Biological aspects of altered bone remodeling in multiple myeloma and possibilities of pharmacological intervention

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The PhD thesis is based on the experimental work performed at the Department of Clinical Cell Biology at Vejle Hospital during my enrolment as a PhD student at the Faculty of Health Science at the University of Southern Denmark in the period between 1/12-2006 and 31/12-2009.

The obtained results are presented in 4 published papers accompanied to the thesis:

Andersen TL, Boissy P, Sondergaard TE, Kupisiewicz K, Plesner T, Haaber J, Kølvraa S, Delaisse J-M. Osteoclast nuclei of myeloma patients show chromosome translocations specific for the myeloma cell clone: a new type of cancer-host partnership? J Pathol. 2006 Nov 3

Boissy P, Andersen TL, Lund T, Kupisiewicz K, Plesner T, Delaisse JM. Pulse treatment with proteasome inhibitor bortezomib inhibits osteoclast resorptive activity in clinically relevant conditions. Leuk Res. 2008 Apr 2

Abdallah BM, Boissy P, Tan Q, Dahlgaard J, Traustadottir GA, Kupisiewicz K, Laborda J, Delaisse J-M, Kassem M. Dlk1 /FA1 regulates the function of human bone marrow mesenchymal stem cells (hMSC) by modulating gene expression of pro-inflammatory cytokines and immune-response-related factors. J Biol Chem. 2007 Mar 9

Kupisiewicz K**,** Boissy P, Abdallah BM, Dagnaes-Hansen F, Savouret J-F, Erben RG, Søe K, Andersen TL, Plesner T, Delaisse J-M. Potential of resveratrol analogues as antagonists of osteoclasts and promotors of osteoblasts (Calcified Tissue International)

SUMMARY

Multiple myeloma is a fatal B cell neoplasm often resulting in focal and in some cases more diffuse destruction of bone. The bone destruction is a result of increased activity of bone resorbing cells – multinucleated osteoclasts emerging through of multiple fusions. In multiple myeloma, clonally expanding cancer cells provide a stimulatory signal for osteoclast recruitment, differentiation and excessive bone resorption. The stimulatory actions of myeloma cells are believed to be mediated via the production of cytokines and local factors or by modulating bone microenvironment in order to stimulate osteoclastic bone resorption. However, our recent study revealed potentially a novel and more intimate contribution of myeloma cells to the bone destruction. Our analysis of the bone biopsies from myeloma patients showed fully integrated malignant nuclei inside osteoclasts, which were transcriptionally active. As a result, about 30% of the osteoclasts in the bone marrow biopsies form myeloma patients were in fact osteoclast-myeloma cell hybrids. As the functional relevance of this novel cell type remained uncertain, the aim of my PhD study became to 1) strengthen the evidence of the existence of hybrid cells, 2) elucidate the functional differences between hybrid cells and non-hybrid OCs and 3) relate these findings to the pathogenesis of osteolytic disease in multiple myeloma. To this end, I developed a culture model of osteoclast-myeloma cell fusion between (pre)osteoclasts already committed to fuse and myeloma cells selected for adherance. The model was applied for testing of the bone resorptive properties of hybrid cells identified by labelling with green fluorescence. When comparing the highly fluorescent and non-fluorescent OCs on bone slices, it seemed that the frequency of highly fluorescent osteoclasts actively resorbing bone was increased as compared with non-fluorescent osteoclasts. This was assessed in two independent ways. Furthermore, these fluorescent osteoclasts appear to resorb deeper compared to non-fluorescent osteoclasts. The preliminary data that need to be confirmed suggest that formation of hybrid cells

by fusion of myeloma cells with osteoclasts may result in reprogramming of the osteoclasts and contribute to the more aggressive bone resorption by osteoclasts as it is typically seen in myeloma patients.

Another aspect of multiple myeloma and associated bone disease is the unmet need for novel and more efficient therapeutic regiments. Resveratrol (*trans*-3, 4', 5-trihydroxystilbene; RSV) is a natural compound shown to target the key players of myeloma bone disease: bone resorbing osteoclasts, bone forming osteoblasts and myeloma cells. Our in vitro study on RSV showed that it possessed this ideal triad of properties appearing and thus might be of interest as a potential drug for the treatment of multiple myeloma. RSV suppresses the growth and survival of myeloma cells, inhibits osteoclasts and stimulates the formation of osteoblasts. However, the need for high concentrations combined with low biological availability after oral administration and risk of important side effects stimulated a search for RSV derivates with the same spectrum of actions but safer and with better bioavailability. As the other task of my PhD, I screened structurally modified RSV analogues in cultures of myeloma cells, osteoblasts and osteoclasts. Compared to resveratrol, some analogues showed an up to 5,000-times increased potency to inhibit osteoclast differentiation and could still promote osteoblast maturation but they did not antagonize myeloma cells. The potency of the best-performing candidate *in vitro* was tested *in vivo* in an ovariectomy-induced model of osteoporosis, but effect on bone loss could not be detected.

During my PhD, I also participated in the studies of the effect of the proteasome inhibitor - bortezomib on osteoclasts conducted at the department. Based on its potent activity in multiple myeloma, bortezomib was accepted as a front-line treatment of myeloma patients by EMEA for the European Union. In our study we assessed the effect of bortezomib on osteoclasts in cultures under the conditions that mimic the pulse-treatment regime used for myeloma patients. The pulse administration of bortezomib significantly inhibited OC activity and, moreover, significantly but transiently reduced levels of two bone resorption markers measured in serum of treated myeloma patients.

In MM the clonal expansion of malignant plasma cells results in the unbalanced bone remodelling, therefore it is essential to understand the molecular mechanisms governing the actions of osteoclasts and osteoblasts. During my PhD, I was involved in the investigations of mesenchymal stem cells over-expressing delta like protein – 1 (Dlk-1) previously shown to inhibit the differentiation of mesenchymal stem cells (MSC) into osteoblasts. In results, the over-expression of Dlk-1 evoked pro-inflammatory phenotype in MSC suggesting the involvement of Dlk-1 in the immune response.

1.1. INTRODUCTION

1.1. BONE STRUCTURE AND REMODELLING

Bones are rigid organs that form part of endoskeleton in vertebrates. They function as [(1)] a mechanical support and protection for various organs of the body, [(2)] main producers of red and white blood cells and [(3)] homeostatic buffers for mineral (mainly calcium) metabolism. Bones of the body could be divided in two types, long bones and flat bones formed by two different mechanisms during embryonic development [49]. Both flat and long bones consist of two morphologically distinct types of bone, cortical bone constituting 85% of total bone in the body and mainly responsible for mechanical protection, and trabecular bone that constitutes 15% of the bone conveying metabolic functions. The spaces between the bone trabeculi in long bones are composed of blood vessels and bone marrow, which is the site of hematopoiesis (Figure 1). Bone marrow contains of two separate and distinct stem cell populations: the hemapoietic stem cells and the mesenchymal stem cells and their respective progeny.

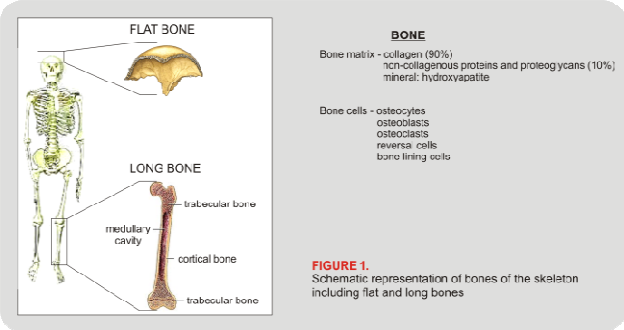

FIGURE 1.
Schematic representation of bones of the skeleton including flat and long bones

Bone consists of bone matrix and bone cells. Bone matrix is composed of an organic component, comprising in 90% of collagen, out of which type I is the most abundant form and represents the majority of bone collagen. Collagen is strengthened by deposits of inorganic calcium salts. The remaining 10% of the organic matrix is composed of non-collagenous proteins and proteoglycans mostly responsible for cell attachment and growth (Figure 1).

1.1.1. Bone cells

Bone cells are responsible for the metabolic activity and constant renewal of bone, and include [(1)] osteocytes, [(2)] osteoclasts, [(3)] osteoblasts, [(4)] reversal cells and [(5)] bone lining cells.

Osteocytes are the most abundant cell type of the bone representing almost 95% of bone cells [164]. Osteocytes descend from osteoblasts that have been embedded in the new deposed bone matrix [73]. They lose a large part of their organelles but gain long, slender cell processes by which they remain in contact with earlier incorporated osteocytes and with other bone cells. Osteocyte function is still poorly understood, although there is increasing body of evidence that they sense the mechanical stress to bone and therefore can regulate bone remodelling process [1;41;42;73].

Osteoclasts are multinucleated cells know to be the only cell type resorbing bone. Osteoclasts differentiate from macrophage-monocyte hematopoietic precursors that undergo multiple cellular fusions forming multinucleated osteoclasts with up to 100 nuclei [21;46;227;228]. The osteoclast differentiation requires different environmental factors, of which macrophage-colony stimulating factor (M-CSF) and receptor activator for NF-κB ligand (RANKL) seem

to play crucial roles. M-CSF signalling is essential for the expansion of osteoclast precursors while RANKL provides a turn-on signal for cell fusion, differentiation and subsequent activation of mature osteoclasts [238]. RANKL signalling is blocked by its decoy receptor osteoprotegerin (OPG) expressed mainly by osteoblasts but also other cells in the bone marrow [43]. Osteoclasts resorb bone by acidification that causes elution of bone minerals followed by digestion of the organic matrix by use of several proteolytic by enzymes, of which cathepsin K is of the greatest importance [184;223].

The formation of new bone is the responsibility of osteoblasts that originate from multipotent mesenchymal stem cells. These precursor cells, in response to various hormones or local factors, may give rise to osteocytes and extracellular matrix, but can also differentiate along other pathways to become adipocytes, chondrocytes, myoblast or fibroblasts [6]. Osteoblasts produce and secrete the major part of the organic bone matrix that in tightly regulated process becomes calcified to form mineralized bone. Osteoblasts do not function individually but are found in clusters along the bone surface depositing the layer of bone matrix that they are producing.

The existence, origin and function of reversal cells attracted a considerable scientific attention since the discovery of osteoblastic cells in resorption pits vacant of osteoclasts. Reversal cells seem responsible for the removal of residual organic matrix and the deposition of primary collagen in the resorption pit, initiating further bone formation [72;149].

In the adult skeleton, the majority of surfaces are covered by flat, thin elongated bone lining cells, which are thought to represent the inactive form of osteoblasts in terms of matrix production. The main function of bone lining cells seems the mechanical protection of bone surface. An important aspect concerning bone lining cells is that the retraction or removal of these cells is a mandatory step in starting osteoclastic bone resorption [246]. In agreement, recent data suggest that bone lining cells can cooperate with osteocytes and sense mechanical strain and translate it to biochemical signal regulating bone remodelling [42].

1.1.2. Bone remodelling

Bone is continuously renewed throughout adult life in a process of bone remodelling implementing the removal of old bone by osteoclasts followed by osteoblast-mediated formation of new bone at the place of removal. This process is believed to occur at discrete sites named basic multicelluar units (BMU), where the actions of osteoclasts and osteoblasts are tightly coupled spatially and temporally to retain balance between resorption and formation of bone [173]. A fully developed BMU consist of bone-resorbing osteoclasts in front, followed by osteoblasts forming bone. Further studies provided evidences that the BMU is not in direct contact with bone marrow but is separated from the bone marrow cavity by the canopy of made up by a single layer of flat osteoblast-like cells. This closed structure was renamed bone remodelling compartment (BRC, Figure 2) [94]. The integrity of the canopy seems to be essential for balanced bone turnover [15]. Bone remodelling occurs in a sequential and cyclical manner referred to as the Activation/Resorption-Reversal-Formation cycle [89]. One of the initial events activating bone resorption is believed to be apoptosis of osteocytes in response to local mechanical stress. Additionally, hypoxic conditions can also act as a local stimulatory factor triggering the formation of osteoclasts and initiating bone resorption [20]. Apoptotic osteocytes send the recruitment signal for osteoclast precursors thus initiating osteoclastogenesis and bone resorption [86;212]. During the resorption phase osteoclasts work in a concentrated fashion, removing both mineral and organic components of bone matrix leaving scalloped erosion areas also called the eroded surface. After the completion of bone resorption, osteclasts undergo apoptosis and this is followed by a reversal phase, during which osteoblast precursors are recruited to bone surface. The first osteoblasts that enter the area are called reversal cells and they prepare and condition the resorbed areas and provide recruitment signals for more osteoblast differentiation and migration into area [72]. The formation phase follows with osteoblasts laying down new bone matrix until the resorbed area is completely replaced. The bone remodelling is believed to be terminated by action of osteocytes producing sclerostin that inhibits bone formation by antagonising Wnt signalling pathway in osteoblasts (Figure 2) [234].

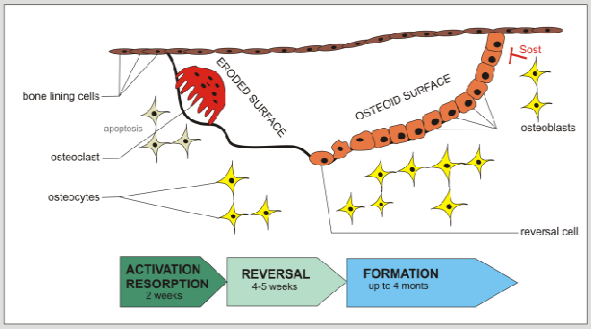

FIGURE 2.
Schematic illustration of bone remodelling compartment with cells involved in bone remodelling (see description in text). Below depiced remodelling sequence (for details refer to the text). Sost - sclerostin

Under the normal circumstances, the actions of osteoclasts and osteoblasts are tightly coupled, and bone resorption and formation occurs in balanced fashion. Abnormalities of bone remodelling can lead to either extensive loss or gain of bone mass as seen in several skeletal disorders (Table 1). Altered bone turn-over with development of osteoporosis is a severe problem in modern Western population with drastic effects on the quality of life and global health.

TABLE 1. Abnormalities of remodelling in metabolic, inflammatory and cancer-induced bone disorders. ++ - definitely increased; - - definitely decreased; + - transiently or variably increased; - - transiently or variably decreased. Modified from [177]

BONE DISEASE	BONE RESORPTION	BONE FORMATION	BALANCED BONE REMODELLING	REFERENCE
Postmenopausal and senile osteoporosis	++	-	0	[65;206]
Glucocorticoid-induced osteoporosis	-	++	0	[240]
Hyperparathyroidism	++	++	+	[94;232]
Hyperthyroidism	++	++	+	[52]
Osteopetrosis	–	+	0	[39;40]
Paget disease	++	++	+	[127]
Inflammation	++	-	0	
Multiple myeloma	++	-	0	[15]
Prostate cancer	+	++	0	[145]
Breast cancer	++	+	0	[12]
Lung cancer	++	+	0	[57]

1.2. MULTIPLE MYELOMA AND ASSOCIATED BONE DISEASE

Multiple myeloma is a clonal malignancy of terminally differentiated plasma cells accumulating in the bone marrow. Myeloma accounts for approximately 1% of all malignant diseases in a Caucasian population with around 300 new cases annually in Denmark. The median age at diagnosis is 68 years and 55% patients who present with multiple myeloma are aged 60 or older whereas only 3% cases are detected in patients younger than 40. Multiple myeloma remains incurable at present with median survival ranging between 3.5 and 5 years.

Multiple myeloma is characterised by the presence of plasma cells in bone marrow (>10%) and the increased levels of monoclonal proteins detected in either blood or urine of more than 95% of patients. The infiltration of myeloma cells in the bone marrow may cause symptoms due to bone destruction, immunodeficiency and renal impairment. Skeletal complications, including bone pain, osteolytic lesions, pathological fractures and hypercalcemia cause significant morbidity in about 80% of patients at the time of diagnosis [16].

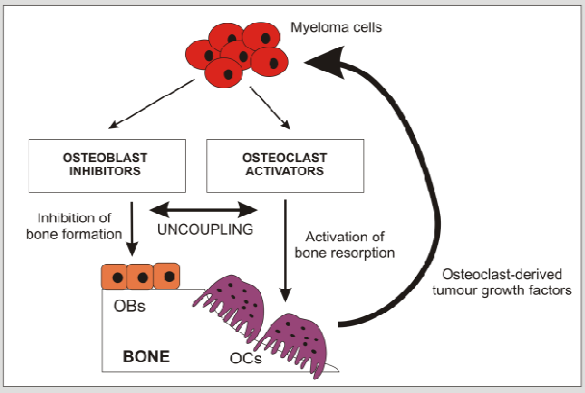

FIGURE 3.
Model for mechanisms of the induction of myeloma bone disease (for detailed description please refer to the text). OBs - osteoblasts, OCs - osteoclasts

Biologically, the infiltration of myeloma cells in the bone marrow results in the activation of osteoclastogenesis and osteoclast-mediated bone resorption in the vicinity of myeloma cells. In return, activated osteoclasts provide a feedback stimulatory signal facilitating proliferation and survival of myeloma cells often referred to as the vicious cycle of myeloma-osteoclast interaction. At the same time, bone formation by osteoblasts is inhibited in the presence of myeloma cells that secrete soluble factors such as Dickkopf-1 protein that antagonise osteoblast activity. Thus, myeloma bone disease develops as a consequence of unbalanced bone remodelling with extensive bone resorption, which is not compensated by increased bone formation (Figure 3) [27;213;233].

1.3. OSTEOCLASTS AND BONE RESORPTION IN MULTIPLE MYELOMA

1.3.1. *Osteoclasts*

The hallmark of multiple myeloma is enhanced bone destruction mediated by multinucleated osteoclasts in areas adjacent to myeloma cells. Bone resorption is a multistep process initiated by the proliferation of immature osteoclasts progenitors, the commitment of these cells to osteoclast phenotype, and finally degradation of inorganic and organic matter of bone by the mature osteoclasts. The functional cycle of the osteoclast consists of matrix adherence and bone resorption followed by detachment and movement to a new site of bone degradation [75]. Actively resorbing osteoclasts are highly polarized cells in contrast to osteoclasts inactive in terms of bone resorption. The membrane areas of actively resorbing osteoclasts can be separated into following domains: $^{(1)}$ the sealing zone, $^{(2)}$ the ruffled border, $^{(3)}$ the basolateral membrane and $^{(4)}$ the functional secretory domain [185]. The sealing zone is a structure rich in F-actin and largely devoid of organelles organised as a ring surrounding the ruffled border [111;226]. In addition to F-actin, the sealing zone contains several matrix-recognizing proteins and integrins such as vinculin, CD44 and $\alpha_v\beta_3$ integrin allowing a tight interaction of the osteoclast with the bone [111]. The ruffled border is a highly convoluted plasma membrane domain under which the actual resorption takes place. Bone degradation occurs in the extracellular space between bone matrix and the ruffled border called the resorption lacuna [186]. In the middle of the basolateral membrane is the secretory domain that appears when collagen degradation is started (Figure 4).

Bone demineralization involves acidification of the isolated extracellular microenvironment between the osteoclast and the bone surface, a process that is mediated by a vacuolar ATPase proton pump located in the ruffled border generating acid pH in the resorption lacuna [97;182;198]. This acidic milieu first mobilizes bone mineral; subsequently the demineralised collagen component of bone is degraded by a lysosomal protease cathepsin K [184;214;223]. The products of bone degradation are endocytosed by the osteoclast, transported to and released at the secretory domain.

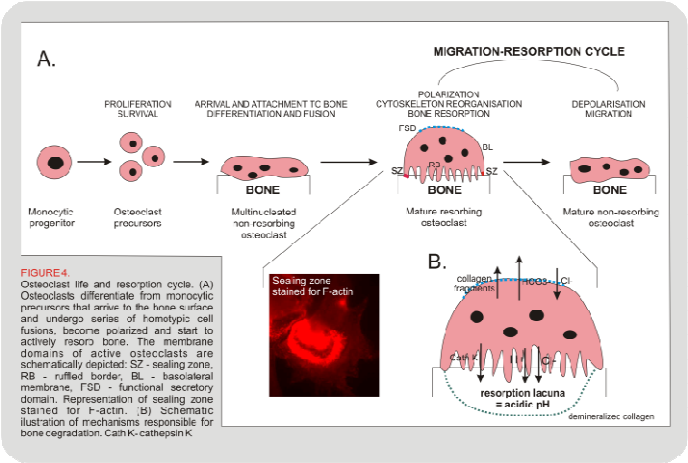

FIGURE 4.
Osteoclast life and resorption cycle. (A) Osteoclasts differentiate from monocytic precursors that arrive to the bone surface and undergo series of homotypic cell fusions, become polarized and start to actively resorb bone. The membrane domains of active osteoclasts are schematically depicted: SZ - sealing zone, RB – ruffled border, BL - basolateral membrane, FSD - functional secretory domain. Representation of sealing zone stained for F-actin. (B) Schematic illustration of mechanisms responsible for bone degradation. Cath K- cathepsin K

1.3.2. The indirect effect of myeloma cells on bone resorption

It is widely accepted that in multiple myeloma cancer cells operate via the production of cytokines and local factors or by modulating bone microenvironment in order to stimulate osteoclastic bone resorption [152]. Osteoclast activating factors were subsequently found to include interleukins (IL)-1β, -6 and -11, tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, hepatocyte growth factor (HGF), RANKL (antagonised by OPG), macrophage inflammatory protein (MIP)-1α and β, stromal derived factor (SDF)-1α and vascular endothelial growth factor (VEGF) but more factors are emerging. The characteristics of the best known osteoclast activating factors are depicted in Table 2.

TABLE 2. Characteristics of myeloma induced cytokines involved in osteoclast activation

FACTOR	SOURCE	MECHANISMS OF ACTION ON OCs	REFERENCE
IL-1β	Myeloma cells	Activation of osteoclasts	[58;59;135]
IL-6	Stromal cells	Growth factor for myeloma cells Recruitment of osteoclast precursors and induction of bone resorption	[64;180]
IL-11	Myeloma cells Stromal cells	Induction of osteoclastogenesis and bone resorption through RANK/OPG pathway	[165]
TNF-α	Myeloma cells Marrow accessory cells	NF-κB activation in osteoclast precursors Stimulation of osteoclast formation	[109;131;169]
TGF-β	Myeloma cells	Triggering IL-6 production by stromal cells	[105]
HGF	Myeloma cells	Upregulation of IL-11 and osteoclast activation	[101;189;190]
RANKL/OPG	Stromal cells Osteoblasts	RANKL induces osteclast differentiation while OPG acts as ite decoy receptor Myeloma cells induce RANK- production by stromal cells and internalise OPG	[79;196;204]
MIP-1α and β	Myeloma cells Bone marrow cells	Induction of osteoclast formation via IL-6	[7;56;82;123;217]
SDF-1α	Stromal and endothelial cells	Increase in osteoclast motility and bone resorbing activity	[224;248]
VEGF	Myeloma cells Stromal cells	Increase in osteoclast motility and bone resorbing activity	[158;159;211]

1.3.3. The direct effect of myeloma cells on bone resorption

Based on these observation, it has been generally accepted that bone resorption in multiple myeloma is mediated by osteoclasts considered to be the only cells that are able to degrade bone. Myeloma cells are believed to play an indirect role, however recent data suggest that myeloma cells may be more directly involved in the generation of osteolytic lesions. Under appropriate culture conditions myeloma cell lines U266 and MCC-2 can phenotypically differentiate into osteolcast-like cells showing to some extend the ability to resorb bone [50;51]. These results were in accordance with those of McDonald and colleagues who showed that myeloma cells of a mouse plasmacytoma model were able to resorb bone directly without the involvement of osteoclasts [143]. A recent study, based on FISH and immunohistochemistry applied on bone marrow biopsies from myeloma patients, demonstrated an unexpected new contribution of myeloma cells to the formation of osteoclasts [14]. Bone-resorbing osteoclasts from myeloma patients contained nuclei with translocated chromosomes of myeloma clone origin: t(4;14) and t(11;14). The level of integration of myeloma nuclei varied from patient to patient but was often around 30% of the osteoclasts, and these myeloma nuclei were demonstrated to be transcriptionally active and integrated amongst the other nuclei. Interestingly, the occurrence of such osteoclast-myeloma cell hybrids correlated with the proximity of myeloma cells to bone resorbing osteoclasts. Similar hybrid cells could be generated in myeloma cell-osteoclast co-cultures under osteoclast-forming conditions. These observations indicate that hybrid cells that appear like bone resorbing osteoclasts can originate through fusion between myeloma cells and osteoclasts both in vitro and in vivo, and suggest a possible novel role of myeloma cells in bone resorption if the fusion results in reprogramming of the osteoclast and render it more aggressive with regard to bone resorption.

1.3.4. Osteoclast-myeloma cell vicious cycle

The fact that myeloma cells grow and expand almost exclusively in the bone marrow suggests the importance of the bone marrow microenvironment in supporting myeloma cell growth and survival. Recently, especially the role of osteoclasts in promoting the growth of myeloma cells became evident. Studies of Yaccoby and colleagues had previously showed that in an animal model of human myeloma using SCID-human host system, myeloma cell growth was suppressed by inhibition of osteoclast activity with bisphosphonates [242]. This complex interdependence was further investigated in a culture system between primary myeloma cells and osteoclasts derived from peripheral blood mononuclear cells [8;95;243]. Growth of myeloma cells was potently enhanced by cell-to-cell interaction with osteoclasts and largely dependent on the increased production of IL-6 by osteoclasts.IL-6, known as myeloma growth factor, is the most potent stimulator of myeloma cell expansion; however there are several candidates that may mediate the cellular interactions between myeloma cells and osteoclasts. B-cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) have been implicated as growth and survival factors [148] and blocking of their actions with a decoy receptor significantly induced apoptosis in myeloma cells [9]. Because myeloma cells become refractory to chemotherapeutic agents in advanced stages, there is a possibility that the interaction with osteoclasts may have a protective role against cytotoxic effects of anti-cancer therapies. Indeed, cell-to-cell interaction with osteoclasts not only enhances myeloma cell growth but also causes marked resistance to doxorubicin [8]. These observations are in agreement with clinical data that myeloma patients at advanced stages with extensive osteolytic lesions show refractoriness to chemotherapies, and suggest that increased osteoclast number and

activity may contribute to aggressiveness and drug resistance of adjacent myeloma cells.

1.4. OSTEOBLASTS AND BONE FORMATION IN MULTIPLE MYELOMA

1.4.1. Wnt signalling in osteoblasts

Deposition of new bone is mediated by osteoblasts that are believed to originate from bone marrow mesenchymal stem cells (MSCs). Due to their pluripotent differentiation ability, MSC are ultimately capable of differencing into cells of different lineages, including not only osteoblasts, but also chondrocytes, adipocytes and myocytes. The commitment and fate of MSCs seem to be governed by canonical and non-canonical Wnt signalling, differently regulated among various MSC-derived cell lineages. Non-canonical Wnt pathway has been less characterised and the molecules and interactions involved are known to be diverse. As oppose to the canonical signalling, it does not operate through β-catenin but invokes several other cascades including PCP (planar cell polarity), JNK (c-Jun N-terminal knase), calcium and Rho signalling [133]. As the relevance of non-canonical Wnt pathway in the osteoblast differentiation is still under extensive investigation and detailed discussion is beyond the scope of the thesis, this chapter will mostly concentrate on the canonical Wnt signalling.

Activation of the canonical Wnt pathway occurs upon binding of Wnt to the frizzled receptor and low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6) co-receptors. Signals are generated through the proteins Disheveled (Dsh), Axin and Frat-1, which disrupt the protein complex and inhibit the activity of glycogen synthase kinase 3 (GSK3), thus causing hypophosphorylation of its substrate β-catenin. Stabilized β-catenin then accumulates in cytosol and translocates to the nucleus where it acts on gene transcription (Figure 5). Wnt signalling is tightly regulated by members of several families of secreted antagonists. Interactions between Wnt and frizzled receptors are inhibited by members of the secreted frizzled-related protein (sFRP) family [34] and Wnt inhibitory factor 1 (WIF-1) [55]. LRP5/6 co-receptor activity is inhibited by the members of the sclerostin (SOST) family [130;191] and Dickkopf-1 (Dkk-1) protein [44]. Interaction of Dkk-1/LRP with internalizes the complex for degradation, thus diminishing the number of Wnt co-receptors available for signalling [139] (Figure 5).

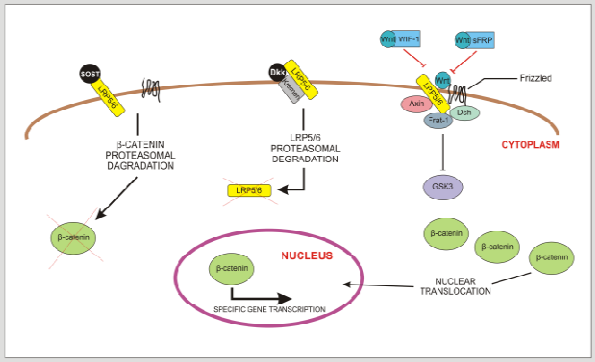

FIGURE 5.
Elements of Wnt signalling (for detailed description see text). Depicited in black and light blue are inhibitors of Wnt signalling and the mechanisms of their actions

1.4.2. Adipocyte/osteoblast transdifferentiation

MSCs can give rise to both osteoclastic and adipogenic cells, and there is a compelling evidence for a reciprocal relationship between these cells. Single MSC-derived clones were shown to have the ability to differentiate into adipocytes, dedifferentiate, and subsequently differentiate into osteoblasts in vitro [202]. Also mature osteoblasts or adipocytes were able to inter-differentiate, when cultured under respectively adipogenic or osteoblast-promoting conditions [112]. Moreover, adipose-derived stem cells isolated from extramedullary fat display differentiation capacity to both adipocytes and osteoblasts [90;250;251]. The differentiation fate of MSC precursors is differently regulated for both osteoblast and adipocytes and there are mutual interactions controlling the MSC development. The factors that induce adipogenesis inhibit osteoblast differentiation and, vice versa, factors that promote osteogenesis suppress adipocyte formation (Figure 6). The overexpression of transcription factors such as: core-binding factor (CBFA1/Runx2) [119], osterix [157] and lipoprotein related receptor 5 (Lrp5) [176] leads to osteoblast differentiation, while peroxisome proliferator-activated receptor gamma 2 (PPARγ2) [128] induces adipocyte lineage. In addition, bone microenvironment facilitates several factors inducing osteogenesis such as bone morphogenic proteins (BMPs) [136] and Wnt [77], or regulating adipogenesis such as Dlk1/Pref-1 [4] and Noggin [181]. However, not only chemical cues, but also physical activation such as cell density and cell shape appear to play a role in lineage commitment. Lower cell densities seem to support osteoblast differentiation of MSCs, whereas higher cell densities cause the cells to condensate, forcing adipocyte formation [142].

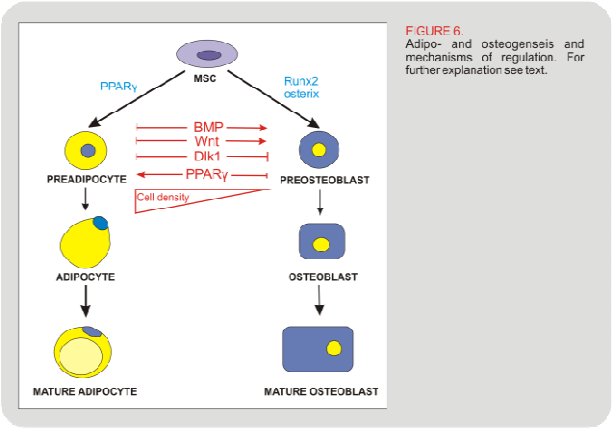

FIGURE 6.
Adipo- and osteogenseis and mechanisms of regulation. For further explanation see text.

1.4.3. Delta-like protein 1

Another regulatory mechanism of osteblast/adipocyte differentiation is mediated via delta-like protein 1 / fetal antigen 1 (Dlk 1/FA1, also named preadipocyte factor 1) signalling. Dlk 1 is a member of the epidermal growth factor-like homeotic protein family, which expression is known to modulate the differentiation fate of MSC in bone marrow [125]. Abdallah and collegues, using a stable retroviral transduction, identified Dlk 1 as a novel factor controlling the differentiation of MSC rending them in the progenitor status and inhibiting the formation of osteoblasts and adipocytes [5]. Dlk 1 has been shown to be highly expressed in preadipocytes during adipogenesis but its expression is abolished after differentiation to adipocytes. Dlk 1 is acting inhibitory for adipocyte differentiation, and only when down-regulated, it allows adipocyte differentiation to occur [201]. These observations suggest that Dlk 1 may be a unique inhibitor of adipogenesis produced and secreted by preadipocytes that keeps the cells in undifferentiated stage and prevents differentiation. Additionally to its prevention actions on adipocyte differentiation, Dlk 1 was also shown to negatively regulate the formation of osteoblasts from MSC precursors [5] thus being an unique endocrine regulator of bone mass. Furthermore, the novel role of Dlk 1 in the modulation of the expression of several pro-inflammatory cytokines by MSC was discovered using DNA microarray technology [2]. This modulator effect of Dlk 1 may further influence MSC differentiation by controlling the composition of their microenvironment.

1.4.4. Bone formation in multiple myeloma

In typical destructive bone lesions of multiple myeloma, enhanced bone resorption is accompanied by impaired bone formation, which is the cause of the “punched-out” lesions visible on X rays. Analyses of bone turnover by biochemical bone markers also suggest imbalance with enhanced bone resorption and suppressed bone formation [215;235]. These and other findings suggest that myeloma cells effected osteoblastic bone formation by mutually blocking the differentiation of osteoblastic precursors and inducing apooptosis in mature osteblasts [82]. In agreement, Tian and coworkers reported a significant increase of the levels of Dkk1 in serum of newly diagnosed myeloma patients [219]. Notably, the severity of bone lesions was correlated with elevated Dkk1 levels in these patients [219]. Interestingly, patients with advanced disease, as well as human myeloma cell lines, did not express Dkk-1, suggesting that Dkk-1 may mediate bone destruction in the early phases of disease [219]. Knowing its role in Wnt-mediated osteoblast development, Dkk1 emerged as one of the most potential mediator of osteoblast dysfunction in myeloma bone disease. Furthermore, secreted Frizzled-related protein-2 (sFRP-2), a soluble antagonist of Wnt signalling, has been shown to be expressed by myeloma cells [162]. In vitro studies proved the role of myeloma-cell derived sFRP-2 in the suppression of bone formation, raising the possibility that sFRP-2 may play a role in the development of lytic lesions observed in multiple myeloma [162]. Moreover, myeloma cells were found to act downstream of Wnt signalling, inhibiting Runx2 activity and reducing osteoblast differentiation mediated by both cell-cell contact and IL-7 [80]. Also cytokines and local factors are implemented in altered bone formation in multiple myeloma. TGF-β, released from bone matrix during osteoclastic bone resorption, has a dual role, not only stimulating osteoblast activation, but negatively effecting osteoclast differentiation [150]. HGF is produced by myeloma cells and increased in the serum of patients with multiple myeloma [190]. Increased levels of HGF are correlated with poor prognosis [13] and negatively correlated with levels of bone specific alkaline phosphatase, a marker of bone formation [203]. In agreement, HGF was found to inhibit BMP-induced osteoblastogenesis and the expression of transcription factors Runx2 and Osterix [203]. In addition to blocking osteoblast differentiation, myeloma cells were shown to induce apoptosis of osteoblasts when cultured with human osteoblastic cells [220].

1.5. TREATMENT OF MULTIPLE MYELOMA

Altered activity of osteoclasts and osteoblasts in multiple myeloma leads to osteolytic lesions and compromised quality of life for myeloma patients. Currently available treatments are only palliative and not curative, extending the life span of myeloma patients to 3 - 5 years but not leading to cure. A very important contribution to anti-myeloma therapy, in terms of prolongation of survival, has been obtained with stem cell transplantation, where the patient receives an autologous or allogenic stem cell graft proceeded by high dose of myelo-ablative chemotherapy. Allogenic stem cell transplantation with use of stem cells from a family or matched unrelated donor remains experimental. However, many myeloma patients are not allocated to stem cell transplantations because of their advanced age, poor performance status, pronounced renal failure or comorbidity. These patients receive a standard dose of conventional chemotherapy or a suitable alternative therapeutic regimen with addition of one and more “novel agents” that have been available in recent years. For decades, an oral regimen of melphalan combined with prednisolone remained the cornerstone of anti-myeloma therapy with the overall response of 50% [155]**.** Many investigators have sought to improve the results obtained with melphalan plus prednisolone by using more sophisticated treatment regimens, but real improvement of the outcome has not been obtained until the

introduction of "novel agents" in the treatment programs (first and foremost thalidomide, lenalidomide and bortezomib).

Bisphosphonates are effective in the treatment of myeloma bone disease, however limited by their inability to promote new bone formation [11;132]. Furthermore, bisphosphonates have recently been associated with the development of osteonecrosis of the jaw, identified in 4-6% patients receiving intravenous bisphosphonates [24]. Introduction of "novel agents" into the treatment programs may offer advantages especially when a rapid reduction of tumour burden is required or in cases with extensive osteolytic bone lesions. In this context bortezomib seems to be of particular interest as shown in Table 3 that gives an overview of actions and major side effects of some of the drugs used for treatment of multiple myeloma with focus on their activity towards osteoclasts, osteoblasts and myeloma cells.

TABLE 3. Treatment options for multiple myeloma and their actions on osteoclast, osteoblasts and myeloma cells, and most severe side effects.

TREATMENT	DRUG (ACTIVE COMPOUND)	OSTEOCLAST	EFFECT ON OSTEOBLAST	MYELOMA CELLS	MOST SEVERE SIDE EFFECTS
PROTEASOME INHIBITOR	Velcade (bortezomib)	Inhibition of maturation and fusion [36;102;225]	Stimulation of osteoblast differentiation [61;96;163]	Suppression of proliferation and induction of apoptosis [83;167;207]	Thrombocytopenia, neutopenia, pneumonia, fatigue, diarrhea
BISPHOSPHONATES	Aredia (pamidronate disodium), Zometa (zoledronic acid)	Inhibition of bone resorption [11;91;113]	No effect on osteoblastic bone formation [1*;182]	Induction of apoptosis [19;84;197]	Osteonecrosis of the jaw
GLUCOCORTICOIDES	(dexamthasone, prednisolone)	Promotion of osteoclastogenesis [108;115;120;210]	Suppression of bone formation, incuction of osteoblast apoptosis [110;150]	Induction of apoptosis [85]	Glucocorticoid-induced osteoporosis
IMIDs	Thalidomid (thalicomice), Revlimid (lenalidonide)	Inhibition of osteoclastogenesis [17;18;47;124]			Fatigue, peripheral neuropathy
CONVENTIONAL CHEMOTHERAPY	Alkeran (melphalan)			Induction of apoptosis	
HIGH DOSE CHEMOTHERAPY AND STEM CELL TRANSPLANTATION	High dose of Alkeran (melphalan), stem cells			Induction of apoptosis	
RADIATION THERAPY	High-energy rays			Induction of apoptosis	Fatigue, local radiation damage to normal tissue
SUPPORTIVE THERAPY	Growth factors antibiotics				

1.5.1. Novel therapeutic targets

Since cure for multiple myeloma still cannot be achieved with currently available treatment modalities, there is an urgent need to search for new therapeutic agents. A detailed and comprehensive review of available therapies is beyond the scope of the thesis; however some areas of particular interest are summarized in Figure 7 and include:

(1) The improvement of existing therapeutic regiments and avoiding side effects – novel group of proteasome inhibitors is being tested in phase I clinical trials [53;121;146].

(2) Tumour neoangiogenesis – neovasuclarization is believed to be critical for growth and metastasis of tumours [93;200]. In multiple myeloma several clinical observations indicate that the presence of myeloma cells within the bone marrow compartment is associated with increased activity of endothelial cells resulting in neo-angiogenesis [153;179;229-231]. The density of newly formed blood vessels in multiple myeloma seems to positively correlate with the expression of VEGF by myeloma cells and number of plasma cells in myeloma bone marrow samples [30]. Therefore targeting angiogenesis has become a promising strategy for multiple myeloma and a variety of therapies directed at interfering this process are currently under development [104;110].

(3) Pro-apoptotic regiments for myeloma cells – inhibiting proliferation and inducing apoptosis of malignant plasma cells seems the key therapeutic regimen for multiple myeloma. Several strategies have been implemented to achieve this goal and the some promising therapies that have emerged recently are kinase- [28;144] and telomerase-inhibitors [194] drugs that modulate the duration of cell cycle or target cell-death receptors [63;76], heat shock proteins [71;161] or environmental stimuli facilitating myeloma cell survival [222;236;237]. Moreover, recently mammalian target of rapamycin (mTOR) has emerged as a critical effector in cell-signalling pathways commonly deregulated in human cancers [88]. Also in vitro and in vivo findings [74;178] support its importance in multiple myeloma and mTOR antagonists are currently in clinical trials [29].

(4) Immunotherapy – since myeloma cells express various potential target antigens, active immunotherapy is being investigated as a novel treatment modality for multiple myeloma. Accordingly, few cell surface molecules have been identified as suitable targets for the development of passive immunotherapy against multiple myeloma. Some of the possible targets for antibody therapy are CD74 [205], CD40 [118;129;171;172] and CD20 [45;114] all involved in the proliferation and survival of myeloma cells. In addition, the immune system is largely impaired in patients with multiple myeloma with significant dysfunction of dendritic cells [48], regulatory T cells [31;174] and natural killer cells [68]. Therefore it may be important to stimulate the host immune response towards myeloma cells. Several vaccination strategies are being explored to achieve this goal.

(5) OPG/RANKL system - RANKL, the main inducer of osteoclast activation during myeloma bone disease, is expressed at the surface of myeloma cells [192;193] and largely induced in bone marrow stromal cells. The therapeutic opportunities that may arise from interfering with RANKL were tested with OPG, a soluble decoy receptor for RANKL and antibody to RANKL, both supposed to regulate osteoclast activation upon the exposure to RANKL. Initial attempts with a recombinant OPG construct (AMGN-0007) were well tolerated and caused a rapid and sustained dose-dependent decrease of bone resorption [36]. More recently, a humanized monoclonal antibody specifically binding to RANKL has been developed (Denosumab, AMG 162), and proved safe and efficient in preliminary studies on

patients with multiple myeloma with radiological confirmed bone lesions [35].

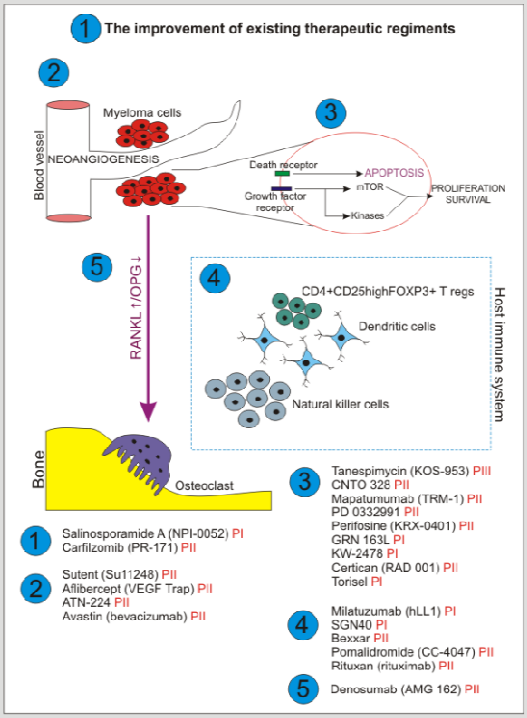

FIGURE 7.
Overview of novel therapeutic targets for multiple myeloma. For general description and numbering refer to text. Below are highlighted currently tested drugs in each category, followed by the indication of phase trial category in red (PI - phase I, PII - phase II and PIII - phase III). The overview of currently used drugs is modified from the Multiple Myeloma Research Foundation: http://www.multiplemyeloma.org/treatments/3.08.php

1.5.2. Bortezomib

The most promising of emerging therapies for multiple myeloma is the inhibition of ubiquitin / proteasome pathway. Proteolysis is a normal cellular process and thus substrates for proteasomes include many cellular proteins that maintain normal cell cycle progression, growth and survival [26]. Conversely, pharmacological inhibition of proteasome function hampers the normal elimination of misfolded proteins, thereby causing a build-up of unwanted proteins and eventual cell death. These laboratory observations were recently translated to the clinical application of proteasome inhibitors as cancer therapies supported by studies suggesting favourable therapeutic index. Indeed, proteasome inhibitors exhibit higher cytotoxicity towards proliferating malignant cells than quiescent normal cells. In context of multiple myeloma, the first proteasome inhibitor bortezomib was shown to target simultaneously the three critical players of myeloma induced bone disease: myeloma cells, OCs and OBs. Bortezomib is a potent inhibitor of myeloma cell growth and survival in vitro seen in both myeloma cell lines, freshly isolated primary myeloma cells [100] and using animal models [167]. Bortezomib triggers apoptosis in myeloma cells mainly by inhibiting the inducible NF-κB activation [98]but, on the other hand, induced the canonical NF-κB activation [99]. However, the actions on NF-κB alone are unlikely to account for the overall anti-myeloma activity of bortezomib. Studies to date suggest that bortezomib affects both various apoptotic signalling cascades and blocks growth/ survival mechanism in myeloma cells. Osteoclasts are also sensitive to bortezomib treatment, and we and other groups have recently shown the inhibitory effects of bortezomib on osteoclasts formation and function [38;249]. Clinical studies show that bortezomib leads to decrease in bone resorption markers regardless of the overall treatment outcome [216]. In addition to inhibiting osteoclasts, bortezomib was demonstrated to possess a beneficial anabolic effects on the skeleton in vitro as it induces the osteoblast differentiation from mesenchymal precursors [81]. In support of this observation, clinical studies have demonstrated significant increases in markers of bone formation, including alkaline phosphatase and osteocalcin in patients responding to treatment [96;247]. In addition to changes in markers of bone formation, bortezomib treatment has also been shown to result in a reduction of serum Dkk1 and RANKL [216].

Bortezomib, has been initially approved as a treatment for relapsed/refractory multiple myeloma patients who already received two of other types of chemotherapy. Recently, bortezomib had been also accepted as a front-line treatment for multiple myeloma independently of the previous treatment history. The drug is administered as a single intravenous injection given at day 1, 4, 8 and 11, and followed by 10-day break in the treatment.

1.5.3. Resveratrol and its analogues

Multiple myeloma remains an incurable disease despite the progress in treatment during recent years. Therefore there is still an urgent need for new drugs with better efficacy and less toxicity. Nature has been a source of medicinal agents for many years and an impressive number of modern drugs have been isolated from natural sources or derived from natural product molecules, especially in cancer therapies. Resveratrol (*trans*-3, 4', 5-trihydroxystilbene; RSV) is a natural compound present in the skin of red fruits, seeds, berries and is concentrated in derived-products such as red wine [87]. RSV is raising a lot of interest because of its possible anti-tumor and cancer-chemopreventive properties, suggested by observations on different cancer cell lines *in vitro*, and also in animal cancer models such as breast cancer [25], skin cancer [106], liver cancer [244], colorectal and intestinal cancers [188;218], lung cancers [117] and neuroblastoma [54]. RSV has been shown to affect a series of critical events associated with tumor initiation and progression, including up regulation of p53 and p21 levels, induction of NO, inhibition of COX, protection against reactive oxygen intermediates, down-regulation of survival factors and proteinases [69;168].

In context of RSV as a potential drug candidate for treatment of multiple myeloma, a recent in vitro study revealed that RSV can induce apoptosis in myeloma cells, prevent osteoclast differentiation and their bone resorption, and promote differentiation of bone mesenchymal stem cells into bone forming osteoblasts [37]. The proapoptotic actions of RSV towards tumour cells were shown to be mediated by antagonising the activation of NF-κB [208] and downstream abrogation of the expression of genes responsible for cell survival [32]. Furthermore, RSV was also shown to potentiate the apoptotic effects of conventional chemotherapeutic agents [23;107], as well as suppressing the

expression of MMPs by myeloma cells [209] and inhibiting bone marrow aniogenesis [103].

In light of these observations, RSV was considered to be a potentially interesting drug that could affect all key aspects of multiple myeloma and the associated bone disease. However, the in vitro studies showed that, in order to elicit its biological effect, RSV must be used at very high concentrations unlikely to be achievable in vivo. The administration of high doses of RSV carries a risk of severe adverse effects observed in rodents [60]. Furthermore, recent studies suggest that the target organs of resveratrol are liver and kidney, where it is concentrated after absorption and is mainly converted to an inactive form - glucuronide conjugate [122]. As RSV shows a very limited therapeutic potential, efforts are directed to search for its natural or synthetic analogues with higher bioavailability and safer [22;154]. Currently, a RSV derivate (STR501) is being tested in phase II clinical trial to assess its safety and efficacy towards multiple myeloma, alone or in combination with bortezomib.

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