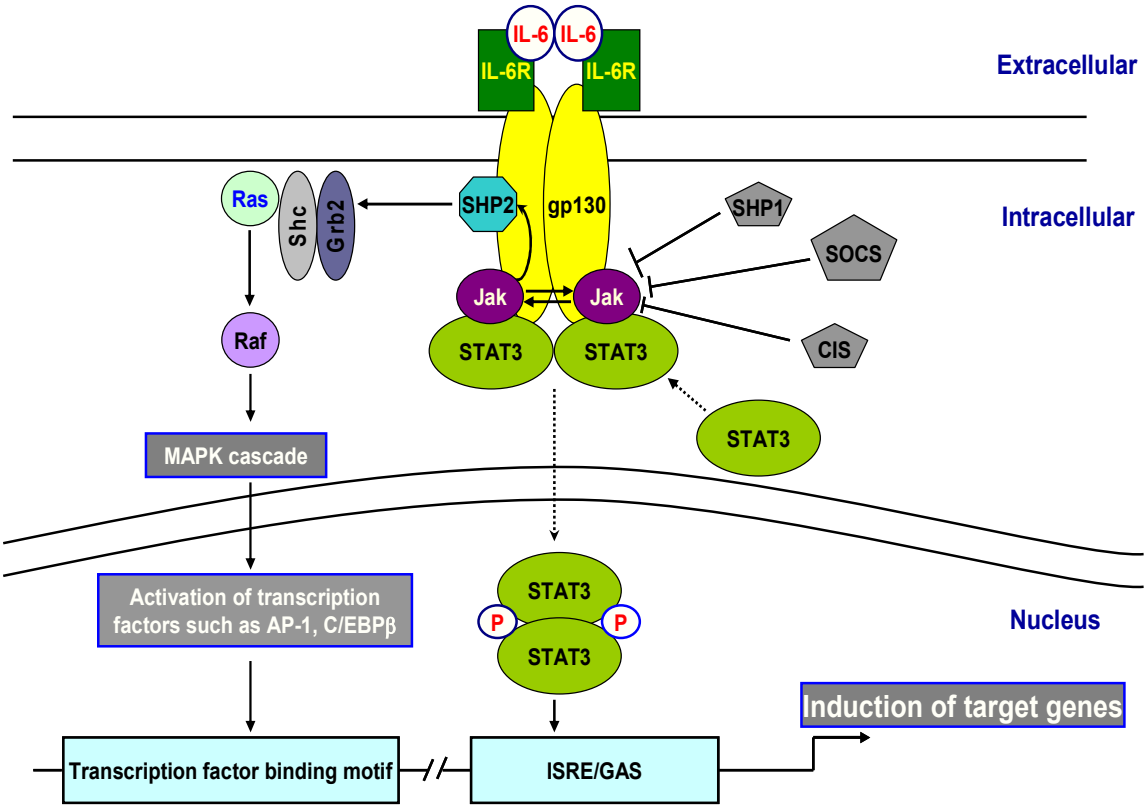

Fig. 1-2. Interleukin-6 mediated signal transduction pathway.

1-3. INTERLEUKIN-6 MEDIATED SIGNAL TRANSDUCTION

The binding of IL-6 to the cognate receptor induces dimerization of the receptors, and triggers activation of intracellular signaling cascades including rapid internalization of the ligand (31). Upon ligand-mediated receptor hexamerization, two JAKs (janus-activated kinase) are brought into close proximity, allowing trans-phosphorylation. Activated JAKs subsequently phosphorylate additional targets, including the receptors and the latent cytoplasmic transcription factor, signal transducer and activator of transcription (STAT), initiating the signal transduction pathways (32). Activated JAK1 is believed to phosphorylate six tyrosines on the cytoplasmic domain of gp130 subunits. Of the six tyrosine residues, the second recruits SH2 domain, and other with the YXXQ motif serve as a docking site for STAT3. Subsequently, JAK1 phosphorylates Tyr^{705} at the C-terminus of STAT3. This phosphotyrosine permits the dimerization and nuclear entry of STATs (33). The nuclear entry of STAT proteins is dependent on importin α-5 (also called nucleoprotein interactor 1) and the ran nuclear import pathway. In the nucleus, STAT proteins recognize and bind to the consensus oligonucleotide sequences TTCC(C or G)GGAA (32, 33).

For the activation of the MAPK (mitogen-activated protein kinase) cascade by IL-6 mediated signaling, the SHP2 (SH2-domain-containing tyrosine phosphatase)-binding site Tyr^{759} of gp130 is crucial (34, 35). SHP2 links the Grb2-SOS (growth-factor-receptor-bound protein/Son of sevenless) complex and/or Gab1 (Grb2-associated binder-1) to gp130 (35-37). Two C-terminal tyrosine residues within SHP2 (Tyr^{542} and Tyr^{596}) are believed to interact with the Grb-SOS complex. Finally, recruitment of SOS to the receptor complex at the membrane allows Ras activation, which in turn leads to the activation of the Ras-Raf-MAPK cascade (38-40). IL-6 can activate PI3K (phosphoinositide 3-kinase) in cardiac myocytes, multiple myeloma and basal cell carcinomas. In these cells, gp130 mediated PI3K/Akt activation involves the direct interaction of the adaptor protein Gab1 with PI3K (2).

1-4. TUMOR NECROSIS FACTOR-α MEDIATED SIGNAL TRANSDUCTION

Tumor necrosis factor-α (TNF-α), first identified and cloned in 1984, is a

pleiotrophic cytokine that elicits a broad spectrum of cellular responses including macrophage activation, fever, acute phase response, as well as cell proliferation, differentiation, and apoptosis (for review, see Ref (41)). There are two TNF receptors of 55 and 75 kDa, both of which exhibit specific and high-affinity binding to TNF-α. Both TNF receptors belong to the TNF receptor superfamily, which also includes the nerve growth factor receptor (NGFR), the Fas antigen, CD40, OX40, CD27, and lymphotoxin beta receptor (LTBR) (42). The TNF-R1 (p55) is responsible for mediating most of the actions of TNF including antiviral activity, apoptosis, cytokine production, and induction of a number of gene products, while the function of TNF-R2 (p75) is mainly related to T cell development and the proliferative action of cytotoxic T cells (for review, see Ref (43)).

The binding of TNF-α to TNF-R1 triggers a series of intracellular events that ultimately result in the activation of several major transcription factors, including nuclear factor-κB (NF-κB) and c-Jun that are responsible for the expression of target genes important for cell growth and death, development, oncogenesis, inflammation, and stress responses (for review, see Ref (44)). The initial step in TNF mediated signaling involves the binding of the TNF to the extracellular domain of TNF-R1 and the release of the inhibitory protein silencer of death domains (SODD) from TNF-R1's intracellular domain (ICD). The resulting aggregated TNF-R1 ICD complex is recognized by the adaptor protein TNF receptor-associated death domain (TRADD), which recruits receptor-interacting protein (RIP), TNF-R-associated factor (TRAF2), and Fas-associated death domain (FADD) that are responsible for initiating signaling events. Then, caspase-8 is recruited to the TNF-R1 complex and becomes activated by FADD, initiating an apoptotic cascade. TRAF2 recruits antiapoptotic proteins named cellular inhibitor of apoptosis protein-1/2 (cIAP-1/2) which also have the ubiquitin protein ligase activity. TRAF2 is also thought to activate a mitogen-activated protein kinase kinase kinase (MAPKKK), such as extracellular signal-regulated kinase kinase kinase 1 (MEKK1) or apoptosis-stimulated kinase 1 (ASK1), in a complex at or near the receptor, thereby activating a cascade of kinases resulting in the activation of c-Jun NH_2-terminal kinase (JNK), a kinase that phosphorylates c-Jun (45). Finally, the

protein kinase RIP is critical for the activation of the transcription factor NF-κB (46).

Fig 1-2

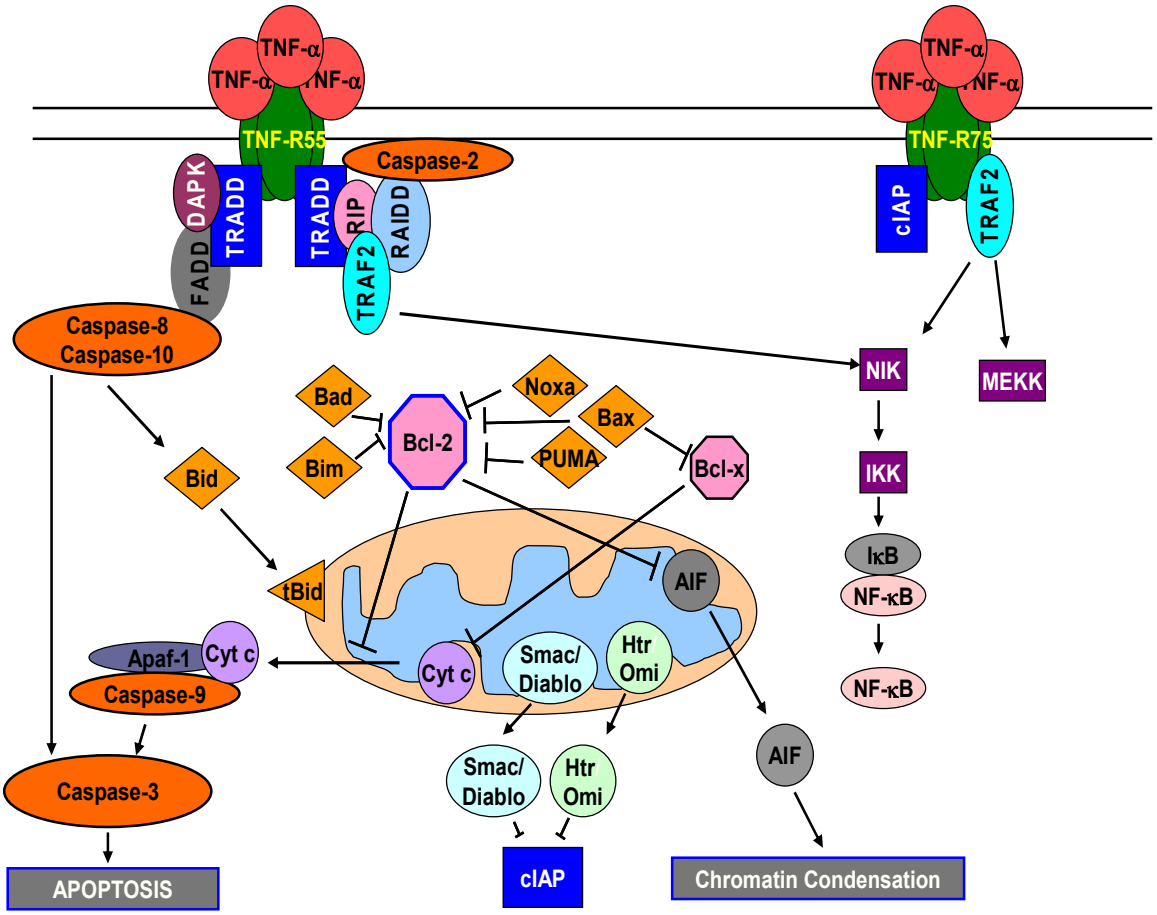

Fig. 1-2. TNF-α mediated signal transduction pathway.

CHAPTER 2

The IL-6/sIL-6R treatment of a malignant melanoma cell line enhances susceptibility to TNF-α-induced apoptosis

2-1. ABSTRACT

Melanoma is an intractable tumor that has shown very impressive and promising response to local administration of high dose recombinant TNF-α in combination with IFN-γ in clinical studies. In this study, we investigated the effect of the IL-6/sIL-6R on TNF-α-resistant B16/F10.9 melanoma cells. A low dose of TNF-α or IL-6/sIL-6R had minimal affect on the cell growth. However, the highly active fusion protein of sIL-6R and IL-6 (IL6RIL6), covalently linked by a flexible peptide, sensitized TNF-α-resistant F10.9 melanoma cells to TNF-α-induced apoptosis. Stimulation of the cells with IL6RIL6 plus TNF-α resulted in both the activation of caspase-3 and the reduction of Bcl-2 expression. Flow cytometry analysis showed that IL6RIL6 upregulated TNF-R55 and TNF-R75 expression, suggesting an increase in TNF-α responsiveness by IL6RIL6 resulting from the induction of TNF receptors. Moreover, exposure of F10.9 cells to neutralizing antibody to TNF-R55 significantly inhibited IL6RIL6/TNF-α-induced cytotoxicity. These results suggest that the IL-6/sIL-6R/gp130 system, which sensitizes TNF-α-resistant melanoma cells to TNF-α-induced apoptosis, may provide a new target and for immunotherapy.

KEYWORDS: IL-6; sIL-6R; TNF-α; TNF-R55; caspase-3; Bcl-2; Apoptosis

2-2. INTRODUCTION

The annual incidence of malignant melanoma is increasing at a faster rate than any other cancer (47). The relative resistance of melanoma cells to chemotherapy has led to a search for novel alternative approaches to treatment in clinical trials, including immunotherapy. Clinical studies on the administration of IL-2, IFN-α/γ, and TNF-α each alone or in combination have been reported (48, 49). TNF-α alone for the treatment of melanoma has not been shown to be sufficient for effective suppression of tumor growth. However, synergistic effects with TNF-α can be achieved in combination with chemotherapeutic agents (i.e., melphalan) or with IFN-γ (50, 51).

TNF-mediated signaling events occur by its binding to two distinct receptors; TNF-R55 and TNF-R75. TNF-R55 is thought to be involved in mediating cytotoxicity, antiviral activity, and fibroblast proliferation; TNF-R75 is involved in the proliferation of thymocytes and cytotoxic T cells (52). The apoptotic response mediated by TNF-α results from the formation of TNF/TNF-R55/TRADD/TRAF/RIP complexes and intracellular signaling transducers, leading to caspases activation (53).

The biological activity of interleukin 6 (IL-6) is initiated by its specific binding to the cognate IL-6 receptor α (IL-6Rα), and this complex then binds to gp130. The IL-6Rα form exists as either a membrane-bound or a soluble form. The soluble form of IL-6R (sIL-6R) can also form a complex with IL-6, in association with gp130, resulting in activation of signal transduction. The sIL-6R is found in serum and urine (54, 55); augmented serum levels have been documented in a variety of diseases (56, 57). IL-6 has been found to be a potent inhibitor of A375 melanoma cell adhesion and proliferation (58, 59). We previously showed that co-treatment of IL-6 with sIL-6R resulted in growth inhibition and morphological change of F10.9 melanoma cells through p21 accumulation (27). Hence, our findings suggest IL-6/sIL-6R treatment affects the cytotoxic responsiveness of melanoma cells to other toxic stimuli.

The highly metastatic F10.9 melanoma cells, derived from a spontaneous B16 melanoma of C57B1/6 mice, have been shown to be insensitive to IL-6; however, this unresponsiveness can be restored by IL-6, in conjunction with sIL-6R (27). IL6RIL6

is a highly active fusion protein of the soluble form of IL-6R and IL-6; it has been shown to be highly active with gp130 positive cells (27, 60).

In the present study, we investigated whether the IL-6/sIL-6R system potentiates the cytotoxic responsiveness of TNF-α in TNF-α-resistant B16/F10.9 melanoma cells. We found that IL6RIL6 sensitized TNF-α-resistant B16/F10.9 melanoma cells to TNF-α-mediated apoptosis; this effect was associated with both the upregulation of TNF receptors and the reduction of Bcl-2 expression in response to IL6RIL6.

2-3. MATERIALS AND METHODS

2-3-1. Cells and reagent

A Murine B16 metastatic melanoma clone, F10.9 cells, was cultured in DMEM with 8% FBS, supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. A fused IL6RIL6 chimera (IL6RIL6), a secreted 85-kDa protein, was obtained from Dr. Michel Revel (Weizmann Institute, Israel). Human recombinant IL-1β, IFN-γ, TNF-α, and IL-6 were obtained from Peprotech (Rehovot, Israel). Anti-mouse gp130 and anti-mouse TNF-R55 neutralizing Ab were purchased from R&D System (Minneapolis, MN). TNF-R75 neutralizing Ab was purchased from Biolegend (San Diego, CA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit/-mouse/-hamster IgGs were purchased from the Jackson ImmunoResearch Lab (West Groove, PA).

2-3-2. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the TRIzol reagent (Life Technologies, Inc., MD) as previously described (61). Ten micrograms of RNA were reverse-transcribed using Oligo(dT) 18mer as a primer and M-MLV reverse transcriptase (Bioneer, Korea) to produce the cDNAs. PCR was performed using selective primers for mouse TNF-R55 (sense primer: 5'-AGTGAGACACACTTCCAG-3'; antisense primer: 5'-ACTACCTCACTGACAGGT-3'), the mouse TNF-R75 (sense primer: 5'-GACAGGAAGGCTCAGATGTGCT-3'; antisense primer: 5'-GCATTTCCGGGAATAGCCAGG-3'), the mouse Bcl-2 (sense primer: 5'-AAGCTGTCACAGAGGGGCTA-3'; and antisense primer: 5'-CCTGAAGAGTTCCTCCACCA-3'), and mouse GAPDH genes (sense primer: 5'-GTCTTCACCACCATGGAGAAGG-3'; antisense primer: 5'-CGTTCAGCTCTGGGATGACCTTG-3'). PCR was carried out for 32 cycles using the following conditions; denaturation at 94°C for 0.5 min, annealing at 60°C for 0.5 min, and elongation at 72°C for 0.5 min.

2-3-3. Measurement of cell viability

The cells were plated on 48 well plates (Nunc, Roskilde) for the assay, and then stimulated with IL-1β, IFN-γ, IL-6, TNF-α, IL6RIL6, and IL6RIL6 plus TNF-α for 72 h. Cell viability was determined by the crystal violet staining as previously described (62). In brief, cells were stained with 0.4% crystal violet in methanol for 30 min at room temperature (RT), and then washed with tap water. Stained cells were extracted with 50% methanol, and dye extracts were measured at a 540 nm wavelength using a microtiter plate reader (Bio-Tek, Vermont).

2-3-4. Western blotting

Cells were lysed in lysis buffer (M-PER® mammalian protein extraction reagent (Pierce, Rockfold, IL), supplemented with 1 mM phenylmethylsulfonylfluoride (PMSF), 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 mM sodium fluoride, and 1 mM sodium orthovanadate. Soluble lysates (40 μg) were subjected to SDS–PAGE and transferred to a nitrocellulose membrane, and then the membrane was probed with specific Abs, followed by HRP-conjugated anti-IgG Ab. ECL was used for the detection.

2-3-5. Determination of caspase-3 activity and of nuclear apoptotic cells by nuclear staining

For detection of caspase-3 activity, cells (2 x 10^5/well) were plated on six-well plates (SPL, Korea), and were incubated with medium alone, IL6RIL6, TNF-α, and IL6RIL6 plus TNF-α for 72 h. Soluble lysates (40 μg) were subjected to SDS–PAGE and transferred to a nitrocellulose membrane, and then the membrane was probed with anti-caspase-3 Ab (Cell Signaling, Beverly, CA).

For the determination of nuclear apoptotic cells, cells (2 x 10^4/well) were plated on 24-well plates (Nunc, Roskilde), and then the cells were incubated with medium alone, IL6RIL6, TNF-α, and IL6RIL6 plus TNF-α for 72 h. Afterwards, the culture medium was replaced with medium containing 10 μg/ml of the DNA-binding fluorochrome PI. Cells were incubated for 15 min at 37°C and thereafter immediately assessed using a

laser scanning confocal microscope (FV300, Olympus, Japan). Nuclear staining and morphology were documented photographically and evaluated by digital image analysis. For each sample, 500–1000 nuclei were counted, and the number of apoptotic cells was expressed as percentages.

2-3-6. Analysis of TNF-R55 and TNF-R75 protein expression by immunofluorescence flow cytometry

F10.9 cells (2 x 10^5/well) were plated in six-well plates (SPL, Korea). Duplicate wells were incubated with medium alone or IL6RIL6 for 48 h. Cells were trypsinized, suspended in PBS containing 5% FBS and 0.02% azide, stained with anti-mouse TNF-R55 or TNF-R75 Ab, washed with cold PBS, and then stained with FITC-labeled anti-hamster IgG. After washing three times, cells were fixed in 1% paraformaldehyde and analyzed for TNF-R55 or TNF-R75 expression on the FACStar (Becton–Dickinson, Mountain View, CA). We have previously demonstrated that trypsinization does not affect cell surface expression of receptor proteins (63). Ten thousand cells were analyzed for each sample.

2-3-7. Neutralization bioassay

F10.9 cells (4 x 10^3/well) were plated in 96-well plates (SPL, Korea). Normal mouse serum (NMS, 10 μg/ml), anti-gp130 (10 μg/ml) Ab, anti-TNF-R55 (5 μg/ml) Ab, and anti-TNF-R75 (5 μg/ml) Ab were pretreated to the cells for 1 h at 37°C, and then IL6RIL6 or TNF-α were added and incubated for an additional 72 h. Cell viability was determined by crystal violet staining.

2-3-8. Statistical analysis

The Student's t-test was used to assess significant differences among the treatment groups. The criterion for statistical significance was set at $p < 0.05$.

2-4. RESULTS

2-4-1. Cell growth inhibitory effects of the IL-6/sIL-6R and TNF-α on melanoma cell growth

We investigated whether melanoma cell growth was affected by cytokines known to be related to melanoma progression. To initiate this study, mouse F10.9 cells were treated with the inflammatory cytokines, TNF-α, IL-1β, IFN-γ, IL-6 (1–100 ng/ml), or IL6RIL6 (10–500 ng/ml) for 72 h, cell viability was assessed by crystal violet staining. As shown in Fig. 2-1, cell viability was decreased with TNF-α (~40% inhibition at 100 ng/ml) or IL6RIL6 treatment (~35% to ~60% inhibition at 100 or 500 ng/ml). However, IL-6 alone did not affect F10.9 cell growth; suggesting the lack of functional IL-6R expression on the cells (27). IL-1β and IFN-γ did not influence cell growth.

2-4-2. Synergistic growth inhibitory effects by IL-6/sIL-6R plus TNF-α and apoptosis induced by IL-6/sIL-6R plus TNF-α

To determine whether the IL-6/sIL-6R in conjunction with TNF-α has a synergistic effect on the F10.9 cells causing cell growth inhibition, we treated F10.9 cells for 72 h with IL-6 (10 ng/ml), IL6RIL6 (100 ng/ml), TNF-α (10 ng/ml), and IL6RIL6 (100 ng/ml) plus TNF-α (10 ng/ml). Interestingly, cell growth was dramatically decreased, by more than 80% with co-stimulation by IL6RIL6 and TNF-α, when compared to IL6RIL6 alone (~40% inhibition), and TNF-α alone (less than 5% inhibition) (Fig. 2-2A). Caspase-3 is one of the key molecules involved in apoptosis and activation of caspase-3 is required for the proteolytic processing of its inactive zymogen into active-19 and 17 kDa (64). To determine if the IL-6/sIL-6R plus TNF-α stimulation could activate apoptotic signaling in F10.9 cells, the cells were incubated with medium alone, IL6RIL6 (100 ng/ml), TNF-α (50 ng/ml), or both (100+50 ng/ml) for 72 h, and then western blot analysis was performed. As shown in Fig. 2-2B, the caspase-3 cleaved forms, 19-kDa and 17-kDa, were observed 72 h after stimulation with IL6RIL6 plus TNF-α, but not with either alone. In order to further confirm the IL-6/sIL-6R plus TNF-α induced apoptosis, a nuclear staining assay was performed using PI.

Chromatin condensation was also observed with IL6RIL6 plus TNF-α stimulation (Fig. 2-2C). As shown in Fig 2-2D, IL6RIL6 or TNF-α alone did not cause apoptosis compared to control cells (~5% and ~2%, respectively). However, the number of cells with strongly condensed nuclei was increased by IL6RIL6 plus TNF-α (~28%). These results indicate that the IL-6/sIL-6R plus TNF cause apoptotic cell death in F10.9 cells by activating caspase-3.

2-4-3. IL-6/sIL-6R-inducible upregulation of TNF-R55 is responsible for the potentiated cytotoxicity

TNF-α-mediated apoptosis is initiated by its binding to TNF-R55 or TNF-R75. To test if the levels of TNF-R55 or TNF-R75 expression on F10.9 cells are altered by the IL-6/sIL-6R, we next performed flow cytometry analyses using specific MAbs that recognize TNF-R55 or TNF-R75, respectively. The cells were incubated with medium alone or IL6RIL6 for 72 h, respectively. Fig. 2-3A and B illustrate enhancement of TNF-R55 and TNF-R75 protein expression by IL6RIL6. We next evaluated the functional consequences of the IL-6/sIL-6R-enhanced TNF-R55 and TNF-R75 on F10.9 cells. To establish the specificity of this effect, neutralizing Abs to the gp130, TNF-R55, and TNF-R75 were used (Fig. 2-3C). Anti-mouse gp130 Ab or TNF-R55 Ab pretreatment abolished IL6RIL6 plus TNF-α-induced apoptosis (lane 6; 36%→96%, lane 7; 36%→56%). However, anti-TNF-R75 Ab pretreatment did not alter the viability of F10.9 cells (lane 8; 36%→40%). As well, the anti-TNF-R75 Ab plus anti-TNF-R55 Ab mix did not demonstrate any significant additive effect compared to the anti-TNF-R55 Ab alone (lane 9; 36%→63%). Therefore, these results suggest that the IL-6/sIL-6R is responsible factor for the TNF-α-inducible cytotoxicity, and that IL-6/sIL-6R-upregulated TNF-R55 is a key receptor for TNF-α mediated apoptosis in F10.9 cells.

Fig. 2-1

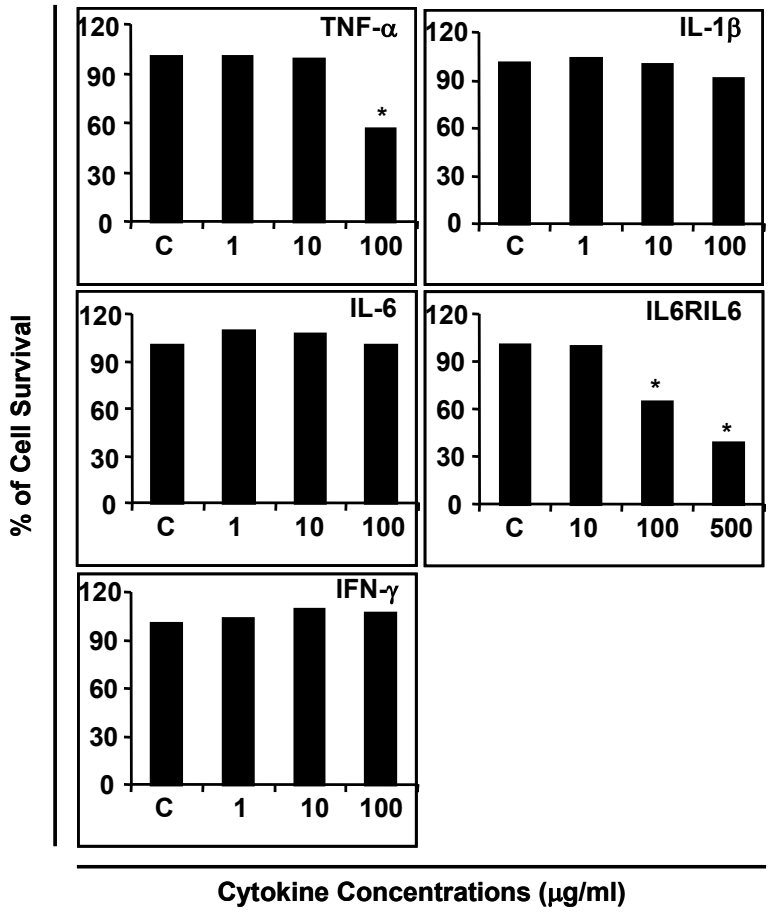

Fig. 2-1. The IL-6/sIL-6R and TNF-α inhibition of F10.9 cell growth. F10.9 cells (2 x 10^4) were incubated with TNF-α (1-100 ng/ml), IL-1β (1-100 ng/ml), IL-6 (1-100 ng/ml), IL6RIL6 (10-500 ng/ml), IFN-γ (1-100 ng/ml) for 72 h, then cell viability was analyzed by crystal violet staining. Control cell growth was set at 100% survival, and each experimental condition was calculated to determine the percentage of survival in the experimental and control cells. Representative of three experiments.

Fig. 2-2A

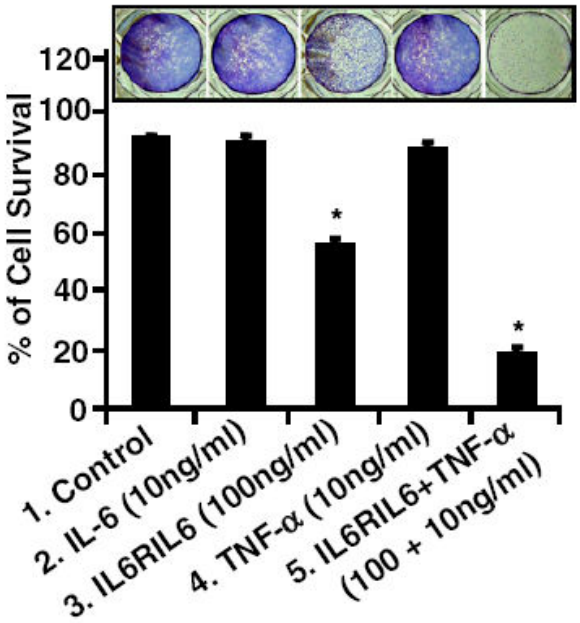

Fig. 2-2B

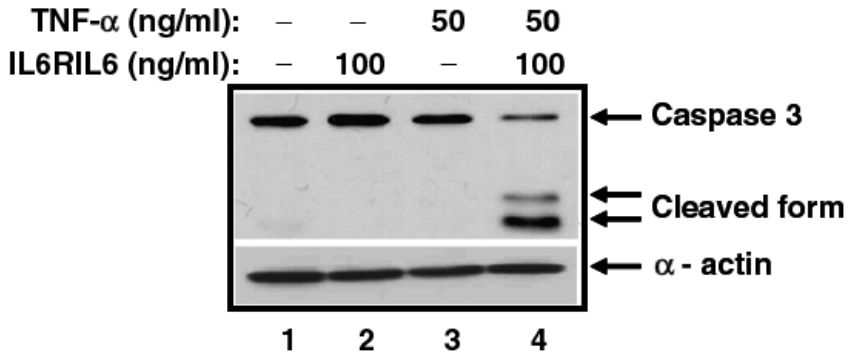

Fig. 2-2C

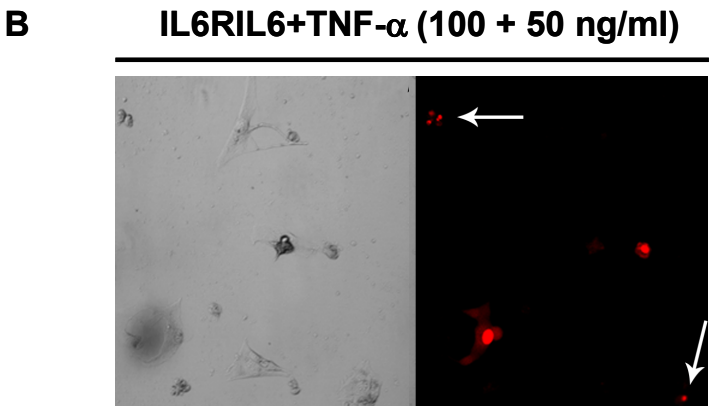

Fig. 2-2D

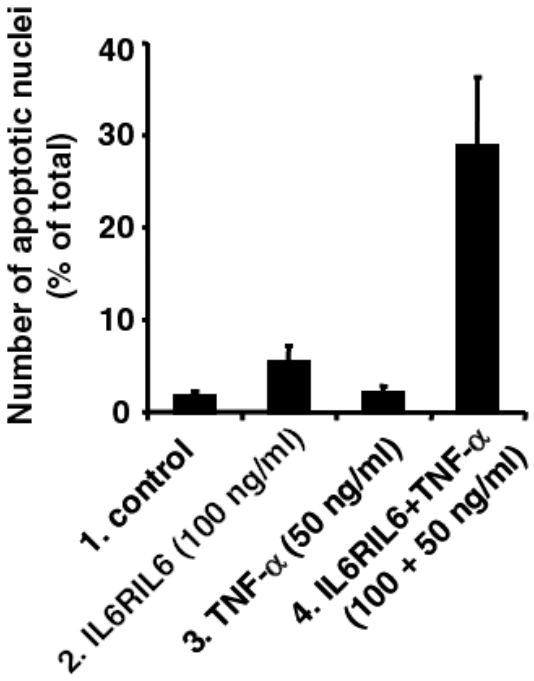

Fig. 2-2. The IL-6/sIL-6R in combination with TNF-α inhibits F10.9 cell growth. **A.** F10.9 cells (2 x 10^4) were incubated with medium alone (lane 1), IL-6 (lane 2; 10 ng/ml), IL6RIL6 (lane 3; 100 ng/ml), TNF-α (lane 4; 10 ng/ml), IL6RIL6 plus TNF-α (lane 5; 100+10 ng/ml) for 72 h. Relative cell viabilities were determined by crystal violet staining. The control cell growth was set at 100% survival, and each experimental condition was calculated to determine the percentage of survival in the experimental and control cells. Results represent the means ± SD of 3 separate experiments (significant versus the control, $*p<0.05$). **B.** F10.9 cells were incubated with medium alone (lane 1), IL6RIL6 (100 ng/ml, lane 2), TNF-α (50 ng/ml, lane 3), IL6RIL6 plus TNF-α (100+50 ng/ml, lane 4) for 72 h, and then the cells were lysed, loaded on 12% SDS-PAGE, blotted with anti caspase-3 Ab. Membrane was stripped, and reprobed for α-actin. **C.** Apoptosis was determined by apoptotic nuclei visualized by propidium iodide (PI) staining. Cells showing condensed nuclei (arrowheads) are indicated. **D.** Cells with apoptotic nuclei were calculated as a percentage of total cell numbers. The results are representative of three experiments.

Fig. 2-3A

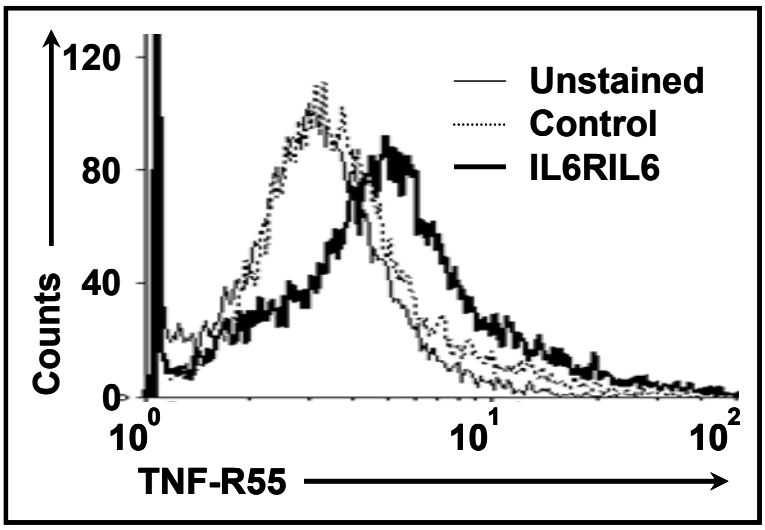

Fig. 2-3B

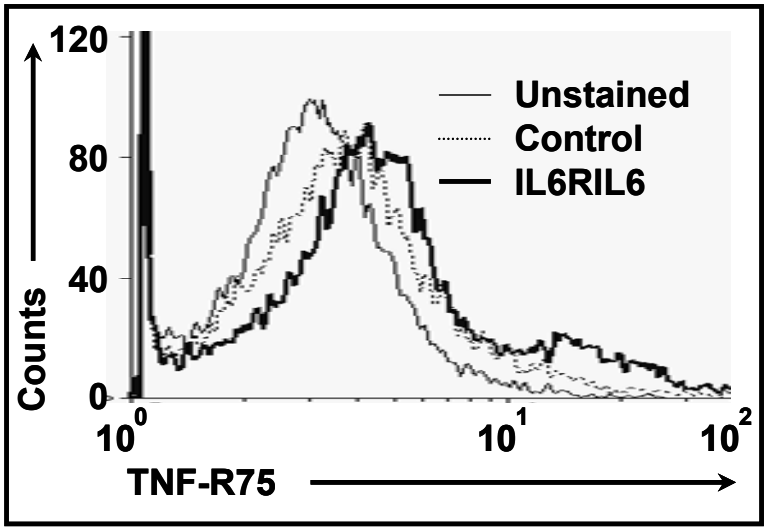

Fig. 2-3C

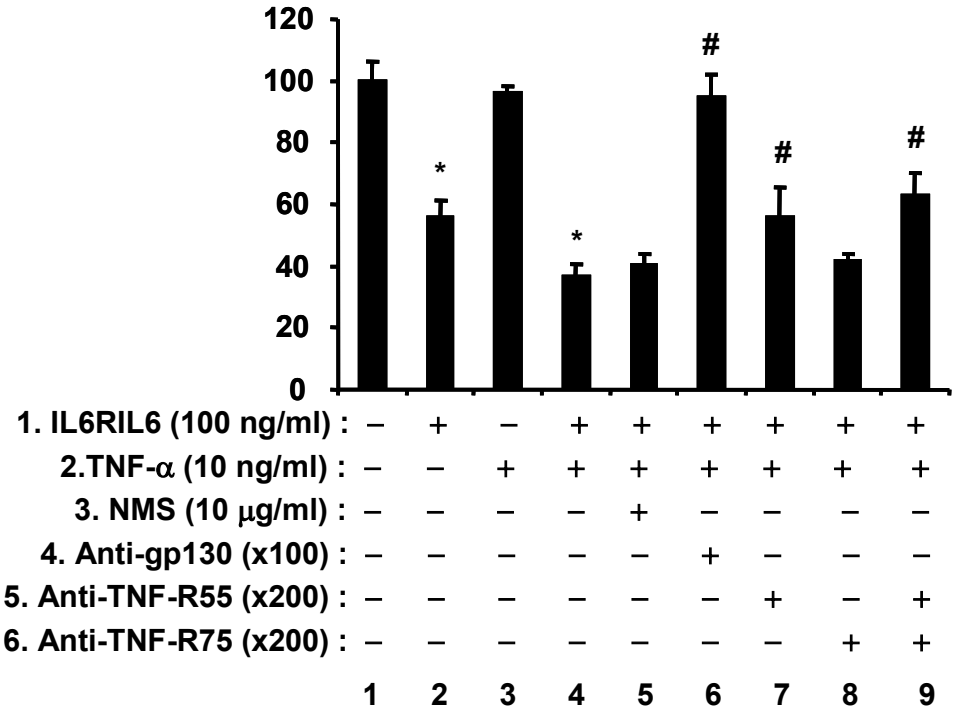

Fig. 2-3. The IL-6/sIL-6R upregulates TNF-R55 and TNF-R75 expression and the IL-6/sIL-6R-upregulated TNF receptor is biologically active. **A** and **B.** The cells were incubated with medium alone or IL6RIL6 (100 ng/ml) for 72 h. The cells were then trypsinized and TNF-R55 (A) and TNF-R75 (B) expression was assessed by FACs analyses. A thin solid line in *A* and *B* shows unstimulated cells, a dotted line shows isotype control in IL6RIL6 stimulated cells, a thick solid line shows positively stained cells with anti-TNF-R55 or anti-TNF-R75 Ab in IL6RIL6 stimulated cells. The results are representative of five experiments as shown. **C.** F10.9 cells (4 x 10^3/well, 96 plate) were preincubated without or with NMS (lane 5; 10 μg/ml), anti-gp130 Ab (lane 6; 10 μg/ml), anti-TNF-R55 Ab (lane 7; 5 μg/ml), anti-TNF-R75 Ab (lane 8; 1 μg/ml), or anti-TNF-R55/75 Abs (lane 9; 5 μg/ml each) respectively for 60 min at 37ºC, then IL6RIL6 (100 ng/ml), or TNF-α (10 ng/ml) was used to treat cells for an additional 72 h. Cell viabilities were determined by crystal violet staining. Control cell growth was set at 100% survival, and each experimental condition was calculated to determine the percentage of survival compared to the control cells. The data is represented by the

mean ± SD of three separate experiments (significance was determined by comparing the experimental and control cells, *p<0.05; significant as compared with IL6RIL6/TNF-α-treated group, $^{\#}p$<0.05).

2-4-4. The IL-6/sIL-6R decreases expression levels of Bcl-2

Several lines of evidence suggest that molecules that induce apoptosis sensitize cancer cells by altering the expression level of Bcl-2 (65, 66). To determine the effects of the IL-6/sIL-6R on Bcl-2 expression patterns in F10.9 cells, we performed RT-PCR and western blot analysis. As shown in Fig. 2-4, the F10.9 cells constitutively expressed Bcl-2 at high levels. However, with IL6RIL6 stimulation, the level of Bcl-2 expression was significantly reduced (30% at the mRNA and 46% at the protein level compared to controls, respectively), implying that Bcl-2 molecule may also be another key factor along with up-regulated TNF-R55 in TNF-α-induced apoptosis.

Fig 2-4A

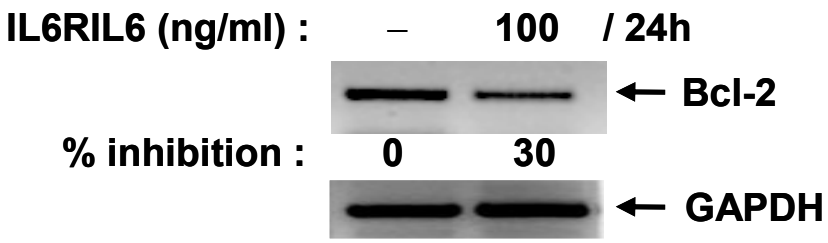

Fig 2-4B

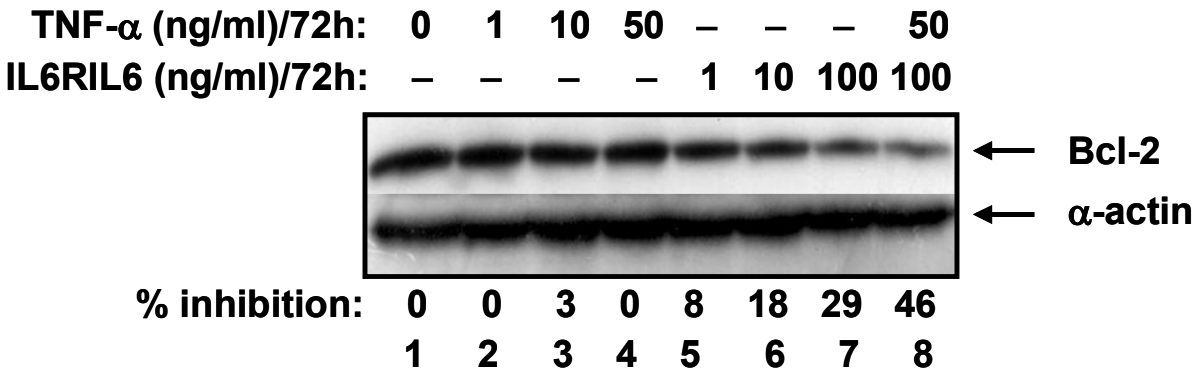

Fig. 2-4. The IL-6/sIL-6R decreased expression level of Bcl-2. **A.** F10.9 cells were incubated with medium alone or IL6RIL6 (100 ng/ml) for 24 h. The preparation of total RNA and RT-PCR was performed. Values for Bcl-2 mRNA were normalized to GAPDH mRNA levels for each experimental condition. Constitutive expression of Bcl-2 mRNA was set at 1; IL6RIL6 treatment was compared with control levels to arrive at the fold induction value. The results are representative of three experiments. **B.** The cells were incubated with medium alone, IL6RIL6 (1-100 ng/ml), TNF-α (1-50 ng/ml), or together for 72 h, and then equal volumes (40 μg) of cell lysate were loaded on 10% SDS-PAGE, blotted with anti-Bcl-2 Ab. Membranes were reprobed for α-actin. The results are representative of three experiments.

2-5. DISCUSSION

In this study, we investigated the possible potentiation of TNF-α-mediated cytotoxicity by inclusion of the IL-6/sIL-6R, and the functional properties of TNF-R55 in this system on B16/F10.9 melanoma cells. In the F10.9 mouse melanoma cells, IL6RIL6 and TNF-α were the only cytokines capable of inducing cell growth inhibition; TNF-α induced apoptosis was enhanced by inclusion of the IL-6/sIL-6R, based on nuclear condensation, induced caspse-3 activity, and reduction of Bcl-2 expression.

In most cases of the melanoma cells studied, TNF/TNF-R55 signaling was found to induce NF-κB activation, resulting in anti-apoptosis and inflammation (45). However, under certain conditions, apoptosis can be triggered by TNF/TNF-R55 signaling-mediated caspases activation or decrease in anti-apoptotic molecule expression when protein or RNA synthesis is blocked by cycloheximide or actinomycin D, or when NF-κB activity is diminished (67, 68). F10.9 melanoma cells are resistant to TNF-α-mediated cell death. However, we previously observed NF-κB activation by TNF-α or the IL-6/sIL-6R plus TNF-α at an early point in time in the F10.9 cells (61). In addition, our study suggests that the IL-6/sIL-6R/TNF-α induction of apoptosis did not occur at an early point in time (0-24 h), but rather occurred at later points in time (48-72 h). These results indicate that activated NF-κB likely causes TNF-α to display anti-apoptotic activity in F10.9 cells at an early point in time. Our results clearly demonstrated that inclusion of the IL-6/sIL-6R in TNF-α treated F10.9 cells triggers nuclear condensation and induced caspase-3 activity at late point in time, resulting in F10.9 cell death. These results suggest the possibility that the IL-6/sIL-6R may provide positive enhancement of TNF-α-mediated apoptosis *in vivo*.

Currently, TNF-α is clinically used for human cancer therapy in combination with with melphalan or with IFN-γ known to upregulate TNF-Rs expression and down-regulate Bcl-2 expression in melanoma (49, 51, 69). In the case of IL-6, studies have established that the IL-6/sIL-6R influences apoptotic processes; Kovalovich et al. reported that IL-6 protects cells from Fas-mediated apoptosis through modulation of Fas and anti-apoptotic gene induction in the liver (70). In Crohn's disease, the IL-6/sIL-6R mediates resistance of T cells to apoptosis (71). By contrast, our results in the

melanoma cell line, F10.9 cells, showed that the IL-6/sIL-6R directly caused cell growth inhibition and together with TNF-α supported recovery of TNF-α-induced apoptosis in TNF-α-resistant cells. In this regard, IL6RIL6-upregulated TNF-R55 expression and the reversal of cell growth inhibition with TNF-R55 Ab; these findings are also important because TNF-R55 upregulation may increase TNF-α responsiveness to TNF receptors (Fig. 2-3C).

We also evaluated the role of the Bcl-2, anti-apoptotic molecule. Bcl-2 is involved in the control of mitochondrial permeability by forming pores in the outer membrane or by regulating the opening and closing of the permeability pores (64). The Bcl-2 is expressed in both normal melanocyte as well as in advanced melanoma cell lines (72). In this study, we observed that constitutive expression of Bcl-2 in F10.9 cells was down-regulated by the IL-6/sIL-6R (Fig. 2-4), suggesting that Bcl-2-mediated apoptosis by the IL-6/sIL-6R/TNF-α could be initiated through caspase-3 activation by activated caspase-9 dissociation from the complex by release of mitochondrial cytochrome *C*. However, the regulation of another anti-apoptotic genes such as Bcl-xL, Mcl-1, c-FLIP by IL-6/sIL-6R/TNF-α may also be considered because anti-TNF-R55 Ab could not completely abrogate the IL-6/sIL-6R/TNF-α enhanced apoptosis; however, anti-gp130 Ab could do (Fig. 2-3C).

A recent report by Micheau et al provides an explanation for TNF/TNF-R55 signaling; after TNF-α binding to the TNF-R55, complex I composed by RIP1, TRADD, TRAF2, and cIAP1 are formed in the early stage leading to NF-κB activation; then RIP1, TRADD, and TRAF2 are dissociated and replaced by FADD and caspase-8 to form complex II in late stages. This complex II initiates caspase-8 activation, resulting in cell death (73). This scenario gives rise to the question, what regulator(s) are involved in stimulating TNF/TNF-R signaling to take alternative directions: cell survival or death? One possible answer may be provided by the IL-6/sIL-6R/gp130 as a coordinated cytokine *in vivo*.

Our results highlight the importance of the IL-6/sIL-6R/gp130 system in the sensitization of TNF-α-resistant F10.9 melanoma cells to TNF-α-mediated apoptosis. These results may help identify a novel target for immunotherapy.

CHAPTER 3

Novel role of IL-6/sIL-6R signaling in the expression of inducible nitric oxide synthase (iNOS) in murine B16, metastatic melanoma clone F10.9, cells

3-1. ABSTRACT

Inducible nitric oxide synthase (iNOS) has been shown to be frequently expressed in melanomas; up-regulation of this enzyme is thought to be associated with tumor progression. In this study, we investigated whether diverse cytokines such as: IL-6, TNF-α, IL-1β, IFN-γ and IL6RIL6 (a highly active fusion protein of the soluble form of the IL-6R (sIL-6R) and IL-6) enhance the *iNOS* gene expression in B16/F10.9 murine metastatic melanoma cells. An increase at iNOS expression and NO production was observed with the co-treatment of IL6RIL6 plus TNF-α. Gel shift and reporter gene analyses revealed that IL6RIL6 selectively activated AP-1; while TNF- α increased the activities of both NF-κB and AP-1. Persistent activation of AP-1 was also seen in cells treated with IL6RIL6 plus TNF-α. Stimulation of cells with IL6RIL6/TNF-α resulted in the activation of mitogen-activated protein kinases (MAPK) such as c-Jun N-terminal kinase (JNK) and p38, and abrogation by pretreatment with JNK or p38 MAPK inhibitor. IL6RIL6 or IL6RIL6/TNF-α- inducible AP-1 binding increase was supershifted by anti-c-Jun or c-Fos antibodies, and the activation of c-Jun and c-Fos was dependent on JNK and p38, respectively. These results suggest that IL-6/sIL-6R/gp130 complex signaling has an unexpected positive effect on *iNOS* gene expression through JNK/p38 MAPK mediated-AP-1 activation in melanoma cells.

KEYWORDS: Nitric Oxide; Cytokines; Transcription Factors; Cell Activation.

3-2. INTRODUCTION

Interleukin 6 (IL-6) is a pleiotropic cytokine that is not only involved in the regulation of inflammatory and immunologic responses, but also acts in other biological systems (74, 75). IL-6 promotes the differentiation of B cells and subsequent Ig production, expands hematopoietic progenitor cells, and induces the expression of a variety of acute phase proteins. IL-6 functions as a proinflammatory cytokine in modulating immune and inflammatory responses by enhancing leukocyte recruitment (75). However, under some circumstances, IL-6 serves as an anti-inflammatory cytokine by inhibiting TNF-α expression (76). Biological activity of IL-6 is initiated by its specific binding to the cognate IL-6 receptor α (IL-6Rα) expressed on the cell surface. The IL-6/IL-6R complex induces the homodimerization of a signal transducing subunit, gp130. These events lead to activation of gp130-associated Janus kinases (JAKs), activation of a variety of signaling pathways (STATs or MAPK), and resulting in subsequent gene expression (74, 75). The IL-6Rα form exists as either a membrane-bound or a soluble form. A soluble form of the IL-6R (sIL-6R) can be generated by shedding of the membrane-bound forms or by mRNA alternative splicing (77, 78). sIL-6R can also form a complex with IL-6, in association with gp130 as a membrane-bound form, resulting in activation of signal transduction. The sIL-6R is found in serum, urine, synovial fluid and cerebrospinal fluid (54, 55), and augmented serum levels have been documented in a variety of diseases (23, 56, 57).

Melanocytes in early stage human melanoma are very sensitive to IL-6; however, in advanced metastatic melanoma the melanocytes lose their sensitivity to IL-6 (79). In this study, the highly metastatic F10.9 melanoma cells, derived from a spontaneous B16 melanoma of C57B1/6 mice, were used. These cells are completely insensitive to IL-6; however, this unresponsiveness can be restored by IL-6, in conjunction with sIL-6R. We have previously showed that co-treatment of IL-6 plus sIL-6R resulted in growth inhibition and morphological change of F10.9 melanoma cells through p21 accumulation (27). IL6RIL6 is a highly active fusion protein of the soluble form of the IL-6R and IL-6; it has been shown to be highly active with gp130 positive cells (27).

Nitric oxide (NO) is an important bioactive material and signaling molecule that

mediates a variety of biological actions such as vasodilatation, neurotransmission, and host defense; it has been proposed to contribute to the pathogenesis of cancer (80). The genes for three isoforms of NOS have been identified; two of these isoforms are calcium-dependent and constitutively expressed; these are endothelial NOS (type III) and neuronal NOS (type I). Type II iNOS is a calcium-independent and an inducible form responsive to LPS and cytokines such as the TNF-α and IFN-γ. iNOS has been shown to be frequently expressed in melanomas; up-regulation of the enzyme has been observed to be associated with tumor progression (80). Although the exact function of iNOS in carcinogenesis remains unclear, NO overproduction may affect the development or progression of melanoma. It has been shown that transfection with the *iNOS* gene in murine melanoma cells suppresses tumorigenicity and abrogates metastasis (81).

In the present study, we explored whether various cytokines increase the expression of the *iNOS* gene in F10.9 melanoma cells. We found that IL6RIL6/TNF-α potently induced iNOS expression. As a key transcription factor for the iNOS induction with IL6RIL6/TNF-α stimulation, we found that IL6RIL6-dependent AP-1 activation is required. In addition, we found that JNK and p38 MAPK were involved in the activation of c-Jun and c-Fos, components of AP-1 activation in response to IL6RIL6/TNF-α.

3-3. MATERIALS AND METHODS

3-3-1. Cells and reagents

Murine B16 metastatic melanoma clone F10.9 cells were cultured at 37ºC, with 5% CO_2, in Dulbecco's modified Eagle's medium with 8% fetal bovine serum, supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were subcultured every 3 days at 10-30% confluence. For all experiments, cells were grown to 80%-90% confluence, and were subjected to no more than 20 cell passages.

A fused IL6RIL6 chimera was obtained from Dr. Michel Revel (Weizmann Institute of Science and Technology, Rehovot, Israel). It was produced using mammalian Chinese hamster ovary (CHO) cells; the secreted 85-kDa protein was purified using an immuno-affinity column. Human recombinant TNF-α, IL-1β, IFN-γ and IL-6 were purchased from Peprotech (Rehovot, Israel). SB202190, PD98059, and SP600125 were purchased from Calbiochem (San Diego, CA). Anti-PCNA, anti-c-Rel (p65), anti-I-κBα, anti-c-Jun, anti-c-Fos, anti-Jun B, anti-Jun D and anti-Fos B antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38, and anti-phospho-I-κBα antibodies were supplied from Cell Signaling (Beverly, CA). Anti-iNOS antibody was obtained from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were supplied by the Jackson ImmunoResearch Lab (West Groove, PA). TRITC-conjugated anti-rabbit IgG was obtained from Zymed Laboratories Inc. (San Francisco, CA). Other reagents for the molecular studies were supplied from Sigma Chemical (St Louis, MO).

3-3-2. Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from cells incubated with each cytokine for 24 h. Briefly, total RNA was extracted using TRIZOL reagent (Life Technologies, Inc., Rockville, MD) as previously described (82). Ten micrograms of RNA was reverse-transcribed using Oligo(dT) 18mer as a primer and M-MLV reverse transcriptase

(Bioneer, Eumsung, Korea) to produce the cDNAs. PCR was performed using the selective primers for the mouse iNOS (sense primer: 5'-CTGCAGCACTTGGATCAGGAACCTG-3'; antisense primer: 5'-GGGAGTAGCCTGTGCACCTGGAA-3') and mouse GAPDH genes (sense primer: 5'-GTCTTCACCACCATGGAGAAGG-3'; antisense primer: 5'-CGTTCAGCTCTGGGATGACCTTG-3'). PCR was carried out for 35 cycles using the following conditions: denaturation at 94°C for 0.5 min, annealing at 60°C for 0.5 min, elongation at 72°C for 0.5 min. The band intensities of the amplified DNAs were compared after visualization with a UV transilluminator.

3-3-3. Preparation of nuclear extracts

F10.9 cells were washed with ice-cold PBS, scraped, and transferred to microtubes. Cells were allowed to swell by adding 100 μl of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet-P40, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride (PMSF)]. Tubes were vortexed to disrupt cell membranes. The samples were incubated for 10 min on ice and centrifuged for 5 min at 4°C. Pellets containing crude nuclei were resuspended in 60 μl of the extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM PMSF, and then incubated for 30 min on ice. The samples were centrifuged at 15,800g for 10 min to obtain supernatant containing the nuclear extracts. The nuclear extracts were stored at -70°C until use.

3-3-4. Western blotting

Cell lysates were prepared as previously described (83). Briefly, cells were lysed in lysis buffer (M-PER® mammalian protein extraction reagent (Pierce, Rockfold, IL), supplemented with 1 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 mM sodium fluoride, and 1 mM sodium orthovanadate. Soluble lysates (40 μg) were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane, then the membrane was probed with specific antibodies, followed by horseradish peroxidase-conjugated anti-IgG antibody. Enhanced chemiluminescence (ECL) was

used for the detection of bound antibody.

3-3-5. Measurement of nitric oxide (NO)

F10.9 cells (2 x 10^4/well) were plated in 48-well plates (Nunc, Roskilde); NO was determined by measuring the levels of nitrite and nitrate in the culture medium using the total nitric oxide assay kit (Assay Designs, Ann Arber, MI). Briefly, 50 μl of culture supernatant was incubated with a mixture containing 25 μl of reduced beta nicotinamide adenine denucleotide (NADH) and 25 μl of nitrate reductase for 30 min at 37°C. Then, 25 μl Griess I solution containing sulfanilamide and 25 μl Griess II solution containing N-(1-Naphthyl)ethylenediamine were added and incubated at RT for additional 10 min. The absorbance of the reaction mixture was read at 550 nm. The nitrite concentration was calculated by comparing samples with a standard curve prepared using sodium nitrate.

3-3-6. Electrophoretic mobility shift assays

DNA binding activity was examined using oligonucleotide probes for nuclear factor-κB (NF-κB) and activated protein-1 (AP-1). A double stranded DNA probe for the consensus sequence of NF-κB, (5'-AGTTGAGGGGACTTTCCCAGGC-3') and for AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3') were used after end-labeling with [γ-^{32}P] ATP and T_4 polynucleotide kinase. The reaction mixture contained 2 μl of 5 × binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly dI-dC and 50 mM Tris-Cl (pH 7.5), 5 μg of nuclear extracts and sterile water in a total volume of 9 μl. Incubation was initiated by addition of a 1 μl probe (2 x 10^4 cpm/sample) and continued for 20 min at RT. For competition assays, an aliquot of nuclear extract (5 μg each) was pre-incubated with a 100-fold excess of unlabeled probe and 1 μg of specific anti-c-Jun, anti-c-Fos, anti-Fos B, anti-Jun B, or anti-Jun D antibodies for 30 min at RT. Samples were loaded onto 4% polyacrylamide gels at 250 V. The gels were removed and dried, followed by autoradiography.

3-3-7. Immunofluorescence microscopy

F10.9 cells (4 x 10^4/well) were plated on Lab-TEK chamber slides® (Nalge Nunc International Corp.) and grown to 80% confluence. At that time, the original medium was aspirated off; fresh 2 % serum medium (200 μl) was added to the wells, and then cells were left overnight. Standard immunocytochemical methods were used as previously described (83). Briefly, cells were fixed at -20°C in 100% methanol for 1 h. The cells then were incubated in blocking buffer (5% BSA in PBS) overnight at 4°C and subsequently incubated for 1.5 h at RT with anti-c-Fos or anti-c-Jun (1:100 dilution) antibodies in 1% BSA containing PBS. Immune complexes were detected with TRITC-conjugated goat anti-rabbit IgG antibodies (1:100 dilution) in 1% BSA containing PBS after incubation for 40 min at RT and examined using a laser scanning confocal microscope (FV300, Olympus, Japan).

3-3-8. Construction of iNOS promoter luciferase construct and NF-κB and AP-1 reporter gene assays

To determine the activity of the *iNOS* gene transactivation, we used the pGL-miNOS-1588 luciferase reporter assay system. To generate miNOS promoter-luciferase construct (pGL-miNOS-1588), mouse genomic DNA was isolated from the mouse tail using the SV genomic DNA isolation kit (Promega, Madison, WI). The miNOS promoter region from –1588 bp to +165 bp was amplified by the polymerase chain reaction (PCR) using specific primers (sense: 5'-GGTACCGACTTTGATATGCTGAAATCCATA-3' antisense: 5'-AGATCTAGTTGACTAGGCTACTCCGTG-3') and ligated into the pGEM-T easy vector (Promega, Madison, WI). The amplified product, whose DNA sequence was verified by an automatic DNA sequencer, was subcloned into the Kpn I/Bgl II site of the pGL3-basic plasmid.

To perform the reporter gene assay, cells were plated onto 12-well plates (Nunc, Roskilde) and transfected on the following day. A dual-luciferase reporter assay system (Promega, Madison, WI) was used to determine promoter activity. Briefly, the

cells were transiently transfected with 1 μg of pGL-miNOS1588, pNF-κB-Luciferase or pAP-1-Luciferase plasmid and 20 ng of the pRL-SV plasmid (Promega, Madison, WI) using Lipofectamine2000® Reagent (Invitrogen, Gaithersburg, MD), and then incubated with medium alone, IL6RIL6, TNF-α, or IL6RIL6/TNF-α for 18 h. Firefly and Renilla luciferase activities in the cell lysates were measured using a luminometer (Turner Designs; TD-20, CA). Relative luciferase activity was calculated by normalizing iNOS, NF-κB or AP-1 promoter-driven firefly luciferase activity versus that of Renilla luciferase.

3-3-9. The Decoy oligodeoxynucleotide technique

Double-stranded oligodeoxynucleotides (ODN) of AP-1 were prepared from complementary single-stranded phosphothioate-bonded ODN (Bioneer, Eumsung, Korea) by melting at 95°C for 5 min and followed by a cool-down phase at ambient temperature. The sequences of the single stranded ODN were as follows; AP-1, 5'-CGCTTGATGACTCAGCCGGAA-3'; and mutant AP-1, 5'-CGCTTGATTACTTAGCCGGAA-3' (84). Transfection of the decoy ODN was performed using Lipofectamine 2000® reagent (Invitrogen, Gaithersburg, MD).

3-3-10. Statistical analysis

Student's t-test was used to assess significant differences among treatment groups. The criterion for statistical significance was set at $p<0.05$.

3-4. RESULTS

3-4-1. Enhancement of iNOS expression by IL6RIL6/TNF-α

We were interested in assessing whether iNOS expression in melanoma cells could be modulated by cytokines known to be associated with the pathogenesis of melanoma (85). Highly metastatic F10.9 melanoma cells, derived from B16 melanoma, was treated with the following proinflammatory cytokines; TNF-α, IL-1β, IFN-γ, IL-6, and IL6RIL6, which is a fusion protein of sIL-6R and IL-6 (27), or LPS for 24 h. The melanoma cells did not constitutively express iNOS mRNA, and the expression level of iNOS mRNA was not detected in the cells treated with these cytokines (Fig. 3-1A). However, co-treatment of cells with IL6RIL6 and TNF-α increased the level of iNOS mRNA expression by 2.6- or 2.3-fold (Fig. 3-1B: lane 3; 1C: lane 4). TNF-α is one of the proinflammatory cytokines to induce iNOS in a variety of cell types (86). Nonetheless, iNOS mRNA expression was not affected by LPS plus TNF-α (1B: lane 6) or the other cytokines (IL-1β, IFN-γ, and IL-6) plus TNF-α (1B: lane 2 and 4-5). Although iNOS mRNA expression was not affected by IFN-γ plus IL6RIL6 (1C: lane 3), the level of iNOS mRNA was slightly enhanced by IL-1β plus IL6RIL6 (1C: lane 2) or LPS plus IL6RIL6 (1C: lane 5). These results suggest that IL-6-mediated signaling pathway may be one of the important pathways for the *iNOS* gene expression in melanoma cells.

The kinetics of *iNOS* gene expression induced by IL6RIL6/TNF-α was further examined. As shown in Fig. 3-1D, iNOS mRNA expression was clearly detected at 12 h, and still enhanced for up to 24 h, upon IL6RIL6/TNF-α stimulation. Western blot analyses confirmed that the iNOS protein level was first detected at 12 h, and increased at 48 h after IL6RIL6/TNF-α stimulation (Fig. 3-2A). To determine whether iNOS expressed in B16/F10.9 cells was functional, we determined NO production by IL6RIL6/TNF-α in melanoma cells. NO production was observed to be ~2.4-fold increased in cells treated by IL6RIL6/TNF-α, but not by IL6RIL6 or TNF-α alone (Fig. 3-2B). The data indicate that IL6RIL6/TNF-α induces iNOS expression at both the mRNA and the protein level, leading to the enhancement of NO production in B16/F10.9 cells.

Fig. 3-1A

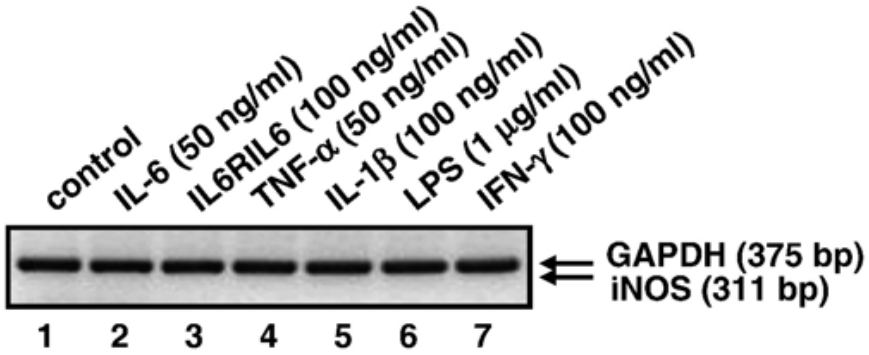

Fig. 3-1B

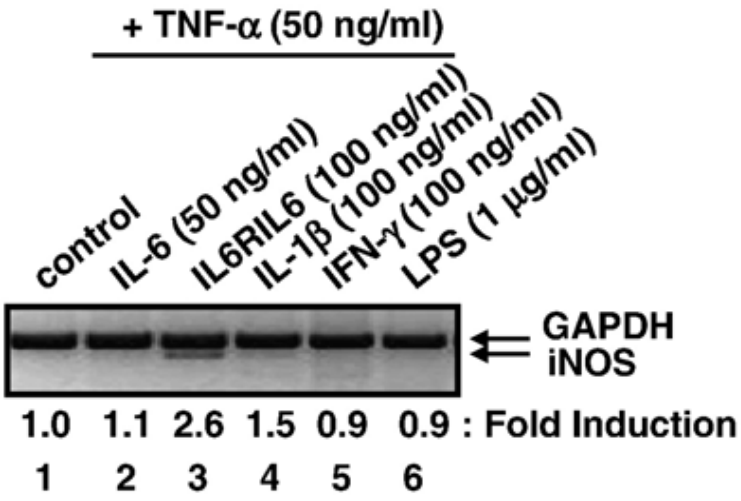

Fig. 3-1C

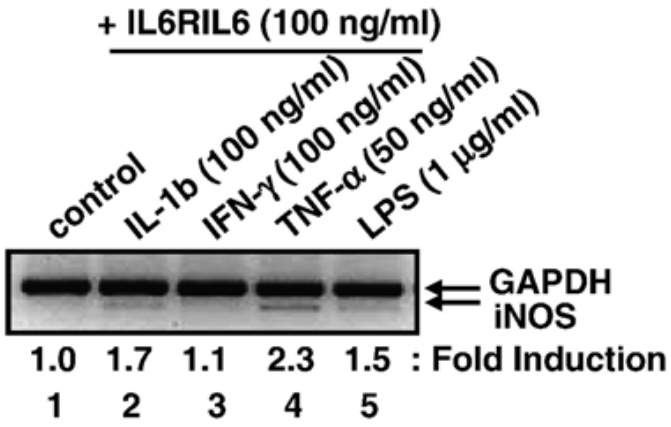

Fig. 3-1D

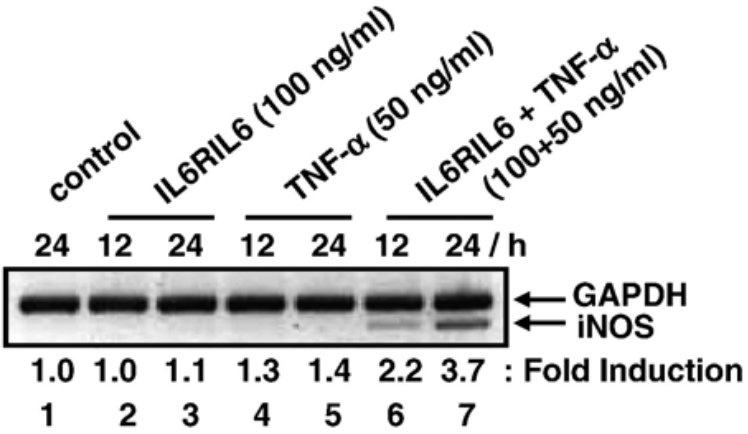

Fig. 3-1. A. Effect of IL-6, IL6RIL6, TNF-α, IL-1β, IFN-γ or LPS on iNOS mRNA expression. B16/F10.9 cells were incubated in a medium containing each cytokine for 24 h and total RNA was extracted using TRIZOL reagent. Duplex RT-PCR was performed as described in the methods section. **B.** Effect of IL-6, IL6RIL6, IL-1β, IFN-γ or LPS on iNOS mRNA expression in B16/F10.9 cells co-treated with TNF-α (50 ng/ml). Duplex RT-PCR was performed as described in panel (A). **C.** Effect of IL-1β, IFN-γ, TNF-α or LPS on iNOS mRNA expression in B16/F10.9 cells co-treated with IL6RIL6 (100 ng/ml). Duplex RT-PCR was performed as described in panel (A). **D.** Time course of iNOS mRNA expression by IL6RIL6/TNF-α. Total RNA was extracted from cells incubated with IL6RIL6 (100 ng/ml), TNF-α (50 ng/ml) or IL6RIL6 (100 ng/ml)/TNF-α (50 ng/ml) for 12 or 24 h. Duplex RT-PCR was performed as described in panel (A).

Fig. 3-2A

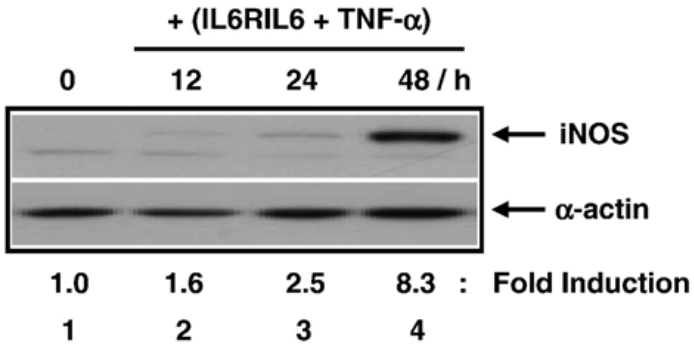

Fig. 3-2B

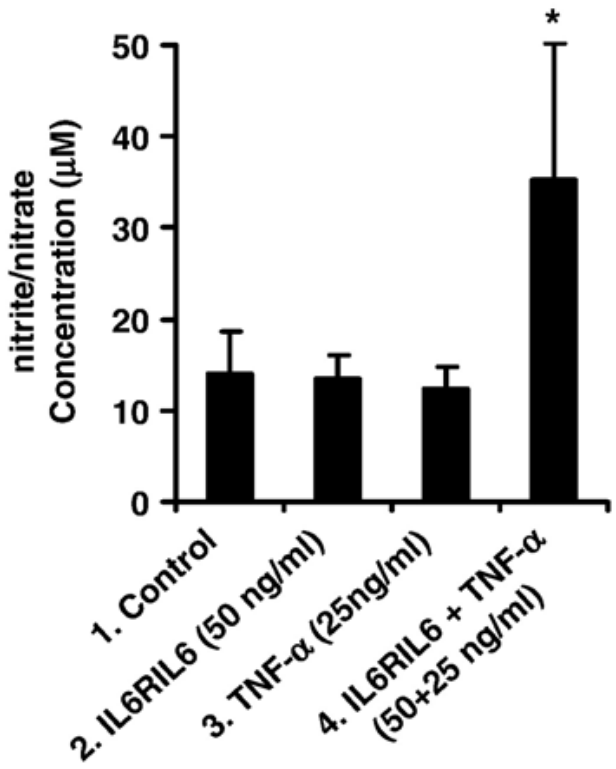

Fig. 3-2. A. Increase in iNOS protein expression by IL6RIL6/TNF-α. iNOS protein levels were monitored for 12, 24 and 48 h after treating cells with IL6RIL6 (100 ng/ml)/TNF-α (50 ng/ml). Relative iNOS protein levels were determined by measuring immunoblot band intensities by scanning densitometry. **B.** NO production by IL6RIL6/TNF-α. The amount of nitrite/nitrate in medium was monitored for 72 h. Data represent means ± SD of 4 separate experiments (significant compared to control group, $*p<0.05$).

3-4-2. Transcriptional activation of iNOS gene by IL6RIL6/TNF-α; Role of AP-1

In order to examine whether the transcriptional activation of the *iNOS* gene was needed for the increase, at the mRNA and protein levels for iNOS, reporter gene assays were performed using B16/F10.9 cells transfected with a mammalian expression vector pGL-miNOS1588, which contains the luciferase structural gene and -1.59 kb miNOS promoter (87). IL6RIL6/TNF-α stimulation of transfected cells for 18 ḥ resulted in a ~2.8-fold increase in the luciferase activity; whereas, IL6RIL6 alone or TNF-α alone had minimal effects; ~1.4-fold and ~1.7-fold, respectively (Fig. 3-3A), suggesting that the transcriptional activation of *iNOS* gene is required for the *iNOS* gene induction.

iNOS gene expression is controlled primarily by the transcription factor, NF-κB (88). In order to determine whether the iNOS expression of IL6RIL6/TNF-α is dependent on NF-κB activation, nuclear extracts prepared from the cells treated with IL6RIL6, TNF-α, and IL6RIL6/TNF-α for 0-6 h were probed with the radiolabelled NF-κB consensus oligonucleotide. NF-κB was activated by TNF-α or IL6RIL6/TNF-α with a band intensity of a slow migrating p65/p50 complex that was increased over a period of time from 1 h to 6 h; however, NF-κB activation was not observed by IL6RIL6 alone (Fig. 3-3B). These results suggest that the main target for IL6RIL6 to induce iNOS expression is unlikely to be the NF-κB pathway in B16/F10.9 cells.

We further wanted to know the nuclear levels of the p65 protein, a component of the activated NF-κB heterodimer upon stimulation. To test this, The B16/F10.9 cells were treated with IL6RIL6, TNF-α and IL6RIL6/TNF-α for 0-60 min, and then western blot was performed with prepared nuclear extracts. As shown in Fig. 3-3C, the level of nuclear p65 protein was enhanced at 15 min, and remained elevated 60 min after TNF-α or IL6RIL6/TNF-α treatment. However, nuclear translocation of the p65 protein did not occur with IL6RIL6 (Fig. 3-3C). Because nuclear translocation of p65 is preceded by phosphorylation and degradation of the I-κBα subunit, we examined I-κBα phosphorylation by TNF-α or IL6RIL6. We found that I-κBα phosphorylation was detected at 30 min, and remains elevated 60-180 min after exposure to either TNF-α or IL6RIL6/TNF-α; while IL6RIL6 alone was without effect (Fig. 3-3D). These results confirm that NF-κB is not activated by IL6RIL6 in B16/F10.9 cells, and that NF-κB is

not associated with IL-6-mediated iNOS expression.

Expression of the *iNOS* gene has been reported to be controlled by the transcription factor AP-1 (84). Thus, we carried out reporter gene analyses using a luciferase plasmid containing the NF-κB or AP-1 consensus sequences to determine whether activation of NF-κB, AP-1 or both is/are required for the IL6RIL6/TNF-α-mediated transcriptional activation of *iNOS* gene expression. As shown in Fig. 3-4A, NF-κB reporter activity was observed to be ~5.6-fold increased after TNF-α treatment, while reporter activity was barely influenced by IL6RIL6. In cells treated with IL6RIL6/TNF-α, NF-κB reporter activity was observed to be ~5-fold increased that was not significantly different from that of the TNF-α treatment; this confirmed no relevance of IL-6-mediated signaling for NF-κB activation. Exposure of cells transfected with pGL-AP-1 reporter plasmid to TNF-α for 18 h showed ~2-fold increase in the luciferase activity (Fig. 3-4B). However, AP-1 reporter activity by IL6RIL6 was observed to be ~4-fold increased, and reporter activity by IL6RIL6/TNF-α was potentiated (~8-fold) (Fig. 3-4B), suggesting relevance of IL-6-mediated signaling for the AP-1 activation.

We next performed gel shift analysis using the AP-1 consensus sequence to confirm whether the IL6RIL6/TNF-α-induced *iNOS* gene expression is mediated through AP-1 activation (Fig. 3-5A). Nuclear extracts from the cells incubated with IL6RIL6, TNF-α, or IL6RIL6/TNF-α for 0-6 h were probed with the radiolabelled AP-1 consensus oligonucleotide, and then the formation of AP-1 complex was analyzed. AP-1 complex was clearly observed by IL6RIL6 treatment, although NF-κB binding activity was not induced by IL6RIL6 (Fig. 3-3B). Moreover, IL6RIL6/TNF-α-induced AP-1 activity was found to be much stronger than treatment with IL6RIL6 alone or TNF-α alone. These data suggest that IL-6 stimulation in these cells regulates AP-1 transcription factor activity, but not NF-κB activity.

To verify whether AP-1 activation by IL6RIL6 was needed for the transcriptional activation of the *iNOS* gene in F10.9 cells, we performed reporter gene analyses using pGL-miNOS1588 and AP-1 decoy ODN (Fig. 3-5B). IL6RIL6/TNF-α-induced luciferase activity was significantly inhibited by AP-1 decoy ODN (63% inhibition),

while the AP-1 mutant ODN had a minimal effect (18% inhibition). Together, these finding provides strong evidence that IL6RIL6 selectively activates AP-1 transcription factor, leading to *iNOS* gene expression.

Fig. 3-3A

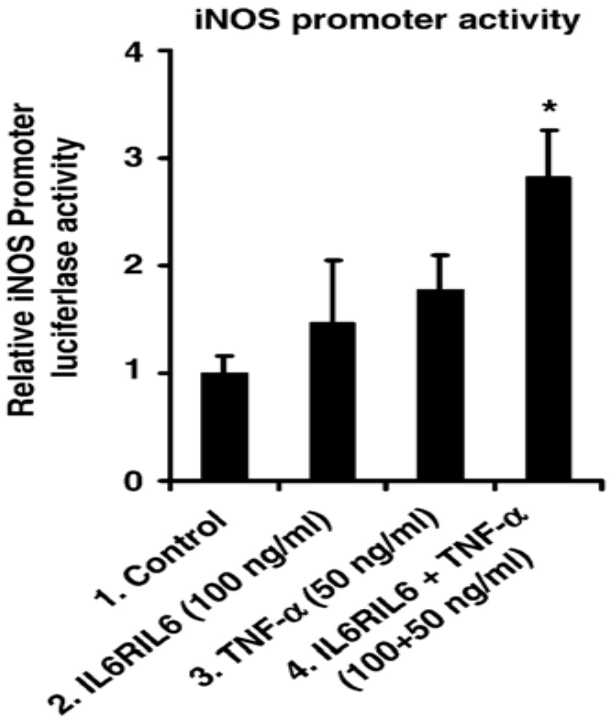

Fig. 3-3B

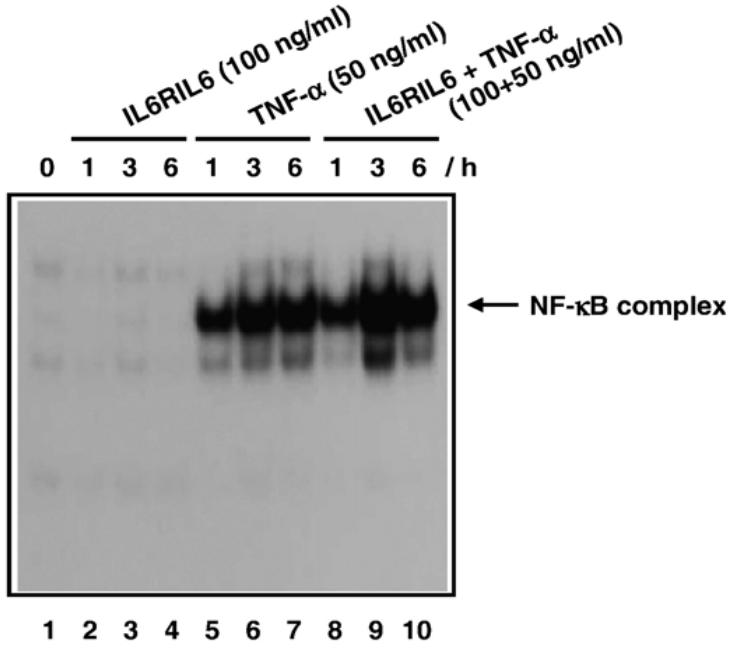

Fig 3-3C

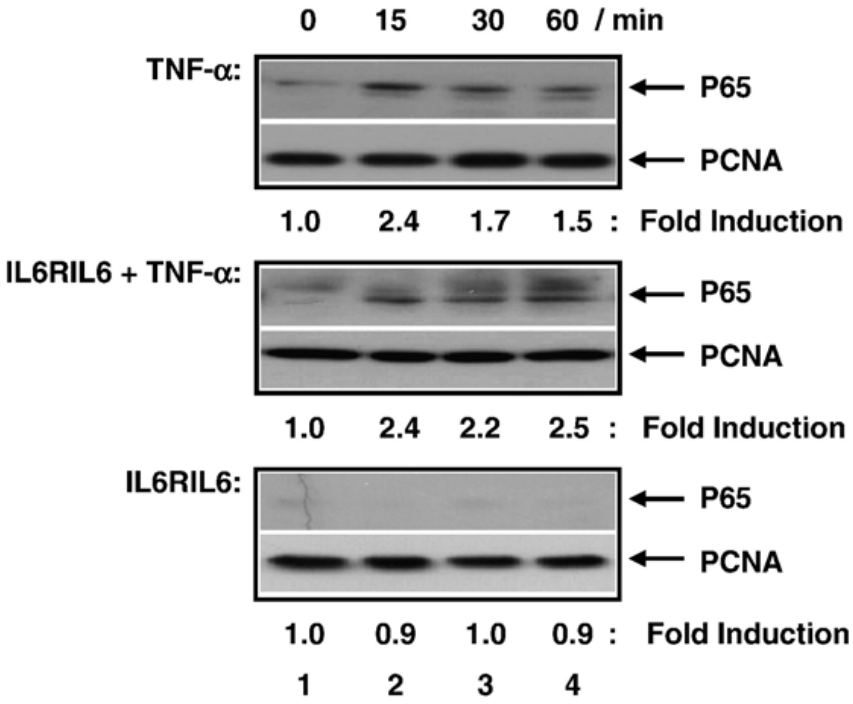

Fig. 3-3D

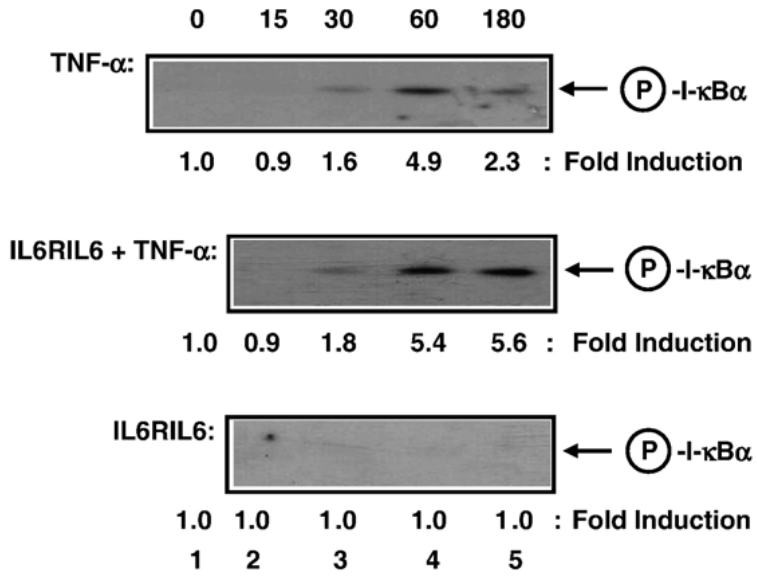

Fig. 3-3. A. Effect of IL6RIL6, TNF-α or IL6RIL6/TNF-α on *iNOS* gene transactivation. Induction of luciferase activity by IL6RIL6/TNF-α in B16/F10.9 cells transiently transfected with pGL-miNOS1588 construct, which contained a 1588 bp iNOS promoter sequence, was confirmed using a luminometer. A dual luciferase reporter gene assay was performed on lysed cells co-transfected with pGL-miNOS1588 (firefly luciferase) and pRL-SV (Renilla luciferase) (in the ratio of 100:1) after exposure to IL6RIL6 (100 ng/ml), TNF-α (50 ng/ml) or IL6RIL6 (100 ng/ml)/TNF-α (50 ng/ml) for 18 h. Reporter gene activations were expressed as changes relative to Renilla luciferase activity. The results shown represent the means ± SD of 4 separate experiments (significant versus the control, $*p<0.05$). **B.** No effect of IL6RIL6 on NF-κB DNA binding. Gel shift assays were performed with nuclear extracts prepared from B16/F10.9 cells incubated with IL6RIL6 (100 ng/ml), TNF-α (50 ng/ml) or IL6RIL6 (100 ng/ml)/TNF-α (50 ng/ml) for each point in time. All lanes were loaded with 5 μg of nuclear extracts and labeled NF-κB DNA consensus sequence. The arrow indicates the NF-κB (p65/p50) binding complex. **C.** Effect of IL6RIL6, TNF-α or IL6RIL6/TNF-α on p65 nuclear translocation. B16/F10.9 cells were treated with IL6RIL6 (100 ng/ml), TNF-α (50 ng/ml) or IL6RIL6 (100 ng/ml)/TNF-α (50 ng/ml) for each point in time, and the protein levels of nuclear p65 was determined immunochemically using specific antibodies. **D.** Effect of IL6RIL6, TNF-α or IL6RIL6/TNF-α on I-κBα phosphorylation. The levels of phosphorylated I-κBα were determined using specific antibodies.

Fig. 3-4A

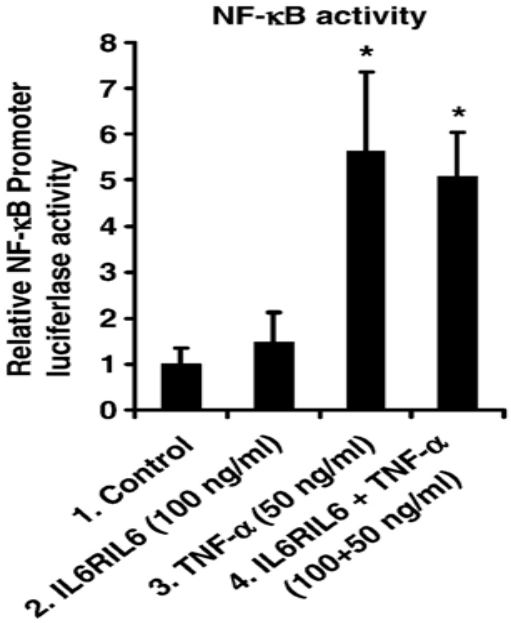

Fig. 3-4B

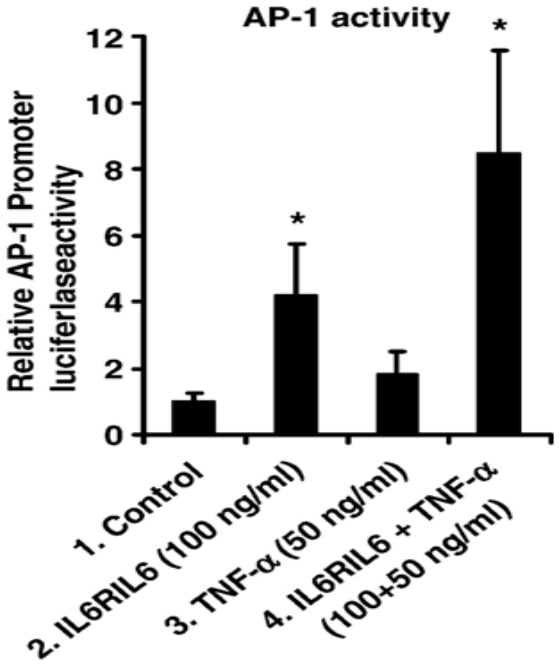

Fig. 3-4. A. NF-κB reporter gene analysis. Cells were transfected with the pNF-κB-Luc plasmid, and reporter gene analysis was performed as described in Fig. 3-3, panel (A). Data represent the means ± SD of 4 separate experiments (significant versus the control, *p<0.05). **B.** AP-1 reporter gene analysis. Cells were transfected with pAP-1-Luc plasmid, and reporter gene analysis was performed as described in Fig. 3-3, panel (A). Results represent the means ± SD of 4 separate experiments (significant versus the control, *p<0.05).

Fig. 3-5A

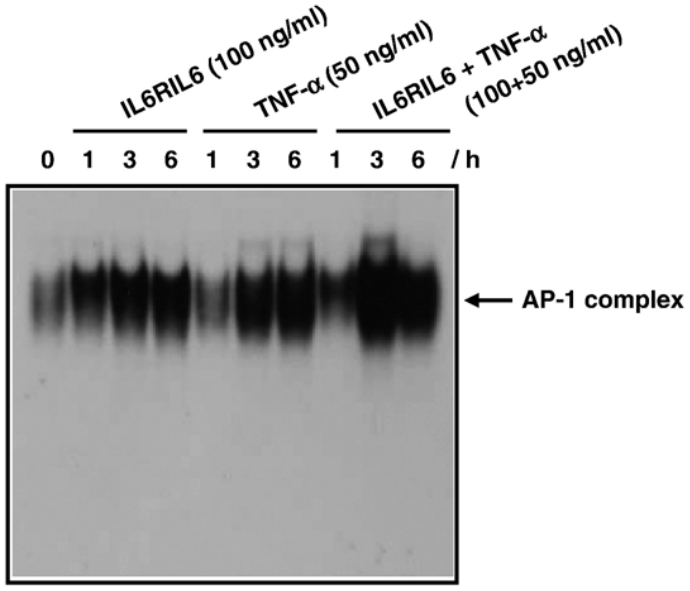

Fig. 3-5B

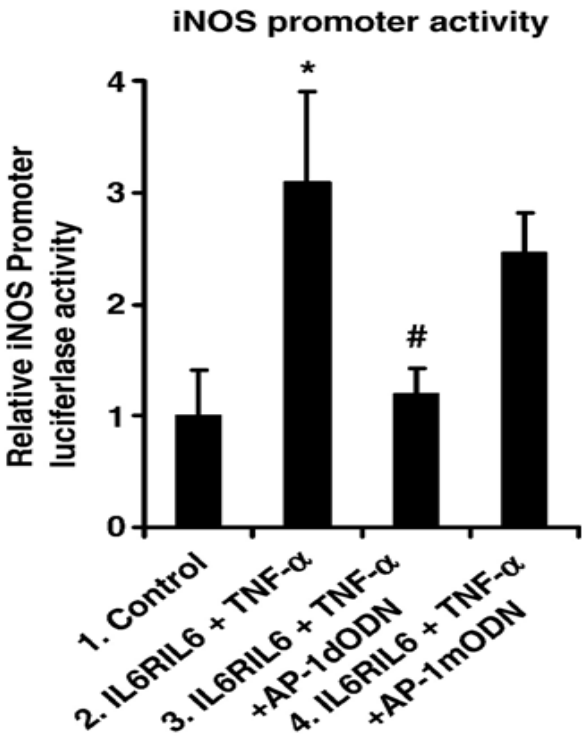

Fig. 3-5. A. Effect of IL6RIL6, TNF-α or IL6RIL6/TNF-α on AP-1 DNA binding. Gel shift assays were performed as described in Fig. 3-3, panel (B). The arrow indicates the AP-1 binding complex. **B.** Effect of AP-1 decoy ODN on the activity of reporter gene containing miNOS promoter. The cells were transfected with pGL-miNOS1588 plasmid in the presence or absence of AP-1 decoy ODN (AP-1 dODN, 10 μM), and the reporter gene analysis was performed as in Fig. 3-3, panel (A). Decoy ODN of mutant AP-1 (mAP-1 dODN, 10 μM) was used to show the specificity of decoy ODN activity. The data is represented as a mean ± SD of 3 separate experiments (significant as compared with the control, $*p<0.05$; significant as compared with IL6RIL6/TNF-α-treated group, $^{\#}p<0.05$).

3-4-3. Involvement of c-Fos/c-Jun in IL6RIL6/TNF-α-mediated AP-1 activation

Fos and Jun family proteins function as dimeric transcription factors that bind to AP-1 regulatory elements in the promoter or enhancer regions of numerous mammalian genes (89). To identify the transcription factors that comprise the inducible AP-1 complex in response to IL6RIL6/TNF-α, specific antibodies directed against individual AP-1 proteins were evaluated for the ability to supershift the retarded band. The cells were incubated with IL6RIL6, TNF-α, and IL6RIL6/TNF-α for 3 h, nuclear extracts were pre-incubated with a 20-fold excess cold probe, normal rabbit serum (NRS), anti-c-Jun, anti-Jun D, anti-Jun B, anti-FosB or anti-c-Fos Ab for 20 min at RT, and then probed with the radiolabelled AP-1 consensus oligonucleotide. The specificity of the DNA probe to the AP-1-binding complex was supported by competition for binding to a radiolabelled AP-1 probe with a 100-fold molar excess of unlabeled AP-1 oligonucleotide. Interestingly, the band of the migrating complex with the AP-1 consensus sequence was supershifted by anti c-Jun, anti-c-Fos Ab (Fig. 3-6A), or anti c-Jun/anti c-Fos Ab mixture (Fig. 3-6B), indicating that the AP-1 complex activated by IL6RIL6/TNF-α is composed of c-Jun and c-Fos proteins.

3-4-4. Role of JNK and p38 MAPK in the IL6RIL6-mediated AP-1 activation

Recent studies have suggested that c-Jun and c-Fos are phosphorylated by MAPKs; extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK (89). Therefore, we wished whether TNF-α, IL6RIL6, or IL6RIL6/TNF-α activates ERK, p38, and JNK. B16/F10.9 cells were stimulated with TNF-α, IL6RIL6, and IL6RIL6/TNF-α for various periods of time (0-180 min), cells were lysed, and subjected to western blot with anti phospho-ERK, anti phospho-p38, anti phospho-JNK Ab. As shown in Fig. 3-7, ERK MAPK phosphorylations were observed to be at maximal levels after a 15 min exposure of the cells to TNF-α, IL6RIL6, and IL6RIL6/TNF-α (lane 2, 6, 10) and then gradually decreased to the control level. p38 phosphorylations were observed after a 15 min stimulation with TNF-α, IL6RIL6 and IL6RIL6/TNF-α, and remained elevated for up to 180 min (B: lane 2-5; lane 6-9; lane 10-13). Interestingly, the phosphorylation levels of p38 MAPK in response to

IL6RIL6/TNF-α were synergistically enhanced, compared to the other; IL6RIL6 alone or TNF-α alone, indicating amplification of p38 signaling cascade after IL6RIL6/TNF-α treatment in these cells. JNK phosphorylations under three different conditions were also observed upon stimulation (C: lane 2-4; lane 6-7; lane 10-11). These results revealed that IL6RIL6 stimulation with or without TNF-α resulted in the activation of ERK, p38, and JNK.

Next, we examined whether inhibition of MAPKs results in abrogation of IL6RIL6/TNF-α-induced AP-1 activation. The cells were pre-treated with MAPK inhibitors for 30 min, and then exposed to IL6RIL6/TNF-α for an additional 3 h. The nuclear extracts prepared from the cells were probed with the radiolabelled AP-1 consensus oligonucleotide. As shown in Fig 3-8A, treatment of SB202190 (p38 MAPK inhibitor) or SP600125 (JNK inhibitor) distinctly inhibited IL6RIL6/TNF-α-stimulated-AP-1 binding activities (lane 4 and 6). However, PD98059 (an ERK inhibitor) did not affect AP-1 binding activity (lane 5). These results suggest that IL6RIL6/TNF-α-induced-AP-1 activation, in part, require the activation of p38 and JNK, but not ERK.

Since c-Jun and c-Fos are major constituents of the AP-1 complex activated by IL6RIL6/TNF-α (Fig. 3-8B), we further assessed whether activation of c-Jun or c-Fos was dominated by JNK and p38 MAPK. Nuclear extracts were pre-incubated with anti-c-Jun or anti-c-Fos Ab for 20 min at RT and probed with radiolabelled AP-1 binding oligonucleotide. The supershifted AP-1 complex by c-Jun Ab (lane 3) was inhibited by SP600125 (lane 7), but not by SB202190 (lane 5). By contrast, the supershifted AP-1 complex by c-Fos Ab (lane 4) was diminished in cells pre-treated with SB202190 (lane 6), but not with SP600125 (lane 8). Immunocytochemistry analyses were performed to confirm these results. As shown in Fig. 3-8C, in the unstimulated B16/F10.9 cells, c-Jun and c-Fos protein are evenly located in both cytoplasm and nucleus. Nuclear translocation of both transcription factors was found in the cells exposed to IL6RIL6/TNF-α. Movement of c-Jun and c-Fos protein into the nucleus by IL6RIL6/TNF-α was specifically blocked by the p38 inhibitor and the JNK inhibitor, respectively. These results combined with the gel shift data indicate that

JNK specifically dominates c-Jun activation with IL6RIL6/TNF-α stimulation, and that simultaneously p38 MAPK dominates c-Fos activation.

3-4-5. The p38 inhibitor SB202190 and JNK inhibitor SP600125 abrogate IL6RIL6/TNF-α-induction of iNOS expression

To test the involvement of MAPKs activation in IL6RIL6/TNF-α-induced iNOS expression, we examined the effect of AG490, an inhibitor of JAK2/STAT3 (90) or SB202190, an specific inhibitor of p38 MAPK (91) or SP600125, an inhibitor of JNK (82) or PD98059, an inhibitor of MEK1/2, an upstream kinase that phosphorylates ERK1/2 (92). The cells were pretreated for 30 min with the indicated amounts of inhibitors and stimulated with IL6RIL6/TNF-α for an additional 24 h, and iNOS mRNA expression was analyzed by RT-PCR. IL6RIL6/TNF-α-induced iNOS expression was inhibited by SB202190 or SP600125 (Fig. 3-9A; lane 4-5). Conversely, IL6RIL6/TNF-α-induced iNOS expression was not inhibited by AG 490 or PD98059 (Fig. 3-9A; lane 3 and 6). To test whether the results at the mRNA level were reflected at the protein level, cells were stimulated with IL6RIL6/TNF-α for 48 h, then cell lysates were analyzed for iNOS protein expression by western blot. The iNOS were induced at the protein level (Fig. 3-9B; lane 2) and pretreatment of the cells with SB202190 or SP600125 inhibited iNOS protein expression (Fig. 3-9B; lane 4-5), but iNOS expression was not inhibited by AG 490 or PD98059, as observed at the mRNA levels (Fig. 3-9B; lane 3 and 6). These results collectively indicate that the p38 and JNK signaling pathways regulate IL6RIL6/TNF-α-induced *iNOS* gene expression through AP-1 activation, but JAK2/STAT3 or ERK1/2 pathway does not regulate.

Fig. 3-6A

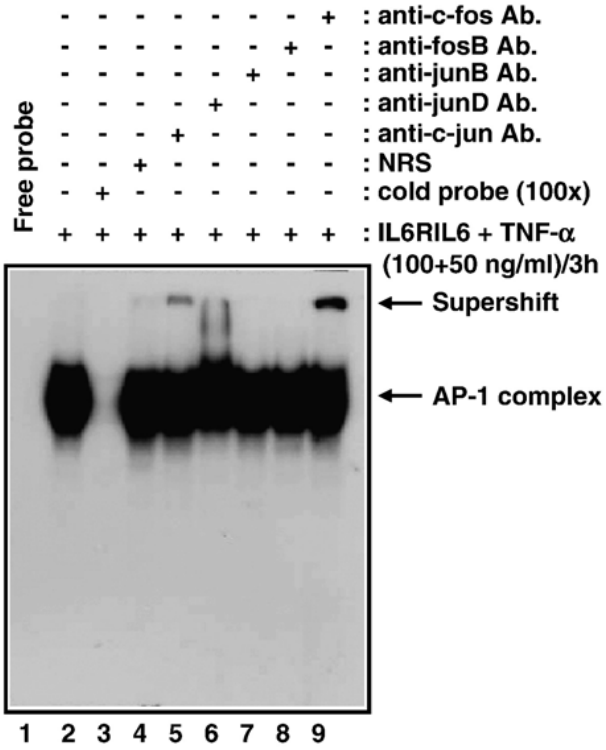

Fig. 3-6B

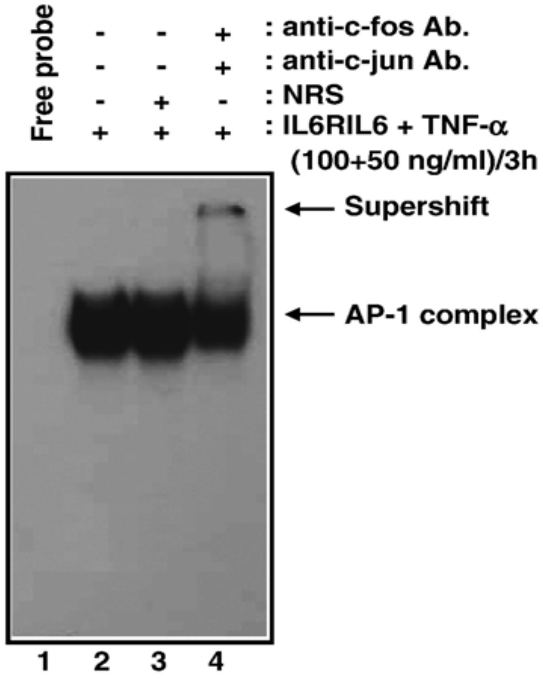

Fig. 3-6. A. Supershift of AP-1 transcription complex by addition of c-Fos or c-Jun antibody. An antibody competition experiment was carried out by incubating the nuclear extracts prepared from cells treated with IL6RIL6/TNF-α for 3 h, with the specific antibody directed against c-Fos, Fos-B, Jun-B, Jun-D or c-Jun protein (2 μg each). Competition study was carried out by adding a 100-fold excess of unlabeled (cold) AP-1 oligonucleotide. Lower arrow indicates AP-1 binding complex and upper arrow the supershifted AP-1 binding complex. **B.** Supershift of AP-1 transcription complex by co-addition of c-Fos and c-Jun antibodies.

Fig. 3-7

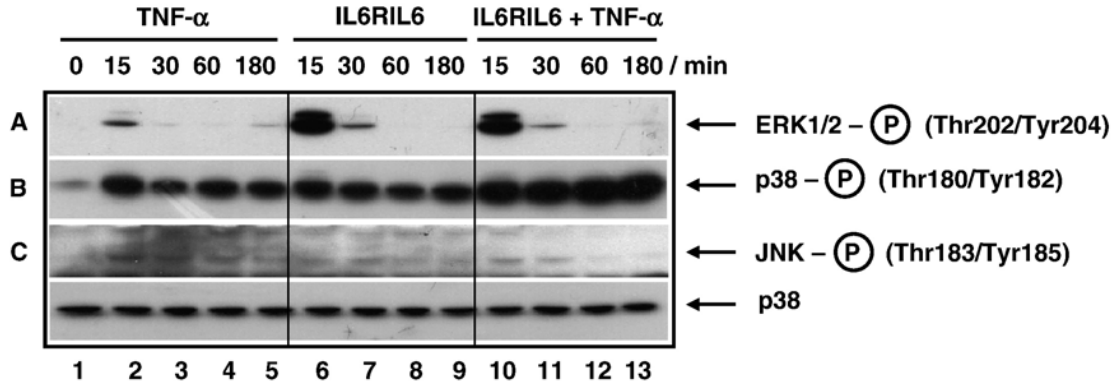

Fig. 3-7. Effects of TNF-α, IL6RIL6, or IL6RIL6/TNF-α on MAPK phosphorylation. B16/F10.9 cells were incubated with TNF-α (50 ng/ml) or IL6RIL6 (100 ng/ml)/TNF-α (50 ng/ml) for each point in time, and the activations of ERK, p38, and JNK MAPK were assessed by immunoblotting the phosphorylated forms of these MAPKs. The levels of p38 MAPK were determined as loading controls.

Fig. 3-8A

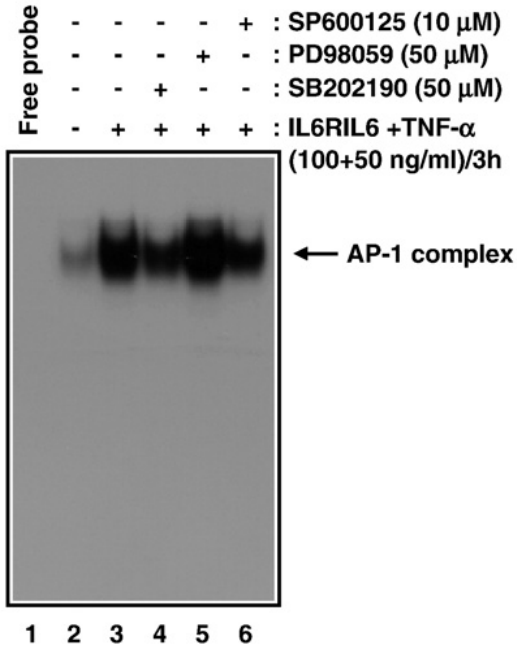

Fig. 3-8B

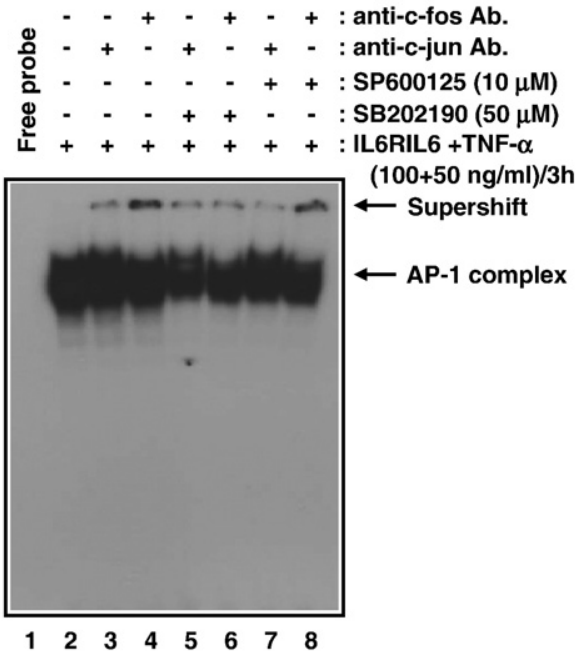

Fig. 3-8C

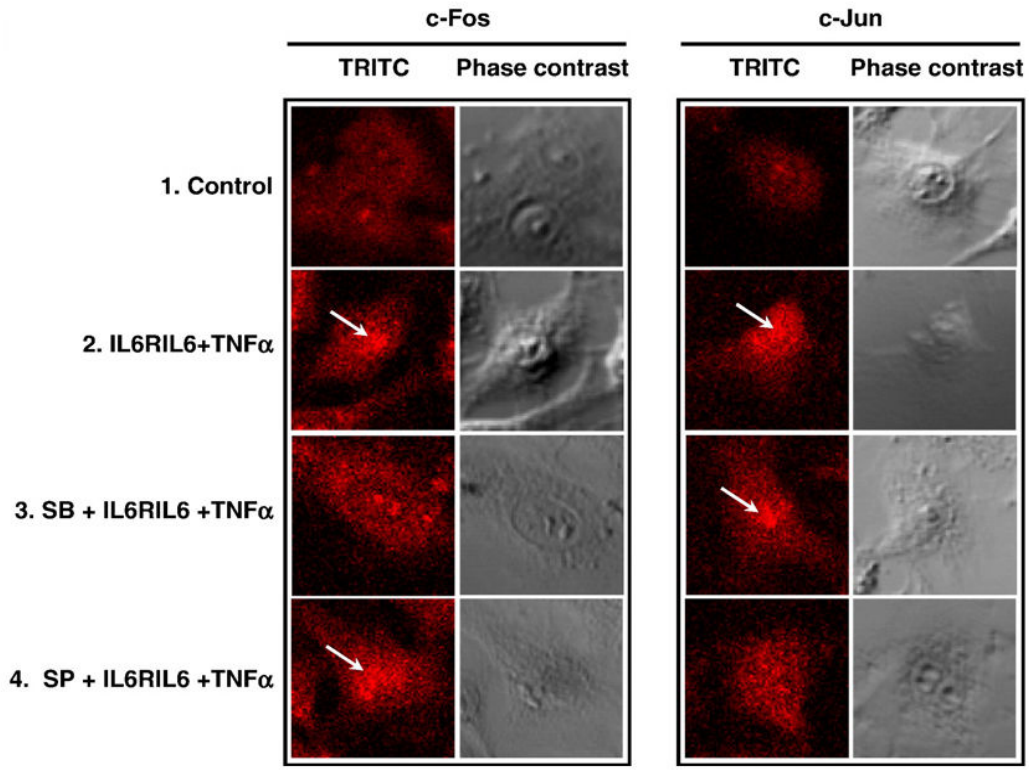

Fig. 3-8. A. Effects of MAPK inhibitors on AP-1 transcription complex. The extent of AP-1 DNA binding activity was assessed 3 h after exposure of each inhibitor (SP600125, 10 μM; PD98059, 50 μM; SB202190, 50 μM)-pretreated (30 min) cells to IL6RIL6 (100 ng/ml)/TNF-α (50 ng/ml). **B.** Effects of MAPK inhibitors on supershifted AP-1 transcription complex. An antibody competition experiment was carried out by incubating the nuclear extracts prepared from cells treated with IL6RIL6/TNF-α for 3 h, with the specific antibody directed against c-Fos or c-Jun protein (2 μg each). The cells were 30 min pretreated with or without JNK (SP600125, 10 μM) or p38 (SB202190, 50 μM) inhibitor. **C.** Immunofluorescence subcellular localization of c-Fos or c-Jun. Each protein was immunochemically detected using anti-c-Fos or anti-c-Jun antibody. IL6RIL6 (100 ng/ml)/TNF-α (50 ng/ml) caused c-Fos (left panel) and c-Jun (right panel) to migrate toward the nucleus. The same fields were examined by phase-contrast filed to verify the location of nuclei. SP600125 (10 μM) or SB202190 (50 μM) was 30 min pretreated before adding IL6RIL6/TNF-α to cells.

Fig. 3-9A

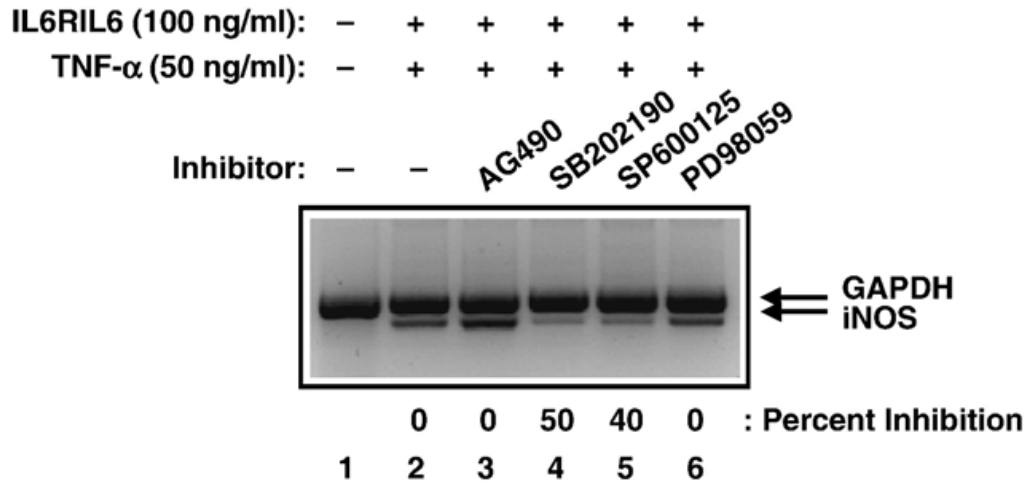

Fig. 3-9B

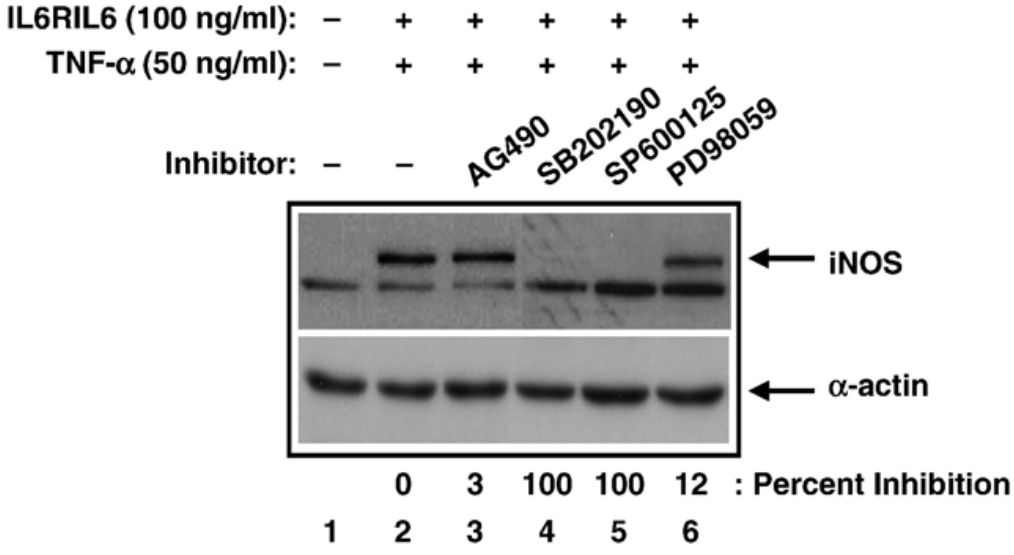

Fig. 3-9. A. Effects of MAPK inhibitors on iNOS mRNA expression. B16/F10.9 cells were treated with IL6RIL6 (100 ng/ml)/TNF-α (50 ng/ml) for 24 h with/without specific inhibitors (SP600125, 10 μM; PD98059, 50 μM; SB202190, 50 μM). To assess possible role of STAT pathway, AG490 (50 μM) was also introduced. **B.** Effects of MAPK inhibitors on iNOS protein expression. B16/F10.9 cells were treated with IL6RIL6 (100 ng/ml)/TNF-α (50 ng/ml) for 48 h with/without specific inhibitors (SP600125, 10 μM; PD98059, 50 μM; SB202190, 50 μM).

3-5. DISCUSSION

The pathophysiological role of IL-6 among a variety of cytokines has been an attractive area of study over the past several decades. A number of studies have implicated pro-inflammatory properties of IL-6 in several pathological conditions, such as rheumatoid arthritis, multiple myeloma, multiple sclerosis, and crohn's disease (75, 93). As well, recent findings indicate that IL-6 has a number of immunosuppressive functions in IL-6-knockout models (94, 95). We previously reported that the IL-6/sIL-6R complex induces an irreversible growth arrest and morphological differentiation in murine B16 metastatic melanoma clone F10.9 cells; IL-6 alone fails to initiate intracellular signaling events within these cells, but the inclusion of sIL-6R restores the ability of IL-6 to activate IRF-1 and p21/waf-1/Cip-1; these findings demonstrate that B16/F10.9 cells lack sufficient membrane IL-6R subunits to transmit IL-6-induced signals (27).

Several proinflammatory cytokines including TNF-α, IL-1β or IFN-γ induce iNOS in a variety of cell types (86, 96-101). In addition, melanocyte and melanoma are well documented to induce iNOS upon diverse immunogenic stimuli such as TNF-α (88, 102, 103). In this study, we demonstrate another novel function of IL-6 and sIL-6R in B16/F10.9 cells, that being the regulation of AP-1-mediated iNOS expression. Most of the cytokines known to be potent iNOS inducers do not increase iNOS expression in B16/F10.9 melanoma cells; whereas, stimulation by IL6RIL6 plus TNF-α was shown to be capable of up-regulating iNOS expression and NO production (Fig. 3-1 and 3-2). The promoter region of iNOS gene contains three sites for STAT binding (104), and the activation of both JAK and STAT is required for the IL-6-inducible transcriptional activation of *iNOS* gene in cardiomyocytes. Moreover, AG490, JAK2/STAT3 inhibitor, completely blocks the induction of iNOS (105). In this study, we observed that inhibition of JAK2/STAT3 pathway by AG490 did not abrogate IL6RIL6/TNF-α-induced iNOS expression. Therefore, our findings support that IL6RIL6/TNF-α-induction of *iNOS* gene expression is not mediated by JAK/STAT signaling pathway in B16/F10.9 cells.

Current evidence suggests that activation of NF-κB, which is a pleiotropic regulator

of many genes involved in immune and inflammatory responses, has a crucial role in iNOS induction (86, 88). NF-κB exists in the cytoplasm of unstimulated cells in a quiescent form bound to its inhibitor such as I-κBα. When dissociated and activated NF-κB complexes translocate to the nucleus where they are involved in many physiological processes, including *iNOS* gene expression (88, 106, 107). In our study, the activation of p65/p50 NF-κB DNA binding, p65 nuclear translocation and I-κBα phosphorylation were occurred by TNF-α alone or IL6RIL6/TNF-α, but not by IL6RIL6 alone. In addition, NF-κB transcriptional activity results obtained from the NF-κB reporter gene assay were also consistent with aforementioned (Fig. 3-3B). Thus, our findings suggest that NF-κB may not be a target of the IL-6 signaling pathway for *iNOS* gene expression since IL6RIL6 alone does not affect NF-κB activity.

The promoter region of the *iNOS* gene contains two putative binding sites for AP-1 (86, 104); the role of this transcription factor in *iNOS* gene expression appears to be cell-type specific; AP-1 mediates *iNOS* gene induction in C6 glioma cells (108) and in human lung epithelial cells (109); while for human colon tumor epithelial cell lines, AP-1 has a repressor function (97, 110, 111). Our findings demonstrate that IL6RIL6/TNF-α stimulation of cells synergistically enhances AP-1 reporter activity (~8-fold), while IL6RIL6 alone and TNF-α alone are ~4-fold and ~2-fold, respectively (Fig. 3-4B) and that IL6RIL6 leads to potent activation of AP-1 (Fig. 3-4B and 3-5A), suggesting that AP-1 stimulated by IL6RIL6/TNF-α functions as a strong inducer of iNOS expression in B16/F10.9 cells (Fig. 3-5B). Concerning the activation modes and functions of AP-1, recent studies reported that various AP-1 complexes, such as JunD/Fra2 (109, 112), JunB/AP-1 (84), Fra1/AP-1 (108) or c-fos/AP-1 (113), are involved in the induction of *iNOS* gene transcription. Our results demonstrate that IL6RIL6/TNF-α-induced AP-1 complex formation for iNOS induction requires the c-fos/c-jun heterodimer. This is inconsistent with a previous report that c-fos/c-jun proteins act as negative regulator of iNOS expression in human DLD cells, upon stimulation of cytokine mixture (97). This discrepancy may be, in part, explained by the possibilities of different repressor(s) involvement to modulate *iNOS* gene transcription in response to a given distinct stimulus.

NF-κB signal transduction pathway has been shown to be directly engaged by the TNF receptor and several downstream kinases including I-κB kinase α and β (114). By contrast, AP-1 activation has been shown to be primarily mediated by MAPK activities (115). Given that MAPKs contribute to AP-1 activation and *iNOS* gene expression, we examined whether IL6RIL6, TNF-α, or IL6RIL6/TNF-α stimulation results in the activation of MAPKs in B16/F10.9 cells. Our data indicates that ERK1/2, p38, and JNK are phosphorylated by IL6RIL6/TNF-α stimulation (Fig. 3-7).

We have shown that MAPKs are associated with the activation of the AP-1 complex, resulting in the induction of the *iNOS* gene. The results of the gel shift and immunocytochemistry analysis, using specific MAPK inhibitors revealed that AP-1 complexes, c-jun and c-fos, were dominated by JNK and p38 MAPK, respectively. In addition, inhibition of JNK or p38 MAPK completely blocked iNOS expression both at the mRNA and protein level by IL6RIL6/TNF-α (Fig. 3-9). These results collectively indicate that AP-1 activation through JNK and p38 MAPK pathways are involved in IL6RIL6/TNF-α-induced iNOS expression.

IL-6 is believed as a functional cytokine during malignant tumor progression in human melanoma cells (116, 117). Both IL-6 receptor and its signal transducer gp130 are known to be detected in primary and metastatic melanoma cell lines (116). sIL-6R is also detected in normal healthy individuals (30-80 ng/ml) (54, 118) and circulating levels of the soluble receptor are significantly elevated during malignant disorders (56, 119). sIL-6R/IL-6 fusion protein has anti-tumoral effects in mice transplanted with B16 melanoma cells (28). In the present study, we showed that IL6RIL6/TNF-α enhanced NO production through iNOS induction in B16/F10.9 cells. Pathological function of NO in melanoma progression is quite controversial (120-122). In general, low levels of NO likely produced by tumor cells themselves act in favor of tumor progression (123, 124), while high levels of NO are tumoricidal (125). However, the data obtained from animal studies are distinct from cell culture studies. Growth rate of B16/F1 melanoma cells was significantly increased in iNOS knock-out mice, presumably through the deregulation of vascular endothelial growth factor (126). *Vise versa*, blood vessel endothelium-derived NO caused spontaneous apoptosis and

metastasis of B16/F1 melanoma cells in C57/Bl6 mice (127). Hence, it could be possible that NO production by IL6RIL6/TNF-α in B16/F10.9 melanoma cells affects cellular phenotypes in a bi-directional manner.

Our results highlight another functional role for the IL-6/sIL-6R/gp130 system; that is *iNOS* gene expression through JNK/p38 MAPK mediated-AP-1 (c-jun/c-fos) activation in B16/F10.9 cells. Since NO production by iNOS induction affect tumorigenicity and metastasis of melanoma, further study is required precisely to understand the relevance of IL6RIL6/TNF-α enhanced iNOS/NO *in vivo*. Taken together, the cellular signaling pathways, by which IL6RIL6 enhance iNOS, are believed to be unique pathways found in melanoma pathophysiology, and may potentially serve as pharmaceutical targets for future melanoma treatment strategies.

CHAPTER 4

Tumor necrosis factor-alpha induced VCAM-1 expression is inhibited by high density lipoprotein in human astroglioma Cells

4-1. ABSTRACT

Astrocytes, the major glial cells in the central nervous system (CNS), can express vascular cell adhesion molecule-1 (VCAM-1) in response to cytokines, such as TNF-α. In CNS, an increased VCAM-1 expression may contribute to inflammatory processes. We, in the present study, have examined the effect of human plasma High Density Lipoproteins (HDL) and other lipoproteins on VCAM-1 expression in astroglioma cells since astrocytes secrete HDL-like lipoprotein particles which contain apo E and cholesterol, phospholipid. The exposure of astroglioma cells to the major plasma lipoprotein fractions (VLDL, LDL and HDL) had no effect on the VCAM-1 expression. However, TNF-α-induced VCAM-l was inhibited by HDL in a dose-dependent manner, but not by VLDL or LDL. The inhibitory effect of HDL on TNF-α-induced VCAM-l was reversed by the inclusion of Apo A-I antibody, the major apolipoprotein of HDL, demonstrating the specificity of this response. Reconstituted HDL (discoidal complex of apo HDL and DMPC), but not apo HDL or DMPC, was effective in suppressing the VCAM-1 expression. RNase protection assay (RPA) revealed that TNF-α-induced VCAM-l mRNA expression was markedly inhibited by HDL (500 μg cholesterol/ml). These results indicate that HDL-like particles in the CNS may function as an immunosuppressive molecule in pathologic conditions of CNS.

KEYWORDS: TNF-α, Astrocytes, Vascular Cell Adhesion Molecule, High Density Lipoproteins.

4-2. INTRODUCTION

Cell adhesion plays a key role in a number of diverse biological processes such as acute and chronic inflammation, autoimmune diseases, and atherosclerosis (79). Specific cellular interactions in these disease processes are mediated by adhesion molecules, a family of proteins that bind specifically to complementary adhesion molecules on other cells.

Astrocytes are the resident glial cells in the CNS that support brain capillary endothelium, and are in intimate contact with brain endothelial cells via perivascular end-feet, and can modulate the endothelial function in response to external stimuli (128). Exposure of astrocytes to inflammatory cytokines, such as TNF-α, IL-lβ, and IFN-γ, induce the expression of adhesion molecules such as VCAM-1, ICAM-1 (129, 130). These adhesion molecules are increased in the CNS particularly during times of inflammation and are thought to contribute to extravasation of leukocytes across the blood-brain barrier (BBB) and into CNS parenchyma (131). In diseased states, ICAM-1 and VCAM-1 expression have been detected on the endothelial cells comprising the BBB, as well as astrocytes and microglia, which contribute to the structural integrity of the BBB (131).

It was demonstrated that astrocytes generate and secrete HDL-like lipoprotein particles, which contain apo E, cholesterol, and phospholipids (132). As well, it has been shown that apo E is abundantly present in the CNS (133). Despite the existence of the BBB, lipoprotein particles have been shown to be also present in the CSF. Several studies demonstrated that these particles originate from CNS, although a portion of their protein components may filter through the BBB by a receptor-mediated mechanism called transcytosis (133). The lipoprotein particles isolated from human CSF showed size, structure, and composition similar to plasma HDL (134). On the contrary, other reports indicated that CSF lipoproteins are very different in virtually all respects from particles found in the plasma (135), but it seems most probable that brain lipoproteins participate in lipid transport and homeostasis in CNS as in the vascular compartment (134). In the present study, we have examined the effect of cholesterol-rich native plasma HDL, cholesterol free reconstituted HDL and lipid-free

apolipoproteins of HDL on the VCAM-1 expression in astroglioma cells. We demonstrate that TNF-α-induced VCAM-l was inhibited by plasma HDL, but not by VLDL or LDL.

4-3. MATERIALS AND METHODS

4-3-1. Cells and reagents

U373-MG human astroglioma cells were obtained from the American Type Culture Collection (Rockville, MD). Human recombinant TNF-α (specific activity: 5.6 x 10^7 U/mg*)* was obtained from Genentech Inc. (South San Francisco, CA). Anti-human VCAM-l Abs (clone IG11B1, IgG1 isotype) were obtained from Serotec Inc. (Washington, DC). FITC-conjugated goat anti-mouse IgG was obtained from the Southern Biotechnology Association (Birmingham, AL). pBluescript SK(+/-) containing a fragment of the human VCAM-l cDNA (bp 1307-2811) was obtained from the American Type Culture Collection (Manassas, VA). pAMP-1 vector containing a fragment of human GAPDH cDNA was obtained from Life Technology Inc. (Grand Island, NY). The RNase protection assay (RPA) kit was purchased from Ambion Inc. (Austin, TX). Fresh normolipidemic human plasma was obtained from the Alabama Regional Blood Bank (Birmingham, AL). Very low density lipoproteins (VLDL), LDL and HDL fractions were isolated from plasma by the sequential flotation method (136) and purified further by another density gradient ultra-centrifugation step (137). Apolipoproteins (apo) of HDL were prepared by delipidating purified HDL with a chloroform:methanol (2:1) mixture. Discoidal reconstituted HDL containing apo HDL and phosphatidylcholine (PC) with an apo HDL to PC ratio of 1:2 was prepared by mixing apo HDL and Dimyristoylphosphatidylcholine (DMPC) liposomes and subsequently sonicating the mixture. Unilamellar vesicles of DMPC were also prepared by suspending dried DMPC in phosphate buffered saline (PBS) with subsequent sonication of suspended DMPC. DMPC was purchased from Avanti Polar Lipid Inc. (Alabaster, AL). Anti-human apo A-I polyclonal antibody was purchased from Boheringer Mannheim Biochemical Co. (Indianapolis, IN).

U373-MG human astroglioma cells were maintained in minimal essential medium (MEM) supplemented with 1 mM Earles BSS media with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and heat-inactivated fetal bovine serum (FBS). For passage, monolayers were rinsed with PBS and then dislodged by 0.25% trypsin. To study the effect of lipoproteins or TNF-α on the VCAM-l expression, cells in 2-3

replicated wells (2 x $l0^5$/well, 6 well plates, Nunc) were incubated with serum-free medium alone, TNF-α (50 ng/ml), VLDL (300 μg cholesterol/ml), LDL (800 μg cholesterol/ml), HDL (500 μg cholesterol/ml), or TNF-α plus VLDL, LDL or HDL for 48 h.

4-3-2. Analysis of VCAM-1

Production and/or expression of VCAM-1 in cultured astrocytes were measured by the immunofluorescence flow cytometry and by RPA as described previously (138).

4-3-3. Flow cytometry

U373-MG cells were harvested by mild trypsinization following aspiration of the culture medium and by subsequent washing of cells with PBS. The trypsinized cells were washed once, suspended in PBS containing 0.5% FBS and 0.02% sodium azide, and then incubated with human VCAM-l Ab (1:500 dilution) for 30 min. at 4°C. The cells were then washed twice and subsequently stained with FITC-labeled goat anti-mouse IgG (1:100 dilution) for 30 min. at 4°C. After washing, cells were fixed in 1% paraformaldehyde, and VCAM-1 expression was measured by fluorescence-activated cell sorting (FACS) (FACStar, Becton Dickinson, Mountain View, CA). Negative controls were incubated with an isotype-matched control anti-mouse IgG. Ten thousand cells were analyzed for each sample. VCAM-1 was expressed as a percentage of positive cells.

4-3-4. RNase protection assay (RPA)

Total cellular RNA was isolated from cell monolayers that have been incubated with VLDL, LDL or HDL in the absence or presence of TNF-α, as previously described (63). Briefly, total RNA was extracted using TRIZOL reagent (Life Technologies, Inc., Rockville, MD). A plasmid containing a fragment of the human VCAM-l cDNA (bp 1307-2811) in pBluescript SK(+/-) was linearized with *SpeI*, which digests within the VCAM-l cDNA insert. The *in vitro* transcription of this linearized vector with T7

RNA polymerase results in a 449-bp antisense RNA probe and a protected fragment consisting of 427 nucleotides. A plasmid containing a fragment of human GAPDH cDNA (bp 43-531) in pAMP-1 was linearized with *NcoI* which digests within the GAPDH cDNA insert. *In vitro* transcription of this linearized vector with T7 RNA polymerase results in a 290-bp antisense RNA probe and protected fragment consisting of 230 nucleotides. GAPDH mRNA was utilized as a "house-keeping gene" since its levels are not affected by TNF-α treatment. RPA was conducted with an RPA kit according to the manufacturer's instructions. Briefly, 15 μg/ml of total RNA was hybridized with VCAM-1 (2.5×10^4 cpm) and GAPDH (2.0×10^4 cpm) riboprobes at 42°C overnight in 20 μl of 40 mM PIPES (pH 6.4), 80% deionized formamide, 400 mM NaOAc, and 1 mM EDTA. The hybridized mixture was then treated with RNase A/T1 (1:200 dilution in 200 μl of RNase digestion buffer) at room temperature (RT) for 1 h, and RNA was precipitated and analyzed by 5% denaturing (8M urea) polyacrylamide gel electrophoresis. The gels were exposed to X-ray film, and quantification of protected RNA fragments was performed by scanning with a PhosphorImager (Molecular Dynamics, Sannyvale, CA). Values for VCAM-l mRNA were normalized to GAPDH mRNA levels for each experimental condition.

4-3-5. Statistical analysis

Values are presented as mean $\pm$ standard deviation. The paired Student's *t-test* was applied to determine whether differences between values were significant. A statistical probability of $p<0.05$ was considered significant.

4-4. RESULTS

4-4-1. Response of human astroglioma cells to plasma lipoproteins

To define functional effect of plasma lipoproteins on astroglioma cells, we examined the effect of several lipoproteins on the expression of an adhesion molecule, VCAM-1. This gene was chosen since it was previously shown that astroglioma cells have the capacity to express VCAM-1 in response to cytokine TNF-α (138). U373-MG human astroglioma cells were treated with TNF-α (50 ng/ml), VLDL (300 μg/ml), LDL (800 μg/ml), HDL (500 μg/ml) or together for 48 h, and then, VCAM-1 expression was assessed by FACS analyses. As a representative histogram of the FACS results (Fig. 4-1A) shows, a physiologic level of VLDL, LDL or HDL alone did not influence on VCAM-l expression (lane 3-5), compared with control (lane 1), and VLDL or LDL showed minimal effects of TNF-α-induced VCAM-l expression (lane 6 and 7). However, HDL significantly inhibited the TNF-α-induced VCAM-l expression (lane 8, 43% inhibition). Flow cytometric profile of control cells and TNF-α treated cells without or with HDL show that the TNF-α treatment enhances the percentage of VCAM-1 positive cells (Fig. 4-1B, profile 3), and inclusion of HDL inhibits the number of TNF-α-induced VCAM-1 positive cells (Fig. 4-1B, profile 2). These results suggest that HDL affects adhesion molecule VCAM-1 expression in astroglioma cells.

4-4-2. Inhibition of TNF-α-induced VCAM-1 expression by HDL

Additional study by FACS analysis with different amounts of HDL (0-1000 μg/ml) was performed. As shown in Fig. 4-2A, HDL reduced the TNF-α-induced VCAM-l expression in a dose dependent manner; VCAM-1 expression was reduced by 56% at a normal physiologic level of HDL (lane 6, 500 μg HDL cholesterol/ml) compared with TNF-α alone (lane 2, 50 ng/ml). HDL (50-1000 μg/ml) alone had no effects on VCAM-l expression, similar with control (lane 1). To test the specificity of these inhibitory effects, antibody against apo A-I, the major apolipoprotein species of HDL, was used. For this test, anti-apo A-I antibody was pre-incubated with HDL for 30 min at RT and these complexes were added to the cells, and then TNF-α was treated for

additional 48h. As shown in Fig. 4-2B, TNF-α-induced VCAM-1 expression was inhibited by HDL (~58%). This inhibitory effect by HDL was significantly abolished (~31%) by the addition of anti-apo A-I Ab, suggesting that inhibition by HDL is specific. Preincubation of HDL with a control IgG had no effect in this response (data not shown).

4-4-3. Inhibition of TNF-α-induced VCAM-1 expression by Reconstituted HDL

Recent studies have shown that reconstituted HDL particles prepared from a major apolipoprotein species of HDL (apo A-I) and phosphatidylcholine (PC), but not apo A-I or PC alone, are effective in inhibiting TNF-α-induced VCAM-l expression in endothelial cells (139, 140). Therefore, we tested the effect of HDL and cholesterol-free reconstituted HDL prepared from delipidated HDL (apo HDL) and DMPC on VCAM-l expression in U373-MG cells. As shown in Fig. 4-3, reconstituted HDL had an inhibitory effect on TNF-α-induced VCAM-l expression (lane 4, 38%), but the effect was lower than that of native HDL (lane 3, 45%). Apo HDL or DMPC liposomes alone had a minimal effect on TNF-α-induced VCAM-l expression (lane 5 and 6).

4-4-4. HDL inhibits TNF-α-induced VCAM-1 mRNA expression

We further examined whether HDL could modulate VCAM-1 mRNA expression. As illustrated in Fig. 4-4A/B, U373-MG cells are constitutively negative for VCAM-l mRNA (lane 1), while TNF-α induces VCAM-l mRNA expression (3h-12h; lane 2, 4, 6, 8), and inclusion of HDL inhibits TNF-α-induced VCAM-1 mRNA expression by more than 80% with the lapse of time (3 h-12 h; lane 3, 5, 7, 9). GAPDH was utilized as a house-keeping gene. These findings at the mRNA level are comparable to what was observed at the VCAM-l protein level (Fig. 4-1 and 4-2).

Fig. 4-1A

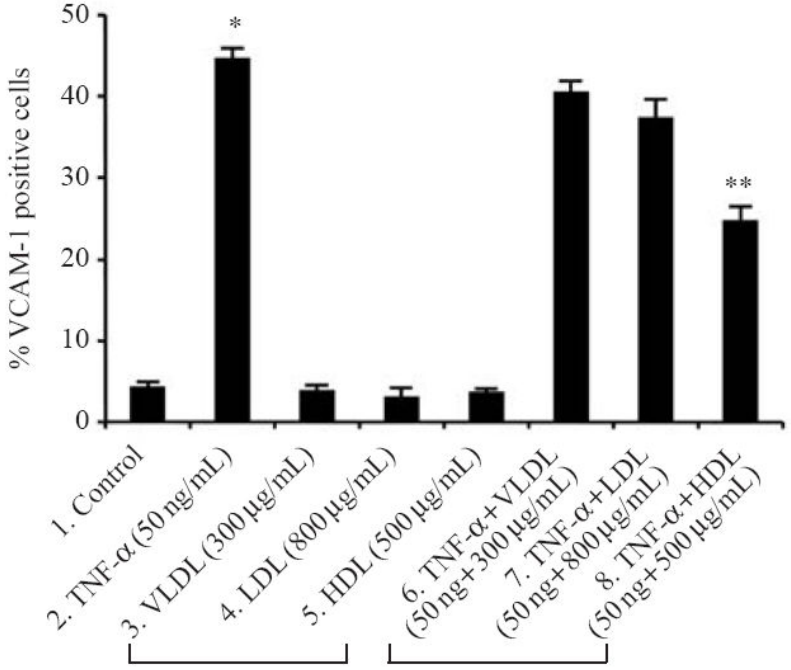

Fig. 4-1B

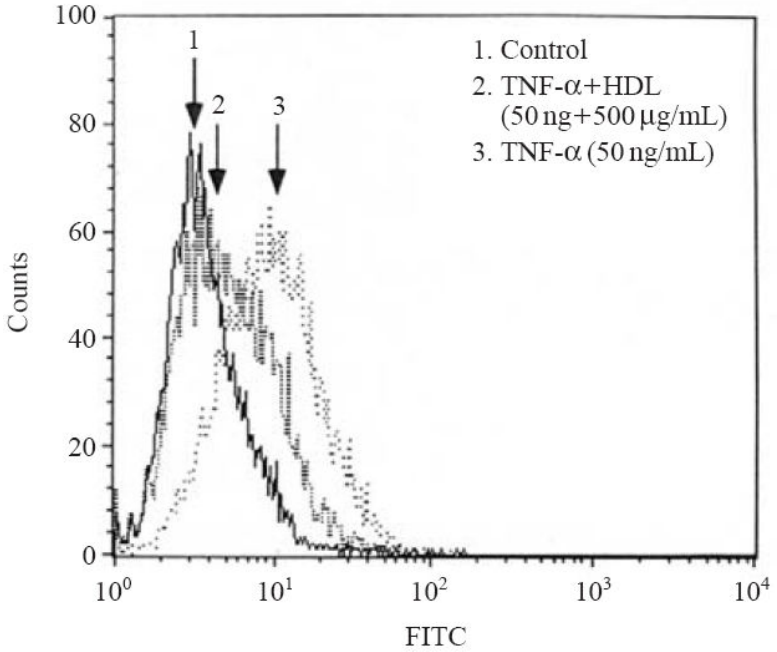

Fig. 4-1. Effect of plasma lipoproteins and/or TNF-α on VCAM-l expression on astroglioma cell lines. **A**. U373-MG cells were incubated with either medium alone, TNF-α (50 ng/ml), VLDL (300 μg cholesterol), LDL (800 μg cholesterol) or HDL (500 μg cholesterol) or TNF-α with lipoproteins (VLDL, LDL or HDL) for 48 h. Cells were harvested and analyzed for VCAM-1 expression by FACS. Values expressed as the means ± SD. $^{**}p<0.01$ versus TNF-α-treated control group. **B.** U373-MG cells were incubated with either medium alone or TNF-α (50 ng/ml) with or without HDL (500 μg/ml) for 48 h. The cells were then trypsinized, and VCAM-1 expression was assessed by FACS analysis. Flow cytometric profile 1 shows control cells, and profiles 2 and 3 show cells incubated with TNF-α with or without HDL, respectively. A representative of three experiments.

Fig. 4-2A

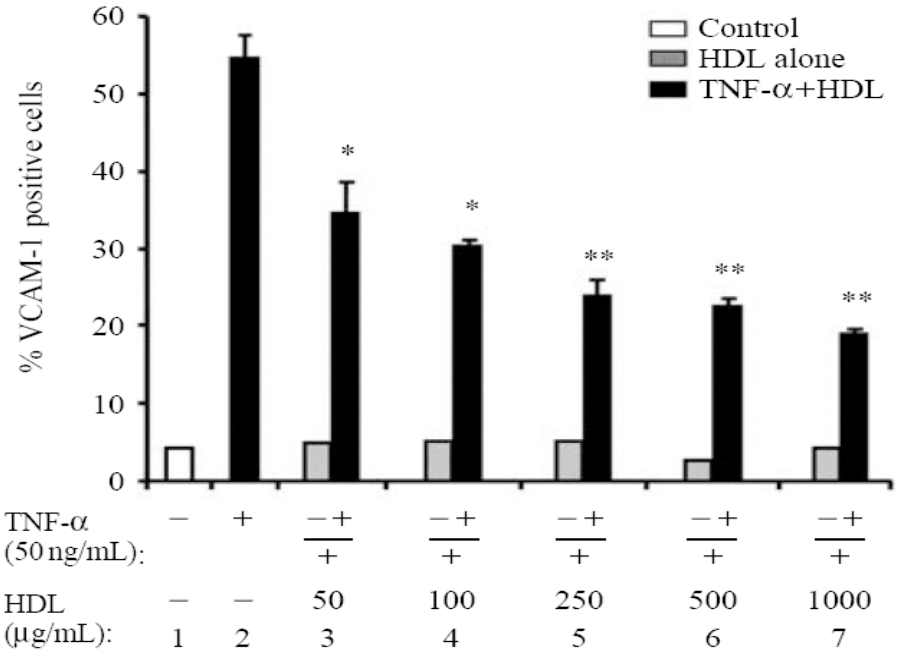

Fig. 4-2B

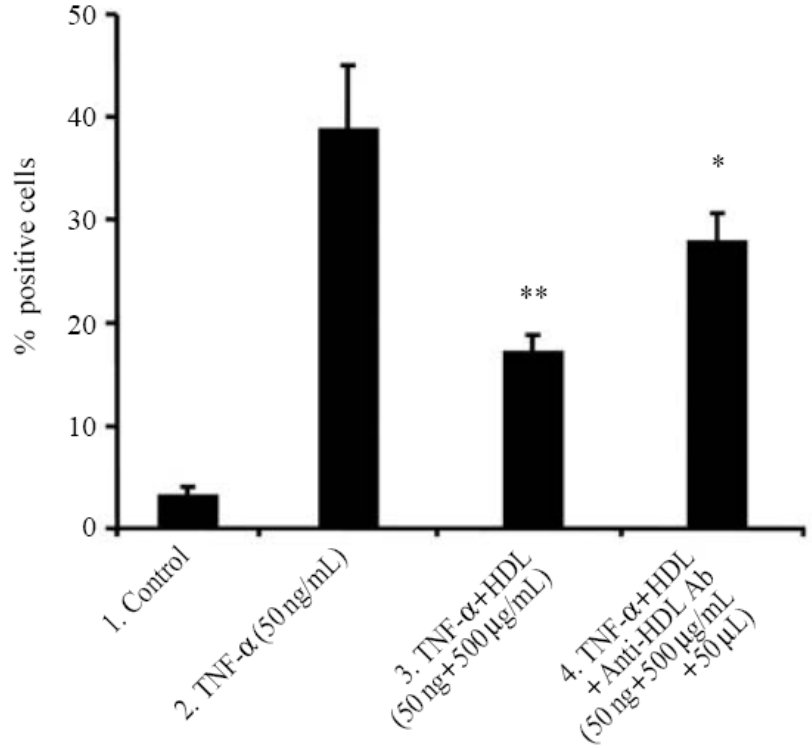

Fig. 4-2. The inhibitory effect of HDL on TNF-α-induced VCAM-1 expression. **A.** U373-MG cells were incubated with either medium alone, TNF-α (50 ng/ml), or increasing concentrations of HDL (50-1000 μg HDL cholesterol/ml) with or without TNF-α (50 ng/ml) for 48 h. Cells were harvested and analyzed by FACS for VCAM-l expression. Values expressed as the means ± SD of three separate experiments. *p<0.05 or **p<0.01 versus TNF-α-treated control group. **B.** U373-MG cells were incubated with either medium alone or TNF-α (50 ng/ml), TNF-α plus HDL (500 μg cholesterol/ml) or TNF-α plus HDL plus anti apo A-I Ab (50 μl of 1:100 dilution) for 48 h. Cells were harvested and analyzed for VCAM-1 expression. Values expressed as the means ± SD of three separate experiments. *p<0.05 or **p<0.01 versus TNF-α-treated group.

Fig. 4-3

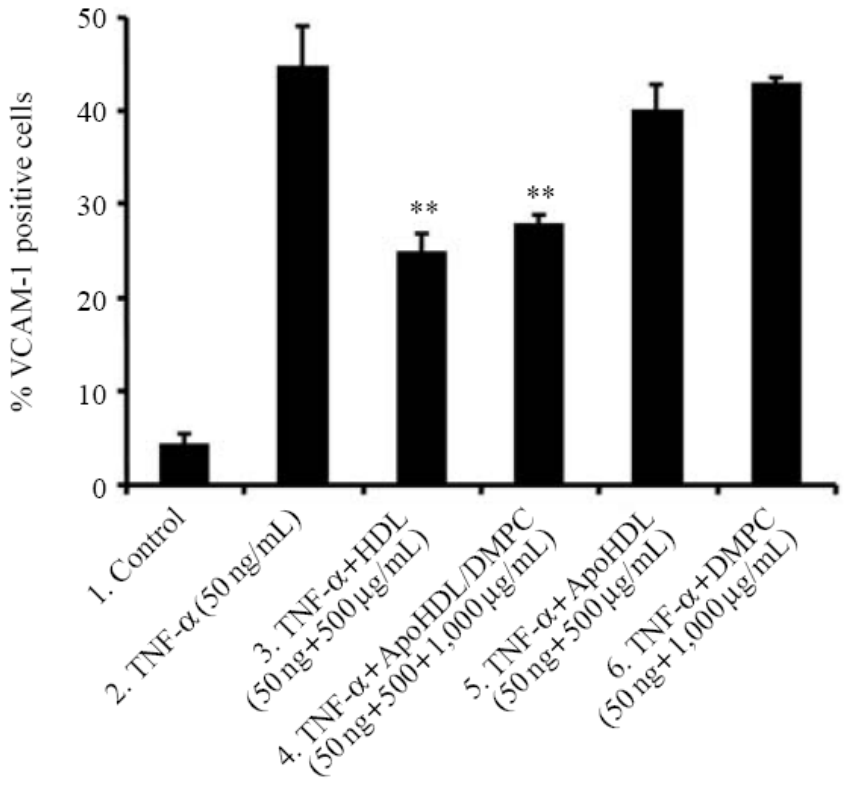

Fig. 4-3. Effect of various HDL forms on the TNF-α-induced VCAM-I expression. U373-MG cells were incubated with either medium alone, TNF-α (50 ng/ml), TNF-α plus native HDL (500 μg protein/ml), TNF-α plus reconstituted HDL (500 μg apo HDL and 1000 μg DMPC/ml), TNF-α plus apo HDL (500 μg protein/ml*)* or TNF-α plus unilamellar DMPC liposomes (1000 μg/ml*)* for 48 h. Cells were harvested and analyzed for VCAM-1 expression. Values expressed as the means ± SD of three separate experiments. **$p<0.01$ versus TNF-α-treated control group.

Fig. 4-4A

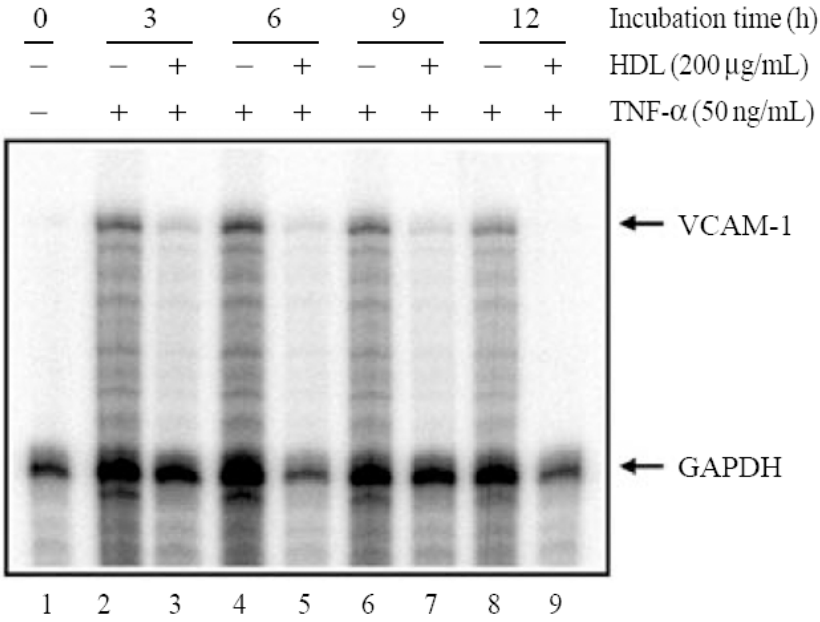

Fig. 4-4B

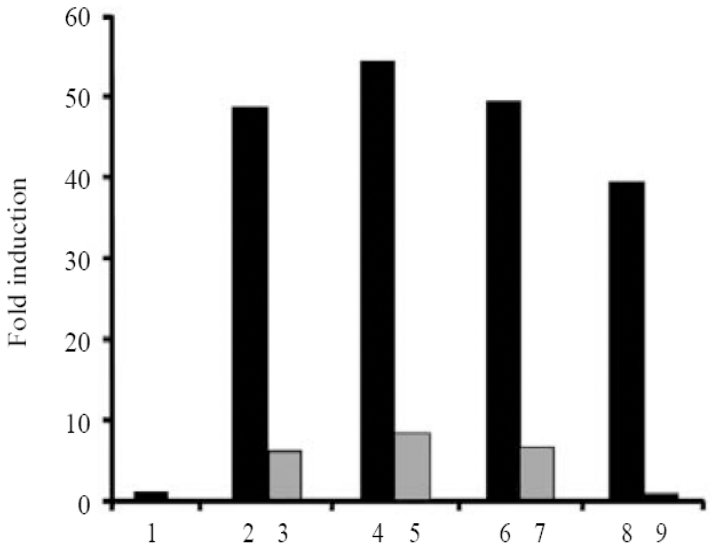

Fig. 4-4. HDL inhibition of TNF-α-induced VCAM-1 mRNA expression. **A.** U373-MG cells were incubated with medium alone for 3 h (lane 1) or with medium containing TNF-α ± HDL for 3 h (lanes 3 and 2), 6 h (lanes 5 and 4), 9 h (lanes 7 and 6) or 12 h (lanes 9 and 8). After incubation, RNA was extracted and analyzed for VCAM-1 and GAPDH mRNA by RPA. **B.** Values for VCAM-1 mRNA expression were normalized to GAPDH mRNA levels for each experimental condition. Quantification of the experiment shown in A is depicted. Constitutive expression of VCAM-1 mRNA was set at 1, and each treatment was compared with control levels to arrive at the fold induction value. A representative of two separate experiments.

4-5. DISCUSSION

In the present study, we investigated whether plasma HDL regulates VCAM-1 expression in human astroglioma cells. Our findings show that HDL alone does not induce VCAM-1 expression; rather it can inhibit TNF-α-induced VCAM-1 expression. Moreover, our results demonstrate a selective inhibitory effect of HDL on TNF-α-induced VCAM-1 expression with specific inhibition observed by anti-HDL antibodies.

HDL exerts their anti-inflammatory properties mediated multiple mechanisms, including G-proteins, MAPKs (141). Furthermore, HDL prevents endothelial cell dysfunction and activation, and reduces the deleterious effect of ox-LDL by regulating membrane cholesterol level (141, 142). Sato et al. also reported that HDL-like lipoproteins derived from CSF had ability to alter the migration of neural cells (143), that is similar function to that of plasma HDL shown in endothelial cells (142), suggesting their functional similarity. Therefore, native plasma HDL inhibition of VCAM-1 expression (in this study) raises a possibility that HDL-like particles in the CSF may play a role of modulating VCAM-l expression in glial cells**.** The apo E and/or apo A-1 containing HDL-like particles in the CNS have been suggested to be formed via a recombinant process involving the uptake of cellular phospholipids by apo E synthesized by astrocytes and other glial cells, and by apo A-I transported from periphery into the brain parenchyma or synthesized by brain endothelial cells via ABCA1 transporter (144). We also observed in this study that plasma HDL was effective in suppressing VCAM-1 expression in cultured astrocytes, suggesting that apo A-I containing HDL-like particles, formed by recombinant process in the CNS, may play a role of regulating VCAM-1 expression.

HDL induces FGF-2 expression in astrocytes, which is associated with ERK activation and PLC activation and Ca^{2+} mobilization (145) and inhibits TNF-α-induced VCAM-1 expression in endothelial cells (146). We demonstrated the ability of natural HDL, their lipids, apolipoprotein components, and reconstituted HDL to inhibit TNF-α-induced VCAM-1 expression in astrocytes. VCAM-1 expression on these cells depends on the activation of transcription factors, in particular NF-κB (146); HDL inhibits the activation of NF-κB induced by TNF-α, resulting in inhibition of VCAM-1

expression in endothelial cells (146, 147). Xia et al. reported that HDL inhibited TNF-α stimulated sphingosine kinase activity in endothelial cells, resulting in a decrease in both sphingosine 1-phosphate production and TNF-α induced VCAM-1 expression, suggesting the importance of sphingosine kinase pathway (147). As well, it is known recently that HDL induces TGF-β expression in endothelial cells (148) and TGF-β inhibits TNF-α induced VCAM-1 expression in astrocytes (149), raising a possibility that HDL inhibition of TNF-α induced VCAM-1 expression shown in this study may be occurred by TGF-β induced by HDL. This possibility is being currently investigated.

Although the mechanism whereby HDL inhibits TNF-α induced VCAM-1 expression in the CNS is not fully understood, in any circumstances, if the integrity of BBB is destroyed by traumatic injury, hemorrhage or neuroinflammatory diseases, plasma HDL and proinflammatory cytokines including TNF-α could infiltrate into the CNS, and HDL may function as an immunosuppressive role in inhibiting cytokine-induced adhesion molecules expression, together with HDL-like particles derived from CNS.

CHAPTER 5

Conclusion

Anti-proliferative effect of IL-6 has been described in melanocytes and early stage of melanoma cells (13). In contrast, most cells from advanced stage of melanoma are resistant to IL-6 (79). Hence, melanoma immunotherapy using IL-6 has been limited *in vivo*. However, in combination with the sIL-6R, the IL-6 resistant cells become more sensitive. Therefore, IL-6 immunotherapy for melanoma utilizes sIL-6R in combination with IL-6. Preclinical studies in mouse model have demonstrated that the IL-6/sIL-6R system based vaccine in association with GM-CSF is able to elicit efficient anti-tumor responses, mediated by $CD8^+$ and NK cells activation, which result in inhibition of the tumor growth, metastasis formation, showing prolonged survival of the animals (28). Promising clinical results and the evidence of immune responses in the vaccinated patients have led IL-6/sIL-6R system based melanoma immunotherapy into phase III clinical trials (150). In these regards, novel alternate approaches to melanoma immunotherapy are being accelerated. To facilitate the *in vitro* studies for melanoma therapy, IL6RIL6, a fusion protein consisting of a part of sIL-6R and IL-6 has been recently developed (27, 28). IL6RIL6 protein has much stronger activity to induce signal transduction than that of IL-6 alone or IL-6 plus sIL-6R. Thus, IL6RIL6 made it possible to extend the functional significance of IL-6 and sIL-6R (29, 151, 152). We also tested IL6RIL6 fusion protein to find novel functionality of IL-6/sIL-6R system in B16/F10.9 melanoma cells.

In chapter 2, our results show that the IL6RIL6 protein directly causes cell growth inhibition of B16/F10.9 melanoma cells, and together with TNF-α enhances apoptosis (Fig. 2-1) by increasing TNF-receptor expression (Fig. 2-3) and downregulating Bcl-2, anti-apoptotic gene (Fig. 2-4). However, several other anti-apoptotic genes such as Bcl-xL, Mcl-1, and c-FLIP may also be involved in this mechanism because TNF-R55 neutralizing Ab could not completely abrogate the IL6RIL6/TNF-α enhanced apoptosis (Fig. 2-3C).

iNOS has been known to be expressed in melanoma, and iNOS up-regulation has been observed to be associated with tumor progression (80). A number of reports have suggested that the constitutive production of low levels of intracellular NO promotes melanoma tumor growth and survival (123, 124, 153), however high levels of

NO are cytostatic or cytotoxic (120). As well, transfection with the *iNOS* gene in murine melanoma cells has been shown to suppress tumorigenicity and abrogate metastasis (81). In chapter 3, we show that IL6RIL6 plus TNF-α potently increase *iNOS* gene expression and NO production (Fig. 3-1 and Fig. 3-2). IL6RIL6-activated AP-1 transcription factor seems to be needed for the iNOS induction by IL6RIL6 plus TNF-α stimulation (Fig. 3-4B and Fig. 3-5B). In addition, we found that activation of JNK and p38 MAPK in response to IL6RIL6 plus TNF-α are also important in the activation of c-Jun and c-Fos, members of AP-1 complex (Fig. 3-8).

The expression of adhesion molecules such as VCAM-1 and ICAM-1 are increased in the CNS inflammation process (129). These adhesion molecules are considered to contribute in extravasation of leucocytes across the BBB and into the CNS parenchyma (131). In chapter 4, we demonstrate that HDL inhibits TNF-α-induced VCAM-1 mRNA and protein expression in a dose-dependent manner (Fig. 4-1 and Fig. 4-4). Our results further demonstrate a selective inhibitory effect of HDL on TNF-α-induced VCAM-1 expression, indicating specific inhibition by anti-HDL antibodies (Fig. 4-2B).

Collectively, our results from chapter 2 highlight the functional importance of the IL-6/sIL-6R/gp130 system in the sensitization of TNF-α-resistant melanoma cells to TNF-α-mediated apoptosis. Also, results from chapter 3 demonstrate the novel functionality of IL-6/sIL6R/gp130 mediated AP-1 activation in *iNOS* gene expression, upon IL6RIL6 plus TNF-α-stimulation. Novel functions of the IL-6/sIL-6R/gp130 system shown in this study may help to find potential pharmaceutical targets for future melanoma treatment strategies. As well, in conditions of neuroinflammation, hemorrhage, or trauma by which integrity of BBB is destroyed, plasma HDL may enter the CNS. At these diseased conditions, plasma HDL together with the CNS-derived HDL-like particles secreted by the astrocytes or endothelial cells may function as an immunomodulator in inhibiting cytokine-induced adhesion molecules expression.

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Wagley Yadav

PUBLICATIONS LISTS

1. **Y. Wagley**, K.W. Kang, H.W. Kim, Y.R. Pokharel, Y.Y. Chung, I.Y. Chang, J.J. Kim, J.S. Moon, Y.K. Kim, S.Y. Nah, H.S. Kang, and J.W. Oh. 2007. Novel role of IL-6/SIL-6R signaling in the expression of inducible nitric oxide synthase (iNOS) in murine B16, metastatic melanoma clone F10.9, cells. *Free Radical Biology & Medicine* 42:215-27.

2. **Y. Wagley**, Y.C. Yoo, H.G. Seo, M.H. Ree, T.H. Kim, K.W. Kang, S.Y. Nah, and J.W. Oh. 2007. The IL-6/sIL-6R treatment of a malignant melanoma cell line enhances susceptibility to TNF-α-induced apoptosis *Biochemical and Biopphysical Research Communications* 354:985–991.

3. **Y. Wagley**, and J.W. Oh. 2008. Tumor Necrosis Factor-Alpha Induced VCAM-l Expression is Inhibited by High Density Lipoprotein in Human Astroglioma Cells. *Korean Journal of Physiology &. Anthropology* 21:225-233.

저작물 이용 허락서

학 과	의과대학 의학과	학 번	20047673	과 정	석사, 박사
성 명	한글: 와글리야다브 한문 :		영문 : Wagley Yadav		
주 소	광주광역시 동구 서석동 375. 조선대학교 의과대학 2호관 해부학교실 2110호				
연락처	E-MAIL: yadavwagley@gmail.com				
논문제목	한글: 피부암세포에서 IL-6/soluble IL-6 Receptor Signaling의 기능연구. 영어: Functional Studies of IL-6/soluble IL-6 Receptor Signaling in Melanoma.				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

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저작자: Wagley Yadav (와글리야다브) (서명 또는 인)

조선대학교 총장 귀하