Review

The role of the bone marrow microenvironment in multiple myeloma

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Summary. Multiple myeloma (MM) is a malignant disease that results from an excess of monotypic plasma cells in the bone marrow (BM). This malignancy is characterised by complex karyotypic aberrancies. In 60% of all MM there are recurrent primary translocations involving the heavy chain gene locus. The MM cells strongly interact with the BM microenvironment, which is composed of endothelial cells, stromal cells, osteoclasts, osteoblasts, immune cells, fat cells and the extracellular matrix. This interaction is responsible for the specific homing in the BM, the proliferation and survival of the MM cells, the resistance of MM cells to chemotherapy, the development of osteolysis, immunodeficiency and anaemia. New therapeutic agents target both the MM, as well as the interaction MM cell – BM microenvironment.

Key words: Multiple myeloma, Microenvironment, Osteoclast, Bone marrow stromal cell

Epidemiology and clinical presentation

Multiple myeloma (MM) is a malignant disease that results from an excess of monotypic plasma cells (PCs) which are usually actively secreting antibody (M protein or paraprotein). The malignant PC cells (MM cells) are mainly localised in the bone marrow (BM), although small numbers of MM cells can be encountered in the peripheral blood circulation. MM comprises about 2.5% of all malignancies and 10% of the haematological malignancies and MM has an annual incidence of 40 per million. The median age at diagnosis is 63 years; fewer than 2% of MM patients are under 40 years of age at diagnosis. It has a twofold higher incidence in Afro-Caribbean ethnic groups than in Caucasians (UK Myeloma forum, 2001). Men are 1.7 times more affected than women. The aetiology of the disease is unknown.

Genetic factors (Grosbois et al., 1999), rheumatoid arthritis, exposure to ionizing radiation, benzene, dioxins, certain herbicides and insecticides are potential risk factors (Kyle, 1999). Multiple myeloma (MM) is a B-cell malignancy which remains largely incurable despite advances in systemic and supportive therapies.

Signs and symptoms of multiple myeloma include bone pain, which may be present in three-fourths of patients. Osteolytic lesions and compression fractures may be seen in the axial skeleton and proximal long bones, the most common being the spine. There is an increased osteoclastic activity in myeloma patients mediated by osteoclastic stimulating factors. This increased bone resorption may lead to hypercalcemia.

Renal insufficiency is often multi-factorial but is predominantly due to the development of “myeloma kidney” in which the distal convoluted tubules and collecting tubules become obstructed with casts consisting mainly of Bence Jones (monoclonal urinary light chain) protein.

Low levels of endogenous erythropoietin and inhibition of erythroid lineage synthesis often result in anemia with manifestations such as weakness, dyspnoea and pallor. Cellular and humoral immune dysfunction are also commonly observed and result in an enhanced susceptibility to bacterial or herpes zoster infections.

Neurological dysfunction is common during the course of the disease and may be from spinal cord compression manifested as back pain, sciatica, or muscle weakness, or from a demyelinating neuropathy (a paraneoplastic manifestation of the myeloma paraprotein) (Zaidi, 2001).

The most common type of monoclonal protein produced is IgG, followed in frequency by IgA, IgD and extremely rarely IgE. IgA MM is more associated with extra-skeletal disease, whereas IgD MM is more commonly associated with plasma cell leukemia (PCL) and renal damage (Durie et al., 2003).

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Part of the MM is de novo and part is preceded by a monoclonal gammopathy of undetermined significance (MGUS). In one Mayo Clinical Study, 58% of the MM had prior MGUS or plasmacytoma (Kyle et al., 1994).
By definition, MGUS patients are asymptomatic and have stable M-protein measurements. Patients with MGUS usually have less than 10% BM plasmacytosis, a serum monoclonal protein level for IgG ≤ 3.5 g/dl and for IgA ≤ 2 g/dl, a urinary Bence-Jones protein ≤ 1 g/24 h. MGUS is much more common than MM occurring in 1% of the population over age 50 and 3% over age 70 (Greipp, 1995). MGUS patients can be safely observed without chemotherapy. During long-term follow-up 25% of patients with MGUS will develop MM or IgM expressing lymphoma, primary amyloidosis, macroglobulinaemia, chronic lymphocytic leukaemia or plasmocytoma, at a rate of 1% per year (Kyle et al., 2002). The transition of MGUS into MM does not always pass through a period of smouldering MM.

Smouldering MM (2% of the MM) is characterised by a stable intramedullary tumour-cell content of ≥ 10% but ≤ 30%, and none of the other complications of MM (Rosinol et al., 2003) (Table 1).

Patients with active MM (Table 1) present with a BM plasmacytosis of ≥ 10%, a serum monoclonal protein of >3.5 g/dl for IgG and >2 g/dl for IgA, a 24-hour urine monoclonal protein of >1 g and lytic bone lesions. Active MM is, during progression, associated with increasingly severe features of lytic bone disease, anaemia, immunodeficiency and renal impairment and also, in a fraction of patients, the occurrence of tumours in extramedullary sites, such as blood, liver, spleen, lymph nodes, pleural fluid and skin. Exceptionally, infiltration of other organs, such as thyroid (personal observation), the gastro-intestinal tract (Amonkar et al., 2003), prostate (Yasuda et al., 1994), and testis (Oppenheim et al., 1991) can be observed in end-stage disease. Extramedullary MM involving the blood in the terminal phase of MM is called secondary plasma cell leukaemia (PCL).

Variants of MM are primary PCL, non-secretory MM and osteosclerotic MM. Primary PCL refers to patients not in the terminal phase of multiple myeloma in which abnormal PCs comprise at least 20% of the differential peripheral blood count. Hepato- and splenomegaly are common. PCL is usually an aggressive illness which has clinical resemblance to acute leukaemia. Compared to MM there is a tendency in PCL for more frequent extramedullary disease, thrombocytopenia, and high serum lactate dehydrogenase (Dimopoulos et al., 1994). Non-secretory MM is defined by the absence of M-protein in the serum and urine. The MM cells either do not produce or do not secrete immunoglobulins. Mutations in genes encoding \( V_L \) (Dul, 1990) and \( C_L \) (Coriu et al., 2004) have been implicated as causal factors in non-secretory MM. Osteosclerotic MM is a very rare entity associated with peripheral neuropathy, organomegaly, endocrinopathy, M-protein and skin changes (POEMS syndrome).

The Durie-Salmon staging system continues to be

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**Table 1. Diagnosis of MM, indolent and smouldering MM.**

<table>
<thead>
<tr>
<th>MM</th>
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<tr>
<td><strong>Major criteria</strong></td>
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<tr>
<td>I. Plasmocytoma on tissue biopsy</td>
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<tr>
<td>II. BM plasmocytosis with &gt; 30% plasma cells</td>
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<tr>
<td>III. Monoclonal globulin spike on serum electrophoresis: &gt; 3.5 g/dl for IgG, &gt; 2 g/dl for IgA, ≥ 1 g/24 h for k or l Bence Jones proteinuria</td>
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<tr>
<td><strong>Minor criteria</strong></td>
</tr>
<tr>
<td>a. BM plasmocytosis with 10-30%</td>
</tr>
<tr>
<td>b. Monoclonal globulin spike: 3.5 g/dl for IgG, 2 g/dl for IgA, ≤ 1 g/24 h for Bence Jones proteinuria</td>
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<tr>
<td>c. Lytic bone lesions</td>
</tr>
<tr>
<td>d. Uninvolved immunoglobulin levels IgM &lt;50 mg/dl, IgA &lt;100 mg/dl, or IgG &lt;600 mg/dl</td>
</tr>
<tr>
<td><strong>Diagnosis of MM</strong></td>
</tr>
<tr>
<td>In a symptomatic patient requires a minimum of one major + one minor criterion:</td>
</tr>
<tr>
<td>I + b, I + c, I + d</td>
</tr>
<tr>
<td>II + b, II + c, II + d</td>
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<tr>
<td>III + a, III + c, III + d</td>
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<tr>
<td>or three minor criteria that must include a + b:</td>
</tr>
<tr>
<td>a + b + c or a + b + d</td>
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<tr>
<td><strong>Indolent MM</strong></td>
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<tr>
<td>No bone lesions or limited bone lesions (≤ 3 lytic lesions)</td>
</tr>
<tr>
<td>No compression fractures</td>
</tr>
<tr>
<td>M protein levels IgG ≤ 7 g/dl, IgA ≤ 5 g/dl</td>
</tr>
<tr>
<td>No symptoms or associated disease features: performance status &gt;70%, hemoglobin &gt;10 g/dl, serum calcium normal, serum creatinine &lt;2 mg/dl, no infections</td>
</tr>
<tr>
<td><strong>Smoulder MM</strong></td>
</tr>
<tr>
<td>Same as indolent MM except:</td>
</tr>
<tr>
<td>no bone lesions</td>
</tr>
<tr>
<td>BM plasmocytosis ≤ 30%</td>
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used worldwide. This system integrates the major clinical parameters in correlation with measured myeloma cell mass (the total number of MM cells in the body) (Durie and Salmon, 1975) (Table 2). Numerous groups have proposed new systems to more accurately and simply stage and/or classify MM patients into prognostic categories. A simple alternative to the Durie-Salmon staging system was developed by the Southwest Oncology Group (SWOG) (Jacobson et al., 2003) (Table 3). This SWOG staging system focused on two common measures with prognostic importance: serum $\beta_2$ microglobulin and serum albumin. $\beta_2$ microglobulin may be a product of MM cells and can be used as a tumour marker to predict the course of the disease (Norfolk et al., 1980). Serum albumin is an indirect indicator of IL-6 levels, liver function and nutritional status of the patients. Low serum albumin correlates with both rapid MM growth and the patients overall performance status.

### Histopathology of MM

The trephine biopsy is helpful for the diagnosis, prognosis, evaluation of the haematopoietic reserve, and finally, for the evaluation of the treatment efficacy, for the detection of therapy-related and other complications (aplasia, fibrosis, amyloidosis, osteopenia, osteosclerosis, secondary myelodysplasia/leukaemia) and for the study of angiogenesis.

The trephine biopsy allows us, in contrast to the aspirate, to evaluate the architectural organisation of BM PCs. The topographic organisation of PCs is useful in distinguishing reactive plasmocytosis from MM. In the former the PCs tend to be arranged along the capillaries. In the latter, the clustering of PCs is randomly distributed between fat cells, without topographic orientation to capillaries, even if the percentage of MM cells in the aspirate smears is below 10%. In MM with a nodular growth pattern of the MM cell, and especially in MM with BM fibrosis, the percentage of PC in the aspirate smears is usually underestimated.

Regarding the PC cell type, the most significant prognostic differences are found when MM cells are subdivided into two broad categories (Bartl et al., 1987): the plasmacytic type, with predominantly non-nucleolated PC (68% of MM, 32 months median survival) and the plasmablastic type, with predominantly nucleolated PCs (32%, 8 months median survival). However, when other characteristics such as cellular size, cytoplasmic structure and nuclear configuration are taken into consideration, the spectrum of MM cells could be divided into six cell types which can be combined into three prognostic grades: MM, low-grade malignancy (Marschalko and small cell type, 70% of MM, 40 months median survival); MM, intermediate-grade malignancy (cleaved, polymorphous and asynchronous, 20% of MM, 20 months median survival) and MM, high-grade malignancy (blastic, 2%, 8 months median survival).

Some unusual cytological variations of MM cells such as the “flaming cells”, “mott, morular or grape cells” and the “thesaurocytes” are well known in the haematological literature. Less well known and therefore not readily recognised, are the MM cells with

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**Table 2. Durie and salmon staging system.**

| STAGE I (low cell mass: 600 billion MM cells/m²) |  
| All of the following: |  
| Haemoglobin value > 10.5 g/dl |  
| Serum calcium value normal or < 10.5 mg/dl |  
| Bone X-ray, normal bone structure (scale 0) or solitary |  
| Bone plasmocytoma only |  
| Low M-component production rates: IgG value < 5.0 g/dl; IgA value < 3.0 g/dl; Urine light chain M-component on electrophoresis < 4 g/24 h |  

| STAGE II (intermediate cell mass: 600 to 1,200 billion MM cells/m²) |  
| Fitting neither stage I or III. |  

| STAGE III (high cell mass: > 1,200 billion MM cells/m²) |  
| One or more of the following: |  
| Haemoglobin value < 8.5 g/dl |  
| Serum calcium value > 12 mg/dl |  
| Advanced lytic bone lesions (scale 3) |  
| High M-component production rates: IgG value > 7.0 g/dl; IgA value > 5.0 g/dl; Urine light chain M-component on electrophoresis > 12 g/24 h |  

**SUBCLASSIFICATION (either A or B)**

A: relatively normal renal function: serum creatinine value < 2.0 mg/dl
B: abnormal renal function: serum creatinine value ≥ 2.0 mg/dl

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**Table 3. Swog classification.**

| STAGE I | $\beta_2$M < 2.5 mg/l |
| STAGE II | 2.5 < $\beta_2$M < 5.5 mg/l |
| STAGE III | $\beta_2$M > 5.5 mg/l & albumin ≥ 30 g/l |
| STAGE IV | $\beta_2$M ≥ 5.5 mg/l & albumin < 30 g/l |
Bone marrow microenvironment in multiple myeloma

Monocytoid features, signet-ring MM cells, histiocytoid MM cells, clear MM cells, MM cells with spindle cell morphology, and MM cells with oncotic changes (Banerjee et al., 2004). The prognostic significance of these unusual cytological variants is not well determined.

The growth pattern in MM is of prognostic significance: the presence of nodules of MM signals a progressive course, osteolytic lesions and an unfavourable prognosis (Bartl and Frisch, 1995).

Although not used in daily practise, the plasma cell burden (tumour load) measured histomorphometrically as the “percentage of infiltration volume” had been shown to have prognostic value (Carbone et al., 1987):
- stage I: minimal infiltration (<5 vol%), in 14% of MM cases at diagnosis, median survival of 86 months.
- stage II: low infiltration (5-19 vol%), in 28% of MM cases at diagnosis, median survival of 46 months.
- stage III: intermediate infiltration (20-50 vol%), in 40% of MM cases at diagnosis, median survival of 25 months.
- stage IV: high infiltration (>50 vol%), in 18% of MM cases at diagnosis, median survival of 15 months.

The PC labeling index (PCLI), a measure of the percentage of PC cells in the S-phase, is determined by measuring the bromodeoxyuridine antibody BU-1. A PCLI higher than 1% is considered high. The PCLI has been demonstrated to be an independent prognostic factor in newly diagnosed patients (Greipp et al., 1988, 1993; Boccadoro et al., 1987). The percentage of abnormal metaphases in conventional cytogenetic analysis correlates with the PCLI as well as with the extent of BM involvement by MM (BM PC percentage) (Rajkumar et al., 1999). This is not unexpected, since chromosomal abnormalities may offer a proliferative advantage to the MM cells, thereby leading to an unfavorable prognosis. Plasma cell type also correlates with PCLI (Schambeck et al., 1996). Although exceptions occur, patients with PC of the Marshalko type or small cell type have generally a low PCLI. A high PCLI in MM patients with apparently stable, plateau phase with minimal numbers of residual monoclonal PC, is an adverse parameter that may predict a short time to disease progression and death (Steenosma et al., 2001). High dose chemotherapy has been shown to improve the overall survival of patients with a high PCLI (>1.2%), whereas it did not prolong overall survival in those with a low PCLI (Boccadoro et al., 1999).

Evolving genetic events in MM

MM cells have extensive somatic mutations of rearranged immunoglobulin (Ig) genes, and in the vast majority of cases, they express an Ig isotype other than IgM, which indicates post-follicular B-cell origin. Further support for the post-follicular origin of MM comes from the analysis of the mutational status of the variable region of the IgH gene. MM is characterised by mutated homogeneous variable gene sequences, which indicates that the B cell has passed the germinal centre and is no longer under continuous influence from the somatic hypermutation mechanism.

The low rate of proliferation of MM cells makes it difficult to perform conventional cytogenetics. Interphase FISH and molecular genetic investigations of MM have provided evidence of a marked karyotypic instability. Numerical chromosome structural changes are present in virtually all MM, and in most, if not all cases of MGUS (Avet-Loiseau et al., 1999; Drach et al., 1995; Flactif et al., 1995; Zandecki et al., 1996, 1997). MM can be subdivided into two cytogenetic categories (Debes-Marun et al., 2003): hypodiploid/pseudodiploid (which also includes the near-tetraploid karyotypes) and the hyperdiploid. The latter is defined by the presence of multiple trisomic chromosomes (mostly chromosomes 3, 5, 7, 9, 11, 15 and 19) associated with a gain of DNA-observed as aneuploidy with flow cytometry. The number of structural abnormalities per cell is lower in the hyperdiploid group (average 5/1) than in the hypodiploid group (average 9/1) but the types of abnormalities are similar in both groups (Smadja et al., 2001). Hypodiploidy is associated with an unfavourable prognosis (Smadja et al., 2001; Debes-Marun et al., 2003).

Unbalanced chromosomal structural changes are present in most MM (Cigudosa et al., 1998). Karyotypic complexity is thought to increase during tumour progression. Chromosomal gains that recur in more than 30% of MM include 1q, 3q, 9q, 11q and 15q. Monoallelic loss of 13q sequences is one of the most frequent abnormalities in MM (50%) and is an independent predictor of poor prognosis (Avet-Louiseau et al., 2000; Kuehl, 2002).

During the pathogenesis of MM, most primary translocations are reciprocal translocations that juxtapose an oncogene and a IgH gene sequence. These reciprocal translocations result in activation of oncogenes, because they come under the influence of enhancer regions at the IgH gene locus. They are mediated mainly by errors in Ig heavy chain switch recombinations, but sometimes by errors in somatic hypermutation during plasma-cell generation in germinal centres (Bergsgel et al., 2001). These translocations are almost universal in MM cell lines and are present in 70% of PCL, in about 60-75% of MM and in 50% of MGUS and smouldering MM (Fonseca et al., 2002). Despite the promiscuity of translocation partners, most Ig translocations involve three groups of genes: the first group contains the cyclins D1 (on 11q13), D3 (on 6p21), and possibly D2 (on 12q13) which are translocated in 25% of tumours. The second group comprises two proteins that are encoded on 4p16: MMSET – a nuclear SET DOMAIN protein – and fibroblast growth factor receptor 3 (FGFR3), an oncogene receptor tyrosine kinase. These are translocated in 15% of MM. The third group of proteins in which encoding genes are translocated in 10% of MM comprises two B-ZIP
transcription factors c-maf (on 16q23) and mafB (on 20q11). The oncogene c-maf transactivates the cyclin D2 promoter and enhances MM proliferation. c-maf driven expression of integrin b7 enhances MM adhesion to BM stroma and increases the production of vascular endothelial growth factor (VEGF) (Hurt et al., 2004). Given the lack of cyclin D1 expression in normal lymphocytes, the occurrence of Ig translocations that deregulate cyclin D1 and D3 in about 25% of the tumours, the expression of cyclin D1 in nearly 40% of tumours lacking a t(11;14) translocation, and the increased expression levels of cyclin D2 in most remaining tumours, it seems apparent that almost all MM deregulate at least one of the cyclin D genes. The deregulation of one of the three cyclin D genes may render the cells more susceptible to proliferative stimuli, resulting in a selective expansion as a result of interaction with BM stromal cells (BMSCs) that produce IL-6 and other cytokines (cfr infra).

On the basis of the expression of cyclin D1, 2 and 3 and on the basis of the five recurrent translocations (11q13 – cyclin D1; 6p21 – cyclin D3; 4p16 – MMSET & usually FGFR3; 16q23 – c-maf; and 20q11 – mafB, recently a translocation and cyclin D (TC) molecular classification was proposed (Hideshima et al., 2004) (Table 4). TC1 MM (18%) express high levels of either cyclin D1 or cyclin D3 as a result of an Ig translocation; TC2 MM (37%) express low to moderate levels of cyclin D1 or cyclin D3 as a result of an Ig translocation; TC3 MM (22%) is a mixture of MM that do not fall into one of the other groups, with most expressing cyclin D2, but a few also expressing low cyclin D1 or D3. TC4 MM (16%) have a t(4;14) resulting in high levels of MMSET and in most cases FGFR3. They also express high levels of cyclin D2. TC5 MM (7%) express the highest level of cyclin D2 in most tumours lacking a t(11;14) translocation, and the increased expression levels of cyclin D2 in most remaining tumours, it seems apparent that almost all MM deregulate at least one of the cyclin D genes. The deregulation of one of the three cyclin D genes may result in a selective expansion as a result of interaction with BM stromal cells (BMSCs) that produce IL-6 and other cytokines (cfr infra).

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Table 4. Translocation and cyclin D (TC) molecular classification of MM.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>PRIMARY TRANSLOCATION</th>
<th>GENES AT BREAKPOINT</th>
<th>D-CYCLIN</th>
<th>PLOIDY</th>
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<tbody>
<tr>
<td>TC1</td>
<td>11q13</td>
<td>Cyclin D1</td>
<td>D1</td>
<td>NH</td>
</tr>
<tr>
<td></td>
<td>6p21</td>
<td>Cyclin D3</td>
<td>D3</td>
<td>NH</td>
</tr>
<tr>
<td>TC2</td>
<td>None</td>
<td>None</td>
<td>D1</td>
<td>H</td>
</tr>
<tr>
<td>TC3</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>H-NH</td>
</tr>
<tr>
<td>TC4</td>
<td>4p16</td>
<td>FGFR3/MMSET</td>
<td>D2</td>
<td>NH-H</td>
</tr>
<tr>
<td>TC5</td>
<td>16q23</td>
<td>c-maf</td>
<td>D2</td>
<td>NH</td>
</tr>
<tr>
<td></td>
<td>20q11</td>
<td>mafB</td>
<td>D2</td>
<td>NH</td>
</tr>
</tbody>
</table>

H: hyperdiploid, NH: non-hyperdiploid

Activating point mutations of N-ras or K-ras oncogenes occur in 40% of MM at the time of diagnosis and in less that 5% of MGUS (Corrandini et al., 1993; Liu et al., 1996).

Mutations and/or mono-allelic deletions of p53 (17p13) are rare in newly diagnosed MM, but are found with increasing frequency in patients with relapsed disease and PCL (Drach et al., 1998).

There is increasing evidence that in addition to genetic aberrations epigenetic processes play a major role in carcinogenesis of lymphoid and haematopoietic malignancies (Takahashi et al., 2004). DNA methylation, catalysed by DNA methyltransferase, involves the addition of a methyl group to the carbon 5 position of the cytosine ring in the CpG dinucleotide, leading to a conversion to methylcytosine. DNA methylation is associated with several changes in chromatin structure, including the regulation of histone methylation and acetylation and recruitment of proteins to the methylated sites. DNA methylation of the CpG dinucleotides in the promoter region of a gene can interfere with the binding of transcription factors, thus suppressing gene expression. Methylated DNA segments can also repress the transcription of genes distant from the methylation site (located on the same chromosome) by altering the structure of chromatin domains (Razin, 1998). Normally, CpG dinucleotides in the 5’ promoter regions are protected from methylation, so that these genes are in a transcription-ready state. Aberrant methylation of gene promoter regions is one of the most important epigenetic changes found in both MM and MGUS. In MM, hypermethylation of genes including p15[INK4b], p16[INK4a] (Juge-Morineau et al., 1997) and p18[INK4c] (Kulkarni et al., 2002), tissue inhibitor of metalloproteinase 3 (TIMP3), E-cadherin (Seidl et al., 2004), death-associated protein kinase (DAPK) (Galm et al., 2004), p73 (Galm et al., 2004), suppressor of cytokine signalling (SOCS)-1 (Galm et al., 2003), the estrogen receptor (Chim et al., 2004) and O6-methylguanine DNA methyltransferase (MGMT) (Seidl et al., 2004) has been observed. p15[INK4b], p16[INK4a] and p18[INK4c] are cell-cycle regulators involved in inhibition of G1 phase progression. They compete with cyclin D1 for binding to CDK4 / CDK6 and inhibit CDK4/6 kinase activity, resulting in dephosphorylation of the retinoblastoma protein and inhibit CDK4/6 kinase activity, resulting in dephosphorylation of the retinoblastoma protein and related G1 growth arrest. Patients with an unmethylated status of p16[INK4a] have a better outcome compared to those with a p16 methylation at diagnosis (Galm et al., 2004). Inactivation of p18[INK4c] occurs at a low frequency, most likely as a late progression event (Kulkarni et al., 2002). DAPK regulates apoptosis induced by interferon gamma (IFN-γ). The SOCS protein is involved in the Janus activating kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. It suppresses signalling through various cytokines such as IL-4, IL-6, leukaemia inhibitory factor, oncostatin M, and interferon.

Seidl et al. (2004) analysed samples of MGUS
patients and consecutive samples of MM patients using a methylation-specific polymerase chain reaction. Methylation frequencies for p16, TIMP3, p15, E-cadherin, DAPK, p73 and MGMT were as follows: 28%, 35%, 10%, 0%, 17%, 21% and 7%, respectively, in patients with MGUS and 36%, 29%, 27%, 27%, 22%, 15% and 4%, respectively, in patients with MM. The E-cadherin gene was unmethylated in all MGUS samples, in contrast to the increasing percentages of E-cadherin gene methylation observed in smouldering MM, active MM, and PCL samples. The transmembrane glycoprotein E-cadherin mediates Ca\textsuperscript{2+}-dependent MM, and PCL samples. The transmembrane gene methylation observed in smouldering MM, active cadherin gene was unmethylated in all MGUS samples, 15% and 4%, respectively, in patients with MM. The E-cadherin gene was unmethylated in all MGUS samples, in contrast to the increasing percentages of E-cadherin gene methylation observed in smouldering MM, active MM, and PCL samples. The transmembrane glycoprotein E-cadherin mediates Ca\textsuperscript{2+}-dependent intercellular adhesion, which is essential for normal tissue homeostasis. In a variety of solid tumours, loss of E-cadherin has been associated with invasion and metastasis, and methylation has been identified as the main reason for loss of expression (Kudo et al., 2004). In agreement with the hypothesis that E-cadherin methylation was associated with advanced disease, in two-patients with follow-up samples, E-cadherin gene was found to be unmethylated at the time of diagnosis, but methylated at the time of disease progression. In most of their cases, by comparing methylation patterns in samples obtained during follow-up, either methylation in both the first sample and second sample or methylation in the second sample without methylation in the first sample was observed. In a study (Takahashi et al., 2004) examining 40 MM cases and 20 MGUS cases, 85% of MGUS cases had one or more genes methylated. Methylation of MGUS was significantly higher than that in control tissue, but significantly lower than that in MM cases. In the same study 48 leukaemias and 42 lymphomas were also analysed. They found a rather similar methylation pattern of the 14 genes studied among the leukaemias and the lymphomas. However, the pattern of MMs varied from the other tumour types for six genes. With microarray studies (Pompeia et al., 2004) in the KAS-6/1 MM cell line, a number of genes (BAD, BAK, BIK, and BAX) involved in apoptosis were found to be suppressed by methylation. With the use of the drug zebularine CpG methylation could be reduced. After removal of the demethylating drug, the addition of IL-6 restored CpG methylation and re-established previously silenced gene patterns, thus implicating a novel role of IL-6 in processes regulating epigenetic gene repression and carcinogenesis.

Acetylation of the lysine residues at the N terminus of histone proteins removes positive charges, thereby reducing the affinity between histones and DNA. RNA polymerase and transcription factors are then able to access the promoter region of active genes. Acetylation of histone tails in combination with DNA hypomethylation correlates with transcriptional activity and has been shown to correlate with cyclin D1 activation in B-cell malignancies (Liu et al., 2004). JAG2, the NOTCH ligand, has been found to be overexpressed in MGUS and MM. This overexpression appears to be a consequence of hypomethylation of the JAG2 promoter (Houde et al., 2004). JAG2 induces via NOTCH the secretion of IL-6, VEGF and IGF-1 in BMSC (Houde et al., 2004).

Global gene-expression profiling (GEP) of MM cells can be obtained by means of nucleic acid microarrays (gene chips) (Sheils et al., 2003). Unsupervised hierarchical cluster analysis of gene expression patterns in CD138-enriched plasma cells from untreated MM patients, allowed the identification of 4 MM subgroups, with the two extremes being similar to either MGUS (MM1 subgroup) or myeloma cell lines (MM4 subgroup) (Zhan et al., 2002). The GEP signatures of these subgroups of patients highly correlated with distinct cytogenetic abnormalities, clinical parameters, and survival after high-dose therapy with peripheral blood stem cell transplant. MM4 subgroup was more likely to have abnormal cytogenetics, increased serum β2 microglobulin (>4 mg/mL), increased creatinine, and deletions of chromosome 13 (Zhan et al., 2002). These data lend credence to the concept that MM consists of multiple disease entities with distinct mechanisms of transformation, as evidenced by the highly coordinated gene expression changes seen in each subtype. It is currently not resolved whether this coordinated subgroup-specific expression occurs through deregulation of distinct molecular pathways. However, if confirmed, these data could provide a framework for the development of novel therapeutic interventions based on the molecular biology of disease subtypes. Another yet unexplained issue is the observation that GEP fails to explicitly distinguish MGUS and the MM1 subgroup, yet the former condition only converts to overt MM at a rate of 1% per year. To define specific pathways important in the multistep transformation process of normal plasma cells (PCs) to monoclonal gammopathy of uncertain significance (MGUS) and multiple myeloma (MM), Davies et al. (2003) have applied microarray analysis to PCs from 5 healthy donors (N), 7 patients with MGUS, and 24 patients with newly diagnosed MM. Unsupervised hierarchical clustering using 125 genes with a large variation across all samples defined 2 groups: N and MGUS/MM. Supervised analysis identified 263 genes differentially expressed between N and MGUS and 380 genes differentially expressed between N and MM, 197 of which were also differentially regulated between N and MGUS. Only 74 genes were differentially expressed between MGUS and MM samples, indicating that the differences between MGUS and MM are smaller than those between N and MM or N and MGUS. Differentially expressed genes included oncogenes/tumor-suppressor genes (LAF4, RB1, and disabled homolog 2), cell-signaling genes (RAS family members, B-cell signaling and nuclear factor (NF)-κB genes), DNA-binding and transcription-factor genes (XBP1, zinc finger proteins, forkhead box, and ring finger proteins), and developmental genes (WNT and SHH pathways).

GEP has also been used to assess the actual origin of the disease. The molecular signatures of cells representing the late stages of human B cell development
were compared to high-risk and low-risk forms of MM. The high-risk form of MM (MM4 subgroup) proved to have a tonsil B-cell-like signature, whereas the MM3 subgroup tended to have tonsil PC-like signature and the low-risk MM2 subgroup tended to have a BM PC-like signature (Zhan et al., 2003). MM1 showed no significant linkage with normal cell types studied.

Using GEP, Moreaux et al. (2004) identified overexpression of 2 receptors for a proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF) in MM cells compared to normal PC. They subsequently confirmed the expression of BAFF and APRIL receptors in a majority of 13 MM cell lines as well as in purified primary MM cells of 11 patients. BAFF and APRIL belong to the TNF superfamily and are critical for normal B cell development, maturation and homeostasis. They are not only involved in B cell autoimmunity (Mackay et al., 2005), but also in the pathogenesis and maintenance of B lineage haematological malignancies (He et al., 2004; Jelinek and Darce, 2005). Binding of APRIL and BAFF to these receptors in MM induces activation of NF-κB, phosphatidylinositol-3 (PI-3) kinase/AKT, and mitogen-activated protein kinase (MAPK) pathways and induces strong upregulation of the Mcl-1 and Bcl-2 antiapoptotic proteins. Serum BAFF and APRIL were shown to be increased in MM patients. This is an example of how GEP can help in finding new molecular pathways and potential therapeutic targets.

Because microarrays are not likely to become routine clinical tests, global GEP studies can be used to identify a small subset of genes whose expression can be applied in the development of gene-based risk-adapted patient stratification. At the American Society of Haematology meeting in December 2004, a preliminary study was presented based on the use of microarrays containing 12,000 genes in a population of 221 patients. Three genes associated with rapid relapse were identified in pretreatment PCs of these patients: RAN, ZHX-2 and CHC1L (Harousseau et al., 2004). Patients with high RAN expression had increased risk of a disease event while patients with high ZHX-2 or high CHC1L had decreased risk of an event (longer event-free survival). Each of these genes had independent prognostic influence, so the combination of RAN overexpression and loss of expression of ZHX-2 and CHC1L was associated with a dismal prognosis. RAN is a member of the Ras family of GTPase proteins that has a role in many aspects of cell biology, including shuttling protein and RNA in and out of the nucleus as well as regulating chromosomal condensation, spindle formation, nuclear assembly, and cell cycle-progression (Quimby and Dasso, 2003). ZHX-2 has been shown to be a negative regulator of the NF-Y transcription factor, which is a transcription regulator of many genes in cell cycle control and proliferation (Kawata et al., 2003). The function of the CHC1L gene is not well established, but it has been proposed to be a candidate tumour suppressor gene.

In an effort to gain insight into the mechanism of action of various single-agent compounds (cfr infra), GEP before and after short-term drug treatment has been performed to identify potential mechanisms of action (Shaughnessy et al., 2002). PECAM1 (CD31), VEGF (vascular endothelial growth factor) and the anti-apoptotic molecule MCL-1 (myeloid cell factor-1) were downregulated after treatment with dexamethasone. PECAM-1 has been shown to have an inhibitory activity on mitochondrial-dependent apoptosis (Gao et al., 2003). Thalidomide induced changes in 57 genes. Revlimid, the thalidomide analogue CC-5013, caused changes in 98 genes. The proteasome inhibitor bortezomib induced changes in 9 genes.

Although the molecular signature of MM cells is identified, the signature of the stromal component of the BM microenvironment and whether this signature is qualitatively altered is not yet fully determined. This knowledge is of clinical relevance, as it is becoming increasingly accepted that an important adjunct to conventional therapies, which target the tumour cell directly, depend on the manipulation of the microenvironment (cfr infra). In a pilot study of Shaughnessy et al. (2002), a pair-wise comparison of normal biopsies and MM biopsies has revealed 146 genes with decreased and 86 genes with increased expression in the MM biopsies. To identify which of the 232 genes were PC specific, a pair-wise comparison of each MM biopsy with purified PCs derived from the aspirate from the same patient on the same day was performed. Substracting overlapping significant genes revealed 75 genes that were significantly different. Of these “microenvironment-associated genes”, 54 showed a decreased expression, and 21 an increased expression. Within the upregulated genes, the four most significant altered genes were a voltage gated K+ channel-related transcript, fibronectin, collagen 3A (adhesion molecule), and matrix metalloproteinase 8 (MMP-8). MMPs play a role in angiogenesis (cfr infra). The expression of the fibronectin and collagen 3A gene was not elevated in MGUS biopsies. The two genes were expressed at several fold higher levels in the MM biopsies with high tumour load compared to those with a low tumour load. The apparent upregulation of two adhesion molecules in cells of the microenvironment is an indication for the important role of the micro environment in MM.

Host interactions in MM

The selective localisation of MM cells in the BM is characteristic of MM. The BM microenvironment provides the appropriate “soil” for intramedullary MM. This microenvironment is a complex structure of various extracellular components and many cell types including the “fibroblastic” BMSCs, vascular endothelial cells (ECs), osteoblasts, osteocytes, and immune cells (Fig. 1). Reciprocal positive and negative interactions among these cells are mediated via a variety of cytokines, receptors and adhesion molecules.
The biological phenomena affected by these tumour-host interactions are: homing of MM cells to the BM, spread of MM via the microvasculature to other sites within the BM and the generation of paracrine factors. These factors are involved in survival (inhibition of apoptosis), differentiation, proliferation, drug resistance, osteoclastogenesis, inhibition of osteogenesis, angiogenesis, humoral and cellular immunodeficiency and anaemia.

**Interaction with the bone marrow endothelial cells**

MM cells are attracted to the BM by chemokines, such as monocyte chemoattractant protein (MCP-1) (Vanderkerken et al., 2002; Vande Broek et al., 2003). The BM endothelial cells (BMECs) are the first cells encountered by the MM cells upon entry into the BM environment from the blood circulation. The ECs contribute to the specific homing through the expression of chemokines and adhesion molecules. The splice variant CD44v10 (Asosingh et al., 2001) is necessary for adhesion to BMEC. The EC also induce the expression of key molecules such as insulin-like growth factor (IGF)-1R (Asosingh et al., 2000), matrix metalloproteinase (MMP)-9 (Van Valckenborgh et al., 2002; Vande Broek et al., 2004), 67kD Laminin receptor (Vande Broek et al., 2001) and CD44v6 (Asosingh et al., 2000) on the MM cells, enabling them to migrate with a higher efficiency. The role of BMECs in MM and other haematological malignancies has been previously reviewed in this journal (De Raeve et al., 2004).

**Interaction with the bone marrow fibroblastic stromal cells and the extracellular matrix**

After interacting with the BMECs, the MM cells are further attracted to the BM through stromal cell-derived factor-1α (Hideshima et al., 2002), insulin-like growth factor 1 (Vanderkerken et al., 1999) and laminin-1 (Vande Broek et al., 2001), a component of the basement membrane beneath the EC layer.

Once arrived in the BM, adhesion molecules mediate homotropic interactions with other MM cells, as well as heterotropic interactions with extracellular matrix and BMSCs (Dalton, 2003). The adhesion of MM cells to BM stroma is mediated by several types of cell membrane receptors. The β1-integrin very late-activating antigen (VLA)-4 (CD49d) binds to fibronectin and vascular cell adhesion molecule (VCAM)-1 (CD106) on other cells (Sanz-Rodriguez et al., 1999). The β1-integrin VLA-5 (CD49e) binds to fibronectin. The αvβ3 integrin lymphocyte function-associated antigen (LFA)-1 (CD11a/CD18) allows homotypic cell aggregation and heterotypic cell binding to intercellular adhesion molecule (ICAM)-1 (CD54) (Kawano et al., 1991). The αvβ3 integrin (CD51/CD61), expressed on the MM cell, binds to vitronectin and fibronectin. The incubation of MM cells with vitronectin or fibronectin results in an increased release of MMP-2 and MMP-9 (Ria et al., 2002). Syndecan-1 (CD138), a heparan sulphate proteoglycan, regulates tumor cell growth and survival, and elevated serum levels correlate with increased tumour cell mass, decreased MMP-9 activity (Kausal et al., 1999) and poor prognosis (Dhodapkar et al., 1998). Furthermore, adhesion of MM cells via syndecan-1 to collagen induces MMP-1, thereby promoting bone resorption and tumour invasion.

Adhesion of MM cells to BMSCs triggers nuclear factor-κB-dependent transcription and secretion of IL-6 in the latter (Uchiyama et al., 1993; Chauhan et al., 1996). IL-6 is the most important growth factor in MM (Kawano et al., 1988), protects the MM cells from
apoptosis (Lichtenstein et al., 1995) and confers resistance to dexamethasone, a common conventional therapy, via the PI3-K/Akt signalling (Hideshima et al., 2001b). Clinically, serum IL-6 and IL-6 receptors are prognostic factors in MM reflective of the proliferative fraction of tumour cells (Stasi et al., 1998). The receptor for IL-6 (gp80, CD80, IL6R) consists of a signal-transducing molecule IL-6Rß and a specific ligand-binding protein IL-6Rα. IL-6Rα can be found on the membrane, but also exists in a soluble form, sIL-6Rα. The binding of IL-6 to IL-6R induces phosphorylation of gp130 (Taga et al., 1989), which in turn activates Ras (rat sarcoma virus)/Raf/MAPK (mitogen activated protein kinase) (MEK)/ERK, PI3-K/Akt and JAK/STAT3 (signal transducer and activator of transcription) downstream signalling pathways in the MM cells. Activation of the JAK/STAT3 pathway induces proliferation and inhibition of Fas-antigen-induced apoptosis (Catlett-Falcone et al., 1999). Genes activated via STAT3 include bcl-xL (Catlett-Falcone et al., 1999) and myeloid cell factor-1 (mcl-1). Mcl-1 has recently been identified as a crucial survival factor for MM (Derenne et al., 2002). IL-6 induces X-box binding protein-1 (XBP-1), a transcription factor which is involved in differentiation of normal B cells to PCs (Wen et al., 1999).

The major source of IL-6 is the BMSC. IL-6 is involved in a number of amplification loops: IL-6 strongly increases the cell surface expression of the heparan sulfate proteoglycan CD44 on MM cells and, in turn, MM cells induce IL-6 secretion by BMSCs (Vincent and Mechti, 2004). MM cells secrete IL-1ß (Thomas et al., 1998), transforming growth factor(TGF)-ß (Carter et al., 1990; Urashima et al., 1996), tumour necrosis factor(TNF)-α (Urashima et al., 1996) basic fibroblast growth factor (bFGF) and VEGF (Dankbar et al., 2000), and in turn, these cytokines induce the secretion of IL-6 by the BMSCs (Fig. 2). Antibodies against the adhesion molecules VLA-4 (CD49d), CD29, LFA-1, CD44 and the 86-kD subunit of Ku autoantigen are able to inhibit the secretion of IL-6 by BM stroma in co-culture with MM cells (Lorkhorst et al., 1994; Teoh et al., 1998). Stromal cell-derived factor (SDF-1α), a chemokine secreted by the BMSCs, induces IL-6 and VEGF secretion by BMSCs.

Other growth factors in MM include granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), stem cell factor (SCF), TNF-α (Hideshima et al., 2001a), CD40 ligand (Westendorf et al., 1994), hepatocyte growth factor (HGF) (Borset et al., 1996; Iwasaki et al., 2002), IGFB-1 (Hallek et al., 1998), IL-3 (Lee et al., 2004), IL-10 (Lu et al., 1995), IL-15 (Tinhofer et al., 2000), IL-21 (Brenn et al., 2002), VEGF (Podar et al., 2001) and osteopontin (Saeki et al., 2003; Caers et al., 2004). IGFB-1 induces proliferation, survival, migration and drug resistance to DEX in MM cells via MEK/ERK and PI3-K/Akt signalling cascades (Georgii-Hemming et al., 1996; Ge et al., 2000; Tu et al., 2000). IGFB-1 upregulates intracellular anti-apoptotic proteins including c-Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein (cFLIPL), survivin, c-inhibitor of apoptosis (XIAP) and increases telomerase activity via induction of PI3-K/Akt/NF-κB (for nuclear factor involved in the transcription of κ light chain gene in B lymphocytes) signalling (Akiyama et al., 2002).

TNF-α protein and mRNA is expressed in both MM cells and BM mononuclear cells (Lichtenstein et al., 1989). TNF-α secretion is higher in those MM patients with bone disease (Davies et al., 2000). TNF-α secretion by MM cells induces NF-κB dependent upregulation of adhesion molecules (CD49d, CD54) on both MM cells and BMSCs. This results in the induction of IL-6, IGFB-1 and VEGF secretion in BMSCs. The increased cell adhesion has been shown in MM to interfere with Fas-antigen (Apo-1/CD95) apoptosis signalling. β1 integrin-mediated adhesion (VLA-4, CD49d) to fibronectin inhibits Fas-induced caspase-8 activation and apoptosis by influencing the trafficking of cFLIPL and increases the availability of cFLIPL. Haematological cancer cell lines that are grown in suspension have a greater pool of membrane-associated cFLIPL. This membrane-association reduces cFLIPL availability for Fas-association death domain (FADD) binding, and CD95 cross-linking by an agonist-acting antibody will result in death inducing signal complex (DISC) formation, activation of procaspase-8 (CASP = cytosolic aspartate-specific protease) and apoptosis. In adherent cell lines there is an enhanced cytosolic cFLIPL. Incorporation of cFLIPL into DISC prevents procaspase-8 processing and activation, thereby inhibiting apoptosis (Shain et al., 2002).

There is also evidence that adhesion of MM cells via VLA-4 (α4β1, CD49d) to fibronectin upregulates p27Kip1 conferring drug resistance (cell adhesion mediated drug resistance) (Damiano et al., 1999; Hazlehurst et al., 2000). Drug-resistant MM cell variants overexpress VLA-4 in comparison with the drug-sensitive cell

![Fig. 2.](image-url) Paracrine interactions between the MM cell and the BMSC.
variants (Damiano et al., 1999). Human 8226 MM cells which pre-adhered to fibronectin are less susceptible to the effects of doxorubicin and melphalan compared with the same cells grown in suspension (Damiano et al., 1999).

**Interaction with the osteoclasts and osteoblasts**

A major determinant of morbidity in MM stems from its residence in the BM. This growth in the BM results in bone pain, osteopenia, “punched-out” lytic bone lesions, hypercalcemia and pathological fractures.

The skeleton constantly undergoes remodelling, in which bone resorption is followed by bone formation. During bone resorption, osteoclasts attach to the bone surface. After resorption, osteoclasts migrate to the recently resorbed area and lay down new bone matrix. A functional balance must be maintained between resorption and formation to keep the bone mass constant. In MM this balance between bone formation and osteoclastic bone resorption is disturbed in favour of net bone resorption through an increased osteoclastic activity together with a decreased osteoblastic activity.

Osteoclasts are the principal resorptive cells of bone, playing a key role in the regulation of bone mass (Blair et al., 2004). They are multinucleated cells formed by the fusion of mononuclear progenitors of the monocyte/macrophage family. The binding of activated osteoclasts to the bone matrix is controlled by integrins, and more particularly the \( \alpha_v \beta_3 \) integrin. \( \alpha_v \beta_3 \) integrins bind to type I collagen and osteopontin (OPN) (Prince et al., 1987; Reinhold et al., 1990), two major bone matrix proteins. OPN is involved in osteoclast differentiation, migration and function (Yamate et al., 1997). Matrix-derived signals are transmitted via the \( \alpha_v \beta_3 \) integrin, causing cytoskeletal organisation and formation of a ruffled membrane. The osteoclast acidifies an extracellular microenvironment (Howship’s lacunae) by means of an electrogenic proton pump (\( \text{H}^+ \)-ATPase) (Li et al., 1999). Carbonic anhydrase II (Sly et al., 1983) helps to maintain the neutral pH inside the osteoclasts. The acidic extracellular milieu mobilises the mineral phase of bone and provides an optimal environment for organic matrix degradation by cathepsin K (Teitelbaum, 2000; Blair et al., 2004). Degradation products (collagen fragments, solubilised calcium and phosphate) are processed within the osteoclast and released into the circulation.

New insights in the pathophysiology of osteoclast differentiation and activation has emerged through the characterisation of three new molecules that belong to the TNF family: receptor activator of NF-κB (RANK), which is expressed by dendritic cells (Bachmann et al., 1999), osteoclast precursors, chondrocytes and mature osteoclasts, RANK Ligand (RANKL), which is expressed by BMSCs, immature osteoblasts and activated T cells (Lacey et al., 1998) and osteoprotegerin (OPG), which is secreted by the BMSCs and is a soluble decoy receptor for RANKL. RANK was initially identified in dendritic cells and seemed to be important in the regulation of interactions between T cells and dendritic cells (Anderson et al., 1997; Josien et al., 1999). The binding of RANKL to RANK also promotes osteoclast maturation and activation (Hsu et al., 1999; Takahashi et al., 1999). OPG blocks the RANK-RANKL interaction and thus inhibits osteoclast differentiation and activation (Simonet et al., 1997) (Fig. 3). TGF-β, a negative regulator of osteoclastogenesis, acts through the induction of OPG by BMSCs (Takai et al., 1998). In vitro, the complete osteoclastogenesis can be achieved with pure populations of macrophages exposed only to monocyte-colony stimulating factor M-CSF and RANKL (Jilka et al., 1992; Quinn et al., 1998): M-CSF causes osteoclast precursors to proliferate and RANKL stimulates the pool of M-CSF-expanded precursors to commit to the osteoclast phenotype. In vivo maturation of macrophages into osteoclasts requires the presence of BMSCs or osteoblasts.

Whether the cytokines already characterised as osteoclast activating factor, exert their effect through the RANK/RANKL pathway is an important issue. TNF-α can directly stimulate osteoclast differentiation in the presence of M-CSF through a mechanism independent of the RANK/RANKL system. In addition, TNF-α stimulates osteoblastic cells to express RANKL (Hofbauer et al., 1999; Yin et al., 1999) and M-CSF (Romas et al., 2000), which in turn prompts macrophages to become osteoclasts. Glucocorticoids (Hofbauer et al., 1999), IL-1β (Hofbauer et al., 1999), IL-11 (Paul et al., 1990), PTH (Koseki et al., 2002), prostaglandin-E2 (Tsukii et al., 1998), vitamin D3 (Yasuda et al., 1998) enhance the expression of RANKL. Hepatocyte growth factor (HGF), an angiogenic factor which is expressed by MM cells, upregulates the IL-11 expression. IL-6 supports human osteoclast formation by a RANKL-independent mechanism (Kudo et al., 2003). The primary effect of IL-6 on osteoclast formation is to increase the pool of the early osteoclast precursors. Therefore, IL-6 stimulatory effects on osteoclast precursors may enhance the effects of osteoclast activating factors that act at later stages of osteoclast differentiation (de la Mata et al., 1995). In co-culture experiments, the addition of blocking antibodies against IL-1β, IL-6 and TNF-α does not prevent RANKL upregulation (Giuliani et al., 2001). Giuliani et al. (2001) have shown that human MM cells up-regulate the expression of RANKL and down-regulate the expression of OPG at mRNA and protein level in pre-osteoblastic or BMSCs in co-culture systems. Initial reports did not find expression of RANKL on the MM cells themselves (Giuliani et al., 2001; Pearse et al., 2001). Later on, Sezer et al. (2002) detected, by the use of flow cytometry, strong expression of RANKL in the cell membrane of BM MM cells. The expression of RANKL on MM cells correlated with osteolytic bone disease in MM patients (Farrugia et al., 2003). Yaccoby et al. (2003) demonstrated in vitro that MM cells attract osteoclast precursors and induce their differentiation, in
the absence of BMSCs or EC, by secreting RANKL. The expression of RANKL by MM cells was also observed in the 5T2MM and the 5T33MM murine MM model (Croucher et al., 2001; Vanderkerken et al., 2003a). Recent data suggest that T cells are in part involved in MM-induced osteolysis (Giuliani et al., 2002). In a co-culture transwell system it was shown that human MM cell lines increased the expression of RANKL in activated T lymphocytes and that purified MM cells stimulated RANKL in autologous T lymphocytes. Moreover, the release of IFN-γ - an inhibitor of osteoclastogenesis - by T lymphocytes was reduced in the presence of MM cell lines or MM cells. RANKL mRNA was up-regulated in BM T lymphocytes of MM patients with severe osteolytic lesions.

In MM, the down-regulation of OPG occurs at the transcriptional and post-translational level. Contact between MM cells and BMSCs results in inhibition of OPG production at the mRNA level (Giuliani et al., 2002). In addition MM cells decrease OPG by
internalizing it through syndecan-1 (CD138). OPG is degraded within the lysosomes of the MM cells (Standal et al., 2002). In the murine 5T2MM model treatment with OPG prevented osteolysis (Croucher et al., 2001) and continuous treatment with OPG of 5T33MM injected resulted in a decreased tumour load and survival (Vanderkerken et al., 2003b). Syndecan-1 (CD138) has by itself a negative regulatory role on osteoclastogenesis and induces osteoblastogenesis (Dhodapkar et al., 1998). OPG expression is reduced by glucocorticoids (Hofbauer et al., 1999), which are included in most MM therapy regimens. The administration of OPG has been shown in an osteolytic sarcoma murine model to block cancer-induced skeletal destruction and to reduce skeletal pain (Honore et al., 2000).

Besides the osteoclast activating factor IL-1β (Cozzolino et al., 1989), TNF-β (Bertolini et al., 1986; Garrett et al., 1987), and IL-6 (Bataille et al., 1989), recently osteopontin (OPN) was reported to be expressed in MM cells, but not in MGUS, myelodysplastic syndrome, idiopathic thrombocytopenic purpura, acute myeloid leukaemia and hereditary spherocytosis (Saeki et al., 2003). Plasma OPN levels in patients with MM were significantly higher than those of patients with MGUS or smouldering MM, or healthy volunteers (Saeki et al., 2003). These plasma OPN levels correlated with both the stage and the degree of bone destruction. OPN can function as an extracellular matrix protein, but also as a cytokine. Next to CD44v6-10, the main receptor of OPN is the αβ3 integrin. This integrin also plays an important role in the angiogenesis. OPN binding to the αβ3 integrin on ECs increases NFκB and inhibits EC apoptosis. VEGF (vascular endothelial growth factor) induces OPN and αβ3 expression in the ECs and stimulates the cleavage of OPN by thrombin, resulting in an enhanced rate of EC migration (Senger et al., 1996). In isolated 5T2MM and 5T33MM cells the presence of OPN mRNA and the secretion of OPN was demonstrated by RT-PCR and ELISA, respectively (Caers et al., 2004). Serum OPN levels were higher in both murine 5TMM models compared to naive mice. Recombinant OPN proved to be a strong inducer of MM cell growth.

Macrophage inflammatory protein (MIP)-1α and MIP-1β are chemokines that significantly participate in MM associated bone disease. MIP-1α is a member of the C-C chemokine family and can interact with three types of chemokine receptors (CCR1, CCR5 and CCR7). MIP-1α acts as chemoattractant and activator of monocytes. Both osteoclast precursors and BMSCs express CCR5. Both MIP-1α and MIP-1β induce RANKL expression by the BMSCs. MIP-1α has been detected in the supernatant of BM cultures of MM patients and also at the mRNA level in MM cells (Abe et al., 2002). MIP-1α levels are elevated in BM plasma of 56% of MM patients and correlate with the stage of the disease (Choi et al., 2000). In MM patients with severe bone disease increased MIP-1α serum levels have been observed (Terpos et al., 2003). In addition, in a gene expression profile study of 92 primary MM, the MIP-1α gene was overexpressed in osteolytic MM (Magrangeas et al., 2003). Since the CCR5 is also expressed by the MM cells, MIP-1α and MIP-1β may have an autocrine effect on MM cells. MIP-1α triggers migration and enhances survival and proliferation in MM cells (Lentzsch et al., 2003). In addition, MIP-1α interacts with osteoprotegerin (OPG) in a bone matrix and in this way a negative feedback loop is created where tumour growth will enhance OPG expression and OPG will induce RANKL secretion by the BM stroma and osteoblasts resulting in osteolysis. In this way a positive feedback loop is created where tumour growth osteolysis results in tumour growth. The increased osteolytic activity in MM causes the release of several cytokines from the bone matrix, including IL-6, IGF-1, TGF-β and bFGF. These factors will enhance directly or indirectly MM cell growth and will in addition induce the release of PTH-related protein by MM cells (Otsuki et al., 2001). This PTH-related protein will induce RANKL secretion by the BM stroma and osteoblasts resulting in osteolysis. In this way a positive feedback loop is created where tumour growth causes osteolysis and osteolysis results in tumour growth (Fig. 3).

Besides the increased osteoclastic activity there is also evidence of a reduced activity of osteoblasts in MM. The function of osteoblasts is dramatically reduced when the proportion of MM cells in the BM exceeds 50% (Bataille et al., 1991).

Tian et al. (2003) compared patterns of gene expression using oligonucleotide microarrays between MM patients with and without osteolytic lesions. They found four genes that were overexpressed in the patients with lytic lesions compared to those without lytic lesions. One of the four genes coded for a secreted protein, dickkopf 1 (Dkk1), that had a role in bone formation. Dkk1 is a Wnt-signalling antagonist. Wnt glycoproteins constitute a major cell-to-cell signalling family that act as ligands to activate receptor-mediated signal transduction pathways that control nearly all aspects of cellular biology during development and beyond. In the classical Wnt-signalling pathway, a Wnt growth factor must bind to both the receptor and either a low density lipoprotein receptor-related protein 5 (LRP5) or LRP6 co-receptor to initiate signalling. In the
Wnt/ß-catenin pathway, Wnt growth factor binding results in stabilisation of cytoplasmic ß-catenin, which is now free to translocate to the nucleus and activate Wnt-responsive genes. Bone morphogenetic protein type 2 (BMP-2) can induce differentiation of the uncommitted mesenchymal progenitor-cell into osteoblasts through this Wnt/ß-catenin signalling pathway. DKK1, as an antagonist of this pathway, binds to Wnt-coreceptor LRP5. DKK1 blocks the terminal differentiation of osteoblasts, and causes, at high levels and with long-term exposure, loss of viability of osteoblast stem cells. Immunohistochemical analysis of BM biopsies showed that only MM cells contained detectable DKK1. Elevated DKK1 levels in BM plasma and peripheral blood from patients with MM correlated with the gene-expression pattern of DKK1 and were associated with the presence of focal bone lesions. Knowledge of the DKK1-mechanism of osteoblastic inhibition helps us to understand how lytic lesions are maintained in MM patients and why osteoblasts do not replace lost bone during bisphosphonate treatment (despite inhibition of osteoclasts) in such patients. Since Wnt signals can directly regulate the capacity of haematopoietic stem cells for self-renewal (Murdoch et al., 2003; Rey et al., 2003), elevated levels of DKK1 may also have a role in immunosuppression and anaemia, a serious complication observed in patients with MM. Given that the haematopoietic stem-cell proliferation is dependent on a BM niche that is created by osteoblasts, the blocking of osteoblast maturation could impede the establishment of this niche. It is possible that DKK1 would interfere with the mobilisation, engraftment, and proliferation of haematopoietic stem cells during the course of autologous transplantation. DKK1 was rarely detected in plasma cells from MGUS patients and patients with end-stage disease or secondary plasma-cell leukaemia indicating that DKK1 expression is limited to a specific stage of the disease. Since immature, but not mature osteoblasts are a source of RANKL (Atkins et al., 2003), the DKK1-mediated block in osteoblast differentiation could result in an increased osteoclastic stimulation.

Close contact between MM cell lines and primary human osteoblasts induces IL-6 production by the latter (Karadag et al., 2000). BMP-2 has been shown to induce apoptosis in MM cell lines and in primary MM cells (Kawamura et al., 2000). Since BMP-2 also exhibit strong bone-inducing properties (Service, 2001), BMP-2 potentially could be of therapeutic benefit (Ducy et al., 2003). Complete remission rates of 25-30% can be obtained with a median survival exceeding 5 years (Zaidi and Vesole, 2001). More detailed information with management guidelines is available in a recently published consensus report (Durie et al., 2003b).

The understanding of the tumour microenvironment has led to the suggestion that the target of therapy should not necessarily be the tumour cell alone, but also the microenvironment that sustains the cell. It is beyond the scope of this general introduction to give an overview of all the new compounds which are currently in a pre-clinical or clinical phase of investigation. At this stage, with the current knowledge of the interactions between MM cells and its microenvironment, it does not appear that any single agent will be able to control MM permanently. The next generation of clinical trials will be designed to combine different molecules to take advantage of non-overlapping toxicities and synergisms between those agents, thus converting the disease process into a chronic phase (Kyle and Rajkumar, 2004). Some non-traditional therapeutic agents will be mentioned here in order to illustrate their combined action on both the microenvironment and the MM cell.

**Thalidomide and analogues**

Although initially evaluated because of its potential antiangiogenic effect (D’Amato et al., 1994), the mechanism of action of thalidomide (Thalidomid®) are not yet fully elucidated. It is likely that its pharmacologic effect may be mediated via a number of pathways involving both the MM cell and the BM microenvironment (Hideshima et al., 2000). Thalidomide can induce apoptosis or G1 growth arrest in MM cell lines and in primary MM cells that are resistant to melphalan, doxorubicin and dexamethasone (Hideshima et al., 2000).

Thalidomide can inhibit angiogenesis and induce apoptosis of established neovasculature in experimental models (Kenyon et al., 1997; Yaccoby et al., 2002). Thalidomide can alter the expression of adhesion molecules such as ICAM-1 (CD54), VCAM-1 (CD106) and E-selectin (Geitz et al., 1996), suppresses the production of TNF-α (Sampaio et al., 1991), increases the production of IL-10 (Corral et al., 1996), augments natural killer cell cytotoxicity (Davies et al., 2001) and enhances cell-mediated immunity by directly stimulating cytotoxic T cells (Haslett et al., 1998) (Fig. 4). Its interaction with type 1 and type 2 helper T cells produces complex effects on the levels of cytokines such as IL-4, IL-5, and IFN-γ (McHugh et al., 1995). Singhal et al. (1999) reported an impressive overall response rate of 32 % in 84 patients with MM of whom 90% had failed after an autologous stem cell transplant. Meanwhile other groups have confirmed these results with an overall response rate of 25-45% and response duration of 9-12 months with 10-20% of the patients free of progression after 2 years (Rajkumar and Witzig, 2000; Rajkumar et al., 2000; Juliusson et al., 2000; Barlogie et
There is some controversy in the literature regarding the effect of thalidomide on the microvessel density (MVD). In the study of Singhal et al. (1999) there was no significant change in the MVD in patients with a response. Only in a few patients with a complete or nearly complete remission a markedly decreased MVD was observed. In a recent study a significant decrease in MVD could be demonstrated in patients who were responsive to thalidomide. No significant change in MVD was seen in those failing to respond to thalidomide (Kumar et al., 2004).

The thalidomide analogue CC-5013 (Revlimid®) is another immunomodulatory agent that exhibits virtually no sedative and only occasionally neurotoxic effects, but causes myelosuppression. CC-5013, like TNF-related apoptosis-inducing ligand (TRAIL), triggers caspase-8 mediated MM cell apoptosis (Mitsiades et al., 2002b). Responses have been reported in one third of patients with advanced and refractory MM (Zangari et al., 2001; Richardson et al., 2002).

VEGFR-inhibitor

Vascular endothelial growth factor (VEGF) is expressed and secreted by MM cell lines, patients’ MM cells, and BMSCs. There is a paracrine tumour-stromal circuit of VEGF/IL-6 production and secretion. The VEGF secreted by MM cells triggers IL-6 production from the BMSC and BMEC, thereby augmenting paracrine MM growth (Podar and Anderson, 2005). Conversely, IL-6 enhances the production and secretion of VEGF by the MM cells. VEGF also induces proliferation and migration of the MM cells via Flt-1 (Fms-like tyrosine kinase-1, VEGFR-1) and not via the kinase insert domain-containing receptor KDR (VEGFR-2). These autocrine effects are mediated by 2 pathways: MEK (for MAP kinase or ERK kinase) / ERK (for extracellular signal-regulated protein kinase) pathway (proliferation) and the protein kinase C-α (PKC-α-dependent kinase) pathway (migration) (Podar et al., 2001). Moreover, VEGF mediates EC proliferation, inhibits maturation of dendritic cells (Gabrilovich et al., 1996), and increases bone-resorbing activity of the osteoclasts by enhancing their survival (Nakagawa et al., 2000). GW654652 is an indazolylpyrimidine belonging to the group of the tyrosine kinase inhibitors. This small molecule blocks VEGF-induced tyrosine phosphorylation of VEGFR-1 and related downstream signalling in MM cells and inhibits MM cell migration, proliferation and survival. It also acts on MM-stroma interactions as shown by inhibition of VEGF and IL-6 production in co-culture model. Secondary to the interference of IL-6 production, GW654652 offers the potential to overcome drug resistance in MM (Podar et al., 2004a).

Arsenic trioxide

Arsenic trioxide (TRISENOX®), an antitumour agent with a multifaceted mechanism of action, is highly effective in the treatment of relapsed/refractory acute promyelocytic leukaemia. In vitro data show that arsenic trioxide at concentrations of 1-5 µM is cytotoxic to MM

![Fig. 4. Mechanisms of action of new agents that target both the MM cell clone and the bone marrow microenvironment. Thalidomide (T), arsenic trioxide (A) and bortezomib (B) inhibit the adhesion between the MM cell and the bone marrow stromal cell (BMSC). B and T inhibit the production of TNF-α and IL-6. The effect of B on MM cells is mediated in part by inhibition of NF-κB. B also causes a decrease in caveolin-1. A and T inhibit angiogenesis and stimulate the immune surveillance by T cells and NK cells. A, B and T also have a direct anti-MM effect.](image-url)
cell lines and is not cross-resistant with dexamethasone or doxorubicin. When used as a single agent at a clinically relevant concentration of 1-5 µM, arsenic trioxide induces cell cycle arrest (by inducing the p21cyclin-dependent kinase inhibitor protein) and apoptosis (by caspase activation) in both MM cell lines and fresh MM cells, which cannot be rescued by BM stroma or IL-6 (Rousselot et al., 1999; Park et al., 2000). Arsenic trioxide treatment also results in MM destruction through modulating integrins (CD54 on MM cells and CD11a on lymphokine-activated killer (LAK) cells), and through overexpression of CD38 and its ligand CD31 on plasma cells and LAK cells, respectively (Deaglio et al., 2001). Arsenic trioxide inhibits angiogenesis and decreases EC response to VEGF (Roboz et al., 2000). Arsenic trioxide may be most beneficial when used in combination with other agents that have non-overlapping or complementary mechanisms, such as dexamethasone. Dexamethasone produces responses in more than half of patients treated, but resistance develops in more than 90% of patients within 2 years of treatment. Resistance to dexamethasone is associated with high bcl-2 levels. It is further known that IL-6 protects MM cells from dexamethasone-induced apoptosis. In a phase 2 study (Hussein et al., 2004), 24 MM patients (eight had relapsed and 16 were refractory to prior therapy) received arsenic trioxide (0.25 mg/kg/d for 5 d/week during the 2 first weeks of each 4-week cycle). Reduction (25% or more) in serum-protein levels occurred in eight of 24 (33%) patients. An additional six (25%) patients had stable disease. The median time to response was 67.5 days, with a median duration of response of 130 d. These data indicate that arsenic trioxide may be of benefit, even in patients with late-stage, relapsed and refractory MM.

**Bortezomib**

The proteasome is a multisubunit enzyme complex that degrades many proteins involved in the regulation of cell-cycle or cell-survival pathways. The proteolytic enzymes recognise the presence of ubiquitin, which marks the proteins (including regulators of cell proliferation) that have been designated for degradation. Selectively inhibiting proteasome activity “stabilises” the proteins that regulate the cell cycle, thus disrupting cell proliferation resulting in apoptosis (Adams et al., 2000). Proteasome inhibitors act against MM cells, at least in part, by targeting X-box protein (XBP)-1, a transcription factor which induces differentiation of normal B cells to PCs (Lee et al., 2003). The inhibition of the proteasome leads to the down-regulation of ligands and receptors that mediate interactions between the BM stroma and the MM cells.

Bortezomib (VELCADE®), formerly PS-341, is a boronic acid dipeptide, approved by the United States Food and Drug Administration for the therapy of MM patients with progressive myeloma after previous treatment (Kane et al., 2003). In addition to inducing apoptosis of the MM cell (by activation of caspase-8, -9 and -3), bortezomib decreases transcription and secretion of cytokines in the BM microenvironment that sustain tumour growth (reduced secretion of IL-6 by stabilizing IkB and inhibiting NF-xB-induced IL-6 secretion). It down-regulates expression of adhesion molecules by MM cells and BMSC (Hideshima et al., 2001a) (e.g. VCAM-1 - CD106) (Read et al., 1995) (Fig. 4). Consequently, bortezomib inhibits IL-6 or cell-adhesion-mediated drug resistance (Hideshima et al., 2003).

Caveolae (“little caves”) represent potential novel therapeutic targets in MM. Caveolae are vesicular flask-shaped invaginations (50 to 100 nm) of the plasma membrane, which have been implicated in transcytosis and signalling processes, including apoptosis. They are composed of cholesterol, sphingolipids, and integral membrane proteins, termed caveolins. Caveolin-1 (Cav-1) is required for caveolae formation and its phosphorylation initiates downstream signalling. Cav-1 colocalises with IL-6R signal transducing chain gp130 and with IGF-1R. Bortezomib both inhibits VEGF-triggered caveolin-1 phosphorylation and markedly decreases caveolin-1. Consequently, bortezomib inhibits VEGF-induced MM cell migration (Podar et al., 2004b). Bortezomib also decreases VEGF secretion in the BM microenvironment and inhibits migration and survival in human umbilical vascular endothelial cells (HUVECs), by inhibiting VEGF-triggered tyrosine phosphorylation of Cav-1 in EC (Podar et al., 2004b).

Gene microarray profiling of bortezomib-treated MM cells revealed induction of heat shock protein 90 stress response (against proteasome inhibition), providing the rationale for the combined clinical use of Velcade® and 17-allylamino-17-demethoxy-geldanamycin (17-AAG) to enhance anti-MM activity (Mitsiades et al., 2002a). Protein profiling of bortezomib-treated MM cells demonstrated cleavage of DNA repair enzymes, providing the rationale for combining bortezomib with DNA damaging agents (conventional chemotherapeutic drugs) to enhance sensitivity to overcome resistance to these conventional therapies (Mitsiades et al., 2003).

**Bisphosphonates**

The role of the osteoclasts as a component of the BM-microenvironment has emerged in the last years. They play a major role in bone pain, hypercalcaemia and pathological fractures, which are a major cause of morbidity and mortality in MM. All bisphosphonates, used as inhibitors of bone resorption, contain two phosphonate groups attached to a single carbon atom, to give a “P-C-P” structure. These compounds are selectively concentrated in the bone, where they interfere with the action of osteoclasts. Long-term therapy with bisphosphonates has been shown to reduce skeletal morbidity, improve quality of life, and reduce the need for surgery and radiotherapy (Bersenson et al., 1996,
Remarkable progress has been made in increasing the potency of bisphosphonates as inhibitors of bone resorption. The most potent compounds in current use are characterised by the presence of a nitrogen atom at critical positions in the side chain, which, together with the “P-C-P” moiety itself, seems to be essential for maximum activity. These more potent nitrogen-containing bisphosphonates, such as zoledronic acid, are inhibitors of the mevalonate pathway, a biosynthetic pathway responsible for the production of cholesterol and isoprenoid lipids such as isopentenyl pyrophosphate, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate. Farnesyl pyrophosphate and geranylgeranyl pyrophosphate are required for the posttranslational modification (prenylation) of small GTPases such as Ras, Rho and Rac, which are prenylated at the cysteine residue in characteristic C-terminal motifs (Luckman et al., 1998). Hughes et al. (1995) observed the characteristic morphologic features of apoptosis in both isolated murine osteoclasts in vitro and in osteoclasts in histological sections from bisphosphonate-treated mice. This apoptosis is mediated by inhibiting the enzymes of the mevalonate pathway. In addition to interfering with the osteoclast function via the mevalonate pathway, bisphosphonates impede osteoclast formation, maturation and fusion of osteoclast precursors (Lowik et al., 1988). Bisphosphonates disrupt the formation of the cytoskeletal actin ring in polarised, resorbing osteoclasts (Murakami et al., 1995) resulting in a lack of ruffled border. The ruffled border of the osteoclasts is a region of convoluted plasma membrane facing the resorption cavity that is dependent on the cytoskeleton. Interestingly, it has recently been shown that bisphosphonates pamidronate and zoledronate stimulate OPG production by primary human osteoblasts (Viereck et al., 2002). Although bisphosphonates have been shown to inhibit proliferation of MM cells and induce MM cell apoptosis in vitro (Shipman et al., 1997; Aparicio et al., 1998), this could not be confirmed in vivo in the murine 5T2MM model (Shipman et al., 1997). Probably the bisphosphonates have an indirect effect on MM cell burden by inducing alterations to the local BM microenvironment.

**Conclusion**

There is more and more convincing evidence from cytogenetic and gene expression profiling studies that MM is a heterogenous disorder with complex genetic aberrations. Recent insights into the physiopathology of MM has found a redundancy of mechanisms by which MM can escape the normal growth control mechanisms and by which MM can acquire drug resistance to the conventional chemotherapeutic agents. The study of the interaction of MM with the BM microenvironment can reveal new therapeutic targets, which not only can result in a more efficient control of MM cell growth, but which will also alleviate MM patients from the notorious MM-associated bone lesions.

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