Chapter 15

NOVEL CYTOKINES IN THE TREATMENT OF MALIGNANCIES

Robin Parihar and William E. Carson, III
Department of Molecular Virology, Immunology and Medical Genetics and Department of Surgery, The Ohio State University Comprehensive Cancer Center and Solove Research Institute, Columbus, OH

1. INTRODUCTION

For the last twenty years, cytokines have been examined as therapeutic agents because of their potential to manipulate the immune response to malignant cells. Typically, cytokine therapy has been aimed at either activating cytotoxicity or cytokine production by existing immune cells, or by increasing the number of immune cells by stimulating their growth and survival. In addition to numerous studies that have attempted to optimize therapeutic strategies of currently known cytokines, recent efforts have concentrated on defining novel cytokines with unique immune modulatory properties. The immune function and anti-tumor activity of these novel agents are currently being investigated in the context of clinical trials.

Interleukin-18 (IL-18), initially described as IFN-γ-inducing factor, is an attractive candidate for the immunotherapy of cancer. In pre-clinical development for almost a decade, IL-18 has now entered the clinical arena for the treatment of patients with solid tumors. Armed with the progress made in pre-clinical models, investigators are hoping to harness the basic biology of IL-18 for enhancement of anti-tumor immunity. IL-21 represents one of the first cytokines isolated strictly from a bioinformatics screening approach after the discovery of its receptor. Since its discovery and characterization in early 2000, IL-21 has undergone rapid pre-clinical development and is now making its way into the clinic. IL-24, first

characterized as the product of a novel tumor suppressor gene, *mda-7*, has now developed into a therapeutic tool because of its potent tumor-specific growth inhibitory properties.

Here we summarize the basic science and pre-clinical evidence supporting the use of IL-18, IL-21 and IL-24 in patients with cancer. In addition, we will also review the phase I/II clinical trials utilizing these novel agents for the treatment of patients with advanced malignancies. Further, we theorize on the potential role for these novel agents in the immunotherapy of cancer and highlight future directions for their clinical application.

2. INTERLEUKIN-18: A POTENT INTERFERON-γ INDUCING FACTOR

2.1 Discovery and Cloning

In 1989, Nakamura *et al.* described an IFN-γ inducing activity in the sera of mice treated with endotoxin that functioned not as a direct inducer of IFN-γ, but rather as a co-stimulant together with IL-2[1]. The inability of neutralizing antibodies directed against IL-1, IL-4, IL-5, IL-6, or TNF to neutralize this serum activity suggested that it was a distinct factor. Subsequent publications reported that the endotoxin-induced co-stimulant for IFN-γ production was present in extracts of livers from mice pre-conditioned with the bacterium, *P. acnes*[2,3]. The factor, named IFN-γ-inducing factor (IGIF), was purified to homogeneity from *P. acnes*-treated mouse livers. Its molecular mass and amino acid sequence were reported by Nakamura and colleagues in 1993[3].

Degenerate oligonucleotides derived from the amino acid sequence of IGIF were used to clone the murine IGIF cDNA[4]. Importantly, induction of IFN-γ was found to be independent of IL-12 (an already known potent inducer of IFN-γ). The human cDNA sequence for IGIF was subsequently reported in 1996[5]. Comparative analysis of the protein-folding pattern of IGIF to that of other cytokines showed the highest homology to mature human IL-1β. Sequence identities were also assembled for the IGIF sequence to other members of the IL-1 family of cytokines. After numerous biochemical approaches determined that IGIF did not bind to the IL-1 type I receptor , IGIF was termed IL-18[5,6].

2.1.1 Receptors and Signaling

The IL-18 receptor (R) complex is a heterodimer containing an IL-1Rrp chain that is responsible for extracellular binding of IL-18 and a non-binding chain (AcPL) responsible for signal transduction[7,8]. Transfection studies in human peripheral blood mononuclear cells (PBMCs) have shown that both chains are required for functional IL-18 signaling[9]. IL-18R is expressed on a variety of cells including NK cells, neutrophils, macrophages, endothelial cells, and smooth muscle cells[10,11]. The IL-18R complex can be up-regulated on naïve T and B cells by IL-12[12]. In contrast, T cell receptor (TCR) ligation in the presence of IL-4 results in down-regulation of the IL-18R[13]. Modulation of this complex during various immune processes is therefore likely to be functionally significant. For example, administration of an anti-IL-18R antibody *in vivo* resulted in reduced mortality upon exposure to a lethal LPS dose and a subsequent shift in balance from a T helper-type 1 to a T helper-type 2 immune response[14].

Upon binding of IL-18, the IL-18R is recruited to form a high-affinity complex, inducing signaling pathways shared with other IL-1R family members. These pathways involve recruitment and activation of myeloid differentiation 88 (MyD88) and IL-1R-associated kinase (IRAK) to the receptor complex[15]. Following activation, IRAK auto-phoshorylates, dissociates from the receptor complex, and interacts with the adaptor protein tumor necrosis factor receptor-associated factor 6 (TRAF6)[16]. Activation of NF-κB-inducing kinase and rapid induction of IκBα degradation, allow NF-κB nuclear translocation and genetic transcription of IL-18-sensitive genes[17]. In addition to IRAK/TRAF6 signaling, recent evidence suggests a role for mitogen-activated protein kinases (MAPK) in IL-18 signaling. Indeed, IL-18-induced activation of the MAPK p38 and the extracellular signal-regulated kinases (ERKs) p44/p42 was detected in a human NK cell line[18]. In addition to IL-18-induced MAPK signaling, diminished NK cell activity and IFN-γ production in response to IL-18 by mice deficient in the transcription factor tyk-2 suggest that, like IL-12, IL-18 may also signal via tyk-2, a member of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) family of signaling proteins[19]. Additional evidence for cooperation between IL-12 and IL-18 signaling pathways has been presented by numerous investigators. Using *in vitro* promoter analysis studies in mouse T cell lines, Nakahira *et al* showed that IL-12-induced STAT4 enhanced IL-18-induced transcription factor activation and binding to IFN-γ promoter response elements[20]. Ongoing studies are investigating the effects of IL-18-dependent signaling on *in vivo* anti-tumor immune responses in several tumor models (see below).

2.1.2 Biological Activity and Rationale for Clinical Development

Although initially regarded as a co-factor for the potent production of IFN-γ by murine and human immune cells, the effector role of IL-18 has gradually expanded. For example, single-agent IL-18 has been shown to enhance T and NK cell cytotoxicity as well as cytokine production[12,21]. IL-18 also increased FasL on NK cells and consequent Fas/FasL-mediated cytotoxicity of both viral and tumor targets[22]. Accordingly, IL-18-deficient mice exhibited reduced NK cell cytolytic activity that could be at least partially restored by administration of exogenous IL-18[23]. In combination with other factors, IL-18 can exert important immune functions. For example, IL-18 in conjunction with IL-2 induced potent IL-13 production from murine T and NK cells[24]. On non-T cell populations, IL-18 in conjunction with IL-3 has been shown to induce type 2 cytokine production and pro-inflammatory mediators from bone marrow-derived basophil[25]. Direct effects on macrophages and dendritic cells (DCs) have also been observed. For example, stimulation of bone marrow-derived macrophages or splenic DCs with IL-12 and IL-18 can induce IFN-γ production[26]. IL-18 also promoted neutrophil activation, reactive oxygen intermediate synthesis, cytokine release, and de-granulation[27]. Recent studies have suggested that IL-18 can up-regulate intracellular adhesion molecule-1 (ICAM-1) and VCAM-1 expression on endothelial cells and synovial fibroblasts, implicating a role for IL-18 in cellular adhesion and trafficking[28].

These potent immune modulatory properties of IL-18 suggest that this cytokine could have strong anti-tumor activity. Indeed, several murine tumor models have given preliminary indications that IL-18 may serve as an immune modulatory agent with potential clinical anti-tumor utility. In a early model of chemically-induced intraperitoneal (IP) sarcoma, IL-18 administration stimulated NK cell-mediated cytokine production, induced cytotoxic $CD8^+$ T cells, and evoked lasting immunological memory (as shown through resistance to re-challenge with tumor cells of mice cured of the chemically-induced sarcoma, but not with a non-relevant carcinoma)[29]. Interestingly, IL-18 administration had little direct effect on the proliferation of tumor cells *in vivo*, indicating an indirect activity through stimulation of the immune system. Subsequently in depth analysis of IL-18-induced immune responses by the same group revealed that IL-18 initially stimulated a non-specific arm of the immune response (activation of NK cells), followed by the development of a specific CTL-mediated anti-tumor response[30]. In accordance with these data, administration of human pancreatic carcinoma cells transfected with the IL-18 gene to T cell-deficient mice did not produce long-lasting anti-tumor immunity, further confirming the requirement of the adaptive immune response for effective tumor

clearance[31]. Interestingly, IL-18 gene-transfected renal cell carcinoma cells demonstrated a reduced tumorigenicity in syngeneic mice[32]. Depletion of both $CD4^+$ and $CD8^+$ T cells markedly attenuated the effects of IL-18, whereas depletion of only $CD4^+$ cells did not. Similarly, blockade of IFN-γ with monoclonal antibodies completely abrogated the anti-tumor effect in a similar in vivo model of IL-18-expressing tumor[33]. The anti-tumor effects of IL-18 were also evaluated in more aggressive tumor models such as the CL8-1 melanoma or MCA-205 fibrosarcoma[34]. IL-18 given as a single agent resulted in the rejection of 80% of CL8-1 tumors when given either pre- or post-tumor inoculation, with induction of tumor-specific immunity. Depletion of NK cells *in vivo* using neutralizing antibodies (anti-asialoGM1) completely abrogated the growth inhibitory effects of IL-18. Investigators are hopeful that these observations will help in developing new strategies aimed at augmenting the successive stages of IL-18's anti-tumor effects.

From the aforementioned studies, it is clear that the ability of single-agent IL-18 treatment to suppress tumor growth in animal models varied depending on tumor type and stage. In contrast, administration of IL-18 in combination with IL-12 has proven to be a highly reliable and effective anti-tumor regimen in pre-clinical models. Combined treatment with IL-18 and IL-12 (at doses of 1 μg and 0.1 μg, respectively) has been associated with dramatic inhibition of tumor growth[35,36]. Numerous potential anti-tumor mechanisms have been proposed for the IL-18/IL-12 combination. More recent reports show that IL-12 and IL-18 synergistically induce tumor regression in a mammary carcinoma model via inhibition of angiogenesis, rather than through an antigen-specific immune response[37]. Histological examination of regressing tumors revealed extensive areas of necrosis with dense infiltrates of polymorphonuclear cells. Inhibition of angiogenesis was more directly demonstrated through destruction of tumor microvasculature via a semi-quantitative *in vivo* matrigel-based assay[37]. Expression of IFN-γ and IP-10 (an antiangiogenic chemokine) were elevated following administration of IL-18 + IL-12, confirming the anti-angiogenic activity of this combination treatment. Unfortunately, the clinical utility of the IL-18/IL-12 regimen has been hampered by the persistence of treatment-limiting toxicities in pre-clinical animal models, including death. Mice treated with high-dose IL-18/IL-12 died of diarrhea and weight loss after development of severe hemorrhagic colitis[34]. Carson *et al* characterized the cells involved in mediating the toxicities associated with administration of IL-12 plus IL-18 as daily therapy[38]. These investigators found that, while the individual cytokines were well tolerated, the administration of IL-12 plus IL-18 induced a potent, systemic inflammatory response characterized by elevated levels of pro-inflammatory cytokines and acute-phase reactants that mediated multi-organ pathology. Interestingly, depletion of NK cells in this

model completely abroaged treatment-induced inflammation, suggesting a critical role for this cell compartment in the fatal systemic response. Subsequent studies utilizing dose reductions (0.2 μg and 0.01 μg for IL-18 and IL-12, respectively) reported 83% of mice with a marked suppression of tumor growth[39]. However, persistent side effects in half of these mice once again prompted early discontinuation of treatment. Importantly, elevated levels of serum IFN-γ did not correlate with the severity of these toxic side effects as mice treated with both low-dose and high-dose cytokine combinations had similar elevations in serum IFN-γ.

Recently, another clinically useful cytokine has been combined with IL-18 for the potential treatment of malignancy. High- to moderate-dose IL-2 has been given to patients with advanced cancers with minimal success, mostly due to severe treatment-limiting toxicities. To reduce related toxicity, low-dose IL-2 has been combined with IL-18 in a murine model of malignant disease[40]. Co-administration of these two cytokines completely eradicated 12-day established fibrosarcomas without notable toxicity. Notably, all treated mice achieved complete and long-lasting protective immunity. Interestingly, anti-tumor immunity correlated with enhanced proliferation, cytolytic activity, and IFN-γ production from murine NK cells. Use of transgenic and knock-out animal strains showed that IFN-γ and Fas ligand-dependent pathways were more important than those of perforin, suggesting that direct cancer cell killing may not have been the primary anti-tumor mechanism. Although combination cytokine immunotherapeutic approaches with IL-18 represent viable strategies for the treatment of cancer patients, their safety and clinical utility in humans has yet to be determined in phase I/II clinical trials.

2.1.3 Clinical Trials

Although IL-18 was discovered and cloned over ten year ago, only a limited number of trials have been attempted in patients with cancer. In 2001, a phase I dose-escalation study of recombinant human IL-18 (rhuIL-18) was initiated in patients with solid tumors to determine safety, define biologically effective dose, and to assess pharmacokinetics, antigenicity, and anti-tumor activity[41]. Cohorts of three patients were given rhuIL-18 as a 2-hour infusion daily for 5 consecutive days at seven different planned dose levels (3, 10, 30, 100, 300, 600 and 1000 μg/kg/day). To date, thirteen patients have been treated up to the 100 μg/kg/day dose level. The most common adverse events have included fever, chills, and nausea. Plasma concentrations of IL-18 increased in a dose-dependent manner, with an average half-life of approximately 36 hours. Dose-dependent increases in GM-CSF, IL-18 binding protein (a negative regulator of soluble IL-18), and

IFN-γ were observed in a majority of treated patients. These preliminary data demonstrate the safety of single-agent rhuIL-18 and suggest immune modulatory activity in the setting of malignancy. However, future studies will need to correlate the induction of IFN-γ and IL-18 binding protein with efficacy in order to establish IL-18 as a viable cancer therapeutic agent.

3. INTERLEUKIN-21: A REGULATORY CYTOKINE FOR T, B, AND NK CELLS

3.1 Discovery and Cloning

The discovery of IL-21 represents the utility of computer algorithm tools for the discovery of sequences that encode orphan receptors. Thus, before the IL-21 protein was even discovered, a receptor subunit was first identified via a bioinformatics approach[42]. This receptor is further characterized below. The ligand for the IL-21R (i.e. IL-21) was found using a functional assay in which the BaF3 cell line (hematopoietic progenitor origin) was stably transfected with full-length IL-21R[43]. Conditioned media from more than 100 primary and immortalized cell lines were tested for the ability to bind IL-21R on BaF3 cells. Interestingly, conditioned media derived from cultures of activated T cells (specifically $CD3^+$ cells activated with PMA and ionomycin) were the only positive source of activity. Subsequent Real-Time PCR data provided definitive evidence that IL-21 is expressed exclusively by activated $CD4^+$ T cells[43]. General activation using PMA and ionomycin enhanced message levels, but higher-level expression was seen in cells stimulated with anti-CD3 monoclonal antibody. IL-21 expression was increased to an even greater extent by treatment with a combination of anti-CD3 and anti-CD28 Abs, indicating that this message is likely up-regulated following T cell activation.

3.1.1 Receptors and Signaling

The full-length cDNA sequence for IL-21R encodes a 538 amino acid cytokine receptor with an extracellular domain consisting of one copy of the conserved WSXWS cytokine-binding domain[42]. This domain is followed by a transmembrane region and then by a large intracellular domain that contains structural motifs previously shown to be important in signal transduction[43,44]. The IL-21R has the highest amino acid sequence similarity to the β subunit of the IL-2R. The functional IL-21R complex consists of a

heterodimeric complex of the IL-21R with the common γ chain of the IL-2/IL-15 receptor[43].

Determination of the tissue distribution of the IL-21R offered a strong indication of the potential sites of action of the IL-21 ligand. Northern analysis revealed transcripts in human spleen, thymus, lymph node, and peripheral blood leukocytes[43]. Flow cytometric analysis using fluorescently labeled IL-21 revealed receptor expression on resting B cells as well as on B cell lymphoma, natural killer-92, and T cell lines[43]. The IL-21R was also detected on human peripheral B cells as well as mouse splenic B cells. In addition, IL-21R expression was detected on the surface of both $CD4^+$ and $CD8^+$ T cells, but only following activation[43]. The very low levels of this receptor on naïve T cells argues that IL-21 may not be involved in the T cell development phase, but rather in modifying T cell responses downstream of antigen activation. Western analysis confirmed that IL-21R protein was expressed in each of these cellular sources.

IL-21 employs signaling elements common to the class of cytokine receptors utilizing the common gamma chain for intracellular signaling, including IL-2 and IL-15[45]. Not surprisingly, IL-21 has been found to have similar immune effects and acts on some of the same cells types on which IL-2 and IL-15 act[46]. IL-21 mediates its immune signal transduction mainly via the JAK/STAT signaling pathway. IL-21 induced the activation of JAK1 and JAK3 receptor-associated kinases[46,47]. Further downstream, IL-21 promoted STAT1, STAT3, and STAT4 activation and their translocation to regulatory sits of IL-21-responsive genes in NK and T cells, most notably IFN-γ[48]. Recently, Strengell *et al* have shown that IL-21 induced the production of critical transcription factors that regulate innate and Th1 adaptive immune responses, most notably MyD88 and T-bet[49]. Current studies are attempting to examine the relative roles of these signaling molecules in diverse immune responses, ranging from INF-γ secretion to direct, tumor-specific cytotoxicity.

3.1.2 Biological Activity and Rationale for Clinical Development

Since their discovery, there has been significant interest in characterizing the immune functions of the IL-21R and IL-21. Studies attempting to describe these effector functions have primarily focused on the cell types known to express the IL-21R, notably T cells, NK cells, and B cells.

One of the first studies examining the biologic roles of IL-21 showed that IL-21 is a direct product of activated $CD4^+$ T cells[50]. This was the first indication that one of the primary functions of this unique cytokine may be associated with T helper immune responses. In this regard, IL-21 has been shown to enhance the proliferative effects of IL-2, IL-15, or IL-7 on

peripheral T cells, even in the absence of TCR-CD3 stimulation[43]. In addition to its role as a helper-like cytokine, evidence is accumulating that IL-21 enhances primary T-cell responses and effector cell differentiation. Kasaian *et al* have reported that IL-21 significantly increased alloantigen stimulation of murine T cells, resulting in increased CTL activity, an effect similar to that achieved with IL-2, IL-15, or IL-12[50]. Furthermore, IL-21 was able to enhance IFN-γ production by T cells, alone or in combination with IL-2 or IL-15. Collectively, these data indicate that IL-21 may be important for the development of T helper cell type 1 (Th1) responses and for augmenting cell-mediated effector functions.

In addition to enhancing a primary antigen response, IL-21 may also modulate memory T cell functions. Recently, it has been shown that IL-21 prevented the proliferation of murine $CD44^+CD8^+$ memory T cells mediated by IL-15 and the subsequent up-regulation of cytokine receptors for IL-2, IL-15, and IFN-γ[50]. This result suggested that IL-15-induced proliferation of memory $CD8^+$ cells is independent of TCR activation and that these T cells display characteristics of innate immune cells. Therefore, the inhibition of these T cells by IL-21, combined with the abrogation of some NK cell responses (see below), has suggested that IL-21 promotes the transition between innate and adaptive immunity. Current studies examining the role of IL-21 in dendritic cell-mediated proliferation of antigen-specific T cells are attempting to provide a mechanism to validate this contention[51,52].

To date, the overall effects of IL-21 on NK cells have been difficult to interpret, mainly because studies with NK cells have shown both positive and negative effects. For example, an early report showed that IL-21 inhibited the IL-15-mediated expansion of naïve mouse NK cells, failed to stimulate the cytolytic activity of freshly isolated mouse NK cells, and antagonized the viability of IL-15-treated mouse NK cells[50]. In contrast to these inhibitory effects, some reports have shown that IL-21 can mediate the rapid maturation of murine NK cells *in vitro*[53,54]. In addition, IL-21 was more recently shown to stimulate cytotoxicity and IFN-γ production in previously activated NK cells and to enhance these responses in combination with IL-15[48,54]. To complicate matters further, somewhat different effects have been observed in human NK cells. For example, IL-21 stimulated the cytolytic activity of freshly isolated, peripheral human NK cells[43]. Moreover, the combination of IL-21 plus IL-15 stimulated expansion of $CD56^+CD16^+$ NK cells from bone marrow cultures[55]. Interestingly, these cells exhibited enhanced effector cell activity as compared to the typical $CD56^+CD16^-$ cells that arise following exposure to IL-15 alone. An intriguing explanation that has been proposed for the apparent species difference in NK cell responses is the relative naïve nature of laboratory mouse NK cells compared with human NK cells, which are exposed to

significantly more environmental pathogens[55]. Thus, the differential effects observed among species may result from differences in IL-21R expression, which is activation-sensitive (see above). Although a direct comparison of conditions between species is difficult, it appears that the experiments described with mouse NK cells were performed using relatively higher doses of IL-21 than those performed on human NK cells. Therefore, the activity of IL-21 on murine and human NK cells may be similar when dose and activation state are matched. During the transition between innate and adaptive immunity, IL-21 is thought to enhance the effector functions of NK cells and $CD8^+$ T cells (as described above), but also limit the expansion of resting and activated NK cells[49,50,56]. Thus, the IL-21 effect on NK cells may vary depending on the timing and magnitude of the T cell response and the subsequent concentrations of IL-21. For example, antigen activation of relatively few T cells may promote NK cell expansion and effector cell function, whereas a larger number of activated T cells may actually down-regulate NK cell expansion and function. These hypotheses, of course, are currently being validated in both human and murine systems.

The effects of IL-21 on peripheral B cell proliferation vary markedly depending on the type of co-stimulus provided to the B cells. For instance, IL-21 inhibits the proliferation of human B cells treated with anti-IgM and IL-4[43]. Thus, IL-21 may down-modulate T-independent, B cell proliferation that is associated with innate immunity. In addition, Mehtta *et al* showed that IL-21 induced the apoptosis of resting primary murine B cells[57]. The activation of these B cells with IL-4, LPS, or anti-CD40 Ab did not prevent the IL-21-mediated apoptosis, suggesting a dominant role for IL-21 in regulating B-cell homeostasis. More recently, numerous studies have elucidated the role of IL-21 on immunoglobulin production by B cells[58]. For example, IL-21 was found to directly inhibit IL-4-induced IgE production from B cells[59]. Pene *et al* further showed that IL-21 specifically induced the production of IgG_1 and IgG_3 Ab isotypes by CD40-activated $CD19^+$ naive human B cells, suggesting that IL-21 acts as a "switch factor" for the production of specific IgG isotypes[60]. In addition to normal B cell function, IL-21 may regulate aspects of B cell tumorigenesis. IL-21R is not expressed on acute B-cell leukemia cell lines, but is readily detectable on many B-cell lymphoma cell lines[43]. IL-21 appears to be a growth and survival factor for myeloma cell lines and some myeloma specimens, which are cancers derived from terminally differentiated B lymphocytes[61]. Current studies are further assessing the relative role that IL-21 plays during the various stages of B cell maturation and, more importantly, during the subsequent processes of transformation.

3.1.3 Clinical Trials

To date, no clinical data exist for IL-21 administration to patients with cancer. However, a phase I trial in patients with metastatic melanoma and renal cell carcinoma has currently been approved by the FDA. Future trials in pre-clinical development plan for the use of single-agent IL-21, as well as combination strategies utilizing IL-21 with chemotherapy or other biological agents (e.g. therapeutic anti-tumor antibodies). Despite the lack of clinical correlates, numerous pre-clinical models have strongly suggested the anti-tumor utility of IL-21 administration in the context of advanced malignancy. Nelson *et al* have shown that treatment of tumor-bearing mice with systemic administration of IL-21 suppressed tumor growth without the toxic side effects commonly seen with other moderate-dose cytokine treatments[62]. Since IL-21 and IL-2 (a commonly utilized anti-cancer cytokine) both signal to the same immune cells via the IL-2 common γ chain receptor subunit, the toxicity profile following IL-21 administration was compared directly to that of IL-2. Vascular leakage, lung and liver inflammation, and systemic pro-inflammatory cytokines all occurred at a lower frequency in IL-21-treated mice, suggesting the potential for anti-tumor efficacy without severe treatment-limiting side effects[62]. A study by Wang and colleagues suggested that the anti-tumor activity of IL-21 *in vivo* is mediated through activation and effector functions of NK cells[63]. Indications that IL-21 could lead to adaptive immune responses were provided by DiCarlo *et al* who showed rejection of a murine mammary adenocarcinoma by specific cytotoxic T cells via IFN-γ-dependent mechanisms[64].

These pre-clinical studies suggest that IL-21 administration can activate immune mechanisms leading to tumor regression. The planned clinical trials of recombinant human IL-21 (rhuIL-21) will attempt to demonstrate safety and confirm these findings of anti-tumor immune activity in human patients.

4. INTERLEUKIN-24: FROM TUMOR SUPPRESSOR GENE TO APOPTOSIS INDUCING CYTOKINE

4.1 Discovery and Cloning

Neoplastic cells often exhibit a less differentiated state resulting in an enhanced proliferative ability and tumorigenic potential[65]. Jiang *et al* treated human melanoma cells with the combination of fibroblast interferon (IFN-β) and the protein kinase C activator mezerein (MEZ) to induce an irreversible

loss in growth potential, suppression of tumorigenic potential, and terminal differentiation in the melanoma cells[66]. Subtraction hybridization analysis using this model system resulted in the identification and cloning of numerous genes regulated during the process of growth arrest and terminal differentiation (i.e. melanoma differentiation associated (*mda*) genes)[66,67]. The expression of one particular gene, *mda-7*, correlated strongly with the induction of irreversible growth arrest, cancer reversion, and terminal differentiation in human melanoma cells[68,69]. It was subsequently determined that *mda-7* was highly expressed in normal melanocytes and its expression decreased progressively during the processes of melanoma transformation and progression to metastatic disease[70]. Interestingly, *mda*-7 expression increased in growth-arrested and differentiation inducer-treated human melanoma cells in a p53-independent manner. The endogenous levels of *mda*-7 was higher in normal melanocytes as compared to levels in metastatic human melanoma cells. To date, differential expression of this gene in human cells has been documented only in the context of melanoma. This is most likely due to its relatively high basal-level of expression in normal melanocytes compared to that in other cell types. For example, only a sub-population of blood cells have been shown to constitutively express the *mda*-7 gene[71]. Although the specific physiological role played by the *mda-7* gene product in normal melanocytes or other cell types has yet to be clearly defined, the gradual loss of expression observed with melanoma progression supports the possibility that *mda-7* might play a tumor suppressive effect in the context of melanoma[70]. Characterization of structural and sequence homology suggested that the *mda-7* gene product belonged to the IL-10 family of cytokines, and was therefore re-designated as IL-24[72].

4.1.1 Receptors and Signaling

Based on the demonstrated homology to IL-10, it was hypothesized that the *mda*-7/IL-24 receptor would share some sequence or structural similarities to the IL-10R. The IL-10 receptor was initially identified as a complex of single-chain R1 type and single-chain R2 type receptor subunits[73]. Other members of the IL-10 family of cytokines, such as IL-20 and IL-22, also bind to and signal through heterodimeric receptors each with a R1 and R2 type of receptor subunit. Similarly, the mda-7/IL-24 receptor complex was found to consist of two chains, IL-20R1 and IL-20R2[74]. Furthermore, mda-7/IL-24 also bound to a second receptor complex, consisting of IL-20R2 and IL-22R1[75]. When activated by their ligands, both IL-24 receptor complexes signal through the JAK/STAT pathway, primarily via STAT3[74]. Although all the signaling pathways involved in mediating the

effects of mda-7/IL-24 have not yet been fully elucidated, current evidence suggests that the protein kinase R pathway, components of the MAPK pathway, PI3 kinase, and angiogenic pathways are involved[76-79]. Interestingly, the activities of these diverse pathways cannot be attributed entirely to the cytokine properties of mda-7/IL-24. In fact, clinical conditions associated with JAK/STAT dysregulation typically involve neoplastic changes and not the anti-proliferative and apoptosis-inducing effects seen following mda-7/IL-24 treatment. For example, STAT3 has been shown to participate most frequently in the development and maintenance of numerous malignancies, including multiple myeloma and chronic myelogenous leukemia (CML)[80,81]. Thus, it remains to be determined whether mda-7/IL-24, which utilizes STAT3 signaling, induces apoptosis through this same pathway. Using cells deficient in JAK/STAT signaling, investigators hope to clarify these issues regarding the effects of mda-7/IL-24.

4.1.2 Biological Activity and Rationale for Clinical Development

Huang *et al* analyzed a large collection of normal and cancer cell types for expression of IL-24[68]. Although most cell types lacked constitutive expression of IL-24, melanocytes expressed constitutive levels of both IL-24 mRNA and protein. To further define the normal tissues that express *mda-7/IL-24*, an extensive northern blot analysis of normal tissues revealed IL-24 expression in tissues of the immune system, including spleen, thymus and peripheral blood leukocytes[68]. Furthermore, activation of human PBMCs *in vitro* with PHA or LPS, or *in vivo* by microbial infection, resulted in secretion of active IL-24 protein[72]. Subset analysis confirmed that IL-24 was up-regulated in monocytic cells after stimulation with LPS. Slight and delayed expression was also apparent in activated T cells cultured on anti-CD3 mAb coated plates or activated by ConA exposure. Current studies are underway to better understand the role of IL-24 in a normal physiological context, and eventually its implications in malignant disease.

IL-24 mRNA can also be induced in cells that are not of the melanocyte or hematopoietic linage. Although induced expression was not apparent in most normal and tumor-derived cells examined, treatment of primary cells as well as cell lines derived from breast, cervical and prostate carcinoma, osteosarcoma, nasopharyngeal carcinoma, as well as normal breast epithelium and cerebellum astrocytes with IFN-α + MEZ induced IL-24 mRNA expression[68]. These results confirmed that IL-24 is not expressed constitutively in most normal and cancer cell types, but that expression can be induced in a spectrum of normal and tumor cell types. Importantly, this induction was independent of alterations in classic tumor suppressor genes

such as retinoblastoma (*Rb*) and/or *p53*[82]. To date, however, the specific cellular cues responsible for IL-24 expression, and the potential patho-physiological role for this molecule in the context of malignancy remain to be elucidated.

Regardless, expression of the IL-24 gene in human melanoma cells following transient or stable transfection has resulted in potent growth suppression[79]. When expressed at super-physiologic levels, IL-24 induced growth suppression and apoptosis in a broad spectrum of human cancers, including melanoma, glioblastoma, and carcinomas of the breast, colon, lung, and prostate[68]. In contrast, IL-24 gene transfection had little effect on the growth and survival of normal breast and prostate epithelium, endothelium, melanocytes, and skin and lung fibroblast cells[69]. Current studies are attempting to find common patterns of gene expression following IL-24 transfection in order to provide clues regarding susceptibility and cell-specific activity. As an *in vivo* proof-of-principle, Su *et al* have shown that IL-24 gene-expressing human breast carcinoma cells engrafted into nude mice grew at a significantly slower rate than non-transfected tumor cells[83]. In another in vivo tumor model, inhibition of lung tumor growth following systemic administration of mda-7/IL-24 was associated with significant decreases in microvessel density and hemoglobin content, indicating an anti-angiogenic mechanism *in vivo*[84].

4.1.3 Clinical Trials

Based on the tumor-selective inhibitory properties of IL-24, as documented in cell culture gene transfer models and animal xenograft models, a phase I dose-escalation study was initiated coordinately by Introgen Therapeutics Inc. and the Baylor Sammons Cancer Center[85]. An adenoviral vector encoding IL-24/*mda*-7, termed Ad.*mda-7* (drug designation: INGN 241), was administered to patients with advanced carcinoma. Patients that had a surgically resectable lesion received a single injection of Ad.*mda-7* directly into tumors at doses ranging from $2x10^{10}$ to $2x12^{12}$ viral particles. Twenty-four hours post-injection, the lesions were surgically removed, serially sectioned, and analyzed for viral vector distribution, IL-24 protein expression, and the level of apoptosis induction. This study demonstrated that intra-tumoral administration of Ad.*mda-7* was safe with only mild toxicities observed, including injection site pain, transient low-grade fever, and mild flu-like symptoms. In addition, a recent update from this phase I study documented that Ad.*mda-7* could induce apoptosis in a large percentage of the tumor volume examined (70%) following intra-tumoral administration[86]. This initial study in patients confirmed the growth inhibitory effects of IL-24 observed in animal models.

In addition, these data have provided rationale for the development of phase II clinical trials attempting to define the potential anti-tumor efficacy of IL-24 expression in tumors of patients with advanced malignancies.

5. CONCLUDING REMARKS

Cancer is a complex genetic disease involving aberrations in multiple pathways that control cellular growth and differentiation (reviewed in Knudson *et al*[87]). Traditional treatment modalities including radiation and chemotherapy have attempted to curb the growth of tumor cells via relatively non-specific mechanisms. However, numerous studies have shown that cancer cells develop multiple mechanisms of resistance to these treatments[88]. Although increasing doses and alterations in treatment regimens have been somewhat successful at circumventing this resistance, long-term anti-tumor efficacy has often been hampered by toxic side effects[89-91]. In contrast, utilization of the host immune system represents an attractive method to combat cancer because of the potential for low treatment-limiting toxicities. Cytokines, the protein hormones of the immune system, have been the most easily utilized immune component for therapy because they can be mass-produced and administered as either systemic or localized therapy.

Over the last fifteen years, however, several cytokines have been used in cancer therapy with only moderate success, the most prominent of which include IL-2, IFN-α, and IL-12. Clearly, induction of optimal immune activation with minimal toxicity following cytokine administration to humans is not a simple matter. Can investigators find a way to harness the potential that IL-18, IL-21, and IL-24 have shown in the pre-clinical arena? Current attempts in phase I/II clinical trials will determine the safety and potential efficacy of these agents for single-agent therapy. In addition, combination of these cytokines with low-dose chemotherapy or therapeutic antibodies directed against tumor-associated surface markers are also being developed for clinical trials. Correlative studies associated with these trials will attempt to define the underlying mechanisms by which these agents mediate their anti-tumor effects. Hopefully, insights from current and future pre-clinical studies combined with the correlative data from humans trials will translate into enhanced clinical activity.

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