Different aspects of Thalidomide treatment and Stem cell transplantation in Multiple Myeloma patients

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Different aspects of Thalidomide treatment and Stem cell transplantation in Multiple Myeloma patients

Verschillende aspecten van Thalidomide behandeling en stamcel transplantatie bij patiënten met de ziekte multipel myeloom

Proefschrift

Ter verkrijging van de graad van doctor aan de universiteit Utrecht op gezag van de rector Magnificus, prof. Dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op donderdag 5 oktober 2006 des ochtends om 10.30 uur.

door

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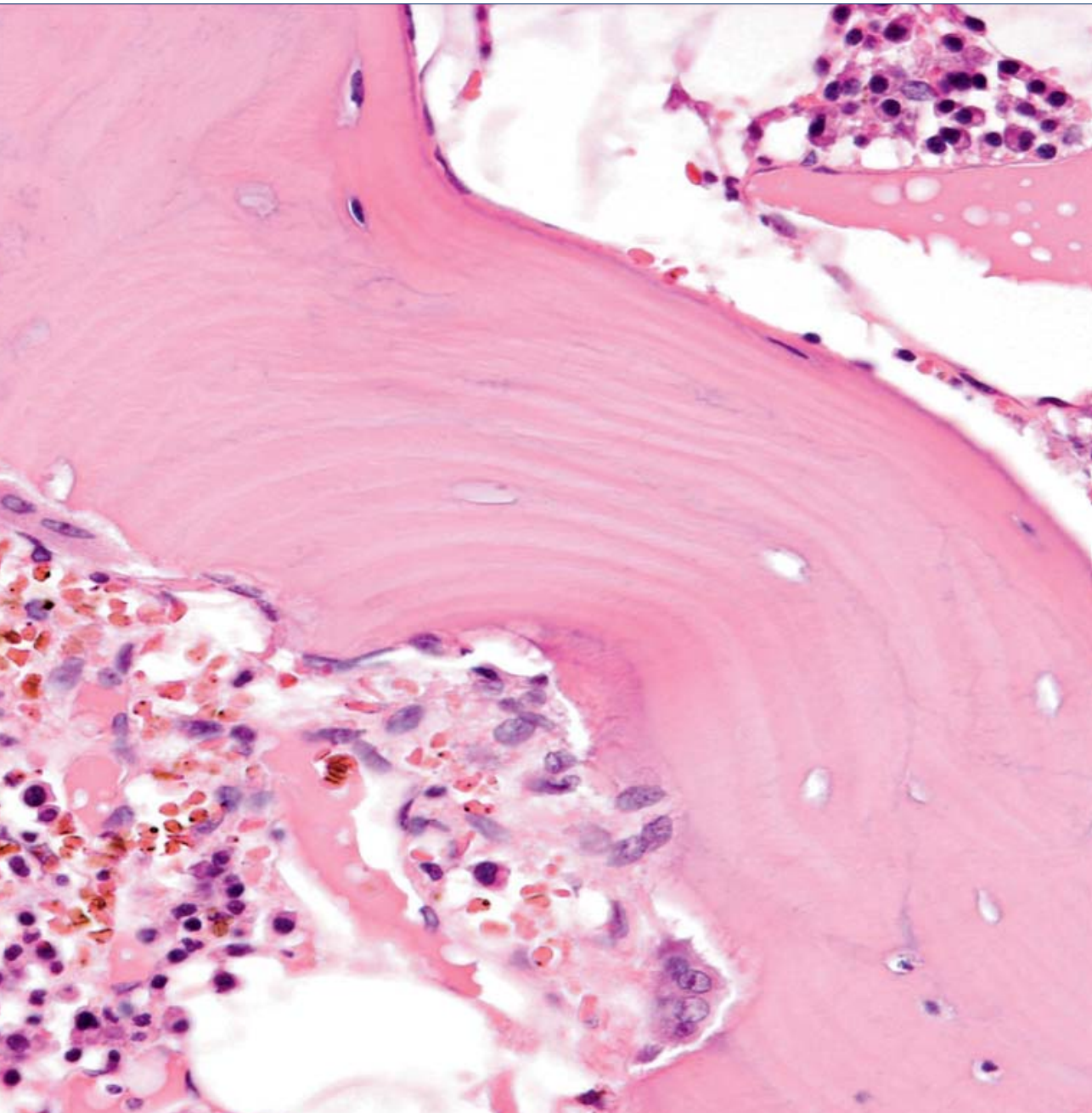
Co-promotores: Dr. H. M. Lokhorst
Dr. T. Lisman

Voor mijn ouders

Contents



General introduction

Multiple myeloma

Multiple myeloma (MM) is an uncommon diagnosis in the general population, nevertheless it accounts for 10% of the haematological malignancies. This malignancy consists of a multifocal neoplastic proliferation of plasma cells. These plasma cells are monoclonally differentiated B-cells infiltrating the bone marrow, and forming multiple tumorous masses scattered throughout the skeletal system. The plasma cells produce osteoclastic factors, induced by interleukin (IL-1), tumor necrosis factor (TNF-β) and IL-6, that cause bone destruction and bone resorption.[1-3] Bone pain is therefore one of the major symptoms, sometimes complicated by pathological fractures or nerve root compression. The bone destruction can radiologically be visualized as punched-out lesions in the bone.[4] The bone resorption, with lytic lesions and bone destruction can cause pathological fractures, including vertebral fractures, sometimes being the primary presenting symptom of the disease. The bone destruction is associated with tumor infiltration and correlates with tumor load. Other complications are renal involvement and neurological abnormalities.

For the diagnosis of MM the bone marrow should be infiltrated with more than 30% plasma cells. The bone marrow can be investigated with a bone marrow aspiration and a bone marrow biopsy. In the WHO classification of multiple myeloma the major and minor diagnostic criteria are summarized and the percentage of bone marrow infiltrating plasma cells is one of the most important diagnostic features.[5] Massive infiltration of the bone marrow can cause bone marrow failure (anemia) and immunodeficiency (hypogammaglobulinaemia).

The disease is generally found in middle-aged and elderly patients, only rarely found in patients under 40 years of age, whereas more males than females are effected.[6]

The clonally transformed plasma cells all produce the same homogeneous immunoglobulin, the so called paraprotein or M-component. This paraprotein can be detected in urine and blood serum, and can cause renal failure. The presence of this paraprotein is indicative for a malignant condition.

In patients presenting with symptomatic disease, the paraprotein, the bone marrow plasmocytosis and the lytic bone lesions are the major diagnostic criteria leading to the diagnosis of MM. To confirm the diagnosis of MM, a bone marrow biopsy is almost always performed.

The bone marrow features of multiple myeloma

Both the pathologist and the hematologist have a role in the evaluation of the bone marrow in MM. The diagnostic features of multiple myeloma in the bone marrow are summarized in chapter 2, where the histology and plasma cell morphology of multiple myeloma in the bone marrow biopsies are described.[7] As a pathologist and a hematologist it is important to be aware of the different histological and cytological patterns of plasma cell infiltration, and of the use of different laboratory techniques such as immunohistochemistry and the presence of accumulating chromosomal abnormalities in the course of the disease.

Also this chapter states the importance of the microenvironment for optimal tumor survival and differentiation. Malignant circulating B-cells home in the bone marrow to find an optimal environment for survival and differentiation. This microenvironment includes

stromal cells, endothelial cells and extracellular matrix. All have their own factors for interaction with the plasma cells. The components of the microenvironment become more and more important for therapeutic options, since they interfere with these factors or (more directly) with the functioning and adhesion of plasma cells with the individual cells of the microenvironment.

Therapy and stem cell transplantation

For many years the standard therapy for multiple myeloma patients consisted of the combination of melphalan and prednisone, with response rates of around 50% and a median overall survival of 3 years.[8] Currently high dose chemotherapy followed by reinfusion of autologous stem cells has become the standard approach for younger myeloma patients. Median overall survival has now improved to approximately 5 years, although this strategy has no curative potential. The role of allogeneic stem cell transplantation is not yet established. Although it may be the only therapy with a curative potential, it is associated with a high treatment related mortality, especially when a myeloablative regimen is used. Following non-myeloablative conditioning the myelosuppression can be reduced, resulting in less morbidity and less treatment-related mortality.[9, 10] However the outcome of ongoing prospective studies with these non-myeloablative regimens has to be awaited, especially to know whether the so called “Graft versus Myeloma effect” of donor T cells makes this a more effective strategy than intensive chemotherapy and autologous SCT. The combination of vincristine, doxorubicin and dexamethason (VAD) is widely used as pre-transplant induction therapy.[11] VAD therapy may be associated with many side effects. Since dexamethason in this regimen is probably largely responsible for the effect of VAD, dexamethason alone appeared to be an alternative which was more convenient for the patient.[12,13] Of course the result of the pretreatment with eradication of the non-clonally transformed bone marrow is different depending whether total myeloablation or non-myeloablation is intended. The regeneration of the haematopoietic bone marrow depends on the type of conditioning pre SCT (non-myeloablative versus myeloablative), but also depends on the method of transplantation (allogeneic or autologous).

In chapter 3 we describe the post-transplantation phases in detail following myeloablative SCT. As a pathologist, but also as a hematologist, it is very important to recognize these phases of regeneration.

Apart from normal regeneration, also the complications that can occur during bone marrow regeneration with their characteristic morphology and significant dyshaematopoiesis are discussed.

Finally in chapter 4 the problems of diagnosing bone marrow regeneration versus the presence of residual tumor cells or of recurrent disease are discussed.

1.1.4 The history of Thalidomide

The treatment of multiple myeloma patients with thalidomide is one of the most important aspects in this thesis. Thalidomide was first synthesized as a sedative and tranquillizer by Chemie Gruenenthal in Western Germany in 1954, at that time called Softenon.

In pregnant women it was very effective as a sedatative and in the treatment of nausea (morning sickness).[17] The drug had an exceptional low toxicity in experimental mammalian species. It was marketed in 1957 and was welcomed by prescribers because of the lower acute toxicity then the alternative drugs such as barbiturates. It was used in many countries all over the world. Following the introduction of softenon was an outbreak of children born with bilateral and symmetrical malformations of the limps and ears, often accompanied by malformations of the internal organs. It took more than four years to detect thalidomide as the cause of these malformations.[14, 15] The mechanisms behind these embryopathic changes are still not understood. The spectrum of malformation attributed to the drug are absence of the auricles with deafness, defects of the muscles of the eye and of the face, absence or hypoplasia of arms, thumbs with three joints, defects of the femur and tibia and malformations of the heart, the bowel, uterus and gallbladder. The malformation differs from child to child, depending on the time of drug used during pregnancy. The sensitive period reaches from day 35 to day 49 after the last menstruation. About 40% of the thalidomide victims die before their first birthday. In 1961 thalidomide was withdrawn in Germany, and approximately 8 months later the malformation outbreak stopped. After many legal acquisitions against Chemie Gruenenthal, in 1970, the court published an evaluation about the whole thalidomide tragedy, confirming the teratogenic effect of thalidomide and stressing that it is important to change the development and marketing of a new drug in general. The thalidomide victims who survived are now between 40 and 50 years old and experience an extremely diminished quality of life. Many genetic conditions produce malformations similar to thalidomide, only the thalidomide malformations can not be passed from one generation to the next. Around the world 12000 children were born with thalidomide malformations, of which less than half are still alive. Thalidomide was consigned as one of the largest medical tragedies in history and was not used anymore for many years.
Besides being used as a sedative, this drug was at that time also tested on different types of cancer, with variable and mostly disappointing results.[16,17] After 25 years a renewed interest in the hypothetical anti-angiogenic effect of this drug developed.[18,19]

Thalidomide, revival of the old drug

In 1999 dr.Singal presented a study in the New England journal of medicine in which he stated that thalidomide was successful in the treatment of refractory and resistant multiple myeloma patients.[20] He wrote:*" When the wife of a patient with progressive and resistant myeloma insisted on a trial with this drug because of its potential in blocking blood flow to tumors (anti-angiogenesis), Dr. Barlogie began clinical studies"*.
After this first publication, thalidomide made a real comeback in the medical world as an anti-tumor drug. Nowadays the drug is tested on many malignant tumors, including multiple myeloma and different kinds of diseases, like leprosy, Behçet syndrome, graft versus host disease and aphthosis in HIV positive patients.[4,21,22]
The anti-tumor effect of thalidomide in multiple myeloma however is one of the most effective ones sofar.[23] The exact working mechanism of this anti-angiogenic drug is still not fully understood, but many studies help us understanding small parts of this puzzle:

Pro-apoptotic effects and G1 growth arrest of malignant plasma cells, down regulation of plasma cell adhesion (via ICAM-1) to the bone marrow stromal cells, inhibition of production/secretion of plasma cell growth factors such as IL-6, and TNF-α, all potentially contribute to the anti-tumor effect of the drug.[24, 25] The anti-angiogenic effect of thalidomide in vitro is shown through inhibition of production of vascular endothelial growth factor (VEGF) and fibroblast growth factor (β-FGF, but also by inhibition of the vascularisation of bone marrow in MM patients.[26-29] The immunomodulatory effect of thalidomide is shown by the upregulation of natural killer cells through the release of interferon gamma (IFN-γ) and IL-2.[30,31] The effects of thalidomide are shown in summary in figure 1.
In our study, discussed in chapter 5, the anti-angiogenic effect of thalidomide in vivo was investigated in bone marrow biopsies, taken at different time points during the treatment and after SCT. Half of the longitudinal biopsies were taken from patients receiving thalidomide treatment (TAD), the other half was randomly selected from a population receiving the more conventional treatment without thalidomide (VAD). This way the effect of thalidomide on the microenvironment like the amount of vessels and the endothelial cells, could be visualized in these bone marrow biopsies
Because thalidomide is known to have teratogenic effects, this drug is contra-indicated in pregnant women. A negative pregnancy test and two effective forms of birth control are prescribed for women. The teratogenic effects of the drug can also pass through the sperm of men so as a consequence when using thalidomide they must practice abstinence or use a condom. The side effects of the drug include sedation, constipation, dry skin, pruritus, and venous thrombosis.

Venous thromboembolism

Many malignancies, including multiple myeloma, are associated with an increased risk for venous thromboembolism. This includes deep venous thrombosis in the leg and life threatening emboli to the lung. In MM patients the combination of regular multi-chemotherapy with adriamycin, thalidomide and prednisone was reported to be associated with the occurrence of venous thromboembolism in 10-30% of the patients, being much higher than in MM patients receiving the more conventional chemotherapy without thalidomide in which the risk for thrombosis is reported to be between 1-5%.[32-35]
From studies in patients with venous thrombosis in the absence of a malignant disorder, it appears that alterations in the coagulation system that promote clot formation or prevent clot degradation (including factor V Leiden, the prothrombin G20210A mutation, high levels of coagulation factors VIII, IX, XI, and plasma hypofibrinolysis) constitute a risk for development of a first venous thrombosis. We therefore hypothesized that similar changes could be induced by thalidomide treatment, and could therefore account for the increased thrombosis risk of this drug. Although multiple alterations in the hemostatic system resulting in a potential hypercoagulable state are known to occur in MM patients, the potentially most important changes are substantial increases in plasma high levels of FVIII and von Willebrand factor (VWF). The extremely high plasma levels of these proteins are presumed to be of clinical importance based on epidemiological studies in

patients with venous thrombosis without underlying malignancy.[36] In chapter 6 and 7 we speculated that the combination of a hypercoagulable or hypofibrinolytic state contributes to the hemostatic dysbalance in MM patients treated with thalidomide. It was already known that especially during treatment with thalidomide the factors FVIII and VWF were elevated in MM patients and might result in a clinically relevant hypercoagulable state. The pathogenesis of these elevated procoagulant factors in MM and their relationship with thalidomide treatment are unknown. We therefore prospectively followed a group of patients that were included in the Hovon 50 study. In the study described in chapter 7, multiple myeloma patients were randomly selected for receiving a thalidomide combination (TAD) or the more conventional chemotherapy (VAD) prior to a SCT followed by maintenance therapy with thalidomide or interpheron. At different time points during the treatment blood samples were taken to determine the blood plasma the levels of VWF and FVIII and. the overall plasma fibrinolytic activity was determined.[36, 37]
The different chapters in this thesis try so give a better understanding of some of the many morphological and physiological changes that occur in patients treated for multiple myeloma.

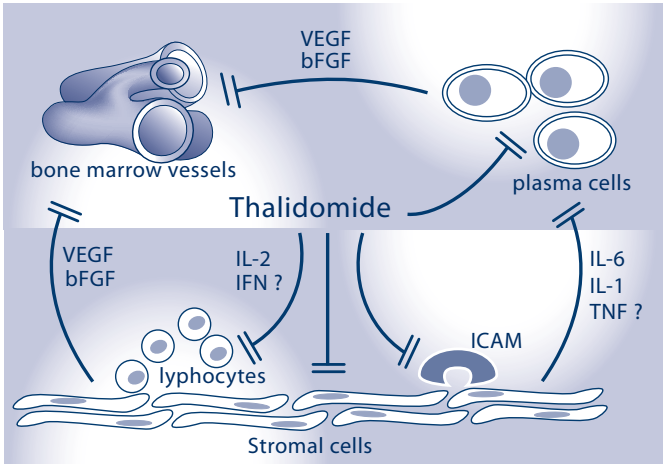


Figure 1
The effects of thalidomide.

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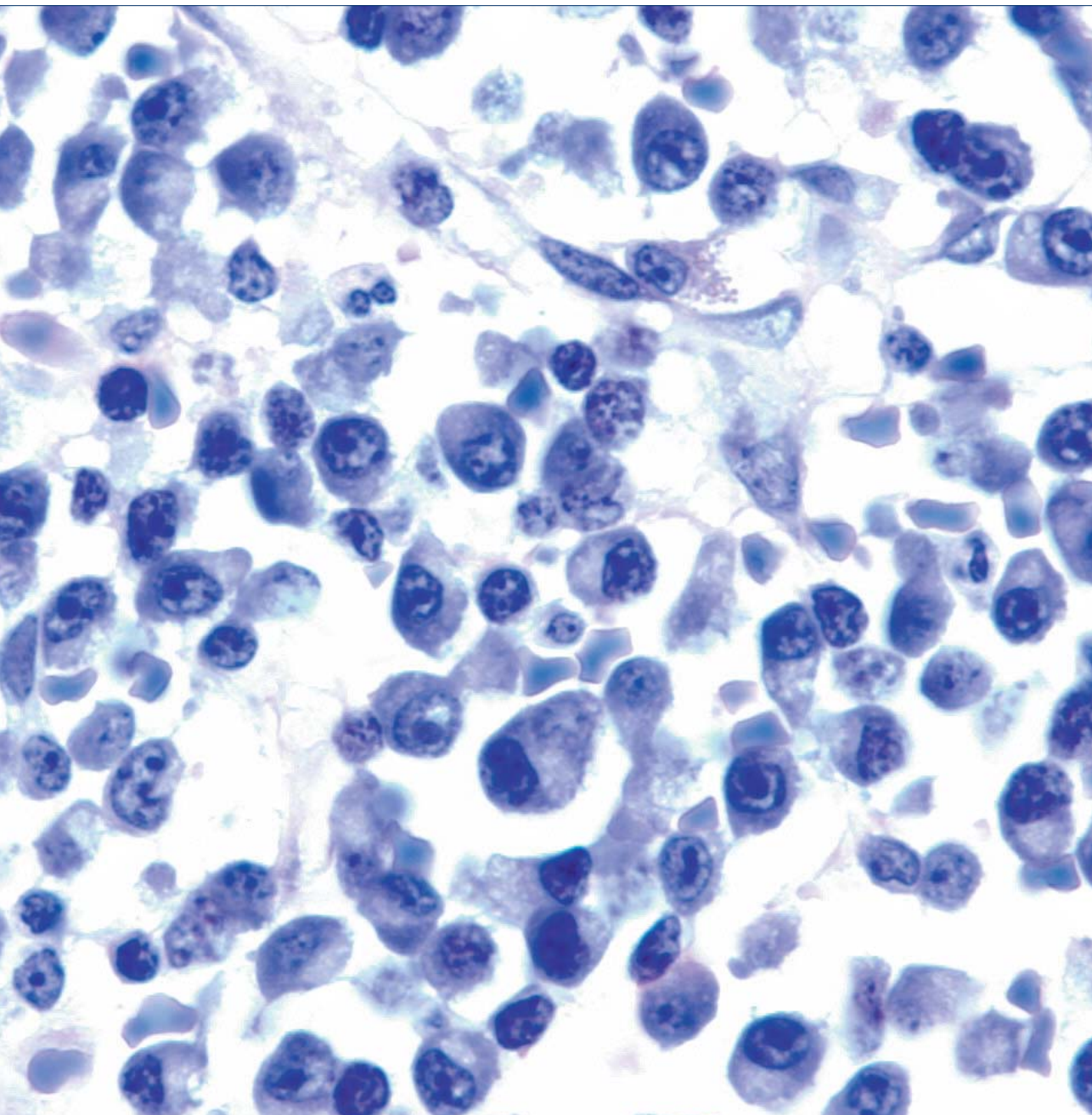

Pathology of multiple myeloma

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Current Diagnostic Pathology 2003; 9: 322-327

Abstract

The diagnosis of multiple myeloma is based on a combination of radiological, laboratory and pathological findings. Bone marrow plasmocytosis is the most important criterion in the diagnosis of multiple myeloma. The pattern of infiltration and the plasma cell morphology have prognostic significance. The immunophenotype of the plasma cells can change after treatment; a polytypic phenotype after treatment has a significantly longer progression free survival. The expression of adhesion molecules and proliferative markers will be increasingly important in predicting the prognosis and providing targets for new treatments. Another growing field is the elucidation of chromosomal abnormalities in the different stages of the disease and development of cytogenetic prognostication.

Introduction

Multiple myeloma (MM) is a haematological malignancy characterized by a multifocal neoplastic proliferation of monoclonal plasma cells in the bone marrow. These endstage B-cells secrete a homogeneous immunoglobulin product known as M-component or paraprotein. Although the M-component can also be found in benign or premalignant conditions (e.g. monoclonal gammopathy of undetermined significance, MGUS), its presence usually indicates a malignant condition such as MM, plasmacytoma or Waldenström's macroglobulinaemia. Most patients show wide spread disseminated disease at presentation. Typical features are bone destruction (causing bone pain and pathological fractures), hypercalcaemia, paraprotein in urine and/or blood, immunodeficiency (because of hypogammaglobulinemia), anaemia, bone marrow failure and renal failure. A combination of radiological, laboratory and pathological findings provides the diagnosis of MM.

According to the new World Health Organization classification,[1] the diagnostic criteria of MM can be divided in major and minor criteria:

A) The major criteria include:
- Marrow plasmocytosis (≥ 30%).
- Plasmocytoma on biopsy.
- M-component: Serum: IgG >3.5g/dl, IgA >2 g/dl.
- Urine > 1 g/24hr of Bence-Jones protein.

B) The minor criteria comprise:
- Marrow plasmocytosis (10-30%).
- M-component- present but less than above.
- Lytic bone lesions.
- Reduced normal immunoglobulins (<50% normal):
  - IgG <600 mg/dl, IgA <100 mg/dl, IgM <50 mg/dl.

The diagnosis can be made when at least one major and one minor criterion or three minor criteria are present.

Histology

The first step in the diagnostic histopathology process is to distinguish reactive plasmocytosis from malignant disease. In reactive plasmocytosis, the plasma cells are interstitially located in small cell clusters, often along vessels. Myeloma-associated plasma cells usually occur in larger foci, nodules or sheets.[1]
There are three major patterns of infiltration in MM.

I Interstitial, with or without paratrabecular accumulation of plasma cells. Infiltration of the interstitial fat and between haematopoietic cells is correlated with an indolent course and with generalised osteoporosis. Location of plasma cells around small blood vessels is seen in both malignant and in reactive disease.
II Nodular or in broad bands. Nodular infiltration is correlated with multifocal osteolytic lesions and progressive disease. Exclusively nodular infiltration is rare.
III Packed marrow. Packed marrow or sarcomatous infiltration is associated with generalised/diffuse osteoporosis with thinning of all trabecula and carries the worst prognosis.

Plasma cell morphology

The plasma cell morphology is important and has prognostic significance. While some patients have a uniform plasma cell morphology, others have a heterogeneous plasma cell aspect within different stages of the disease. Three different types of plasma cells can be recognized; mature, intermediate and immature or plasmablastic cells (figure 1 and 2). The immature plasma cells are associated with proliferation and poorer prognosis,[2] as are cases with multilobulated polyploid plasma cell nuclei. These unusual plasma cell features are excluded as criteria from the current morphologic classifications, despite the fact that a morphologic plasma cell abnormality may indicate the presence of multiple myeloma, regardless of the percentage of plasma cells. Recognition of the morphologic variants of neoplastic plasma cells, associated with poorer prognosis, is important to alert the clinician to possible aggressive behaviour of the tumour. Three cytological features of plasma cells are important in this aspect.

A Nuclear pleomorphism

This feature rarely occurs in reactive plasma cells and is a good indicator of neoplastic disease. Normal cells show a eccentric wheel-spoke pattern of the nucleus. In malignant disease, this can change into many different shapes and prominent large nucleoli can be found. Some of the nuclear variations described in literature are: cytoplasm invaginations in the nucleus, invaginations of the inner nuclear membrane, Dutcher bodies, nuclear lobulation and multinuclearity (figure 3). Nuclear lobulation and mitotic figures give rise to clinically aggressive disease.[3-6] Nucleoli in plasma cells indicate ribosomal activity rather than immaturity and proliferative activity.

B Cytoplasmic clearing

The normal cytoplasmic clearing paracentral of the nucleus, typical for the Golgi zone, can disappear in malignant disease causing variations in cytoplasm staining. Cytoplasm anomalies are often seen and the cytoplasm of the malignant plasma cell contains the produced immunoglobulins in a variety of forms in the endoplasmatic reticulum: Russell bodies, signet-ring-like cells, grape cells, mott cells, flaming cells (the cytoplasm is eosinophilic or has 'flaming' eosinophilic margins), tadpole like cells, thesaurocytes/ Gaucher-like cells) and crystalline rods can be distinguished [7, 8,43] (figure 4 and 5).

C Nuclear-cytoplasmic asynchrony/high nucleo-cytoplasmic ratio

This can be present, where the nucleus contains prominent nucleoli or a diffuse chromatin and the cytoplasm seems normally mature (figure 6). The prognosis is clearly related to nuclear changes and nuclear-cytoplasm asynchrony. No prognostic value is related to cytoplasm anomalies alone.[9]

According to Frisch and Bartl, these variations in plasma cell morphology can be summarized into three prognostic grades according to the predominant plasma cell type: Marschalko or low grade (small cell), intermediate (cleaved, polymorphous, asynchronous) and high grade (blastic).[7] The plasmablastic morphology of tumour cells occurs in almost 10-15 % of MM patients during the initial phase of disease, but this percentage may increase at the time of bone marrow relapse and is prominent in extramedullary relapse. It is clearly associated with poorer prognosis.[1,6,7,11-14]

Micro-environment

MM is a bone marrow disorder in which malignant monoclonal B-cells differentiate into plasma cells. The bone marrow predilection suggests that circulating malignant B cells transit in the bone marrow to find an optimal environment for surviving and differentiation [15]. It suggests a need for external support not found elsewhere in the body. The malignant monoclonal B cells differentiate into plasma cells by interaction with stromal cells, endothelial cells, the extracellular matrix and by interaction with other malignant plasma cells. The adhesion molecules are being shown to be of importance in MM and play a role in the connection and interaction of all these cells and components. Myeloma cells express a specific receptor for interleukin-6, which is the major growth and survival factor for MM cells. Interleukin-6 is produced by cells in the bone marrow micro-environment and is one of the main examples of the importance of the interaction of the microenvironment and the tumour cells in MM. [12,16-18] Stromal cells and extracellular matrix are activated by tumour cells and also produce cytokines, including IL-6, VEGF, IGF1, SDF-1 and TNF-α, determining growth, differentiation and adhesion of the tumour and therefore regulating tumour survival, maturation, migration and growth.[15,19,20] Syndecan-1, one of the most important adhesion molecules in the myeloma cell, accumulates in myeloma bone marrow and high levels of syndecan-1 in the serum are an indicator of poor prognosis.[21] Novel therapeutic treatments (such as thalidomide and its immuno modulatory drugs) target on MM cells but also on the BM micro-environment and can

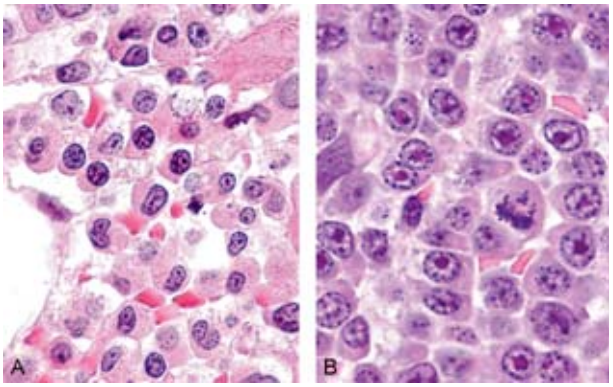

Figure 1
Three different types of plasma cells; A mature, B intermediate. H&E staining 600x.

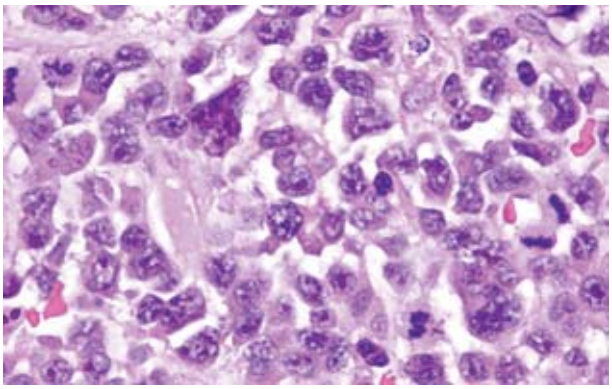

Figure 2
Three different types of plasma cells; Plasmablastic type H&E staining 600x.

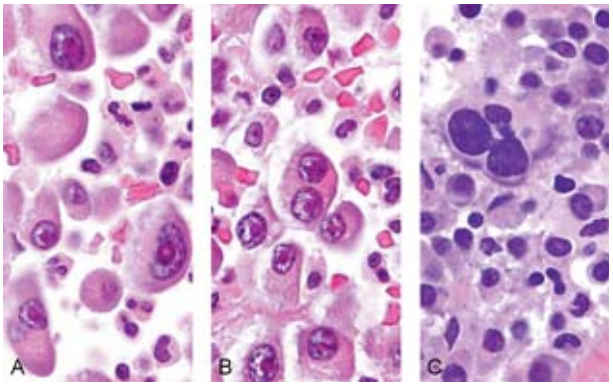

Figure 3
Nuclear pleomorphism; A prominent nucleoli, B multinuclearity, C nuclear lobulation. H&E staining 600x.

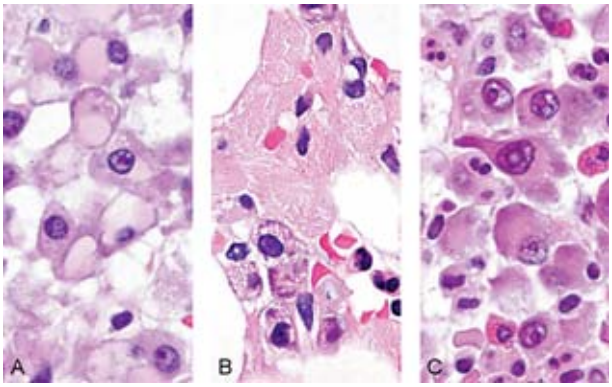

Figure 4
Cytoplasmic anomalies; A Russel bodies, B Gaucher cells, C Tadpole like cells. H&E staining 600x.

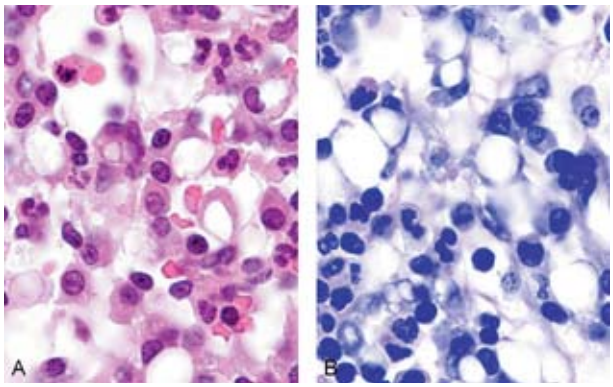

Figure 5
Cytoplasmic anomalies: Signetring-like cells; A H&E 600x, B giemsa staining 600x.

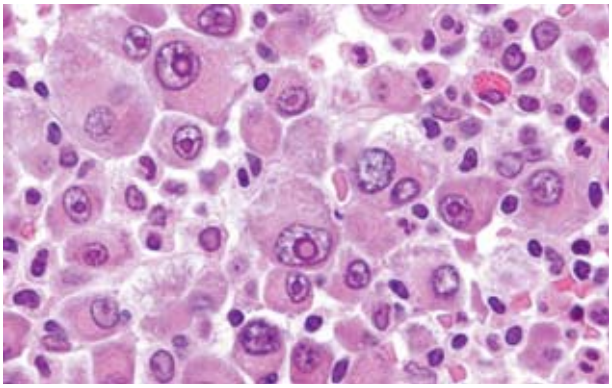

Figure 6
Nuclear-cytoplasmic asynchrony. H&E staining 600x.

therefore overcome drug resistance.[19, 22] Other alterations in bone and bone marrow are high osteoclastic index, fibrosis, marrow atrophy, dyshaematopoiesis, amyloidosis, angiogenesis and secondary neoplasia (figure 7 and 8). New therapeutic targets may aim at the interaction of the malignant cells and the micro-environment of the bone marrow.[20] Discrepancies between numbers of plasma cells in aspirates and biopsies may occur due to fibrosis or nodular infiltration, preventing aspiration of plasma cells. The development of coarse fibrosis usually leads to an unfavourable prognostic outcome. Also the presence of many osteoclasts resorbing bone, is prognostic for an aggressive tumour behaviour.[23, 24] Bisphosphonate therapy reduces osteoclastic resorption and stimulates osteoblastic repair. [7] Non specific alterations due to the therapy include absence of heavy infiltration, bone marrow lymphocytosis and granulomas.

Immunohistochemistry

Plasmablastic myeloma may be indistinguishable from an immunoblastic lymphoma or an anaplastic carcinoma. For differentiation, immunohistochemical staining is very helpful. In most cases, the malignant plasma cells in MM produce paraproteins representing the immunoglobulin heavy chain or the immunoglobulin light chain. In 60% of patients the paraprotein secreted is IgG; IgA is secreted in 20% of patients whereas in 15-20% of patients, only light chains are produced. The monoclonal immunoglobulins can be detected in the serum and/or as Bence-Jones proteins in the urine. In trephines they can be visualised by immunohistochemistry. In the de-novo plasma cell leukaemia, the cells often express only light chains, IgE or IgD and the absolute number of these cells correlates with disease activity.

In addition to cytoplasmic immunoglobulines, myeloma cells usually express several membrane markers detectable in immunohistochemical stainings. In the initial phase of disease CD38, CD79a, CD56, CD54 and CD40 can be found. Usually, pan-B markers, CD19, CD20 (L26) and CD45, are negative. Especially after treatment, the immunophenotypic evaluation is very important, since a normal polytypic phenotype of the plasma cells after treatment has a significantly longer progression-free survival.[25] At the time of medullary relapse, CD 28 expression may appear and at time of extramedullary relapse CD 56 may be lost.[12]

The importance of adhesion molecules in MM pathogenesis is becoming increasingly recognized. Molecules like CD44, CD 56, CD 58, VLA 4, VLA 5, RHAMM and CD 138 (syndecan-1) play a role in homing, growth, survival and spread of MM cells.[26] Detection of these molecules will become increasingly important for predicting prognosis and providing targets for new treatments. Ki67 (MIB-1) is a nuclear antigen of proliferating cells in the S-phase of the cell cycle and can be useful in the differential diagnosis of MM and MGUS, although only 1-2 % of plasma cells in MM are usually positive .

Chromosomal abnormalities

Chromosomal abnormalities are present in 20-60% of newly diagnosed patients and in 60-70% of patients with progressive disease, indicating an ascending scale of chromosomal abnormalities in the pathogenesis of the disease.[1,27] Gains, losses,

translocations and mutations are all common findings and also multiple chromosomal gains and losses can be found in many cases.[28] These genetic changes may prevent the differentiation and normal death of the myeloma cells, which continue to proliferate and accumulate in the bone marrow. Translocations and rearrangements of the BCL-2 and cyclin D1 gene are common findings. Abnormalities of chromosomes 1, 11, 13 and 14 and immature morphology of the plasma cells, correlate with resistance to treatment and are seen as unfavourable prognostic factors representing aggressive disease.[12,18,28-33] Translocations of the immunoglobulin heavy chain locus (IgH) results in upregulation of oncogenes and are frequently unbalanced with loss of one of the chromosomes and can be associated with shorter survival. Fonseca et al.[30] found subgroups and classified patients for prognostication according to molecular cytogenetic identification into three distinct categories: A poor prognostic group with (t(4;14)(p16;q32), t(14;16)(q32;q23), and –17p13) has a median survival of 24.7 months. An intermediate prognostic group (-13q14), with a median survival of 42.3 months and a good prognostic group (all others,

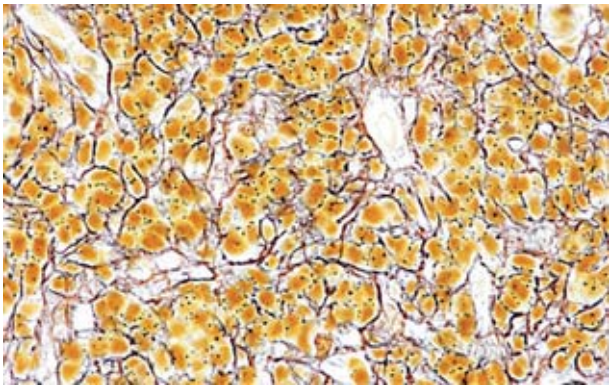

Figure 7
Unfavourable prognostic outcome: Coarse fibrosis, Gomori staining 600x.

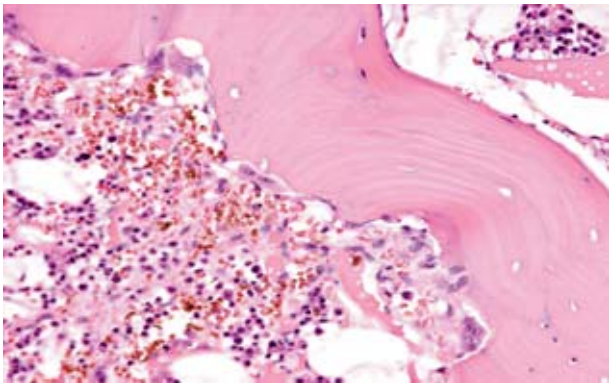

Figure 8
Unfavourable prognostic outcome: Osteoclasts resorbing bone. H&E staining 200x.

with median survival of 50.5 months) are also recognized.[30,34-36]
The p53 point mutations are rarely seen at time of diagnosis but can occur in progressive disease in 10 % of the patients and are associated with aggressive and leukaemic behaviour of the MM. P53 is thought to inhibit BCL2 and thus apoptosis. It also seems to be important in the pathogenesis of extramedullary relapse.[12,18,37,38] N-ras and K-ras mutations contribute to a higher proliferation rate and are associated with shorter survival; in particular, they are seen in times of medullary relapse.[12,18] In MGUS numerical alternations are found, indicating cytogenetic patterns characteristic of more than one tumour cell clone. This differs from the one clone in MM cells and plasma cell leukaemia.[39]

The mechanisms involved in chromosomal instability may be associated with decondensation of pericentromeric heterochromatin, which permits recombination and translocation of the chromosome and thus promotes proliferation. The chromosomal translocations can cause secondary chromosomal changes and can even multiply during progressive disease. The progression of genomic destabilization affects growth control and might influence sensitivity to therapies like drugs and radiation.[28] This theory is supported by newer techniques such as microarray analysis. More and more new genes are being identified as being involved in the development of the malignant plasma cell.[40] The expression patterns in MM cells are involved with adhesion, apoptosis, cell cycle, drug resistance, growth arrest, oncogenesis, signalling and transcription.[41] In the future, MM may even be classified by a personal gene expression profile, leading to an individual gene-based treatment.

Extramedullary lesions
Extra medullary plasmacytomas often occur in the head and neck region, but also in the blood, the pleura, the skin and in many other sites. Also in these tumours Bartl's histologic grading criteria are useful in significant relationship between higher tumour grading and shorter disease-specific survival and regional lymph node involvement.[12,42] In many patients, treatment should be deferred until there is evidence of progressive disease, in a similar manner to solitary plasmacytoma of bone and asymptomatic MM.[43] In contrast, involvement of the central nervous system in MM is associated with cytogenetic abnormalities of chromosome 13 (in particular), tumour bulk, plasmablastic morphology, additional extramedullary myeloma and circulating plasma cells, and the outcome of these patients is extremely poor. The clinician should be alert for signs of CNS involvement.[14] The disease usually terminates in refractory disease or changes into an immunoblastic lymphoma or a secondary myelodysplastic syndrome.

Practice points

- The percentage of plasma cells in the bone marrow is the mostimportant diagnostic criterion for multiple myeloma.
- Nuclear pleomorphism and nuclear-cytoplasmic asynchrony are histological markers of poor prognosis.
- Multiple chromosomal abnormalities are found in most cases of multiple myeloma, and the number of abnormalities increases with disease progression.

Research directions

- Use of DNA micro-arrays to identify specific gene over- or underexpression in the pathogenesis and progression of multiple myeloma.
- Further research into the role of cell adhesion molecules in multiple myeloma.

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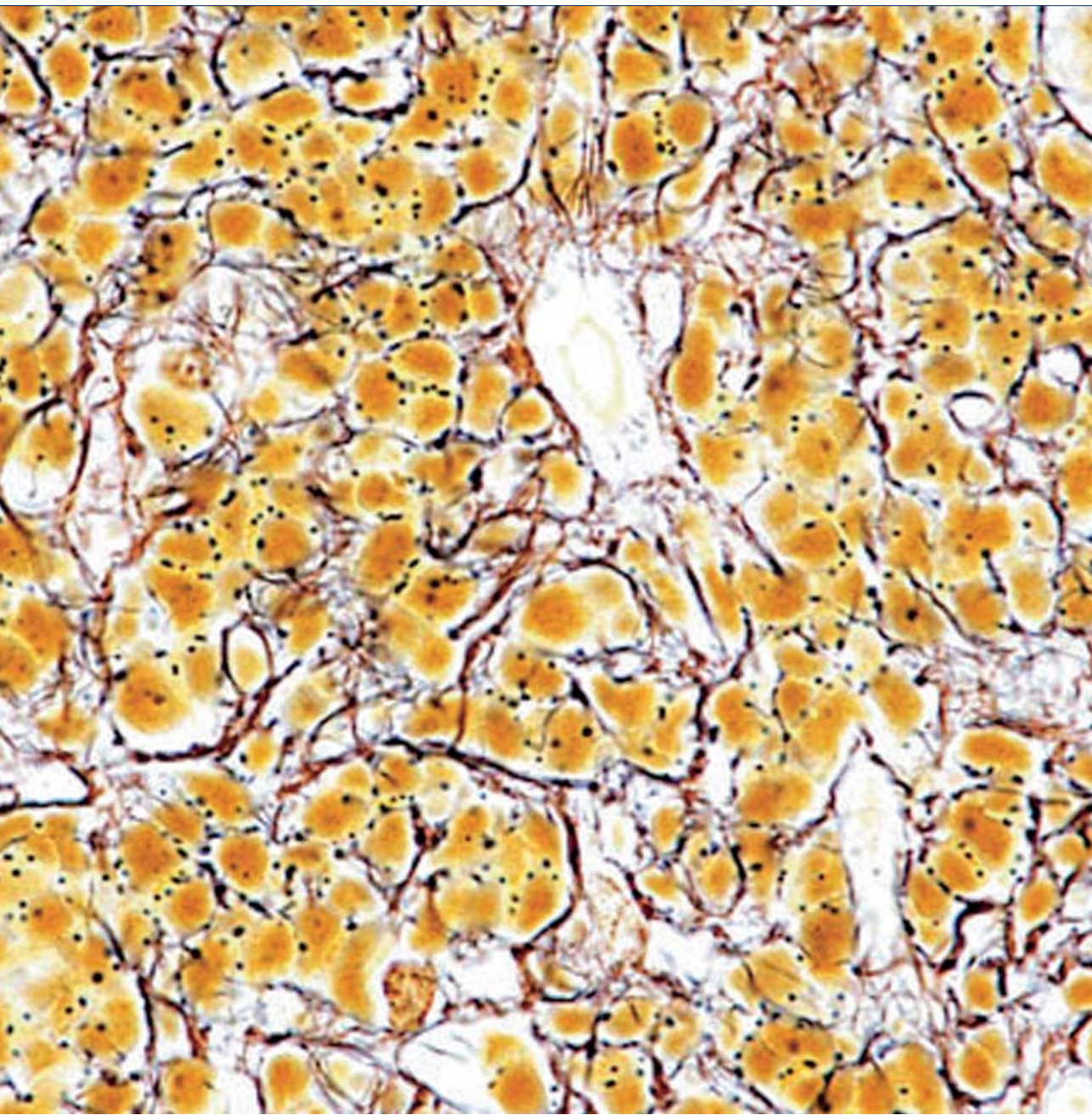

Morphology of the bone marrow after stem cell transplantation

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Abstract

In many haematological conditions the only curative option is stem cell (SCT) or bone marrow (BM) transplantation. Little information exists about BM morphology following non-ablative engraftment. During the pretransplantation period and depending on the kind of pre-treatment, there may be hypoplasia, residual disease and varying degrees of fibrosis. In the post transplantation period, after 1-3 weeks of severe transfusion-dependent pancytopenia, first signs of a successful engraftment are indicated by the recurrence of neutrophils, monocytes and erythrocytes in the peripheral blood. In the BM there is slow regeneration of erythropoiesis, followed by the other lineages of haematopoiesis and increase in reticulin fibres or even a resolution of fibrosis.
Diagnostic problems arise when neoplastic lympho- or haematopoesis are still maintained following transplantation. Moreover, there may be a significant graft versus tumour response reaction or an already relapsing disease needing aggressive treatment. On the other hand, a conspicuous dyshaematopoiesis should not be mistaken as presenting a myelodysplastic syndrome. The presence of granulomas being treatment-related or a manifestation of intercurrent granulomatous disease has to be considered. More advanced knowledge of the histological features of regenerating BM will certainly aid the recognition of relapsing disease and is needed for the adequate reporting of post-transplant alterations associated with a successful or failing engraftment.

Abbreviations

BM, bone marrow; SCT, stem cell transplantation; HLA, histocompatible leukocyte antigen; GVHD, graft versus host disease; PCR, polymerase chain reaction; STR, short tandem repeat; CMV, cytomegalovirus; ALL, acute lymphocytic leukaemia; G-CSF, granulocyte-colony stimulating factor; MDS, myelodysplastic syndromes; TBI, total body irradiation

Introduction

Bone marrow (BM) or, more recently, stem cell transplantation (SCT) is used as a curative therapy for a variety of haematological and non-haematological disorders.[1-7] Among them are leukaemias, myelodysplastic syndromes (MDS), multiple myeloma, malignant lymphomas and some solid tumours. Although a large proportion of these conditions can be ameliorated or even cured by a variety of treatment options including cytostatics, interferon or molecularly targeted therapy (imatinib) in patients with high-risk disease or resistance to chemotherapy, a SCT will improve the chances of survival.

SCT requires the pretreatment of the patient with chemo- and radiotherapy to eliminate the tumour and the patients' immune system. These conditioning regimens may be varyied and, especially in elderly patients, nowadays dose-reduced or low-intensity regimens are generally preferred.[8-12] They exert a significant impact on haematopoietic cells and the microenvironment of the BM stroma and in particular, influence on the extent of chimerism.[13] Stem cells are collected from the peripheral blood or the BM of a suitable related or unrelated donor and transfused to the patient after the pre-treatment to replace the deficient patient's haematopoietic and immune system. The SCT can be autologous (from the patients' own stem cells), syngeneic (from an HLA identical twin) and allogeneic (from an HLA-identical sibling or matched unrelated donor). In the latter incidences the donors are pre-treated with cytokines [granulocyte-colony stimulating factor (G-CSF)] to increase the amount of stem cells. In patients receiving an allogeneic graft the treatment related morbidity and mortality are significantly higher, due to graft versus host reaction and infections, compared with patients receiving an autologous transplantation.[14,15]

In this review, we will discuss the histological features seen in the BM during the different periods after SCT, including stromal changes, regeneration of haematopoiesis, detection of residual disease and characteristics for intercurrent disease. It is important that pathologists dealing with post-transplantation BM biopsies are aware of those features to recognize properly total or partial haematological reconstitution or alterations associated with relapsing disease.

Pre- versus post-treatment period

Preceding SCT, a variety of high- or low-dose chemotherapies is usually given, occasionally in combination with total-body irradiation (TBI) depending on the age of the patient and/or the underlying neoplastic disorder. This pre-treatment is myelotoxic and applied not only to eradicate the patient's own (non-clonally transformed) haematopoiesis and immune system but, more important, to eliminate the malignancy. The pretreatment is different when total myelo-ablation is intended, versus the non-myelo-ablative situation where there is no full eradication of the patients own BM and where there is an important role for a graft versus tumour effect.[8,10,12,16-18] During the period of pancytopenia the patient also receives prophylactic oral antibiotic, antifungal, antiemetic and antiviral therapy. This more or less aggressive pretreatment of the patients induces numerous serious changes in the BM involving haematopoiesis, the stromal cells and the interstitial fibrous matrix and is also related to the underlying lympho- or haematopoietic malignancy or a number of other disorders.[19-22]

A significant reduction of the erythropoiesis is usually observed. If this is accompanied by fibrosis, it is indicative of a more advanced stage of the disease, impairs engraftment after the transplantation and may result in an unfavourable (transfusion dependent) recovery, especially in the myeloproliferative disorders.[23,24] The number of macrophages in the pre-transplantation BM does not differ from that seen after myelo-ablative treatment.[25,26]

Normal cellularity, with a normal aspect of haematopoietic cell lineages, except slight dysmegakaryocytopoiesis and a reticulin fibre density not exceeding grade II, are indicative of normal engraftment after transplantation even in patients with preceding chronic idiopathic myelofibrosis.[24,27-29]

In patients with delayed engraftment after transplantation, the pretransplant BM may show little to marked fibrosis, even with reticulin fibrosis grade III/IV and a hypocellularity with a patchy aplastic BM, accompanied by dysmegakaryocytopoiesis and dyserythrocytopoiesis. Therefore, an acellular or hypocellular BM and a high degree of reticulin-collagen fibrosis are signs of late or failing engraftment after transplantation.[20,21,24,30-32]

The regeneration of haematopoiesis

Speed and quality of haematopoietic regeneration are variable and generally depend on the nature of the underlying disease and on the type of the conditioning regimen and method of transplantation. Usually, BM reconstitution is better and faster when a non-myelo-ablative treatment is applied compared with aggressive myelo-ablative strategies, where severe immunosuppression, slower haematopoietic regeneration and longer lasting pancytopenia are seen. In this context it is noteworthy that SCT results in faster recovery than the full BM transplantations used in the past.[14,15] Also, the age and physical status of the patient play an important role. Younger and healthier patients have a better and faster recovery / regeneration than older patients or patients with longstanding and advanced stages of disease.[33-36]

The regenerative capacity in the post SCT samples in many cases does not seem to be related to the donor-recipient relationship, the HLA match or the graft versus host disease (GVHD) prophylaxis.[37-40] The marrow pluripotent stem cells are certainly the most

important factor in engraftment of haematopoiesis.[41,42] A subtype of this population, the CD34 progenitors, are not only precursors of haematopoiesis, but also seem to be important in producing endothelial cells.[43-47] Donor/host chimerism is determined by the methods of transplantation and the ratio of donor to host stem cells and the varying phases of the cell cycle.[13,40,42]

In case of full chimerism (only donor cells), a more effective conditioning regimen has been used than when a mixed chimerism is detectable.[13] Although mixed chimerism is not necessarily associated with a poor outcome, many authors have suggested that this feature bears an increased risk of developping leukaemia.[48-51] Also, reappearance or increase of recipient chimerism is indicative of relapsing disease.[47,52-54] Although we will not concentrate on the topic of chimerism, nowadays it is very helpful, when looking through the microscope, to understand the features in a BM biopsy of a transplanted patient.[55]

The post-transplantion phase in detail

Rapid recovery of the microenvironment is essential for an undisturbed engraftment of haematopoiesis following SCT. In the sequel of myeloablative treatment we recognize chronologically the following phases of haematopoietic reconstitution during the early and late post-transplantation (PT) period:

First week PT

In the first days PT biopsies are almost never performed, except in post-mortem studies. This is one of the reasons for a lack of knowledge of the early stages of haematopoietic recovery. From our own experience there is a conspicuous decrease in cellularity with extensive cell necrosis associated with a marked oedema and expansion of the adipose tissue (Figure 1a) or even so-called scleroedema (Figure 1b,c), creating a morphological situation that is very difficult to evaluate and resembling features often encountered in radiation and/or drug-induced aplasia (severe toxic myelopathy).[56] Initially there may be a slight decrease in reticulin fibres.[26,27] *In vitro* studies have shown adhesion of engraftable haematopoietic CD34 progenitor cells to stromal cells within one hour of contact; however, the timing of marrow infusion after conditioning seems to be important.[57-61] Angiogenesis seems the most important factor for restitution of the haematopoiesis. Survival of a considerable number of host endothelial cells after myeloablative transplantation suggests a persistence of host-derived stromal vascularisation, followed by significant disturbances of vascular architecture.[55,62] Also the differentiation of endothelial cells from haemangioblasts is also implied, resulting in a formation of a vascular plexus. Moreover, during the PT period the engrafted CD34 progenitors seem to act as precursors for both haematopoietic cells and endothelial cells, generating a mixed chimeric state after transplantation.[43-47,63-65] Finally, a close functional association is detectable between endothelial and progenitor cells regarding trafficking and expansion, and finally homing.[66-68]

The distribution of engrafting cells is not random. In the first hours after engraftment the donor stem cells are equally located in the highly vascularized central regions of the

BM and adjacent to the endosteal (paratrabecular) borders. In the following hours the number of donor cells in the endosteal region decreases and the proportion of cells in the central regions increases.[32,69] The Distribution of the progenitor cells can be visualized by the immunohistochemical CD34 staining, where they are differenciated from endothelial cells by their histological characteristics including distribution (histotopography).[26,27,70,71] The induced BM changes become more evident at the end of the first week. Initially there is a proteinaceous interstitial/stromal oedema and between the oedema and the fat tissue there are small erythroid precursor islands with centrally localized macrophages.[19-21,24] There is a significant relationship between the number of erythroid cells and the amount of macrophages, containing cell debris and iron to a large extent.[23,25] Macrophages constitute an important part of the bone marrow microenvironment, responding to fat cell necrosis, and play a key role in the regulation and differentiation of progenitors of all lineages.[72-75] The CD68+ macrophages add to the creation of erythroblastic islets and seem to mediate the complex mechanism of generating mature erythrocytes.[23,25] Erythropoietic regeneration in the ensuing days may show marked megaloblastic features accompanied by nuclear abnormalities signalling an arrest of maturation, due to the adverse effects of chemotherapeutic agents on DNA synthesis and can easily be detected using glycophorin C immunostaining.[23,76] Regenerating erythropoietic islets are especially located between the restored fat cells, providing the microenvironment for erythroid recovery. Among other changes found are, single megakaryocytes often with dysplastic features (Figure 1d), granular eosinophilic exudation and multiloculated fat cells. The latter can be very numerous.

Second week PT

Together with the continuous increase in the number of erythropoietic islets and the intensive transfusion therapy, there is a corresponding rise of haemoglobin/hematocrit values. As already indicated, erythropoietic regeneration in the ensuing days may show marked megaloblastic features due to the effect of the aggressive chemotherapeutic agents (low versus high intensive conditioning regimens) on the DNA synthesis (Figure 1e). This effect may be accompanied by nuclear abnormalities.[23] Comparable to the recovery of the erythropoiesis, there is regeneration of the megakaryopoiesis.[24,26,77] A few myeloid colonies can also be observed, usually around the bony trabeculae. From this week on until 45 days after transplantation, there is a decrease in the amount of macrophages by about 40-50 %. It is presumed to be associated with the degradation of cells and debris following pretransplantation therapy (scavanger function). Patients with delayed engraftment may show clusters of large macrophages.[24,25] There is also a slight inflammatory infiltrate consisting of lymphocytes, some mast cells and plasma cells with a perivascular preference, as is usually the case in reactive situations or toxic myelopathy.[19,56,78-80] The stromal cells have to remain functional in order to provide the appropriate environment for regeneration or engraftment. After initial regression of the fibres a mild transient reticulin increase is an important feature in this period, it is part of the healing process of the damaged microenvironment of the BM, functionally associated with adhesion properties that are essential for a proper homing of stem cells.[81,82]

Third week PT

From the third week on, mixed erythroid and myeloid colonies are seen (Figure 2a,b). The amount of lymphocytes is still as low as in the second week. In the second and third week after transplantation there is still a clear increase in reticulin fibres in almost all patients with pretransplant fibrosis, but it is also observed in some patients without increased pregraft fibrosis. This finding is in contrast to the significant resolution of fibrosclerosis in responding patients with chronic idiopathic myelofibrosis at a later stage.[26-29]

The development and severity of acute graft versus-host-disease (GVHD) correlates significantly with the number of CD45RO lymphocytes in the marrow.[83] In chronic myeloid leukaemia, severe acute GVHD also correlates with a larger amount of reticulin fibres in the early PT period and with a delay in achieving transfusion independence.[32] Patchy regeneration of megakaryocytes occurs (Figure 2c), including dysplastic megakaryocytes.[24,77] Normalization of megakaryocyte size and cytological appearance are hallmarks of successful engraftment. CD61 immunohistochemistry facilitates the detection of smaller elements of the megakaryocytes, especially the immature precursor cells.[26,55,76,77] The reconstitution of the lymphoid population (T lymphocytes) also starts at this point (Figure 2e). Although the absolute peripheral lymphoid counts are decreased in the PT period, there is no significant relationship with the total amount of marrow lymphocytes at the different end points.[83] The total amount of lymphocytes in the marrow is between five and 20 cells per mm^2.[84,85]

Fourth week PT

Three weeks after transplantation the BM shows at least 50% of the normal cellularity with conspicuous regeneration of erythropoiesis and neutrophil granulopoiesis occasionally forming loose clusters and sheets in responding patients (Figure 3a,b). From the first month after transplantation the amount of lymphoid cells increases. Especially in cases where GVHD occurs, the number of lymphocytes is high, specially of CD45RO lymphoid cells.[83]

Second month PT

After more than a month the bone marrow cellularity should be normal in uncomplicated cases.[24] The PT quantity of lymphocytes is comparable to that of the normal bone marrow, in contrast to the depression of the amount of lymphocytes in the peripheral blood.[83] Focal necrosis of the bone associated with reactive bone formation may be seen; the newly formed bone will usually disappear within a few months. In children with acute lymphocytic leukaemia (ALL) a vascular osteonecrosis has been described.[86]

Late PT period

After 3 months the T and B cells are present in normal numbers in the BM and in the peripheral blood. However, the quality and function of the lymphoid cells still remains underdeveloped, resulting in frequent impairment of immune reconstitution [87] There seems no relation between the lymphocyte repopulation in the peripheral blood and the engraftment status of the BM. According to sequentially performed BM biopsies

in responding patients with gross fibro-osteosclerotic changes a total regression of the pretransplant increased fibrosis was completed (Figure 3c,d) after about six months.[12,26,27,29] The number of endothelial cells is suggested to play an important role after SCT in, for example, multiple myeloma patients, with decrease of BM microvessel density after transplantation.[88] Since the CD34+ endothelial cells in the BM have both a host and graft origin, they show mixed chimerism after the third month post transplantation, with donor derived elements of 18-25%. When there is disease relapse, there is an almost complete conversion of the endothelial cells to a host type, pointing to a CD34 progenitor cell origin of the endothelial cells.[47,52,55]

Complications of regenerating haematopoiesis

In the PT period a number of various complications can occur that are associated with a characteristic BM histopathology.

Significant dyshaematopoiesis

Significant dyshaematopoiesis, but especially dysmegakaryopoiesis (Figure 4a,b) can be present the first months after transplantation, with conspicuous cytoplasmic and nuclear abnormalities due to the chemotherapeutic or transplantation-related effects on the DNA synthesis. One has to be very reluctant to make a diagnosis of MDS in this period.

Acellular or hypocellular marrow

Some patients with post SCT are left with an acellular or hypocellular BM showing a conspicuous scleroedema and adipocytosis associated with primary engraftment failure (Figure 4c). This situation may also develop in the BM marrow, generated by induction treatment, by infectious agents such as viruses or perhaps due to the ensuing treatment. Also an insufficient quantity and quality of engrafted stem cells may be the reason for a decreased cellularity.

Residual neoplastic haematopoiesis or tumour cells

A diagnostic problem in the first weeks, occasionally also later, is the presence of the original malignancy in the BM. This is especially the case in non-myelo-ablative conditioning regimens. These are performed to reduce the mortality and morbidity of allogeneic transplantation and do not completely destroy the host BM. The aim is to induce enough immunosuppression to allow engraftment of donor cells with the hope that the graft antitumour effect will eradicate the residual tumour cells. This, however, will take some time and the presence of recognizable tumour cells in a biopsy raises the question of recurrent disease. As a general rule one can state that the presence of (monotypic) tumour cells in the first weeks after transplantation can be regarded as residual and that no extra therapeutic steps have to be considered in this situation. Substantial residual infiltrate can be detected by morphology alone. Small clusters, or even single tumour cells may be detected by their distinct morphology, although immunohistochemistry may be helpful (Figure 4d,e). It is more difficult to differentiate leukemic blasts from regenerating myeloid blasts and even immunohistochemistry will not be helpful in these

cases. In malignancies characterized by distinct genetic alterations polymerase chain reaction or short tandem repeat (STR) microsatellite markers may be applied, to look for lineage-specific chimerism and minimal residual disease. Using these techniques a reappearance or increase of the distinct genetic alterations was indicative of relapsing disease. [49,54,89-95]

Recurrent disease

Recurrent disease is unfortunately frequently encountered in patients after SCT. It may be very evident or it may be hard to distinguish from residual disease and may be encountered in an early phase where the pathologist has to rely upon immunohistochemical or molecular techniques to prove the existence of a malignancy. It may be very difficult and, for example (monoclonal) multiple myeloma cells may be present for a considerable time before they disappear. However, an increase in the number of cells suggestive of the original disease is nearly always a sign of recurrent disease (Figure 4f). Delayed engraftment is not significantly related to outcome or even recurrence of the disease.
Residual clonally transformed CD34 endothelial cells and myofibroblasts may be able to survive myeloablative treatment and thus are thought to be the source for a later relapse. Conversion of lineage-specific mixed chimerism in megakaryocytes might be due to the polyploidy status of these cells, but abrupt changes of the donor to host type of erythroid precursors, megakaryocytes or CD34 precursor cells are associated with recurrent disease.[52,53,55] The changes of the donor to host origin of the mature endothelial cells are also seen in patients with evolution into recurrent haematological malignancies.[47,52,53] Leukaemic relapse is characterized by a lesser degree of host retrieval in the mature BM macrophage population.[96]

Intercurrent disease

PT BM specimens may reveal intercurrent disease. One of the most frequently occurring features is the presence of granulomas in the early post-therapeutic and post-transplant period. Granulomas are considered to be therapy induced and may persist for a longer period. If granuloma formation is extensive, the differential diagnosis with a granulomatous infection has to be considered. The correct interpretation of the granulomas is only possible in the correct clinical setting. Focal necrosis of the bone associated with reactive bone formation may be seen; the newly formed bone will usually disappear within a few months. Except in children with ALL, where avascular osteonecrosis has been described, bone changes do not seem to have clinical implications.[97]
Some patients develop opportunistic infections that may also involve the BM. This is, however, an unusual finding. Cultured stromal cells infected with Epstein-Barr virus fail to support haematopoiesis and also cytomegalovirus (CMV) infection can also affect stromal cells.[98,99] The changes in the BM seen during intercurrent of opportunistic infection are generally non-specific and accompanied with an increase in lymphoid cells or macrophages. When erythrophagocytosis is seen, one has to consider CMV infection or toxoplasmosis.[100]

Discussion

SCT is more and more used as a treatment in haematological and sometimes also non-haematological diseases. If there is an undisturbed recovery, usually no BM biopsy is taken. This is one of the problems in reading the BM biopsy specimen at different times after transplantation. Biopsies are usually taken if the clinical signs and peripheral blood reconstitution fail and the BM shows a disturbed recovery and/or delayed engraftment of the donor stem cell graft. The CD34 progenitor cells are thought to present the common stem cells, able to engraft the BM and to develop into haematopoietic cells and endothelial cells, whereas the fibrous matrix and their cellular constituents (myofibroblasts, fibroblasts, fibrocytes) seem to be non-transplantable, i.e. host derived.[101-103] Except for the fact that the CD34 cells are pluripotent progenitor cells, their nature is not precisely known. Some acute myeloid leukemia (AML) patients have CD117+ precursor cells instead of CD34+ progenitors in primary and during relapsing disease.[104] According to the World Health Organization criteria, however, in the definition of AML the number of CD34 cells is one of the most important parameters, even without knowing the exact origin of these cells. Besides the CD34 cells, lymphocytes are also important cells after SCT, specially in GVHD.[105] This is a well-known reaction after allogeneic stem cell transplantation and clinically the acute GVHD is called chronic 100 days after the SCT. Based on histological features, the acute and chronic grading is different and not always comparable with the clinical picture. As well as GVHD, graft versus tumour effect is also a recognized entity. Some authors believe in the graft versus tumour effect and the effect of donor lymphocyte infusion is based on the idea that donor T/NK-cells can kill the residual tumour.[106,107] Others also believe that donor B cells are important in a clinically more autoimmune setting with antibody production by donor B cells. These (auto-) antibodies are directed to the internal organs of the host, such as the skin, liver and BM.[87,108,109] In conclusion, BM biopsies are important tools to obtain information about the haematopoietic status of postSCT patients. Mixed chimerism is a striking phenomenon of reconstituting haematopoiesis after SCT and has been shown by a combined immuno- and genotyping involving all cell lineages derived from the CD34 progenitor cells.[47,52,54,55,96] The main clinical goal will be the detection of residual or recurrent malignant disease. Knowledge of the underlying condition and of the features of the regenerating BM is a prerequisite for adequate reporting of such marrow changes and a challenging task for the pathologist.

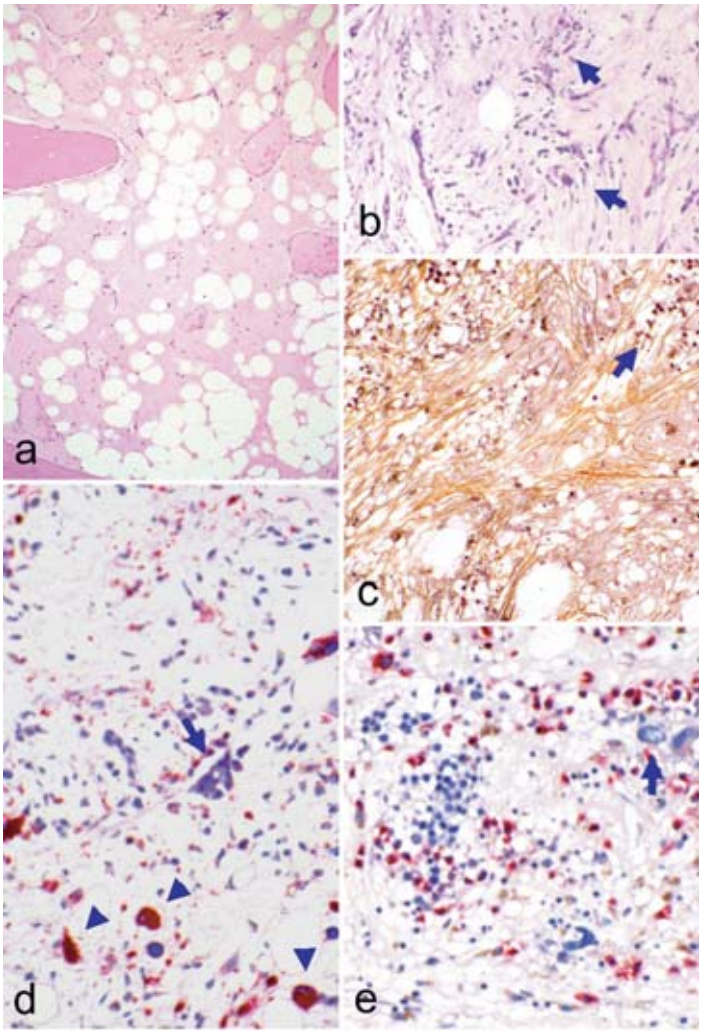

Figure 1

First and second week after transplantation. a, day 5 with oedema and adipose tissue due to myeloablative therapy. b and c, day 7 in a patient with myelofibrosis a striking sclerosis is still maintained and an initial perivascular generation of haematopoiesis detectable (arrows). d, day 7 with single regenerating small erythroid islets (arrow) are associated with a very few tiny megakaryocytes (arrow heads). e, day 14 with increase in (macrocytic) erthropoiesis and neutrophil granulopoiesis adjacent to small dysplastic megakaryocytes (arrow). (a, H&E, b, PAS, c, silver impregnation after Gomori, d, CD 61 immunostaining, e, chlororacetate esterase) a x 90, b,c x 180, d,e x 380.

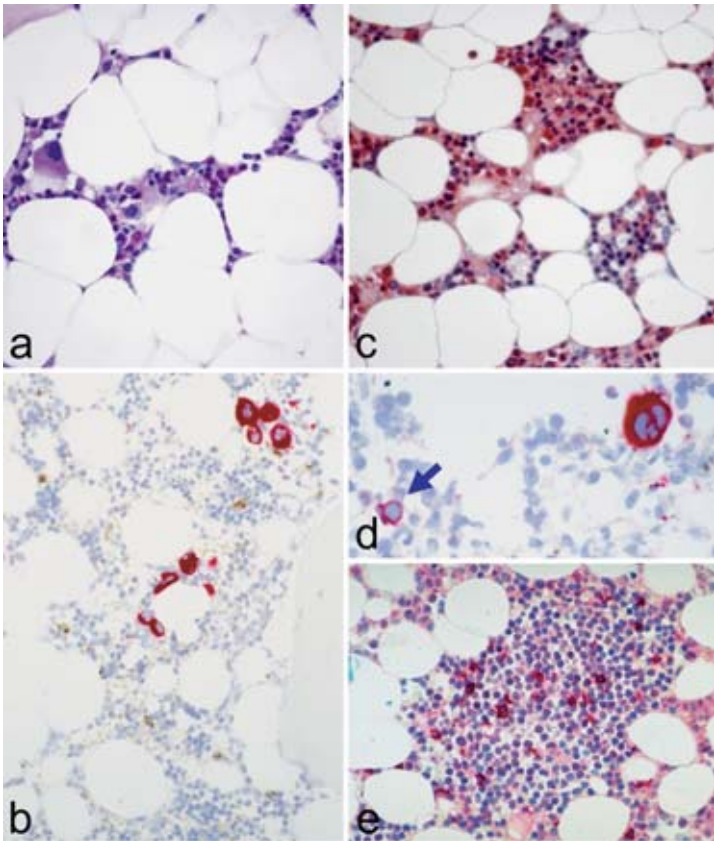

Figure 2

Second to third week after transplantation. a,b, day 15 to day 20 reveals larger groupings of mixed, often macrocytic erythroid and especially granulocytic colonies between the adipose tissue. (b). c,d, Tiny assemblies of small to medium sized megakaryocytes, in particular dysplastic micromegakaryocytes and precursors (d) do occur (arrow). e, Cluster of lymphocytes revealing a predominance of T-lymphoid cells in GVHD. (a, PAS, b, chlororacetate esterase, c,d, CD 61 immunostaining, e, CD 20 immunostaining), a,b x 380, c,e x 180, d x 570.

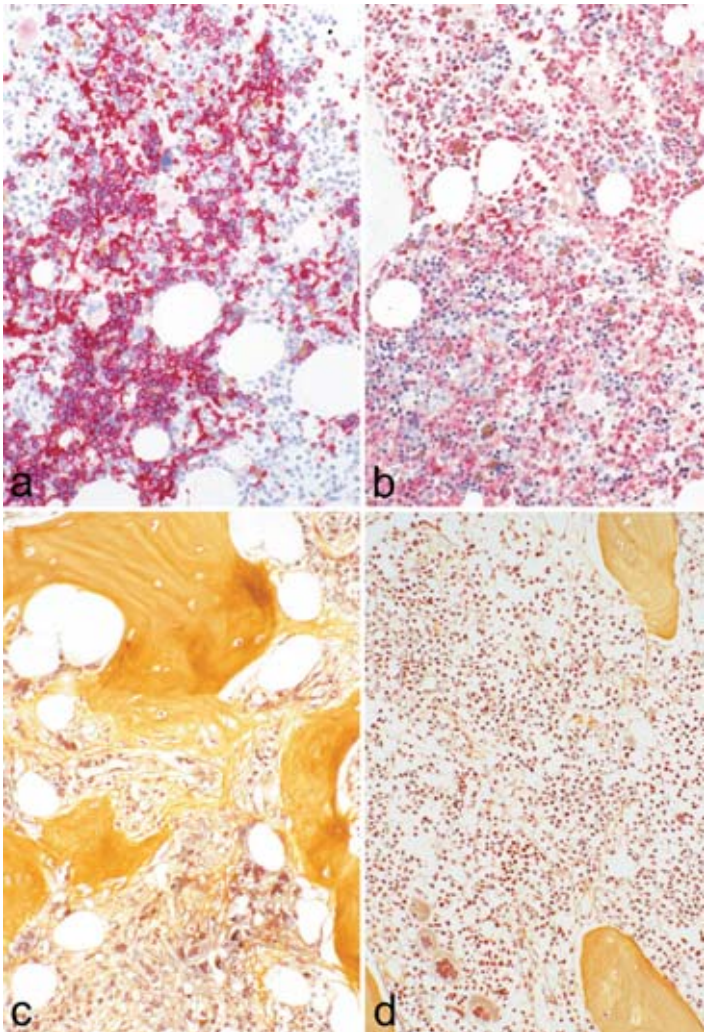

Figure 3

One to six months after transplantation. a, conspicuous regeneration of erythropoiesis with confluent islands between the 5 to 6 weeks after transplantation, b, During the same period neutrophil granulopoiesis is left-shifted and markedly expanding throughout the marrow. In patients with pretransplant excessive osteomyelofibrosis (e) a significant resolution of collagen between month four to six is observable (d). (a, antiglycoperin B immunostaining, b, chlororacetate esterase, c,d, Gomori=s silver impregnation) a-d x 180.

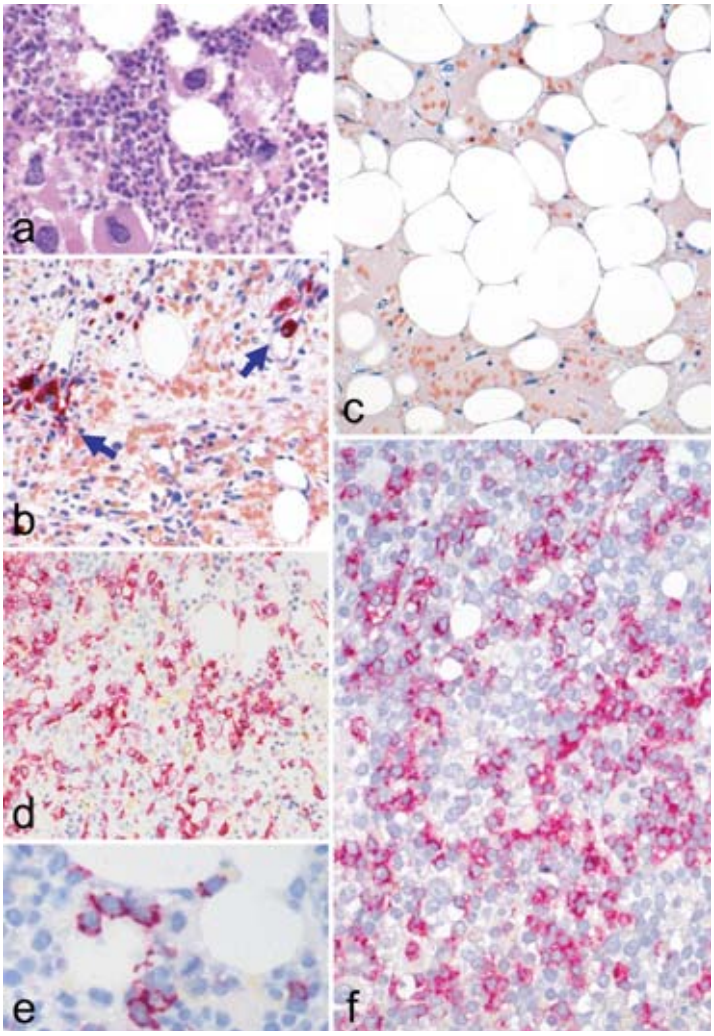

Figure 4

Late post transplant complications (at about day 100). a,b, Severe maturation defects of megakaryopoiesis displaying hypolobulated, cloud-like nuclei with hyperchromatic chromatin pattern may be present (a) or small clusters (arrows) of dysplastic megakaryocytes (b) in a hypoplastic marrow. c, Primary engraftment failure is characterized by an aplastic marrow with no myeloid cells and oedema d,e, Blastic infiltrates may initiate with dispersed tiny clusters of CD 34 positve precursors (e). f, Graft versus host leukaemia (manifest relapse) displays an overgrowth of (myeloid) blasts expressing partially lysozyme (myelomonocytic origin) and effacing any regenerating haematopoiesis (a, H&E, b, CD 61 immunostaining, c, chlororacetate esterase, d,e, CD 34 immunostaining, f, lysozyme immunostaining) a-d x 180, e x 570, f x 380.

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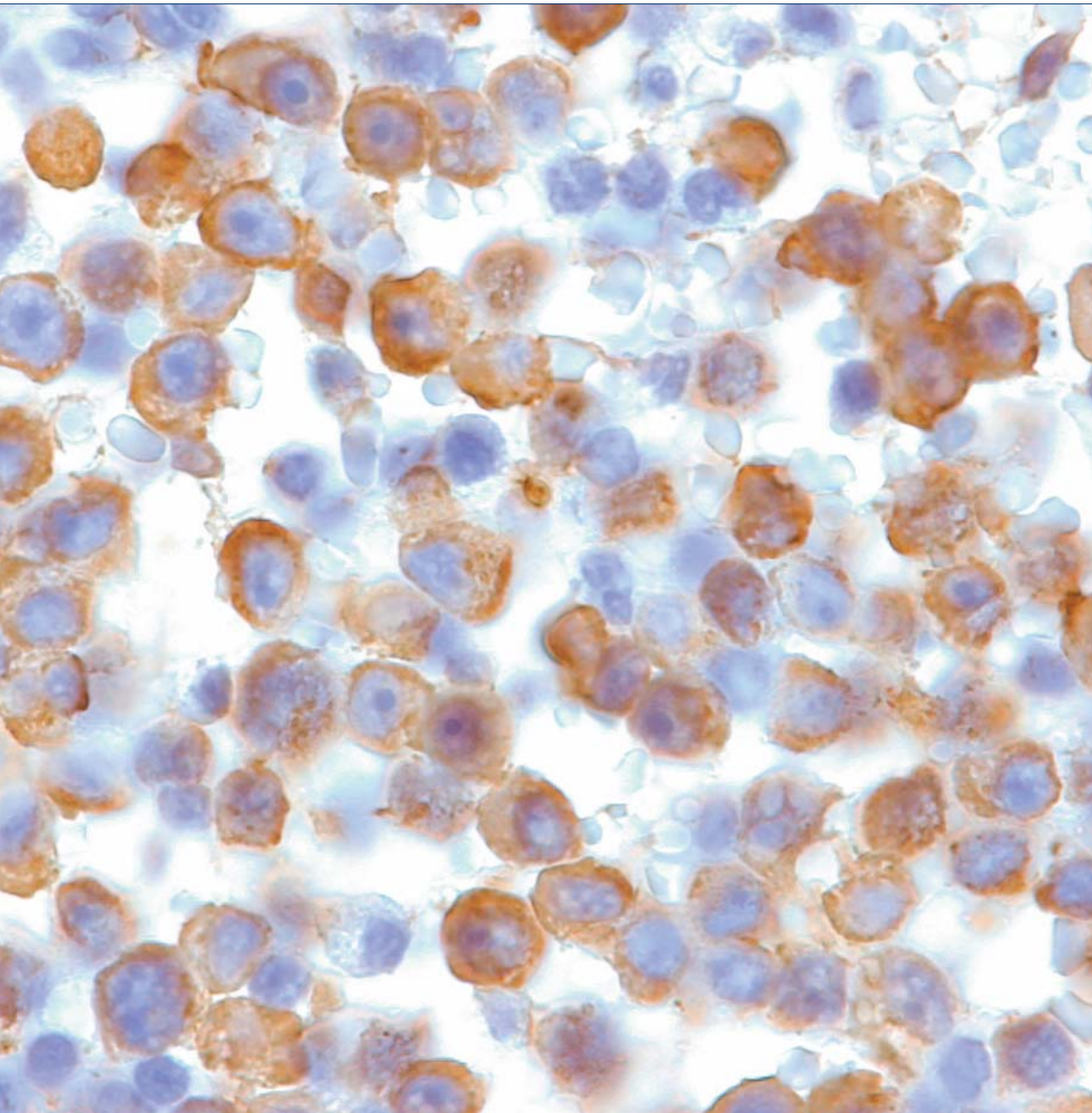

The significance of monoclonal plasma cells in the bone marrow biopsies of patients with multiple myeloma following allogeneic or autologous stem cell transplantation

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Abstract

High dose chemotherapy followed by reinfusion of autologous or allogeneic haematopoietic stem cells is a widely used treatment option for patients with Multiple Myeloma (MM). Bone marrow aspirates and bone marrow biopsies are part of the diagnosis and in follow-up procedures to determine therapy effect and remission status. In this study we evaluated retrospectively the prognostic significance of the presence of monotypic or polytypic plasma cells in bone marrow biopsies following transplantation, in terms of progression free and overall survival. A total of 205 MM patients were evaluated, including 108 patients receiving an allogeneic and 97 patients receiving an autologous stem cell transplantation. In the patients with available post transplant biopsies the polytypic group (n=82) showed a significantly better progression free survival (P=0.0042) than the monotypic group (n=66). However the overall survival (P=0.62) was comparable. The same results were found when the allogeneic and autologous transplants were evaluated separately. No plateaus were observed in the progression free survival curves suggesting that no cure is achieved even in patients with “clearance” of myeloma plasma cells in bone marrow biopsies. The definite prognostic value of immune phenotyping of bone marrow biopsies in myeloma needs to be determined in prognostic studies in comparison with standard methods to determine minimal residual disease, including plasma cell phenotyping of bone marrow aspirates, immune fixation of serum and urine, measurement of circulating serum free light chains and molecular analysis with patient specific primers.

Introduction

Multiple myeloma (MM) accounts for 10% of the haematological malignancies and is characterized by an expansion of monoclonal plasma cells. It is typically found in middle-aged and elderly patients. Most patients present with symptoms and require treatment.[1] High dose chemotherapy followed by infusion of autologous haematopoietic stem cells has become the standard treatment for MM patients.[2] Allogeneic stem cell transplantation, although associated with a higher treatment related mortality, may be the only strategy with a curative potential in MM due to the additional Graft versus Myeloma effect.[3] When these approaches are applied as part of first line treatment, complete remission (CR) rates varying between 20 and 50% may be achieved. Achievement of CR is an important prognostic criterion for progression free survival and overall survival.[2-4] The myeloma subcommittee of the European Group of Bone Marrow Transplantation (EBMT) has defined criteria to evaluate response following transplantation in myeloma patients, which have become widely accepted and applied. The criteria for a CR include absence of myeloma proteins in serum and urine and less than 5 % plasma cells in a representative bone marrow aspirate or biopsy.[5] Absence of clonal plasma cells in the bone marrow is not required. In this study, we evaluated retrospectively the prognostic significance of the presence of monotypic or polytypic plasma cells in bone marrow biopsies after stem cell transplantation (SCT) and during post-transplantation follow-up, for outcome of the disease in terms of progression free and overall survival.

Materials and methods

Patients:

Between 1986 and January 2006, 205 MM patients were transplanted. The mean age at the moment of SCT was 54 years (range 32-70, median 55 years). A total of 108 patients (52%) received an allogeneic SCT (Allo-SCT), with a median follow-up after SCT of 104 months (range 32-175 months). All patients received a transplant from a matched sibling donor; 60 patients first received myeloablative conditioning with cyclophosphamide and total body irradiation (TBI), 48 patients received nonmyeloablative conditioning with fludarabin and low dose TBI. The other 97 patients (48%) received an autologous SCT (Auto-SCT) as part of first line treatment and had a median follow-up of 65 months (range 40-89 months). Thirty five of them were transplanted after conditioning with cyclophosphamide and TBI and 62 patients after conditioning with high dose Melphalan 200 mg/m^2.

Bone marrow biopsies

Bone marrow biopsies were part of the evaluation of the response and were performed routinely between 3 and 6 months following transplantation. Bone marrow biopsies were available from all 205 patients before transplantation and from 148 patients both before and after transplantation. The trephines were formalin fixed and always partially EDTA decalcified and processed to paraffin wax. Sections were cut at 4 μm and routinely stained with H&E, Giemsa and a reticulum staining. Plasma cells were immunohistochemically stained for kappa, lambda, IgG, IgM, IgA, IgD and CD138 (syndecan-1), using a standard avidin-biotin complex technique (figure 1A-D). Five fields of 100 cells were counted, thereby giving a reliable estimation of the percentage monotypic or polytypic plasma cells.

To evaluate the polytypic or monotypic nature of the plasma cell population, we distinguished two groups of immunohistochemical plasma cell responses:

1. Polytypic. Polytypia was defined as a ratio of kappa:lambda positive cells of 60:40 (range 50:50 to 70:30), without a monotypic heavy chain expression. In this group were also included patients with less than 5% plasma cells and a subsequent changing expression from monotypic to polytypic in the first months after transplantation.
2. Monotypic. These patients showed a dominant immunoglobulin expression of one type in the bone marrow, due to the presence of monotypic plasma cells.

Statistics

Comparisons were made between patients receiving allogeneic and autologous SCT. The overall survival and progression free survival were computed by Kaplan-Meier survival analysis. The log-rank test was used to test for differences in survival analysis. The log-rank test was also used for differences in survival between groups. P-values <0,05 were considered statistically significant.

Results

Outcome of transplantation

One hundred and eight patients received an allogeneic SCT. Overall median survival after Allo-SCT was 104 months (mean 82 months). Treatment related mortality (TRM) occurred in 11 patients (10 %); in 1 patient (1%) TRM occurred before the plasma cell response could be measured.
The median overall survival of patients receiving an autologous transplant was 65 months (mean 73 months). Two patients died because of TRM (3%).

Significance of the presence of clonal myeloma cells after transplantation

In 148 patients biopsies were available after transplantation, in 84 of them following allogeneic SCT and in 64 following autologous SCT. The patients with polytypic plasma cells after transplantation had a significantly prolonged progression free survival as compared to patients with residual monoclonal plasma cells, 54 versus 28 months ($p=0.0042$, figure 2). However overall survival in both groups was not significantly different (65 versus 60 months, $p=0.62$, figure 3), although after prolonged follow-up patients with polytypic plasma cells tended to have a better chance of surviving.
From the 84 allogeneic transplanted patients 48 patients (58 %) were polytypic, and 36 patients (42 %) were monotypic after transplantation. Progression free survival was significantly longer in the polytypic group as compared to the patients with persistent monotypic plasma cells ($p=0.02$, figure 4). Overall survival in both groups was comparable ($p=0.52$, figure 5). However, the 5 year survival in patients with polytypic plasma cells was 55 %, compared to 45 % in patients with a monotypic plasma cell population.
From the 64 autologously transplanted patients with bone marrow biopsies available after SCT transplantation, 34 patients (53 %) were polytypic and 30 patients (47 %) monotypic. Although not statistically significant, the overall survival ($P=0.94$, figure 6) and progression free survival ($P=0.07$, figure 7) tended to be longer in the polytypic group, especially during longer follow-up. The 5-year overall survival in the polytypic group was 52 % and the monotypic group was 46 %.

Discussion

Response criteria defined by the myeloma subcommittee of the European Group of Bone Marrow Transplantation (EBMT) have become the standard for the evaluation of the response following SC transplantation in multiple myeloma. These criteria require for a CR, absence of myeloma proteins in serum and urine and less than 5 % plasma cells in a representative bone marrow aspirate or biopsy.[5] Probably because immune phenotyping is not routinely possible on a wide scale, the presence or absence of clonal bone marrow plasma cells is not part of the EBMT response evaluation.

In this study we retrospectively evaluated the prognostic significance of plasma cell immune phenotyping in bone marrow biopsies following stem cell transplantation. Progression free survival both in patients with an autologous and an allogeneic SCT was significantly longer in the patient groups with a polyclonal plasma cell population after SCT, as compared with the monoclonal groups. Duration of PFS following allogeneic transplantation appeared longer, however due to heterogeneity of the patient groups no conclusion is possible about a potential additional efficacy of allo-reactivity. Disappointing is also the finding that after prolonged follow-up there is no plateau in the PFS curves, indicating that even after allo-SCT no cure is achieved. Presence or absence of clonal plasma cells had no impact on overall survival. Several reasons may be responsible for this. The first reason could be that complete tumor eradication following transplantation is not achieved, and in patients with morphological absence of clonal plasma cells there is still a hidden tumor load. This may be due to the fact that the sensitivity to detect clonal plasma cells in bone marrow biopsies is low compared with immune phenotyping of aspirates using myeloma cell specific markers or by molecular analysis with patient specific primers.[6] Another additional reason may be the high efficacy of modern salvage therapy which may include a second autologous transplant, application of novel agents like Thalidomide and Bortezomib and donor lymphocyte infusions in patients with a relapse after Allo-SCT.[7-14]

In conclusion the significance of plasma cell immune phenotyping as part of response evaluation of stem cell transplantation seems limited. Although absence of clonal myeloma cells in bone marrow biopsies is associated with a prolonged remission, no patients with sustained remissions are identified and there is no impact on overall survival of patients. However more definite conclusions may be possible when the significance of plasma cell immune phenotyping is evaluated in prospective myeloma trials in comparison with the other sensitive tumor load evaluations like immune fixation of serum and urine, the assay to measure serum free light chains and plasma cell immune phenotyping of aspirates.

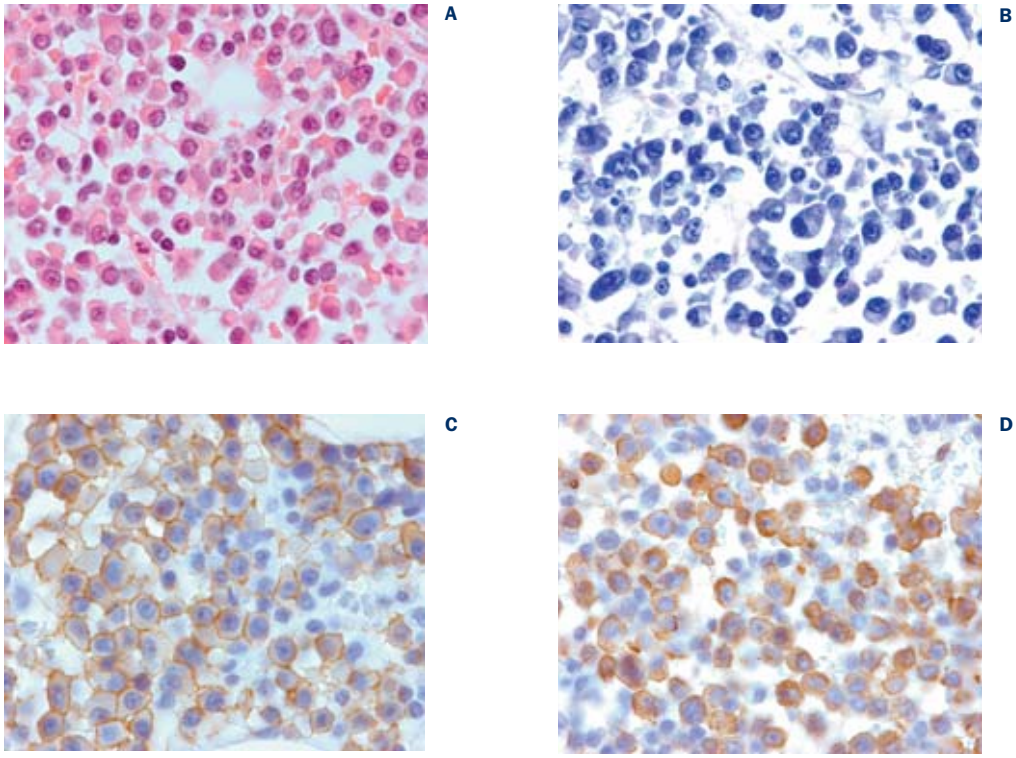

Figure 1

(A) shows an H&E staining of malignant plasma cells in a bone marrow biopsy of a multiple myeloma patient. The same bone marrow was stained with a Giemsa staining (B), CD138 (C) and Lambda (D).

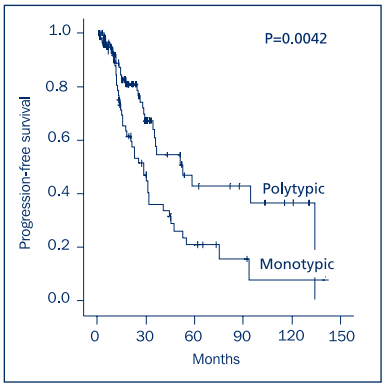

Figure 2

Progression-free survival in the total population of multiple myeloma patients, regarding the monotypic and polytypic nature of the plasma cells following transplan.

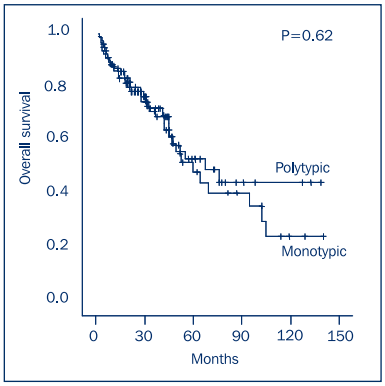

Figure 3

Overall survival in the total population of multiple myeloma patients, regarding the presence of monotypic and polytypic plasma cells in bone marrow biopsies following transplantation.

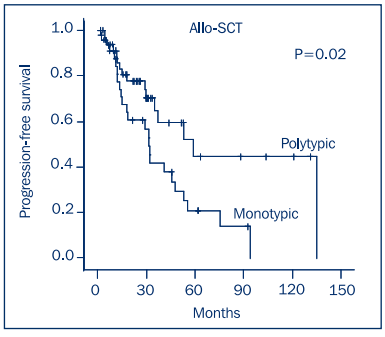

Figure 4

Progression free survival in patients treated with an allogeneic transplantation, regarding the polytypic and monotypic nature of the plasma cells in the bone marrow biopsy after transplantation.

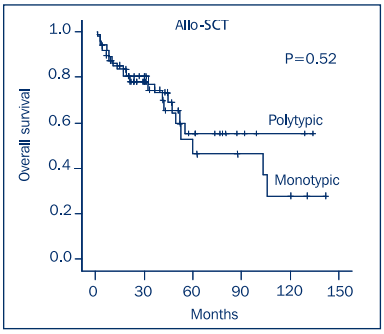

Figure 5

Overall survival in patients treated with an allogeneic transplantation, regarding the polytypic and monotypic nature of the plasma cells in the bone marrow biopsy after transplantation.

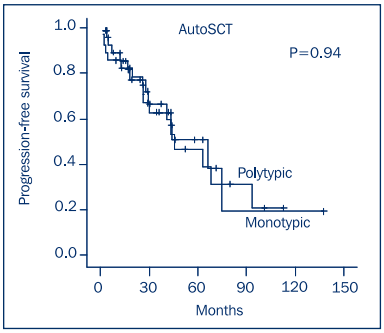

Figure 6

Overall survival in patients treated with an autologous transplantation, regarding the polytypic and monotypic nature of the plasma cells in the bone marrow biopsy after transplantation.

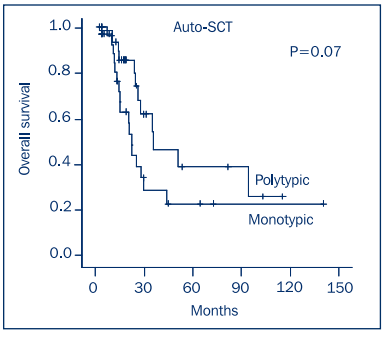

Figure 7

Progression-free survival in patients treated with an autologous transplantation, regarding the polytypic and monotypic nature of the plasma cells in the bone marrow biopsy after transplantation.

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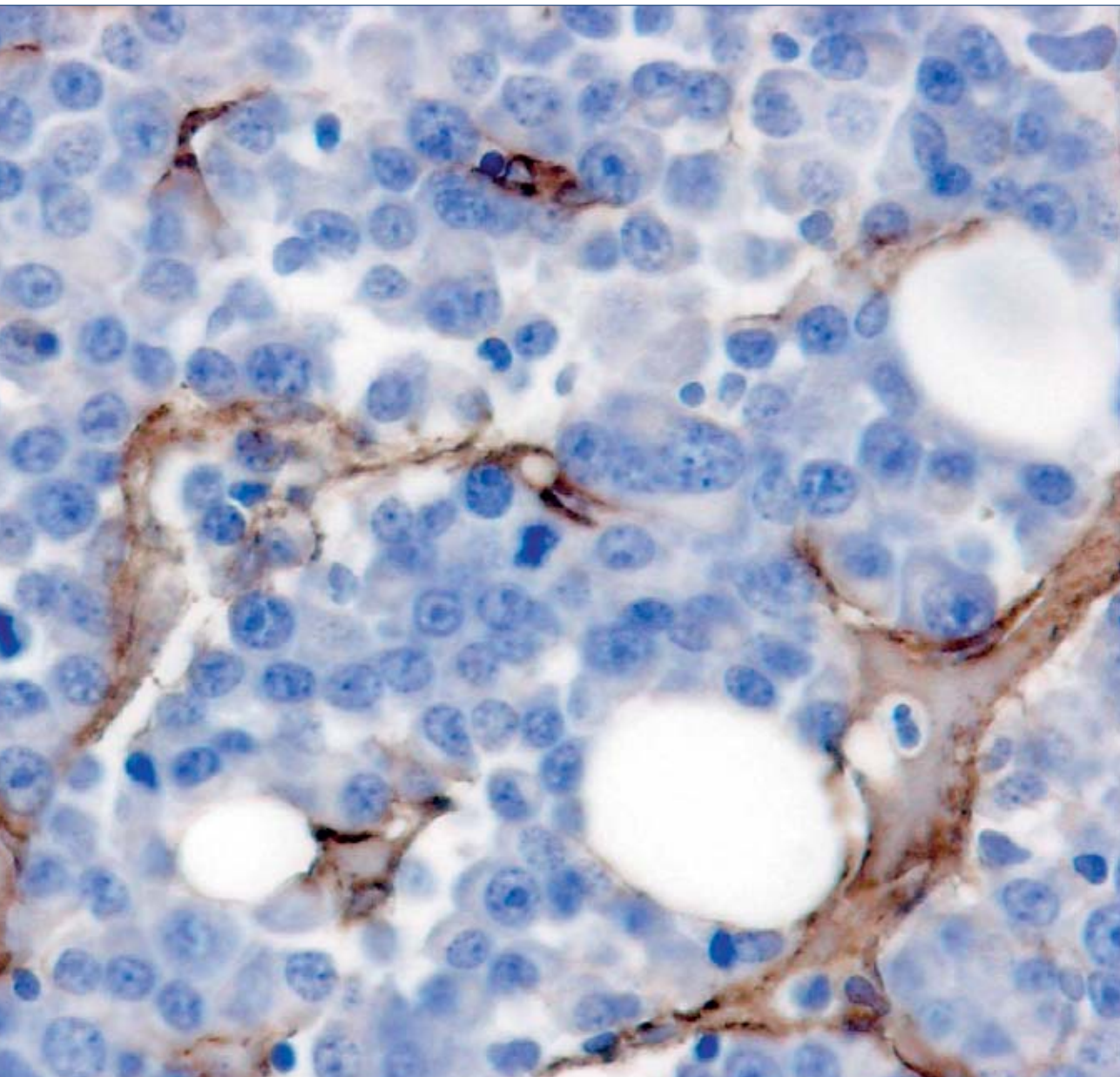
chapter 5



Reflection of the anti angiogenetic potential of Thalidomide in the bone marrow of multiple myeloma patients

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Submitted

Abstract

Bone marrow angiogenesis progressively increases along the spectrum of plasma cell disorders; it correlates with increasing tumor cell infiltration, and decreases after effective chemotherapy. The increasing amount of vessels in the bone marrow is therefore a reliable marker for tumor aggressiveness. The anti-angiogenic drug thalidomide is effective in patients with refractory and relapsed multiple myeloma. The effect of thalidomide as part of first line treatment was evaluated in the Hovon 50 study, in which previously untreated myeloma patients under 66 years were randomized between induction therapy with vincristine, adriamycin, and dexamethasone, or vincristine, adriamycin and thalidomide. After induction all patients received high dose melphalan with autologous stem cell rescue. During treatment we longitudinally compared the microvascular density in the bone marrow of 14 patients by multiple biopsies. Follow-up bone marrow biopsies were compared at time of diagnosis, after induction therapy with VAD or TAD, and after stem cell transplantation. A significantly increased microvascular density was found during induction chemotherapy with VAD, compared to the patients receiving TAD, indicating that the anti-angiogenic effect of thalidomide is most effective in the period of induction therapy.

Keywords
Anti-angiogenesis, bone marrow biopsy, multiple myeloma, thalidomide

Abbreviations
BM, Bone marrow; CAD, Cyclophosphamide, Adriamycin, Dexamethasone; HDM, High Dose Melphalan; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; MVD, micro vascular density; SCT, stem cell transplantation; TAD, Thalidomide, Adriamycin, Dexamethason; VAD, Vincristine, Adriamycin, Dexamethason; VEGF, vascular endothelial growth factor; vWf, von Willebrand factor.

Introduction

Bone marrow biopsies from patients with monoclonal gammopathy with uncertain significance (MGUS) show an increased amount of vessels , whereas more vessels are seen in trephines of multiple myeloma (MM) patients. In MM patients the amount of vessels correlates with the percentage of tumor infiltration.[1-10] This indicates that bone marrow angiogenesis progressively increases along the spectrum of plasma cell disorders, from the more benign MGUS stage to advanced myeloma.[11,12] The neoplastic plasma cells induce bone marrow angiogenesis by stimulating the production of angiogenic cytokines such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).[8,13-20] Increased bone marrow angiogenesis also has a prognostic significance. It is correlated with increased beta2-microglobulin levels and reduced patient survival.[1,2,12,13,21,21-25] Effective chemotherapy is accompanied by a significant decrease in bone marrow angiogenesis in MM. [26-28]

One of the best known anti-angiogenic drugs in the treatment of multiple myeloma is thalidomide. After the first publication of the use of thalidomide in a series of multiple myeloma patients in 1999, thalidomide is nowadays widely used in patients with relapsed disease and it's effectiveness as part of first line treatment is tested in several ongoing phase 3 multi-centre studies, including trials in which thalidomide is combined with melphalan and prednisone or thalidomide is combined with antracyclines and or dexamethasone.[4,29,30,30,31,31-34] The anti-angiogenic effect of thalidomide is well established, both in animal models of angiogenesis and from inhibitory effects of proliferation in cultured endothelial cells.[35-39]

Thalidomide also has strong procoagulant effects. This is illustrated by the high incidence of deep venous thrombosis in up to 30% of MM patients, particularly when thalidomide treatment is combined with dexamethasone or anthracyclines.[40-42] The occurrence of venous thrombosis suggests that the suppression of angiogenesis by thalidomide is accompanied by an increased procoagulable state. Clinically, it has already been demonstrated that multiple myeloma patients have high levels of factor VIII and von Willebrand factor (vWf), which is a prominent risk factor for the development of deep venous thrombosis and pulmonary embolism.[43-47] The mechanism behind the increase in vWf levels is uncertain, but it may involve activation of endothelial cells.

Previous studies on the effect of thalidomide did not study sequential bone marrow biopsies. Therefore in this study we prospectively evaluated the effect of different treatment strategies (one of which includes thalidomide) on bone marrow angiogenesis by measuring bone marrow micro-vascular density in patients included in the Hovon 50 study. The amount of vessels in the bone marrow biopsies was determined longitudinally and correlated with the therapy strategy, stage of treatment and the response.

Methods

Patients and tissue samples

We studied newly diagnosed multiple myeloma patients who were included in the HOVON 50 study, a prospective Dutch randomized phase III study on the effect of thalidomide combined with adriamycin, dexamethasone and high dose melphalan.

We included the fourteen patients with at least 3 sequential biopsies in this study. According to the Declaration of Helsinki, the protocol was approved by the Research Ethics Board. In this prospective phase 3 study, the effect of thalidomide as part of first line treatment before and as maintenance after intensive therapy was evaluated.

Patients were randomly assigned to induction chemotherapy consisting of 3 cycles of vincristine, adriamycin and dexamethasone (arm A, VAD). Patients assigned to arm B (TAD) received thalidomide (200 mg orally) instead of vincristine. Stem cells were mobilized after cyclophosphamide, adriamycin, dexamethasone (CAD) and G-CSF until collection. After this induction therapy all patients received 1 course of High Dose Melphalan (HDM) followed by autologous stem cell rescue. Patients randomized to arm A received maintenance therapy with α-interferon and patients randomized to arm B received thalidomide 50 mg/daily without VTE prophylaxis. During induction therapy with TAD low molecular-weight heparin was given to prevent venous thromboembolism.[41,42]

Bone marrow biopsies were taken at three different stages of the treatment: the first biopsy at diagnosis, the second after VAD or TAD treatment and the last one between one and eight months after the autologous stem cell transplantation. We investigated serial biopsies of seven patients receiving TAD treatment, and of seven patients receiving VAD treatment. All patients reached partial remission after the treatment, both in arm A and arm B. Informed consent was obtained from all patients.

Immunohistochemistry

The bone marrow biopsies were fixed in 4% formalin, deparaffinized and decalcified using EDTA and stained immunohistochemically with anti-CD34 (diluted 1:400, monoclonal, Immunotech)), being a golden standard for identification of endothelial cells, and anti-vWf (diluted 1:1600, polyclonal vWf, Dako cytomation. The anti-CD34 and anti-vWf immunohistochemical staining was performed with a labeled streptavidin-Biotin peroxidase method, in a standard 3-step method. Although also the stem cells in the bone marrow stain positive for CD34 staining, these cells can easily be distinguished from the vessels in the bone marrow.

The amount of vessels was counted by two trained microscopists in ten 400x magnification fields ($2mm^2$), equally distributed among microscopic fields with and without tumor infiltration, depending on the amount of tumor infiltration. The mean number of microvessel cross-sections per area in each sample was determined as the microvascular density (MVD). The amount of vessels was corrected for the cellularity of the bone marrow to 100% and therefore also for the amount of myeloma cell infiltration.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat (GraphPad, San Diego, CA) software package. Since experiments were performed with serial biopsy specimens, analyses were performed using repeated measures analysis of variance (ANOVA) with Dunn's post test. P values <0.05 were considered statistically significant.

Results

Seven patients with multiple myeloma randomized to receive VAD, and seven randomized to receive TAD were included in this study. From all patients 3 sequential bone marrow biopsies were available, which were taken at the time of diagnosis, after VAD or TAD induction treatment and after stem cell transplantation. Patients were comparable with respect to age and disease stage at diagnosis. All patients reached partial remission after induction therapy.

All biopsies were stained for CD34 and vWf, after which the amount of vessels identified by either CD34 or vWf staining were counted. The number of vessels in 10 representative 400x magnification fields were determined. This number was corrected for the cellularity of the bone marrow sample.

Figure 1 shows typical examples of the staining patterns observed in the myeloma population at the time of diagnosis. Figures 1A and 1B shows examples of CD34 (A) and vWF (B) staining of a bone marrow sample at low magnification in a patient. Figure 1C and 1D shows a typical example of CD34 (C) and vWF (D) staining in the bone marrow from a patient with substantial tumor cell infiltration, whereas in figures 1E (CD34) and 1F (vWF) a typical staining pattern from a patient with low cellularity is shown.

At the time of diagnosis, the number of vessels in the bone marrow, corrected for the cellularity, was similar in patients in the VAD and TAD arm (table 1 and figure 2). When the amount of vessel cross-sections was determined according to CD34 positivity, patients in the VAD arm (figure 2A) had 432 vessels per 10 fields (range 89-1108), whereas the patients in the TAD arm (figure 2B) had 444 vessels per 10 fields (range 143-892). Also, when the amount of vessels was determined by vWF positivity, no difference in amount of vessels was present between the VAD (mean 340, range 39-1136;figure 2C) and TAD (mean 185, range 86-309; figure 2D) arm.

After chemotherapy, the amount of vessels in the VAD arm as measured by CD34 staining significantly increased compared to the amount of vessels at diagnosis (mean 951, range 260-2000, $p<0.05$). In contrast, in the TAD group the amount of vessels as measured by CD34 staining did not increase after chemotherapy (mean 314, range 73-650). Consequently, the amount of vessels after chemotherapy in the VAD group was significantly higher compared to the amount of vessels in the TAD group after chemotherapy ($P=0.03$).

After stem cell transplantation, there was a significant decrease of the amount of vessels identified by CD34 staining in the VAD arm, but it was not different from the amount of vessels at the time of diagnosis, in both the VAD and TAD arm.

In contrast to the vessel counts performed on basis of CD34 positivity, the amount of vessels as determined by vWF staining did not increase during chemotherapy in the VAD arm. When vessels were identified by vWF positivity, no significant differences in vessel density were observed after chemotherapy and stem cell transplantation for both the VAD and TAD arm.

Discussion

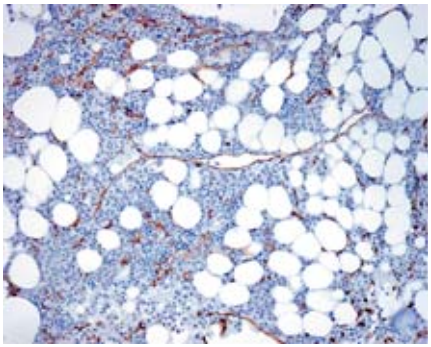
Angiogenesis is the process of new blood vessel formation, which is essential for the proliferation and growth of most malignant neoplasms. Also in multiple myeloma, increase in angiogenesis is associated with the increase of the tumor load and this is important for disease progression. Recent studies suggest that angiogenesis is more prominent in multiple myeloma than in monoclonal gammopathy of undetermined significance (MGUS).[2,11,12] Thalidomide is a well known anti-angiogenetic drug and is successfully used in multiple myeloma patients, but also has procoagulant effects, with high factor VIII and vWF, resulting in thrombosis and embolism.[43-47] This suggests a direct effect on endothelial cells with anti-angiogenic results, which can be measured by counting the amount of vessel cross-sections, lined by endothelial cells, in the bone marrow biopsies. Although the micro-vascular density was measured in other studies, it was never before measured prospectively and longitudinally in the different stages of treatment of thalidomide (TAD), compared to more conventional treatment with VAD, and after the stem cell transplantation.
Although a total of 450 patients were included in this study, we could only evaluate 14 patients with multiple bone marrow biopsies during the treatment. The clinician can evaluate the response and follow-up by less invasive methods like measuring urine and serum M-proteins, since bone marrow biopsies are usually not taken for the purpose of research only. This is most probably the reason for the small amount of sequential biopsies in this study.
In spite of the small amount of bone marrow biopsies our data clearly demonstrate a temporary increase in the amount of blood vessels during the VAD chemotherapy. We also demonstrated a relative decrease of vessel density in the TAD treated patients compared to the VAD treated patients after induction therapy. On the other hand we did not observe a significant change in the amount of vessels during thalidomide treatment. These observations indicate that bone marrow angiogenesis continues during VAD treatment, and that administration of thalidomide prevents the formation of new vessels in the bone marrow. In other words, the anti-angiogenic potential of thalidomide is reflected in the vessel density of the bone marrow of treated patients.

The observation that patients treated with TAD show a significantly reduced bone marrow angiogenesis compared to other treatment regimens is in line with observations by Kumar.(4) However, in that study, MVD measured after completion of induction therapy, decreased with thalidomide treatment, but did not change after treatment with VAD, whereas in our study an increase in MVD after VAD and no change after TAD was observed. The discrepancy between these results is unknown, but may be related to the method by which the MVD was determined. In our study we corrected the counted amount of vessels for the cellularity of the bone marrow, to be able to make a correct comparison between the different biopsies. This way we could strictly compare the effect of the treatment between TAD and VAD on the amount of endothelial lined vessels and expression of vWF, without the effect of tumor reduction itself.

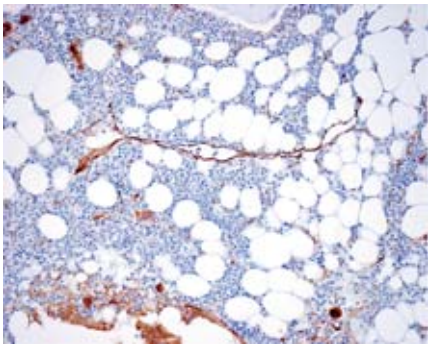
Recently, it was also shown that thalidomide down regulates the expression of pro-angiogenic genes in endothelial cells isolated from the bone marrow of MM patients which again supports the hypothesis that thalidomide inhibits bone marrow angiogenesis in MM patients.[39]
Surprisingly, we only observed a significant increase in the number of vessels in the VAD arm during chemotherapy when the vessels were identified by CD34 staining. Although both anti-CD34 and anti-vWF are used in daily practice as golden standard for the identification of endothelial cells, their staining pattern is different. Both stain blood vessels, but the background staining differs considerably. When staining bone marrow specimens with an antibody against vWF, there is much more background because of serum, which contains remnants of platelets, and free vWF. Moreover, megakaryocytes also stain for vWF. Furthermore, using vWF, the identification of small individual microscopic vessels is more difficult than when CD34 staining is used. With CD34 staining the presence of small individual vessels is much more prominent. Anti-CD34 also detects blasts, but these can clearly be distinguished from the bone marrow vessels.

Although the results of the vWF and CD34 staining methods lead to different conclusions in this study, because the increased amount of vessels after VAD treatment is only observed with CD34 staining, the agreement between the two staining methods is not as poorly as the present results suggest. In fact, if we pool all samples in which vessels were counted in this study (n=42, fourteen patients at three time points), we observe a strong correlation between the amount of vessels counted with CD34 staining and the amount of vessels observed with vWF staining ($r=0.85$, $p<0.0001$). A major limitation of our study is the limited amount of longitudinal samples, which were investigated. However it is very difficult to obtain more than 2 biopsies in most patients as discussed before. Based on the excellent correlation between the amount of vessels measured by CD34 and vWF staining, it is likely that both staining methods would have revealed an increased number of vessels after VAD treatment in a larger population.

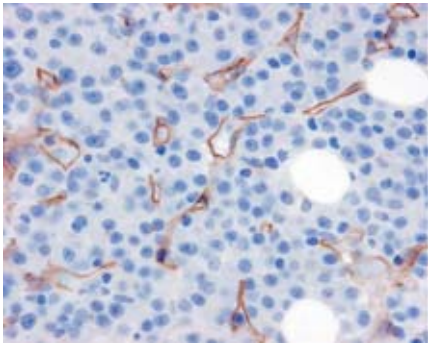
In conclusion, the number of CD34 positive blood vessels in the bone marrow of multiple myeloma patients is significantly increased after induction chemotherapy with VAD, whereas no increase in vessel density is observed after TAD treatment. These results demonstrate that the anti-angiogenic potential of thalidomide is reflected in the vessel density of the bone marrow.



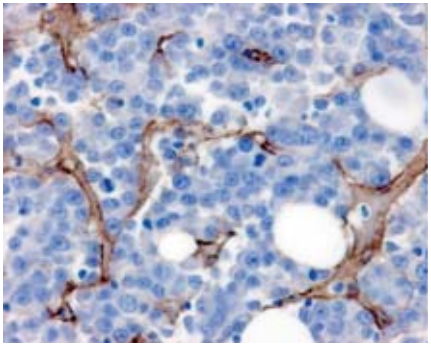
A, CD34



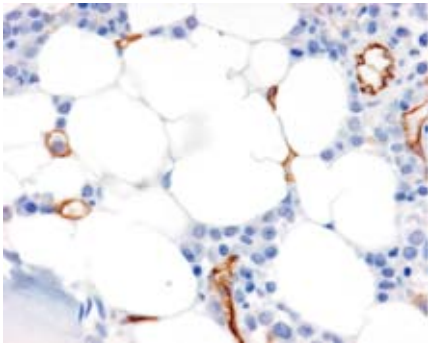
B, vWF



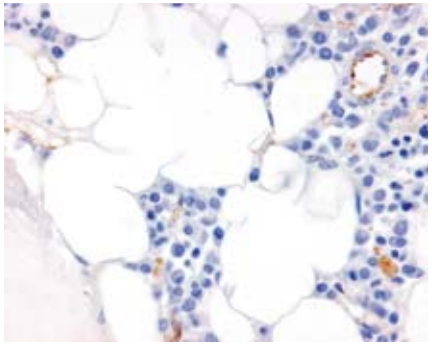
C, CD34



D, vWF



E, CD34



F, vWF

Figure 1

Typical examples of CD34 (A,C,E) and vWF (B,D,F) staining of bone marrow biopsies in 3 different patients. A and B: an overview of vessel staining at a 100x magnification. C and D: vessel staining at a 400x magnification in a patient with substantial tumor infiltration. E and F: vessel staining at a 400x magnification in a patient with low bone marrow cellularity.

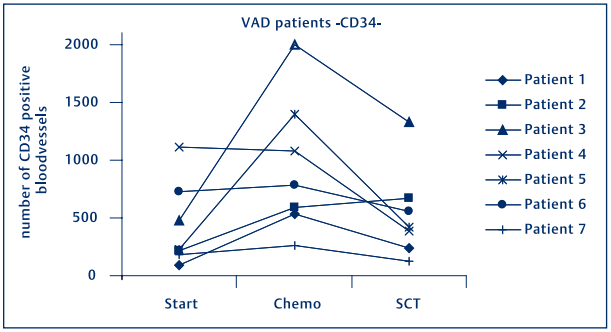
VAD	CD34			vWF		
naam	Start	Chemo	SCT	Start	Chemo	SCT
Patient 1	89	534	238	39	316	166
Patient 2	215	590	673	183	310	345
Patient 3	482	2000	1333	349	1600	747
Patient 4	1108	1084	388	1136	888	258
Patient 5	224	1400	420	63	300	133
Patient 6	722	789	560	492	254	345
Patient 7	185	260	130	118	117	128
Mean	432	951	535	340	541	303

TAD	CD34			vWF		
naam	Start	Chemo	SCT	Start	Chemo	SCT
Patient 8	892	650	265	232	285	131
Patient 9	592	548	278	286	222	180
Patient 10	143	73	520	86	65	423
Patient 11	711	323	210	309	197	135
Patient 12	393	171	418	178	156	100
Patient 13	219	253	267	110	231	146
Patient 14	160	180	173	100	255	113
Mean	444	314	304	186	202	175

Table 1

The amount of vessels corrected for the cellularity of the bone marrow in the patients treated with VAD or TAD.

A



B

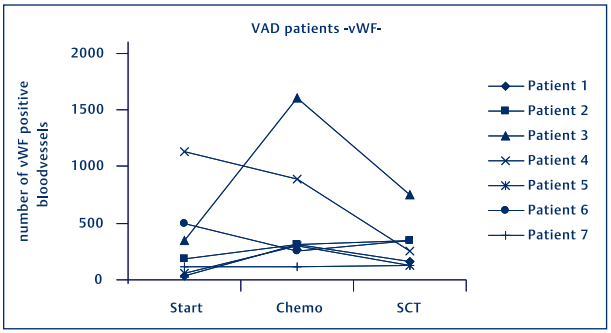
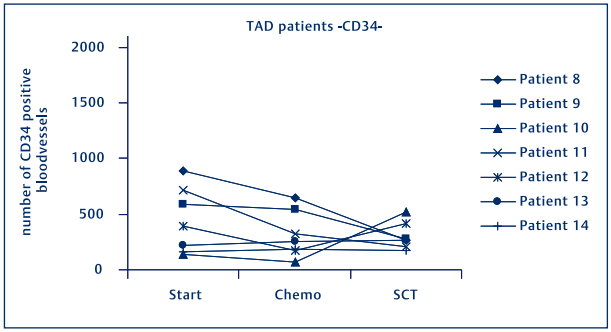


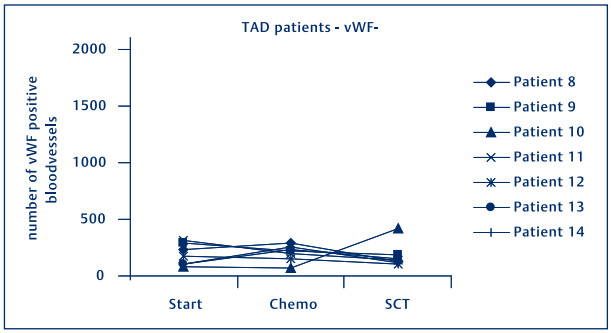
Figure 2

Microvascular density in the bone marrow of MM patients treated with eighter VAD (A,B) or TAD (C,D) staining. Vessels were counted in 10 microscopic fields, and the microvascular-density was corrected for the cellularity of the sample. After chemotherapy the amount of vessel cross-sections in the VAD treated MM patients increased significantly and decreased again after SCT to the starting level. In the TAD treated patients there is no significant change in the amount of vessels during the stages of treatment.

C


TAD patients -CD34-
number of CD34 positive bloodvessels
2000
1500
1000
500
0
Start
Chemo
SCT
Patient 8
Patient 9
Patient 10
Patient 11
Patient 12
Patient 13
Patient 14

D


TAD patients - vWF-
number of vWF positive bloodvessels
2000
1500
1000
500
0
Start
Chemo
SCT
Patient 8
Patient 9
Patient 10
Patient 11
Patient 12
Patient 13
Patient 14

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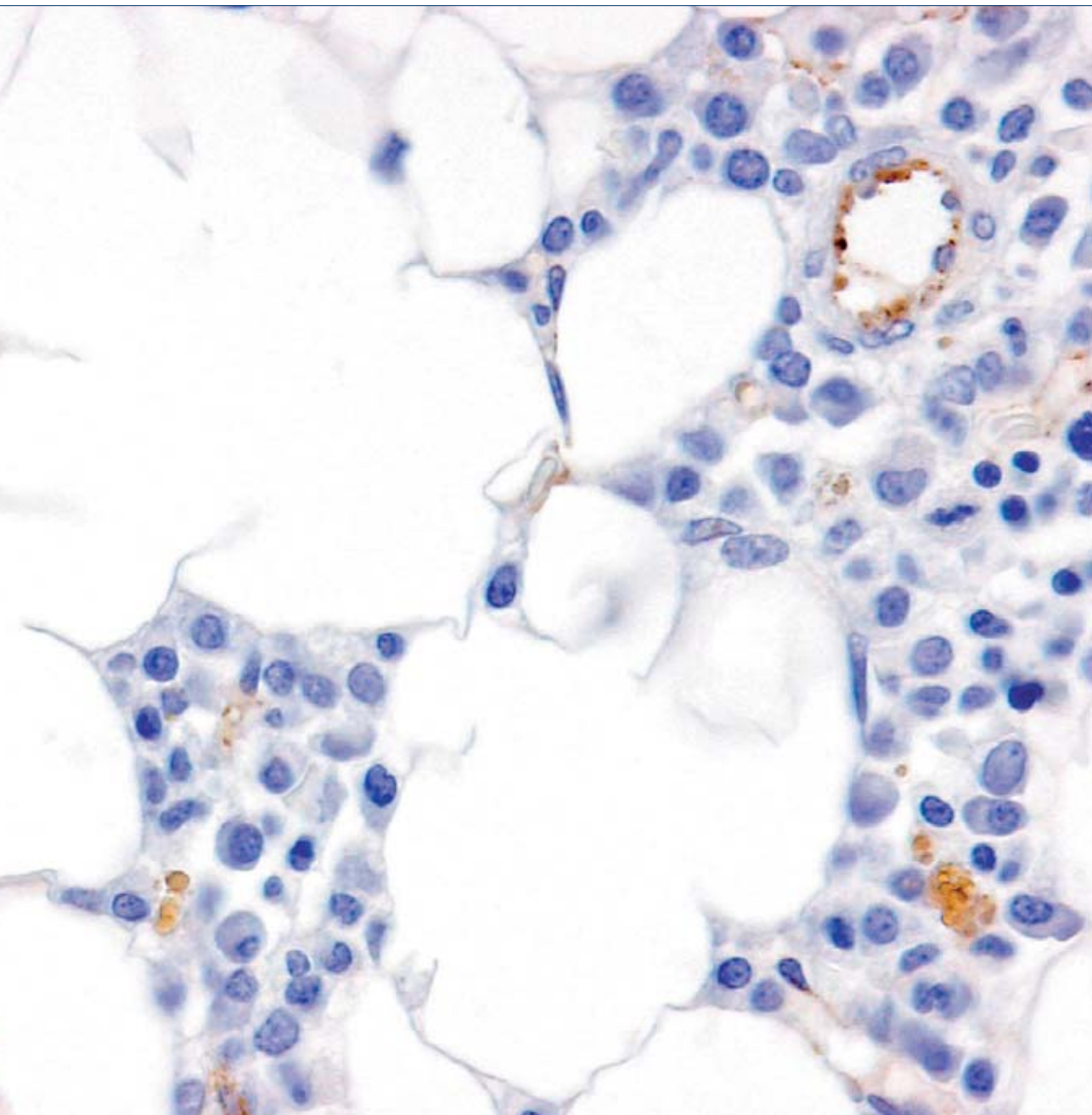

Hypofibrinolysis during induction treatment of Multiple myeloma may increase the risk of venous thrombosis

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Summary

Multiple myeloma patients are at risk for venous thrombosis. Previously, we have demonstrated that hypofibrinolysis constitutes a risk factor for venous thrombosis in otherwise healthy individuals. We measured plasma fibrinolytic potential in a large number of patients at time of diagnosis, during chemotherapy with either vincristine or thalidomide in combination with adriamycin and doxorubicin, and after autologous stem cell transplantation. No evidence for hypofibrinolysis in myeloma patients at the time of diagnosis was observed. A significant, although minor, increase in clot lysis time occurred during both types of chemotherapy, which may result in a higher risk of venous thrombosis.

Keywords
Fibrinolysis, multiple myeloma, venous thrombosis, thalidomide

Introduction

As in many other malignancies, Multiple Myeloma is associated with an increased risk for venous thrombosis. The risk for venous thromboembolism is even more elevated in patients receiving multi-agent chemotherapy and/or prednisone combined with anti-angiogenic drugs. The combination of thalidomide and doxorubicin was reported to be associated with the occurrence of venous thrombosis in 10-30% of the patients, whereas venous thrombosis occurs in 1-5% of the patients receiving more conventional medication.[1,2] Multiple hemostatic alterations promoting coagulation have been found in myeloma patients. These include high levels of factor VIII and von Willebrand factor, acquired APC resistance, and the formation of procoagulant autoantibodies.[3,4,5] Furthermore, it has been described that high levels of serum M-proteins and increased blood viscosity may interfere with fibrin polymerization, resulting in a fibrin clot which is more resistant to fibrinolysis.[6] Finally, high levels of plasminogen activator inhibitor type I (PAI-1) have been found in myeloma patients, which also results in inhibition of fibrinolysis.[7]

We recently reported that overall plasma hypofibrinolysis, as measured by a tissue factor and tissue plasminogen activator (tPA)-induced clot lysis assay, constitutes a risk factor for venous thrombosis in otherwise healthy individuals.[8] In this study, we measured plasma fibrinolytic potential in myeloma patients during the course of therapy including either Vincristine, Adriamycin, and Dexamethasone (VAD) or Thalidomide, Adriamycin and Dexamethasone (TAD) to investigate whether TAD treatment is associated with plasma hypofibrinolysis, and may therefore be an explanation for the increased incidence of venous thrombosis.

Introduction

We studied newly diagnosed multiple myeloma patients who were included in the HOVON 50 study, a prospective randomized phase III study on the effect of thalidomide combined with adriamycin, dexamethasone and high dose melphalan. Informed consent was obtained from all patients. According to the Declaration of Helsinki, the protocol was approved by the Research Ethics Board of each participating hospital. Inclusion criteria were Salmon & Durie stage II or III, age 18-65 years, WHO performance status 0-3 and a negative pregnancy test at inclusion.

The study protocol has been described previously.[9] In short, patients were randomly assigned to induction chemotherapy consisting of vincristine, adriamycin, and dexamethasone (VAD, arm A), or vincristine, adriamycin and thalidomide (TAD, arm B). After induction therapy, all patients received 1 or 2 courses of high dose melphalan (HDM) with autologous stem cell rescue. Patients randomized to arm A received maintenance therapy with interferon alpha and patients randomized to arm B received thalidomide without VTE prophylaxis. During induction therapy with TAD, low molecular-weight heparin was given to prevent venous thromboembolism.[9,10]

From a subset of patients plasma was available. Blood samples from 77 patients at time of diagnosis, during or directly after induction therapy with VAD (45 patients) or TAD (32 patients), and from 35 patients after SCT in the TAD arm and 47 after SCT in the VAD arm were studied. Clot lysis time was measured as described previously.[8] As it is known that clot lysis time strongly increases with age, we used data obtained from 133 healthy controls above 55 years of age which were published previously.[8] The average age of our patient group was 57, while the age of the control group averaged 62. Statistical analysis was performed using the GraphPad InStat (GraphPad, San Diego, CA) software package. As the difference in standard deviations between the groups was significant, we analyzed data using the non-parametric Kruskal Wallis one-way analysis of variance (ANOVA) test. P values <0.05 were considered statistically significant.

Results

Figure 1 shows clot lysis times of plasma samples obtained from myeloma patients at time of diagnosis, during VAD or TAD, and after SCT during maintenance therapy with thalidomide or interferon alpha, and from age-matched healthy controls. There was no significant difference in clot lysis time between the normal control group and the patients tested at time of diagnosis (controls median [range]: 63 [44-91] min vs patients 65 [38-108] min). However, there was a significant increase of clot lysis time during both VAD (median [range]: 68 [44-122] min) and TAD (median [range]: 70 [49-107] min) as compared to clot lysis times of the control subjects indicating the development of hypofibrinolysis during chemotherapy ($p<0.05$, Kruskal Wallis ANOVA with Dunn's post test). After SCT, the clot lysis times were again not different from the control group in both the VAD (median [range]: 67 [43-113] min) and TAD (median [range]: 65 [47-105] min) arm.

Discussion

In this study we found no evidence of hypofibrinolysis in patients with multiple myeloma at the time of diagnosis. A hypofibrinolytic state did develop during both TAD and VAD therapy, although the differences in clot lysis times were small, and after SCT the hypofibrinolytic state was again absent. The specific induction of hypofibrinolysis during chemotherapy might indicate that the increased thrombosis risk associated with chemotherapy might be explained in part by defective clot lysis. However, no increased hypofibrinolysis associated with thalidomide treatment was observed.

In this study we used a plasma-based clot lysis assay, which was previously demonstrated to be of clinical relevance, since hypofibrinolysis as detected with this assay was found to be a clear, and independent risk factor for the development of a first venous thrombosis in otherwise healthy individuals.[8] Patients receiving thalidomide were also receiving low molecular weight heparin as thromboprophylaxis, which could potentially have obscured clot lysis results.[11] However, at the time of sampling, none of the patients had detectable plasma levels of low molecular weight heparin (as measured by an anti Xa assay, data not shown).

In conclusion, in this study we found an induction of hypofibrinolysis in patients with multiple myeloma during VAD and TAD treatment. These results may explain the elevated thrombosis risk in patients receiving induction chemotherapy with VAD or TAD. However, hypofibrinolysis does not explain the extra increased risk of VTE during thalidomide treatment. Presumably other hemostatic alterations are responsible for the thrombotic risk in myeloma patients during thalidomide therapy.

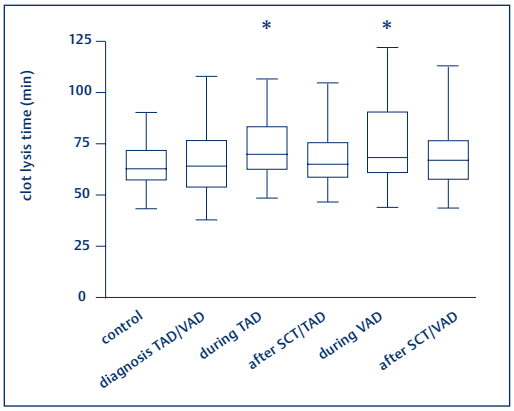

Figure 1

*Box and whisker plot showing clot lysis times of plasma samples obtained from healthy controls compared to myeloma patients at time of diagnosis, during VAD or TAD induction therapy, and after SCT while receiving thalidomide (TAD) or interferon-alpha (VAD) maintenance therapy. The data in box represents the interquartile range with the median shown as line. The total range of data is demonstrated by the high/low bars. Groups significantly different to the control group are marked with * where $P < 0.05$.*

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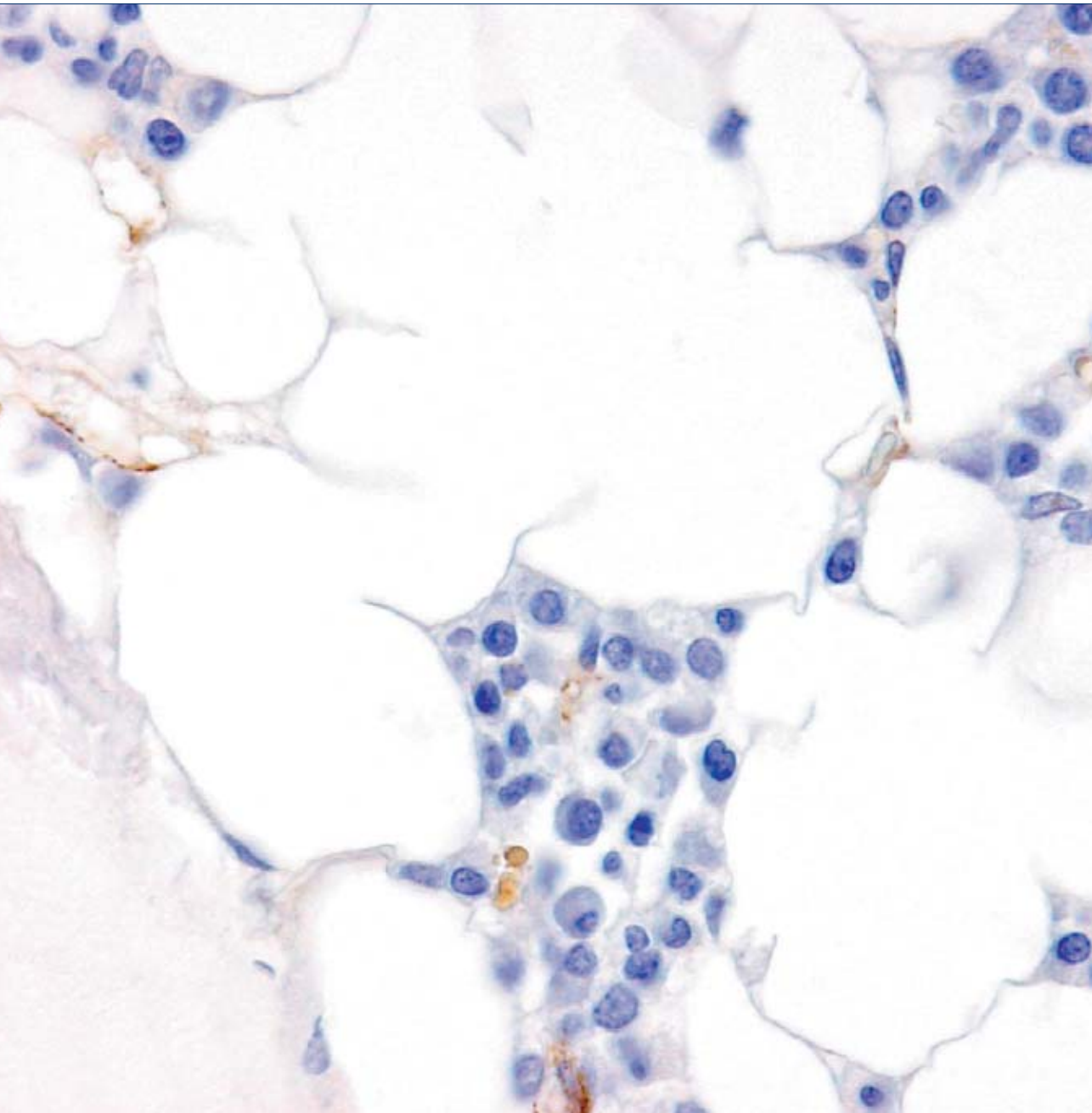

Evaluation of von Willebrand factor and Factor VIII levels in multiple myeloma patients treated with Thalidomide

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Submitted

Abstract

Venous thrombo-embolism (VTE) is a major complication of thalidomide therapy in multiple myeloma (MM) patients, especially when combined with anthracyclines and/or dexamethasone. Levels of factor VIII and von Willebrand factor (VWF) were evaluated during consecutive treatment phases of MM patients, randomized to receive TAD (thalidomide, adriamycin, dexamethason) or VAD (vincristine), followed by high dose therapy (Melphalan), and autologous stem cell transplantation. Levels of VWF and FVIII increased to a similar extent during both VAD and TAD treatment. This may explain the increased thrombotic risk during induction treatment, but does not explain the increased incidence of thromboembolic events during thalidomide treatment.

Introduction

Multiple Myeloma is associated with an increased risk for venous thromboembolism (VTE), which is even more elevated when multi-agent chemotherapy and/or prednisone are combined with anti-angiogenic drugs, such as thalidomide.[1-4] The combination of thalidomide and doxorubicin is associated with the occurrence of venous thrombosis in 10-30% of the patients, whereas venous thrombosis occurs in 1-5% of the patients receiving more conventional medication.[5-8] We recently described that low dose low molecular-weight heparin (nadroparine) effectively reduces the risk of VTE during thalidomide treatment.[9]

Multiple haemostatic alterations promoting coagulation have been found in myeloma patients, including elevated levels of factor VIII and VWF and a hypofibrinolytic state.[3,10,11] Altogether the combination of a hypercoagulable and hypofibrinolytic state causes a hemostatic dysbalance in MM patients treated with chemotherapy, thalidomide and stem cell transplantation (SCT).[12]

We evaluated VWF and FVIII levels longitudinally in patients included in the Hovon 50 study in which patients are randomized to receive thalidomide (TAD) or vincristine (VAD) during induction treatment followed by intensive treatment to investigate a possible relation between FVIII and VWF levels and the increased risk of VTE during thalidomide treatment.

Materials and methods

We studied newly diagnosed multiple myeloma patients included in a Dutch prospective randomized phase III HOVON 50 study on the effect of thalidomide combined with adriamycin, dexamethasone and high dose Melphalan (HDM). Informed consent was obtained from all patients. According to the Declaration of Helsinki, the protocol was approved by the Research Ethics Board of each participating hospital. Details of this study were published elsewhere.[9,13] From a subset of patients, included in two centres (Erasmus MC, Rotterdam and UMC Utrecht) plasma was available for this study. The number of patients studied at each time-point is indicated in figure 1.

Methods:
Blood samples were collected at time of diagnosis (time point 1), during or directly after therapy with VAD or TAD (time point 2), 3 months after high-dose Melphalan (HDM) treatment (time point 3) and after SCT (6 months after HDM, time point 4). Blood was centrifuged and plasma was stored at Levels of clotting factor VIII were measured with a one-stage clotting assay or a commercial coagulation method, Boehringer Mannheim, Mannheim, Germany and VWF antigen by using the LIA test (Boehringer Mannheim, Mannheim, Germany) or an in house developed sandwich ELISA. VWF collagen binding activity was measured using an in house EIA using type I collagen and vWF ristocetin cofactor activity was measured by an aggregometric method using formalin-fixed platelets and ristocetin.

Statistical analysis:
Patients in arm A and B were comparable with respect to stage of disease, age and response to treatment. Statistical analysis was performed using the linear mixed-effects model fit by REML.[14] This model also takes in account the course of the VWF and FVIII levels within the same patient. The nonparametric Friedman test was used to compare the differences between multiple time points per group and the Wilcoxon signed rank test to compare two mutual time points. P values <0.05 were considered statistically significant.

Results

We found substantially elevated levels of both VWF and factor VIII in MM patients at the time of diagnosis.[10] During treatment, both VWF ($p=0.03$) and factor VIII ($p=0.003$) levels show a significant parabolic course during the treatment phases with the maximal values at time point 2 in both the VAD and TAD group (indicated in box-plots in figures 1A and 1B). This indicates a significant increase in VWF and FVIII levels between time points 1 and 2 (before treatment and after VAD/TAD), and a subsequent significant decrease of VWF and FVIII levels after HDM treatment. No differences in VWF and FVIII levels or in the course of the levels during treatment between the VAD and TAD group

could be demonstrated. Most patients (85%) reach a partial remission , and there was no relation between the remission status and the levels of VWF or FVIII. We observed a strong correlation between VWF and FVIII levels ($P<0.0001$), and also VWF levels were strongly correlated with VWF collagen binding and ristocetin cofactor activity ($P<0.0001$, data not shown).

Discussion

In accordance with our previous study, VWF and FVIII levels were elevated in the majority of patients at the time of diagnosis.[10] Both in the VAD and TAD arm, VWF and FVIII levels increased during chemotherapy, and decreased again after HDM treatment. However, no differences in VWF and FVIII levels between VAD and TAD treatment were observed. VWF antigen levels correlated strongly with FVIII levels and VWF functional activity, indicating that the VWF in MM patients, although present at elevated levels, is functionally normal.

These results implicate that although increased VWF-Ag and FVIII levels may contribute to the increased risk of VTE in myeloma patients, these levels are unrelated to the increased thrombosis risk associated with TAD as compared to VAD. Similarly, we recently described an induction of hypofibrinolysis during treatment of MM, which may contribute to the increased thrombotic risk, but also in this study, no differences between VAD and TAD treated patients were found.[11] Alternative mechanisms explaining the additive thrombosis risk of TAD thus still remain to be identified.

The levels of VWF in MM patients could be elevated by increased production of VWF in bone marrow endothelial cells because of tumor angiogenesis. However, we did not find a correlation with tumor load, as VWF-Ag and FVIII levels were not significantly higher in untreated patients as compared to levels measured in patients in remission after high dose therapy.[13]

In conclusion, the results of this study indicate that although high levels of VWF and FVIII could contribute to the increased risk of a venous thrombo-embolism in MM, especially during the induction therapy, other mechanisms must be responsible for thalidomide specific venous thrombo-embolism.

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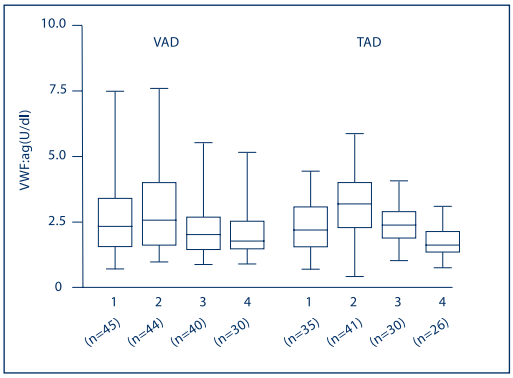

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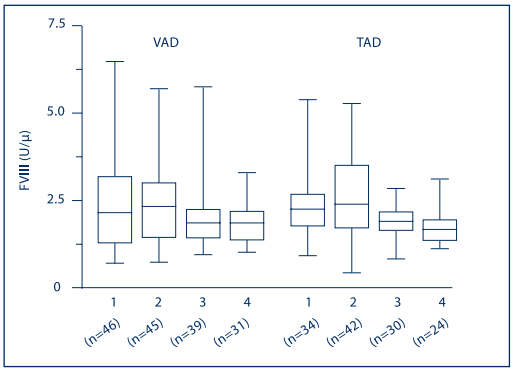

Figure 1A

Levels of VWF:Ag at the four different timepoints during the VAD or TAD treatment. Shown are box and whisker plots in which the data in box represents the interquartile range with the median shown as line. The total range of data is demonstrated by the high/low bars.
The number between brackets indicates the number of patients studied at each timepoint.

Figure 1B

The levels of FVIIIc at the four different time points are shown during the treatment. Shown are box and whisker plots in which the data in box represents the interquartile range with the median shown as line. The total range of data is demonstrated by the high/low bars. The number between brackets indicates the number of patients studied at each timepoint.

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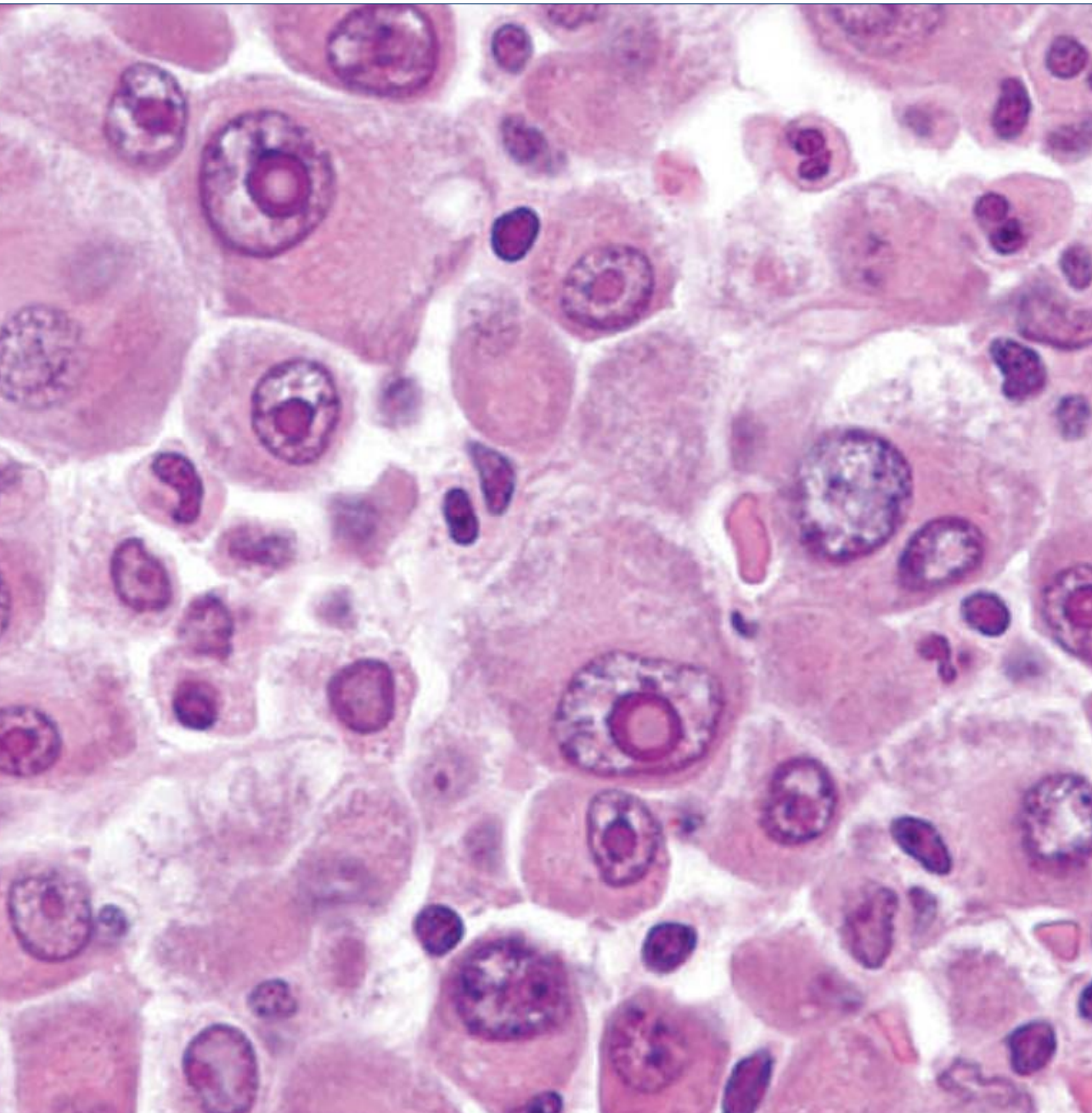
chapter 8



General Discussion

Diagnosis

Multiple myeloma (MM) is a malignancy consisting of a neoplastic proliferation of plasma cells in the bone marrow of generally middle aged and elderly persons. The formation of multiple tumorous masses in the bones, with subsequent activation of osteoclasts, results in lytic bone lesions, with a variety of clinical symptoms and ultimately a life-threatening bone marrow failure combined with a possible immunodeficiency status. These bone lesions are part of the minor criteria in the diagnosis of the disease. Among the major criteria are the presence of a significant amount of paraprotein in urine and serum and a bone marrow plasmocytosis of 30% or more. Since the presence of plasma cells in the bone marrow is of major importance for the diagnosis of the disease, a diagnostic bone marrow biopsy is performed in almost every patient. When diagnosing the bone marrow biopsy, not only the percentage of plasma cells is important, but also some other morphological features, even when the required plasma cell percentage is not achieved. Considering the different histological patterns of plasma cell infiltration, a nodular infiltration for instance is associated with more pronounced osteolytic lesions and progressive disease, whereas in patients with interstitial infiltration more generalized osteoporosis is seen (Chapter 2).
Also recognition of the abnormal plasma cells with nuclear pleomorphism and high nuclear-cytoplasmic ratios are important prognostic clues, even when only a small percentage of plasma cells is present in the bone marrow. However in these cases when the WHO diagnostic criteria of MM are not met, future bone marrow biopsies are required to confirm the diagnosis.[1]

Treatment

Chemotherapy with alkylating agents is the treatment of choise for symptomatic multiple myeloma in elderly patients or patients not able to receive high-dose therapy. The combination of melphalan and prednisone in intermittent courses has been the standard therapy for the last 30 years.[2] Before the introduction of melphalan around 1950, the mean survival of MM patients was only 7.1 months.[3] The response to the treatment of melphalan plus prednison might take 6 to 12 months. Although complete response occurs in less than 5% of the patients, the mean survival of patients treated with Melphalan and Prednison is approximately 3 years. On the other hand a quick response to treatment is not always a positive sign and might indicate a high proliferative activity of the myeloma cells that can result in an early relapse.[4]
In the early seventies the combination therapy was introduced in the treatment of multiple myeloma. Since many agents had shown individual potency to reduce myeloma cell growth, the combination of these chemotherapeutics was a logical option. Melphalan, corticosteroids, cyclophosphamide, antracyclines and interferon-α were explored in different combinations, with the aim to accomplish high remission rates. Responses were obtained in 70% of the myeloma patients, but the survival rates were not improved,

compared to patients treated with Melphalan and Prednison alone.[5] Nowadays the advantage of combination therapy over the therapy with Melphalan and Prednisone still has not been proven.[6] There is no survival difference between the two approaches.[7] In the 1980's the VAD (vincristine, adriamycin, dexamethasone) regimen was introduced. Although 10-15% of previously untreated patients now achieve complete remission and the response rate is around 60-80%, also this treatment option has no serious benefit on survival rates.[8,9] The same is true for the combination of INF-α with dexamethasone and the addition of INF-α to the VAD regimen.[10,11] In recent years thalidomide was found to be a new promising drug, with a total response rate of around 32%, also being active in previously treated MM patients.[12,13]
In conclusion we can state that although the response rates to the different kinds of therapies are excellent, non of them is curative and in the large majority of the multiple myeloma patients the disease finally relapses.

Stem cell transplantation

Autologous stem cell transplantation (SCT) has become the standard treatment for the younger (under 65 years) myeloma patient. The overall survival rates of MM patients treated with induction chemotherapy followed by autologous bone marrow transplantation (BMT) are superior over prolongation of the treatment with chemotherapy alone.[14] The addition of Melphalan to the VAD regimen made the latter more successful.[15]
For stem cell transplantation we have the choice between bone marrow stem cells and peripheral blood stem cells. Peripheral blood-derived stem cells result in faster recovery of the peripheral blood values than do marrow derived stem cells, whereas overall- and event-free survival are identical.[16,17] Moreover the amount of tumour cells harvested from the bone marrow may be higher than from the blood cell harvest.[18,19] The stem cells are collected after G-CSF stimulation using the CD34 marker for cell separation. The speed of haematological recovery in the bone marrow is correlated with the amount of stem cells that is infused.[20,21] The CD34 pluripotent progenitor cells are thought to represent the common stem cells, able to engraft the BM, developing into haematopoietic cells and endothelial cells, whereas the fibrous matrix seems to be non-transplantable, i.e. host derived.[22] Some authors state that also in the peripheral blood stem cell transplants the stem cell harvest might contain contamination of residual tumour cells.[19,23] The presence of monoclonal plasma cells in the blood stem cell harvest is associated with shorter relapse-free survival after transplantation.
Autologous transplants are associated with a high engraftment rate, a low mortality rate and a quick return to normal activity levels. They therefore provide an alternative for conventional treatment. Despite the prolongation of survival and a good quality of life, there is no plateau in the survival curves, and autologous SCT is therefore probably not curative. Taking also in account the risk of contamination by residual tumour cells and the beneficial effect of the graft versus tumour reaction in the allogeneic situation, allogeneic sources for stem cell harvesting are an alternative.[24-28] Allogeneic SCT however

is associated with a higher transplantation related mortality, such as graft versus host disease, opportunistic infections and secondary malignancies.[28] The place of allogeneic SCT in myeloma is still not established.
In chapter four our experience with autologous and allogeneic transplanted multiple myeloma patients are overviewed. We found that patients with clearance of monotypic plasma cells following allogeneic and autologous SCT enjoyed a longer progression free survival, although overall survival was not prolonged when compared to patients with residual monotypic plasma cells. The question arises whether immune typing of bone marrow biopsies is indicated in the follow- up of patients following transplantation. Probably more sensitive molecular techniques using patient specific primers are needed to identify patients with "true" complete remissions especially following allogeneic transplants.

The bone marrow after SCT

After transplantation the response status of the patient and the repopulation of the bone marrow should be checked carefully. Bone marrow biopsies and bone marrow aspirates show us the features of the regenating marrow, complicating diseases and presence or absence of the malignant plasma cells. In patients with normal recovery after SCT the peripheral blood shows normal returning values of the different cell lines. One of the problems of an in depth study of the BM after SCT is the lack of BM biopsies taken in patients with an undisturbed bone marrow recovery. Only when the clinical features require so and peripheral blood reconstitution fails, a BM biopsy is performed. In chapter 3 we present an overview of the normal recovery of the bone marrow and its complications after stem cell transplantation. After an initial recovery of the erythropoieis, the granulopoieis and the megakaryopoiesis follow shortly later. After three months the recovery of the marrow should be completed.
Bone marrow biopsies are important tools to obtain information about the status of haematopoiesis post SCT. In the case of multiple myeloma the examinations are of course also focused on the detection of residual or recurrent malignant disease.

Angiogenesis

In the bone marrow of multiple myeloma patients it is not only important to focus on the cellular components, but also on the microenvironment. The micro-vessel density is important in relation to angiogenesis and is associated with the increase of the tumor load, which in turn is important for disease progression. Therefore angiogenesis is suggested to be more prominent in multiple myeloma than in monoclonal gammopathy of undetermined significance (MGUS) or indolent myeloma.[40-42] Thalidomide is a well known anti-angiogenic drug and is successfully used in the treatment of multiple myeloma patients. It also has procoagulative effects resulting in thrombosis and pulmonary embolism.[43-47]

In chapter 5 we studied prospectively the amount of vessel cross-sections in bone marrow biopsies before and after VAD treatment and compared the results with thalidomide (TAD) treatment and after the stem cell transplantation.
Only a small number of patients could be included in this study, since the clinicians can evaluate the response to therapy by less invasive methods as by measuring urine and serum M-proteins. Bone marrow biopsies are usually not done for the purpose of research only.
Nevertheless our data clearly demonstrate an increase in the amount of blood vessels during the VAD chemotherapy, decreasing again after the SCT. We also demonstrated a relative decrease of vessel density in the TAD treated patients compared to the VAD treated patients after induction therapy. This supports the hypothesis that the anti myeloma effect of thalidomide is, at least in part, based on an anti-angiogenic effect. On the other hand we did not observe a significant change in the amount of vessels during thalidomide treatment, contradicting this hypothesis. These observations can also indicate that bone marrow blood vessel formation is stimulated in this phase of the disease. In this scenario Thalidomide thus prevents the stimulation of new vessel development. The reduction in the amount of vessels during thalidomide treatment is also confirmed by other authors.[48] However, the increase in MVD after VAD was not observed in most other studies. The discrepancy may be related to the method by which the MVD was determined, since we corrected the number of vessels for the cellularity of the marrow, while others did not.
Recently it was shown that thalidomide down regulates the expression of pro-angiogenic genes in endothelial cells isolated from the bone marrow of MM patients, which again supports the hypothesis that thalidomide inhibits bone marrow angiogenesis.[49] In our study there was a significant increase in the number of vessels in the VAD arm identified by CD34 staining, however this findings could not be confirmed in a study using an anti-VWF antibody. Although both anti-CD34 and anti-VWF are used in daily practice for the identification of endothelial cells, the background staining differs considerably, probably because of the presence of serum, which contains remnants of platelets, megakaryocytes and free VWf. In the anti-VWF staining the identification of small individual microscopic vessels is more difficult than in the CD34 staining .
The agreement between the two staining methods regarding the presence of vessels is not as poor as the present results suggest, as is shown by the correlation between the amount of vessels counted with CD34 staining and the amount of vessels observed with VWF staining. Based on this correlation it is likely that both staining methods would have revealed an increased number of vessels after VAD treatment in a larger population.

Coagulation

Multiple myeloma is associated with an increased risk for thrombosis. This includes deep venous thrombosis in the leg and subsequent lung emboli. The combination of multi-chemotherapy with adriamycin, thalidomide and prednisone was reported to be

associated with the occurrence of venous thromboembolism in 10-30% of the patients, being much higher than in MM patients receiving the more conventional chemotherapy (1-5%).[50-53] Although multiple haemostatic alterations leading to increased fibrin clot formation are known to occur in MM patients, the extremely high levels of von Willebrand factor (VWF) found in these patients are presumed to be of particular clinical importance. Except for VWF, also FVIII is elevated in these patients.[43] The elevation of these factors presumably leads to a hypercoagulable state which could very well be of clinical importance for these patients. To study the reason for this elevation of FVIII and VWF and the relationship with thalidomide treatment, we desbribe in chapter 7 a large group of MM patients, randomly selected for receiving a thalidomide combination (TAD) or the more conventional chemotherapy (VAD) before receiving a SCT. During the treatment blood samples were taken and the plasma the levels of VWF and FVIII were measured. The results implicate that, although increased VWF-Ag and FVIII levels may contribute to the increased risk of thrombosis in myeloma patients, these levels are unrelated to the increased thrombosis risk associated with TAD as compared to VAD treatment. The levels of VWF in MM patients could be elevated by increased production of VWF in bone marrow endothelial cells because of tumor angiogenesis. However, we did not find a correlation with tumor load, as VWF-Ag and FVIII levels were not significantly higher in untreated patients as compared to levels measured in patients in remission after high dose therapy.[54] Although high levels of VWF and FVIII could contribute to the increased risk of a venous thrombo-embolism in MM, especially during the induction therapy, other mechanisms must be responsible for thalidomide specific venous thrombo-embolism.

We speculate that besides a hypercoagulable state also a hypofibrinolytic state (or a combination of the two) could contribute to the hemostatic dysbalance in MM patients treated with thalidomide. In chapter 6 we described an induction of hypofibrinolysis during treatment of MM, which may contribute to the increased thrombotic risk, but also in this study, no differences between VAD and TAD treated patients were found.[55] We found no evidence of hypofibrinolysis in patients with multiple myeloma at the time of diagnosis. Although a hypofibrinolytic state did develop during either TAD or VAD therapy, the differences in clot lysis times were small, and after SCT the hypofibrinolytic state was again absent. This might indicate that an increased thrombosis risk associated with chemotherapy, is explained in part by defective clot lysis. However, no increased hypofibrinolysis associated with thalidomide treatment was observed. Therefore the reason for additional thrombosis risk of thalidomide thus still remains unclear and other hemostatic alterations must be responsible for this phenomenon in myeloma patients during thalidomide therapy.

Prevention of venous thrombo-embolism

The increased venous thromboembolic (VTE) risk during thalidomide therapy in combination with dexamethason, can be prevented by low dose heparin.[56] Patients

receiving thalidomide, nowadays also receive low molecular weight heparin as thromboprophylaxis, which could potentially have obscured clot lysis results, and may also have affected VWF and FVIII levels by unknown mechanisms. There is no discussion about the effect of low molecular-weight heparine (Nadroparine) during thalidomide therapy to prevent a deep venous thrombosis in multiple myeloma (MM) patients.[56] MM patients treated with thalidomide should always receive low molecular-weight heparine during the treatment. More debateble is the question whether the procoagulant factors in the plasma of MM patients should be monitored during the chemotherapy. As described before, the only pro-coagulation factors that seemed to be important are von Willebrand factor (VWF) and Factor VIII (FVIII).[43] In chapter 7 we could confirm that the VWF and FVIII levels are elevated in a majority of the patients. However, this elevation is not related to thalidomide, and the increased thrombosis risk during thalidomide treatment is not explained by the elevated levels of VWF or FVIII. The monitoring of these coagulation factors during the thalidomide treatment therefore does not seem to necessary. The same can be stated when the hypofibrinolytic activity is measured in MM patients during thalidomide treatment (chapter 6). Although hypofibrinolysis is induced in MM patients during the VAD and TAD treatment, it does not explain the extra increased risk of VTE during thalidomide treatment. Moreover, since LMWH abrogates the Thalidomide-induced thrombosis risk, the question whether it would be useful to monitor hypercoagulability or hypofibrinolysis during therapy to identify patients at risk is no longer relevant.

A positive argument for the importance of hypercoagulability in the occurrence of VTE is the positive effect of low molecular-weight heparin (LMWH) on the thrombosis risk.[56] It was already known that the treatment with LMWH is as effective as oral anticoagulants in the prevention of VTE in cancer patients, without elevating bleeding rates.[57] It is also known that heparin decreases the VWF release from cultured endothelial cells. This effect is not described when using LMWH.[58,59] The outcome of our study in chapter 7, showing no differences in the level and time effect of VWF and FVIII while using LMWH, can therefore be accepted as reliable. The occurrence of VTE in the patient population using TAD or VAD was comparable, meaning that LMWH reduces in the patient the extra risk factor when using thalidomide, but does not prevent the occurrence of the VTE in myeloma patients in general. Still a reliable number of patients are faced with a life threatening VTE and it is important to eliminate this risk factor.

There are indications that the pathways of blood coagulation and angiogenesis are linked. LMWH is not only an anti-coagulant, but is also an anti angiogenic medication. LMWH improves survival times in cancer patients receiving chemotherapy.[60] Many angiogenic factors are heparin binding proteins that in their functions may be affected by treatment with LMWH. These include fibrin formation, binding to fibroblast growth factor (FGF2) and to vascular endothelial growth factor.[61] LMWH inhibits angiogenesis and this effect seems independent of the anti-coagulation actions.[62]

Although the elevated factors VIII and VWF do not seem to be as important for the increased VTE as we first thought, the mechanism behind this elevation remains very interesting. Where does it come from? The VWF could be produced directly or indirectly

by the tumor itself. However, in chapter 5 we studied the bone marrow biopsies of MM patients and there seems to be no relation between tumor load and the amount of VWF. So it is not likely that VWF is produced by the myeloma cells. Consequently there is likely to be another mechanism.

The combination of pro-coagulation and hypofibrinolysis could be an explanation for the increased risk of VTE in patients treated with thalidomide, but it is very likely that other factors that we have not studied are more important. It is possible we have not examined the right coagulation factors, but it is also likely that the changes that are induced by the tumor cells itself, not related to the coagulation system (changes in the vessel wall or the endothelial cells), play a role in this process.

Thalidomide is also used in solid tumors, with varying and generally disappointing results, also resulting in an increased amount of VTE in these patients.[63,64] This emphasizes the fact that it is thalidomide itself that is, at least in part, responsible for the thrombosis risk.

Concluding, when treating patients with thalidomide, we should be alert for the occurrence of a venous thrombotic event that can be a life threatening disease and should be treated.

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Summary

Introduction

Multiple myeloma (MM) is an haematological malignancy caused by an unrestrained proliferation of plasma cells. These plasma cells are monoclonally differentiated B-cells, and part of the white blood cell count. This proliferation of cells infiltrates the bone, forming multiple tumorous masses scattered throughout the blood forming skeletal bone marrow. These plasma cells produce osteoclastic factors, causing local bone destruction and bone resorption, causing bone pain as one of the major symptoms. This bone resorption and bone destruction can cause pathological bone fractures and the amount of bone destruction is associated with tumor infiltration and correlates with tumor load. Other symptoms are fatigue, wasting, renal and neurological abnormalities. The disease is generally found in middle-aged and elderly patients, only rarely found in patients under 40 years of age.

The bone marrow biopsy

The clonally transformed plasma cells all produce the same homogeneous immunoglobulin, the so called paraprotein or M-component. This paraprotein can be detected in urine and blood serum, can cause renal failure and is indicative for a malignant condition. These malignant cells are located in the blood forming bone marrow and can be detected by a bone marrow biopsy. Anemia or hypogammaglobulinaemia (immunodeficiency) can be caused by bone marrow failure due to massive infiltration of the blood forming bone marrow.

The diagnosis of multiple myeloma is based on a combination of radiological, laboratory and pathological findings. For the diagnosis of MM more than 30% bone marrow infiltration with plasma cells should be present according to the WHO classification of multiple myeloma and the percentage of bone marrow infiltrating plasma cells is one of the most important diagnostic features. The bone marrow can be investigated with a bone marrow aspiration and a bone marrow biopsy and both the pathologist and the hematologist are important for the judgment of the bone marrow features in multiple myeloma. The types of plasma cell infiltration, the immunophenotype, the accumulating chromosomal abnormalities and the microenvironment are important for tumor survival and differentiation (chapter 2). This microenvironment includes stromal cells, endothelial cells and extra cellular matrix, interacting with adhesion of the plasma cell component.

Chemotherapy and stem cell transplantation

After making the diagnosis a therapy should be started. The combination of melphalan and prednisone has been standard therapy for many years. Although not proven, the only possible curative option for multiple myeloma patients is chemotherapy followed by a stem cell transplantation (SCT). A SCT can be autologous (stem cells from the patient itself) or allogeneic (stem cells from a HLA-identical family member or a matched unrelated donor). The chemotherapy combination of vincristine, doxorubicin and dexamethason (VAD) is used in many cases. Right now autologous peripheral blood stem cell transplantation - supported high-dose melphalan is considered standard therapy for multiple myeloma.

The treatment with chemotherapy is applied to eliminate the malignant plasma cells. Except for the plasma cells it also eradicates the patients own blood forming haematopoiesis, resulting in bone marrow failure with slow regeneration. The regeneration of the haematopoietic bone marrow depends on the type of conditioning pre SCT (non-myeloablative or myeloablative), but also depends on the method of transplantation (allogeneic or autologous). It is important to be familiar with the normal regeneration of the blood forming bone marrow, but complications can occur during regeneration with characteristic bone marrow morphology and significant dyshaematopoiesis (chapter 3). During the pretransplant period and depending on the kind of pre-treatment there may be hypoplasia, residual disease and fibrosis. In the post transplanted period, after a period of transfusion-dependent pancytopenia, first signs of a successful engraftment are indicated by the recurrence of neutrophils, monocytes and erythrocytes in the peripheral blood along with the regeneration of erythropoiesis, followed by the other lineages of haematopoiesis and fibrosis. Conspicuous dyshaematopoiesis after transplantation should not be mistaken as a myelodysplastic syndrome and the presence of granulomas is more likely to be treatment-related than a manifestation of intercurrent granulomatous disease.
Also the presence of residual tumor cells in the bone marrow after the autologous or allogeneic stem cell transplantation is important, as their presence can indicate recurrent or refractory disease (chapter 4). On the other hand the presence of a low percentage of tumor cells after the transplantation can be cleared due to graft versus tumor response and no therapy is needed.

Thalidomide therapy

Except for VAD therapy and melphalan, thalidomide is more and more used in multiple myeloma patients. Although presented as a new active immunomodulatory and anti-angiogenic drugs, thalidomide already has a long and impressive history. In 1954 Thalidomide was first synthesized as a sedative and tranquillizer, at that time called Softenon. In pregnant women it worked very well to treat morning sickness and help them sleep. It was welcomed by prescribers, because of less acute toxicity then the alternatives. It took more than four years to find out that the drug interfered with the development of the fetus and an epidemic of children followed, born with severe malformations of the limps, ears and internal organs. Around the world 12000 children were born with thalidomide malformations, of which less than half of them are still alive. Thalidomide was at that time also tested on different kinds of cancer, but because it was consigned as one of the largest medical tragedies in history, it was not used anymore for many years. After more than 25 years thalidomide made a comeback and the drug is tested on many malignant tumors. The anti-tumor effect in multiple myeloma however is one of the most effective ones sofar. In the bone marrow the anti-angiogenic effect of thalidomide could be shown in bone marrow biopsies, taken at different time points during the treatment with VAD or TAD. Although only a very small amount of patients, the anti-angiogenic effect of thalidomide on the microenvironment like the amount of vessels and the endothelial cells could be visualized in these bone marrow findings (chapter 5).

Thalidomide side effects

As expected thalidomide treatment also has side effects, including sedation, constipation, dry skin, itching and others. Most important the combination of regular multi-chemotherapy with adriamycin, thalidomide and prednisone in multiple myeloma patients was reported to be associated with increased life threatening venous thromboembolism (deep venous thrombosis and lung embolism), being much higher than in patients receiving the more conventional chemotherapy. Although multiple pro-coagulating haemostatic alterations are known in MM patients, high levels of FVIII and von Willebrand factor (VWF) and a possible disturbance in fibrinolysis were likely to be of clinical importance. Hypofibrinolysis was known to contribute as a risk factor for venous thrombosis in otherwise healthy individuals and could be important in thalidomide treatment. The plasma hypofibrinolytic activity potential was measured in a large number of multiple myeloma patients at time of diagnosis, during chemotherapy with either vincristine or thalidomide in combination with adriamycin and doxorubicin, and after autologous stem cell transplantation, as the fibrinolysis of an already formed thrombus could be reduced. No evidence for hypofibrinolysis in myeloma patients at the time of diagnosis was observed (chapter 6). Although a significant increase in clot lysis time occurred during both types of chemotherapy, which may result in a higher risk of venous thrombosis, there was no extra increase due to thalidomide treatment compared to the more conventional VAD treatment. But besides the hypofibrinolysis also high levels of coagulation FVIII and von Willebrand factor were shown to be important, especially when thalidomide was combined with anthracyclines and/or dexamethasone. The levels of factor VIII and von Willebrand factor (VWF) were evaluated in the blood of a large series of patients, during consecutive treatment phases of MM patients, randomized to receive TAD (thalidomide, adriamycin, dexamethason) or VAD (vincristine), followed by high dose therapy (melphalan), and autologous stem cell transplantation. Levels of VWF and FVIII increased to a similar extent during both VAD and TAD treatment (chapter 7). This may explain the increased thrombotic risk during induction treatment, but also this does not explain the increased incidence of thromboembolic events during the thalidomide treatment. Although the cause of this elevated thromboembolic risk is not known, we know now that the extra thrombotic risk in practice can be prevented by using low dosed heparin during thalidomide treatment.

Concluding we can say that thalidomide in combination with dexamethason followed by stem cell transplantation is an effective therapy for MM patients. The elevated thrombotic risk due to this treatment can cause life-threatening situations. Although we found that hypofibrinolysis, coagulation factor VIII and VWF are elevated in multiple myeloma patients, these factors do not explain the extra increased risk for this side effects during the thalidomide treatment, compared to the more conventional chemotherapy. Other explanations must be responsible for the increased risk of thrombo-embolic events in multiple myeloma patient treated with thalidomide therapy and are waiting to be investigated.

Samenvatting

Inleiding

Multipel myeloom, in Nederland ook wel de 'ziekte van Kahler' genoemd, is een kwaadaardige hematologische tumor, veroorzaakt door een ongeremde groei en deling van plasmacellen. Plasmacellen ontstaan uit B-lymfocyten, deze zijn een onderdeel van onze witte bloedcellen. De plasmacellen produceren de verschillende soorten antilichamen ten behoeve van onze afweer. Iedere plasmacel kan slechts een type antilichaam aanmaken. Inmiddels zijn meerdere chromosomale afwijkingen en delingsfouten beschreven die ten grondslag kunnen liggen aan een ongeremde groei van één type plasmacel, waardoor een plasmacel kloon ontstaat. Zo'n klonale proliferatie van eenzelfde type plasmacellen produceert een abnormaal grote hoeveelheid van hetzelfde antilichaam, ook wel homogeen immuunglobuline, paraproteïne of M (Myeloom)-component genaamd. Paraproteines zijn niet specifiek voor het multipel myeloom, ze kunnen ook gemaakt worden door andere tumoren die uit B-lymfocyten ontstaan. Hun aanwezigheid is als regel een teken van een abnormale toename van plasmacellen of de voorlopers daarvan. Dit paraproteïne kan bij patiënten worden teruggevonden in het bloed en in de urine. Het kan zich ophopen in de nier en zo nierfunctiestoornissen veroorzaken.
Plasmacellen voelen zich thuis (en nestelen graag) in het bloedvormende beenmerg dat zich bevindt in de lange pijpbeenderen van armen en benen, evenals in de platte botten als bijvoorbeeld de schedel, heupbeenderen en het borstbeen. Een clonale proliferatie van plasmacellen in het kader van een multiple myeloom zal zich dan ook in die gebieden bevinden en deze tumor is dan ook vaak uitgebreid in de botten van het lichaam aanwezig, in de vorm van vele tumoreuze haarden. Deze plasmacellen produceren ook osteoclastische factoren, dat wil zeggen dat ze stoffen produceren die het bot ter plaatse afbreken. Dat is ook de reden waarom patiënten met multiple myeloom zich nogal eens presenteren met botpijn. Deze botafbraak kan zo uitgebreid zijn dat ze radiologische afwijkingen geeft en zelfs botbreuken veroorzaakt. Dit laatste is sterk geassocieerd met tumor infiltratie en correleert met de hoeveelheid kwaadaardige plasmacellen ter plaatse. De ziekte kan zo uitgebreid aanwezig zijn in het bloedvormende beenmerg, dat het verdringing geeft van de normale bloedvorming, met als gevolg dat er te weinig bloedcellen worden aangemaakt (beenmergfalen) en er anemie (bloedarmoede) of immuunstoornissen kunnen ontstaan. Andere symptomen waarmee patiënten zich kunnen presenteren zijn onder andere moeheid, gewichtsverlies en neurologische afwijkingen. De ziekte komt met name voor bij ouderen, zelden bij mensen onder de 40 jaar.

Het beenmerg biopt

Omdat de ziekte zich bevindt in het bloedvormende beenmerg, kan het worden opgespoord middels een beenmerg biopt (weefselstukje) of een beenmerg aspiratie (waarbij losse cellen worden opgezogen). In Nederland wordt het beenmerg biopt meestal beoordeeld door een patholoog en de beenmerg aspiratie meestal door een hematoloog. Een samenwerking tussen beide specialismen is dan ook noodzakelijk voor het stellen van de diagnose. De diagnose multiple myeloom is gebaseerd op een combinatie van radiologische, chemische en microscopische bevindingen. Volgens de WHO (World Health Organisation) classificatie is meer dan 30% tumor infiltratie in het beenmerg nodig voor het stellen van de diagnose, dit percentage is een van de belangrijkste diagnostische

criteria. Daarnaast is ook de vorm van de plasmacellen belangrijk voor het type multiple myeloom. In hoofdstuk 2 van dit proefschrift wordt een overzicht gegeven van de meest voorkomende typen plasmacellen bij het multipel myeloom, evenals van de verschillende typen infiltratie, het antilichaam profiel (immuunfenotype), de opeenstapeling van verschillende chromosoom afwijkingen en het belang van het omgevende bindweefsel en de bloedvaten. Deze factoren zijn belangrijk voor de nesteling en het vasthechten van de plasmacellen en dus ook belangrijk voor tumor groei en de prognose van de patiënt.

Chemotherapie en stamcel transplantatie

Na het stellen van de diagnose is het belangrijk om snel een juiste therapie te starten. Sinds vele jaren wordt chemotherapie met melphalan en prednison als standaard therapie gebruikt. Ook de combinatie chemotherapie met vincristine, adriamycine en dexamethason (VAD) wordt vaak gebruikt. Hoewel de ziekte met deze vorm van therapie langdurig onder controle kan worden gehouden, betreft het in principe toch een ongeneeslijke ziekte. De enige mogelijke genezing zou een behandeling met chemotherapie kunnen zijn, gevolgd door een stamcel transplantatie. Deze stamcellen kunnen worden verkregen uit het bloed van de patiënt zelf (autologe stamceltransplantatie) of van een familielid of een onverwante donor (allogene stamceltransplantatie). Op dit moment is de combinatie met autologe perifere stamcel transplantatie in combinatie met hoge dosis melphalan een standaard therapie voor het multipel myeloom. De behandeling met chemotherapie heeft als doel het volledig elimineren van de kwaadaardige plasmacellen. Echter als gevolg van deze therapie worden niet alleen de plasmacellen uitgeschakeld, maar ook het eigen bloedvormende beenmerg van de patiënt. Dit resulteert in beenmergfalen, met lage hoeveelheden cellen in het bloed, welke een langzaam herstel vertonen in de weken na het teruggeven van de autologe of de allogene stamcellen. De snelheid van het herstel is afhankelijk van het type chemotherapie en het type transplantatie. Het is belangrijk om als patholoog bekend te zijn met de fasen van het normale herstel van het beenmerg na de stamcel transplantatie. Echter gedurende dit herstel kunnen ook complicaties optreden, met karakteristieke beenmerg veranderingen. Zowel de normale regeneratie van het beenmerg, als ook deze complicaties, worden besproken in hoofdstuk 3: In de eerste periode na de stam cel transplantatie zijn patiënten afhankelijk van bloedtransfusies omdat er nog geen bloedcellen worden aangemaakt. Vervolgens begint als eerste de aanmaak van rode bloedcellen, gevolgd door herstel van de witte celreeks en de bloedplaatjes.

Behalve dat er complicaties in dit herstel mechanisme kunnen optreden, is ook de aanwezigheid van kleine of grote aantallen tumorcellen na de transplantatie belangrijk. In hoofdstuk 4 van dit proefschrift wordt de betekenis van deze cellen na chemotherapie en stamcel transplantatie behandeld voor de prognose en overleving van de patiënt. De aanwezigheid van plasmacellen kan natuurlijk duiden op het niet volledig verdwenen zijn van de tumor of duiden op een snelle terugkeer van de ziekte, waarbij de prognose ongunstig is. Echter het is ook goed mogelijk dat deze resterende tumor cellen in de eerste maanden na de transplantatie nog zullen verdwijnen als gevolg van een graft versus tumor effect. Dat wil zeggen dat de donorcellen van de transplantatie (oorspronkelijk afkomstig uit een ander lichaam) de tumorcellen zullen doden, omdat ze deze cellen herkennen als ‘lichaamsvreemd’. In dat geval is geen snelle therapie vereist en de prognose gunstig.

Thalidomide therapie

Behalve de therapie met VAD en melphalan, is nu thalidomide een regelmatig gebruikt middel in de behandeling van het multipel myeloom. Hoewel thalidomide werd gepresenteerd als een nieuw middel, dat zowel het immuunsysteem kan beïnvloeden als ook nieuwvorming van bloedvaten kan remmen, heeft het al een lange en indrukwekkende geschiedenis. In 1954 werd thalidomide voor het eerst gesynthetiseerd als een slaapmiddel, maar het werkte bij zwangere vrouwen ook uitstekend tegen ochtendmisselijkheid. In die tijd droeg het middel de naam Softenon. De doktoren schreven het middel graag voor, omdat het minder acute bijwerkingen had dan de alternatieven in die tijd en het middel in proefdieren geen bijwerkingen bleek te hebben. Het duurde meer dan 4 jaar voordat duidelijk was dat het gebruik van softenon tijdens de zwangerschap bij mensen verantwoordelijk was voor de epidemie van aangeboren afwijkingen bij pasgeboren kinderen. De afwijkingen betroffen met name ernstige misvormingen van de ledematen, oren en interne organen. In totaal zijn ongeveer 12000 kinderen geboren met deze thalidomide malformaties. Minder dan de helft van hen is nog in leven. Ook in die tijd was thalidomide al getest op verschillende vormen van kanker, waarbij het effect in het multiple myeloom een van de meest succesvolle resultaten boekte. Omdat het middel echter beschouwd werd als een van de grootste medische tragedies uit de geschiedenis, is het middel volledig in diskrediet geraakt en vele jaren niet meer gebruikt.
Totdat de vrouw van een uitbehandelde multipel myeloom patiënt aandrong op een proefbehandeling met thalidomide omdat zij over de remmende werking op bloedvaten bij deze ziekte had gelezen. Het succesvolle resultaat van deze proefbehandeling resulteerde in een grotere studie, die in 1999 werd gepubliceerd. Als gevolg hiervan maakte het middel na meer dan 25 jaar een ware comeback als een anti-tumor middel. In het beenmerg kan het groeiremmende effect op de bloedvaten worden aangetoond door de hoeveelheid vaten in het beenmerg te bestuderen. In hoofdstuk 5 van dit proefschrift wordt gekeken naar het aantal vaten ten tijde van de diagnose van het multipel myeloom en vervolgens vergeleken met de vaatdichtheid na chemotherapie en na stam cel transplantatie. Hierbij wordt een kleine groep patiënten vergeleken die behandeld werd met VAD chemotherapie, tegenover de patiënten behandeld met TAD (waarin de vincristine werd vervangen door thalidomide) voor de autologe stam cel transplantatie. Hierbij werden de beenmergbiopten beoordeeld ten tijde van de diagnose, vergeleken met de biopten van na de chemotherapie en na de stamcel transplantatie. Hoewel het slechts een kleine groep patiënten betrof, was er een duidelijk minder aantal vaten tijdens de thalidomide (TAD) therapie, passend bij de vaatgroei remmende werking, ten opzichte van een duidelijke stijging van het aantal vaten tijdens de VAD therapie. Na de stam cel transplantatie wordt dit verschil echter weer opgeheven. De remmende werking lijkt in dit geval dus slechts tijdelijk te zijn.

Bijwerkingen van thalidomide

Zoals te verwachten heeft ook de thalidomide behandeling bijwerkingen. De meest voorkomende bijwerkingen betreffen slaperigheid, constipatie, een droge huid en jeuk. Thalidomide wordt echter veelal gebruikt in combinatie met andere middelen in een multi-chemotherapie behandeling met adriamycine en prednison waarbij door het optreden

van trombose, verhoogde stollingneiging en een longembolie een levensbedreigende situatie kan ontstaan. Deze ernstige bijwerking komt veel meer voor bij patiënten met thalidomide combinatie behandeling, dan bij patiënten met een meer conventionele combinatie therapie.
Inmiddels zijn al vele stollings bevorderende mechanismen bekend in multipel myeloom patiënten. De hoge bloedwaarden van stollingsfactor VIII en Von Willebrand factor (VWF) en een verstoring in de afbraak van een reeds gevormd stolsel (fibrinolyse) zouden ons inziens een verklaring kunnen zijn voor dit duidelijke toegenomen risico op trombotische processen.
Het is reeds bekend dat verminderde fibrinolyse een risicofactor vormt voor trombose in anderszins gezonde mensen. Het zou dus goed mogelijk kunnen zijn dat deze verminderde fibrinolyse een verklaring vormt voor het toegenomen stollings risico tijdens thalidomide behandeling. De mate van verminderde afbraak van een stolsel in het bloedplasma werd gemeten bij een groot aantal patiënten ten tijde van de diagnose, tijdens de chemotherapie en na de autologe stam cel transplantatie en beschreven in hoofdstuk 6 van dit proefschrift. Hoewel er inderdaad een verminderde afbraak was van een stolsel tijdens de chemotherapie in beide groepen, was er geen verschil tussen de patiënten met VAD therapie en TAD therapie. Dit mechanisme lijkt dus geen verklaring voor het extra risico dat multipel myeloom patiënten lopen tijdens thalidomide behandeling vergeleken met de patiënten behandeld met VAD.
Behalve de verminderde fibrinolyse in multipel myeloom patiënten, was ook bekend dat stollingsfactoren VIII en VWF bij hen verhoogd aanwezig zijn, met name als thalidomide wordt gecombineerd met andere vormen van chemotherapie en/of prednison. De hoeveelheid VWF en FVIII in het bloed werd daarom eveneens gemeten bij patiënten gerandomiseerd voor TAD of VAD, gevolgd door hoge dosis melphalan en autologe stamcel transplantatie, zoals beschreven in hoofdstuk 7. Ook hier was er een duidelijke stijging van zowel stollingsfactor VIII, als ook van VWF in beide groepen tijdens de chemotherapie. Hoewel dit zeker een verklaring kan zijn voor het toegenomen risico op trombose tijdens chemotherapie in het algemeen, is het niet verantwoordelijk voor het extra risico bij patiënten behandeld met thalidomide. De oorzaak voor het verhoogde risico op trombose bij thalidomide behandeling is op dit moment nog niet duidelijk. Inmiddels is wel bekend dat dit extra risico op trombose in de praktijk kan worden voorkomen door het gebruik van lage dosering heparine, een algemeen gebruikt antistollingsmiddel.
Concluderend kunnen we zeggen dat combinatie therapie met thalidomide gevolgd door een stamcel transplantatie een effectieve behandeling is bij patiënten met multipel myeloom. Het verhoogde risico op trombose waarmee deze combinatie therapie gepaard gaat, kan echter een levenbedreigende situatie opleveren. De geremde afbraak van een stolsel of de hoge stolfactoren VWF en FVIII lijken echter geen verklaring voor dit extra trombose risico tijdens thalidomide therapie in multipel myeloma patiënten in vergelijking tot meer conventionele chemotherapie. Er moeten andere verklaringen zijn voor het verhoogde trombose risico tijdens thalidomide therapie. Meer wetenschappelijk onderzoek in deze richting is dan ook een uitdaging voor de toekomst, waaraan ik de komende jaren mijn bijdrage hoop te leveren.

Dankwoord

Curriculum Vitae

List of publications

Dankwoord

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Marco en Gerdy van Ee-van Amerongen.
We hebben elkaar ontmoet tijdens een duikvakantie in Egypte en bleken niet ver van elkaar te wonen. Inmiddels hebben we vele avonturen met elkaar beleefd. Toen we zonder huis zaten, hebben we bij jullie voor vele maanden onderdak gekregen. Vele mensen zeiden tegen ons: 'Als dat maar goed gaat'. Maar niets is minder waar. Er is een warme vriendschap van overgebleven en van jullie kennis van alle groen hebben wij erg veel geleerd. De gezellige etentjes, het opkweken van plantjes, het delen van de zorg voor de paarden en het werken in de tuin, waren een stabiele basis in die periode en veel van wat er in dit boekje is geschreven, is bij jullie in huis tot stand gekomen. Daarmee hebben ook jullie een waardevolle bijdrage geleverd aan dit proefschrift. Marco en Gerdy, bedankt voor alles.

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Als laatste natuurlijk Wouter Hartmann, allerliefste Wouter. Bedankt dat je het mogelijk hebt

gemaakt om mijn werk en promotieonderzoek te combineren met een leven samen. Mijn lange werkdagen en mijn liefde voor het buitenleven maken dat het huishouden nogal eens op jou neer komt. Als de gemoederen eens hoog oplopen, ben je altijd in staat om me weer te kalmeren en je vrolijke aard werkt aanstekelijk. Uit het vertrouwde Utrecht verhuizen naar Limburg is niet niks en betekende weer een beetje opnieuw beginnen. Hopelijk gaan we samen nog een mooie toekomst tegemoet.

Curriculum Vitae

Ik ben geboren op 3 oktober 1970 in Dordrecht. Na enkele jaren verhuisde ik met mijn ouders naar Roermond. Hier doorliep ik de lagere school en in 1990 behaalde ik het examen VWO aan de Rijks Scholen Gemeenschap te Roermond. Van 1990 tot 1998 studeerde ik geneeskunde aan het Universitair Medisch Centrum Utrecht, met een stage dermatologie, een extra stage pathologie en een keuze co-schap dermatologie. Vanaf 1998 ben ik werkzaam geweest op de afdeling pathologie van het Universitair Medisch Centrum Utrecht, waar ik in 2004 mijn opleiding tot patholoog afronde. In 2002 en 2003 ben ik in het laboratorium werkzaam geweest aan een onderzoeksproject dat door KWF kankerbestrijding werd gefinancierd. De titel van dit project was: Onderzoek naar de procoagulante werking van Thalidomide bij de ziekte van Kahler. In de daaropvolgende jaren werd het onderzoek voortgezet. Na mijn opleiding ben ik als patholoog werkzaam gebleven op de afdeling pathologie van het Universitair Medisch Centrum Utrecht. Naast mijn promotieonderzoek heb ik mij vaak bezig gehouden met mijn hobby's, waaronder paardrijden, duiken en het werken in de tuin. Sinds 1 maart 2006 ben ik werkzaam als patholoog op de afdeling pathologie van het Academisch ziekenhuis Maastricht.

Ariënne van Marion was born on the 3rd of October 1970 in Dordrecht, the Netherlands. In 1990 she graduated from highschool at the Rijks Scholen Gemeenschap in Roermond. She started her medical study in Utrecht and as she was already interested in pathology during her study, in 1996 she did an extra teaching practice for 7 months at the pathology department in Utrecht. Her other topic of interest was dermatology, were she passed her scientific practical training and a teaching practice. She graduated as a medical doctor in 1998.
In 1998 she started her clinical work combined with her research. She worked as a resident in pathology in de department of pathology in Utrecht (head Prof. Dr. J.G. van den Tweel and Prof. Dr. P. van Diest). The research was performed in the Multiple Myeloma laboratory in Utrecht (head Dr. H. Lokhorst) and supported by the Dutch Cancer Society (KWF). She became a pathologist in september 2004 and worked in Utrecht until march 2006. From that date she is working in the Academic hospital in Maastricht.

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