IL-6 as a drug discovery target

Bernd Stein and May S. Kung Sutherland

Interleukin 6 (IL-6) is a cytokine that functions as a trophic and differentiating factor in cells of many types. Because of its wide-ranging expression and effects, the inappropriate expression and modulation of IL-6 production has important physiological consequences. In this review, the authors examine the role of IL-6 under physiological and pathophysiological conditions, and its feasibility as a drug discovery target.

Cytokines, together with hormones and neuronal signals, are part of an important communication system within eukaryotic cells. Interleukin 6 (IL-6) is a cytokine that is produced by many cell types. Originally, IL-6 was independently described as interferon-β_2, T-cell replacing factor, B-cell stimulating factor, 26 kDa protein, hybridoma/plasmacytoma growth factor, hepatocyte stimulating factor and bone marrow stromal cell derived growth factor[1]. These early studies also led to the conclusion that IL-6 is involved in various cellular processes. The following sections describe the biological activities of IL-6, its role in the development of several pathological conditions and the therapeutic approaches that have been developed.

IL-6 protein and its receptor

The protein

Human IL-6 is a single-chain protein with a molecular mass ranging from 21 to 28 kDa. IL-6 is modified by *N*- and *O*-glycosylations, as well as by phosphorylation on serine residues. The cytokine is synthesized as a precursor protein of 212 amino acids with a hydrophobic 28-residue signal sequence. Its closest homolog is granulocyte colony-stimulating factor (G-CSF). The gene for human IL-6 is located on chromosome 7p21 (for review, see Ref. 2).

IL-6 receptor

IL-6 binds to the cell surface via an interaction with the extracellular region of the α-chain of the IL-6 receptor (80 kDa subunit, IL-6Rα). This complex then associates with the gp130 receptor. The gp130 subunit undergoes ligand-dependent dimerization with subsequent activation of the JAK/STAT signaling cascade (for review, see Ref. 2). The intracellular domain of IL-6Rα does not play a role in signal transduction. The soluble IL-6 receptor, which lacks the transmembrane and cytoplasmic domain of IL-6Rα, is also responsive to IL-6 and acts as an agonist.

A few other cytokines, such as IL-11, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor and cardiotropin, can also signal through gp130 but bind to their own receptor subunit[3].

Regulation of IL-6 production

IL-6 is produced by a large variety of cells, including fibroblasts, endothelial cells, keratinocytes, monocytes, T cells, mast and tumor cell lines, and cells of neural origin. The IL-6 promoter, which is highly conserved between mouse and human over the first 300 bp, contains binding sites for several known transcription factor families. IL-6 gene expression is modulated through at least three *cis*-acting elements: NF-κB, C/EBPβ and AP-1 binding sites. IL-6 is upregulated by a variety of stimuli, including cytokines (IL-1 and tumor necrosis factor, TNF-α), forskolin, phorbol esters and lipopolysaccharide (LPS) (for a recent review, see Ref. 2). Some of the more important inhibitors of IL-6 gene expression appear in the steroid family, and include estrogens, androgens and glucocorticoids (for a recent review, see Ref. 2).

Activation of the JAK/STAT pathway

The IL-6 activated gp130 receptor associates with members of the Janus kinase (JAK) family of cytoplasmic tyrosine kinases. This family is characterized by proteins with a C-terminal protein tyrosine kinase domain and an adjacent

Bernd Stein* and **May S. Kung Sutherland**, Signal Pharmaceuticals, Inc., 5555 Oberlin Drive, San Diego, CA 92121, USA. *tel: +1 619 558 7500, fax: +1 619 558 7513, e-mail: bstein@signalpharm.com

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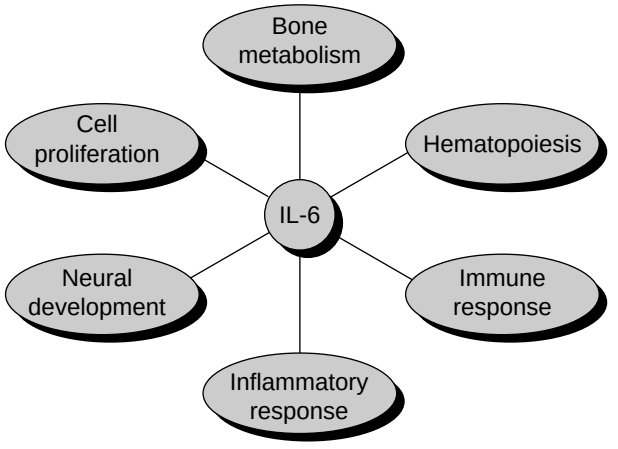

Figure 1. *Biological activities of interleukin 6 (IL-6).*

kinase-related inactive domain[4]. Different cytokines activate different JAKs. IL-6 activates JAK1, JAK2 and Tyk2. The activation of JAK family members results in phosphorylation of the IL-6 receptor, as well as the association and activation of STAT1 and STAT3 (signal transduction and activators of transcription) family members. STAT family members are characterized by SH2 and SH3 (*src*-homology) domains. The phosphorylated, active form of STAT acts as a transcription factor, binding to DNA. Phosphorylation of the IL-6 receptor also provides the opportunity for *src*-homologous collagen (SHC) to associate with it. This triggers the activation of the Ras signaling pathway, which ultimately results in activation of the mitogen-activated protein kinases[5].

Biological activities of IL-6

The following sections briefly describe the role of IL-6 in the development of the inflammatory response, the differentiation and activation of cells of the hematopoietic lineage, and the regulation of nerve cell and bone cell functions (see Figure 1).

Immune response

IL-6 stimulates the production of immunoglobulins and the activation of killer cells[6]. For B cells, IL-6 has been shown to be vital for the final differentiation of activated cells to become immunoglobulin-secreting plasma cells[7]. In addition, IL-6 stimulates the proliferation of thymic and peripheral T cells[8] and, in conjunction with IL-1, induces the formation of cytolytic T cells[9].

Hematopoiesis

An important role of IL-6 in hematopoiesis has been demonstrated by the observation that the administration of IL-6, in conjunction with IL-3, stimulated the proliferation of primitive hematopoietic progenitor cells, myeloid cells and megakaryocytes[10]. Further, IL-6 increased the size of megakaryocytes in bone marrow and increased the platelet count in mice and primates[11]. In support of previous reports, the administration of IL-6 to mice with radiation-induced hematopoietic suppression facilitated the recovery of myeloid and megakaryocytic cells[12].

Inflammatory response

The role of IL-6 in the induction of the acute phase response in liver has been known for many years. This reaction is nonspecific and occurs in response to injury, trauma or infection. The result is fever, leukocytosis, changes in plasma cortisol levels and increased plasma levels of proteins such as C-reactive peptide (CRP) and antiproteases[13]. In rodents, IL-6 induced the release of ACTH and corticosteroids[14]. Intracerebroventricular administration of IL-6 to rodents elicited fever and acute-phase proteinemia[15], indicating that the hypothalamus may also play a role in the response to trauma.

Neural development

In situ hybridization demonstrated that IL-6 is expressed at high levels during the development of the brain[16]. The highest mRNA levels were localized to the hippocampus, hypothalamus and subcortical structures of the rat brain[17]. It is thought that IL-6 may function as a neurotrophic factor by stimulating the release of nerve growth factor[18] because IL-6 stimulated the growth of PC12 cells[19] and catecholaminergic and cholinergic neurons[20].

Bone metabolism

The effect of IL-6 in regulating bone cell function has been studied by many investigators. The bone-forming cells (osteoblasts) and the bone-resorbing cells (osteoclasts) arise from different precursors in the bone marrow. Both cell types have been shown to express receptors for IL-6 as well as to secrete IL-6 in response to inducers, including IL-1β and TNF-α (Refs 21–24; S.K. Sutherland *et al.*, unpublished). *In vitro*, IL-6 and its inducers promote osteoclastogenesis and increase bone resorption in human and murine bone cell models (Refs 25–27; S.K. Sutherland *et al.*, unpublished). Antisense oligonucleotides and antibodies to

IL-6 abrogated these responses[25–27]. Further, the formation of osteoclastic cells in a human co-culture system, which more closely reflects the physiological environment in bone, was reduced by administration of an anti-IL-6 antibody, supporting the role of IL-6 in osteoclastogenesis (S.K. Sutherland *et al.*, unpublished).

A significant correlation between bone mineral density and polymorphic variations of the IL-6 gene in humans was recently reported[28], supporting the role of IL-6 in regulating bone mass. Elevated serum levels of IL-6 and increased bone marrow cell production of IL-6 have been found in postmenopausal women, women with postmenopausal osteoporosis and orchidectomized males[29–31]. Furthermore, genetically engineered IL-6-deficient mice were protected from the extensive bone loss associated with ovariectomy[31]. These findings support the premise that IL-6 plays an important role in maintaining bone homeostasis (this is discussed in more detail in the section on bone resorption below).

IL-6 knockout mice

Mice with a disruption of the IL-6 gene in both alleles develop normally, but are protected from the increased osteoclastogenesis and extensive bone loss exhibited by their normal littermates after ovariectomy[32] or orchidectomy[33]. These mice exhibited small decreases in the absolute numbers of hematopoietic stem cells and progenitors[34], and slightly impaired accumulation of leukocytes in subcutaneous air pouches[35]. There was severe impairment of the macrophage and neutrophil response and hence the acute phase response[36] to infection by influenza pneumonitis[37], *Candida albicans*[38] and vaccinia virus[39]. Overall, these studies suggest that, with the exception of osteoclastogenesis after ovariectomy and the immune response to certain infectious agents, most of the functions of IL-6 can be taken over by other cytokines. This is no surprise, given the number of cytokines that signal through the gp130 receptor subunit.

In comparison, the genetically engineered deficiency of the gp130 signal transducing element in mice was a lethal event, resulting from abnormal development of the ventricular myocardium and a reduction in the numbers of pluripotent hematopoietic stem cells and primordial germ cells (for review, see Ref. 40). There was no apparent abnormality in the formation of the skeleton[41]. It should be noted that the lethality was to be expected since gp130 (as stated above) is a common signal transducer for several cytokines. No knockouts of the IL-6Rα subunit have been described, to our knowledge.

IL-6 transgenic mice

Genetically engineered mice that overexpressed IL-6 exhibited elevated levels of acute phase reactant proteins (α_2-globulins, hypergammaglobulinemia), mesangial cell proliferative glomerulonephritis and massive polyclonal plasmacytosis with autoantibodies in the spleen, lymph nodes and thymus[42]. Chronic expression of IL-6 in the astrocytes and glial cells of the nervous system resulted in lack of formation of the blood–brain barrier, extensive neurodegeneration in the hippocampus and cerebellum, and an inflammatory response associated with increased cytokine expression, synthesis of acute phase proteins and gliosis[43]. Skeletal analysis of these mice revealed decreased bone formation and resorption, supporting a role of IL-6 in bone metabolism[44]. These phenotypes are expected from the participation of IL-6 in many physiological processes, as described above.

IL-6 in disease

As mentioned previously, IL-6 plays a vital role in the proliferation and differentiation of many tissues, including the liver, brain and bone marrow cells. Additionally, inappropriate expression and production of IL-6 is thought to be involved in the pathogenesis of numerous diseases, including inflammatory conditions (encephalitis, rheumatoid arthritis) and cancer (leukemia, renal cell carcinoma) (see Figure 2). Several of these conditions are described below.

Infection/inflammation

Chronic heart disease. Chronic heart failure[45,46] and coronary artery disease[47] are two diseases for which a correlation has been found between the levels of IL-6 and the severity of the condition. Elevated levels of IL-6 were also associated with changes in hemodynamic parameters and depressed cardiac function[45]. In animal models of myocardial ischemia and inflammatory injury, IL-6 mRNA levels were increased and correlated with increased mRNA levels of an intercellular adhesion molecule (ICAM-1)[48]. Induction of IL-6 in vascular cells may be the consequence of a hypoxia-mediated event involving the nuclear transcription factor, NF-IL6 (Ref. 49), triggering the adhesion of neutrophils to the myocardial tissue and causing severe tissue damage and cell death[48].

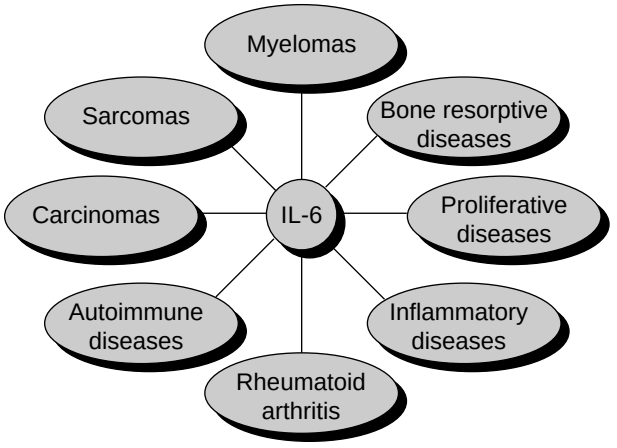

Figure 2. *Pathological conditions in which interleukin 6 (IL-6) has been shown to play a role.*

Inflammation of the central nervous system. As mentioned above, IL-6 is expressed in the neurons and glia of subcortical areas, cerebellum and brain stem[17]. Further, IL-6 has been detected in the cerebrospinal fluid but not in the serum of subjects with inflammation of the CNS, including meningitis and encephalitis[50,51]. Elevated IL-6 levels were also found in plasma and cerebrospinal fluid of humans with multiple sclerosis, as well as in a mouse model of multiple sclerosis[50,52,53]. These findings support the notion that IL-6 is an initiator of the inflammatory response in the brain[15].

Inflammatory bowel disease. Inflammatory bowel disease (IBD) is associated with systemic endotoxemia and severe inflammation and scarring of the intestinal tissues. Elevated levels of the receptor antagonist to cytokines IL-1β, IL-2, IL-6 and IL-8, the IL-1 receptor and the soluble IL-6 receptor have been found in colonic mucosa from IBD patients[54,55], the highest levels being associated with the diseased areas of the colon. The sources of IL-6 were infiltrating T cells, macrophages and B cells[56]. Using an *in vitro* model, IL-6 was also found to activate the synthesis of acute phase proteins (such as fibrinogen and α_1-antitrypsin) in the human intestinal epithelial cell line Caco2 (Ref. 57).

Autoimmune diseases

AIDS is an immune deficiency syndrome resulting from infection by HIV, and is characterized by decreased T-cell numbers and a paradoxical rise in immune activation. HIV infection results in an increase in the serum levels of a number of cytokines, including IL-6. Elevated serum or plasma levels of IL-6 were found in the majority of HIV-infected donors, as well as increased production of IL-6 by peripheral blood monocytes[58,59]. Exposure of healthy blood monocytes to HIV enhanced the production of IL-6 by these cells[60,61]. IL-6 appeared to have an autocrine (or paracrine) effect on the replication of HIV, because blocking the production of IL-6 dramatically reduced the viral titer[62]. One of the possible mechanisms of how HIV infection upregulates IL-6 expression is through the viral *trans*-activating protein, HIV-1 Tat. Tat interacts with the IL-6 promoter and increases C/EBPβ binding, resulting in increased IL-6 transcription[63]. These findings suggest an important mechanism for IL-6 in maintaining HIV infection.

Cardiac myxoma is the most common primary heart tumor in adults. Patients with cardiac myxoma exhibit a variety of autoimmune symptoms such as hypergammaglobulinemia and elevated acute phase proteins (for review, see Ref. 64). Several studies have reported a significant correlation between the presence of tumor tissue and elevated serum levels of IL-6 or increased tissue expression of IL-6 mRNA (Refs 65,66). These reports suggest that IL-6 plays a role in the inflammation of cardiac tissue.

Castleman's disease is a clinically and histologically heterogeneous disease characterized by giant lymph node hyperplasia, fever, anemia, hypergammaglobulinemia and increased plasma levels of acute phase proteins[67]. Increased production of IL-6 was associated with hyperplastic lymph nodes and peripheral blood mononuclear cells[67,68]. Additionally, increased IL-6 receptor levels were found on peripheral B cells, further enhancing the sensitivity of the system to IL-6 (Ref. 69). Overexpression of IL-6 in mice leads to the development of a syndrome similar to Castleman's disease[70], supporting a causative role for IL-6 in the disease.

Endometriosis is a gynecological disorder characterized by the implantation and growth of endometrial tissue outside of the uterine cavity. This tissue appears to trigger the host immune response, resulting in humoral and cell-mediated inflammatory responses, and eventually chronic inflammation and infertility. Increased levels of cytokines, including IL-1β (Ref. 71) and TNF-α (Ref. 72), have been found in the peritoneal fluid from patients with chronic inflammation, whereas there was no significant difference in the

amount of IL-6 (Ref. 73). However, there was a positive correlation between the amount of IL-6 and the number of peritoneal macrophages present in the peritoneal fluid, with the production of IL-6 being greatly enhanced by these macrophages in response to LPS (Ref. 73). Endometrial stromal cells from subjects with endometriosis also expressed higher basal levels of IL-6, with the production of this cytokine being greatly enhanced by IL-1β (Ref. 74). Further research is needed to establish whether there is a strong correlation between increased production of IL-6 and the inflammation of the endometrial tissues contributing to the severity of the disease.

Liver cirrhosis. Plasma levels of IL-6 and TNF-α and mRNA levels for IL-6, IL-2 and IL-8 were elevated in subjects with severe cirrhosis of the liver[75]. These patients showed a positive correlation between IL-6 levels and organ failure[76], as well as increased secretion of IgA and IgG by peripheral blood monocytes[77]. IL-6 upregulates the expression of fibrinogen mRNA in rodent livers[78]. These changes are thought to play a role in the development of fibroid tissue in diseased liver, which eventually leads to liver cirrhosis.

Rheumatoid arthritis is a chronic inflammatory joint disease in which persistent synovitis leads to bone and cartilage damage. Elevated levels of several cytokines, including IL-6 and its soluble receptor, IL-1, IL-11, oncostatin M and TNF-α, were found in synovial fluid from patients with rheumatoid arthritis[79–81]. These cytokines stimulated chondrocytes, synoviocytes and osteoblasts to secrete prostaglandins, plasminogen activator, collagenases and other proteases, resulting in the breakdown of extracellular matrix and extensive tissue destruction[82]. However, increased serum levels of IL-6 only were found to correlate with increased levels of acute-phase reactant proteins – such as C-reactive protein (CRP), fibrinogen and α_1-acid glycoprotein – in arthritic patients[81,83].

Elevated levels of IL-6 and soluble IL-6 receptors also correlated with the increased number of osteoclast-like cells present in the synovium and with the enhanced bone resorption[80]. The same study showed that addition of synovial fluid from arthritic patients to co-cultures of murine osteoblasts and bone marrow cells stimulated the formation of osteoclasts[80]. Synovial macrophages from arthritic patients were capable of differentiating into osteoclast-like cells, and they resorbed bone *in vitro* when co-cultured with rat osteoblastic cells[84]. In conclusion, although a variety of cytokines of the gp130 family are produced in large quantities in the inflamed joints of arthritic patients, only IL-6 produced by synovial fibroblasts appears to be the mediator of the induction of acute phase protein synthesis[81].

Proliferative diseases

IL-6 promotes the growth of many cell types, including lymphocytes, keratinocytes and mesangial cells[85]. Overexpression of IL-6 is thought to contribute to the pathogenesis of disorders such as psoriasis and mesangial proliferative glomerulonephritis.

Psoriasis. Hyperproliferation of keratinocytes and enhanced expression of the keratin K17 protein causes the skin disease known as psoriasis. Interactions between T cells and keratinocytes are important for the pathogenesis of psoriasis and for the generation of growth factors and the proinflammatory cytokines IL-2, IL-8, interferon-γ (IFN-γ), transforming growth factor-α (TGF-α) and IL-6. Peripheral blood monocytes and skin fibroblasts from psoriatic patients were found to produce increased levels of IL-6 (Refs 86,87), whereas keratinocytes expressed low levels of IL-6 (Ref. 87). However, TGF-α has been shown to increase the production of IL-6 in human keratinocytes through activation of NF-κB and NF-IL6 binding[88]. IFN-γ and IL-6 have been shown to increase the synthesis of the K17 keratin protein through a mechanism involving the phosphorylation of the STAT1 transcription factor[89]. While controversy exists over the production of IL-6 by keratinocytes, the data clearly suggest that the production of IL-6 by various cell types (fibroblasts and circulating monocytes) impact upon the function of keratinocytes.

Glomerulonephritis. Increased proliferation of the intrinsic mesangial cells, plasmacytosis and progressive renal tissue damage are characteristic of mesangial glomerulonephritis. IL-6 is thought to play a major role in the development of the disease. Renal cells expressing IL-6 were localized to the interstitial tissue (tubules) and glomeruli, including the mesangial cells, glomerular epithelial cells and Bowman's capsule[90]. Soluble aggregates of IgA and IgG stimulated the synthesis of TNF-α and IL-6 and increased the proliferation of mesangial cells in culture; this effect was blocked by an anti-IL-6 antibody[91]. Mesangial cells treated with IL-1 or IL-6 exhibited increased proliferation and altered morphology, as well as releasing increased levels of platelet-activating factor and peroxides[92]. Transgenic mice overexpressing

IL-6 exhibited plasmacytosis and progressive kidney pathology leading to glomerulonephritis[42]. Treating the transgenic mice with anti-IL-6 antibodies suppressed the plasmacytosis and improved the renal pathology[93]. While these findings support the premise that IL-6 plays a major role in the development of glomerulonephritis, some investigators[94,95] have disputed a role for IL-6 since they were unable to induce the disease by dosing rodents with high quantities of IL-6 or to reproduce the findings of others[93] by blocking the development of the disease with an anti-IL-6 antibody.

Malignancies

Kaposi's sarcoma/multiple myeloma. Multiple myeloma, the second most frequent malignancy of the blood in the USA, is characterized by the accumulation of malignant plasma cells in the bone marrow and the presence of monoclonal immunoglobulin produced by these cells in the serum and urine[96]. Kaposi's sarcoma associated herpesvirus (KHSV) was found in the bone marrow stromal cells of patients with multiple myeloma, and is thought to play a role in advancing the malignant stage of the disease[96]. The genome of KHSV contains coding regions for proteins similar to the human macrophage inflammatory protein, interferon regulatory factor (IRF) and IL-6 (Ref. 97). IL-6 has been reported to act as an autocrine/paracrine growth factor for myeloma and Kaposi's sarcoma cells[98–100]. IL-6 and its viral homolog are thought to stimulate the growth of these cells while preventing apoptosis of malignant plasma cells[96–98]. Patients with multiple myeloma can exhibit bone lesions caused by intensive bone resorption and increased formation of osteoclasts expressing high levels of IL-6 (Ref. 101). These findings strongly suggest a role for IL-6 in the development of multiple myeloma.

Renal carcinoma cells secrete IL-6 and express receptors for IL-6 (Refs 102,103). IL-6 inhibits apoptosis of these cells and stimulates the activity of superoxide dismutase; both of these actions overcome the activity of anticancer drugs and enhance the survival of the cancer cells. Increased serum levels of IL-6 correlated with the severity of the disease and the production of acute phase proteins[103]. A common complication of renal cell carcinoma is hypercalcemia, which is thought to be caused by the production of parathyroid-hormone-related protein (PTHrP) by a subpopulation of cells in the tumor, the growth of which is stimulated by IL-6 (Ref. 104). De La Mata and coworkers[105] showed that IL-6 enhanced the effect of PTHrP-induced hypercalcemia and bone resorption in mice inoculated with CHO cells stably expressing PTHrP. Thus, IL-6 plays a role in the survival of renal carcinoma cells and contributes to the development of hypercalcemia of malignancy.

Prostate cancer. Increased levels of IL-6 and IL-6 receptors have been measured in cancer of the prostate[106]. IL-6 has been shown to stimulate the proliferation of prostatic carcinoma cell lines while blocking apoptosis induced by factors such as p53 (Ref. 107). The proliferation of these cells was blocked by an anti-IL-6 antibody or an IL-6 receptor antagonist[108], clearly supporting the role of IL-6 in prostatic cancer.

Antitumor effects of IL-6

IL-6, like interferon, has been reported to exhibit antitumor effects. IL-6 showed both direct action on the growth of tumors as well as induced host defenses via its pleiotrophic actions on the immune system and hematopoiesis (for review, see Ref. 85). As such, IL-6 inhibits the growth of leukemic cells, breast carcinoma, colon carcinoma and endothelial cells.

Mammary carcinoma. Normal mammary epithelial cells produce IL-6 and IL-8, which are involved both in the differentiation of these cells as well as in enhancing the production of immunoglobulins in the milk supply[109]. Significant amounts of IL-6, IL-8 and IFN-γ have been found in human milk. However, IL-6 was not detected in breast carcinoma and was reduced in mammary cells infected with c-Ha-*ras* or c-*erb*β2 oncogenes[109]. In support of a protective role of IL-6 in mammary carcinoma, IL-6 exhibited a dose-dependent inhibition of the growth of breast carcinoma cells as well as affecting the morphology and phenotype of these cells, resulting in the disruption of tumor formation (for review, see Ref. 85).

Leukemia. IL-6 is capable of reducing the growth and inducing differentiation of certain myeloleukemic cell lines. This process has been shown to occur via the transcription factors c-Jun, Jun B and c-Fos (through AP-1 sites) and NF-IL6 (for review, see Ref. 85). We and others have shown that the differentiation of myeloid lymphoma U937 cells can be induced by IL-6 (Ref. 110; S.K. Sutherland *et al.*, unpublished). Other cell lines, such as acute promonocytic leukemic THP-1 cells, AML-193 monoblastic leukemic cells and ML-1 myeloblastic cells, also differentiated in the

presence of IL-6 (Refs 85,110). By contrast, IL-6 inhibited the growth of B leukemic and lymphoma cells (for review, see Ref. 85). These findings have been extended to *in vivo* models in which IL-6 caused a rapid decrease in the number of preleukemic cells and inhibited the development of acute myeloid leukemia in SJL-J mice[111].

Bone resorption

As described above, IL-6 plays a critical role in the formation of osteoclastic cells. As such, dysregulation of IL-6 activity in bone cells may lead to the development of pathological diseases.

Osteoporosis. Estrogen plays an important role in maintaining bone mass in men and women by controlling bone loss or resorption. This protective effect is particularly evident when comparing the bone mass of premenopausal and postmenopausal women. Three to seven years after the menopause, the rate of loss of bone mass may be as much as 10% per year, followed by a steady loss of at least 1% per year for the rest of a woman's life[112,113]. Women between the ages of 50 and 80 years may lose as much as 30–50% of their bone mass. Although some bone formation occurs in these women, the amount of bone lost outpaces the amount of bone formed. Indeed, postmenopausal osteoporosis is characterized by fragile brittle bones and an enhanced susceptibility to fracture.

The production of IL-1, TNF-α and IL-6 was found to be elevated in bone marrow cells from postmenopausal women and those who had discontinued estrogen replacement therapy[30], as well as in blood cells from women with postmenopausal osteoporosis[31]. However, other studies, including that of Kassem and coworkers[114], did not find such an increase. The reasons for this discrepancy are unclear, but likely reflect the heterogeneity of the populations being studied, the methodologies being utilized and the circulating levels of estrogen in these women.

In mice, estrogen suppressed IL-6 production and protected ovariectomized mice from developing osteoporosis[26]. Stromal cells from ovariectomized, but not sham-operated, mice greatly induced the formation of osteoclastic cells in co-cultures with bone marrow cells[25]. In normal fetal human osteoblasts (S.K. Sutherland *et al.*, unpublished) and fetal osteoblasts overexpressing the endogenous estrogen receptor[115], estrogen treatment decreased the production of IL-6. Estrogen downregulates IL-6 gene expression at the transcriptional level in the absence of an estrogen receptor binding site in the promoter. The ligand-activated estrogen receptor interacts with the transcription factors NF-κB and C/EBPβ and thereby reduces IL-6 promoter activity[116]. This novel nonclassical estrogen effect opens up the possibility to detect selective estrogen receptor modulators.

A human bone cell co-culture model to study bone formation and bone resorption in a more physiologically relevant system has recently been developed (S.K. Sutherland *et al.*, unpublished). By co-culturing immortalized human osteoblasts with monocytic cells it was demonstrated that, in the presence of agents such as 1,25-dihydroxyvitamin D_3, the monocytic cells differentiated into osteoclast-like cells, as determined by the expression of several phenotypic markers associated with true osteoclasts. The formation of these cells was dependent upon IL-6, because inhibiting IL-6 levels by the addition of estrogen or with an anti-IL-6 antibody reduced the number and activity (as measured by resorption) of these cells. These studies correlate with the *in vivo* results mentioned above.

Supporting these results, other investigators have shown that the administration of an anti-IL-6 antibody reduced the number of osteoclasts in trabecular bone of ovariectomized mice[26] as well as the ability of human giant cells to resorb dentine[27]. Antisense oligonucleotides to IL-6 also decreased the amount of dentine resorbed by human osteoclast-like giant cells[117]. In the mouse model of bone metabolism, a good correlation was reported between the levels of IL-6 and soluble IL-6 receptors and osteoclastogenesis[80,118]. The role of IL-6 in enhancing bone resorption was highlighted in the IL-6 knockout mouse model[32]. Compared to wild-type or heterozygous mice, the knockout mice were protected from the detrimental effects of ovariectomy.

Interestingly, some investigators[119–121] reported that IL-6 does not appear to play a role in osteoclast differentiation and bone resorption in rodent models. Although these investigators did not observe any effect of exogenously added IL-6 on the resorption of neonatal mouse calvariae, IL-1 and parathyroid hormone (PTH) stimulated bone resorption in their studies. Since PTH and IL-1 are known stimulators of the production of IL-6 as well as the soluble IL-6 receptor (which is crucial for the IL-6 response in rodents), it is possible that the resorption observed by these investigators was mediated indirectly by IL-6.

The role of androgens in maintaining bone mass is under investigation by many researchers. Earlier results indicated that androgens in addition to estrogen may also serve to

protect the skeleton. Androgen deficiency in mice is associated with bone loss, increased production of IL-6 by bone marrow cells and increased osteoclastogenesis[33]. In support of this, orchidectomy of the IL-6 knockout mouse did not increase bone resorption[33]. Interestingly, severe osteoporosis was found in a young human male who had normal androgen levels but a mutant, nonfunctional estrogen receptor[122], indicating a role for estrogen in men. Estrogen has also been shown to prevent bone loss in men with prostate cancer who had been orchidectomized[123]. While it may be possible that androgens operate through the estrogen receptor (after conversion to estrogen by aromatase), other studies have shown that androgens can downregulate the expression of the IL-6 promoter through their own receptor and NF-κB (Ref. 124).

Paget's disease is a bone disorder characterized by increased bone resorption followed by abundant bone formation, the new bone formed being abnormal and structurally unsound (for review, see Ref. 125). While the osteoblasts are normal, the osteoclasts are abnormally large, containing as many as 100 nuclei per cell compared with the 3–10 nuclei found in healthy adult cells. Pagetic osteoclasts were found to contain viral inclusions and express paramyxoviral proteins[125,126]. *In vitro*, these osteoclasts expressed increased protein levels of IL-6 and mRNA levels of IL-6, IL-6 receptor and NF-IL6 (Refs 127,128). Serum levels of IL-6 were also elevated in patients with Paget's disease[128]. Therefore, along with osteoporosis, Paget's disease is another bone disease in which IL-6 plays a critical role.

Primary hyperparathyroidism is characterized by increased bone turnover and elevated serum levels of calcium, PTH and 1,25-dihydroxyvitamin D_3 (Ref. 129). Circulating levels of IL-6, soluble IL-6 receptor, and TNF-α were increased in these patients, and serum IL-6 levels correlated with biochemical indices of bone resorption and serum PTH levels[129]. PTH has been shown to stimulate the production of IL-6 and leukemia inhibitory factor in both *in vitro* and *in vivo* mouse models[22,127] via protein kinase A and the formation of cAMP (Ref. 130). Estrogen had no effect on PTH-stimulated production of IL-6 (Ref. 131). Interestingly, *in vitro*, anti-IL-6 receptor antibody only partially blocked PTH-stimulated bone resorption[132], suggesting that cytokines other than IL-6 might be involved.

Therapeutic intervention

Several approaches have been taken that have not only established the role of IL-6 in a disease but have also been considered as possible means by which the condition could be controlled. These include blocking or interfering with the action of IL-6 using antibodies against IL-6 itself or its receptor, small peptides capable of competing with IL-6 for binding to the receptor, antisense constructs to IL-6, and small molecules that interfere with the production of IL-6 (Figure 3). These examples are discussed below.

IL-6 receptor antagonists

Targeting the α-chain of the IL-6 receptor may offer a way of specifically inhibiting the effect of IL-6 activities. Further, such

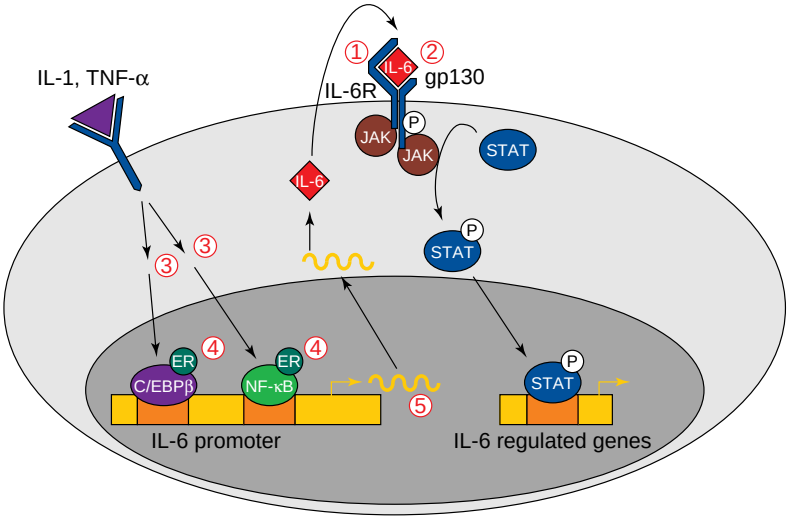

Figure 3. *Simplified scheme of interleukin 6 (IL-6) gene regulation and IL-6 signaling with site of action of potential therapeutic agents of IL-6 function. 1, IL-6 receptor (IL-6R) antagonist, IL-6R antibody; 2, anti-IL-6 antibody, minibody polypeptide; 3, signal transduction inhibitor; 4, estrogen mimic; 5, IL-6 antisense. IL-1, interleukin 1; TNF-α, tumor necrosis factor-α; JAK, Janus kinase; STAT, signal transducer and activator of transcription; ER, estrogen receptor; C/EBPβ, CCAAT-enhancer-binding protein β; NF-κB, nuclear factor.*

an approach would also cover the soluble IL-6 receptor, which has been shown to increase the sensitivity of cells to IL-6. Based on structural information available from human growth hormone, a computer model of the interaction of IL-6 with IL-6Rα and gp130 was constructed. Mutants of IL-6 were found that still associated with IL-6Rα but prevented the association with gp130 (Refs 133,134). These IL-6 receptor antagonists dissociate the IL-6Rα binding from the signal transduction. Further, construction of a bifacial mutant that increased the binding of IL-6 to IL-6Rα but still prevented the association with gp130, resulted in an IL-6 receptor superantagonist that blocked IL-6 action at very low dosages[134].

This IL-6 receptor superantagonist was tested in prostate carcinoma cell lines and was found to inhibit their growth as well as alter their resistance to certain anticancer chemotherapeutic agents[135]. The effects were comparable with those obtained with the neutralizing antibody to IL-6. The receptor antagonists and superantagonists were also successfully tested in multiple myeloma cell lines[136].

IL-6 receptor antibodies

Antibodies to the IL-6 receptor bind to the 80 kDa receptor subunit and probably alter its conformation, thus preventing the binding of IL-6. This approach has been used both *in vitro* and *in vivo*. Patients with rheumatoid arthritis exhibit destruction of the cartilage and bone in their joints. Anti-IL-6 receptor antibody blocked the formation of osteoclastic cells in co-cultures of murine osteoblasts and bone marrow cells treated with synovial fluid from arthritic patients[80]. Treatment of arthritic patients with anti-IL-6 receptor antibodies demonstrated rapid anti-inflammatory effects with symptomatic improvement[137]. Another example is human renal cell carcinomas, which lost their resistance to certain anticancer chemotherapeutic agents after they had been treated with anti-IL-6 receptor antibodies[102]. As expected from the stimulatory effect of PTH on IL-6 production, anti-IL-6 receptor antibodies reduced PTH-stimulated bone resorption in some patients that had elevated serum levels of PTH (Ref. 132).

Neutralizing antibodies to IL-6

Commercially available polyclonal antibodies to IL-6 act by sequestering extracellular levels of the cytokine, thus reducing the effective concentration of IL-6 in circulation. This approach has been used in both *in vitro* and *in vivo* rodent studies to establish the role of IL-6 in the pathogenesis of a disorder. For example, in AIDS, the anti-IL-6 antibody abolished the ability of IL-6 to induce HIV replication in B-cell cultures[62] and blocked the rise in serum IgG levels[85]. In murine models of hypercalcemia associated with renal cell carcinoma, the neutralizing antibody to IL-6 normalized serum calcium and PTHrP levels and inhibited tumor growth[104]. Human renal cell carcinomas also lost their resistance to certain anticancer chemotherapeutic agents after they had been treated with anti-IL-6 antibodies[102]. Similarly, the growth of human prostate carcinoma cell lines was inhibited, with the cells losing their resistance to certain anticancer chemotherapeutic agents[138]. Transgenic mice overexpressing IL-6 generally die from renal complications through mesangioproliferative glomerulonephritis. However, administration of the anti-IL-6 antibody suppressed plasmacytosis and reduced the extent of renal disease[93].

The neutralizing antibody to IL-6 has been used in the treatment of a human patient presenting with multiple myeloma. A two-month treatment with the antibody resulted in the blocking of the proliferation of the myeloma and reduction of serum IgG and acute phase CRP levels[139]. In a murine model of myeloma, the antibody to IL-6 also reduced the growth of the tumor and the bone resorption associated with the disorder[101].

In studies of relevance to osteoporosis, the neutralizing antibody to IL-6 reduced the number of osteoclasts and its precursor cells in ovariectomized mice but not in normal mice[26]. *In vitro*, the antibody to IL-6 blocked the TNF-α induced production of IL-6 in cultures of murine osteoblasts and suppressed the formation of osteoclasts in primary bone cell cultures[25]. Blocking the production of IL-6 in human osteoclast-like giant cells using an anti-IL-6 antibody decreased the ability of these cells to resorb bone[27]. IL-6 was confirmed to play a role in the generation of osteoclast-like cells in our human co-culture system as the addition of an anti-IL-6 antibody reduced the formation of these cells (S.K. Sutherland *et al.*, unpublished).

A major disadvantage of these protein-based drugs is that they cannot be administered orally, thus making the treatment more expensive.

Minibody polypeptide

Phage display technology has been used to identify peptides that bind to IL-6 and alter its biological activity. The isolation and characterization of such a peptide has been described[140]. The 7 kDa peptide binds specifically with micromolar affinity to IL-6 and prevents its interaction with IL-6Rα. The peptide blocked the induction of acute phase proteins in a

hepatoma cell line. A major challenge will be to generate biologically active non-peptide analogs of these peptides.

Antisense constructs

Expression of an RNA or DNA fragment that is complementary to the RNA of IL-6 results in the formation of unusual dimeric, or even trimeric, structures. These complexes are not only unable to be translated into protein but are also subject to rapid degradation. Therefore, antisense constructs are an effective way of specifically blocking the expression of a gene. The major drawback is that they have no effect on gene products already produced. Some gene products are so stable that antisense constructs are not effective. Further, antisense constructs are nucleotide-based molecules that are relatively unstable and require special formulation for delivery to be therapeutically effective.

In vitro, antisense constructs to IL-6 inhibited the proliferation of Kaposi sarcoma cells[141] and also inhibited the ability of osteoclast-like human giant cells to resorb bone[117]. Antisense constructs to the IL-6 receptor with chemical modifications to increase their stability have been suggested for use as therapeutics.

Estrogen and estrogen mimics

Estrogen blocks the production of IL-6 at the transcriptional level through an estrogen-receptor-dependent mechanism[116] (see section on osteoporosis). Hormone replacement therapy (HRT), consisting of conjugated equine estrogens alone or combined with progestogens for nonhysterectomized women, is regarded as a preventative treatment for postmenopausal osteoporosis. The short-term effect of HRT on bone mass has been well studied. In general, a 5–7% increase in spinal bone mass occurs within one year after starting therapy and plateaus within two to three years[31]. Very few long-term studies have been done. One recent study showed that vertebral bone mineral density in women treated with HRT was 14.5% greater than those not receiving therapy; the greatest effect observed was on vertebral bone[113]. Other benefits of HRT include reduced risk for myocardial infarction and possible protective effects against neurodegenerative diseases such as Alzheimer's disease. Side effects of HRT include increased risk of endometrial and breast cancer and cyclic or irregular bleeding, depending on the regimen[142] – these are the major reasons for the low acceptance of this kind of treatment. HRT is typically administered orally, but trials are under way in which estrogen alone or in combination with progesterone is available in patch form.

Raloxifene belongs to a rapidly growing family of new anti-estrogens that include droloxifene and idoxifene. They exhibit high affinities for the estrogen receptor and combine some of the beneficial effects of estrogen on bone with lower estrogenic actions on reproductive tissues[143,144]. Raloxifene and droloxifene decreased the rate of bone loss and serum cholesterol associated with ovariectomy in laboratory rats while exhibiting minor effects on the uterus[143,144]. In a pilot study in healthy postmenopausal women, raloxifene decreased the expression of bone turnover markers and serum cholesterol levels, but appeared to increase the incidence of vasodilatation[143]. *In vitro*, raloxifene at high doses reduced the release of IL-6 by cultures of human preosteoclastic cells[22]. We have found that raloxifene inhibited the production of IL-6 in a dose-dependent manner in cultures of human osteoblasts alone and in co-cultures of osteoblasts and preosteoclastic cells (unpublished data).

Androgens have also been shown to exert protective effects on the skeleton (see section on osteoporosis) but are not currently used as therapeutics for the treatment of osteoporosis.

Small-molecule inhibitors

The ideal inhibitor of IL-6 is a small molecule that is orally active and stable in the human body. Furthermore, the drug should be specific for IL-6, specific for the disease tissue and exhibit no side effects. Signal Pharmaceuticals is developing two classes of compounds that block IL-6 gene expression. These compounds are small molecules that interfere with the signaling cascade leading to IL-6 gene expression. One class blocks IL-6 gene expression through the estrogen receptor utilizing the novel, nonclassical estrogen mechanism. These compounds are targeted for the treatment of osteoporosis. The other series works independently of the estrogen receptor and is of particular interest for treatment of IL-6 dependent diseases, such as multiple myeloma and cancer. Both classes of compounds have demonstrated efficacy and safety, both *in vitro* and in a human bone model system preventing bone resorption, without generating undesired estrogen responses. Proof-of-principle studies in a rat ovariectomized bone resorption model have also demonstrated the *in vivo* efficacy of these compound classes.

Summary

Studies with the IL-6 receptor antibody and antagonist as well as the IL-6 neutralizing antibody correlate quite well

and serve as proof-of-principle that blocking IL-6 may beneficially affect certain diseases. The widespread use of the peptide-based drugs in the clinic is still questionable. While antisense constructs and estrogen or estrogen mimics have been shown to block the production of IL-6, the former compounds must be further modified to improve pharmacokinetics and the latter compounds exhibit undesirable side effects (hot flushes, increased incidence of cancer).

Conclusion

IL-6 has widespread biological activities and inappropriate expression of this cytokine results in profound pathophysiological effects. Many approaches have been used to confirm the role of IL-6 in cell function or pathophysiological disorder, including the use of a neutralizing antibody to IL-6, antisense constructs, antibody to the IL-6 receptor, estrogen or estrogen mimics, minibody polypeptides and small-molecule inhibitors. These approaches have yielded not only valuable basic research tools but also therapeutic agents. Early clinical data and data from animal models clearly suggest that IL-6 is a validated drug discovery target. The challenge is to develop therapeutics that are easily delivered to the target site and retain biological activity with minimal side effects. In particular, small-molecule inhibitors acting on novel molecular targets may ultimately provide hope for the more-effective treatment of IL-6-dependent diseases.

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