HENRIK HJORTH-HANSEN

NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA

Publication from The Norwegian University of Science and Technology, Faculty of Medicine, Institute of Cancer Research and Molecular Biology, N-7489 Trondheim, Norway.

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ABBREVIATIONS

MGUS	monoclonal gammopathy of undetermined significance
MM	multiple myeloma
HGF	hepatocyte growth factor
SF	scatter factor
IL	interleukin
Ig	immunoglobulin
mAb	monoclonal antibody
BMP	bone morphogenetic protein
BMU	basic multicellular unit
Oc	osteoclast
Ob	osteoblast
TNF	tumour necrosis factor
MMP	matrix matalloprotease
PTHrP	parathyroid hormone-related peptide
IGF-1	insulin-like growth factor-1
TRANCE	TNF-related activation-induced cytokine
OPG	osteoprotegerin
RANK	receptor activator of NF-κB
RANKL	RANK ligand
VEGF	vascular endothelial growth factor
FGF	fibroblast growth factor
SDF-1	stromal cell-derived factor-1
RT-PCR	reverse transcription polymerase chain reaction
SCID	severe combined immunodeficiency
NOD/SCID	non-obese diabetic SCID
ADCC	antibody-dependent cellular cytotoxicity
JAK	Janus kinase
MAP kinase	mitogen-activated protein kinase
STAT	signal transducer and activator of transcription

MULTIPLE MYELOMA: DISEASE CHARACTERISTICS, CLINICAL MANAGEMENT, EPIDEMIOLOGY AND ONCOGENESIS

1.1 HISTORICAL OVERVIEW, CLINICAL FEATURES AND EPIDEMIOLOGY

Multiple myeloma (MM) is a disease characterised by the accumulation of slowly dividing, terminally differentiated malignant plasma cells in the bone marrow compartment. The condition was partially described already in the 1840s with the detection of abnormal proteinuria and softenings of the bone (mollities ossium), documented in the review on the history of MM by Kyle.[1] In more than 99% of patients these malignant plasma cells secrete a single molecule of immunoglobulin (Ig) of IgG, IgA, IgD or IgE classes (very rarely IgM). By serum or urine electrophoresis a single spike monoclonal component is detectable, which represents the entire Ig or the Ig light chain.[2;3] Secretion of light chains in the urine was first described in 1845 and was analysed by Sir Hugh Bence Jones and his colleague William McIntyre, hence the name ”Bence Jones protein” [*].[4] Light chains were divided into two separate classes according to their chemical properties and were named κ and λ in reference to the surnames of the discoverers Korngold and Lipari.[5] Detection of the monoclonal component by electrophoresis provided medicine with its first tumour marker from biological fluids and provided a strong indication that cancer arises from a single cell, i.e. monoclonality.

Clinically, multiple myeloma frequently presents with anemia, lethargy, infections, kidney failure, bone pain, hypercalcemia or fractures.[6] Symptoms may rarely arise from hyperviscosity or amyloidosis. In a Norwegian screening programme, however, 44 % of patients diagnosed with MM were asymptomatic, but from the onset of symptoms and need for treatment, no difference in survival was seen between asymptomatic and symptomatic patients.[7] The main staging system for MM was developed by Durie and Salmon in 1975 and relies on the radiographic extent of bone disease, Ig levels in serum and urine, and serum levels of hemoglobin, calcium, and creatinine.[8] Although the course of disease in individual patients is variable, the prognosis of MM patients as a group is dismal with a median survival of

[*] In his account of the case Bence Jones wrote: ”January 2nd. -The patient died. The following day I saw that the bony structure of the ribs were cut with the greatest ease and that the bodies of the

approximately three years from diagnosis on standard therapy.[9;10] Despite considerable research effort the condition still is incurable. Already in 1958 the first report on the efficacy of alkylating agents in MM was published.[11;12] In the late 1960s the combination regimen of the alkylating agent melphalan and the glucocorticoid prednisone was introduced.[11;13] This regimen increased survival from 12-17 months to 24-39 months. In later years, with the advent of stem cell supported high dose chemotherapy an additional improvement of the prognosis was found in three separate studies.[14-16] Thalidomide, an inhibitor of angiogenesis, has been shown to induce remissions and to prolong survival in a proportion of heavily pre-treated patients.[17]

The incidence of MM increases steadily with age, and is extremely rare before the age of 40.[6;18;19] The age-adjusted incidence of MM has been unchanged over a span of 45 years of follow up in Minnesota, USA, but in Sweden an increase was observed among men from the 1950s to the 1970s. The age-adjusted incidence in Sweden is 3.2/100.000 inhabitants and in Minnesota 4.1/100.000, in both countries the male/female ratio is 2:1. There is a difference in the incidence of MM between different populations, exemplified by the high incidence found in the black population of the USA and the low incidence found among the Japanese and Chinese.[20] Interestingly, among the Chinese and Japanese immigrants to the USA the incidence of MM remains low in the new country. This indicates that observed differences in incidence of MM stem from genetic differences between the populations rather than environmental factors.

1.2. GENETIC ABERRATIONS IN THE ONCOGENESIS OF MM

Antigenic stimulation of B cells leads to generation of either memory B cells or plasmablasts in germinal centres of lymph nodes. Both types of cells display hypermutation of the variable regions of the Ig gene, and plasmablasts undergo isotype switching and migrate to the bone marrow where they survive for about 30 days.[21-23] In multiple myeloma, malignant plasma cells have a prolonged life span as compared to normal plasma cells, and in general proliferate very slowly with a plasma cell labelling index rarely higher than 2% except in advanced disease. There is also evidence for considerable loss of tumour cells in vivo.[24;25] The oncogenesis of MM is best understood as a multistep transformation process.[21] Monoclonal gammopathy of

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vertebrae were capable of being sliced off with the knife. The pericardium contained an ounce or two of fluid……. The kidneys were healthy to the naked eye and to the microscope."

undetermined significance (MGUS), which designates the finding of a small monoclonal component in serum in the absence of criteria for MM, represents the earliest recognisable step in myelomagenesis. MGUS is frequent in the population, with a prevalence of about 1% in 50-year-olds and 3% in 70-year-olds. It progresses over decades, and after 25 years of follow-up 40% of MGUS patients had developed MM or other lymphoproliferative disease.[26] Examined by sensitive fluorescence in situ hybridization (FISH) techniques, about 50% of MGUS patients display karyotypic abnormalities, and nearly 100% of MM patients.[27-29] The genes that are dysregulated due to these chromosomal abnormalities are likely to explain biological behaviour of myeloma cells, and some translocations involve the Ig locus. The Ig isotype-switching is likely a perilous event in the evolution of a plasma cell, in similarity with abnormalities in the process of Ig VDJ recombination, which is found in B cell lymphomas. Translocation and overexpression has been described in MM cell lines for cyclin D1 (cell cycle protein), c-maf (proto-oncogene), interferon regulatory factor –4 (transcription factor), fibroblast growth factor receptor-3, the MM-SET gene (putative transcription factor) and *myeov* (unknown function).[21;30] Some of these translocations have also been detected in original tumour material from patients and in MGUS, which shows that they occur early in MM.[21] Furthermore, deletion of parts of chromosome 11 or chromosome 13 as well as the existence of any translocation, is associated with an adverse prognosis.[31] Bi-allelic loss of the retinoblastoma gene, which is located on chromosome 13, is however rare in MM.[32] Loss of the p53 tumour suppressor gene has been found in late stage MM and indicates an adverse prognosis.[33] Activating mutations of *ras* are frequently found in terminal disease and extramedullary myeloma, but are seldom found in the early stages of disease or MGUS.[21]

Our present knowledge of the nature of genetic disturbances, their functional consequences and the time perspective of early and late genetic events is fragmentary. I have high hopes for the future application of novel techniques based on molecular biology (i.e. fluorescent in situ hybridization, microarray and differential display techniques) and advanced protein separation (proteinomics). I believe application of these techniques will dramatically contribute to our understanding of the genetic and functional disturbances in myeloma.

1.3 INTERACTIONS BETWEEN STROMA AND MYELOMA CELLS

Myeloma cell adhesion to stromal cells and extracellular matrix proteins is important for cell survival and is specific for the bone marrow microenvironment. Myeloma cells seem dependent on a supportive microenvironment as exemplified by the growth of human myeloma cells only in the human bone implant and not in mouse bone marrow in the SCID-hu mouse model (see section 3).[34;35] IL-6 is held to be of prime importance in survival and growth of myeloma cells, and adhesion of myeloma cells to stromal cells induces stromal IL-6 secretion and increases the sensitivity of myeloma cells to IL-6.[21;36-45] Stromal adhesion also inhibits myeloma cell apoptosis partially by IL-6-related mechanisms.[40;45] Adhesion of myeloma cells to stromal components also induces drug resistance.[46].

In a study using IL-6 independent myeloma cell lines seeded on normal or myeloma stroma, a 80-90% inhibition of DNA synthesis was observed on normal stroma, but not on myeloma stroma.[39] In the same study, adhered IL-6-dependent and highly sensitive B9 cells showed a meagre three-fold increase in DNA synthesis in spite of high concentration of IL-6 in the media. This demonstrates that although IL-6 secretion may be augmented by myeloma cell adhesion, the stromal cells also harbour growth inhibitory properties, which may be lost in tumour progression. The nature of such inhibitory mechanisms are unknown

Myeloma cell adhesion is mediated by adhesion molecules, such as the integrins LFA-1, VLA-4 and VLA-5, by ICAM-1, CD56 (NCAM), CD44 (hyaluronan receptor), CD58, CD138 (syndecan-1) and Ku antigen. [47-50] The counterparts of these molecules, such as fibronectin, laminin, hyaluronan, collagen, and VCAM-1 are all present in bone marrow microenvironment.[36;47;48] Blocking experiments with antibodies do, however, fail to abrogate more than half of this binding, indicating that other molecules significantly contribute to stromal adhesion of myeloma cells.[47-49]. In a recent report, murine myeloma cells adhered to VCAM-1 through VLA-4 produced high concentrations of an osteoclast activating factor (OAF), which was not the case in the non-adhered state[51]. These observations demonstrate an alteration of the biological behaviour of myeloma cells when adhered to stroma, and experiments to describe the myeloma cell in this state should be performed.

Chemotaxis of myeloma cells in response to stromal cell-derived factor (SDF-1) and insulin-like growth factor (IGF)-1 has been demonstrated. [52;53] These studies

support the notion that myeloma cells may be attracted to the bone marrow by an active process, chemotaxis, and not only seed in favourable soil. This may help to explain why myeloma cells spread to skeletal sites and seldom elsewhere. Motile myeloma cells have a polarised morphology with cellular protrusions called uropods. Myeloma cells adhere to stromal cells by their uropods and uropods sequester adhesion molecules and growth factors. This has potential bearing on presentation and function of growth factors in myeloma.[54]

1.4 ANGIOGENESIS

Angiogenesis is a prerequisite for growth of solid tumours beyond the size of approximately 2mm.[55] Blockage of angiogenesis offers possibilities to inhibit tumour growth by a general mechanism common to all malignant tumours. Experiments have demonstrated that angiogenesis inhibitors cause involution of tumours in mice. Treatments could be repeated, showed no signs of drug resistance, and induced tumour dormancy.[56] Angiogenesis is not only increased in solid tumours, but also in hematological malignancies including in myeloma.[57;58] Increased angiogenesis has been demonstrated in active myeloma, and the microvessel area correlated excellently with the plasma cell labelling index.[57] One possible mediator of angiogenesis in MM is fibroblast growth factor (FGF)-2.[59] Thalidomide is efficacious in advanced MM and inhibits angiogenesis.[17] Clearly, inhibition of angiogenesis has therapeutic potential in MM.

1.5 MYELOMA FOUNDER CELLS: WHICH CELLS CONSTITUTE THE PROLIFERATIVE POOL

This is one of the most controversial issues in myeloma biology. Myeloma cells are derived from cells that have been stimulated by antigen, since hypermutation of the Ig heavy chain without intraclonal variation has been demonstrated[22;23]. There exists a pool of CD19+ clonotypic preswitched B lymphocytes in peripheral blood of myeloma patients, but the size of this fraction is a matter of debate.[60-63] As plasma cells themselves have a low proliferation rate except in advanced stages of disease, the proliferative compartment of the myeloma clone has been postulated to be found among these CD19+ B lymphocytes. Such CD19+ clonotypic cells have a drug resistant phenotype and hyperploidy of DNA.[64] The controversy concerns the self renewal capacity of these CD19+ B lymphocytes. One view advocates that they are

important whereas the other view holds that only myeloma cells have a self-renewal capacity, and that pre-switch B lymphocytes are non-essential for propagation of the clone. If so, the main oncogenic events should happen during and after switching of the Ig heavy chain locus. The latter theory may be supported by the fact that proliferative IL-6 responsive myeloma cells are found predominantly in the immature plasma cell fraction.[40;65] Moreover, given that myeloma cell lines are examples of the proliferating pool in myeloma, their phenotype is plasma cell and not B cell.[66]

With the advent of myeloma mouse models (see section 3) the question of tumour renewal capacity in different subpopulations of the myeloma clone may be addressed experimentally. Severe combined immunodeficiency (SCID) mice carrying human fetal bone implants (SCID-hu mice) were injected intraosseously with primary CD38+CD45-myeloma cells or plasma cell depleted bone marrow or peripheral blood.[67] Only plasma cells gave myeloma in these hosts, and solely in human bone. In contrast, Pilarski et al demonstrated myeloma growth in mouse bone marrow of NOD/SCID mice injected intracardially or intraosseously with peripheral blood from patients with aggressive myeloma.[68] Osteolytic lesions also developed in a high proportion of mice injected with G-CSF-mobilized blood from patients with minimal disease. There were virtually no mature myeloma cells in these apheresis products. In spite of a lack of histological or flow cytometrical evidence for engraftment of human cells radiolucencies of bone developed. Since it is not excluded that radiolucencies may develop in NOD/SCID hosts engrafted with normal blood, unfortunately no firm conclusion can be based on this interesting experiment. Inoculation of phenotypically specified subpopulations is necessary to clarify the issue of myeloma founder cells. To conclude, the self-renewing and tumorigenic capacity of plasma cells in immunodeficient mice has been demonstrated, whereas the capacity of clonotypic B cells as founder cells for myeloma still is an open question

1.6 MORPHOLOGICAL AND PATHOPHYSIOLOGICAL ASPECTS OF MYELOMA BONE DISEASE

1.6.1.Normal bone remodelling

Much of the knowledge about the nature of the remodelling process is derived from morphometric examinations of bone, i.e. bone histomorphometry.[69;70] Normal bone undergoes remodelling to replace old, redundant or brittle bone continuously

throughout life in a tightly regulated, balanced process.[71] The basic multicellular unit (BMU), which consists of osteoclasts (Oc), osteoblasts (Ob), their precursors, matrix, vascular supply and nerves, executes this task. Oc resorb bone in the front of the BMU and Ob fill the resorbed defect with unmineralized matrix (osteoid) which is subsequently mineralised. The bone remodelling cycle is probably initiated by osteocytes, which are embedded in the bone canaliculi. They form a network throughout the bone connected by gap junctions. These cells are thought to continuously inhibit the bone remodelling cycle, but when the osteocyte network is interrupted, e.g. by a microfracture, inhibition will be lost and the remodelling ensues.[72] Pre-osteoblastic cells prepare a cleansed surface for myeloid cell-derived Oc precursor cells by use of proteases like matrix metalloproteases (MMP). Subsequently Oc precursors fuse with one another to form multinuclear mature Oc. These cells degrade bone with different enzymes (MMP) and hydrochloric acid, which is generated by an extremely efficient proton pump. Once committed, Oc have a life span of approximately 16 days and subsequently apoptose, which means that during the resorptive process there is continuous need for recruitment of new Oc to the BMU. [71] Differentiating and chemotactic substances from the resorbing Oc stimulate the generation of synthetically active Ob. Ob may adopt several cell fates after they have covered the resorptive pit. They may be engulfed in bone substance to become quiescent osteocytes, lining cells, or apoptose.[73]

Generally, the pit generated by the resorbing Oc is filled with an equal amount of bone substance, i.e. the osteoclastic resorption is coupled with osteoblastic formation of new bone. In states of disease, bone formation may be inadequate to fill resorbed bone defects, i.e. the bone remodelling cycle is uncoupled.

1.6.2 Bone remodelling in multiple myeloma

In 1974, Mundy found increased numbers of Oc in bone biopsies from MM patients and a soluble osteoclast-activating factor (OAF) in supernatants from bone marrow cultures (see section 2).[74] Bone histomorphometric examination of MM patients showed that the disease is characterised by an increase in Oc numbers and resorptive activity, notably in the vicinity of myeloma cells.[75] In this study the tetracycline-labelled and osteoid surfaces frequently were increased, but the thickness of osteoid and the calcification rate were reduced, suggesting that each individual Ob had decreased activity. In a study of patients with and without osteolytic lesions, both

forms of disease had equally increased bone resorption. Patients with lytic disease had clear decrease in Ob indices, as opposed to patients with non-lytic disease.[76] In early MM increased osteoblastic activity is found, mainly caused by recruitment of new Ob. In overt disease, however, there is decreased Ob activity caused by a combined effect of decreased recruitment and synthetic activity of the individual Ob.[77] In conclusion, histomorphometric data show that MM is characterised by a uniformly increased bone resorption, and that in the earlier stages of disease the bone formative capability compensates bone loss, but in later stages of disease uncoupling of the bone remodelling process occurs.

2. CYTOKINES INVOLVED IN GROWTH CONTROL AND BONE DISEASE IN MULTIPLE MYELOMA

A summary of important data on cytokines relevant to myeloma growth control and bone disease is given in Tables I and II (end of Chapter 2). My interpretation of these data is presented in the columns denoted “Importance in MM”.

2.1 IL-6

The myeloma-stimulating effect of IL-6 was discovered in the late 1980s.[78;79] IL-6 has been shown to be the main growth and survival factor of myeloma cells in vitro and possibly also in vivo. The transmembrane signal of IL-6 IL-11, cardiotropin-1, ciliary neurotropic factor, leukemia inhibitory factor and oncostatin M is transduced by the gp130 protein, and these proteins are hence named the gp130 superfamily. IL-6 first binds to its specific α-receptor subunit (gp80) and the complex of IL-6 and IL-6R in turn recruits gp130. The signal from gp130 involves two downstream pathways: the Ras-dependent mitogen-activated protein (MAP) kinase pathway and the Janus kinases/signal transducer and activator of transcription (JAK/STAT) pathway.[21;80] Activation of MAP kinase has been demonstrated to be important for proliferation, whereas activation of JAK/STAT has been implicated both in proliferation and prevention of apoptosis. IL-6 stimulation of myeloma cells leads to tyrosine phosphorylation of JAK1, JAK2 and Tyk kinases, which in turn leads to tyrosine phosphorylation of STAT1 and STAT3 proteins. Constitutive phosphorylation of STAT3 has been demonstrated in primary myeloma samples, and could be reversed by incubation with the IL-6R antagonist Sant7.[81]

IL-6 seems to be essential in plasma cell biology as shown by the lack of inducibility of plasmocytoma in IL-6 knockout mice.[82] Furthermore, transgenic overexpression of IL-6 gives either polyclonal plasmocytosis or plasmocytomas.[83] Double overexpression of IL-6R and IL-6 leads to rapid plasmocytoma growth.[84] Because there is a strain-dependent variability in plasmocytomagenesis by IL-6 overexpression or pristane oil injection, IL-6 seems to be a necessary but not sufficient factor for plasmocytomagenesis in mice. A role for T cells in the development of plasma cells is likely, since nude mice injected with a vector harbouring viral transforming oncogenes developed lymphomas, whereas normal mice developed plasmocytomas.[85] In human myeloma, cellular responsiveness to IL-6

is correlated with aggressive disease and the plasma labelling index.[86] IL-6 responsiveness seems to be a property of immature plasma cells, since more differentiated cells do not proliferate in response to IL-6.[65;87] IL-6-dependent cell lines may be regularly obtained from patients with extramedullary disease in the presence of GM-CSF, which increases cellular sensitivity to IL-6.[88]

Concluding remarks on the importance of IL-6 in the growth control of myeloma is given in section 6.3. In relation to bone disease, IL-6 appears to be of relatively little importance. It is a weak Oc activator but may play a role in the coupling of remodelling by also stimulating Ob formation and differentiation.

2.2. IL-11

Interleukin-11 is a gp130 family cytokine. The main interest in IL-11 has been in the field of thrombocytopoiesis, and the Federal Drug Agency has approved IL-11 for treatment of thrombocytopenia.[89] IL-11 is also involved in other lineages of hematopoiesis. IL-11 has potent anti-inflammatory properties in vitro and in vivo by decreasing production of pro-inflammatory cytokines like IL-1, IL-6, IL-12 and TNF-α.[89] In animal models, IL-11 is efficacious in the treatment of graft versus host disease, arthritis, inflammatory bowel disease and inflammatory skin diseases. In myeloma, IL-11 has a proliferative effect in selected cell lines either directly or after induction of IL-11 responsiveness by IL-10.[90-93] The potency of IL-11 is however unimpressive, and data from patients show little IL-11 responsiveness in primary samples. Relatively low levels of IL-11 in serum and lack of IL-11 production in bone marrow culture supernatants adds to the picture that IL-11 is probably not an important cytokine in myeloma cell proliferation.[94;95]

IL-11 seems to have a specific role in bone metabolism, as it is a product of osteoblasts and may increase osteoclastogenesis.[96;97] In analogy with IL-6, IL-11-induced osteoclastogenesis is mediated through Ob-like cells. Furthermore, also IL-1, TNF-α, PTH and vitamin D3 may induce the production of IL-11 and antibody to gp130 or IL-11R blocked the osteoclastogenic effects of these substances, suggesting IL-11 as the common mediator.[96-98] Our finding that myeloma-derived HGF induces IL-11 production in osteoblasts is in analogy with the above mentioned findings.[99] IL-11 effects are in part mediated via prostanoids, since indomethacin partially blocks its effect, but also through RANK ligand which is upregulated in IL-11-stimulated

Ob.[96;100;101] IL-11 may also act as a differentiating agent of Ob.[102] Its role in MM bone disease is unclear.

2.3 Hepatocyte growth factor (HGF)

HGF is a pluripotent cytokine, and may act as a mitogen, motogen, and morhogen.[103] HGF has been implicated in diverse biological processes such as tissue repair after damage of parenchymatous organs, in hematopoiesis and in the formation of the embryo.[104-110] HGF was discovered as a potent stimulant of hepatocyte growth after hepatectomy and was isolated from rat thrombocytes in 1986.[111] Another group found a factor, which disrupted epithelial junctions and induced scattering, which they termed scatter factor (SF).[112] Its genetic sequence was characterised in 1989, and the identity of HGF and SF was confirmed in 1991.[113;114] HGF is homologous with plasminogen, and contains four kringle domains, bridged by several disulfide bonds, which makes the molecule remarkably stable. Its high affinity receptor is c-met, a protein tyrosine kinase type receptor. Heparin and heparan sulphates bind HGF with approximately 1 log lower affinity than c-met.[115;116] A heparan sulphate-containing proteoglycan, syndecan-1 (CD138), which is specifically expressed on myeloma cells, also binds HGF.[117] Cell surface binding of HGF to proteoglycans may modulate its function.[99;117-119]

HGF has been implicated in tumour biology both as a growth modulator in itself or as an inductor of growth factors.[120;121] In the process of metastasis, HGF may induce cancer cell motility (scattering), be involved in the degradation of matrix by induction of proteolytic enzymes, may induce angiogenesis and affect adhesion and homing of tumour cells.[121-126] Elevated HGF levels in serum has been demonstrated in several malignancies as exemplified by our study in acute myelocytic leukemia.[127]

HGF production in myeloma cells was discovered in 1993 as an inhibitor of transforming growth factor (TGF) -β in a mink lung cell bioassay.[128] HGF and its receptor c-met were co-expressed in myeloma cell lines.[129] An autocrine circuit was postulated, since tyrosine phosphorylation of c-met could be regulated by exogenous HGF or HGF antiserum.[129] Production of HGF and expression of c-met was also found in primary cells, and elevated levels of HGF were found in serum of myeloma patients (Paper1). HGF was significantly elevated in a large MM patient material and correlated with the course of disease and prognosis.[130] Its association to other

biological important parameters was weak, but the highest correlation was found with collagen degradation products. HGF concentrations in bone marrow serum are 4-fold higher than in serum from peripheral blood, which indicates that HGF is produced within the bone marrow compartment.[117;131]

Of relevance to bone, the HGF receptor c-met is expressed both on Ob and Oc.[132] HGF induced DNA synthesis in both human Oc and Ob, and HGF production was detected in Oc-like cells. Therefore a role for HGF as a coupling factor in bone remodelling was postulated.[132] In co-culture system of hematopoietic blast cells and the HGF-producing stromal cell line (MC3T3-G2/PA6), HGF blockage abolished increase in Oc numbers and bone resorption.[133] In a rat co-culture system HGF weakly promoted Oc bone resorption.[134] HGF also stimulates formation and proliferation of Oc precursors, CFU-GM.[135] Altogether, these studies indicate a role of HGF in Oc formation, but it seems in itself to be insufficient to mediate resorption. Possibly, HGF may induce resorption by induction of stroma-derived factors like IL-11 or the RANKL system.[99]

2.4 IL-15

IL-15 is a member of the helix-bundle-helix cytokine superfamily. It is structurally similar to IL-2 and shares many of its effects, like the induction of T cell-mediated cytotoxicity and natural killer (NK) cell activation.[136;137] IL-2 and IL-15 also block apoptosis in both these cell types and both have been implicated as therapeutic agents in cancer, since they may augment the immunologic response to tumours.[137;138] Both cytokines stimulate B lymphocyte proliferation and Ig synthesis, and primary samples from patients with B cell and NK malignancies are frequently responsive to IL-15.[139-142] IL-15 has been implicated in the generation of dendritic cells and intraepithelial lymphocytes.[143] The three dimensional structures of IL-15 and IL-2 are similar, but the amino acid and DNA sequences are not homologous.[136;144] The IL-15 and IL-2 receptors share gamma and beta subunits, but each cytokine receptor has its unique alpha subunit.[136;145] Mice deficient in IL-15 and IL-15Rα lack development of NK cells and intraepithelial γδ-cells.[146;147] Furthermore, animals display lymphopenia, particularly in the CD8+ subset and defective lymphocyte homing to lymph nodes. IL-15 induces a motile phenotype in T lymphocytes.[148] IL-15 transgenic animals have a normal life span, and display an expansion of memory type T lymphocytes.[149] IL-15

stimulates the formation of Oc in vitro in a manner distinct from IL-2, has been implicated in bone loss associated with rheumatoid arthritis, and angiogenesis.[150-152]

How IL-15 works is still enigmatic, since IL-15 protein seldom is detectable in supernatants, in spite of the existence of IL-15 mRNA transcripts in many cell types. In contrast, IL-2 expression is confined to the immune system and is readily secreted, suggesting distinctive biological roles of these cytokines.[153] IL-15 is located both in the Golgi apparatus, cytoplasma, cell surface and nucleus, indicating an intracellular, still obscure role for IL-15.[153;154] IL-15 secretion is hampered both by the existence of multiple stop codons in the downstream sequence, regulatory elements upstream and the existence of two unusual signal peptides which both are relatively inefficiently sectreted.[153]Possibly IL-15 plays a role in a state of cell-cell contact since IL-15 has a "juxtacrine" effect in dense melanoma cell cultures. This effect could be blocked with IL-15 antibody in spite of undetectable IL-15 in supernatants.[154;155] A membrane-bound form of IL-15 has been demonstarted in macrophages.[156] An autocrine anti-apoptotic effect has also been demonstrated in myeloma cells.[157]

2.5 Bone morphogenetic proteins (BMP)

To date, more than fifteen different BMP proteins have been identified.[158;159] A bone-inducing activity from demineralized bone implanted subcutaneously in rats was described by Urist in 1965[160] A protein which induced bone formation in vivo was purified and cloned in 1988.[161;162] BMP-2 through -7 induce bone formation in extraskeletal sites including the formation of a bone marrow cavity with hematopoiesis.[160;163] Other BMPs serve other functions, like the formation of tendons and ligaments (BMP-12, -13 and –14).[164] BMPs are important in fracture repair, a process that recapitulates embryonic enchondral bone formation[158;165]. Carrier-bound BMP may bridge large bone defects and be used clinically in the future.[158] BMP-2 also activates Oc.[166] BMPs are essential in embryological development by inducing apoptosis, migration, proliferation and differentiation of different cell types.[167-170] BMPs may act as cofactors in erythropoiesis and BMP-2 maintains the stem cell phenotype of CD34+ cells.[171;172] One report shows inhibition of proliferation and induction of apoptosis by BMP-2 in murine HS72 hybridoma cells[173]

The TGF-β superfamily consists of TGF-β1 through 5, activins, inhibins, Müllerian inhibitory factor, bone morphogenetic proteins (BMP) and growth and

differentiation factors (GDF). These cytokines exert diverse and essential biological functions by controlling cellular proliferation, apoptosis, motility and differentiation in many organ systems. Proteins of the TGF-β superfamily utilize type I (ALK-1 through -6) and type II receptors (TGF-βRII, ActivinRII, ActivinRIIB and BMPR II) for their signalling.[158;159] The utilization of these receptors is promiscuous and complex, since several combinations of receptor subunits may transduce the signal from one individual cytokine and receptor-bound cytokines may or may not elicit transmembrane signalling. Cytokines bind type II receptors initially and recruit type I receptors to form tetrameric receptor complexes. The type I receptor is the transmembrane signalling unit with serine/threonine kinase activity.[174;175] BMP activities may also be enhanced by the formation of heterodimers of BMP molecules.[176] Downstream, Smad transcription factors are phosphorylated and activated in a cytokine-restricted manner, since TGF-β and activins signal through Smad-2 and -3, whereas BMPs use Smad-1, -5 and –9. These Smads recruit the common mediator Smad4 to form a heterodimeric complex and are shuttled to the nucleus. Transcription is further regulated by the joining of the pathway-restricted Smad/Smad4 complex associating with modulator proteins like FAST-1 and-2. Smads-6 and –7 inhibit Smad signalling. Finally, BMP may be inhibited through extracellular binding by noggin, chordin and gremlin proteins.[167]

BMPs and activins are secreted in their active forms.[163;177] In contrast, TGF-β is secreted in its latent form and consequently needs to be activated by acidification or by proteolytic cleavage by the plasminogen system.[178] TGF-β superfamily proteins commonly are deposited in extracellular matrices like in bone, bound to different matrix proteins. TGF-β superfamily proteins also bind proteoglycans such as heparan sulphates either matrix-bound or on the cell surface. Such surface binding modulates the local concentration and high-affinity receptor binding of growth factor and hence its activity.[179] To summarize, regulation of the proteins of the TGF superfamily takes place at several levels, indicating that this system is strictly organized and controlled.

2.6 RANKL/RANK/OPG

The RANKL/RANK/OPG system was recently discovered as a fundamental mechanism in osteoclastogenesis.[180] RANKL (also called TRANCE) is a protein in the TNF superfamily, which signals through the RANK receptor. Osteoprotegerin

(OPG) functions as a soluble decoy receptor, which potently inhibits effects of RANKL. RANKL and RANK is expressed on Ob and Oc respectively. Cell-cell contact between stromal cells and Oc or its precursors has been shown to be necessary for OAF-induced Oc formation and activation. Addition of M-CSF and RANKL to culture media abolishes the need for stromal help in osteoclastogenesis, implying a pivotal role for these cytokines. Mice engineered to lack or overexpress RANKL, RANK or OPG have intriguing phenotypes. RANKL knockout mice demonstrate deficiencies in early development of B and T lymphocytes, disturbed lymph node architecture in addition to severe osteopetrosis accompanied by a lack of osteoclasts.[181] The phenotype of RANKL and RANK knockout mice was strikingly similar.[182] OPG knockout mice demonstrate extreme osteopenia and arterial calcification, whereas OPG overexpressing mice demonstrate non-lethal osteopetrosis.[183] M-CSF knockout mice (op/op mutation) also demonstrate osteopetrosis. M-CSF is essential for the formation of Oc by promoting survival and proliferation of precursors, and by maintaining their potential for differentiation into Oc, in part by upregulation of RANK.[184] RANKL is important for Oc terminal differentiation, fusion and resorption.[184;185] The timing of these factors for osteoclastogenesis is crucial. Mice deficient in downstream signalling molecules of RANK, such as TRAF6 and double deficient mice for NF-κB subunits p50 and p52, also demonstrate severe osteopetrosis.[186-188]

Almost all previously identified OAFs activate the RANKL system, indicating this system as a final common mediator for osteoclastogenesis, although IL-1 and TNF-α may also activate Oc independently.[101;180;185;189-193] Many of these factors, including PTH, IL-11, and IL-6, in part or entirely exert their effects through stromal cell synthesis of prostaglandin E2, which may be blocked by indomethacin or other cycloxygenase inhibitors.[185;194;195] Prostaglandin E2 in turn up-regulates RANKL, but importantly also decreases OPG synthesis to tilt the balance of OPG/RANKL in the direction of Oc formation and bone resorption.

At present, the relevance of this system to myeloma is unknown, but may be of considerable importance. In a mouse model of sarcoma-induced osteolytic disease, OPG injections not only decreased osteolysis in mice with sarcoma-invaded bone, but also modulated pain-induced behaviour favourably.[196] This experiment calls for testing of OPG in myeloma models or patients.

2.7 Other novel factors

MIP-1α and -1β has earlier been found to stimulate Oc activity and has recently been implicated in myeloma bone disease.[197;198] First, MIP-1α seems to be the main OAF in the ARH-77 mouse model (see section 3).[199] Second, production of MIP-1α and -1β also has been detected in MM patients and cell lines.[200] MIP-1α protein is increased in bone marrow plasma from patients with myeloma, and the OAF activity of two patients could be blocked by neutralizing MIP-1α antibody.[198] IL-17, IL-18 and VEGF have recently been demonstrated to modulate Oc function.[201-204] All these factors should be further explored in MM. VEGF induces stromal production of IL-6 in vitro and myeloma-derived VEGF could induce a paracrine stimulatory circuit.[205] However, only very low concentrations of VEGF are present in body fluids from myeloma patients.[131]

Table I Cytokines in growth control of myeloma cells

	Cell proliferation	Cell survival	Serum levels	Patient prognosis	gp130-dependent	Source	Comments	Importance in MM
IL-6	Stim [78;79;254]	Stim [21;255-257]	Elevated IL-6, IL-6R and gp130 [245;246;258-260]	Prognostic value of serum IL-6 and IL-6R[245]	Yes	Autocrine and paracrine [94]	IL-6 blockage gives little clinical benefit in patients [250;251]	**Yes**
IL-10	Stim [91;261]	Stim [91;261]	Normal [262]	No [262]	(Yes)/No [91;261]	T-cells? [263]		**Minor**
TNF-a	Stim [92;264-266]	Stim [92;264-266]	Elevated [258;267]	No [267]	No [268]	Several		**Minor**
IGF-1	Stim [269-271]	Stim [269-271]	Normal serum levels approx 20nM		Increased sensitivity to IL-6 [271]	Ubiqutous autocrine [272]	Role in homing of myeloma cells [53]	**Possible**
IFN-a	Stim/No/ Inhib [92;273;274]	Stim/No/ Inhib [92;273-275]	?		Partially [92;273-275]		Little or no effect of treatment [9]	
IFN-g	Inhib [276-279]	Inhib [276-279]	?		(Yes) [276-279]		Little or no effect of treatment[280]	
TGF-b	Stim/No/ Inhib [37;92]		Elevated [281]	No [281]	No	Stroma and myeloma cell [37;282]	Role in immunoparesis? Induces stromal IL-6 production [281;282 37]	**Unclear**
Activin A	Inhib [255;283;284 285]	Inhib [255;283]	Unknown in MM, (normals 5 ng/ml) [286]		Inhibits gp130 signalling [285]	Stroma [177]	Unexplored in human myeloma	**Unknown**
IL-11	Minor [90-94]	Stim?	Low [94;95]		Yes	Osteoblast		**Minor**
IL-15	Stim[157] Paper IV	Stim[157] Paper IV	Normal or low Paper IV		No	Autocrine and paracrine[157] (IV)		**Unclear**
HGF	No/Yes (discussion)	No/Yes	Elevated [130]	Yes[130]	No	Autocrine Paper I [129]		**Unclear**
BMP	Inhib Paper V	Inhib Paper V	Unknown		Inhibits gp130 signalling (V)			**Unclear**

Cellular effects of cytokines were found in both cell lines and primary samples except for activin A (murine hybridoma/plasmocytoma cells) and TNF-α (cell lines only).
Stim= stimulatory or antiapoptotic, Inhib= inhibitory or apoptosis inducing where applicable.

Table II Cytokines in myeloma bone disease

	Osteoblast	Osteoclast	RANK-dependent	Bone marrow serum in MM	Serum levels in MM	Importance in MM bone disease
IL-6	Promotes differentiation, anti-apoptotic [73;102]	Weak activator, non essential for Oc-genesis Potentiates or mediates effects of other OAFs [287 288 289;290]	Yes	Low levels not different from normal controls [198;241;291]	Elevated IL-6, IL-6R and gp130 [246;259;260 245]	**Minor**
IL-1	Inhibits differentiation and prolifertion [292;293]	OAF activity in vitro and in vivo [294 295-297]	Yes and no	Low levels [198;241]	Normal [267]	**Minor**
TNF-a	Inhibits differentiation and prolifertion [292;293]	OAF in inflammatory bone resorption [98;193;298]	Yes and no	Low levels [241]	Elevated [258;267]	**Minor**
PTHrP	Inhibition [299]	Rare OAF in MM [300;301]	Yes		Elevated [300]	**Very minor**
IFN-a IFN-g	Inhibition (IFN-γ) [293]	Inhibits Oc resorption [302]	Unknown			
LT-a	Inhibition [293]	Rare OAF activity [295;296;303]	Unknown	Low [198]		**No**
IGF-1	Bone formation [178]	Activator [178]	Unknown			**Unclear**
HGF	Proliferation [132]	Oc formation [134;135]	Unknown	Elevated [117]	Elevated [130]	**Unclear**
IL-11	Differentiation [102]	Activator [96;97]	Yes	Low [94]	Low [94;95]	**Unclear**
IL-15	Unknown	Activator [152]	Unknown		Normal or low Paper IV	**Unclear**
BMP	Bone formation [160;161;167;178]	Activator [166;304]	Indirecly through PG[304]			**Unclear**

PG= prostaglandin. For references to the column “RANK-dependent” see Chapter 2.6.

3. MOUSE MODELS OF MULTIPLE MYELOMA

Animal models are useful and necessary tools to study the biology or therapeutics of disease. Good myeloma models have therefore long been sought for. In 1960, Potter et al reported development of plasmocytomas after injection of paraffin oil mixed with heat killed staphylococcal mixtures. These tumours could be passaged intraperitoneally from generation to generation, but mostly did not grow in bone marrow.[206] The relevance of studies on artificially created murine plasmocytoma to human myeloma is questionable and this objection called for better model systems. Infrequently intravenous injection of plasmocytoma cell lines may infiltrate the bone marrow and cause osteolysis.[207;208]

3.1 Murine myeloma

In 1985 Radl et al described spontaneous occurrence of myeloma in the C57BL/KaLwRij mouse strain. This was characterised by bone marrow infiltration, osteolysis and splenic involvement. Cell lines had to be propagated by passage from generation to generation in syngeneic mice.[209;210] The cell line 5T33, which grows autonomously in vitro and produces autocrine IL-6, has been used to simplify this model, and leads to extensive osteolytic disease in vivo.[211;212] These model systems appear to be relevant since they share many properties with human MM. IL-1α-transfected IL-6 dependent B9 hybridoma cells, denoted B9/BM1, grow readily in bone marrow and induce osteolysis.[213]B9/BM1 cells retain IL-6 dependency in vitro. However, since myeloma-derived IL-1 production is likely to be low or non-existent, the B9/BM1 system appears artificial, and hence of questionable relevance to human myeloma.[214;215]

3.2 Human myeloma cells in mouse recipients

Myeloma cells do not readily grow in nude mice. SCID mice have a defect in recombination of B and T cells and hence no adaptive immune response manifested by propensity to infection, lack of B and T lymphocytes, and agammaglobulinemia. SCID mice are good recipients of transplants since they have reduced capacity to reject allo- or xeno-grafts.[216] Two early studies demonstrated limited localised take in SCID mice injected with primary myeloma cells intraperitoneally.[217;218] In 1993, Huang et al injected ARH-77 cells intravenously in irradiated SCID hosts with

consequent disseminated spread including bone marrow take and osteolysis.[219] SCID mice have normal natural killer (NK) cells and conditioning by irradiation is thought to eliminate the remainder of the immune function of SCID mice. Conditioning based on inhibition of NK function by cytotoxic asialo-GM1 antibody has also been effective in preventing graft rejection in myeloma models.[220] NOD/SCID mice are even more immunodeficient than SCID mice, since their NK and macrophage function is reduced.[221] In a comparative study, NOD/SCID mice were the best recipients of different B cell tumours.[222]

Researchers have previously not succeeded in achieveing growth of primary myeloma cells by i.v. injection, but in Pilarski's work two important improvements in the methodology were introduced.[68] First, irradiated NOD/SCID mice were used. Second, left ventricular injection bypasses the problem of first pass entrapment of myeloma cells in the lungs, and may drastically increase the number of tumour cells which reach the bone marrow. Take in mouse bone marrow by primary myeloma cells and human myeloma cell lines argues against the notion that murine stroma is a poor recipient of primary human myeloma.[67]

The use of human fetal bone implants as an injection site probably represents the best presently available simulation of human myeloma using mouse recipients (SCID-hu model).[34;35] It allows the in vivo growth of myeloma from most patients, in marked contrast to the in vitro situation.[35]

Table III summarizes main features of the different human myeloma models in immunodeficient mice. It is presently premature to conclude on the usefulness of each these models in myeloma research. First, data from cell line models will all be hampered by problems of generalization, since the biology of one cell line may not apply to patients in general. Second, the scarcity of primary material poses major limitations to experimental activities. Last, the use of human fetal bone in the SCID-hu model is ethically controversial.

3.3 Biological questions addressed in myeloma models

IL-6 dependency: The in vivo dependency of human IL-6 of in vitro IL-6-dependent cell lines has been demonstrated in several instances.[220;223;224] Since murine IL-6 does not stimulate human cells, cells either have to secrete human IL-6 to survive, or depend on an exogenous stimulus[220;223;224]. Blocking the IL-6 system inhibits or delays growth of tumours both subcutaneously and intravenously.[220;223]

Additionally, Burger et al used the strictly IL-6-dependent cell line INA-6, which grows intraperitoneally in SCID mice. Ex vivo cells are again strictly IL-6 dependent, and in vivo INA-6 cells survive because they are induced to secrete autocrine human IL-6.[225]

Immunotherapy. The surface antigen HM1.24 is almost exclusively expressed on plasma cells and by use of an antibody against this antigen, a single injection of antibody on the day of inoculation cured animals injected intravenously with ARH-77 cells. When antibody treatment commenced two weeks after inoculation, impressive effects including growth inhibition and cure of both subcutaneously (RPMI 8226) and intravenously inoculated mice (ARH-77) was achieved. The mechanism of this anti-myeloma effect was through ADCC and complement-mediated cytotoxicity. Antibody was given 1-5mg/kg body weight per week, which is similar to dosages currently used for the treatment of lymphoma and CLL by the commercially available CD20 mAb rituximab.[226] Consequently, targeting the HM1.24 antigen by an antibody strategy in myeloma patients should be technically possible to perform. Finally, targeting of the MUC-1 antigen has been tested.[227]

Tumourigenicity: See section 1.5 for a review of the experiments on the myeloma founder cells in mice. Reintroduction of CD19 expression by transfection of the KMS-5 myeloma cell line decreases its tumorigenicity and proliferation.[228]

Drug testing: The phenomenon of chemoresistance has been addressed.[229] Furthermore, studies showing reduced osteolysis after administration of pamidronate (APD) and ibandronate have been performed.[210;230] High concentrations of bisphosponates in vitro induce apoptosis and growth arrest in myeloma cell lines.[231] In vivo, however no anti-myeloma effect was found using the 5T33 cell line.[230] In apparent contrast, an anti-tumour effect was demonstrated in primary samples grown in the SCID-hu model.[232]

Homing: ICAM-1 (CD54) is involved in adhesion of myeloma cells. Using CD54 blocking antibody in the ARH-77 model tumour formation was delayed or blocked. The mechanism for this effect was obscure and was by exclusion attributed to interference with homing. The antibody neither inhibited stromal attachment of ARH-77 cells, nor caused ADCC or complement-mediated lysis.[233]

Table III Human myeloma models in immunodeficient mice

	Mouse strain	Condi-tioning	Cell type	Injection Route	Take	Osteolysis	Hyper-calcemia	Cytokine dependence
Huang[219] Alsina[305] 1993 and 1995	SCID	Irradiation	ARH-77	i.v.	Bone marrow, liver, lung, meninges, brown fat	Yes Oc activation by MIP-1α Increased CFU-GM	Yes	No
Tsunenari [223] 1997	SCID	None	KPMM-2	i.v.	Bone marrow	Yes, Oc activation	Yes	IL-6 (autocrine)
Rebouissou[224] 1998	SCID/ Bg	None	XG-1 XG-2	i.p. in Matrigel™	Local (XG-1+XG-2) Bone marrow (XG-2)			gp130 agonist mAb
Urashima[34] 1997	SCID	Fetal bone	ARH-77 RPMI8226	i.o.	Local+contralateral implant	Yes	Yes	No
Yaccoby[35] 1998	SCID	Fetal bone	Primary 80% take	i.o.	Local+contralateral implant	Yes Human Oc activated	Yes	No
Pilarski[68] 2000	NOD/ SCID	Irradiation	Primary	i.c. i.o.	Bone marrow spleen	Yes		No
Burger[225;306] 1996	SCID	?	INA-6	i.p.	Local	No		IL-6 (autocrine)
Hjorth-Hansen 1996	SCID	Irradiation	JJN-3 JJN-3 T1	i.v. s.c.	Bone marrow,brown fat, meninges, liver	Yes Osteoblastopenia	Yes	No

i.v.=intravenous, i.p.=intraperitoneal, i.c.=intracardiac, i.o.=intraosseous, s.c. subcutaneous

4. AIM OF THE STUDY

The aim of the Trondheim Myeloma Group is to study cellular and molecular properties of the myeloma cell in its microenvironment, in order to design rational strategies for treatment of myeloma.

1. Following the discovery of HGF secretion in myeloma cell lines, we studied HGF and c-met in purified primary samples and patient serum.
2. To study the effect of HGF on myeloma biology including bone disease, we needed a model system with HGF-secreting myeloma cells in the orthotopic situation. In 1996, only the ARH-77 mouse model was developed for studies of myeloma bone disease. First, this model did not produce HGF, but more importantly, we were skeptical to the relevance of lymphoblastoid (Epstein Barr virus positive) ARH-77 cells to myeloma. Lymphoblastoid cells differ considerably in biological properties compared to myeloma cells.[66] We therefore decided to develop our own model system using HGF-producing JJN-3 cells.
3. The biological role of HGF in MM was unclear, and we fruitlessly tested whether HGF had effects on a number of biological properties of myeloma cells. Observing osteoblastopenia in the mouse model, we turned to studies of HGF and cells of the microenvironment of myeloma cells. We found that myeloma-derived HGF increased IL-11 production in osteoblasts and explored this phenomenon.
4. In studies of B lymphocytes from normal blood donors and chronic lymphocytic leukemia patients, I detected transcripts of IL-11 and IL-15 by RT-PCR. These findings were extended to myeloma cells and we therefore sought to elucidate the role of IL-15 in myeloma.
5. Studying BMP-induced osteoblast differentiation, we discovered that BMPs potently inhibited growth of OH-2 cells. This phenomenon was explored.

5. SUMMARY OF THE WORK

Paper 1 extends the findings of HGF and c-met in myeloma cell lines[129]. By use of highly purified B-B4-selected primary myeloma cells we demonstrated the presence of HGF and c-met transcripts by RT-PCR in all patients, but not in normal bone marrow and normal peripheral B cells. By an in-house ELISA, we were able to show that HGF was secreted in supernatants by 17 out of 20 myeloma primary samples. In contrast, IL-1 secretion was not detected in culture supernatants from B-B4-selected plasma cells. HGF concentrations in serum were elevated in relation to healthy control subjects. The expression of c-met protein was detected by flow cytometry and Western blot from cell lines and pleural effusions of patients.

Paper 2 demonstrates the disseminated spread of JJN-3 cells in irradiated SCID mice. We found that mice developed hind limb paralysis due to compression of the spinal cord, myeloma growth in bone marrow, osteolysis, spread to brown fat and modest spread to the liver. Mice were hypercalcemic, and osteolysis was demonstrated on radiographs. HGF production was detected in serum and bone marrow plasma. Bone histomorphometric examination revealed the near total demise of osteoblasts, both functionally and morphologically. Osteoblast counts were reduced by 99% and bone formation rates were halved in end stage disease.

Paper 3 demonstrates a function of myeloma-derived HGF. IL-11 is an Oc activator and we found that HGF markedly up-regulated IL-11 in the SAOS-2 osteosarcoma cell line. Further studies in the HOS osteosarcoma cell line and normal human osteoblast-like cells (hOb) confirmed this effect of HGF. The effect of myeloma-derived HGF was also tested in a co-culturing system. HGF effects clearly increased by cell-cell contact, and this effect could be blocked by prewashing JJN-3 cells with heparin. This study and data from the litterature show that HGF is cell surface-bound to proteoglycans, and that this binding augments HGF–induced IL-11 secretion. Using primary myeloma cells in the co-culture system we repeated findings from HGF-secreting cell lines. Finally we found that IL-11 secretion was augmented in a synergistic manner with IL-1 and TGF-β, and in an additive manner with TNF-α, all factors of potential importance in MM bone disease.

Paper 4 demonstrates effects and production of IL-15 in myeloma cells. IL-15 induced proliferation in the cytokine-sensitive OH-2 cell line, but not in autonomously growing cell lines. IL-15 furthermore counteracted apoptosis quite

potently in OH-2 cells. Effects of IL-15 seemed unrelated to the gp130 system in blocking experiments in contrast to other myeloma-stimulating cytokines like GM-CSF and IL-10 which act through induction of gp130 superfamily cytokines. The study establishes IL-15 as a novel and independent factor in growth and survival of myeloma cells. IL-15 acted in a synergistic manner with TNF-α, but in an additive manner with IL-10, IGF-1 and IL-6. By RT-PCR, IL-15 transcripts were detected in most cell lines and primary samples, but protein was detectable in only one out of twenty supernatants. We compared the proliferative capacity of IL-6 and IL-15 in B-B4-purified primary samples and found weak stimulatory effects in four out of six samples by each cytokine. This finding strengthens the notion of IL-15 as a potentially important factor in growth and survival of myeloma cells, and demonstrates yet another factor which is capable of substituting for the effect of IL-6.

Paper 5 demonstrates the effect of BMP–4 on myeloma cells. We found that BMP-4 inhibited DNA synthesis in OH-2, ANBL-6 and the newly isolated IH-1 cell line, which are all IL-6-dependent. In three autonomously growing myeloma cell lines, little or no effect of BMPs was found. The decrease in DNA synthesis was found to have two causes. First, apoptosis was induced as measured by the annexin V/propidium iodide method. Second, cell cycle arrest in the G0/G1 phase was demonstrated.. Findings were extended to patient samples, again showing a relation to IL-6 stimulation. The interaction of BMP-4 and IL-6 was investigated further. We demonstrated that BMP-4 downregulated constitutive and IL-6-induced phosphorylation of tyrosine residue 705 of STAT3 in IL-6-dependent cell lines. Our finding calls for further studies of BMP biology in myeloma. BMPs, BMP analogues or pharmaceutical agents capable of inducing BMP production in vivo such as statins, could conceivably both inhibit growth of myeloma and stimulate bone formation simultaneously[234]. Hence, BMPs seem attractive molecules to evaluate for future therapy.

6. DISCUSSION

6.1 The JJN-3 mouse model and osteoblastopenia

In the main methodological work of this thesis I demonstrated that intravenous injection of JJN-3 cells resulted in disseminated spread in a partially orthotopic manner. After the initial studies, however, I performed two studies, in which the bone marrow take was less conspicuous and fewer animals became paralytic. Still, all animals had tumour take, but frequently in brown adipose tissue. To circumvent this variability, I used the cultured cells from bone marrow of one mouse, JJN-3 T1 cells. With this cell population, paralysis and bone marrow take was to a greater deal restored. This and later unpublished studies using JJN-3 T1 cells in a subcutaneous model have shown a large variability in tumour growth, demonstrating that the JJN-3 system is hampered with a considerable inherent biological variability both in growth and homing to the bone marrow. This limits its usefulness, in part through the need of large study groups to attain statistically significant results. The reason for this variability is unclear. Growth of JJN-3 T1 cells in bone marrow, paralysis and osteolysis has been independently reproduced by Dr K.Vanderkerken, Brussels, Belgium (personal communication).

The main methodological novelty of this study was to use bone histomorphometry in a mouse model of myeloma and the main biological finding was osteoblastopenia in tumour-invaded bone. Osteoblastopenia was also demonstrated in non-invaded bone, demonstrating the existence of a distant effect on Ob counts by JJN-3 cells (unpublished observations). In comparison to data from bone histomorphometry in human myeloma, our data show a very profound effect on osteoblasts and bone formation. In normocalcemic myeloma patients with high tumour burdens, the Ob counts are actually moderately increased compared to controls, but their synthetic activity is low.[235] In JJN-3 T1 mice, Oc counts were also decreased compared to controls, apparently in discordance with the situation in human myeloma. However, osteoclastogenesis is dependent on functional Ob and the low Oc counts may therefore be a direct consequence of the demise of Ob. Clearly the lower trabecular number and bone volume must be a result of higher bone resorption than formation. We conclude that the model seems to reflect myeloma bone disease quite well, particularily in the aspect of osteoblast function and late stage disease.

The mechanism of osteoblastopenia is still obscure. Possibilities include lack of Ob formation or differentiation due to effects on precursor cells, detachment of Ob or shortening of the lifespan of Ob e.g. by induction of apoptosis. In vitro studies of JJN-3 cells and hOb cells in co-culture, show that the number of alkaline phosphatase-positive colonies actually increased, speaking against a direct effect of JJN-3 cells on Ob (PI Croucher, Sheffield, UK personal comm and my own results). This is in apparent contradiction with the in vivo data, but may be explained if myeloma cells induce one or more substances in the SCID hosts which in turn interferes with Ob. Similar extreme osteoblastopenia has also been described to occur in other tumour models.[236;237] In vitro low-grade inhibition of proliferation of hOb cultured in myeloma-conditioned medium has been found.[238] In addition, a myeloma-derived soluble factor decreases osteocalcin production in osteosarcoma cell lines, a sign of lacking differentiation.[38] To study HGF in relationship to osteoblastopenia, we performed several negative studies in the aspect of adhesion, detachment and differentiation of Ob (unpublished). HGF induced scattering, migration and improved wound healing of confluent layers of osteosarcoma cell lines, as had in part been described before, but not in normal Ob.[239] In conclusion, HGF seemed to be of little relevance to osteoblastopenia in the systems we explored.

6.2 The role of HGF in myeloma

These studies assign a role for HGF in myeloma by induction of IL-11 in osteoblasts in vitro. Although the data we provide to demonstrate this is strong, we have as yet not been able to demonstrate osteoclast activation, due to lack of an Oc assay. Such studies are underway. Our theory is that myeloma–derived HGF induces IL-11, which in turn may upregulate RANKL expression to increase Oc activity.[97;101] The relevance of IL-11 to myeloma bone disease in vivo is, however, uncertain, since little IL-11 may be detected in serum and bone marrow plasma of patients (our unpublished results).[94;95]

An interesting observation in Paper 3 is that cell-cell contact may increase the biological effect of HGF, probably by improved presentation of HGF to c-met. Based on this and other studies, it must be assumed that HGF on myeloma cell membranes is mainly proteoglycan-bound. It is interesting that serum levels of syndecan-1, a myeloma-derived proteoglycan, was a good predictor of prognosis of MM patients[240] Furthermore, purified syndecan-1 modulated IL-11 secretion in SAOS-2 cells in a

manner dependent on the stoichiometric relation between HGF and syndecan-1.[117] A wide array of growth factors such as FGF, HGF, TGF-βs and BMPs bind heparin, heparan sulphates or proteoglycans.[119;162;179] Thus, interactions between proteoglycans and cytokines appear not only important in MM, but also in a broad biological perspective.

Tumour growth is dependent on angiogenesis and HGF has been implicated in this process.[55;125] HGF anti-serum alone was insufficient to block tumour growth in subcutaneously injected JJN-3 T1 SCID mice (unpublished), suggesting that angiogenesis in this model depends on other angiogenetic factors.

To conclude, the role of HGF in myeloma bone disease and myeloma growth remains to be elucidated. The scarcitiy of effects of HGF in vitro and in vivo is puzzling, since HGF from myeloma patients is bioactive and present in bone marrow plasma in biologically relevant concentrations (median 6 ng/ml). This contrasts the low concentrations of other cytokines implicated in myeloma, such as FGF, VEGF, IL-6 and TNF. [131;241] Very recent work from our lab using fibrinonectin-adhered myeloma cell lines indicates that HGF has a growth stimulatory effect. I predict that we still have not investigated HGF in its appropriate context, and suggest further studies, particularly concerning bone resorption and osteoclast activation, and its role in growth of matrix- or stroma-adhered myeloma cells.

6.3 Growth control of myeloma; more than IL-6

Work in this thesis establishes BMPs and IL-15 as novel factors involved in the regulation of life and death of the myeloma cell in vitro.

Concerning IL-15, a confirmatory study recently appeared.[157] In addition to our findings, low-grade presence of intracellular IL-15 protein was reported. Interestingly, evidence was found for an anti-apoptotic IL-15 autocrine circuit in low serum conditions. As in our studies, IL-15 could substitute for IL-6 in the prevention of apoptosis by growth factor deprivation, but also apoptosis induced by Fas ligation and cytostatic drugs, but not dexamethasone. It is too early to say how important the IL-15 mechanism may be in the biology of MM, but these findings underline the fact that cytokines operate in redundant networks. Targeting of the IL-6 mechanism alone in the treatment of myeloma appears to be insufficient, perhaps because other

cytokines like IL-15, IL-10, TNF-α and IGF-1 may substitute. Further studies on the role of IL-15 in the microenvironment of the myeloma cell should be carried out

The finding of BMP-induced apoptosis is novel, both in the context of myeloma and in malignancies at large. Furthermore, the mechanism s seems to be mediated by interference with the IL-6 pathway and dephosphorylation of STAT3. In analogy, the effects of cytokines such as IL-2, IL-5 and IL-12 may be inhibited by TGF-β through decreased phosphorylation of JAK kinases (and as a consequence, STAT proteins downstream).[242] This inhibition of cytokine effects seems likely to be caused by induction of one or more tyrosine phosphatases and dephosphorylation of important residues on JAKs. Studies are underway to clarify if BMP effects are mediated by phosphatases, and to determine if the effect is located at the level of STAT proteins or upstream (JAKs). As the BMP effect in myeloma cells in some cases exceeded the effect of IL-6 starvation, the existence of additional mechanisms for its effect in myeloma are likely to be present. Either, the intracellular signalling pathways of BMPs mediate a direct pro-apoptotic effect, or alternatively, BMP may affect other autocrine or paracrine cytokines in a similar manner as it has been shown to block IL-6. [174;175;243;244] We have experimental data (unpublished) to support the latter notion, since BMP-4 also inhibit the anti-apoptotic effects of TNF-α, IL-10, IL-15 and IGF-1 in OH-2 cells. Thus, in MM, as in other systems, TGF-β superfamily proteins appear to inhibit the signalling of multiple cytokines, which points to a biologically important mechanism by which these proteins exert growth control.[242]

IL-6 is probably a necessary factor for myelomagenesis. A biological role for IL-6 has been established in cell lines and a proportion of primary samples, but many studies leave an impression that IL-6 has limited overall importance in patients. Data on serum levels of IL-6 and IL-6R are relatively unimpressive and the prognostic significance of these factors is present but modest.[130;245;246] Some studies even show a lack of prognostic importance.[247;248] In the bone marrow compartment low levels of IL-6 may be detected, not higher than levels in normal bone marrow[241;249]. Furthermore, there is no evidence that IL-6 blockage in patients is therapeutically beneficial, although such effects have been found in mice injected with IL-6-dependent cells.[220;223;224;250;251]. These considerations and the lack of IL-6 effects in many primary samples imply that additional mechanisms must be important in growth of myeloma cells.

The biology of the myeloma-stromal interaction appears insufficiently explored, probably for methodological reasons. The microenvironment is, however, very important since primary myeloma cells grow poorly in vitro, whereas such cells grow in SCID-hu hosts from a majority of patients.[35;88] In Fig 1, a model for the growth control of myeloma cells in the microenvironment is depicted. The production of stroma-derived soluble factors, apart from IL-6, has not been specifically investigated in myeloma, but has been shown in other contexts. As discussed, myeloma cells alter the properties of the stroma towards a more favourable soil for the malignant cells by increasing IL-6 production and decreasing unidentified growth inhibitory mechanisms (section 1.3). The complexity of the proposed model increases if we also consider that the hematopoietic compartment contributes to the myeloma microenvironment. To address biological questions in this broad scope is obviously difficult, but may prove necessary to be able understand the pathophysiology of MM and to design rational therapy for the disease.

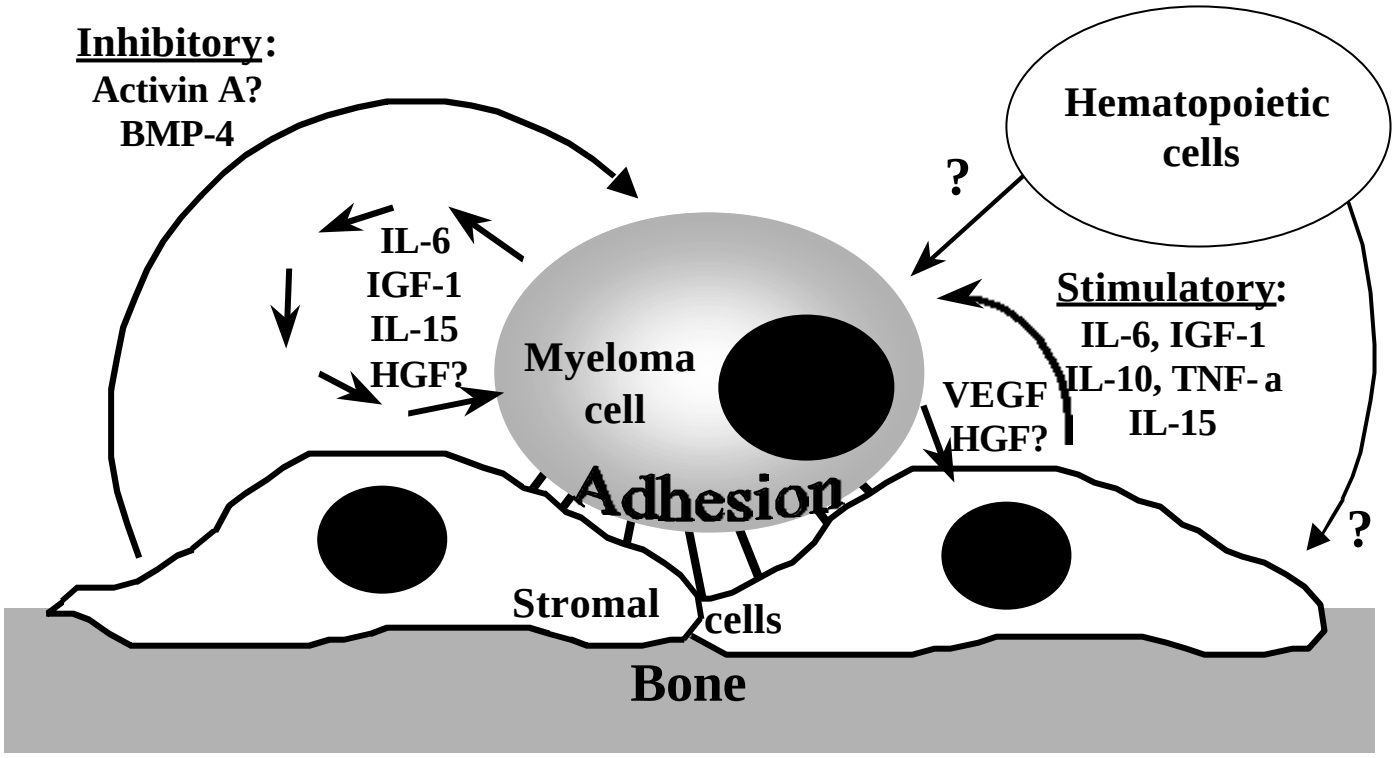

Fig 1. **Growth control of myeloma cells in the microenvironment.** First, adhesion to stromal cells by adhesion molecules prevents apoptosis probably both through ligation of these adhesion molecules themselves and by the effect of stromal- and myeloma-derived cytokines. These cytokines may be presented on cell surfaces or in soluble forms. As the disease progresses, the function of the stromal cells is altered, both by increased production of growth promoting cytokines (like IL-6), but also by decreased adhesion-induced inhibition of growth, conceivably by decreased production of BMPs or activin A. Different cytokine stimuli from the hematopoietic cell compartment may also modulate growth or survival of myeloma cells, but very little is known about the effect of hematopoietic activity on the biology of plasma cells.

6.4 A hierarchy of cytokines in myeloma bone disease

As shown in section 2, a considerable number of molecules may affect the function of Ob and Oc. In Fig 2, I have attempted to depict this information with bearing to Oc activation. The role of the RANKL/RANK/OPG system is still virtually unknown in myeloma, but with availability of research tools this highly interesting system may be explored. In a preliminary report, up-regulation of RANKL mRNA in bone marrow biopsies was demonstrated.[252] In analogy, increased osteoclastogenesis with concurrent up-regulation of RANKL expression and decrease of OPG expression has been demonstrated using cancer cell lines grown in co-culture with bone marrow mononuclear cells.[253] However, since IL-1 and TNF-α have been demonstrated in bone marrow plasma albeit in low concentrations, both RANKL-dependent and -independent Oc activation may be operational in myeloma. Therefore, it is far from self-evident that blockage of the RANKL system will solve the problem of myeloma bone disease. Since HGF induces Ob secretion of IL-11 and other studies show that IL-11 in turn may activate the RANKL system, I postulate the existence of a hierarchy of OAFs in MM as depicted in Fig 2.[97;101]

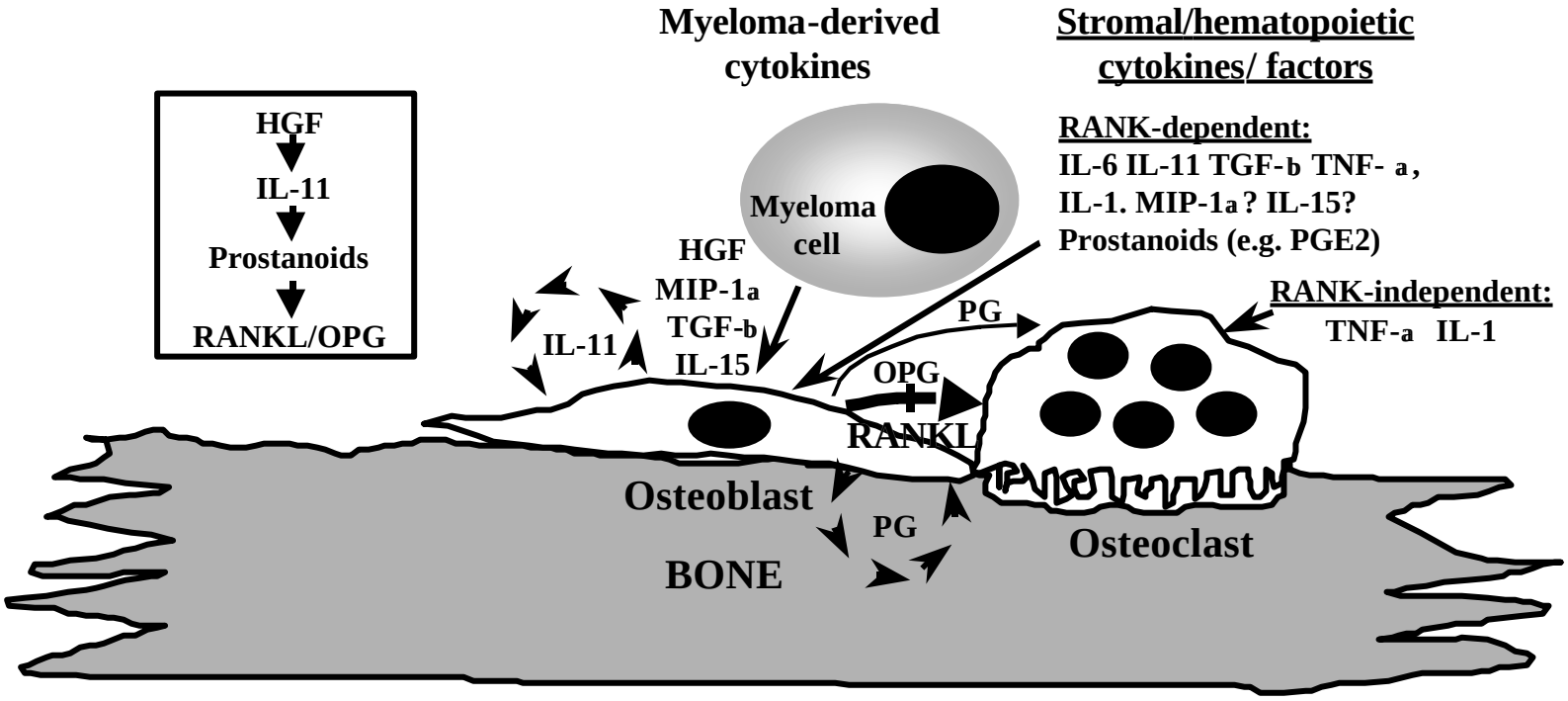

Fig 2. Cytokine mediators of myeloma bone disease. Most of these cytokines probably activate osteoclasts through the RANK/RANKL system, although IL-1 and TNF may also activate osteoclasts RANK independently (however at very high and probably biologically irrelevant concentrations). The cytokines may be produced by the myeloma cells or stromal/hematopoietic cells as depicted. Some factors also induce prostaglandins or leukotrienes which in turn tilt the balance between RANKL and osteoprotegerin in favour of Oc activation. The hierarchy by which HGF may affect the RANK/RANKL system is depicted in the box on the left.

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