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Apoptosis Regulation in Multiple Myeloma

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Abstract

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Multiple myeloma (MM) is a virtually incurable B cell malignancy of the bone marrow. One important part of tumor progression and an obstacle for successful therapy is resistance to apoptosis. To combat this resistance, the mechanisms of apoptosis and survival in MM must be better defined.

In this thesis, we identified Fas up-regulation as a mechanism underlying interferon (IFN)-mediated sensitization to Fas-induced apoptosis in the MM cell line U-266-1970. IFN treatment induced activation of signal transducer and activator of transcription (Stat)1 but, intriguingly, also attenuated activation of MM survival factor Stat3.

Exploring the role of Stat1 further, we established sub-lines of U-266-1970 with a stable over-expression of Stat1 and of its active mutant Stat1C. These sub-lines displayed a decreased expression and activation of Stat3, and an altered expression of apoptosis-related genes Harakiri, Bcl-2 and Mcl-1. In a drug library screening, Stat1 over-expression was associated with an increased sensitivity to Fas-induced apoptosis and, conversely, an increased resistance to several drugs, including the cyclin dependent kinase (cdk)1 inhibitor CGP74514A. We conclude that Stat1 over-expression does not confer a general resistance or sensitivity to apoptosis in MM, but may strongly affect the response to some specific drugs.

We also explored the effects of picropodophyllin (PPP), an inhibitor of the insulin-like growth factor I (IGF-I) receptor tyrosine kinase (RTK), in MM. PPP selectively inhibited the IGF-I RTK activity without inhibiting the insulin RTK activity. Furthermore, PPP potently induced cell cycle arrest and apoptosis in all MM cell lines and patient samples tested, also in the presence of survival factors IGF-I and IL-6. We conclude that PPP has great therapeutic potential in MM.

Finally, we examined the expression and regulation of the inhibitors of apoptosis proteins (IAPs) in a panel of MM cell lines and patient samples. The glucocorticoid dexamethasone, which is used in MM therapy, induced a transient up-regulation and a subsequent down-regulation of c-IAP2, as well as a down-regulation of XIAP, possibly influencing the sensitivity to apoptosis induced by this drug. Supporting this notion, abrogation of IGF-IR signaling by PPP, which sensitizes MM cells to dexamethasone-induced apoptosis, enhanced the down-regulation of c-IAP2 and XIAP.

Keywords: multiple myeloma, apoptosis, survival, IFN, Stat1, Stat3, IGF-I, RTK inhibitor, PPP, IAP, Fas/CD95, dexamethasone

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To Andy, Saskia, Iris and our Baby ♥

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals

- I **L. Y. Dimberg***, A. I. Dimberg*, K. Ivarsson, T. Strömberg, A. Österborg, K. Nilsson, F. Öberg, H. Jernberg Wiklund. Ectopic and IFN-induced expression of Fas overcomes resistance to Fas-mediated apoptosis in multiple myeloma cells. *Blood*. 2005;106:1346-54. Reproduced with permission.
- II **L. Y. Dimberg**, K. Ivarsson, A. I. Dimberg, G. Tobin, S. Ekman, U. Gullberg, R. Larsson, K. Nilsson, F. Öberg, H. Jernberg Wiklund. The effect of Stat1 over-expression on the apoptosis-related gene profile and drug sensitivity of multiple myeloma cells. Manuscript.
- III T. Strömberg, S. Ekman, L. Girnita, **L. Y. Dimberg**, O. Larsson, M. Axelson, J. Lennartsson, U. Hellman, K. Carlson, A. Österborg, K. Vanderkerken, K. Nilsson, H. Jernberg Wiklund. IGF-1 receptor tyrosine kinase inhibition by the cyclolignan PPP induces G2/M-phase accumulation and apoptosis in multiple myeloma cells. *Blood*. 2006; 107:669-678. Reproduced with permission.
- IV A. Hammarberg*, **L. Y. Dimberg***, K. Ivarsson, T. Strömberg, K. Carlson, A. Österborg, K. Nilsson, H. Jernberg Wiklund. Sensitization to dexamethasone-induced apoptosis by abrogation of insulin-like growth factor (IGF)-I receptor signaling is associated with regulation of inhibitors of apoptosis proteins (IAPs) in human multiple myeloma cells. Manuscript.

* equal contribution

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Abbreviations

α IR3	Anti-IGF-I receptor antibody
BMSC	Bone marrow stromal cells
Dex	dexamethasone
EMSA	electrophoretic mobility shift assay
FasL	Fas ligand
GAS	gamma activated site
IAP	inhibitor of apoptosis protein
IFN	interferon
IGF-I	insulin-like growth factor I
IGF-I RTK	IGF-I receptor tyrosine kinase
IL	Interleukin
MM	multiple myeloma
NF- κ B	nuclear factor κ B
PI3K	phosphoinositol 3-kinase
PPP	picropodophyllin
RPA	ribonuclease protection assay
Stat	signal transducer and activator of transcription
TRAIL/ Apo2L	TNF-related apoptosis-inducing ligand/ Apo2L

Introduction

In any multicellular organism, law and order is crucial. Each of the approximately 10^{14} cells that make up an individual human body has to remain in its designated spot and perform its appointed duties, including proliferating and dying in an orderly manner. A single rebellious cell that manages to break free from its predestined fate can give rise to a growing clone of dissidents that will, eventually, take over and lead to the death of the individual. This is what happens in cancer.

A normal cell is subjected to several constraints that prevent this unwanted scenario. For instance, it is dependent on growth factors and oxygen supplemented from surrounding cells and from the blood for its survival. Attachment to neighboring cells and to the extracellular matrix forces the cell to remain in the right place and prevents it from invading the surrounding tissue. Finally, the normal cell has a limited replicative potential and a limited lifespan. If the right requirements for survival are not fulfilled or if the cell has completed its lifetime, the cell's own suicide program, apoptosis, is induced.

During the course of transformation from a normal cell to a cancer cell, the cell acquires capabilities that allow it to break free from these restrictions. Perhaps the most important of these capabilities is the capacity to evade apoptosis. Importantly, evading apoptosis does not only allow a cancer cell to escape from the control of the organism it resides in, but also makes it insensitive to drug therapy. An increased understanding of the mechanisms of apoptosis resistance and how resistance can be circumvented is imperative to learn how to combat cancer more efficiently.

This thesis concerns the regulation of apoptosis in multiple myeloma (MM), a virtually incurable cancer of bone marrow plasma-blasts/plasma cells that afflicts approximately 550 Swedes every year.

Multiple myeloma in brief

Multiple myeloma (MM) is a tumor (*-oma*) that arises at multiple sites in the bone marrow (*myelo-*), usually in the spine, skull, pelvic bone, rib cage, shoulders and hips. The malignant cell in MM displays features of normal plasmablasts/ plasma cells. Non-neoplastic plasma cells are antibody-producing mature B cells that are the principal effector cells of humoral immunity. Accumulation of monoclonal antibodies, the M-protein, in the serum and/or in the urine is a hallmark of MM, but also of the related conditions MGUS (monoclonal gammopathy of undetermined significance) and asymptomatic or smouldering myeloma. For the diagnosis of symptomatic MM, there must be evidence of related organ or tissue impairment such as elevated blood calcium, renal insufficiency, anemia and/or bone lesions, easily remembered by the mnemonic CRAB.¹

Bone lesions, leading to bone pain and bone fractures, are very common in MM. In contrast to most other cancers that metastasize to bone, MM induces bone destruction that is not followed by new bone formation. The reason for this is that the MM cells secrete factors that stimulate the osteoclasts (bone destructing cells) while secreting another factor that inhibits the osteoblasts (bone-producing, bone healing cells).² Calcium is released from the damaged bone and enters the bloodstream, where the excess levels give rise to kidney damage, dehydration, fatigue, nausea and mental confusion. The high production of M-protein by the malignant plasma cells may slow down the blood circulation and reduce blood and oxygen supply to the nerve tissue, resulting in a variety of neurological symptoms. When M-protein is present in the urine, kidney damage can occur. As the malignant cells grow they displace red blood cells and excrete inhibitory factors that prevent erythropoiesis, leading to anemia. MM patients are also more susceptible to infections due to suppression of normal immune functions.

MM is a disease of the elderly: the median age at onset is 68 years and MM is rarely diagnosed before the age of 40. There is a higher incidence of MM in men than in women, and it is more common in the African-American than in the Asian-American population. The etiology of MM is far from being completely understood. Exposure to certain factors such as agriculture chemicals, radiation and viruses such as HIV and hepatitis C virus have been associated with an increased risk of developing MM, and genetic factors

have also been suggested. The first well-documented description of MM in a patient is from the first half of the nineteenth century (reviewed in³) and one study even demonstrates the evidence of MM in Egyptian mummies from 1500-500 BC!⁴

There are several treatment options for MM patients. The first matter to consider is whether the patient is young (generally <65) and healthy enough to receive high-dose chemotherapy and stem cell transplant. This is the most successful treatment so far. Allografting, as opposed to autografting, stem cells induces a graft-versus-myeloma effect that increases the efficacy of treatment even further, but this method involves a high risk of transplant-related death, and there is a large group of patients for whom this treatment is never an option. Other treatment strategies are steroids such as dexamethasone (Dex), radiation therapy or conventional (standard-dose) chemotherapy with e.g. melphalan, vincristine, and prednisone. Several drugs are often combined or used sequentially, for instance VAD: vincristine, adriamycin (doxorubicin), dexamethasone. Some new promising drugs are the immunomodulatory drug thalidomide, the proteasome inhibitor Velcade, histone deacetylase (HDAC) inhibitors, and arsenic trioxide, alone or in combination with conventional drugs.⁵

All of the above mentioned therapy options have one thing in common: although the quality of life may be increased and survival may be prolonged, none of these treatments lead to complete eradication of the disease, except in very rare cases.

One major obstacle in MM treatment is that the tight interaction between the MM cells, the bone marrow stromal cells and the extracellular matrix provides the MM cells with important growth- and survival factors that allow the MM cells to escape from apoptosis. In addition, MM tumors often harbor genetic alterations such as translocations that e.g. promote the expression of oncogenes. By identifying the factors and signal transduction pathways that participate in apoptosis resistance towards different stimuli and finding ways to circumvent their actions, MM therapy could be more efficient. A biological approach to MM therapy that takes apoptosis-resisting factors into account may be the key to combating the disease.

Before we start to discuss some of the factors that may have a large impact of survival and apoptosis resistance in MM, a background of the concept of apoptosis is warranted.

Apoptosis – a short introduction

Historical perspective

The field of apoptosis is one of the “hottest” research areas in biology today, accounting for more than 2% of all life science publications. However, the concept of apoptosis was actually introduced almost 150 years ago (reviewed in⁶).

In 1858, Virchow characterized the changes occurring in cells shortly after death as either necrosis, where “the mortified cell is left in its external form” or “necrobiosis or shrinkage necrosis being where the cell vanishes and can no longer be seen in its previous form”.⁷ The term “necrobiosis” was succeeded by the term “chromatolysis” 26 years later, when Flemming described the morphological changes taking place during regression of the epithelium in mammalian lymphoid follicles. In this study, the first drawings illustrating what we now recognize as apoptosis were produced.⁸ The theory of cell death as a mechanism involved in development, maintenance of homeostasis, control of organ size, and elimination of dysfunctional cells evolved over the following decades. In the late 60s, apoptosis research was greatly facilitated by the use of electron microscopy. Now, the morphological changes occurring in apoptosis could be studied in much greater detail.^{9,10}

A scientific landmark in apoptosis research occurred in 1972, when Kerr and colleagues published a paper in which they coined the term “apoptosis” (derived from a Greek word for “dropping off”, as in falling leaves) and defined this phenomenon as a type of orderly, active process by which cells undergo a series of morphological changes, ultimately leading to recognition and engulfment by phagocytes. They provided evidence that this built-in death program was not only evident during development or during pathological conditions, but also in the normal mature organism, continuing throughout life. The authors defined an important role for apoptosis in homeostasis and suggested that deregulation of apoptosis could lead to pathological conditions such as cancer.¹¹

At the beginning of the following decade, the interest for apoptosis was greatly increased with the discovery that glucocorticoids induce apoptosis

and endonuclease activation in lymphocytes.¹² A few years later, the activation of endonucleases in apoptosis was demonstrated by gel electrophoresis, providing the first clear biochemical evidence for apoptosis.¹³ An understanding of apoptosis process at the genetic and molecular level was initiated in 1986, when Horvitz, Nobel Prize laureate of 2002, and Ellis discovered a set of genes in the nematode *C. elegans* that were involved in apoptosis.¹⁴ These genes were later found to have homologues in a vast number of organisms, including humans. Since then, the list of apoptosis-related genes has expanded and our understanding of the complex network of pathways forming the apoptosis machinery has increased dramatically.

Induction of apoptosis

Apoptosis is usually considered somewhat synonymous with caspase activation. Caspases are cysteine proteases that can be viewed upon as the “executioners” of the apoptosis machinery. They are present as non-active zymogens, pro-caspases, until proteolytic cleavage transforms them into active caspases. Some caspases, such as caspase 8 and 9, are initiator caspases that cleave and activate other caspases, such as caspase 3, known as effector caspases. The precise order in which these caspases are activated is still obscure. Once activated, effector caspases cleave a vast number of cellular proteins (reviewed in¹⁵) including apoptosis regulators, cell cycle regulators, structural proteins, and proteins involved in DNA replication and repair. The cleavage of these proteins ultimately gives rise to the functional and morphological changes - membrane blebbing, DNA cleavage, cell shrinking, and pro-engulfment-signals - that are hallmarks of apoptosis. Finally, the apoptotic cells are recognized and ingested by phagocytes. This final step in apoptosis was previously thought of as mere waste disposal. However, increasing evidence (reviewed in¹⁶) suggests that the engulfment of apoptotic cells by phagocytes also leads to suppression of inflammation, regulation of immune response and modulation of cell killing.

The end result of apoptosis is universal, but apoptosis can be induced by a multitude of stimuli, engaging many different signaling pathways. In principle, there are two alternative major pathways through which apoptosis can be initiated: the mitochondrial (intrinsic) pathway, and the death receptor (extrinsic) pathway. These pathways will be discussed below.

The mitochondrial pathway

The mitochondrial pathway of apoptosis is activated by DNA damage, cell cycle deregulation, hypoxia, and growth factor withdrawal. Signals induced by these stimuli lead to permeabilization of the outer membrane of the mito-

chondria, promoting the release of cytochrome *c*. When cytochrome *c* is released, it associates with apoptotic protease activating factor 1 (Apaf-1), allowing for the recruitment of an inactive initiator caspase, pro-caspase 9. The resulting protein complex, the apoptosome, enables the activation of procaspase 9 into caspase 9. Caspase 9 then activates executioner caspases such as caspase 3, 6, and 7.

The events leading up to the permeabilization of the mitochondrial membrane and the subsequent formation of the apoptosome are not completely elucidated, but it is clear that the Bcl-2 protein family plays a crucial role (reviewed in¹⁷). These proteins are recognized by the presence of one or more Bcl-2 homology (BH)-domains, which allow them to dimerize in a homo- and heterodimeric manner and which define their function.

Anti-apoptotic Bcl-2 family members such as Bcl-2, A1, Bcl-X_L and Mcl-1 have four BH domains, BH1-BH4. Pro-apoptotic members can be divided into the multidomain group harboring domains BH1-BH3 (Bax, Bak, Bcl-rambo) and the BH3-only group consisting of Bim, Bik, Bad, Bid, Harakiri, Noxa and Puma. The anti-apoptotic members associate with the mitochondrial membrane and maintain its integrity, thereby preventing cyt. *c* release. Death signals promote the activation of the BH3-only proteins, allowing these pro-apoptotic proteins to dimerize with anti-apoptotic Bcl-2 members. This dimerization counteracts and neutralizes the pro-survival function of the anti-apoptotic Bcl-2 members, thus promoting apoptosis. The multidomain Bcl-2 proteins Bax and Bak promote apoptosis by homo-oligomerizing in the outer membrane of the mitochondria and promoting its permeabilization, possibly by forming pores or by facilitating the opening of already existing pores (reviewed in^{17,18}).

The death receptor mediated pathway

The family of death receptors is a sub-group of the Tumor necrosis factor receptor (TNFR) family, a number of homotrimeric proteins with a structurally similar extracellular domain and a cytoplasmic domain. The death receptors differ from the other family members in that the proteins display a so-called death domain (DD), a cytoplasmic domain containing a conserved stretch of approximately 80 amino acids, that has been attributed an essential function in apoptosis signaling (reviewed in¹⁹). Most of the death receptors' cognate ligands are trans-membrane proteins that also exist in soluble forms. Like the death receptors themselves, the death receptor ligands are homologous to each other. The principal mechanism of apoptosis induction through death receptors is caspase-8 recruitment and activation following ligand binding. Two of the death receptor systems that have been most extensively

studied in the past years are the Fas/Apo-1/CD95 system and the TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) system.

Fas/Apo-1/CD95

Fas/Apo-1/CD95 is perhaps the best-characterized death receptor. It is expressed as a pre-associated homotrimer on the surface of many cell types, with a particularly high expression in thymus, liver, heart and kidney. The expression of its ligand (L) FasL is restricted to lymphocytes and a few other cell types. Binding by FasL induces clustering of Fas, which attracts the adaptor protein Fas associated death domain protein (FADD) to the death domain at the intracellular part of the receptor. FADD facilitates the recruitment of pro-caspase 8, which together with the receptor and FADD forms the so-called death-inducing signaling complex (DISC).²⁰ In analogy with the previously mentioned apoptosome, the formation of the DISC facilitates the auto-proteolytical cleavage and activation of pro-caspase 8. Caspase 8 activates downstream initiator caspases, which in turn activate the effector caspases that mediate the end point of apoptosis. By an alternative route, caspase 8 also activates the Bcl-2 member Bid, promoting apoptosis via the mitochondrial pathway.

Apoptosis induced by the Fas system has rendered much interest in light of its involvement in several physiological and pathological functions (reviewed in²¹⁻²³). The Fas system plays an important role in maintaining homeostasis in the immune system by inducing apoptosis in activated cytotoxic T cells at the end of an immune response, and eliminating auto-reactive lymphocytes.^{21,24} Fas/FasL interaction is also believed to be the mode of action for elimination of inflammatory cells at “immune-privileged” sites, such as the testis or the retina of eye, where destructive inflammatory responses cannot be tolerated.^{25,26} In addition, Fas/ FasL-induced apoptosis is associated with inflammatory tissue damage in alcohol-induced hepatitis,²⁷ graft-versus-host-disease²⁸ and autoimmune diseases.^{29,30}

In addition to the perforin/granzyme system, T cells and NK cells utilize the Fas system to induce apoptosis in virus-infected cells and tumor cells in the process of immune surveillance.²¹ It is therefore not surprising that resistance to Fas-induced apoptosis is frequently seen in the progression of cancer. According to the somewhat controversial Fas counterattack model, Fas-resistant tumor cells can actively kill T cells by utilizing FasL expressed on the tumor cell (reviewed in²²). This phenomenon has also been described in MM cell lines.³¹

Resistance to Fas is not only a mean to evade immune surveillance, but may also have important implications for the sensitivity to therapy (reviewed in³²). Several reports have indicated that the Fas system is a mediator of

apoptosis induced by several chemotherapeutic drugs, including bleomycin, cisplatin, methotrexate and doxorubicin. These drugs up-regulate the expression of FasL, and antagonistic FasL antibodies abrogates drug-induced apoptosis.³³⁻³⁵ However, the view that death-receptor signaling is involved in drug-mediated cell death has been challenged by other reports in which abrogation of Fas signaling did not confer drug resistance.^{36,37} The discrepancies between these studies may be explained by the relative contribution of the death receptor mediated pathway and the mitochondrial pathway, respectively, depending on the particular drug and type of cell. Scaffidi et al. have proposed that there are two different cell types, I and II. In type I cells, Fas-induced apoptosis is independent on the mitochondria since caspase-8 is more efficiently activated. In type II cells, on the other hand, less caspase-8 is recruited to the DISC and therefore apoptosis induced in these cells is dependent on Bid cleavage and the activation of the mitochondrial pathway.³⁸

Resistance to Fas-induced apoptosis is commonly seen in MM and has been associated with variability in Fas expression in some cases^{39,40} but not in others.⁴¹ A decreased sensitivity to Fas-induced apoptosis has also been connected with IL-6 mediated down-regulation of the pro-apoptotic MAPK pathway component c-Jun,⁴² decreased Bax expression,⁴⁰ and Stat3 dependent up-regulation of Bcl-X_L.⁴³ We have previously demonstrated that the sensitivity to Fas-induced apoptosis can be restored by administering type I and type II interferon (IFN) prior to stimulation by Fas-agonistic antibodies.⁴⁴ A continuation of this study is presented in paper I in this thesis.

Apo2L/TRAIL

At the dawn of death receptor research, TNF was a promising anti-cancer agent. Unfortunately, catastrophic side effects such as septic shock prevented the clinical use of TNF. When Fas was discovered, it was suggested that FasL could potentially be used in cancer therapy *in vivo*. Again, this view had to be revised after the discovery that when administered systemically in tumor-bearing mice, recombinant FasL or agonistic anti-Fas antibody induced apoptosis in normal hepatocytes.^{45,46} With the identification of another TNF member, TRAIL, the hope of exploiting death receptors in cancer therapy rose again.

TRAIL was discovered as a result of the Human Genome Project and was defined purely on the basis of its sequence homology to the other TNF family members, with the closest resemblance to FasL. In contrast to FasL, TRAIL is highly promiscuous. Its ability to engage as many as five different proteins, TRAIL-R1-4 and the soluble TNFR member osteoprotegerin (OPG), is not only exceptional in comparison with other TNF family members, but actually makes it the most promiscuous cytokine known. Intrigu-

ingly, two of the receptors that are bound by TRAIL, TRAIL-R3 and TRAIL-R4, lack a cytoplasmic domain and may act as “decoy receptors” (reviewed in⁴⁷). These decoy receptors have been proposed to mediate another main difference between the Fas system and the TRAIL system, namely the differential sensitivity to TRAIL in tumor cells as compared with normal cells.

Much excitement was raised when it was reported that TRAIL was a potent inducer of apoptosis in tumor cells, but not in normal cells.^{48,49} Since then, numerous reports have demonstrated the efficacy of TRAIL as an apoptosis-inducing agent in cancer, including MM.^{50,51} Originally the idea was that normal cells, in contrast to tumor cells, express nonfunctional decoy receptors that bind TRAIL without transmitting a death signal, thus lending the normal cells protection from TRAIL. This theory has not been validated since no clear correlation between decoy receptor expression and TRAIL sensitivity has been found.

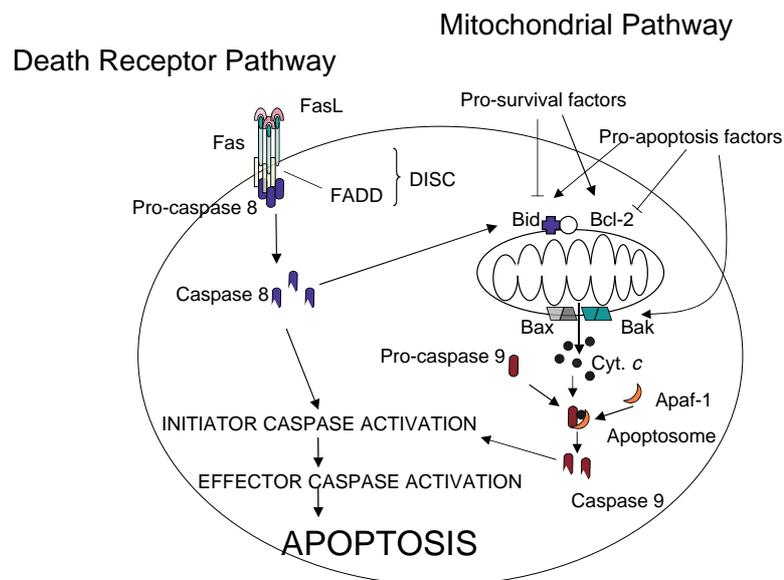


Figure 1. The death receptor pathway, exemplified by the Fas system, and the mitochondrial pathway. Binding of the FasL to its receptor Fas induces receptor clustering and the recruitment of FADD and pro-caspase 8, forming the DISC. Pro-caspase 8 is cleaved into caspase 8, which induces apoptosis through the activation of other caspases. Caspase 8 also activates Bid. Bcl-2 resides in the mitochondrial membrane and maintains its integrity. Bid can heterodimerize with Bcl-2, counteracting its pro-survival function. Bax and Bak homodimerize and promote permeabilization of the mitochondrial membrane, resulting in cyt. c. release. Cyt. c and Apaf-1 form a complex with pro-caspase 9 designated the apoptosome, inducing the activation of pro-caspase 9 into active caspase 9 and subsequent activation of downstream caspases.

The initial excitement over TRAIL as the perfect anti-cancer drug has faltered somehow after reports of toxic effects of some TRAIL variants on normal hepatocytes.^{52,53} However, promising trials in non-human primates indicating that short-term intravenous administration of a non-tagged, Zn-bound TRAIL is well tolerated^{54,55} have offered new hope for TRAIL as a possible therapeutic drug target. Agonistic antibodies against the TRAIL receptor and a soluble, truncated TRAIL are currently in phase I/II trials.⁵⁶

Inhibition at the level of caspase activation: IAPs

Both the mitochondrial pathway and the death receptor mediated pathway ultimately lead to the activation of caspases. This implies that apoptosis induced by either of these pathways can be blocked by direct inhibition of caspase activation. A protein family capable of such an inhibition, the Inhibitors of Apoptosis (IAPs) family of proteins, has rendered much interest in the past years.

The IAPs were originally identified in baculoviruses.⁵⁷ At least eight human homologues of IAPs have so far been identified, including cellular IAP1 (c-IAP1), c-IAP2, X-chromosome-linked IAP (XIAP) and survivin (reviewed in⁵⁸). The common structural feature of the members of the group is at least one highly conserved zinc-binding domain, the baculovirus IAP repeat (BIR). IAPs may also possess CARD (caspase activation and recruitment domain) and RING (really interesting new gene) domains. The BIR domain is probably essential^{59,60} for the ability of IAPs to bind directly to caspases 3, 7 and 9 and inhibit their activation.^{61,62} In addition to directly inhibiting caspases, IAPs can influence cell cycle progression, protein degradation and caspase-independent signal transduction cascades (reviewed in⁶³).

Several studies suggest a role for IAPs in tumor progression. Survivin has clearly been associated with the malignant phenotype by its expression in transformed cell lines and many cancers but not in normal adult tissue.⁶⁴⁻⁶⁶ c-IAP1 is over-expressed in esophageal squamous cell carcinomas⁶⁷ and the c-IAP2 locus is translocated in mucosa-associated lymphoid tissue (MALT) lymphoma.⁶⁸ In addition, high expression levels of XIAP have been correlated with poor prognosis in patients with acute myelogenous leukemia.⁶⁹

In vitro experiments have shown that over-expression of XIAP and survivin confers resistance to chemotherapy and to apoptosis induced by both the mitochondrial and the death receptor mediated pathway. Accordingly, knocking out these IAPs induces apoptosis and sensitization to chemotherapy.⁷⁰ In animal models, XIAP and survivin antisense oligonucleotides delay tumor growth in lung⁷¹ and gastric cancer xenografts⁷², respectively. More-

over, XIAP- and survivin inhibitors do not seem to be toxic to normal adult cells.⁷⁰ All these findings point to IAPs as interesting therapeutic targets. Different XIAP targeting approaches are currently in clinical trials phase I for solid tumors.⁷³ In Paper IV of this thesis, we have examined the expression and regulation of IAPs in MM

Factors influencing MM survival

There are two special features of MM biology that strongly influence the susceptibility of MM cells to apoptosis. One is the high degree of genetic aberrations in MM cells and one is the close contact with the bone marrow microenvironment.

The unusually high degree of genetic aberrations is a consequence of the fact that the MM cell is derived from a post germinal center B cell that undergoes VDJ recombination, somatic hypermutation and immunoglobulin heavy chain (IgH) switch recombination as part of the process of differentiating into a plasma cell. These DNA modification events will often give rise to errors such as double-strand DNA breaks that mediate translocations to the heavy chain IgH locus. IgH translocations are present in 65-75% of biopsy specimens of MM. Ig loci have very strong promoters and consequently, genes that are translocated to these loci will be strongly over-expressed. Some common IgH translocation partners that have been defined are Cyclin D1, D2, and D3, FGFR3, and c-MAF. Some non-B cell specific translocations of e.g. c-myc, IRF4 and MafB that do not involve the IgH locus are present in approximately 5% of MM patients.^{74,75} De-regulations of N-ras and K-ras,⁷⁶ and inactivation of p53 and Rb1⁷⁷ have also been described.

MM cells reside almost exclusively in the bone marrow. The bone marrow microenvironment consists of the extracellular matrix and bone marrow stromal cells (BMSC): fibroblastic stromal cells, osteoblasts, osteoclasts, lymphocytes, and vascular endothelial cells. The reciprocal interaction of MM cells with this microenvironment provides the malignant cells with important growth- and survival signals, mediated by cytokines, adhesion molecules and cell-to-cell-contacts (reviewed in⁷⁸).

Importantly, the genetic heterogeneity of MM and the fundamental importance of the microenvironment are factors that put high demands on the availability of relevant model systems. At present, approximately 80 MM cell lines have been established, representing many different genotypes. The difficulty in establishing MM cell lines lie in the slow proliferative activity of the cells and the dependence of the MM cells on the BMSC. Therefore, in the majority of cases, these cell lines have been established from advanced disease stages of MM and plasma cell leukemia in which the cells have dis-

seminated from the bone marrow. If Epstein Barr Virus (EBV)-transformed lymphoblastoid cells are present in the primary culture, there is a risk that these cells will quickly outgrow the MM cells due to less stringent growth requirement. Therefore, the authenticity of MM cell lines is a crucial issue.⁷⁹ A few in vivo MM models that are suitable for the study of tumor-host interactions have been generated. The 5TMM mouse models originate from spontaneously developed MM in aged C57B1KaLwRij mice.⁸⁰ These models represent human MM well concerning the localization to the bone marrow as well as clinical, biological and genetic characteristics.⁸¹ Another in vivo model is the severe combined immune deficient human (SCID-hu) myeloma model in which primary human MM cells grow in a human fetal bone implanted into a SCID mouse host.^{82,83}

The genetic aberrations present in a MM cell and the microenvironment that the MM cell is exposed to are factors that highly influence the sensitivity to apoptosis signals and thereby contribute to tumor progression and therapy resistance. An account of all the specific factors that may contribute to MM death and survival is clearly beyond the scope of this thesis. However, I will give a brief overview of some of the factors that have been the focus of the investigations presented here.

IL-6

Interleukin (IL)-6 was originally identified as a factor that induces differentiation and immunoglobulin production in normal B lymphocytes.⁸⁴ There is now ample evidence that IL-6, released from the surrounding bone marrow stromal cells,⁸⁵ is a major regulator of growth and survival in MM (reviewed in^{86,87}). Treatment with IL-6 antibodies potently inhibits proliferation of MM cells in vitro⁸⁸⁻⁹⁰ and suppresses the growth of MM cells in vivo.⁸⁵ Furthermore, plasma cell tumors fail to develop in IL-6-null-mice.^{91,92} In addition, IL-6 protects MM cells from apoptosis induced by serum starvation,⁹³ by the glucocorticoid dexamethasone (Dex)^{93,94} and by Fas.⁹⁵

IL-6 signaling activates both the Ras-dependent mitogen-activated protein kinase (Ras/MAP kinase) pathway and the janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (reviewed in⁹⁶). The pro-survival effects of IL-6 in MM have been attributed to activation of Stat3 (discussed below) and up-regulation of Bcl-X_L^{43,97} and Mcl-1,⁹⁷ whereas IL-6 dependent activation of the Ras/MAPK pathway has been implicated in MM growth.⁹⁸

Although IL-6 is considered to be a main MM growth and survival factor, only a subset of primary myeloma cells and cell lines respond to IL-6.^{89,99-101}

In a few cases, it has been suggested that unresponsiveness to IL-6 may also be acquired during the course of tumor progression as a consequence of the development of an autocrine IL-6 loop.^{90,99,100} Accordingly, the U-266-1970 cell line, which is the model system used in paper I and II of this thesis, is IL-6 dependent, whereas U-266-1984, a later passage of the same cell line, has acquired IL-6-independency during in vitro progression.⁹⁰

Interferons

Type I (predominantly α and β) and type II (γ) interferons (IFNs) were first recognized for their anti-viral effects. In their role as the first line of defense against viruses in higher vertebrates, these cytokines have evolved numerous ways to inhibit virus replication (reviewed in¹⁰²). To name but a few of these mechanisms, anti-viral effects mediated by interferons include induction of transcriptional inhibition, RNA cleavage, and inhibition of protein synthesis. In addition, interferons have profound effects on many biological processes, including immune response, proliferation, differentiation and apoptosis.¹⁰²

Interferons have been widely used in the treatment of cancer, including MM. However, the benefit of IFN treatment in MM is under debate. Although IFN- α is widely used as maintenance therapy following conventional chemotherapy and stem cell transplantation, the therapeutic effectiveness varies greatly¹⁰³ and the adverse effects, including cases of development of plasma cell leukemia,^{104,105} are substantial. Nevertheless, IFNs are interesting effectors in light of their ability to increase the sensitivity to apoptosis by death-receptors and other apoptosis-inducing agents. IFNs have been shown to sensitize cells for apoptosis induced by Fas,^{44,106} TRAIL,^{107,108} TNF- α ¹⁰⁹⁻¹¹¹ and doxorubicin.¹⁰⁷ These studies point to the possibility that IFN pathways or components thereof could be exploited to increase the efficacy of therapy in MM. In paper I of this thesis, the mechanism underlying IFN-mediated sensitization to Fas-induced apoptosis in MM is examined.

Stat1 and Stat3

Both IFNs and IL-6 activate signal transducers and activators of transcription (STATs), a family of transcription factors involved in regulation of growth and survival of hematopoietic cells. As the name implies, STATs do not only transduce signals from the cells surface into the cell, but also participate directly in gene regulation. To date, seven STAT proteins have been discovered: Stat1-4, Stat5a, Stat5b, and Stat6. Upon ligand binding of cytokine receptors, STAT proteins are tyrosine phosphorylated by Janus Activated Kinases (JAKs) associated with the receptor. The tyrosine phosphory-

lation allows the STAT proteins to hetero- or homodimerize, translocate to the nucleus, and activate transcription of specific genes.¹¹² Additional transcriptional control is mediated by phosphorylation of a conserved serine residue.¹¹³

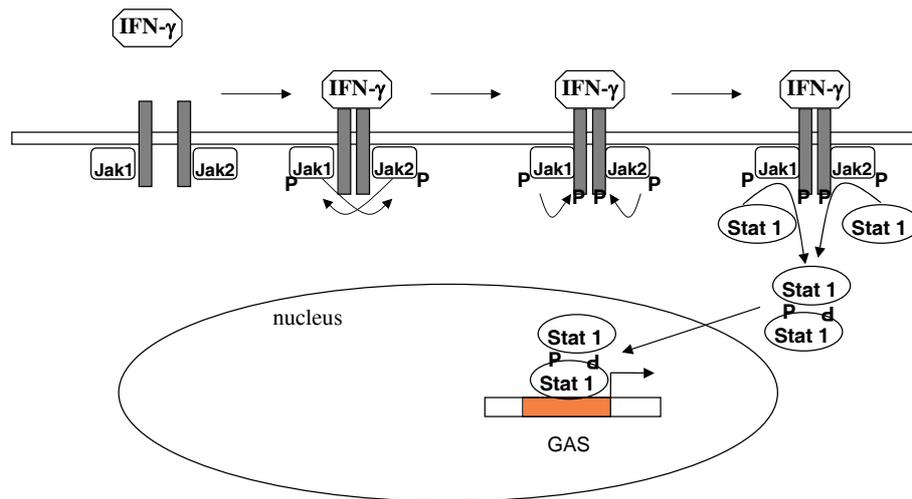


Figure 2. JAK/Stat signaling, exemplified by Stat1 activation by IFN- γ signaling. Jak tyrosine kinases Jak 1 and Jak 2 are non-covalently attached to the cytoplasmic part of the IFN- γ receptor. When IFN- γ binds, the receptor dimerizes, bringing the Jaks in close proximity to each other and enabling them to phosphorylate one another as well as tyrosines on the C-terminal part of the receptor. The phosphorylated tyrosine residues attract SH2 domains on Stat1 molecules. The Stat1 molecules, in turn, become tyrosine phosphorylated, which allows them to form homodimers in which each Stat1 molecule uses its SH2 domain to bind to the phosphotyrosine of its partner. These dimers can translocate into the nucleus and influence the transcription of specific genes through interaction with gamma activation sites (GAS) on the promoters of these genes. For full transcriptional activity, the homodimers are also phosphorylated on serine residues (not shown).

Stat3 is reportedly activated in a number of human cancers (reviewed in¹¹⁴) including MM^{43,115} and has been defined as an oncogene in light of its ability to mediate cellular transformation and block apoptosis.¹¹⁶ Stat3 activation is associated with up-regulation of cyclin D1,¹¹⁷ c-Myc,¹¹⁸ and Mcl-1¹¹⁹ (reviewed in¹²⁰). In MM, Stat3 has been demonstrated to mediate the pro-survival effects of IL-6. Blocking Stat3, either by transfection with a dominant negative form or by use of the JAK-inhibitor AG490, induced down-regulation of the pro-survival protein Bcl-X_L and spontaneous apoptosis.^{43,121} In addition, MM cells can be sensitized to therapeutic drugs including cis-

platin, fludarabine, adriamycin, and vinblastine by inhibiting Stat3.¹²² These studies point to a crucial role of Stat3 in promoting IL-6-induced MM survival.

In contrast to Stat3, Stat1 has been suggested to be a potential tumor suppressor. Targeted disruption of Stat1 promotes spontaneous tumor formation in mice,¹²³ whereas activation of Stat1 has been associated with growth suppression^{124,125} and down-regulation of c-Myc.¹²⁶ In addition, Stat1 has been shown to be critically involved in ischemia-induced apoptosis in cardiomyocytes,¹²⁷ IFN- γ -induced apoptosis in ME180 cells¹²⁸ and TNF- α -induced apoptosis in fibroblasts.¹²⁹

Tumors that express activated Stat3 often express activated Stat1 as well.¹³⁰ Also, extrinsic signaling by some cytokines, such as IL-6, induces activation of both Stat1 and Stat3.¹³¹ It has been suggested that the levels of activated Stat1 and Stat3 balance each other in normal cells, and that a shifted balance towards Stat1 or Stat3 can lead to enhanced apoptosis or survival, respectively. Thus, Stat1 and Stat3 may be able to counteract each other's effects. Several reports provide support for this theory. Stat3 expression and activation is enhanced in Stat1 null cells¹³² and, conversely, Stat1 expression and activation is enhanced in Stat3 null cells.¹³³ IFN- γ -induced, Stat1 mediated apoptosis of ME180 cells can be counteracted by transfection with a constitutively activated Stat3.¹³⁴ Furthermore, Stat1 and Stat3 play opposing roles in cardiomyocytes exposed to ischemia. Stat1, which is associated with ischemia-induced apoptosis, down-regulates the anti-apoptotic proteins Bcl-2 and Bcl-X_L, while Stat3, induced by a cardioprotective factor, up-regulates these genes.¹³⁵ In addition, Hong et al. recently demonstrated a causal relationship between Stat1 activation and apoptosis, and between Stat3 activation and survival, in T cell mediated fulminant hepatitis.¹³⁶

We hypothesize that in MM, pro-survival, IL-6-induced Stat3 can be counteracted by pro-apoptotic, IFN-induced Stat1, and that this may be a mechanism underlying the previously described IFN-induced sensitization to Fas-induced apoptosis.⁴⁴ In paper I in this thesis, we have shown that IFN-mediated up-regulation and activation of Stat1 is accompanied by an attenuation of Stat3 expression and activation. We explore the role of Stat1 in MM further in paper II, in which we demonstrate the establishment and characterization of sub-lines of the MM cell line U-266-1970 with an ectopic over-expression of Stat1.

Bcl-2 family proteins

The influence of growth factors such as IL-6 and IGF-I, as well as genetic alterations that accumulate in the MM cells as the tumor progresses, may lead to an altered expression and activation of several pro- and anti-apoptotic proteins of the Bcl-2 family. For instance, IL-6 deprivation down-regulates the expression of Bcl-2, Mcl-1 and Bcl-X_L, whereas pro-apoptotic Bax is increased.¹³⁷

Bcl-2 itself was the first Bcl-2 protein to be recognized as an important mediator of survival in MM. Bcl-2 is highly expressed both in primary patient MM cells and in MM cell lines.¹³⁸⁻¹⁴¹ In the MM cell line U-266-1970, Bcl-2 expression is increased due to a gene amplification of the Bcl-2 gene.¹⁴¹ Bcl-2 over-expression has been associated with MM drug-resistance towards dexamethasone,^{142 143} paclitaxel,^{143,144} doxorubicin and etoposide,¹⁴⁵ and bortezomib.¹⁴⁶ Bcl-2 is an attractive therapeutic target, and many different approaches to inhibit its expression and activity are currently being explored in MM research. Clinical phase II trials have suggested that Bcl-2 antisense oligodeoxynucleotides (Genasense, G3139, oblimersen) may potentiate the response to vincristine, adriamycin and dexamethasone (VAD)¹⁴⁷ as well as the combination of dexamethasone and thalidomide in MM patients.¹⁴⁸ However, in a randomized phase III trial there was no significant difference in time-to-disease-progression when Bcl-2 antisense was added together with dexamethasone.⁷³

Bcl-X_L has been suggested to mediate the pro-survival effects of the MM survival factor IL-6 downstream of Stat3 activation.⁴³ In addition, ectopic expression of Bcl-X_L induces resistance to Fas-induced apoptosis in MM.¹⁴⁹ In biopsies from MM patients, high expression levels of Bcl-X_L correlate with decreased response to several chemotherapeutic agents.¹⁵⁰

More recently, Mcl-1 has emerged as an important survival factor in MM. It is over-expressed in MM cells as compared to normal cells^{137,151} and like Bcl-X_L, it is induced by Stat3.⁹⁷ When comparing the effect of antisense oligonucleotides against Bcl-2, Bcl-X_L and Mcl-1 in MM cell lines and primary cells, Mcl-1 antisense induced a stronger reduction in viability than any of the other proteins.¹⁵² Interestingly, the Mcl-1 gene can undergo differential splicing, yielding a shorter fragment, Mcl-1 short (Mcl-1_S) where the BH3-domain remains intact but the BH1-, BH2, and transmembrane encoding domains are destroyed. Thereby, a pro-apoptotic BH3-only protein is formed.¹⁵³

Insulin-like growth factor (IGF-I)

Next to IL-6, IGF-I is likely to be the most important growth- and survival factor in MM. The role of IGF-I as a MM survival factor is highly relevant, since IGF-I is produced by, among others, the BMSCs in the MM microenvironment.

IGF-I signaling occurs mainly through the type I insulin-like growth factor receptor (IGF-IR). This receptor is highly homologous to the insulin receptor (IR), especially in the receptor tyrosine kinase (TK) domain. The IGF-IR was originally viewed upon as a somewhat inferior cousin to the IR, used by the cells only when signaling from the insulin receptor was defective or absent.¹⁵⁴ However, there is now ample evidence that although the IGF-IR and the IR are functionally and structurally similar, they have some important functions that clearly distinguish them from each other. Whereas IR-signaling regulates glucose uptake and metabolism, the IGF-IR plays an important role in regulating mitogenesis, protection from apoptosis, and the establishment and maintenance of the transformed state in cancer cells (reviewed in¹⁵⁴).

IGF-I has been identified as a factor mediating survival and resistance to cytotoxic drugs in MM.¹⁵⁵⁻¹⁵⁷ IGF-I stimulation mediates protection from apoptosis induced by serum starvation,¹⁵⁵ Apo2L/TRAIL,¹⁵⁸ and the glucocorticoid dexamethasone.¹⁵⁹⁻¹⁶² In addition, it has been suggested that the levels of IGF-I in serum is a prognostic factor in MM patients.¹⁶³

Binding of the IGF-I ligand to the IGF-IR induces transphosphorylation of the receptor units and tyrosine phosphorylation of the adaptor proteins Shc and the IR substrate (IRS)-1 and -2. This in turn leads to activation of two main signaling pathways, the Ras/MAPK pathway and the phosphoinositol 3-kinase/Akt (PI3K/Akt) pathway, as well as the stress activated protein kinase pathway. The relative contribution of these different pathways in mediating the proliferative and anti-apoptotic effects of IGF-I in MM has been addressed in several reports.¹⁶⁴⁻¹⁶⁶ IGF-I-activated downstream components of the PI3K/Akt pathway which have been implicated to influence MM survival include GSK3 β , p70S6 kinase, Forkhead transcription factor FKHL1¹⁶⁴ and NF- κ B.¹⁵⁸

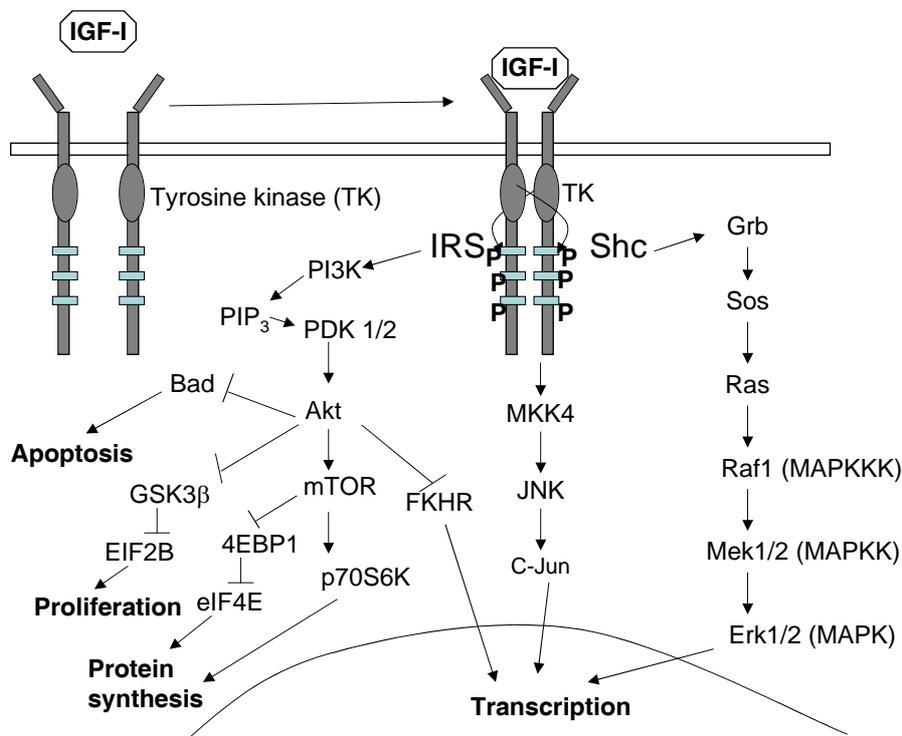


Figure 3. Schematic overview of some of the pathways activated by the IGF-I receptor. In the presence of IGF-I, the receptor dimerizes and the tyrosine kinase domain of each receptor subunit transphosphorylates the C-terminal part of the other subunit. When the receptor is phosphorylated, the adaptor proteins IRS and Shc are recruited. The PI3K pathway is activated downstream IRS proteins, and the Ras pathway is activated downstream Shc. The IGF-IR can also activate the stress activated protein kinases including JNK.

The IGF-IR is an attractive therapeutic target due to its prominent role in promoting growth and inhibiting apoptosis in malignant cells (reviewed in ^{154,167-169}), including MM, ^{155,157,159,162,170} versus its relatively weak involvement in the regulation of the normal cell. ^{154,171}

Strategies aimed at inhibiting IGF-IR signaling include neutralizing antibodies toward the receptor, ¹⁷² dominant-negative receptor mutants ¹⁷³ and anti-sense RNA. ¹⁷⁴ All these strategies have shown that inhibiting IGF-I signaling can induce apoptosis in tumor cells and inhibit tumorigenesis and metastasis. However, they all have drawbacks that may hamper clinical use. Small molecules targeting the tyrosine kinase activity of the receptor ¹⁷⁵⁻¹⁷⁷ have the

advantage of a better bioavailability but must be highly selective for the IGF-IR in order to avoid cross-reactions with the IR and the risk of inducing diabetic responses.

Recently, the cyclolignan picropodophyllin (PPP) has been identified as a selective IGF-IR tyrosine kinase inhibitor that does not co-inhibit the IR. PPP induces apoptosis and cell cycle arrest in malignant cells derived from solid tumors but displays almost no toxicity in normal cells.¹⁷⁸ The effect of PPP in MM is the subject of paper III of this thesis.

The present investigation

Aims

In the studies presented in this thesis we have investigated different aspects of MM death and survival. Our specific aims were

- To identify downstream targets of IFNs in MM that may contribute to sensitization to Fas-induced apoptosis in MM (Paper I)
- To investigate the role of Stat1 in influencing Stat3 activation and drug sensitivity in MM (Paper II)
- To evaluate the effects of the IGF-IR receptor tyrosine kinase inhibitor PPP in MM cells (Paper III)
- To determine the expression of IAPs in primary MM cells, MM cell lines, and other B cell lines, and to investigate whether IAPs are regulated by dexamethasone and IGF-I in MM (Paper IV)

Results and discussion

Paper I: Ectopic and IFN-induced expression of Fas overcomes resistance to Fas-mediated apoptosis in multiple myeloma cells

Although MM cells have a high surface expression of the Fas receptor, they are quite insensitive to apoptosis induced by the Fas/FasL system. In 1998, our group reported that in the MM cell line U-266-1970, the sensitivity to Fas-induced apoptosis could be enhanced if the cells are pre-treated with IFNs.⁴⁴ The year after, Catlett-Falcone et al. published a paper stating that constitutively active Stat3 mediates survival in MM cell line U-266 by inducing Bcl-X_L expression.⁴³ It is well known that IFNs induce Stat1, which in contrast to Stat3 has been suggested to promote apoptosis rather than survival. Several reports suggest that the balance between activated Stat1 and Stat3 may be an important determinant of survival.¹³⁴⁻¹³⁶ In view of these findings, we hypothesized that the Stat1-activating actions of IFNs may counteract IL-6-induced Stat3 and thereby shift the balance from survival to apoptosis by influencing the gene expression of apoptosis-related genes. The object of this work was to investigate this hypothesis, and to identify downstream IFN targets participating in such a sensitization process.

In order to search for downstream targets of IFN stimulation involved in apoptosis regulation, we analyzed the mRNA expression in untreated and IFN-treated U-266-1970 cells using ribonuclease protection assay (RPA). Bcl-X_L, identified by Catlett-Falcone as a mediator of Stat3-induced survival,⁴³ was unaffected by IFNs. Similarly, we could not find evidence for a regulation of any other Bcl-2 member by IFNs. However, two genes were found to be up-regulated following IFN-treatment, the Fas receptor and Apo2L/TRAIL. The up-regulation of these genes was also observed in IFN-treated primary cells from MM patients. We set out to determine whether any of these genes were important for the IFN-mediated sensitization to Fas-induced apoptosis.

TRAIL is structurally similar to FasL and the pathways employed by these inducers share many downstream components, suggesting a plausible basis for synergy between these two systems. Furthermore, both Fas and TRAIL

are likely to be affected by a shift in Stat activation. Fas is transcriptionally up-regulated by Stat1 and down-regulated by Stat3,¹⁷⁹⁻¹⁸¹ and the TRAIL promoter contains putative Stat1 binding sites.¹⁸² To find out whether TRAIL was involved in IFN-mediated sensitization of Fas-induced apoptosis, we treated the cells with IFN in the presence or absence of a TRAIL inhibitor. The proportion of apoptotic cells following treatment with the agonistic Fas antibody CH-11 was then assayed using flow cytometry. We found that the TRAIL inhibitor did not affect the susceptibility to Fas-induced apoptosis, implying that IFN-mediated sensitization to Fas-induced apoptosis is likely to be independent of TRAIL.

The high surface expression of Fas in U-266-1970 MM cells implies that the supply of Fas receptors may not be the limiting factor in apoptosis sensitivity. In addition, previous studies have failed to find a correlation between Fas expression and Fas sensitivity in MM cell lines.^{41,183,184} However, we found that U-266-1970 cells expressing particularly high levels of Fas after IFN treatment were more prone to undergo apoptosis following FasL exposure. In addition, in untreated cells that had been sorted according to Fas expression using FACS, the group exhibiting a high Fas expression was more sensitive to FasL than the low-expressing group. Importantly, ectopic expression of Fas was coupled to a 50 % increase in apoptosis sensitivity, further demonstrating the functional importance of an elevated Fas expression.

Next, we wanted to examine the impact of IFN treatment on the activation of Stat1 and Stat3. To this end, we assayed the quantity of phosphorylated, activated Stat proteins in IFN-stimulated U-266-1970 cells by Western blot. In addition, the binding of Stat proteins to a gamma-activated site (GAS) DNA probe was quantified using electrophoretic mobility shift assay (EMSA). We found that IFNs actually attenuated Stat3 phosphorylation and activation in addition to inducing Stat1 activation. To our knowledge, deactivation of Stat3 by IFNs has not previously been described. When we compared protein complex binding to a GAS site in cells that were treated with IL-6 to cells treated with both IL-6 and IFN, we found that IFNs shifted the predominance of Stat3 homodimers in favor of Stat1 homodimers and, to a lesser extent, Stat1/Stat3 heterodimers.

Based on the data presented in paper I, we propose that one mechanism by which IFN increases the susceptibility to Fas induced apoptosis in U-266-1970 cells is by an up-regulation of the Fas gene. We were also intrigued by the fact that long-term IFN-induced activation of Stat1 was accompanied by deactivation of Stat3. This finding prompted us to further investigate the importance of Stat1 activation in MM apoptosis sensitivity. The role of Stat1 in MM is the subject of paper II.

Paper II: The effect of Stat1 over-expression on the apoptosis-related gene profile and drug sensitivity of multiple myeloma cells

In the study presented in paper I, we had demonstrated that IFN-induced Stat1 expression and activation was associated with attenuation of Stat3 activation. Given the established role of Stat3 activation in MM survival, we hypothesized that an increased Stat1 activation per se would be predictive of an increased sensitivity to apoptosis induced by e.g. Fas. To investigate this hypothesis, we established sub lines of the MM cell line U-266-1970 with a stable over-expression of the wild type Stat1 protein (U-266-1970-wtStat1pCI-neo) and of Stat1C (U-266-1970-Stat1C), an active mutant of Stat1 in which Arg-656 and Asn-664 in the SH2-domain have been exchanged for cysteine in order to promote spontaneous dimerization.¹⁸⁵

The sub-lines exhibited a higher expression of Stat1 and of Interferon regulatory factor IRF-1 (a known target of Stat1) as shown by Western blot. We then examined the DNA binding activity and the transcriptional activating properties of the sub-lines in electrophoretic mobility shift assays measuring Stat1 binding to a GAS-site DNA probe, and in luciferase assays using a GAS-site-containing reporter plasmid. We could conclude that the transfected sub-lines exhibited an increased Stat1 over-expression coupled by an enhanced Stat1-mediated DNA binding activity and, accordingly, a more profound effect on the transcription of genes containing a GAS-site as compared to control cells stably transfected with an empty vector. The effect was dose-dependent: the Stat1C-transfected sub-line differed more from the empty vector-transfected cells U-266-1970-pCI-neo than the wtStat1-transfected sub-line did.

We then wanted to evaluate whether an over-expression of Stat1 would influence the expression of genes involved in apoptosis. We quantified the mRNA levels of a panel of 39 apoptosis-related genes using Multiplex Ligation-dependent Probe Amplification (MLPA) analysis. Two genes were consistently up-regulated in the Stat1-over-expressing sub-lines: pro-apoptotic Bcl-2 member Harakiri and the short, pro-apoptotic form of Mcl-1. The pro-apoptotic Bcl-2 protein Noxa was up-regulated in the Stat1C-transfected sub-line but down-regulated in the wtStat1-transfected sub-line, and the anti-apoptotic genes c-IAP1 and Bfl-1/A1 were up-regulated in the wtStat1-expressing sub-line only. On the protein level, we also observed an up-regulation of anti-apoptotic member Bcl-2. All these proteins are likely to influence the sensitivity to drugs that operate through the mitochondrial pathway. In addition, c-IAP1 expression could potentially influence death-receptor mediated apoptosis as well.

In paper I, we had demonstrated an association between IFN-mediated up-regulation of Stat1 and an attenuation of IL-6-induced Stat3 expression and activation. To investigate whether attenuation of Stat3 would be more prominent in Stat1-over-expressing cells, we analyzed the expression of total Stat3 protein and phosphorylated Stat3 protein by Western blot. In concordance with our hypothesis, U-266-1970-Stat1C and U-266-1970-wtStat1pCI-neo exhibited an attenuated basal expression of Stat3 as well as a lower level of Stat3 phosphorylation following IFN- γ -treatment.

Finally, we analyzed whether the enhanced Stat1 expression and activation, altered Bcl-2 protein expression, and attenuated Stat3 expression and activation of the U-266-1970-Stat1C and the U-266-1970-wtStat1pCI-neo sub-line would give rise to a more apoptosis-sensitive phenotype. First, we analyzed the response to FasL-induced apoptosis. We found that in both the Stat1-over-expressing sub-lines, the response to Fas was increased by approximately 50% as compared to the parental cell line, suggesting that Stat1 over-expression may indeed be predictive of increased Fas-sensitivity.

We also analyzed the response to a panel of drugs, some of which are used in MM therapy and some of which have been previously associated with Stat1- or Stat3-regulation, in an FMCA assay. We did not find any significant differences in the sensitivity to any of these drugs, although Stat1-over-expressing cells appeared slightly more resistant to etoposide. We went on to study the effect of Stat1 over-expression in a high-throughput screening of 1266 drugs included in Sigma's LOPAC library. Of these drugs, 18 elicited a survival index of 50% or less. Some of these drugs – disulfiram, iodoacetamide, mitoxantrone, brefeldin A – induced a stronger response in U-266-1970-wtStat1pCI-neo and U-266-1970-Stat1C than in U-266-1970-pCI-neo. Conversely, U-266-1970-wtStat1pCI-neo and U-266-1970-Stat1C were highly resistant to neostigmine bromide, pentamidine isethionate and CGP-74514A as compared to U-266-1970-pCI-neo.

Taken together, our data suggest that Stat1 over-expression alters the expression of several genes that could potentially influence drug sensitivity. Furthermore, we have demonstrated that Stat1 over-expression per se sensitizes MM cells to Fas-induced apoptosis. This finding lends further support to the notion that IFN- γ -mediated sensitization to Fas-induced apoptosis is likely to involve Stat1. However, we conclude that Stat1 over-expression is not associated with a generally increased drug sensitivity, since several of the drugs tested actually were more efficient in U-266-1970-pCI-neo than in the Stat1-over-expressing cell-lines. This seemingly paradoxical finding is in line with the previous observation by Oshiro et al.¹⁴⁹ that the JAK kinase inhibitor AG490, which down-regulates the activity of both Stat1 and Stat3, sensitizes MM cells to Fas-induced apoptosis but induces resistance to topoisomerase

II-inhibiting agents such as mitoxantrone, etoposide, and doxorubicin. Since Stat1 over-expression was associated with Stat3 down-regulation in our cell lines, it is possible that the observed effects are Stat3-dependent. Nevertheless, the findings presented here open up for the possibility that not only Stat3, but also Stat1 may have an influence on the regulation of apoptosis in MM.

Paper III: IGF-I receptor tyrosine kinase inhibition by the cyclolignan PPP induces G₂/M-phase accumulation and apoptosis in multiple myeloma cells

The cyclolignan picropodophyllin (PPP) has recently been described as a potent and selective inhibitor of the IGF-IR in cell lines originating from melanoma, sarcoma, breast sarcoma, and prostate sarcoma.¹⁷⁸ It binds selectively to IGF-IR, specifically blocking phosphorylation of the residue Tyr1136 in the activation loop of the IGF-IR,¹⁸⁶ without inhibiting the IR, FGF-R, PDGF-R or EGF-R.¹⁷⁸ Moreover, despite being a potent inducer of apoptosis in tumor cells, PPP has a very low toxicity in vivo (LD₅₀ > 500 mg/kg in rodents).¹⁷⁸

Together with the study by Menu et al.¹⁸⁷ which was performed in collaboration with our group and which was published in the same issue of *Blood*, Paper III is the first study of PPP in a hematological tumor. In order to evaluate the effect of this inhibitor in MM, we exposed 13 different cell lines, 10 IL-6-independent and 3 IL-6-dependent, to varying concentrations of PPP. We found that every cell line tested responded to PPP treatment by growth inhibition, with an IC₅₀ ranging from 0.2 μM to 1 μM. The IL-6-dependent cell lines were less sensitive to PPP than the IL-6-independent cell lines. PPP could also induce varying degrees of growth-inhibition in primary MM cells from 10 patients.

The growth inhibitory effect was due to both apoptosis and cell cycle arrest. PPP treatment increased the amount of AnnexinV-positive/PI-negative cells more than six-fold and induced a five-fold enhancement of DNA fragmentation. In addition, PPP induced cleavage of caspases 3, 8, and 9 as demonstrated by Western blot. The cell cycle arrest was associated with an accumulation of cells in the G₂/M phase. This is in concordance with previous findings that IGF-I is needed for progression through the cell cycle, especially in the later phases.¹⁸⁸⁻¹⁹⁰

The bone marrow microenvironment provides the MM cells with growth- and survival factors that may limit the response to therapy. To investigate whether the effect of PPP would be decreased in the presence of such fac-

tors, we pre-incubated MM cell lines with IL-6, IGF-I, IGF-II, or the IGF-I analog Long R3-IGF-I prior to PPP exposure. In addition, we tested the effect of PPP on primary MM cells that were cultured on top of bone marrow stromal cells. The effect of PPP was not diminished in any of these cases, suggesting that the survival-promoting effect of the bone marrow microenvironment may not be able to circumvent the effects of PPP.

In an effort to delineate the signal transduction pathways involved in PPP-mediated inhibition of IGF-I signaling, we monitored the effects of PPP on the expression and phosphorylation of several kinases downstream of IGF-I: Erk1/2, Akt, GSK3 β and p70S6K. IGF-I-mediated phosphorylation of Erk1/2 was clearly inhibited by PPP treatment after 2 hours. The IGF-I-mediated phosphorylation of the kinases GSK3 β and p70S6K (Thr389) was attenuated by PPP, but only after 24 hours stimulation, indicating that the effect of PPP on these kinases is probably not directly downstream of ERK1/2.

The anti-apoptotic proteins Mcl-1 and survivin were transiently down-regulated by PPP, whereas anti-apoptotic Bcl-X_L and heat shock proteins Hsp 60, 70, and 90 were unaffected.

Cell cycle progression is dependent on the differential expression and activation of cyclins, which in turn are regulated by cyclin dependent kinases (cdk). PPP inhibited IGF-I-induced phosphorylation of the cdk 1, a cyclin dependent kinase that mainly associates with the cyclin B1 and facilitates cell cycle progression, especially in the G₂ and M phases (reviewed in¹⁹¹). Cdk1 is held in an inactive state by phosphorylation, therefore dephosphorylation of this kinase should be predictive of an increased activity of cdk1. However, using an in vitro kinase assay with histone H1 as a substrate we found that PPP-treatment actually decreases cdk1 activity as compared to IGF-1 treated controls.

Inhibition of cdk1 by using the inhibitor CGP74512A induced a dose-dependent reduction in the relative number of viable cells, suggesting that cdk1-inhibition may be a plausible mechanism of PPP-mediated apoptosis and cell cycle arrest.

As mentioned previously, the high degree of homology between the IGF-IR and the IR is a potential hazard when targeting the receptor tyrosine kinase, since inhibition of the IR could lead to diabetogenic effects.^{169,192} The selectivity of PPP is therefore a crucial issue. Importantly, the previously observed selectivity for IGF-IR in cell lines derived from solid tumors¹⁷⁸ could be demonstrated in MM as well. Autophosphorylation of the IGF-IR was

clearly inhibited by PPP whereas autophosphorylation of the IR was unaffected.

Previous reports have demonstrated that inhibition of IGF-IR can sensitize MM cells to cytotoxic drugs.^{159,162,177} Accordingly, pre-incubation with PPP enhanced the response to dexamethasone, doxorubicin, melphalan, rapamycin and SB203580. Interestingly, in the case of melphalan and doxorubicin, the sensitizing effect was dependent on the time of addition of PPP: when the drugs were added before PPP there was no sensitizing effect, and when PPP and the drug were added simultaneously the response was actually lower than to PPP alone. We are currently evaluating the combinatorial effects of PPP further, using a large panel of cytotoxic drugs

In conclusion, the findings in Paper III indicate that the selective IGF-IR tyrosine kinase inhibitor cyclolignan PPP induces cell cycle arrest and apoptosis in MM cells. PPP-treatment is associated with a decreased phosphorylation of ERK1/2 and a reduced CDK1 activity. Neither the presence of MM survival factors IGF-I and IL-6 nor the presence of bone marrow stromal cells attenuated the effect of PPP, lending further support to the relevance of PPP as a highly promising therapeutic agent in MM.

Paper IV: Sensitization to dexamethasone-induced apoptosis by abrogation of insulin-like growth factor (IGF)-I receptor signaling is associated with regulation of inhibitors of apoptosis proteins (IAPs) in human multiple myeloma cells

In Paper IV, we set out to investigate the role of IAPs in MM. Firstly, we wanted to establish as to what extent these proteins are expressed in MM cell lines and in MM patient material. Secondly, we wanted to explore a possible link between IAP expression and the effects of the MM survival factor IGF-I and the glucocorticoid dexamethasone (Dex), which is frequently used in MM therapy.

Previous reports have suggested the possibility of such a link. IAP members c-IAP1, c-IAP2 and XIAP can be transcriptionally activated by NF- κ B.¹⁹³⁻¹⁹⁵ This transcription factor can, in turn, be activated by the MM survival factor IGF-I.¹⁵⁸ In parallel, inactivation of NF- κ B is a pro-apoptosis mechanism attributed to some therapeutic agents, including Dex.¹⁹⁶ IGF-I rescues MM cells from Dex-induced apoptosis,^{160,197} and, conversely, abrogating IGF-I signaling by use of the antagonistic antibody α IR3¹⁶² or the IGF-R inhibitor PPP¹⁹⁸ enhances Dex-induced apoptosis. Collectively, these findings form a basis for the hypothesis that Dex-induced apoptosis and IGF-I-mediated protection from Dex may affect IAP expression.

Using RPA and RT-PCR to study mRNA expression and Western blot to study protein expression, we could conclude that the IAPs c-IAP1, c-IAP2, XIAP and survivin were expressed in all studied MM cell lines and in cell lines of other B cell origin. The one exception was c-IAP2, which was not expressed in the MM cell line Karpas 707. When studying IAP mRNA expression in primary MM cells from three different patients using RPA, we could detect c-IAP1, c-IAP2 and XIAP, but not survivin mRNA. However, using the more sensitive method of RT-PCR, survivin was detected in three other MM patients.

The expression of survivin is known to be restricted to the G₂/M phase of the cell cycle.¹⁹⁹ Survivin was down-regulated by Dex in MM cell line LP-1 but not in Karpas 707. We found that one difference between these two cell lines is that in LP-1, Dex does not only induce apoptosis, but also cell cycle arrest in the G₀/G₁ phase of the cell cycle. The cell cycle phase restricted expression of survivin was further confirmed by the finding that PPP, that induces cell cycle arrest in the G₂/M phase,¹⁹⁸ induced an up-regulation of survivin.

c-IAP2 mRNA and protein were transiently up-regulated in LP-1 cells following Dex treatment, possibly representing a counteraction of the apoptotic response. To test whether the Dex-mediated transient up-regulation of c-IAP2 took place at the transcriptional level, we performed reporter gene assays using c-IAP2 promoter constructs coupled to the luciferase gene. Dex-treatment failed to induce activation of the c-IAP2 promoter. This indicates that the observed up-regulation of c-IAP2 mRNA and protein following Dex-treatment may not take place on the level of transcription but instead involve other mechanisms, such as stabilization of c-IAP2 mRNA and protein. Dex could up-regulate c-IAP2 in the presence of the translational inhibitor cyclohexamide but not in the presence of transcriptional inhibitor actinomycin D, further supporting post-transcriptional mechanisms of c-IAP2 regulation.

Both c-IAP2 and XIAP protein levels were down-regulated by Dex at 48h. IGF-I did not induce an up-regulation of XIAP or c-IAP2, implying that the protective effect of IGF-I against Dex-induced apoptosis is unlikely to be mediated by c-IAP2. However, abrogation of IGF-IR signaling by PPP or by α IR3 enhanced the down-regulation of c-IAP2 and XIAP.

In conclusion, paper IV demonstrates the basal expression of apoptosis inhibitors c-IAP1, c-IAP2, XIAP and survivin in a panel of MM cell lines and cell lines of other B cell origin, as well as in primary cells from MM patients. Survivin was down-regulated following Dex-induced cell cycle arrest in G₀/G₁, but up-regulated following PPP-induced cell cycle arrest in G₂/M,

reflecting this IAPs cell cycle phase-restricted expression. The down-regulation of c-IAP2 and XIAP protein following Dex-treatment and IGF-IR abrogation suggests that the IAPs may contribute to the sensitization of MM cells to Dex.

Condensed summary

The common theme in this thesis is factors that may influence the sensitivity to apoptosis in MM.

The first paper involves a physiological apoptosis-inducer, the Fas/FasL system. We demonstrate that interferon treatment up-regulates Fas and alters the balance between activated Stat1 and Stat3, thereby enhancing the sensitivity to this type of apoptosis in MM.

In the second paper, we have explored the impact of an altered Stat1/Stat3 balance further by over-expressing Stat1 in an MM cell line. The findings of this study confirm and extend the conclusions of Paper I, but also complicate the picture further by showing that Stat1 may sometimes be predictive of resistance rather than sensitization to drugs depending on the context.

The third paper brings us closer to the clinic by demonstrating how a new promising drug, the cyclolignan PPP, induces apoptosis and cell cycle arrest in MM cells by selectively targeting signaling through the IGF-IR.

Finally, in the fourth paper we have determined the basal expression of the IAP proteins in MM cell lines and primary cells and found that IAPs are widely expressed. In addition, we have identified a plausible role for IAPs in mediating the sensitization to dexamethasone-induced apoptosis in MM.

The data presented in this thesis have brought us yet another little step closer to understanding the complex biology of MM and learning to combat the disease.

Populärvetenskaplig sammanfattning på svenska

Multipelt myelom är en för närvarande obotlig cancerform som drabbar ca 550 svenskar varje år. Tumören uppstår i benmärgen och består av plasmablaste/plasmaceller, en typ av celler vars normala motsvarighet har till uppgift att tillverka antikroppar för kroppens immunförsvar. Skelettsmärter, frakturer, blodbrist, njurskador och infektionskänslighet är vanliga komplikationer under sjukdomsförloppet. Konventionell behandling med cellgifter kan ofta bromsa tumörens tillväxt till en början. Förr eller senare blir dock behandlingen verkningslös till följd av att tumören blir okänslig, drogresistent.

Grunden till resistens mot droger ligger i cancercellens förmåga till okontrollerad tillväxt och överlevnad. I varje flercellig organism finns det en rad kontrollmekanismer som har till syfte att förhindra enskilda celler från att växa ohämmat. Cancerceller uppstår när normala celler genomgår slumpmässiga genetiska förändringar, som gör dem okänsliga för dessa kontrollmekanismer.

Apoptos, eller programmerad celldöd, är ett exempel på en kontrollmekanism som en cancercell ofta är okänslig för. Enkelt uttryckt är apoptos en typ av självmordsprogram som sätts i funktion när en cell är onödig eller skadlig för resten av organismen. Apoptos kan induceras av flera olika sorters stimuli, exempelvis strålning, DNA skada, och brist på särskilda överlevnadsfaktorer. Därtill kan en cell uppmana en annan att genomgå apoptos genom att binda till den dödsdömda cellens så kallade dödsreceptorer, särskilda proteiner på cellytan som fungerar som förmedlare av apoptos signaler. Man vet att många cellgifter verkar genom att inducera apoptos, vilket alltså innebär att en tumör som är okänslig för apoptos är svår att komma åt.

Oavsett hur apoptos induceras så innebär processen alltid aktivering av en särskild grupp av proteiner, så kallade caspaser. Caspaser har förmågan att specifikt bryta ned en rad livsviktiga proteiner, vilket har till följd att cellen dör på ett mycket kontrollerat sätt. Caspasernas verkningsor sakar exempelvis att cellen skrumplar snarare än faller isär, och att särskilda proteiner på cellytan förändras så att cellen blir igenkänningsbar för städare som

snabbt åter upp den. På så vis undviker kroppen att innehållet från döda celler signalerar vävnadsskada och framkallar inflammation.

En strategi för att lyckas bättre med cancerterapi är att ta reda på mer om hur apoptos och överlevnad regleras i cancerceller. Det är här vår forskning kommer in.

I det första arbetet i denna avhandling har vi studerat hur interferoner, en typ av kroppsegna proteiner som ibland utnyttjas i cancerbehandling, kan göra myelomceller mer känsliga för apoptos. Vi visar att interferonbehandling ökar mängden av en viss dödsreceptor på cellytan och att ett särskilt signaleringsprotein, Stat1, troligen är inblandat i processen. Vi tror att Stat1 härigenom kan fungera som en apoptosfrämjande motpol till ett annat närbesläktat signaleringsprotein, Stat3, som sedan tidigare är känt för att motverka apoptos och främja cellöverlevnad i multipelt myelom.

I det andra arbetet har vi arbetat vidare utifrån denna hypotes. Genom att föra in Stat1-DNA i en myelom-cellinje skapade vi celler som har mer Stat1-protein än ursprungscellerna. Dessa celler var mer känsliga för vissa droger jämfört med den ursprungliga cellinjen, men de var också, paradoxalt nog, mer resistenta mot andra droger. Vi upptäckte också att celler med mer Stat1-protein hade mindre Stat3-protein, vilket skulle kunna stämma med vår hypotes att det råder en viss balans mellan dessa båda proteiner som kan påverka överlevnad om den förskjuts.

I det tredje arbetet har vi undersökt om en ny drog, PPP, skulle kunna fungera mot multipelt myelom. PPP motverkar signaler från IGF-I, ett insulinbesläktat protein som främjar bland annat myelomcellers överlevnad och motverkar apoptos. PPP är en mycket lovande cancerdrog eftersom tidigare försök har visat att PPP gör att tumörceller går i apoptos medan normala celler skonas. Vi kunde konstatera att PPP-behandling gav apoptos i myelomcellinjer och i celler som isolerats från myelompatienter. Dessutom kunde överlevnadsfrämjande proteiner inte motverka PPPs effekt. Sammantaget visar det tredje arbetet att PPP kan ha stor potential i behandlingen av myelom

I det fjärde och sista arbetet har vi kartlagt förekomsten av en sorts apoptoshämmande proteiner, IAP:ar, i ett antal myelomcellinjer samt i myelomceller från patienter. Vi har också visat att mängden av vissa proteiner i IAP-familjen minskar vid dexametason-behandling, särskilt om den ovannämnda drogen PPP, som ökar dexametasonkänslighet, tillsätts samtidigt. Detta skulle kunna betyda att IAP-proteinerna har betydelse för drogresistens mot dexametason.

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