CHAPTER I

INTRODUCTION

The IL-10 cytokine family and the related receptors

IL-10 family includes MDA-7 (IL-24), IL-19, IL-20, IL-TIF (IL-22), and AK155 (IL-26) (F. Sheikh V. et al., 2004; H. A. Whittington et al., 2004; Dumoutier L. et al., 2002). The IL-10 family's history provides insight into the use of, and need for, new topological maps and sequences for the identification of new molecule superfamilies. MDA-7, IL-19 and IL-20 form a subfamily within the IL-10 family, based on conserved amino acid sequences, and on the use of shared receptor complexes. These factors don't mimic IL-10 activities but they are involved in inflammatory processes and show a distinct expression pattern (Nagalakshmi et al., 2004). IL-28A, IL-28B, and IL-29 have been included into this family due to sequence homology rather than functional similarity.

Much is already known about the functions of some of the IL-10 family members. IL-10 is well established as a major anti-inflammatory and immunosuppressive mediator (Sung Woo Lee et al., 2002; Moore K. W. et al., 2001). In contrast to IL-10, the biological roles of IL-19, IL-20, IL-24 and IL-26 are less clear, but the current data imply that IL-19, IL-20 and IL-24 possess certain similar attributes. Among immune cells, IL-19, IL-20 and IL-24 are preferentially expressed by monocytes; IL-24 is additionally expressed by activated T cells (Wolk K. et al., 2002). Structurally and functionally, IL-24 is highly conserved across species. The potential roles of IL-24 as part of a complex cytokine network in wound healing, psoriasis and cancer are discussed. IL-24 is an immediate target gene of the ras oncogene as its expression was

induced by both oncogenic h-ras and k-ras in rat embryonic fibroblasts (Rat1) and rat instinal epithelial (RIE) cells through a mitogen-activated protein (MAP) kinase pathway (Zhang R. et al., 2000). Moreover, IL-24 is also increased in colorectal cancers with microsatellite instability (Allen M. et al., 2004) and in the infiltrating monocytes in the dermis of psoriasis skin lesions (Romer J. et al., 2003). Interestingly, murine IL-24, also called IL-4-induced secreted protein (FISP), was postulated to be a type 2 cytokine (T2) (Schaefer G. et al., 2001). Apart from its expression in immune cells, human IL-24, formerly designated as melanoma differentiation-associated gene 7, has also been shown to be expressed by normal melanocytes (Ekmekcioglu S. et al., 2001).

The receptors for the IL-10 family are all members of the class II cytokine receptor family (CRF2). All functional receptors are heterodimers composed of an alpha or type 1 (R1) chain and a beta or type 2 (R2) chain. Traditional nomenclature has often defined the alpha subunit as being the ligand-binding molecule and the beta subunit as being the signal transducing subunit. Some IL-10 family members share single receptor chains or whole receptor complexes (Table 1). This sharing does not appear to be associated with binding competition for these receptors or mutual limitation of their biological effects. After binding of ligand to the extracellular receptor domains, the signals are predominantly transduced via Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways.

IL-19, IL-20 and IL-24 are distinct from classical ILs

It is well established that the newly discovered IL-20 family of cytokines, all signal through heterodimerc cell surface receptors consisting of 4 types of receptor subunits, IL-20R1, IL22R1, IL-10R2 and IL-20R2 (Wang M. et al., 2005). Unlike heterodimeric receptors with IL-10R2 subunit, heterodimeric receptors consisting of IL-20R2 are quite promiscuous, with

IL-19, IL-20 and IL-24 all being able to bind to and signal through IL-20R1/IL-20R2 (Table 1), while IL-20 and IL-24 also being able to signal through IL-22R1/IL-20R2 (Wang M. et al., 2001; Sa SM. et al., 2007). Upon binding to its receptors, IL-24 induces rapid activation of Stat-1 and Stat-3 transcription factors, which appear to play a role in cell survival and proliferation (Fig. 1).

The significant receptor sharing among these cytokines based on *in vitro* biochemical assays with cultured cells thus has raised questions about whether the three cytokines have redundant biological functions *in vivo* or whether they use the same receptors for different biological end points in a tissue specific or temporally regulated manner.

Under physiological conditions, the major sources of IL-24 are the activated monocytes and T helper 2 cells, whereas the major IL-24 target tissues, based on the receptor expression pattern, are non-haematopoietic in origin, and include skin, lung and reproductive tissues. Supported by the evidence in receptor expression patterns, keratinocytes have been identified as a major target cell type for IL-20 family of cytokines (Wang M. et al., 2001; Sa SM. et al., 2007; Kunz et al., 2006). *In vitro* assay using reconstituted human epidermis has demonstrated that IL-19, IL-20 and IL-24, in addition to IL-22 which signals through IL-22R1/IL-10R2, all could induce acanthosis, S100A7 (psoriasin) expression, and Stat3 activation (Sa SM. et al., 2007). *In vivo*, abnormal over-expression of IL-19, IL-20 and IL-24 has been detected in human psoriatic skin tissues where IL-19 and IL-20 appeared to be expressed by the keratinoytes, while IL-24 was made by infiltrating monocytes (Romer et al., 2003; Wei et al., 2005).

Epidermal hyperplasia induced by intradermal injection of IL-23, a major cytokine implicated in psoriasis, was shown to be mediated by both IL-19 and IL-24, but not IL-20, in an IL-20R2 dependent manner (Chan et al., 2007). Direct intradermal administration of IL-23 in

Table 1. Composition of receptor complexes of IL-10 family members.

	IL-10R1	**IL-20R1**	**IL-22R1**
IL-10R2	IL-10	IL-26	IL-22
IL-20R2	_	IL-19, IL-20,IL-24	IL-20,IL-24
Each receptor complex is composed of two different chains, an R1 and an R2 chain (3–5). IL, interleukin.			

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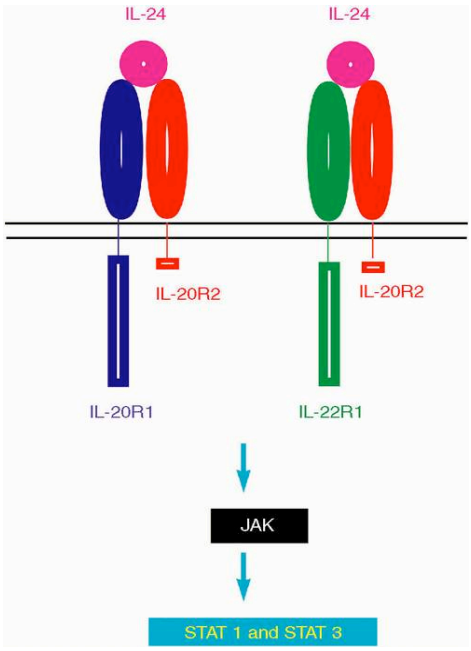

Figure 1. Schematic diagram of interleukin (IL)-24 binding to its two receptors (IL-20R1/ IL-20R2 and IL-22R1/IL-20R2). Wang M. et al., 2005

mouse skin, but not IL-12, initiates a tumor necrosis factor–dependent, but IL-17A–independent, cascade of events resulting in erythema, mixed dermal infiltrate, and epidermal hyperplasia associated with parakeratosis. However, convincing functional evidence *in vivo* from transgenic mouse model so far has only proved role of IL-20 in epidermal proliferation and differentiation (Bloomberg et al., 2001), while IL-19 transgenic mice purportedly did not manifest any overt skin phenotype (Parrish-Nvak et al., 2002). In contrast, structural basis for receptor sharing among IL-19, IL-20 and IL-24 has not been investigated in detail. Overall amino acid sequence homology among these three cytokines is only about 20-30 percent. While IL-24 was also shown to be able to bind to IL-20R2 alone expressed on the cell surface without the ability to signal (Wang M. et al., 2002), both IL-19 and IL-24, but not IL-20, reportedly could bind to a dimeric soluble IL-20R2-Fc fusion protein with varying degrees of affinity (Parish-Novak et al., 2002). However, apparently conflicting data from *in vitro* biochemical binding studies using purified monomeric soluble IL-20R2 receptors demonstrated that IL-20 could form a high affinity binary complex with sIL-20R2 (Pletev et al., 2003).

Twenty five human tissues for gene expression of the corresponding receptor chain were screened (Table 2). The components of the type I IL-20 receptor complex (IL-20R1/IL-20R2), which mediate the effects of IL-19, IL-20 and IL-24, were mainly expressed in the skin, lung and reproductive organs as well as in various glands. Simultaneous expression of the chains of the type II IL-20 receptor complex (IL-22R1/IL- 20R2), which mediates only the effects of IL-20 and IL-24, was more restricted with high levels found only in the skin. This agrees with the semi-quantitative data previously published by Parrish-Novak et al. According to the results obtained from blood immune cell subpopulations, these data demonstrate that receptor

complexes of all novel members of the IL-10 family including IL-19, IL-20 and IL-24, in contrast to IL-10 itself, are scarcely expressed in tissues containing high numbers of immune cells. However, many other tissues have been found to be targets of these novel cytokines. Interestingly, the skin appears to be a very important target for IL-19, IL-20 and IL-24.

In this study, we show that IL-24 transgenic mice targeted to the skin exhibit many phenotypes similar to that of IL-20 with profound effects on epidermal proliferation and differentiation. Furthermore, we demonstrate that soluble IL-20-Fc fusion protein can bind to IL-24 and IL-20, with high affinity and is a potent blocker of both cytokine functions.

Table 2. Tissue distribution of the receptor chains for the IL-10 family members in human tissues.

Tissue	IL-10R1	IL-10R2	IL-20R1	IL-20R2	IL-22R1		
Adrenal gland	++	+++	+++	+	−		
Bone marrow	++	+++	−	+	−		
Brain	+	++	++	+	−		
Cerebellum	+	++	++	+	−		
Colon	++	+++	++	++	++		
Heart	+++	+++	++	+	+		
Kidney	++	+++	+	+	++		
Liver	++	++++	++	++	+++		
Lung	+++	++++	+++	++	++		
Mammary gland	++	+++	+++	++	+		
Pancreas	+	+++	+++	+	++++		
Peripheral blood mononuclear cells	++++	++++	−	+	−		
Placenta	+++	+++++	+++	+++	++		
Prostate	++	++++	+++	++	+		
Salivary gland	++	++++	+++	++	+		
Skeletal muscle	+	+++	++	++	−		
Skin	+	++++	+++	++++	+++		
Small intestine	+++	+++	++	+	+++		
Spinal cord	+	+++	++	+	−		
Spleen	++++	++++	+	++	+		
Stomach	+++	+++	++	++	++		
Testis	−	++	++	++	−		
Thymus	+++	++++	++	++	−		
Thyroid gland	+	+++	++	+	++		
Uterus	++	+++	+++	+	−		

IL, interleukin. Gene expression data are presented relative to those of hypoxanthine phosphoribosyl-transferase 1 and transformed into symbols as follows: (–) 0 to <0.001; (+) 0.001–0.01; (++) 0.01–0.1; (+++) 0.1–1; (++++) 1–10; (+++++) 10–100.

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Evidence of anti-inflammatory effects of HDL

Physical properties such as charge, density, or particle size distinguish four classes of lipoproteins: high-density lipoprotein (HDL), lipoprotein (LDL), very-low-density lipoprotein (VLDL), and chylomicrons. LDL is known as "bad" cholesterol as it contributes to atherosclerosis. HDL is known as "good" cholesterol as it carts excess cholesterol off to the liver for excretion though the bile. HDL has a core with triglyceride and cholesteryl ester, surrounded by the surface of phospholipids and free cholesterol. ApoA1 wraps the HDL particle as the major protein component of HDL plasma, and promotes cholesterol efflux from tissues to the liver for excretion in bile. The precise mechanism of the ability of HDL to protect against atherosclerosis (Gordon T. et al., 1977; Castelli WP. et al., 1986) is uncertain, although it is believed to involve the consequence of one or more the reported actions of HDL (Barter P. et al., 2003). The most well known mechanism of these is that HDL promotes the efflux of cholesterols from cells, minimizing the accumulation of foam cells in the artery wall (Zhang Y. et al., 2003).

Studies of CETP inhibition in rabbits have consistently shown a reduction in atherosclerosis (Rader D. J. et al., 2004). CETP is a plasma glycoprotein that facilitates the transfer of cholesteryl esters from HDL cholesterol to apolipoprotein B–containing lipoproteins (Sirtori CR et al., 2006; Thompson JF et al., 2003). A new drug candidate called Torcetrapib that can enhance HDL was withdrawn. The problem is that despite significantly raising HDL, Torcetrapib failed to slow the progression of coronary plaques. CETP inhibitor, torcetrapib, increased HDL-C levels in healthy volunteers (Clark R. W. et al., 2004) and in subjects with low

baseline HDL-C levels (Brousseau M.E. et al., 2004). Reductions in CETP activity resulted in significantly increased HDL cholesterol concentrations (Davidson M. H. et al., 2006).

The question from Torcetrapib is if increased HDL cholesterol (HDL-C) could not slow down the progression of coronary plaques, which component in HDL may contribute to the antiinflammatory functions of HDL? The hypothesis is that the major protein ApoA1 of HDL negatively regulates IL-24 signaling pathway through direct binding to inflammatory cytokines and thereby inhibiting their signaling. Identification of ApoA1 binding domain (ABD) of IL-24 and of the other cytokines or proteins may ultimately lead to development of novel therapeutics for the treatment of inflammation, psoriasis and other cancers. It is possible that these studies will serve as the foundation upon which more rigorous anti-inflammatory studies of ApoA1/HDL can be conducted.

CHAPTER II

MATERIALS AND METHODS

Cell culture and Cell lines

HEK293T cells were obtained from GenHunter Corporation (Nashville, TN), and were cultured at 37^0C in Dulbecco's Modified Eagle's Media (DMEM, GibcoBRL) containing 10% bovine calf serum (Hyclone) and 1 % penicillin (10,000 U/ml)-streptomycin (10mg/ml) (GibcoBRL) under 10% CO2. BHK21 (Baby Hamster Kidney) cells were obtained from American Type Culture Collection (ATCC), and were maintained in DMEM containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 1% penicillin-streptomycin, and 1mM Sodium Pyruvate (Biowhittaker). BHK21 cells transfected with IL-20R1 and IL-20R2 were established previously and were cultured in the same medium used for BHK21 cells (Wang M. et al., 2002). CHO-s cells were obtained form GenHunter Corporation (Nashville, TN) and were cultured in IMDM, containing 10% fetal bovine serum (Atlanta Biologicals), 1% penicillin-streptomycin. The following cells were established previously and were cultured in DMEM containing 10% bovine calf serum and 1% penicillin-streptomycin, including HEK293T cells stably expressing AP, human IL-24-AP and rat MOB-5-AP, human IL-19-AP or human IL-20-AP.

Constructs Establishment

Soluble receptor psIL-20R2-Fc

The extracellular domain (690bp) of IL-20R2 (NM_144717) was amplified by PCR using two pairs of primers based on both ends of extracellular domain of IL-20R2.

1st primer pair, L: 5'- AAGCTTAGTCTACCAAATGCAGAC-3' (native Kozak sequence included), and R: 5'-AGATCTCTCTCCTTGCACCTCCACAC-3',

2nd primer pair, L: 5'-AAGCTTCACCATGGAGACTTTCACAATGGTTC-3'(optimized Kozak sequence included), R: 5'-AGATCTCTCTCCTTGCACCTCCACAC-3'.

The sequences after PCR were subcloned into pGH-hFc vector obtained from GenHunter Corporation (Nashville, TN) betweem Bgl II and Hind III sites.

Secreted pApoA1-Fc

The full-length sequence (803bp) of Apolipoprotein A1 (NM_000039) from pDNR-lib-ApoA1 (ATTC) was amplified by PCR using the following pair of primers:

L: 5'- AAGCTTCACCATGGAAGCTGCGGTGCTGACC-3'

R: 5'-AGATCTCTGGGTGTTGAGCTTCTTAG-3'.

The sequence after PCR was subcloned into pGH-hFc obtained from GenHunter Corporation (Nashville, TN) between Hind III and Bgl II siste.

pK5-FISP construct

The mouse homology of human IL-24, FISP (NM_053095), from pFLC III was purchased from RIKEN Mouse FANTOM (G4D0005G19) and amplified by PCR using a pair of primers:

L-FISP: 5'-GCTAGCTTCCCACCCAGCAGAAGATC-3'

R-FISP: 5’-GCTAGCAAAGGAGGAAGTTATCCAATC-3’.

The full-length FISP was subcloned into keratinocyte-specific expression vector pK5 obtained from Dr. Ramires (Ciemat, Spain) at Nhe I site. The correct insertion (720bp of full length FISP) was digested with Stu I and Snab I double digetion to obtain a fragment of 430 bp or screened by PCR using the primers,

L-K5: 5’-CGTGCTGGTTATTGTGCTGTCTC-3’ derived from vector pK5 and,

R-ext: 5’-GAATGACCTCAAGACCTTGAATTTG-3’, derived from FISP to obtain a 450bp fragment. As a control, the 130bp genomic DNA locus of BID was obtained by PCR using the primers, 17B14: 5’-CCGAAATGTCCCATAAGAG-3’ located in murine Bid intron 3 near E3/I3 boundary and 17B12: 5’-GAGATGGACCACAACATC-3’ located in exon 3.

pCI-Neo-FISP construct

FISP purchased from RIKEN Mouse FANTOM (G4D0005G19) was amplified by PCR using a pair of primers:

L: 5’-GCTAGCTTCCCACCCAGCAGAAGATC-3’,

R: 5’-TCTAGAGCAAAGGAGGAAGTTATCCAATC-3’.

The PCR product was subcloned into pCI-neo mammalian general expression vector (Promega Corporation) between Nhe I and Xba I site. The correct construct was screened by either digesting it with NheI and XbaI, or transiently transfecting into 293T cells. The medium was collected after 72hr transfection and subjected to Western Blot using anti-rat IL-24 (1:1000).

pIL-19- AP and pIL-20-AP constructs

Full length of IL-19 (533bp) and IL-20 (530bp) were obtained from pcDEF3-IL-19 and pcDEF3-IL-20 kindly provided by Dr.Kotenko from NIHand amplified by PCR using primers:

For IL-19,

L: 5'-AAGCTTGGTACCACGGTG-3',

R: 5'-AGATCTAGCTGAGGACATTACTTC-3',

For IL-20,

L: 5'-AAGCTTGGTACCTTAG-3',

R: 5'-AGATCTTTCTGTCTCCTCCATCC-3'.

The products were subcloned into pAPtag2 expression vector obtained from GenHunter Corporation (Nashville, TN) between Hind III and Bgl II sites and sequence verified.

Transfections

Transient transfecion

Transient transfection was performed with Fugen 6 transfection reagent (Roche) according to the manufacture's protocol. 1μg of plasmid with 3μl of Fugen 6 was used for transfecting 70% confluent cells in 6-well plates (equal to 35mm plates), and 2μg of plasmids with 6μl of Fugen 6 were used for transfecting 70% confluent cells in 60mm plates. Cells were used for experiments 48 hr post-transfection.

Stable transfection

HEK 293T and CHO-s cells stably expressing soluble receptor IL-20R2-Fc were established, using the plasmid construct described above. HEK 293T and CHO-s cells were

seeded in 60mm plates and grown until 70% confluent. Both cells were co-transfected with 2μg of the pIL-20R2-Fc expression plasmid along with 0.2μg of pBabe-puro and Fugen 6 (Roche). 24 hr after transfection, 800μg/ml G418 (Gibco BRL) was added into CHO-s-sIL-20R2-Fc and 10μg/ml puromycin (Sigma) was added to 293T- sIL-20R2-Fc cells, and the selection was carried out for 4 days before drug resistant cells were pooled. The stable transfected cell lines were maintained in the same medium, with 500μg/ml G418 for CHO-s-sIL-20R2-Fc and 5μg/ml puromycin for 293T- sIL-20R2-Fc.

HEK293T cells stably expressing secreted ApoA1-Fc, IL-19-AP and IL-20-AP were established, using the plasmid construct described above. The stable transfected cell lines were maintained in the same medium with 5μg/ml puromycin for 293T-ApoA1-Fc, 293T- IL-19-AP and 293T-IL-20-AP cells.

Histological analysis and immunohistochemistry

Skin samples from the backs of IL-24 TG pups (neonatal lethal) and normal control littermates (1 day old) were fixed in 10% neutral buffered formalin, paraffin-embedded and sectioned at 5μm and stained with hematoxylin and eosin. For immunohistochemistry, the sections were rehydrated and placed in heated Target Retrieval Solution (Dakocytomation, Carpinteria, CA) for 20 minutes. Endogenous peroxidase activity was quenched with 0.03% hydrogen peroxide and a protein block treatment (Dako, Inc.) was performed prior to primary antibody addition. Tissues were incubated with anti-Keratin 6 antibody (Covance Research Products) at 1:2000, anti-Keratin 5 antibody (Covance Research Products) at 1:500, or anti-Ki67 antibody (Vector Laboratories, Burlingame, CA) at 1:2000, for 60 minutes or rat anti-

mouse F4/80 (Serotec Inc., Raleigh, NC) diluted 1:50 overnight. The Rabbit Envision+ / DAB+ system (Dako, Inc) and Dako Envision+ HRP/DAB System (DakoCytomation) were used to produce localized and visible staining. Slides were lightly counterstained with Mayer's hematoxylin, dehydrated and mounted with cover-slip. Tissues incubated with goat anti-CD3 (Santa Cruz Biotechnology, Inc, cat. # SC-1127) were diluted 1:500 for 60 minutes. The Vectastain ABC Elite (Vector Laboratories, Inc) System and DAB+ (DakoCytomation) or Nova Red was used for staining.

Formalin-fixed, paraffin sections of were obtained from the Mouse Pathology at Vanderbilt University Medical Center. The sections were deparaffinized by incubating on a slide heater at 60^0C for 60min, then were subjected to two 10-min buffer changes in 100% xylene. The sections next were rehydrated by a series of incubations in 100%, 100%, 95%, and 85% ethanol, followed by ddH_2O, for 5 min each. To optimize signal, the solution then were subjected to an antigen retrieval procedure with 10mM sodium citrate solution (pH 6.0). The slides were placed in the sodium citrate solution, micromaved for 45 sec at full power, and heated in a pre-warmed steamer for 25min. After cooling at room temperature for 15min, the slides were washed three times with PBS. After antigen retrieval, the specimens were treated with 3% H_2O_2 (DAKO) for 5 min to quench endogenous peroxidase activity. The samples were washed twice with PBS, and nonspecific binding sites were blocked with serum-free blocking reagent (DAKO) for 10min at room temperature. Rat IL-24 monoclonal antibody (GenHunter Corporation) was used as the primary antibody. The optimal antibody concentration (1:100) was determined by testing a serial of dilutions of the antibody at 1:100, 1:200, 1:500, and 1:1000 with antibody diluent (DAKO). The specimens were incubated with monoclonal IL-24

antibody in a covered humidified chamber for 60 min. To evaluated nonspecific staining and allow better interpretation of specific staining, non-immune mouse serum (DAKO X0910) with the same total protein concentration and the same dilution as the primary antibody was applied to the sequential sections as a negative control. After primary antibody incubation, the slides were washed three times with PBS. The specimens were then incubated for 10 min at room temperature with biotin labeled goat anti-mouse immunoglobulin (DAKO). The samples were washed three times with PBS. Next, Streptavidin-HRP (streptavidin conjugated to horseradish peroxidase, DAKO) was applied to the sections, and the incubation was carried out for 10 min. After washing three times with PBS, the specimens were incubated with substrate chromogen DAB (DAKO). DAB solution was applied on each pair of specimens (the testing sample and negative control) individually, and sections were monitored under a microscope for color development. Color development time was in the range of 5 to 10 min. The reaction was stopped by rinsing off the excessive DAB solution with PBS. The specimens then were counterstained with hematoxylin (Mayer's, VWR) for nuclear staining. Afterwards, the slides were dehydrated by sequential incubation in 95% ethanol, 100% ethanol and 100% ethanol for 5 min each, before transferring to xylene. The slides were mounted with Cytoseal XYL (Stephens Scientific).

Immunoprecipitation (IP)

Conditioned medium (800 μl)of AP or human IL-24-AP, IL-19-AP and IL-20-AP were collected and incubated with anti-AP sepharose beads (GenHunter) for 1hr to pull down AP and cytokine-AP fusion proteims. The beads were then individually incubated over night with 1ml

conditioned medium of sIL-20R2-Fc at 4^0C with gentle shaking. The supernatant (unbound sIL-20R2-Fc) was removed. After 3X washing with PBS, the beads were resuspended in 20μl PBS. The solution was mixed with equal volume of 2×SDS sample buffer (125mM Tris-HCL pH 6.8, 20% glycerol, 4% SDS, 2% β-mercaptoethanol, 0.001% bromophenol blue) and boiled for 10 min. The samples then were separated on 10% SDS-PAGE and transferred to PVDF membranes (Amersham). After the transfer, the membrane was washed once with PBS-T (1×PBS, 0.1% Tween-20) for 5 min, and incubated in blocking buffer (PBS-T, 5% w/v nonfat dry milk) for 1 hr at room temperature. The incubation with the first antibody against AP (1:1000 diluted in blocking buffer) was carried out with gentle agitation at room temperature for 1hr. Blots were then washed three times with PBS-T, 5 min for each time, and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000 diluted in blocking buffer) (Amersham) for 1 hr at room temperature. Following three 10-min washes with PBS-T, blot was developed by chemiluminescence (Amersham). The anti-human lgG-Fc antibody (1:5000 diluted in blocking buffer) (Amersham) was applied to the same samples for 2 hr at room temperature. Following the three 10min washed with PBS-T, blot was developed using ECL (GE health Sciences) as described by the manufaturer.

Reciprocally, conditioned media sIL-20R2-Fc was collected and purified by Protein A beads. The beads were incubated overnight at 4^0C with 1ml conditioned media of either AP, or human IL-24-AP at 4^0C with gentle shaking. The supernatant was removed and the beads were resuspended with 20μl PBS after three times 10 min wash with 1ml PBS. The pull-downed samples were analyzed by Western blot using either anti-human IgG-Fc antibody (1:5000) or polyclonal antibody against AP (1:1000) respectively under the same condition as described.

Generation of soluble sIL-20R2-Fc and ApoA1-Fc Fusion Protein

For generating soluble IL-20R2-IgG Fc fusion protein, the extracellular domain of the full-length human IL-20R2 (Bloomberg et al., 2000) was PCR amplified and subcloned into the Bgl II and Hind III sites of pGH-hFc vector (GenHunter Corp. Nashville, TN) to allow in-fame fusion of sIL-20R2 with human IgG Fc. The expression vector was verified by sequencing and transfected into CHO cells for serum free production of the fusion protein.

Protein purification

The sIL-20R2-Fc or ApoA1-Fc was buffered with 10mM HEPES, pH 7.9, filtered with a uStar filter (0.45μm pore size), and stored in -20^{0}C in aliquots. The sIL-20R2-Fc or ApoA1-Fc was purified to homogeneity from the culture media using MabSelect (GE Health Sciences) as instructed by the manufacturer. The beads were washed with 20ml 100mM Tris (pH 8.0) followed by 20ml 10mM Tris (pH 8.0) and eluted with 100 mM glycine (pH 3.0). The eluted solution was collected in 1.5ml conical tubes containing 50μl of 1M Tris (pH 8.0). The protein concentration was determined using Bio-Rad system as instructed by the manufacturer. The purified Fc fusion proteins were analyzed by SDS-PAGE for purity and correct molecular weight.

ELISA Assay for determining affinity between sIL-20R2-Fc and IL-19, IL-20 and IL-24

100μl of anti human IgG-Fc (Amersham) at the concentration of 1μg/μl was coated on the Immuno-Plate (NUNC-*MaxiSorp*^{TM}) for 1hr at room temperature. The wells were then washed with PBS for 5 times. 1% BSA 100μl (dissolved in PBS and stored at 4°C) was utilized

to block the unspecific binding for 1hr at room temperature (optional). 100μl sIL-20R2-Fc at the concentration of 10μg/μl was added into wells and incubated for 2hr at room temperature. The solution was discarded and wells were thoroughly washed with 1× Hanks Buffer for 3 times. Hanks Buffer that contains Mg^{2+} and Ca^{2+} are essential to ionic interaction. 100μl of either AP, IL-19-AP, IL-20-AP or IL-24-AP (all at 0.5 unit/ml AP activity) were added into wells to incubate 1hr at room temperature. After washing with 1× Hanks Buffer for 3 times, 100μl of AP substrate 1× AP assay A reagent (GenHunter, Nashville,TN) was added to each well. After incubation at 37 C, the reation was stopped by adding 100μl of 0.5M NaOH. The solutions were transferred into tubes and added 300μl ddH_2O to make it into 0.5ml. OD_{405} was measured with spectrophotomer.

Determination of inhibitory function of sIL-20R2-Fc on IL-24's binding to its membrane receptor

Competitive Receptor Binding Assay

Competitive receptor binding studies was carried out using AP assay reagent A (GenHunter Corp.), following the manufacturers instructions (Wang M. et al., 2002). Briefly, BHK21 and BHK21 (IL-20R2/IL-20R2) cells were seeded at the density of 1×10^5/well in 12-well plates and grown to 90% confluence. The assay was carried out in duplicate with 0.5 ml/ well of either human IL-24-AP, IL-19-AP, IL-20-AP or AP alone conditioned media produced by 293T cells as previously described. The AP activities of conditional media were kept equal at 1.0 unit/ml. The sIL-20R2-Fc was added at increased dose ranging from 0 to 90μM (Baker PW et al., 1999) in both cell lines. Receptor specific ligand binding was determined by

subtracting nonspecific IL-24-AP, IL-19-AP and IL-20-AP binding to BHK21 cells and the ODs were recorded in a microtiter plate reader at a 405-nm wavelength.

Measurement of alkaline phosphatase activity

5μl of conditioned medium was diluted to 50μl with ddH_2O and incubated with 50μl 2×AP substrate buffer (GenHunter Corp.) at 37^0C for about10 min. The reaction was stopped by adding 100μl of 0.5 M NaOH. The sample volume was adjusted to 1 ml with ddH_2O for the following measurement. The activity was measured by the change in absorbance at 405nm (optical density (OD)) in a spectrophotometer. To convert from OD_{405} to enzyme activity (u/ml; 1 unit of enzyme hydrolyzes 1mmol of p-nitrophenyl phosphate at 37^0C per min (GenHunter, Corp.)), the following calculation was used:

AP activity (unit/ ml) = OD_{405}×54/ (N×M).

N: reaction time in min. M: sample volume in μl.

To convert from AP activity (unit/ml) to fusion protein concentration (mg/ml), the following calculation was used:

AP fusion protein concentration (mg/ml) = AP activity/{750×[67/(67+W)]}

W: molecular weight of the protein interest.

Quantitative assay for ligand-AP in situ on paraffin-embedded sections

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated as described for immunohistochemical staining. The sections were equilibrated in HBHA for 5 min, before incubation with 1unit/ml AP or rat IL-24-AP for 90 min at room temperature. After

washing six times with HBHA, the tissue sections were fixed for 30 sec with acetone-formalin fixative. The slides were washed twice for 5 min with HS. Endogenous alkaline phosphatase activity was inactivated by 60-min incubation in HBHA at 65^0C. After cooling to room temperature, the tissue sections were stained for 30 min with BCIP/NBT Alkaline Phosphatase Substrate in the dark. The slides were washed in ddH_2O to stop the reaction, and mounted with the water-based SHUR/MOUNT (Triangle Biomedical Sciences).

Generation of IL-24 transgenic mice

For creating IL-24 transgenic mice targeted to the skin, full-length FISP cDNA clone (Genbank accession No. BB867056) was purchased from RIKEN Institute & Centers, Japan. The complete coding region of mouse IL-24 was PCR amplified and subcloned into the NheI site of pK5 mammalian expression vector under bovine Keratin 5 promotor control (Murillas et al., 1995). The construct was verified by sequencing before micorinjection. Generation of IL-24 transgenic mice was carried by Vanderbilt Transgenic Mouse /ES Cell Shared resource. Briefly, female B6D2 mice were obtained from Harlan (Indianapolis, Indiana) at 3-5 weeks of age. These females were super-ovulated and mated with B6D2 males for embryo production. The embryos that survived injection were transferred into the oviduct of CD-1 pseudo plugged females (Charles Rivers).

Genotying for IL-24 transgenic mice

0.5 cm of pup tails were digested by 100μl DNA digestion buffer (50mM Tris-HCL pH 8.0, 100mM EDTA pH 8.0, 100mM NaCl, 1% SDS) with proteinase K at the final concentration

of 20mg/ml, and incubated overnight at 50-55^{0}C with gentle shaking, followed by adding 100μl isopropanal with finger mix. The supernatant was discarded after spin down 10min. 1 ml 70% ethanol was added and spun down with 10min. Dry pellet was dissolved in 100μl Tris-HCL (100mM, pH 8.0), and was carried out at 37-50^{0}C for over 2 hr. The approximate 450pb fragment from FISP was obtained by PCR using the primers,

L: 5'-GCTAGCTTCCCACCCAGCAGAAGATC-3' based on 5' end of FISP consensus and R-ext: 5'-GAATGACCTCAAGACCTTGAATTTG-3', derived from vector bovine K5.

The 130bp genomic DNA, locus BID was obtained by PCR using the primers, 17B14: 5'-CCGAAATGTCCCATAAGAG-3' located in murine Bid intron 3 near E3/I3 boundary and 17B12: 5'-GAGATGGACCACAACATC-3' located in exon 3.

Polymerase Chain Reaction (PCR)

PCR (polymerase chain reaction) was utilized to amplify the genes that are interested. The template DNA was first denatured at 94^{0}C for 2 min before stepping into 30 cycle amplification, which was different as indicated below (Table 3). Followed was an extension period at 72^{0}C for 5 min and holding of PCR products at 4^{0}C for further steps.

Table 3. PCR conditions for target genes.

30 cycles	Extracellular domain of IL-20R2	Full length ApoA1	Full length FISP	Full length BID	Full length IL-19	Full length IL-20
94^{0}C	30 sec	30 sec	20 sec	20 sec	20 sec	20 sec
52^{0}C/55^{0}C	52^{0}C 40 sec	52^{0}C 40 sec	55^{0}C 40 sec	55^{0}C 40 sec	55^{0}C 40 sec	55^{0}C 40 sec
72^{0}C	1 min	1.5 min	1 min	20 sec	1 min	1 min

CHAPTER III

SOLUBLE RECEPTOR IL-20R2-Fc INHIBITS THE IL-24 AND IL-20 INDUCED SIGNALING PATHWAY *IN VITRO*

Introduction

IL-24 proteins across species are highly homologous in amino acid sequence (Table 4). Without counting the divergent signal peptide sequences, the homology in mature cytokine sequences across species could be much higher. The mature IL-24 from both rat and mouse, when expressed by HEK-293 cells, has a molecular weight (MW) of ≈ 23 000 (Schaefer G. et al., 2001; Zhang R. et al., 2000), whereas the secreted human IL-24 has an MW of 33 000 (Wang M. et al., 2002) as a result of asparagine-linked (N-linked) glycosylation of the cytokine. However, the differential glycosylation between rodent and human IL-24 had little effect on receptor binding and activation. Importantly, the rodent IL-24 can bind to and activate the human IL-24 receptors. IL-24 binding to either its endogenous receptors on human keratinocytes or to ectopically expressed receptors on baby hamster kidney cells leads to activation of the signal transducers and activators of transcription (STAT) factors and induces expression of other cytokines, such as TNF-α, by PBMC. There is 26% aa identity between human IL-20 and IL-10, and 41% aa identity between IL-20 and IL-19 with conservation of all six cysteines. The IL-20 gene maps to chromosome 1 with IL-10, IL-19 and IL-24/mda-7. Mouse and human IL-20 show 76% aa identity in the mature segment. Cells known to produce IL-20 are monocytes43 and (perhaps) keratinocytes. Until now only IL-20 has been proven to play an important role *in vivo* in the epidermis where both receptors are expressed.

Table 4. Conservation of the interleukin (IL)-24 primary sequence across species.

			Amino acid sequence homology		
Gene	Species	Alternative name	MDA-7	Mob-5	FISP
hIL-24	Human	MDA-7	100%	68%	69%
rIL-24	Rat	MOB-5, C49A	68%	100%	93%
mIL-24	Mouse	FISP	69%	93%	100%

h, human; m, murine; r, recombinant. Mai Wang and Peng Liang, 2004

While the intracellular domains are vary in length and do not show obvious sequence similarity, the extracellular domains of Class II cytokine receptors are rather conserved. These receptors are characterized by the presence of two fibronectin type III (FNIII) domains in their extracellular region. These typically are 210 aa globular modules that consist of seven beta-strands that form two beta-sheets (reminiscent of Ig domains). These domains are typically associated with cell surface adhesion molecules.

Among the receptor subunits within this family, IL-20R2 is 311 aa in length and contains the typical FNIII domain. It is also known as DIRS1 in the patent literature (Parnam C.L., 1999). Over the entire ORF, there is 28% aa identity, human IL-10R2 to human IL-20R2. In combination with IL-20R1, IL-20R2 is a functional part of both the IL-20 and IL-24 receptor.

In previous study, the soluble receptors were reported. The extracellular region of the human interleukin-10 (hIL-10) receptor was expressed using a myeloma cell line. Soluble hIL-10 receptor was able to inhibit the binding of I-hIL-10 to the full-length receptor and was able to antagonize the effect of human IL-10 in cell proliferation and cytokine synthesis inhibition.

Here we show homodimeric IL-20R2 soluble receptor can bind to both IL-20 and IL-24, with high affinity and potently blocks their binding to the native receptors. These results support a redundant role for IL-20 and IL-24 in epidermal functions and a potential therapeutic use of soluble IL-20R2-Fc in autoimmune diseases such as psoriasis where both IL-20 and IL-24 levels are elevated.

Results

Production and purification of soluble receptor sIL-20R2-Fc

For generating soluble IL-20R2-IgG Fc fusion protein, the extracellular domain of the full-length human IL-20R2 (Bloomberg et al., 2000) was PCR amplified and subcloned into pGH-hFc vector (GenHunter Corp. Nashville, TN) to allow in-fame fusion of sIL-20R2 with human IgG Fc. The expression vector was verified by sequencing and transfected into 293T or CHO-s cells for serum free production of the fusion protein. The sIL-20R2-Fc was purified to homogeneity from the culture media using MabSelect (GE Health Sciences) as instructed by the manufacturer. The conditioned media obtained from HEK 293T- sIL-20R2-Fc was analyzed by Western Blot. The medium yielded larger amount of sIL-20R2-Fc when the gene was previously amplified by optimized Kozal sequence containing primer (Fig. 2A, 2B) and that produced by serum free medium yielded more as well (Fig. 2C). Soluble receptor sIL-20R2 contains Fc fragment of lgG and was purified by Beads Protein A (Fig. 2D). Most rat monoclonal antibodies cannot be purified using Protein A columns.

SIL-20R2-Fc inhibits IL-24 to bind to its membrane receptor in vitro

The homodimer sIL-20R2-Fc was demonstrated to interact with human IL-24-AP but not AP alone (Fig. 3). IL-24-AP pulled down by monoclonal α-AP interacted with sIL-20R2-Fc, which was detected by human α-lgG-Fc. Moreover, sIL-20R2-Fc first bound to Protein A was incubated with IL-24-AP and AP, with only IL-24-AP precipitated by sIL-20R2-Fc-Protein-A complex. SIL-20R2-Fc also interacted with the rat IL-24 demonstrated by western blot using antibody against AP (Fig. 4).

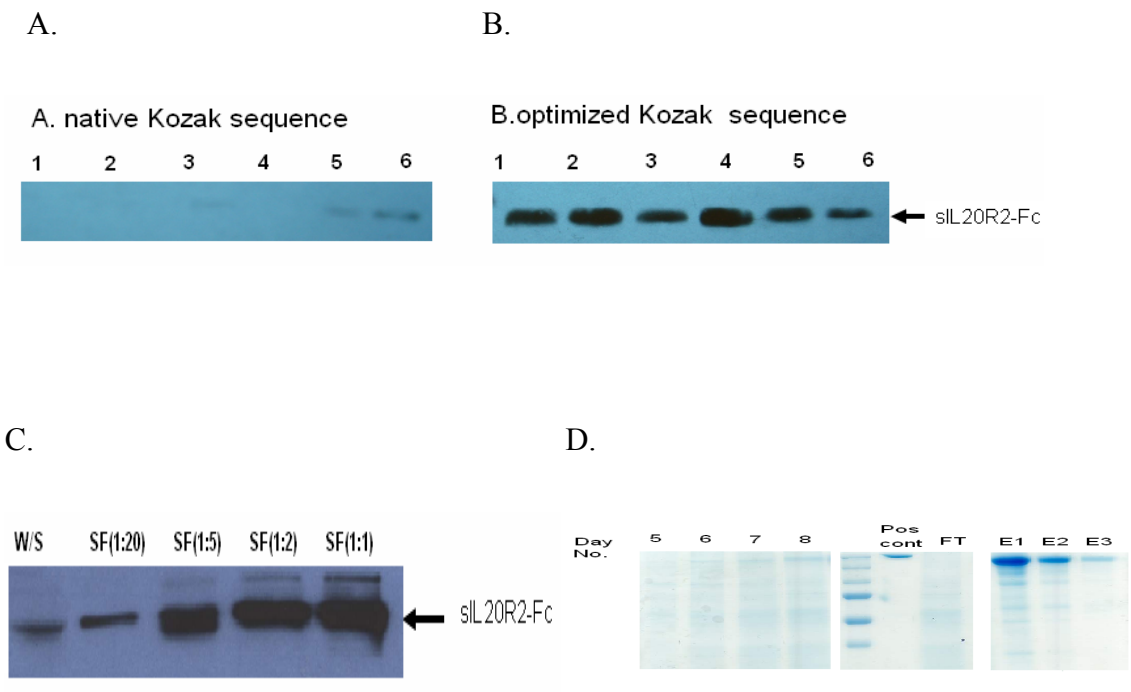


Figure 2. Production and purification of soluble receptor sIL-20R2-Fc.

HEK 293T-sIL-20R2-Fc conditioned media were analyzed by Western Blot with human anti-lgG-Fc (1:5000) (A) sIL-20R2-Fc amplified by primers containing native Kozak sequence and, (B) sIL-20R2-Fc amplified by primers containing optimized Kozak sequence were indicated. (C) sIL-20R2-Fc conditioned media obtained from serum free culturing medium (HyQ 293T). Human anti-lgG-Fc (1:5000) was used to determine the expression of soluble receptor diluted at different times (Lane 2,3,4,5), compared with the expression of that cultured in serum containing medium (Lane1). (D) CHOs cells transfected with sIL-20R2-Fc were cultured in HyQ CHO medium and media were collected each day at Day 6, 7 and 8. Purified the sIL-20R2-Fc with Column Protein A. Generation of sIL-20R2-Fc was observed from Day 6 and the product was mostly eluted in E1, E2 and E3 and flow through (FT) left no sIL-20R2-Fc.

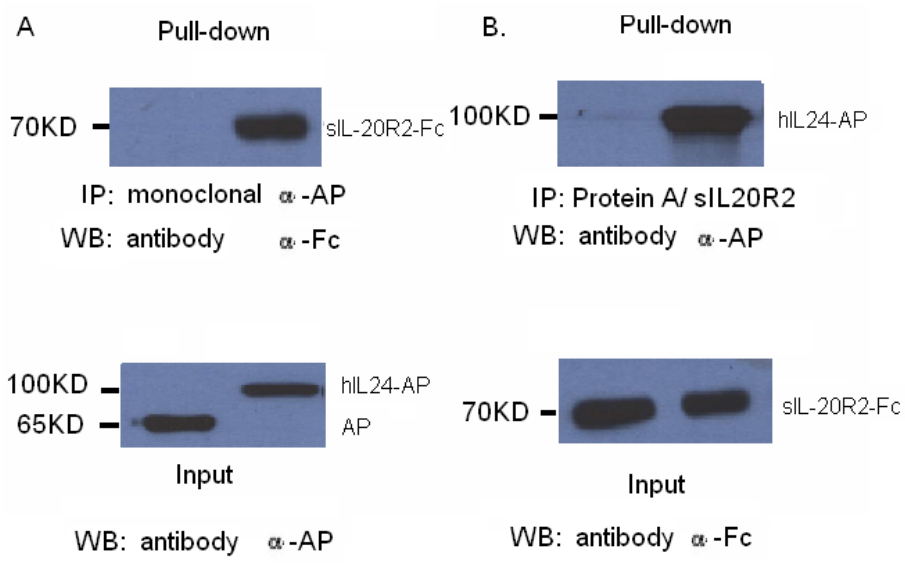


Figure 3. Immunoprecipitation of sIL-20R2-Fc and hIL-24-AP.

(A)Both AP and hIL-24-AP in the conditioned medium were pulled down by the monoclonal antibody AP (α-AP), which were indicated in the lower column of A, and subjected to the sIL-20R2-Fc conditioned medium. Only hIL-24-AP interacted with sIL-20R2-Fc that was determined by antibody against human lgG-Fc (1:5000). (B) SIL-20R2-Fc were pulled downed by the antibody against Fc (Protein-A) which indicated in the lower column in B while separately subjected to the AP and hIL-24-AP. The later was detected by the polyclonal antibody against AP (1:1000) at the upper column.

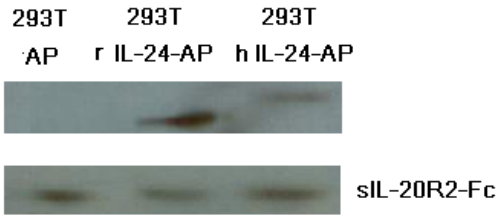


Figure 4. sIL-20R2-Fc interacts with both human IL-24 and rat IL-24.

sIL-20R2-Fc was purified by Protein A and was incubated with 1ml rat IL-24-AP or human IL-24-AP conditioned media with AP activity at 1unit/ml. Conditioned AP medium was used as negative control.

A typical ligand-receptor interaction is expected to be saturated as the concentration of the ligand is raised to excess, and is expected to be of reasonably high affinity. To future characterize the interaction between IL-24 and the soluble receptor, inhibition binding kinetics of IL-24 to the soluble receptor was determined (Fig. 5). BHK 21 (IL-20R1/IL-20R2) cells incubated with IL-24-AP and increased dose of soluble receptor sIL-20R2-Fc indicated that binding of IL-24 to IL-20R1/IL-20R2 was inhibited by the increased dose of soluble receptor. The affinity of sIL-20R2-Fc to IL-24 was determined as Kd=11nm compared with the affinity of heterodimers (Ki= 8nm).

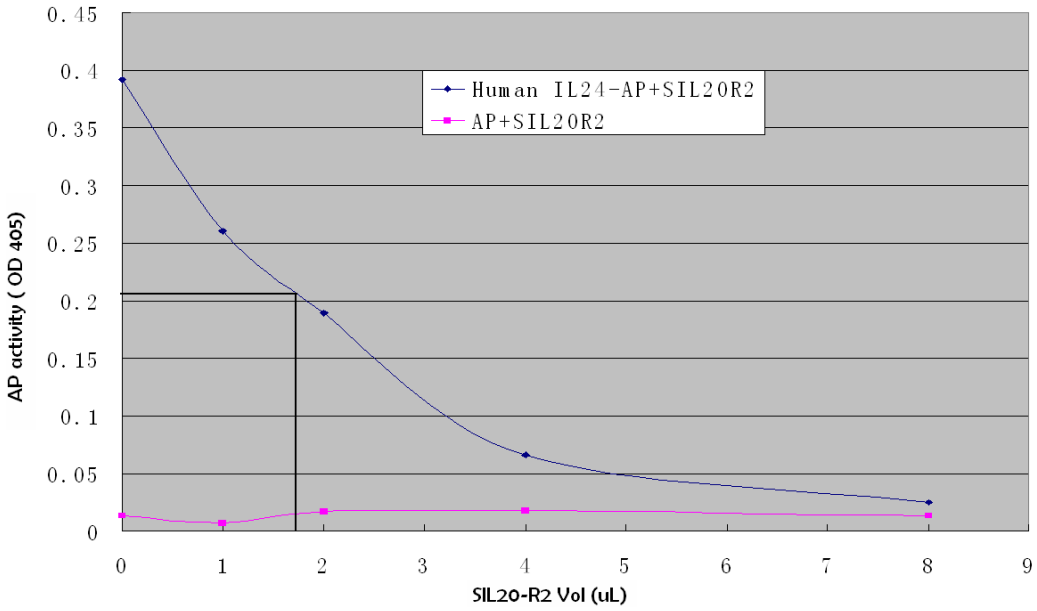


Figure 5. Soluble receptor sIL-20R2-Fc inhibited IL-24 to bind its membrane receptor.

BHK21 (IL-20R1/IL-20R2) cells were subjected to both AP and hIL-24-AP conditioned media with adding increased dose of sIL-20R2-Fc. The affinity was at peak when no sIL-20R2-Fc was added, but at near basal level when sIL-20R2-Fc was increased beyond 20nM. All the data was duplicated.

Discussion

Soluble cytokine receptors regulate inflammatory and immune events by functioning as agonists or antagonists of cytokine signaling. Another important role of soluble cytokine receptors in regulating host defense mechanisms is evidenced by viruses that encode soluble homologues of mammalian receptors and thereby evade innate host immune responses via the sequestration of essential cytokines.

In our study, the soluble receptor sIL-20R2-Fc inhibited the IL-24 induced signaling, especially acted on the ligand-receptor binding. The Kd was determined as 11nM, which was similar to the affinity between IL-24 and its membrane receptors (Ki= 8nM). This implied that sIL-20R2-Fc was a potent inhibitor in the IL-24 pathway. We also demonstrated *in vitro* that soluble IL-20-Fc fusion protein can bind to IL-20 with high affinity, suggesting that it is a potent blocker of both cytokine functions.

Recently developed mouse model for skin inflammation in which IL-23 induced epidermal hyperplasia is mediated by IL-24 and IL-19 in a IL-20R2 dependent manner (Chan et al., 2006) is used to provide a supporting evidence that our generated IL-24 antagonist sIL-20R2-Fc functions *in vivo*. Future experiments may include simultaneous injection of sIL-20R2-Fc to observe the inhibition of the epidermal hyperplasia induced by IL-24, which can supply a strong supports that sIL-20R2-Fc is a potent blocker of IL-24 not only *in vitro*, but also *in vivo*.

CHAPTER IV

OVEREXPRESSION OF IL-24 IN TRANSGENIC MICE CAUSES NEONATAL LETHALITY WITH SKIN ABNORMALITY

Introduction

In the studies of transgenic mice of IL-20 by Chandray group, both human and mouse IL-20 were overexpressed in TG mice using a variety of promoters. In all cases, TG pups expressing the IL-20 transgene were smaller than non-TG (NTG) littermates, had a shiny appearance with tight, wrinkled skin, and died within the first few days after birth (Blumberg, H. et al., 2001). These mice had swollen extremities, tail, nostril, and mouth regions and had difficulty moving. In addition, the mice were frail, lacked visible adipose tissue and had delayed ear and toe development. Low expression levels in liver (less than 100 mRNA molecules/cell) were sufficient for both the neonatal lethality and skin abnormalities. TG mice without a visible phenotype did not either express the transgene, express it at detectable levels, or were mosaic.

IL-20 was found to consist of a 176 aa polypeptide that contains a 24 aa, a 152 aa mature segment, six cysteines, and no potential N-linked glycosylation sites. Histologic analysis of the skin of the IL-20 TG mice showed a thickened epidermis, hyperkeratosis and a compact stratum corneum compared to NTG littermates. Electron microscopic (EM) analysis of skin from TG mice showed intramitochondrial lipoid inclusions, mottled keratohyaline granules, and relatively few tonofilaments similar to that observed in human psoriatic skin. The

histological and EM results supported and extended the observed gross skin alterations. Immunohistochemistry showed IL-20 TG mice results in aberrant expression of several keratins indicative of altered epidermal differentiation. Positive staining for K5 and K14 was detected in the suprabasal layer of the epidermis in addition to the expected basal layer, while staining for the hyperproliferation marker K6 in the suprabasal layer was detected in TG but not in NTG littermate skin.

To study the biological function of IL-24 *in vivo*, the IL-24 TG mouse was generated using mouse the full-length IL-24 cDNA under bovine keratin 5 (K5) promoter control. There is 33% aa identity between human IL-20 and human IL-24 in amino sequence. Both cytokines signal through same receptors IL-20R1/IL-20R2 and IL-22R/IL-20R2. Here we showed IL-24 TG mouse shared similar phenotypes of IL-20 TG mice and had its specific phenotypes that may be related with affinity difference of IL-24 and IL-20 to soluble receptor sIL-20R2-Fc indicated in the previous chapter.

Results

Establishment of expression construct of pK5-FISP

To establish the keratinocyte-specific expression vector of pK5-FISP, full-length FISP cDNA clone (Genbank accession No. BB867056) was purchased from RIKEN Institute & Centers, Japan. It was amplified by PCR (Fig. 6A, 6B) and subcloned into pPCR-TRAP for further amplification (Fig. 6C). The keratinocyte-specific expression vector bovine K5 was obtained from Dr. Ramires (Ciemat, Spain) and the correct insertion was digested by both Nhe I and the combination of Stu I and Snab I enzymes to obtain 720bp and 430 bp fragment (Fig. 6D) or screened by sequencing (Fig. 7).

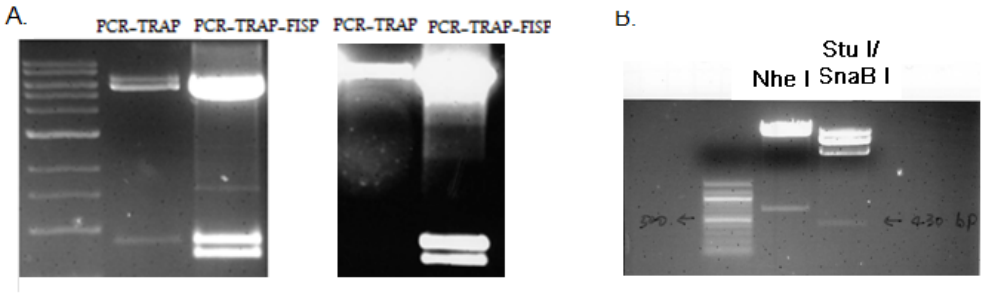


Figure 6. Establishment of expression construct of pK5-FISP and pC1-Neo-FISP.

(A) FISP amplified by PCR from template pFLC III (from RIKEN Mouse FANTOM) was prepared to subcloned to pK5 vector (No.1) and pC1-Neo vector (No.2). (B) Full length of FISP (about 720bp) was amplified by pPCR-TRAP followed manufacturer's instruction. 4 positive clones with NheI-NheI sites (No. 1,2,4,6, uppper) and 2 positive clones with NheI-XbaI sites (No. 3. 4, lower) were indicated on the 1.5% agarose gel. (C) pPCR-TRAP-FISP was respectively digested by Nhe I (left) and Nhe I/Xba I (right). The lower bands were the FISP to be cloned into pK5 and pC1-Neo. pPCR-TRAP was used as vector control. (D) pK5-FISP was digested by both Nhe I and enzymes of Stu I and SnaB I to determine the direction of self-ligated pK5-FISP. The clone generating the 430bp segment was as expected to be the right pK5-FISP construct.

Figure 7. Validation of pK5-FISP by sequencing.

Three pairs of primers designed in establishing pK5-FISP construct were indicated above. The first pair was used to amplify the FISP from template pFLC III.
L-FISP: 5'-GCTAGCTTCCCACCCAGCAGAAGATC -3'
R-FISP: 5'-GCTAGCAAAGGAGGAAGTTATCCAATATC-3'
The second pair was used to sequence the up-and down-stream of the FISP.
L-FISP ext: 5'-GGTTCTCCCAGAACTGTGGGAGGCC-3'
R-FISP ext: 5'-GAATGACCTCAAGACCTTGAATTTG-3'
The third pair of primers was used to determine the right direction of interested gene FISP.
L-K5: 5'-CGTGCTGGTTATTGTGCTGTCTC-3'
R-K5: 5'-AGGCAGCCTGCACCTGAGGAGTG-3'
The combination of primers was used to determine the correct direction of pK5-FISP.
L-FISP: 5'-GGTTCTCCCAGAACTGTGGGAGGCC-3'
R-FISP ext: 5'-AGGCAGCCTGCACCTGAGGAGTG-3'

IL-24 induces abnormality in epidermal differentiation and proliferation

To understand the biological functions of IL-24 *in vivo*, we have generated IL-24 transgenic mice (TG) using mouse the full-length IL-24 cDNA under bovine keratin 5 (K5) promoter control (Fig. 8A). As expected, IL-24 TG mice resembled many phenotypes of that of IL-20, including neonatal lethality within hours upon birth, smaller body size, shiny and dark appearance with wrinkled skin (Fig. 8B). Histological analysis of the skin revealed compact stratum corneum and marked hyperplasia in the epidermis (SS: stratum spinosum) of TG mice compared to wild-type litermates (Fig. 9), again similar to that of IL-20 TG mice.

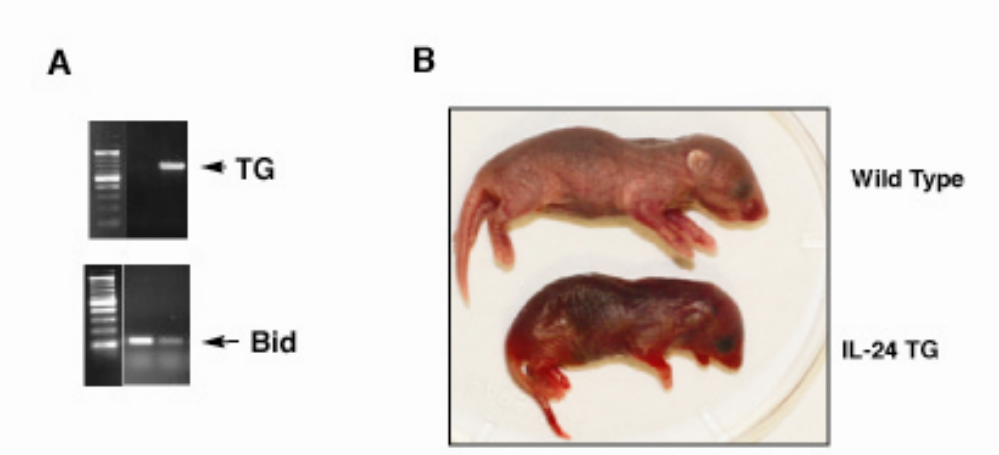


Figure 8. Genotyping for IL-24 transgenic mice and skin phenotype of IL-24 TG mice.

(A) Genotyping for kerotinocyte specific expression of IL-24 TG mice with PCR using the primers specific to IL-24 transgene and a control genomic locus (Bid) from tail DNA of the dead TG pup and control littermate. (B) The IL-24 TG pup (under Bovine K5 promotor control) was slightly smaller than that of WT control littermate, and had shiny, darkened and wrinkled skin.

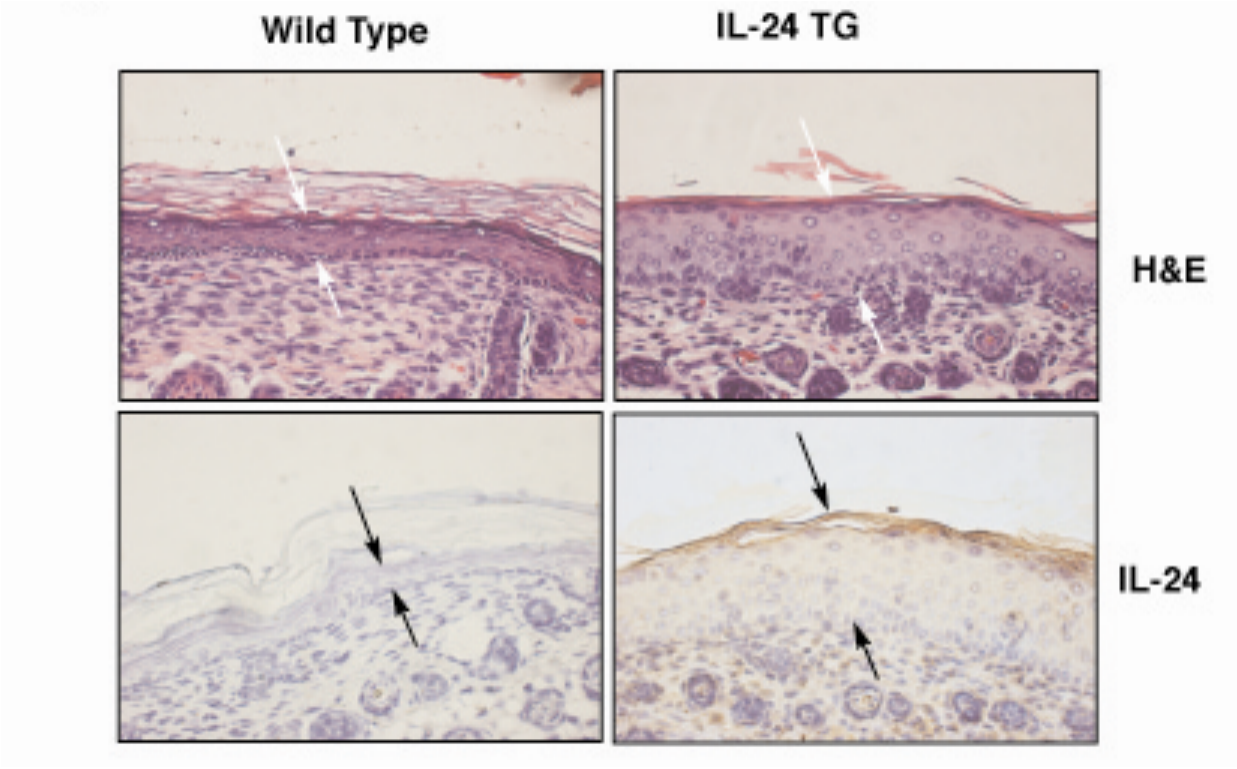


Figure 9. Histological analysis of IL-24 TG mice.

Histological analysis of paraffin-embedded skin sections (H&E, 40X) reveals that the TG mice has compact stratum corneum and markedly thickened epidermal layer (SS: stratum spinosum (between the two arrows). Immunohistochemical staining with polyclonal antibody against murine IL-24 (Zhang et al., 2000) showed positive and diffusive IL-24 expression throughout the skin of the TG mice.

IL-24-AP ligand-affinity staining showed that IL-24 receptor expression pattern was unaltered in the epidermis of IL-24 TG mice (Fig. 10), despite marked epidermal hyperplasia. Furthermore, the epidermis of IL-24 TG mice retained keratin 5 (K5) expression in both suprabasal and basal layers in additional to hair follicles, as in the normal controls (Fig. 11). To provide further evidence for IL-24-induced keratinocyte proliferation, keratin 6 (K6) which is often associated with keratinocyte proliferation, such as during wound healing, as well as Ki67 which is a marker for proliferating cells, were also analyzed by immunohistochemistry. Compared to wild type controls, IL-24 TG skins exhibited strong positive staining for K6 through out suprabasal and basal layers, much like in IL-20 TG mice (Bloomber et al., 2001); whereas Ki67 staining was restricted mostly in the basal layer (Fig. 11). These results suggest that, like IL-20, IL-24 is a potent mitogen for karatinocytes *in vivo* and the abnormal keratinoyte proliferation appears to be originated from the basal layer of the epidermis.

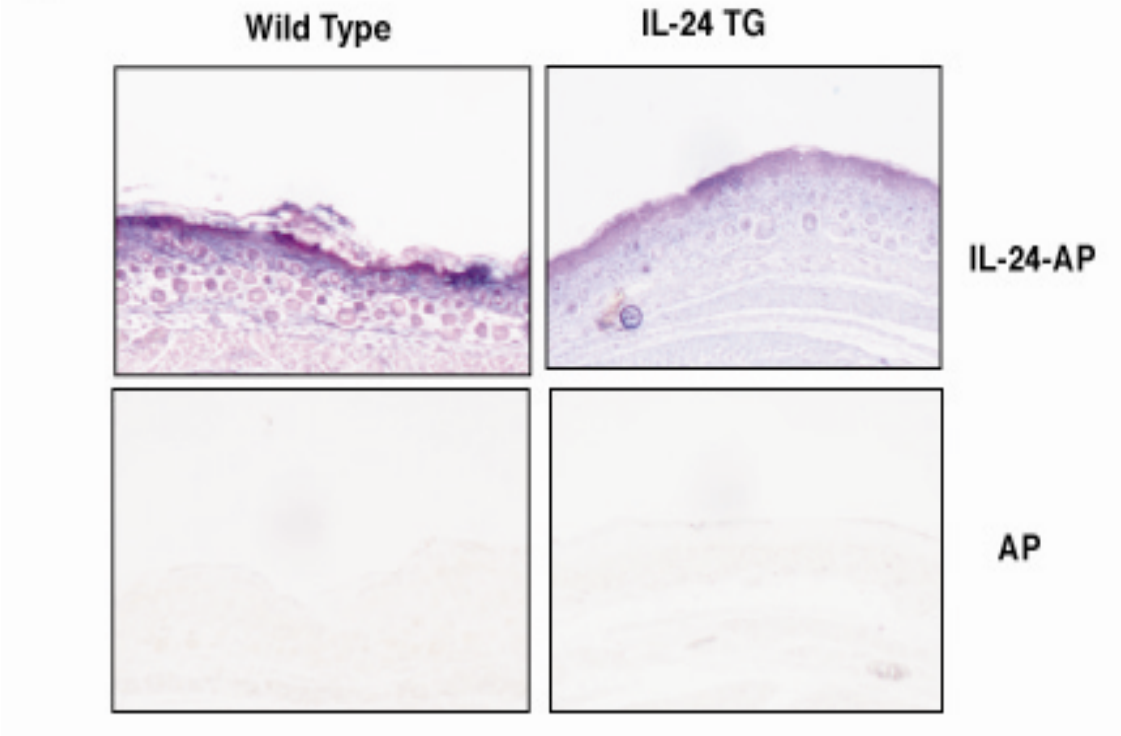


Figure 10. Comparison of IL-24 TG and wild-type control skins in IL-24 receptor expression.

Paraffin embedded skin sections (5X) were analyzed with either ligand IL-24-AP or AP control to detect cell-types expressing IL-24 receptor. Note the intense staining of the epidermis.

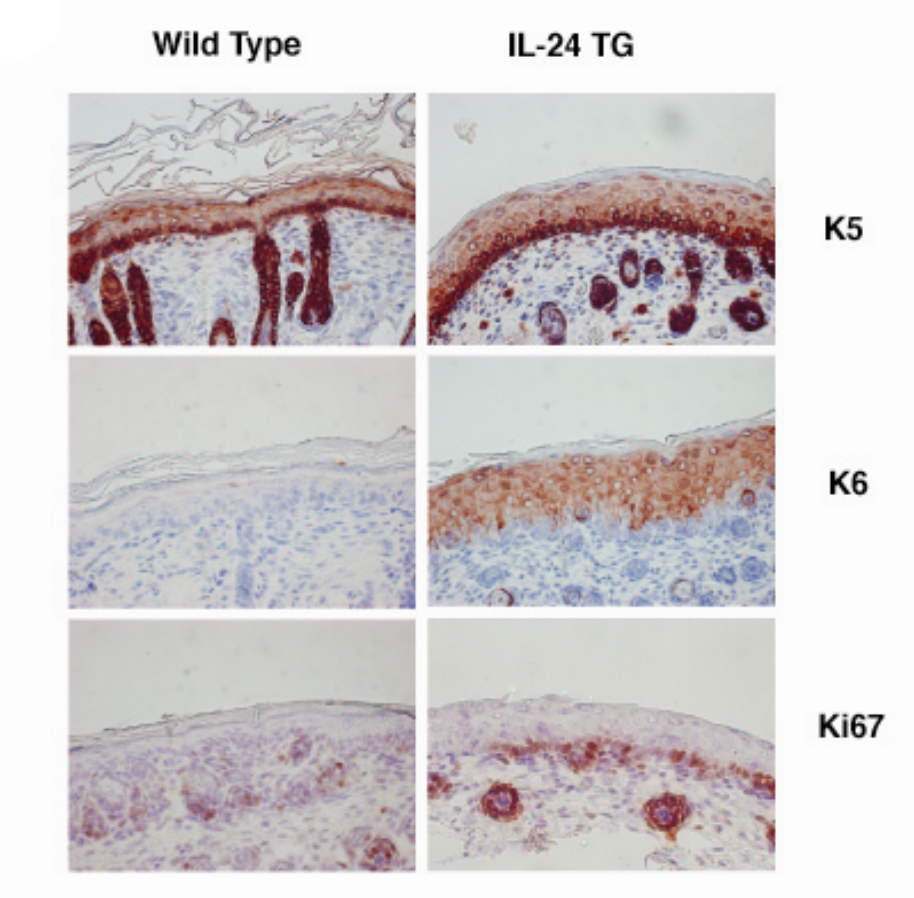


Figure 11. Aberrant Expression of Keratin Proteins Demonstrated by Immunohistochemical Analysis.

K5 was detected in the basal layer of the epidermis in NTG mice. Similar sections obtained from TG mice derived from mouse IL-24 expressed from the K5 promoter show positive staining in the suprabasal of the epidermis as well. Also shown is positive staining for K6 in both the suprabasal and basal layers in TG mouse skin but not in NTG mouse skin. Ki67 staining was positive in the basal of epidermis in TG mouse skin but not in NTG mouse skin.

IL-24 induces macrophage infiltration in the dermis

In addition to the dramatic effects of IL-24 on keratinocytes in the epidermis, some of which resemble phenotypes observed in psoriatic skins, we also examined whether there were any immune infiltrates in the TG skins. Immunohistological analysis was performed with antibodies against CD3 and F4/80 to detect any differences in infiltrating T cells and macrophages, respectively. While little difference in CD3 positive T cell counts were visible, significant number of F4/80 positive cells were detected in the dermis of IL-24 TG pups (Fig. 12). Apart from nearly all the phenotypes examined, infiltrating macrophages appears to be the major difference observed in the skin between IL-24 and IL-20 TG mice where no immune infiltrates were detected (Blumberg et al., 2001). Otherwise, the essentially identical skin phenotypes in transgenic mouse models suggest that IL-20 and IL-24 play a redundant role in keratinocyte differentiation and proliferation.

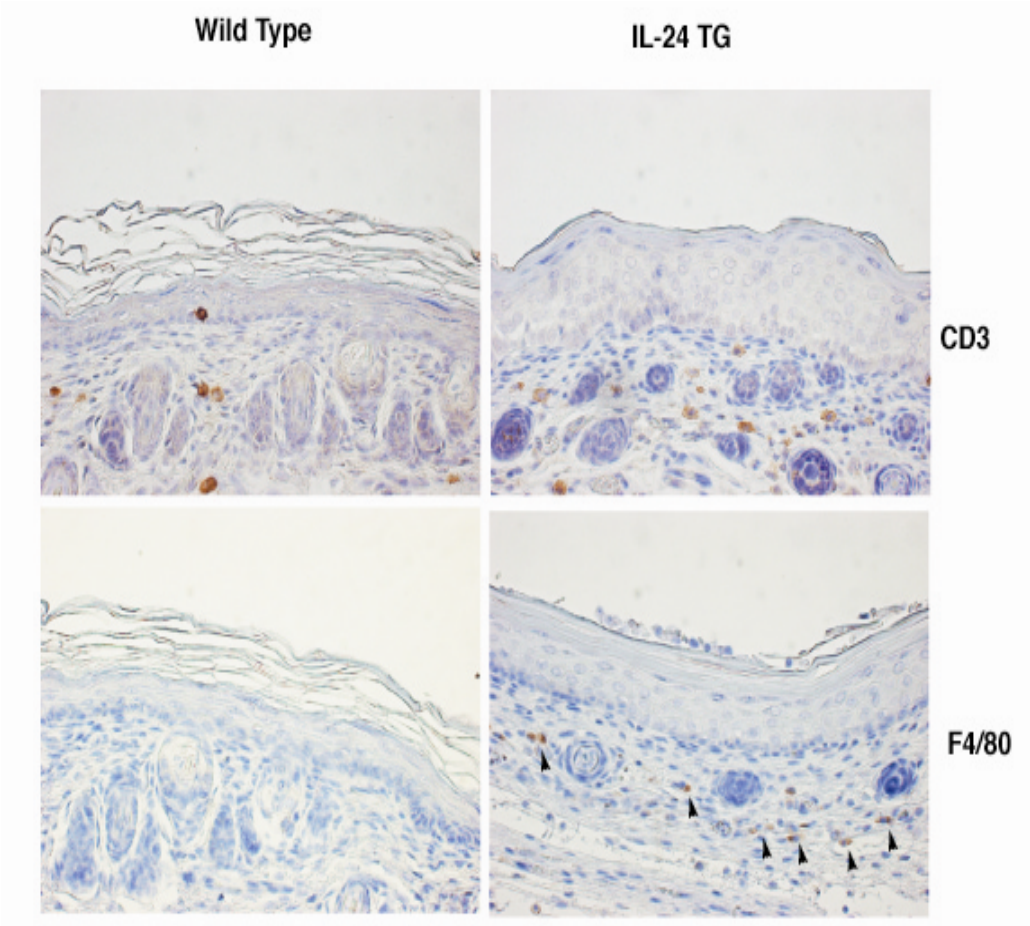


Figure 12. Analysis of immune infiltrates in IL-24 transgenic skins.

Paraffin embedded skin sections (40X) from both IL-24 TG pups and control littermates were analyzed with both CD3 and F4/80 specific antibodies to visualize the presence of T cells and infiltrating monocytes (macrophages). F4/80 positive macrophages were detected in the dermis in TG mouse but not in NTG mouse. Note the presence of macrophages (indicated by arrowheads) in the dermal region of IL-24 TG skins. CD3 staining was similar for both TG and NTG mouse skin (40X).

Discussion

The psoriasis is multigenic skin disease characterized by increased keratinocyte proliferation, altered keratinocyte differentiation, and infiltration of immune cells into the skin (Bos and De Rie, 1999). The evidence suggested a role for IL-24 and receptor in psoriasis was for a role of IL-24 in psoriasis that the observed hyperkeratosis, thickened epidermis, and proliferation in the suprabasal layer in the TG mice that resemble human psoriatic abnormalities. Changes detected in the epidermal protein expression in IL-24 TG mice include the presence of the basal K5 and K6 in both the basal and suprabasal layers, similar to the changes observed in human psoriatic skin (Sun et al., 1985; Castelijns et al., 1999). IL-24 TG mice also express the hyperproliferative-associated Ki67, present in human psoriatic but not normal skin. Immunostaining with anti-murine IL-24 antibody confirmed diffusive pattern of IL-24 protein expression through out the skin (both epidermal and dermal layers) of the TG mice, consistent with the nature of IL-24 being a secreted cytokine.

Apart from nearly all the phenotypes examined, infiltrating macrophages appears to be the major difference observed in the skin between IL-24- and IL-20 TG mice where no immune infiltrates were detected (Blumberg et al., 2001). In contrast, if IL-19 TG mice indeed failed to manifest any overt skin phenotype as reported (Parish-Noval et al., 2002), our results would support that the epidermal hyperplasia caused by both IL-20 and IL-24 is likely mediated through IL-22R1/IL20R2 receptor through which IL-19 fails to signal.

CHAPTER V

APOA1/HDL INTERACTS WITH IL-24

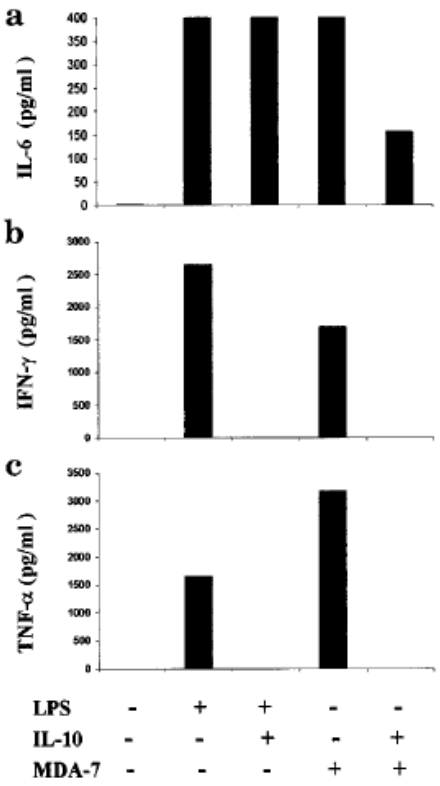
Introduction

There are several well-documented functions of high-density lipoprotein (HDL) that may explain the ability of these lipoproteins to protect against atherosclerosis. The best recognized of these is the ability of HDL to promote the efflux of cholesterol from cells. HDL has additional properties that may also be antiatherogenic, such as an effective antioxidant. The major proteins of HDL, apoA-I and apoA-II, as well as other proteins such as paraoxonase are well-known to have antioxidant properties. As a consequence, HDL has the capacity to inhibit the oxidative modification of low-density lipoprotein (LDL) in a process that reduces the atherogenicity of these lipoproteins. HDL also possesses other antiinflammatory properties. By virtue of their ability to inhibit the expression of adhesion molecules in endothelial cells, they reduce the recruitment of blood monocytes into the artery wall. These antioxidant and antiinflammatory properties of HDL may be as important as its cholesterol efflux function in terms of protecting against the development of atherosclerosis.

HDL has a core with triglyceride and cholesteryl ester, surrounded by the surface of phospholipids and free cholesterol. ApoA1 wraps the HDL particle as the major protein component of HDL plasma, and promotes cholesterol efflux from tissues to the liver for excretion in bile. In some dyslipidemic individuals, HDL particles may be transformed to dysfunctional, lipid-poor, small HDL with diminished residence time in the plasma

compartment. Such small dense HDL of abnormal composition (high triglyceride/CE ratio) has a shorter half-life in plasma and, as a result, HDL levels decrease (Lewis et al., 2005). Small dense HDL particles in hypertriglyceridemic states also exhibit impaired anti-oxidative activity versus larger HDL particles (Hansel et al., 2004) because patients exhibit moderately elevated levels of glucose in plasma due to glycation of apoA-I. This abnormality of apo A-I, along with the potential for elevated oxidative stress, impairs the anti-atherogenic properties of HDL (Decossin et al., 1995; Therond et al., 1999).

Abnormalities associated with inflammation comprise a large group of human diseases. In the immune system, it is often involved with inflammatory disorders, demonstrated in allergic reactions and some myopathies (E. Gallardo et al., 2001). Non-immune diseases with aetiological origins in inflammatory processes are thought to include cancer, atherosclerosis and ischaemic heart disease (Porto I. et al., 2005). IL-24 and IL-10 have effect on inflammatory cytokine secretion from PBMC (Fig. 13) (Eva G. Caudell et al., 2002). High levels of IL-6, IFN-γ and TNF-α were secreted by PMBC in response to IL-24 in the representative sample. IL-24 was a more potent inducer of TNF-α than LPS, which is a known inducer of inflammatory cytokines used as a positive control while IL-10 completely abrogated IL-24-induced secretion of TNF-α and IFN-γ. Interestingly, we found that IL-24-AP conditioned medium when purified by monoclonal antibody against AP, could pull down ApoA1/HDL, which was identified by MALDI-TOF, suggesting the interaction between IL-24 and ApoA1/ HDL through the way of protein-protein interaction or another.



Eva G. Caudell et al., 2002

Figure 13. Effect of IL-24 and IL-10 on IL-6, TNF-α, and IFN-γ inflammatory cytokine secretion from PBMC.

Two milliliters per well (2×10^6 cells/ml) PBMC were plated in a 24-well plate and cultured untreated or with indicated amounts of IL-24 (a, 2 ng/ml; b and c, 20 ng/ml), 5 μg/ml LPS, 500 U/ml IL-10 (~17 ng/ml; R&D Systems), or a combination of IL-10 and IL-24. Supernatants were harvested at 48hr and analyzed for cytokine content by ELISA (Endogen, Woburn, MA) according to the manufacturer's instructions. Data from one representative sample are reported.

Result

The 293T-AP, 293T-rIL-24-AP or 293T-hIL-24-AP cell lines were previously established (Zhang R. et al., 2000) in the medium as described. The conditioned media were collected and subjected to monoclonal α-AP (GenHunter, Nashville, TN). Following resuspending the beads and SDS-PAGE, coomassie blue staining showed that rat IL-24 and human IL-24 but not AP alone, interacted with ApoA1/HDL in the cow serum (Fig. 14).

To generate ApoA1-Fc fusion protein, the full length human ApoA1 (Bloomberg et al., 2000) was PCR amplified and subcloned into pGH-hFc vector (GenHunter Corp. Nashville, TN) to allow in-fame fusion of ApoA1 with human IgG Fc. The expression vector was verified by sequencing and transfected into 293T cells for serum free production of the fusion protein (Fig. 15). The ApoA1-Fc was purified to homogeneity from the culture media using MabSelect (GE Health Sciences) as instructed by the manufacturer. However, the ApoA1-Fc fusion protein indicated no significantly difference in binding with IL-24-AP and AP alone (data not shown).

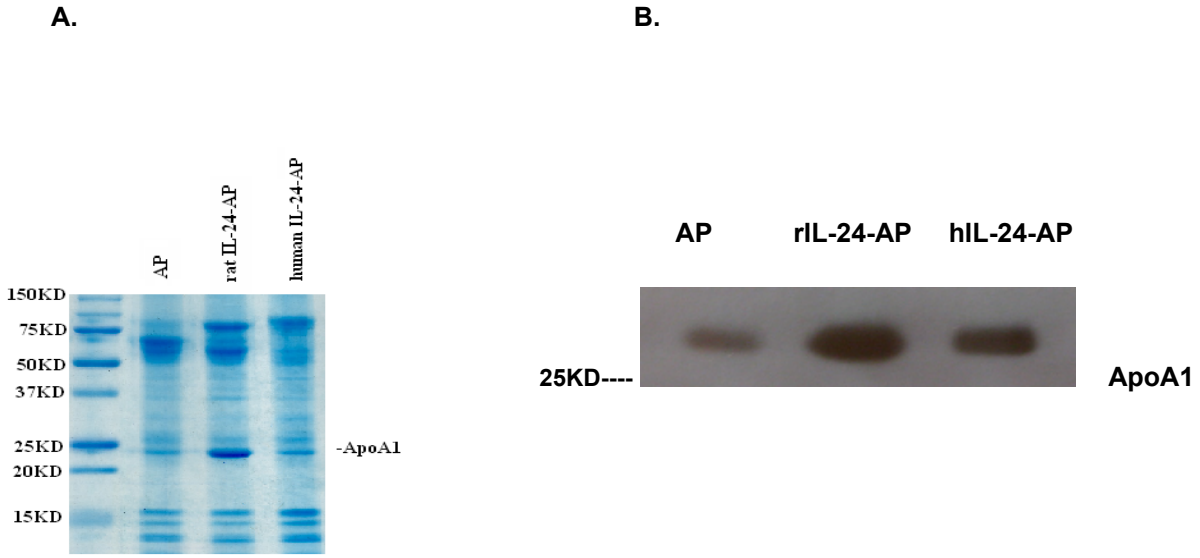


Figure 14. Binding of rat IL-24 and human IL-24 to ApoA1/HDL.

(A) Coomassie Blue Staining analysis of conditioned medium containing AP, rat IL-24-AP or human IL-24-AP. ApoA1/HDL was pulled down by rat IL-24-AP and human IL-24-AP. AP was used as a negative control. (B) AP, rIL-24-AP and hIL-24-AP purified by AP beads were subjected to Western Blot. Anti-ApoA (1:1000) was used to detect bovine ApoA1, followed by the secondary anti-rabbit antibody (1:5000).

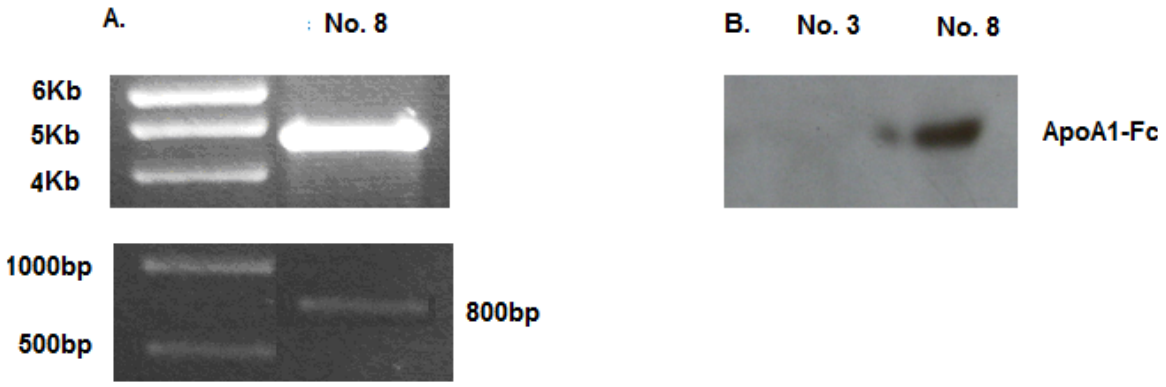


Figure 15. Expression of ApoA1-Fc fusion protein.

(A) ApoA1-Fc construct was digested by Bgl II and Hind III to isolate 800bp ApoA1 in clone No. 8. (B) 293T cells stably transfected with ApoA1-Fc produced the fusion protein detected by anti human lgG-Fc (1:5000) in No.8 while the No. 3 clone digested by enzymes but not isolated the ApoA1, produced no ApoA1-Fc fusion protein on the western blot.

Discussion

The binding of ApoA1/HDL to IL-24 may affect IL-24 signaling through inhibiting the ligand-receptor binding, the activation of transcription factors and the expression of secondary cytokines. The mechanism of binding is remained to be investigated. However, the interaction between IL-24 and ApoA1/HDL implied that ApoA1/HDL may inhibit the IL-24 to interact with both heterodimeric receptors through blocking its binding sites or changing its conformation. Interestingly, the ApoA1 molecular alone produced from HEK 293T transfected with ApoA1-Fc fused construct indicated no visible interaction with IL-24, suggesting that the nature structure of ApoA1/HDL particles is the essential for ApoA1/HDL binding to inflammatory cytokines or proteins. This was supported by the molecular structure obtained from infrared spectroscopy developed by Dr. Paul H. Axelsen, that the lipid molecules comprised a two-layered disk, but that the proteins surrounding the disk were wrapped around the disk's perimeter in a "belt" formation. Other studies showed reconstituted HDL (Calabresi L, F. G. et al., 1997) containing only ApoA-I and phosphatidylcholine, can inhibit the cytokine-induced expression of endothelial cell adhesion molecules to inhibit inflammation.

Whether the interaction between ApoA1 and IL-24 was through protein-protein binding remained to be identified. Further experiments may include chemical crosslinking or immunoprecipitation. A series of C-terminal truncated IL-24-AP fusion protein that contains ApoA1 binding domain (ABD) can be identified followed by bioinformatics used to search for the similar cytokines/proteins that have the ABD.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Cytokines are proteins that are secreted by numerous cell types and affect many of their functions. Interleukins are cytokines that classically serve to establish communication particularly between immune cells and are key players in physiological and pathological processes in multicellular organisms. The most successful strategy for the therapy of autoimmune diseases was to repress the function of ILs constitutes. For example, the neutralization of TNF-α is currently the fastest and most effective therapy for patients with rheumatoid arthritis, Crohn's disease and psoriasis (Olsen N. J. et al., 2004; Schottelius A. J. et al., 2004; Suryaprasad A. G., 2003). Unfortunately, the neutralization of ILs is often bound to blocking various parts of the immune system (Suryaprasad A. G., 2003). Therefore, there is an increasing interest in immune mediators with causal roles in the pathogenesis of a specific disease that only regulate functions of a restricted number of cell populations.

In the process of tumor development, IL-24 may also function as a cytokine to enhance cancer cell survival and growth rather than tumor suppression. First, expression of the IL-24 receptors was upregulated by mutant k-ras, and second, IL-24-induced JAK/STAT signaling was observed in oncogenic ras transformed RIE cells. Although future studies are required to determine the role of deregulation of IL-24 signaling in cancer, the effect of soluble receptor sIL-20R2-Fc on the IL-24-induced JAK/STAT signaling and regulations of IL-24 across species will serve as a solid biochemical foundation for such endeavors.

The effect of MDA-7 on NF-kappaB activation and on TNF-induced NF-kappaB activation and apoptosis in human embryonic kidney 293 cells was reported. Stable or transient

transfection with mda-7 into 293 cells failed to activate NF-kappaB. However, TNF-induced NF-kappaB activation was significantly enhanced in mda-7-transfected cells, as indicated by DNA binding, p65 translocation, and NF-kappaB-dependent reporter gene expression. Thus, the further study will involve in the inhibition of sIL-20R2-Fc on the mda-7 enhanced TNF-induced NF-kappaB activation.

As the IL-24 TG mice resembled many phenotypes of that of IL-20, including neonatal lethality within hours upon birth, smaller body size, shiny and dark appearance with wrinkled skin. However, the IL-24 TG mice were neonatal lethality. The future study would establish the expression construct of IL-24 which is regulated to be turned off before IL-24 TG mice grow to adults. IL-19 TG mice indeed failed to manifest any overt skin phenotype as reported (Parish-Noval et al., 2002), our results would support that the epidermal hyperplasia caused by both IL-20 and IL-24 is likely mediated through IL-22R1/IL20R2 receptor through which IL-19 fails to signal. Although IL-24 shares two heterodimeric receptors with IL-19 and IL-20, the R2 subunit of the receptors, namely IL-20R2, is shared by both and is absolutely required for receptor activation. Thus, IL-20R2 knockout mice should yield much needed information regarding the biological functions of all three cytokines.

Furthermore, the biological functions of soluble cytokine receptors in regulating key inflammatory and immune responses need to be further elucidated. These studies should provide new insights into disease pathogenesis and generate novel therapeutic approaches.

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