XIIth International

MYELOMA WORKSHOP

26th February – 01 March 2009

Washington, DC, U.S.A.

EDUCATIONAL MATERIALS

Organizing Committee

Nikhil C. Munshi, MD
S. Vincent Rajkumar, MD
Vinod Raina, MD
Sundar Jagannath, MD

Welcome Letter

Dear Attendees of the XII International Myeloma Workshop

We are delighted to welcome you to the XII International Myeloma Workshop. Although the workshop had to be moved at a very late stage from New Delhi to Washington DC, your support has been overwhelming, and we are looking forward to a highly successful event.

The two main goals of the workshop are to stimulate debate and discussion among experts and advance the science to provide continuing medical education. To this end, this meeting features scientific presentations from leading myeloma researchers worldwide, covering all aspects of the disease from fundamental basic concepts to current clinical trials. As in past workshops, we have oral and poster presentations of new original research being presented by scientists, clinicians, and others interested in advancing our understanding of myeloma. There are several important new aspects of this workshop that we would like to highlight. First, we have organized 3 consensus panel presentations on some of the most pressing issues that demand uniformity and cohesiveness. The consensus panels that produced these reports have been at work for over a year to develop these guidelines. Second, we have organized a series of debates featuring respected leaders in the fi eld to address some of the most contentious and controversial topics in the field. Third, we have sought to minimize duplication, by integrating the sponsored symposia into the meeting agenda, and have specifi cally designed these sessions to be of continuing medical education value to the practicing clinician. Finally, the workshop features a special section in which we have asked leaders of various cooperative groups from around the world to update us not only on recently completed trials but also give us an overview of ongoing and planned trials so that participants and researchers can grasp the specifi c questions being addressed worldwide, develop intergroup efforts, and be able to minimize unnecessary duplication of trials.

Finally, this workshop will inaugurate the formation of the International Myeloma Society. The aims of the Society are to promote research, education, clinical studies (including diagnosis and treatment), workshops and symposia on all aspects of multiple myeloma and related disorders worldwide. Besides the fairly intense scientifi c content of the workshop, we recognize that a key goal of the meeting is to inspire new collaborations and networking. To this extent we have a number of social events, including a trip to the Newseum in Washington DC. Of particular note is the opening ceremony during which time we will present the Waldenstrom Award for lifetime contribution to Myeloma Research, and several other awards.

We specifi cally like to thank our industry and non-profi t foundation sponsors who have contributed generously to this meeting, and our meeting organizer J Spargo in the United States who helped us organize this meeting, in such a short notice. This meeting would not be possible without them. We also thank Creative Travel in India who helped with the organization of the workshop while the meeting was planned to be held in New Delhi, but continued to provide assistance after the move to ensure a smooth transition. We look forward to your full support in making this meeting a grand success.

Nikhil C. Munshi, MD
S. Vincent Rajkumar, MD
Sundar Jagannath, MD
Vinod Raina, MD

An Invitation to Join the International Myeloma Society

You are invited to become a member of the International Myeloma Society (IMS). This is a new organization which fosters scientific exchange and will be responsible for the final selection of the organizer and the site of the XIV International Myeloma Workshop in 2013.

The dues will be $100 per year. The dues will be used to suppport future International Workshops and probably provide travel grants to the Workshops for young investigators.

For more information please contact Dr. Morie Gertz (gertz.morie@mayo.edu).

To Join the Society Now

Please fill out the form below and turn it in at *(someplace on site?)* or mail it, with your check or money order to: **International Myeloma Society,** c/o Morie Gertz, M.D., Siebens – Department of Medicine, Mayo Clinic, 200 1st St SW, Rochester MN 55905, USA

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Role Of FISH In Myeloma Risk Stratification

Rafael Fonseca MD

Mayo Clinic, Scottsdale, AZ

The study and treatment of multiple myeloma (MM) now requires integration of data arising from clinical trials, and associated correlative laboratory studies. It has become obvious that genetic features are key drivers for disease classification, prognosis and prediction of benefit from therapy. These genetic abnormalities describe the major subtypes of the disease, genetic progression events, features of disease aggressiveness and responsiveness to treatment. Integration of this information into routine clinical care is now considered routine by many, and certainly should be considered a must for all ongoing and future clinical trials. While the subdivision of the disease into several entities poses significant challenges regarding statistical power, it cannot be ignored, and without it we face the possibility of forgoing important clinical observations. With this background our group and others have proposed some general guidelines in the utilization and application of risk stratification strategies for MM patients (www.msmart.org). Here follows a summary of the possible applications of FISH technology for disease classification.

1. Background and techniques

Almost all, if not all, MM cases harbor genetic aberrations when studied by FISH. The query of the MM cells by FISH for abnormalities must be done in conjunction with plasma cell identification by cytoplasmic light chain staining (*e.g*. cIg-FISH) or using methods for cell sorting (*e.g.* CD138 beads). In the absence of these strategies (*e.g*. nuc-FISH) the results are less sensitive, and not reliable enough to be used in clinical practice.

2. Diagnosis (*Biologic Classification*)

In almost all cases MM can be diagnosed without the need for flow cytometry or genetic markers. However, knowing the specific genetic subtype of MM can have very significant implications for determining long-term outcome and prognosis. Despite recent treatment advances, namely novel therapeutics overcoming some of the negative prognostic implications for patients with high risk genetic features (*e.g.* t(4;14)(p16;q32)), these patients still tend to have more aggressive clinical courses with overall worsened prognosis from the time of diagnosis. While these associations are not absolute but their portray an overall perspective of disease biology. In some rare instances FISH can serve as a diagnostic tool. It is know that Waldenström macroglobulinemia never harbors IgH translocations and that almost all IgM MM cases have a t(11;14)(q13;q32). Therefore the presence of a t(11;14)(q13;q32) can help in distinguishing these two pathologically indistinguishable entities.

3. Prognosis (*Prognostic Classification*)

Many systems exist that can reliably predict prognosis for MM patients. These include systems that employ simple laboratory tests (*e.g.* such as the ISS using albumin and β_2-microglobulin) to the more sophisticated ones that employ modern genomic tools (*e.g.* GEP for high-risk signatures). Needless to say, no prognostic system is perfect and can accurately predict an individual patient outcome. Therefore the ability to predict prognosis is truly more of a positioning of a system (a specific set of factors) along a spectrum of relative strength in predicting outcome (*e.g.* RR or the R^2 as a reflection of outcome variability). Increasingly genetics and genomics have been used for prediction of patient outcomes. Genetics have been used by several large studies to predict the outcome of patients treated with conventional and high dose chemotherapy. While only limited data exists regarding the value of prognostic factors for novel therapeutics, it appears that bortezomib can neutralize the negative effect of some of the high-risk markers (minimal data exists regarding thalidomide and lenalidomide). All of the studies addressing the role of proteasome inhibitors in patients with high-risk disease have been relatively underpowered to address the question conclusively, and most have been done in the setting of relapsed and refractory MM, with only the VISTA trial

addressing the question in the upfront setting. It is also important to note that the value of prognostic factors needs to be validated according to the specific stage of the disease; prognostic factors validated in the upfront treatment of the disease may not have similar effects in previously treated patients.

4. Predictive capacity

In some instances new genomic markers may be used to predict responsiveness to specific therapy. The question of what to do with this information is still unanswered, but potentially could be used for treatment selection (*e.g.* useful or not) or prioritization (*e.g.* initiate a bortezomib containing regimen earlier or not). An example of the former could be the use of an FGFR3 inhibitor only in cases with t(4;14)(p16;q32), or FGFR3 positive MM only. If such therapy was truly targeted it would be likely only to benefit such patients, and at least 3 clinical trials of these compounds are ongoing or have been completed. An example of the latter, still to be validated, could be the use of TRAF3 deletions predicting heightened response to bortezomib based combinations. Perhaps of even greater benefit could be the identification of markers that accurately predict lack of efficacy of a specific drug; a hypothetical example could be the presence of proteasome mutations predicting a refractory state to bortezomib, something already shown in preclinical models.

5. Practical aspects of genetic testing

So what is the best way to employ this technology? In my opinion the technology will be useful if it provides clinically useful information for patient care, usually but not always "actionable." These tools should be reliable in predicting outcome and as clinical laboratory test performance (*i.e.* reproducible and accurate test results). Ideally they should be widely available and easy to interpret. Prognostic estimation with FISH based strategies fulfills many of these requisites, albeit with GEP having a greater ability to discern outcome. If current genomic platforms can be employed in determining the outcome in a community setting for MM patients they are likely to replace FISH as the diagnostic tool of choice. However, the hurdles faced by the application of genomic tools including; availability of enough sample, validated and credentialed clinical laboratory testing, insurance coverage for the indication and provider acceptance remain significant. Until these issues are resolved FISH will continue to be the preferred method of risk assessment.

Other methods for disease classification and prognosis have employed novel techniques such as DNA copy number changes as seen in amplifications and deletions. These have employed both aCGH and SNP microarray platforms. These have identified certain genomic regions that could potentially predict outcome (16q deletions, 1q gains, 12p deletions and 5q amp). In some of the proposed prognostic models there variable surpass the prognostication ability seen for markers such as t(4;14)(p16;q32). Most of these markers need to be validated. Ironically all of these regions can be queried via interphase FISH based strategies.

Features	FISH based	Genomic based
Power to predict outcome	++	+++/++++
Clinical test	Yes	Not yet
Information obtained	Limited	Global genomics
Expense	Similar	Similar*
Can do overnight testing?	Yes	Yes
Can be done by smaller laboratories?	No	No
Ease of interpretation	Simple	Moderate**
Number of cells needed for analysis	100	>100,000
Requires cell enrichment/purification?	No for cIg	Yes
Suitable for cases of minimal plasmacytosis?	Yes	No
Number of cases with informative results	Most (>90%)	Low (<50%)
Covered by insurance	Yes	No

** Cost may be less for GEP depending on the number of probes used for FISH*

*** Needs metadata generation and interpretation.*

6. Recommended FISH clinical testing

The recommended clinical testing will undoubtedly change over time if one uses FISH as a primary diagnostic tool, as new probes and markers will be discovered and validated (and some eliminated). Some of the testing could be done only once as the information provided will not change, and some can be repeated since there may be changes over time.

7. Conclusion

Until the time comes that genomic tools become standard clinical tools the application of FISH techniques are likely to remain as the main way of determining the prognostic outcome of MM based on genomics. While in other realms (e.g. breast cancer) genomic results have been employed to determine outcome, it is because that information has been translated to other readily available clinical tools (*e.g.* RT-PCR, IHC, etc). There is no "better test" but rather each comes with its own limitations and advantages.

Level	FISH tests	Recommended testing frequency	Validation
Minimal proposed testing (essential testing)			
Established markers	*Primary events* t(4;14)(p16;q32) t(14;16)(q32;q23)	Once	Validated by several studies
	Progression events 17p deletion	Can be repeated	Validated by several studies
Expanded panel			
Markers with modest effects	Hyperdiploidy t(11;14)(q13;q32)	Once	Almost always favorable but weak effect
	Chromosome 13	May be repeated	Always negative but effect weak
Emerging markers			
Other	Other translocations	Once	Rare events and not routinely tested
Chromosome 1	1q deletion 1p amp	May be repeated	While conflicting studies, seem to predict outcome
SNP/aCGH	16q deletion 12p deletion 5q amplification	May be repeated?	Data not validated yet. Could convert markers of aCGH and SNP into FISH testing

Genetic Changes In Myeloma: Prognostic Implications

Hervé Avet-Loiseau, MD, PhD,

Hematology Laboratory, University Hospital, and INSERM U892, Nantes, France.

Multiple myeloma is characterized by a huge clinical heterogeneity, with survival extending from a few months or even weeks, to more than 10 years. A part of this heterogeneity has been analyzed by the International Staging System. However, this system, based on albumin and β2-microglobulin levels, does not evaluate a major factor of heterogeneity, ie, the chromosomal changes. In the past 7-8 years, many reports have demonstrated the high prognostic value of genetic aberrations in myeloma. The first reports did show that some chromosomal changes observed on the karyotypes were associated with different outcomes.[1] For instance, whereas hyperdiploidy was associated with longer survivals, hypodiploidy and loss of chromosome 13 predicted with a poorer outcome. However, karyotype is hampered by a low mitotic rate in myeloma, preventing informativity for the majority of the patients. For now 7-8 years, many investigators have used interphase fluorescence in situ hybridization to analyze chromosomal changes within plasma cells. Several chromosomal changes have been associated with a shorter survival: del(13), t(4;14), del(17p), 1q gains, t(14;16) are the most widely recognized poor prognostic parameters,[2,3] but many other chromosomal changes have been proposed to impair the prognosis. A matter of debate is the value of del(13). In univariate analyses, del(13) appears as associated with shorter event free survival and overall survival. However, more recent data did show that its prognostic value is totally related to other abnormalities frequently concomitantly observed, such as t(4;14), del(17p) or t(14;16). Thus, if del(13) is probably not so important for prognosis, t(4;14) and del(17p) are widely accepted as poor prognostic parameters, and should be investigated in prospective trials.[3]

More recently, a few teams have investigated the role of gene expression profiling (GEP) in the prognostication of myeloma.[4-6] Based on different platforms, and on different patient populations, these studies demonstrated the high reliability of those techniques to identify patients with a poor outcome. In the pioneer work from Arkansas,[4] GEP focused on a set of 70 (or even 17) genes whose deregulation identified a small group of patients (13%) with a very poor outcome. In the IFM study,[6] a different approach identified a set of 15 genes whose deregulation was associated with a short survival, observed in 25% of the patients. Interestingly, although no single gene was common to the 2 lists, the sets were predictive of survival in independent cohorts of patients, even in patients analyzed at the time of relapse. This is a clear demonstration that the technique is extremely powerful to predict patients who will present a short survival, independently of the treatment received.

Even more recently, some studies using CGH- or SNP-array have demonstrated that all the patients present chromosomal changes, and that a lot of unbalanced chromosomal changes are recurrent among patients[7,8]. Among these different changes, some are associated with a specifically poor outcome: del(12p), 1q gains. Interestingly, one of these studies identified the gain of chromosome 5 as a particularly good prognostic feature, summarizing the prognostic value of hyperdiploidy.[8]

In conclusion, genetic changes display such a high prognostic value that it is unconceivable to not include them in the prognostic models in myeloma. Whether we should use FISH, GEP or CGH/SNP is an unresolved question. The advantage of FISH is that it is available for almost 100% of the patients, independently of the quality of the samples. Array-based technologies require a certain amount of sorted plasma cells, preventing their use for all the patients. The future will probably in the miniaturization of the technology, on a small number of plasma cells.

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Redefining Risk Using Cytogenetic Changes

Johannes Drach

*Medical University of Vienna, Department of Medicine I, Clinical Division of Oncology, Vienna, Austria (*johannes.drach@meduniwien.ac.at)

In an attempt to provide a relatively simple prognostic classification of newly diagnosed MM patients, the Mayo Group has defined two patient groups, which can be categorized as high-risk or standard risk (mSMART classification, see Table 1).[1] At this stage, this information can provide the framework for an individualized treatment approach, but it needs to be considered that current prognostic information is predominantly based upon patient populations treated with chemotherapy. Therefore, any prognostic classification needs to be reconsidered and is likely to be changed as soon as more data with novel agents become available.

One of the most frequent structural abnormalities observed in MM karyotypes involves the Ig heavy-chain (IgH) gene locus on 14q32, which is usually part of a translocation. Heterogeneous translocation partners have been described, with 11q13, 4p16.3, 16q23, 20q11 and 6p21 being recurrently involved in 14q32 translocations of primary MM tumor specimens. These 5 types of primary IgH-translocations, which are mutually exclusive, comprise about 60% of all IgH-translocations, and are mediated primarily by errors during IgH switch recombination. With respect to biology and prognosis, relevant correlations have emerged: Whereas t(11;14)(q13;q32) resulting in upregulation of cyclin-D1 characterizes a group of MM patients with neutral or rather favorable prognosis, presence of a t(4;14)(p16;q32) or a t(14;16)(q32;q23) identifies subsets of MM patients with short survival, even in the context of autologous transplantation.[2-6]

By metaphase cytogenetics, a chromosome 13q abnormality can be found in about 15% of MM patients at diagnosis, whereas interphase FISH studies have shown a higher frequency of 13q deletions in MM, occurring in 39 – 54% of newly diagnosed cases. Karyotypically defined chromosome 13 abnormalities are a more powerful prognostic indicator than FISH-defined chromosome 13 deletions, which are predominantly associated with other abnormalities carrying prognostic information, particularly t(4;14) and t(14;16).[7] An additional genetic high-risk category is defined by deletions of 17p13 at the *TP53* locus, with similar observations for patients receiving standard-dose and high-dose therapy.[3,5,8] Comprehensive analyses of cytogenetic abnormalities in MM identified patients with a t(4;14), t(14;16) and/or 17p-deletion as the group of patients with the worst prognosis suggesting that novel approaches are required for the treatment of such high-risk patients.

An important advantage of novel agents in the treatment of MM is in the area of high-risk disease as defined by chromosomal abnormalities. Clinical evidence suggests that bortezomib may overcome the poor prognosis associated with such chromosomal abnormalities, both in the setting of relapsed/refractory MM and newly diagnosed disease. With respect to untreated patients, promising results have been reported from the VISTA trial: Patients treated with MP + bortezomib had a 32% CR rate independent of the cytogenetic risk category (high-risk [t(4;14), t(14;16), deletion 17p] versus standard-risk [all others]). Moreover, time-to-progression and overall survival were also similar in both risk groups.[9]

Lenalidomide may also be beneficial for MM patients with such high-risk chromosomal features: A retrospective analysis of relapsed/refractory patients treated with lenalidomide/dexamethasone showed no impact of parameters like t(4;14), del(13q), and high beta-2-microglobulin on survival.[10] Lenalidomide plus low-dose dexamethasone demonstrated efficacy in previously untreated MM patients independent of cytogenetics and beta-2-microglobulin. On the other hand, high-risk patients as defined by the mSMART criteria had inferior progression-free survival versus standard risk patients when treated with lenalidomide/dexamethasone.[11] With current follow-up, no significant impact on overall survival was seen.

Gain of chromosome 1q21 has been reported to be a negative prognostic factor in MM patients treated with chemotherapy and autologous transplantation.[12] We have recently investigated whether or not bortezomib may overcome the negative prognostic impact of a chromosome 1q21 (*CKS1B*) gain in MM. We studied 109 pts with relapsed/refractory MM: 49 pts who were treated with single-agent bortezomib, and 60 pts treated with a bortezomib combination (44% combined with conventional chemotherapy, 38% with dexamethasone, 18% with thalidomide and dexamethasone). Among patients treated with single-agent bortezomib, gain of 1q21 was observed in 43%. Treatment outcome was negatively affected by presence of a 1q21 gain: Overall response rate was 34% (versus 53% in pts with normal 1q21; P = 0.16). Moreover, gain of 1q21 was associated with shortened time to treatment failure (TTF) (median, 2.5 versus 6.6 months; P = .02) and overall survival (OS) (median, 5.0 versus 32.4 months; P = .002) compared to pts with normal 1q21. Beta-2-microglobulin and 14q32 translocations were unrelated to treatment outcome after single-agent bortezomib, but median OS was short in the presence of low serum albumin (4.9 versus 29.7 months; P = .01). In the group of pts treated with a bortezomib-combination, 45% had a 1q21 gain. There were no significant differences between pts with 1q21 gain and normal 1q21 regarding overall response (63% versus 58%; P = 0.67), median TTF (12.2 versus 8.9 months; P = .50) and median OS (not reached versus 23.3 months; P = .50). These results provide further evidence for the efficacy of bortezomib-combinations in MM patients with high-risk cytogenetic features.

Additional biological features of the myeloma clone may determine the biology of the disease. The microenvironment of tumor cells in the bone marrow may be actively involved in the progression of malignant diseases. In MM, interactions of bone marrow stromal cells with the malignant plasma cells have gained significant importance as targets for novel therapeutic agents. Based upon these observations, we aimed at analyzing in detail the secretory capacity of bone marrow fibroblasts obtained from patients with MM in order to better understand their contribution to disease progression. We therefore analyzed the secretome of primary bone marrow fibroblasts of MM patients by proteome profiling based on highly sensitive mass spectrometry. Normal skin fibroblasts were found to secrete various extracellular matrix (ECM) proteins including fibronectin, collagens and laminins, in addition to some chemokines and cytokines including CXCL12, follistatin-like 1, insulin-like growth factor binding proteins 4, 5 and 7; and SPARC. In contrast, bone-marrow-derived fibroblasts from MM patients secreted increased amounts of ECM proteins and alpha-fetoprotein in addition to insulin-like growth factor II, stem cell growth factor and matrix metalloproteinase-2. Co-culture of primary MM cells with these fibroblasts further stimulated the secretion of ECM proteins, of cytokines such as inhibin beta A chain and growth factors such as connective tissue growth factor, which might be relevant to support the malignant clone. Proteome profiling of secreted proteins may thus help to identify relevant tumor-associated proteins, to increase our understanding of cell cooperativity and thereby increase our understanding of progression events in monoclonal gammopathies.

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Table 1. mSMART classification of symptomatic MM[1]

	High-risk (25% of patients)	**Standard-risk (75% of patients)**
FISH:	Deletion of 17p	All others including
	t(4;14)	Hyperdiploid
	t(14;16)	t(11;14)
Cytogenetic deletion 13		t(6;14)
Cytogenetic hypodiploidy		
Plasma cell labelling index $\geq$ 3%		

Standard risk also requires a beta-2-microglobulin < 5.5 and LDH < upper limit of normal

High-Throughput Molecular Profiling; Insights into Myeloma Pathogenesis And Clinical Outcome

Professor Gareth Morgan

The Institute of Cancer Research, London, United Kingdom.

Cytogenetics provided the initial impetus for understanding the molecular pathogenesis of myeloma and exploitation of positional cloning to identify genes deregulated by the IgH translocations has without doubt greatly informed our understanding of the molecular biology of myeloma. More recently the development of CHIP based high-throughput profiling technologies has given us a new tool with which to investigate the biology of myeloma.

One of the most important molecular lesions associated with progression of myeloma is loss of genetic material. SNP based gene mapping has given us a high resolution tool able to interrogate the genome for such regions of loss. Results of such analyses have allowed us and others to define the boundaries of deleted regions and the genes contained within them. Narrowing down the deleted regions and regions with uniparental disomy has allowed us to define critically deregulated tumour suppressor genes. These genes deregulate pathways critical to malignant plasma cell survival, cell division and differentiation which is allowing us to generate new rationales for how to target therapy.

Haplo-insufficiency can be an important mechanism underlying tumour progression but mutation of the residual allele is a frequent event in a relevant TS gene and some of the candidates fulfil such a criterion. Methylational inactivation of the residual allele can also lead to inactivation of the residual allele and global methylation analysis combined with gene mapping can give a better insight into critically deregulated genes as can the study of micro RNAs.

There is a tendency to concentrate on acquired tumour specific changes but there is no doubt that inherited variation can impact on both the behaviour of tumour cells and the side effects induced by treatment exposure. We have been addressing this issue by mapping inherited genetic variation at the same time as tumour specific acquired evens and by integrating this data gaining insight into these complex interactions.

While studying biology is important great insights can be gained by combining tumour characterisation with clinical treatment and outcome data. Using his strategy we have been able to define inherited associations with risk of VTE and peripheral neuropathy. At the same time we have been able to define the very poor prognostic impact of 17p- and to define an expression based signature, based on homozygous deletions in cell survival genes, that predicts high risk cases.

New Insights into The Molecular Basis Of MM Pathogenesis And Prognosis

John D. Shaughnessy, Jr. and Bart Barlogie

University of Arkansas for Medical Sciences, Little Rock, AR

High-risk multiple myeloma (HRMM) is routinely defined by laboratory parameters alone or in combination in the Durie-Salmon and, more recently, the ISS staging systems. The Bartl grade, a cell morphology-based staging system, has seen limited use. The presence of abnormal cytogenetics, high labeling index, interphase FISH abnormalities and recently defined flow cytometric measures have also been used. A molecular-based classification and risk stratification of MM may improve the definition of HRMM. Here we focus on the results from our program, while briefly discussing published reports from other groups. Global gene expression profiling (GEP) with Affymetrix U133Plus2.0 microarrays of CD138-selected plasma cells from over 788 patients with newly diagnosed disease undergoing multi-agent chemotherapy and autologous stem cell transplantation (ASCT) has now been performed. Using unsupervised hierarchical cluster analysis we showed that MM comprises a spectrum of seven distinct reproducible subtypes. A validated molecular classification schema has emerged related to recurrent oncogene-activating translocations (**MS** = t(4;14), **MF** = t(14;16) or t(14;20), **CD-1** = t(11;14) or t(6;14) and **CD-2** = t(11;14) or t(6;14) with high *CD20* and/or *VPREB3*), hyperdiploidy (**HY** = high expression of genes from chromosomes 3, 5, 7, 9, 11, 15, 19), low bone disease (**LB** = NF-κB signature, high *CCND2, CST6* and *IL6R*) and proliferation (**PR** = previous subtypes with high *MIK67, CCNB1, CCNB2, TOP2A,* and *TYMS*).

Correlating GEP with outcome, independent of these groups in two independent cohorts, allowed us to identify and validate a high-risk signature (UAMS 17-gene model), present in approximately 15% of newly diagnosed disease. GEP and high-resolution comparative genomic hybridization in 92 cases confirmed that the altered expression of the 17 genes in the model is driven by 1q gains and 1p losses (our unpublished data). This high-risk signature is evident in a subset of all 7 molecular subtypes and negatively influences outcome. For example, low-risk MS disease fares much better than high-risk MS disease. When subjected to multivariate analysis including the International Staging System (ISS) and a gene expression-based proliferation index (GEP PI), the UAMS 17-gene model remained a significant predictor of outcome. Using U133A microarray data, Mulligan and colleagues developed response and survival classifiers for relapsed disease treated with single agent bortezomib or high dose dexamethasone that were significantly associated with outcome that improved upon the risk stratification provided by the ISS. These predictive models showed some specificity for bortezomib. Using U133A data from newly diagnosed disease treated with ASCT, the Mayo clinic group validated the UAMS 17-gene model, but also showed that the t(4;14) translocation remained a significant variable. Using data from a custom cDNA microarray, the IFM recently reported on a 15-gene model of high-risk (IFM 15-gene model) related to cell proliferation. Multivariate analysis performed by this group to compare the UAMS and IFM models showed that the UAMS model was significant in all datasets tested (TT2, TT3, Mayo, CREST, SUMMIT, APEX [bortezomib], and APEX [dexamethasone]); the IFM 15-model was significant in the Mayo and Millennium bortezomib trials only. Together with our internal studies, these data suggest that the UAMS 17-gene modal captures more outcome variability than models of cell proliferation. However, it must be noted that while providing a considerable improvement over standard variables, the R^2 for the UAMS model is only ~30%.

GEP on 71 paired diagnostic and relapse samples indicate that the UAMS 17-gene model score increases in 80% of the cases and a low-risk to high-risk conversion in 14 of 24 (58%) severely impacted post-relapse survival (our unpublished

data). This quantifiable increase in the high-risk score over time, combined with our published report of an increase in the percentage of cells with +1q over time, suggests this reflects the expansion of a dominant clone with survival and/or proliferation advantages. The almost universal increase in this risk score during disease evolution suggests this traceable molecular signature or a flow-based surrogate may play an important role in minimal residual disease evaluation. An urgent task is to now determine whether there is a baseline GEP signature that can prospectively identify the low-risk cases that will convert to high-risk at relapse. It is obvious that new therapeutics need to be developed that effectively target high-risk cells. New data from the Staudt group at NIH suggests that IRF4 depletion may be one approach. We have reported that expression of *TP53* is a surrogate for 17p13 deletion and *TP53* expression below a specific threshold (Affymetrix GCOS Signal < 727), seen in approximately 10% of newly diagnosed disease, imparts a poor prognosis in low-, but not high-risk defined by the UAMS 17-gene model. We recently reported that the addition of bortezomib to TT3 has significantly improved outcome in low-risk MS disease, thereby demonstrating the value of GEP in evaluating benefits of new treatments that might be otherwise masked.

In conclusion, while the majority of patients with MM can anticipate long-term disease control with TT-like regimens, approximately 25% of patients with HRMM do not benefit from current approaches. Molecular-based, risk-adapted therapy designed to reduce toxicities in low-risk and to test new treatment strategies in high-risk are now being employed at our center. Whether molecular-based risk stratification will be adopted on a larger scale will depend on the perceived value of these advances as well as the feasibility and availability of such tests.

Modulation Of Signaling Cascades By Inhibitors of Histone Deacetylase And Akt.

Teru Hideshima, Hiroshi Ikeda, Constantine Mitsiades, Dharminder Chauhan, James E. Bradner, Nikhil C. Munshi, Paul G. Richardson, Kenneth C. Anderson

Jerome Lipper Myeloma Center, Dana-Farber Cancer Institute, Boston, MA, USA

In multiple myeloma (MM), the BM microenvironment plays a crucial role in promoting tumor cell proliferation, survival, migration, and drug resistance. It is composed of different types of cellular components: including hematopoietic stem cells, progenitor and precursor cells, immune cells, erythrocytes, bone marrow stromal cells (BMSCs), BM endothelial cells, as well as osteoclasts and osteoblasts. These cells not only physically interact with MM cells, but also secrete growth and/or anti-apoptotic factors, including interleukin (IL)-6, insulin-like growth factor (IGF)-1, vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α, stromal cell-derived factor (SDF) 1α, and B-cell activating factor (BAFF). These factors trigger several proliferative/anti-apoptotic signaling cascades in MM cells: phosphatidy-linositide-3 kinase (PI3K)/Akt; Ras/Raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-related kinase (ERK); Janus kinase (JAK) 2/signal transducers and activators of transcription (STAT)-3; and nuclear factor (NF)-κB [1]. Therefore, cytokines, their receptors and their downstream protein kinases and transcription factors represent potential therapeutic targets in MM.

Histone deacetylases (HDACs) are divided into four classes: class I, HDAC1, 2, 3, and 8; class II, HDAC 4, 5, 6, 7, 9 and 10; class III, SIR1, 2, 3, 4, 5, 6 and 7; and in class IV, HDAC11. Importantly, class I and IV HDACs are constitutively localized in the nucleus, whereas class II HDACs can shuttle between the nucleus and the cytoplasm interacting with 14-3-3 protein. HDAC inhibitors have shown promise as novel anti-tumor agents for many malignancies, including structurally diverse HDAC inhibitors purified from natural sources or synthetically developed. Hypoacetylation of histones is associated with condensed chromatin, resulting in repression of gene transcription, whereas acetylated histones are associated with open chromatin structure and activation of transcription; therefore, HDAC inhibitors trigger transcription. However, inhibition of HDAC activity ultimately triggers growth arrest and/or apoptosis of tumor cells. Although possible mechanisms of HDAC inhibitor anti-tumor activities have been extensively studied, their growth inhibitory effects in MM cells have not yet been fully characterized. Importantly, recent studies have shown that acetylation of proteins besides histones may be altered by HDACs and their inhibitors.

Histone deacetylase 6 has an essential role in recruitment of ubiquitinated proteins for transport to aggresomes, ultimately leading to lysosomal protein degradation [2]. We have shown that combined inhibition of proteasomes with bortezomib and of aggresomes with HDAC6 specific inhibitor Tubacin [3] triggers synergistic cytotoxicity in MM cell lines and MM patient tumor cells in vitro [4]. We further examined whether inhibition of HDAC6 modulates acetylation of proteins. Interestingly, HDAC6 was constitutively associated with heat shock protein (Hsp) 90, which was enhanced by Tubacin treatment. Consistent with previous studies showing that HDAC6 modulates Hs90 acetylation [5],Tubacin enhanced acetylation of Hsp90 in MM cells. Since Akt is a client protein of Hsp90, we next examined whether acetylation of Hsp90 via inhibition of HDAC6 could modulate activity of Akt. Tubacin enhanced phosphorylation of Akt; conversely, Hsp90 inhibitor 17-AAG inhibited its effect. Interestingly, enhanced interaction of Hsp90 with Akt was blocked by Tubacin. Our results therefore suggest that HDAC6 negatively regulates phosphorylation of Akt.

LBH589 (Novartis Pharmaceuticals) is a hydroxamic acid analog which blocks class I and II HDAC and has significant anti-MM activities [6]. Importantly, LBH589 synergistically augments

MM cell cytotoxicity induced by bortezomib, and an international multicenter clinical trial of LBH589 with bortezomib in MM is ongoing. We found that LBH589 significantly inhibited phosphorylation of JAK2 and its downstream molecule STAT3, without inhibiting Akt or ERK phosphorylation. Interleukin-6 (IL-6) and BMSC co-culture markedly augmented JAK2/STAT3 phosphorylation; however, LBH589 completely abrogated this effect. It has also been shown that acetylation of p65 (RelA) is crucial for its transcriptional activity. We therefore examined whether LBH589 modulates NF-κB activity in MM cells. Importantly, LBH589 strongly enhanced NF-κB activity and LBH589-induced cytotoxicity was augmented by IKKβ inhibitor MLN120B. Our results demonstrate that HDAC inhibitors modulate acetylation not only of histones, but also of other proteins mediating MM cell survival and anti-apoptosis.

Among major signaling cascades, PI3-K/Akt signaling can be activated in MM cells by many cytokines in the BM milieu mediating growth and drug resistance. Perifosine (Keryx Biopharmaceuticals) is a synthetic alkylphospholipid which targets cell membranes and inhibits Akt activation. Perifosine inhibits baseline and cytokine-induced phosphorylation of Akt and its downstream molecules in MM cells. Perifosine induced apoptosis is mediated by JNK activation, followed by caspase-8/9 and PARP cleavage; conversely, JNK inhibition blocks perifosine-induced apoptosis[7]. Perifosine also downregulates protein expression of β-catenin and survivin [8]. Perifosine augments cytotoxicity of both conventional (doxorubicin, melphalan) and novel (bortezomib) agents. Specifically, the addition of perifosine blocks bortezomib–induced Akt activation and upregulation of β-catenin and survivin in MM cells, resulting in synergistic cytotoxicity. Finally, perifosine demonstrates significant anti-tumor activity in vivo, evidenced by downregulation of p-Akt in tumor in a xenograft model of human MM in SCID mice. Importantly, clinical trials of perifosine and bortezomib in relapsed refractory MM, based upon these studies, have shown that perifosine can enhance sensitivity or overcome resistance to bortezomib, with significant and durable responses even in the setting of bortezomib resistance. Finally, we have also examined class-I PI3K expression in MM cells and found that p110δ is expressed in the majority of patient MM cells, but not cell lines. Inhibition of p110d with CAL-101 (Calistoga Pharmaceuticals) inhibits baseline Akt activation in MM cells, associated with cytotoxicity. Neither IL-6 and IGF-1 nor binding of MM cells to BMSCs blocked CAL-101 induced anti-tumor effects. Finally, CAL-101 induced autophagic tumor cell death, evidenced by increased LC3-II expression and increased autolysosomes identified by acridine orange staining and electron microscopy. Taken together, our results show that inhibition of signaling cascades by specific inhibitors of HDAC is a promising therapeutic option in MM.

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Molecular Pathways Induced By Tumor – Microenvironment Interactions In Myeloma; Regulatory Role Of Heat Shock Proteins

Constantine S. Mitsiades, Douglas W. McMillin, Jake Delmore, Joseph Negri, Steffen Klippel, Melissa Ooi, Nikhil C. Munshi, Paul G. Richardson, Kenneth C. Anderson

Department of Medical Oncology, Jerome Lipper Multiple Myeloma Center, Dana Farber Cancer Institute, Harvard Medical School, Boston MA 02115, USA.
constantine_mitsiades@dfci.harvard.edu

Interaction of multiple myeloma (MM) cells with bone marrow stromal cells (BMSCs) and/or other accessory cells of the bone microenvironment confers cytokine- and cell adhesion-mediated acquired tumor cell resistance to conventional therapies, such as glucocorticoids, alkylators and anthracyclines. In contrast, novel therapies, such as proteasome inhibitors, are capable of overcoming the protective effect of stromal cells on MM cells. This contrast, coupled with the significant improvement in outcome of MM patients since the introduction of these novel therapies, have further fueled efforts to identify in an open-ended manner new classes of investigational agents capable of circumventing the proliferative/anti-apoptotic stimulation conferred to MM cells by BMSCs, in particular, or the bone microenvironment more generally. To facilitate this effort, we have developed a series of preclinical models which allows us to characterize in vitro and in vivo the molecular sequelae triggered by tumor – microenvironment interactions; as well as identify and validate molecular targets for therapeutic interventions that can neutralize the impact of the bone microenvironment. These models are based on selective and stable marking of MM tumor cell compartment with fluorescent and/or bioluminescent markers, which allows for sensitive detection and quantification of only the viable tumor cells as they interact in vitro or in vivo with their local microenvironment.

Through the use of these models, transcriptional profiling and cell signaling studies of MM cells co-cultured with stromal cells have revealed that their interaction triggers in MM cells a pleiotropic cascade of proliferative/anti-apoptotic molecular events, including upregulation of MM cell growth factors, caspase inhibitors, DNA repair genes, oncogenic kinases, ubiquitin/proteasome pathway members and heat shock proteins. These events are associated with activation of the PI-3K/Akt/mTOR, Ras/Raf/MAPK, IKK/NF-κB signaling cascades; activation of hTERT, HIF-1a and myc; as well as modulation of a series of other transcripts associated with increased proliferation, survival and/or drug resistance in MM and/or other neoplasias.

Within the multitude of these molecular events, an intriguing role for heat shock proteins, such as hsp90, is highlighted. Many of the aforementioned stroma-induced sequelae in MM cells involve molecules known to be chaperoned by hsp90. Indeed, work from our group and others has shown that selective small molecule inhibitors of hsp90, such as the ansamycins 17-allylamino-17-demethoxy-geldanamycin (17-AAG), abrogate multiple molecular levels of cytokine- and adhesion-triggered signaling cascades that mediate proliferative and/or anti-apoptotic effects of stromal cells. For instance, hsp90 inhibition suppresses cell surface expression of IGF-1R and IL-6R; as well as expression and/or function of Akt, Raf, IKK-α, and $p70^{S6K}$. These events, which decrease the amplitude of signaling via the PI-3K/Akt/mTOR, Ras/Raf/MAPK, IKK/NF-κB pathways, lead, in turn, to diverse downstream pro-apoptotic molecular changes, including increased nuclear translocation of pro-apoptotic Forkhead transcription factors; decreased levels of caspase inhibitors; and suppressed activity of NF-κB, telomerase, HIF-1a and 20S proteasome. These results are consistent with the experience that hsp90 inhibition circumvents the protective effects conferred to MM cells by BMSCs or other accessory cells of the BM milieu.

These data do not imply that hsp90 is the sole or the most important regulator of the biological

sequelae mediating tumor-microenvironment interactions. It is instead likely that other heat shock proteins may play a similar role at a post-translational level, while ongoing studies are addressing the role of other groups of candidate regulators which have emerged from our profiling and functional studies. However, these data do support the notion that, given the pleiotropic nature of cascades modulated by tumor-stromal interactions, hsp90 inhibitors emerge as a drug class that merits further evaluation for its ability to modulate the biological behavior of tumor cells within their local milieu. Furthermore, the experience acquired from ongoing clinical trials of tanespimycin (17-AAG) and other hsp90 inhibitors is expected to inform the "bedside-to-bench" development of alternative schedules and new hsp90 inhibitor-based combination regimens oriented towards improving the profile of this drug class.

RNAi Vulnerability Map in Multiple Myeloma

A. K. Stewart, R.E. Tiedemann, Y. Zhu

Mayo Clinic, Scottsdale, AZ

We have conducted systematic RNA interference (RNAi) lethality screening of the 'druggable genome' in human myeloma cell lines (HMCL) to functionally generate a comprehensive map of molecular vulnerabilities in human myeloma tumor cells.

KMS11 human myeloma cells were screened in the absence and presence of titrated bortezomib (IC_{10}-IC_{80}) with a 13,984-oligo library targeting the druggable genome (6,791 genes) using optimized conditions that resulted in >95% transfection efficiency and less than 5% background toxicity. Universally lethal and non-silencing siRNA were employed as controls. Viability was measured at 96h by ATP-dependent luminescence, normalized by B-score. Bortezomib chemo-sensitization was assessed by Bliss independence. Candidate targets (>300) were validated in secondary and tertiary studies using 4 siRNA per gene. Validated targets were further examined using adenoviral and lentiviral vectors for function and portability in multiple cancer cell lines and primary myeloma samples.

Approximately 14% of the human kinome and 5% of druggable genome siRNA cause reductions in HMCL viability (>3 standard deviations from non-silencing siRNA). Validated myeloma survival targets include 24 kinases and 39 other genes. Similar numbers of bortezomib modulators were selected for detailed analysis. Directly vulnerable kinases are concentrated within cytokine pathways (VEGFR, FGFR3, IGFR/IL6R signaling) or are involved in the regulation of cell cycle, apoptosis or metabolism. While many targets such as polo-like kinase 1 (PLK1) appear universally lethal, other targets such as G-protein receptor coupled kinase (GRK6) show specific cytotoxicity in Myeloma. Non-kinase vulnerabilities are centered on the proteosome, mitotic spindle, transcription, protein anabolism, and regulation of apoptosis. Notable modulators of bortezomib sensitivity included proteasome subunits, ER stress transducing and cyclin dependant kinases, members of the NFkappaB signaling pathway as well as some novel pathways.

We have identified approximately 60 critically vulnerable targets essential for proliferation or survival of Myeloma cells and have additionally defined a similar number of targets that both confer chemosensitization and chemoresistance to bortezomib. Small molecule inhibitors that replicate inhibition of RNAi identified targets are being examined in vitro and in early clinical trials targeting Aurora Kinase A and CDK5 in patients also receiving bortezomib.

Targeting NFkβ Signaling In Multiple Myeloma

P. Leif Bergsagel, J. Jonathan Keats , Marta Chesi and W. Michael Kuehl

Mayo Clinic, Scottsdale, AZ

Multiple myeloma (MM) is a tumor of isotype-switched plasma cells that accumulate in the bone marrow, replacing normal plasma cells and leading to bone destruction and marrow failure. It is often preceded by a stable monoclonal gammopathy (MG) with limited (<10%) bone marrow plasmacytosis that progresses stochastically to MM at a rate of between 1-2% per year. **Primary genetic events** thought to be associated with disease initiation are present at similar frequencies in both MG and MM. In approximately 40% of cases there are recurrent chromosome translocations involving the immunoglobulin heavy chain gene that have the hallmarks of being mediated by errors in switch recombination (90%) or somatic hypermutation (10%). These involve recurrent loci that fall into three categories: **Cyclin D** (15% CCND1 at 11q13, <1% CCND2 at 12p13, 2% CCND3 at 6p21), **MAF** (5% c-maf at 16q23, 2% MAFB at 20q12, <1% MAFA at 8q24.3) and **MMSET** (usually with FGFR3 at 4p16, 15%)[1]. Approximately one half of cases are **hyperdiploid** (48-75 chromosomes) with trisomies involving chromosomes 3, 5, 7, 9, 11, 15, 19 and 21. A unifying feature of MM is dysregulated expression of a cyclin D gene, either directly by Ig gene translocation, by transactivation by MAF (CCND2), or by unknown mechanisms (MMSET – CCND2, Hyperdiploid – CCND1)[2]. Interestingly about 2% of patients express no cyclin D, and one half of these patients have bi-allelic deletion of RB, highlighting the critical role of the CCND/RB axis in the pathogenesis of MM. Deletion of chromosome 13 is seen in one half of MM, but is much more common in the tumors that express CCND2

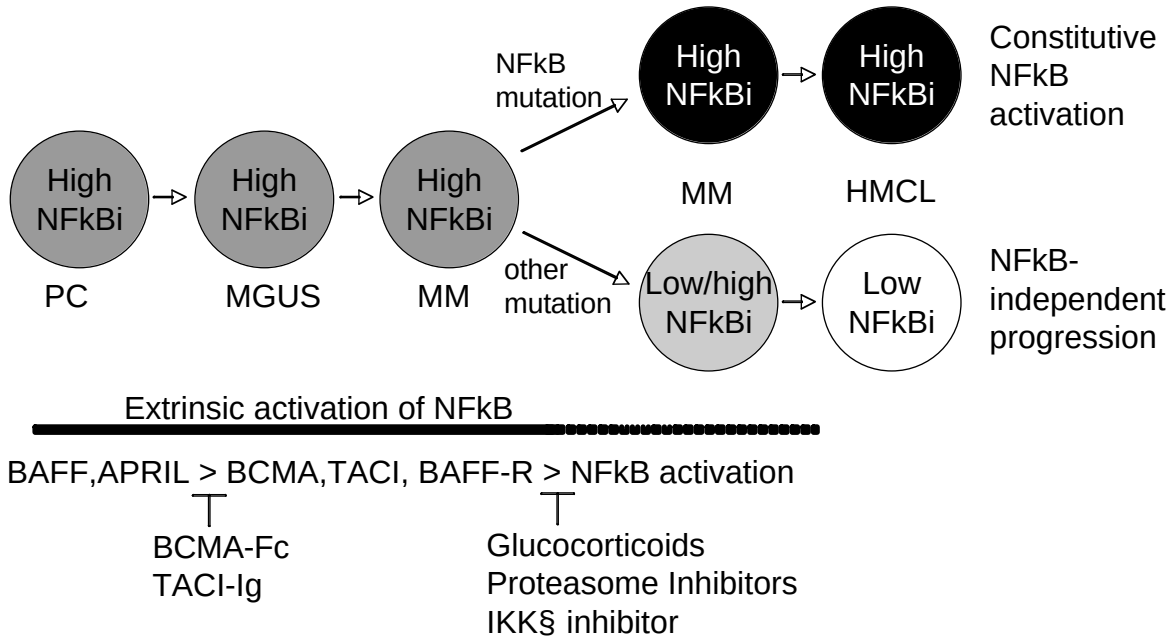

Figure 1. NFkB-dependent and independent tumor progression in MM

Ligand-dependent (BAFF, APRIL) activation of surface receptors (BCMA, TACI, BAFF-R) leads to NFκB activation, resulting in high-levels of expression of NFκB target genes (NFκB index - NFκBi) in normal bone marrow plasma cells (PC), MGUS and some MM. Somatic mutations that result in constitutive activation of the NFκB pathway are present in ~ 25% of MM at diagnosis and 40% of HMCL that have the highest NFkBi. In contrast, other MM and HMCL have progressed independently of NFκB and have lower levels of NFκBi. NFκB signaling may be inhibited at the cell surface with decoy receptors (BCMA-Fc, TACI-Ig) or with agents that target various components of the signaling pathway (glucocorticoids, proteasome inhibitors, IKKB inhibitors).

(>80%) then in those that express CCND1 (~30%). Although with largely redundant functions, CCND1 has additional ability to antagonize RB not shared by CCND2, suggesting that CCND2 tumors may be more critically dependent on the absolute level of RB protein. A variety of **secondary genetic events** associated with disease progression occur with a similar frequency in hyperdiploid and non-hyperdiploid tumors (17p loss or **p53** mutations, **RAS** mutations, secondary Ig translocations, **MYC** translocations, and inactivation of **p18INK4c** or **RB1**. In contrast, a promiscuous array of mutations that activate the **NFκB** pathway are less common in hyperdiploid MM, which nonetheless has high levels of presumably ligand-dependent NFκB activity and appears particularly dependent on the bone marrow microenvironment[3,4].

Activation of the NFκB pathway occurs in the bone marrow as a result of ligand-dependent interactions[5]. The TNF family ligands **BAFF** and **APRIL** are elevated in the serum of MM patients and are produced mainly in the BM microenvironment by monocytes and osteoclasts. MM cells express the receptors **BCMA** and **TACI**, but not BAFF-R[6]. The transcriptional regulation of NFkB target genes is mediated by an array of homo- and hetero-dimers containing p50 and p52, the active isoforms of **NFκB1**(p105) and **NFκB2**(p100) respectively. The function of these dimers is dependent on ubiquitin mediated proteasomal degradation; however, the regulation of this process is different for dimers containing p50 or p52. The classical or "canonical" NFκB (c NFκB) pathway is dependent on two different proteasome dependent degradation steps. The first step is the co-translational degradation of p105 into p50. The second and rate-limiting step is the degradation of the inhibitors of NFκB (IκBs) complex, which retain p50-containing complexes in the cytoplasm. In contrast, the "non-canonical" NFkB (ncNFκB) pathway is regulated by a single proteasome dependent degradation step that mediates the processing of p100 into p52. The activation of both pathways is dependent on extra-cellular stimuli that initiate the degradation of the IκBs or the processing of NFκB2. In some cases a single stimuli, such as cross-linking of CD40 by CD40L, can activate both pathways while in other instances the stimuli activate either the canonical or non-canonical pathway, such as TNF-a or BAFF respectively. Although the series of events linking receptor engagement and the degradation of the Ikβ complex is well documented, the process regulating the non-canonical pathway is not clearly defined. Several proteins are clearly required for the processing of NFκB2 including the absolute requirement of both NIK and IKKa that are dispensable for NFκB1 processing. In the absence of extra-cellular stimuli **NIK** is co-translationally degraded in a **TRAF3** dependent manner to prevent unwarranted activation of the pathway. We have proposed the existence of a cytoplasmic **NIK** regulatory complex containing, at a minimum, **TRAF2**, **TRAF3**, **cIAP1**, and **cIAP2** (all genes found deleted or mutated in MM) that is inactivated by either receptor engagement or mutational inactivation of any of the required components. This places **NIK as a central mediator of non-canonical activity**. We postulate that constitutive activation of NIK in plasma cells may induce MM progression.

The importance of the NFκB pathway in MM is clearly demonstrated by the exquisite sentitivity of MM cells to the proteasome inhibitor Bortezomib and to an IKKB inhibitor[3,7-9]. The relative contribution, however, of the two NFκB pathways in MM is under active investigation. Although the majority of mutations identified in MM specifically target the ncNFκB pathway[4], it has recently been shown that ncNFκB activation can also lead to increased c NFκB activity, raising the possibility that the transcriptional activity of both pathways is implicated in MM tumorigenesis. Furthermore it has been shown that the survival of the MM with activating mutations is reduced by a drug that inhibits IKKB kinase activity (MLN120B), but is independent of a highly-specific genetic knockdown (shRNA) of IKKa[3]. The implication of these results is that it is sufficient to target the cNFκB pathway in order to obtain a therapeutic effect in MM. Analysis of the APEX clinical trial suggests that patients with mutations that inactivate TRAF3 are both particularly sensitive to bortezomib, and resistant to dexamethasone, highlighting the importance of studying the clinical implications of these mutations[4]. Eventually the use of specific biomarkers together with specific targeted

therapies will allow the treatment of MM patients to be individually tailored to the common genetic mutations unique to each patient.

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Cytokine Pathways In Myeloma Growth And Survival

Anne Catherine Sprynski[1], Dirk Hose[2,3], Jérôme Moreaux[1,4], Thierry Reme[1,4], Michel Jourdan[1], Philippe Bourin[5], Jil Corre[5], Bart Barlogie[6], John Shaughnessy[6], Jean François Rossi[1,4,7], Golsdchmidt Harmut[2,3], Bernard Klein[1,4,7]

1 *INSERM, U847, Montpellier, F-34197 France;*
2 *Medizinische Klinik V, Universitätsklinikum Heidelberg, INF410, Germany;*
3 *Nationales Centrum für Tumorerkrankungen, INF350, D-69115 Heidelberg, Germany;*
4 *CHU Montpellier, Institute of Research in Biotherapy, F-34285 France;*
5 *EFS, Toulouse, France*
6 *Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences, Little Rock, AR, USA*
7 *Université MONTPELLIER1, UFR Medecine, Montpellier, France;*

Adress for correspondence. Pr Bernard Klein, INSERM U847, Institute for Research In Biotherapy, CHU Montpellier, Hospital St Eloi, Av Augustin Fliche
34295 Montpellier –FRANCE, tel +33(0) 4 67 33 04 55, bernard.klein@inserm.fr

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Introduction

Numerous myeloma cell growth factors (MGF) have been reported in human multiple myeloma (MM) using in vitro studies. These MGF are IL-6, IGF-1, HGF, BAFF/APRIL, the EGF family, IL-10, IFN-alpha, Wnt family, IL-21 [1]. Their in-vitro efficacy was proven using the stimulation of growth human myeloma cell lines (HMCLs), of the survival of primary multiple myeloma cells (MMC), of the reversal of MMC apoptosis induced by cytotoxic drugs. These MGF have been shown to trigger a restricted set of signaling transduction pathways: MAPK and JAK/STAT for IL-6 and IFN-alpha, MAPK and PI-3/AKT for IGF-1, HGF or EGF family and MAPK, JAK/STAT and NF-□B for BAFF/APRIL. These MGF firstly promote MMC survival, partly by upregulating MCL1 expression and secondly, further promote cell cycle transition that is already deregulated by various translocations or gene amplifications resulting in at least one cyclin D gene overexpression. Using IL-6 dependent cell lines, we have shown that the IL-6 concentration required to promote cell survival was 100 fold higher than that required for cell cycle transition [2].

The ability of these MGF to trigger MMC growth is largely a reflection of their ability to support the proliferation of normal polyclonal plasmablastic cells (PPC) and the long-term survival of bone marrow plasma cells (BMPC). BMPC may survive for more than 20 years in the bone marrow in contact with a BM niche that is close to the hematopoietic stem cell niche [3]. Several studies have shown in mice the important role of IL-6, APRIL, IL-10 and IL-21 to trigger normal PC differentiation and survival.

The involvement of these MGF in the development and acute phase of MM disease in vivo is accumulating. Whether expression of MGF receptors on MMC has prognostic value is not clear presently. Challenging results were published for IGF-1R expression using small patient cohorts, one study showing some prognostic value [4], another one a lack of prognosis value [5]. No data were reported for IL-6R, gp130 IL-6 transducer, IL-21R, c-met (HGF receptor), the 3 BAFF and/or APRIL receptors – BAFF-R, BCMA, TACI -, or other MGF receptors. Serum levels of IL-6 and sIL-6R (that has an agonist activity), or of CRP, a surrogate marker of IL-6 activity, are associated with a poor prognosis.

Treatment with anti-IL-6 antibodies results in a blockade of MMC proliferation and disease stabilization throughout treatment [6]. But disease resumes rapidly after treatment stop.

Treatment with BAFF/APRIL inhibitors also results in disease stabilization, with a marked decrease on the levels of polyclonal immunoglobulins [7].

Results and Discussion.

Our aim was to rank 5 well-studied MGF – IGF-1, IL-6, HGF, BAFF/APRIL, EGF family - according to their in-vitro efficacy and the prognostic value of their receptor expression on multiple myeloma cells (MMC) in vivo [8].

Using a set of 9 human myeloma cell lines (HMCLs) cultured in serum-free culture medium, we found that IGF-1 is a major MGF stimulating the growth of 8/9 of HMCLs. IL-6 is also an important MGF but requires a production of autocrine IGF-1 to stimulate MM cell growth. The other studied MGF stimulated only a part of the HMCLs, 2 of 9 for HGF, 2 of 9 for HB-EGF, and 1 of 9 for APRIL.

Of interest, this ranking of MGF according to their ability to simulate HMCL growth in vitro fits well with their respective importance in vivo. Looking for the prognostic value of MGF receptor expression on MMC, only *IGF-1R* and *IL-6R* gene expressions were proven to have bad prognosis value in two independent series of respectively 168 and 345 newly-diagnosed patients. The HGF receptor – c-met -, the BAFF and APRIL receptors, TACI or BCMA – and the EGF family receptors had no prognosis value. *IGF-1R* gene was expressed in 31% and 50% of MMC of the 2 independent patients series using Affymetrix call. *IL-6R* was expressed in all MMC. Patients with $IGF\text{-}1R^{present}$ MMC had a decreased event-free survival and overall survival compared to patients with $IGF\text{-}1R^{absent}$ MMC. Patients with MMC whose *IL-6R* expression was above the median ($IL\text{-}6R^{high}$ MMC) had also a decreased EFS and OAS compared to patients with $IL\text{-}6R^{low}$ MMC.

The frequency of patients with $IGF\text{-}1R^{present}$ MMC and $IL\text{-}6R^{high}$ MMC was increased in the poor prognostic MM subgroups, according to the Little Rock classification [9], the proliferating group, the t(4;14) translocation group and the group with Maf overexpression. Noteworthy, the prognostic value of $IGF\text{-}1R^{presence}$ remains in patients without t(4;14) translocation.

Regarding the production of the growth factors in vivo, we look for the expression of the genes in MMC and the various bone environment populations. *IGF-1* gene was mainly expressed by osteoclasts and MMC, *IL-6* by stromal cells, *HGF* by MMC, *EGF* family members by monocytes and MMC and *APRIL* by osteoclasts and monocytes.

Given the importance of bone environment in MM disease, we looked for the cell communication signals produced by osteoclasts and bone marrow stromal cells (BMSCs). 557 genes were upregulated in osteoclasts compared to the other cell components of the bone marrow (monocytes, neutrophils, T cells, BMSCs, normal B cells, plasmablasts, BMPC, and MMC). These genes encode for cell communication signals. In particular, we found 4 genes coding for chimiokines targeting CCR2 receptor. Osteoclasts can attract MMC and an anti-CCR2 antibody blocks this attraction. Noteworthy, this in vitro finding has in vivo relevance. *CCR2* gene expression is increased in MMC compared to normal PPC, BMPC or B cells. In addition, patients with $CCR2^{high}$ MMC had increased numbers of bone lesions compared to patients with $CCR2^{low}$ MMC.

Regarding BMCS, they highly expressed the gene for SDF-1 that recruits CXCR4 expressing MMC and they produce largely IL-6. In addition, comparing the GEP of BMSCs from patients with newly-diagnosed stage 3 MM and from age-related healthy individuals, we found that BMSCs of patients with MM had a specific GEP, although these cells were cultured for 20 doubling time in vitro. Using unsupervised hierarchical clustering, MM BMSCs and healthy donor BMSCs cluster in 2 well-separated groups, and MGUS BMSCs distributed in these 2 clusters. A supervised analysis indicated that MM BMSCs overexpressed genes coding for GDF15, amphiregulin, IL-1 and IL-6. Of interest, GDF15 stimulated the growth of stroma dependent HMCL [10]. Thus, BMSCs and osteoclasts produce a complementary network of communication signals attracting MMC close to the endosteum and promoting MMC growth.

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Novel Antigenic Targets For Immunotherapy In Myeloma

Qing Yi, MD, PhD

Department of Lymphoma and Myeloma, Division of Cancer Medicine, and the Center for Cancer Immunology Research, The University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA.

Multiple myeloma (MM) is still a fatal disease. Even with advances in high-dose chemotherapy and autologous stem-cell support, relapses of the underlying disease remain the primary cause of treatment failure. Novel therapeutic approaches that have a mode of action different from and non-cross-resistant with cytotoxic chemotherapy are required to eradicate tumor cells that have become multidrug resistant. To this end, immunotherapy aimed at inducing or enhancing myeloma-specific immunity in tumor-bearing patients may be desirable. Indeed, in the post-allograft relapse setting of MM (in which patients are chemotherapy refractory), long-lasting disease remission has been achieved after infusion of donor lymphocytes, suggesting that chemotherapy and T-cell–mediated cytolysis kill myeloma cells by different modes of action that are non-cross-resistant.

Idiotype (Id) proteins are tumor-specific antigens, and active immunization against these Id determinants on malignant B cells has been shown to produce resistance to tumor growth in transplantable murine B-cell lymphoma and plasmacytoma. Early clinical trials of Id-pulsed dendritic cell (DC) vaccination in MM by many groups including ours reported disappointing results. Fewer than 50% of patients mounted an Id-specific immune response, and clinical responses have rarely been observed. To improve the efficacy of DC vaccination in MM, we investigated the use of Id-pulsed mature DCs administered subcutaneously. Five patients with stable partial remission following high-dose chemotherapy were vaccinated with DC vaccines starting at least 4 months post-transplantation. After 3 DC vaccinations, Id-specific T-cell responses were elicited in 4 patients (4 by ELISPOT assay and 2 by proliferation assay), and anti-Id B-cell responses were elicited in all 5 patients. The cytokine-secretion profile of activated T cells demonstrated a type-1 response. A 50% reduction in serum Id protein was observed in one immunologically responding patient that persisted for more than one year, and stable disease resulted in the other 3 patients. The remaining patient without an immune response to the vaccination relapsed. These results are promising but also call for additional improvements to optimize DC-based immunotherapy in this disease. One of the strategies is to search for and use other myeloma antigens for immunotherapy in patients.

The identification of novel tumor-associated antigens, particularly those shared among patients, is urgently needed to improve the efficacy of immunotherapy for MM. We examined whether Dickkopf-1 (DKK1), a protein expressed by the tumor cells of nearly all myeloma patients, but not expressed in most normal tissues, is a candidate antigen. We identified and synthesized DKK1 peptides for HLA-A*0201 and confirmed their immunogenicity by in vivo immunization in HLA-A*0201 transgenic mice. We detected low frequencies of DKK1 peptide-specific $CD8^+$ T cells in myeloma patients by using peptide-tetramers and generated peptide-specific T-cell lines and clones from HLA-A*0201^+ blood donors and myeloma patients. These T cells efficiently lysed peptide-pulsed but not unpulsed T2 or autologous dendritic cells, $DKK1^+$/HLA-A*0201^+ myeloma cell lines U266 and IM-9, and more importantly, HLA-A*0201^+ primary myeloma cells from patients. No killing was observed on $DKK1^+$/HLA-A*0201^- myeloma cell lines and primary myeloma cells or HLA-A*0201^+ normal lymphocytes, including B cells. Moreover, after adoptive transfer, these T cells were therapeutic against established myeloma in SCID-hu mice. These results indicated that these T cells are potent cytotoxic T cells and recognize DKK1 peptides naturally presented by myeloma cells in the context of HLA-A*0201 molecules. Hence, our study identifies DKK1 as a potentially important antigen for immunotherapy in MM.

Tumor cell-derived heat shock proteins (Hsps) such as gp96 have been used as vaccines for immunotherapy of cancer patients. However, current approaches for immunotherapy are individualized and require the generation of a custom-made product. To improve the applicability and feasibility of Hsp-based immunotherapy in cancers such as MM and to enhance clinical efficacy, we have explored using pooled allogeneic cancer cell line-derived gp96 as a universal vaccine for cancer immunotherapy. We modeled this in a myeloma setting and clearly demonstrated that gp96 vaccination is able to protect mice from tumor challenge and rechallenge and that pooled allogeneic gp96 was as effective as autologous gp96. Pooled gp96BCD plus CpG in combination with anti-B7H1 or anti-IL-10 antibodies were much more effective at eradicating established myeloma than that in combination with anti-CD25 antibody in myeloma-bearing mice. We showed that IFN-γ and $CD8^+$ T cells were required for gp96-induced antimyeloma immune and clinical responses and that pooled allogeneic gp96BCD was as effective as autologous gp96A to induce a potent cytotoxic T-cell response capable of killing unrelated myeloma cells. Together, our study lays the basis for future clinical trials in MM and possibly other cancers by using pooled allogeneic gp96 from human tumor cell lines as a universal vaccine.

β_2-microglobulin (β_2M) is the invariant chain of the MHC class I molecule on the surface of nucleated cells. Free β_2M is found in body fluids under physiologic conditions as a result of intracellular release. Elevated levels of soluble β_2M are present in hematologic malignancies, including MM, lymphomas, and leukemias, and correlate with a poor prognosis regardless of a patient's renal function. This observation suggests an important, yet unidentified, role of this protein in MM. While examining the effects of β_2M on myeloma cells, we made a novel and exciting discovery, namely that monoclonal antibodies (mAbs) against human β_2M have a remarkably strong apoptotic effect on myeloma cells. Anti- β_2M mAbs induced apoptosis in as many as 90% of cells in a 48-hour (h) culture in all tested human myeloma cell lines (n = 8) and primary myeloma cells from patients (n = 10). The mAbs also kill β_2M/MHC class I-bearing lymphoma and leukemia cells. Anti-MHC class I mAbs (LY5.1, IgG1 or W6/32, IgG2a), purified mouse IgG and IgG1 had no effect. Cell death occurred rapidly, without the need for exogenous immunological effector mechanisms (e.g., complement or NK cells) or secondary cross-linking. Anti- β_2M mAb-induced apoptosis in myeloma cells were not blocked by soluble β_2M (10–100 μg/mL, 3- to 30-fold higher than the levels in most MM patients, which is about 3 μg/mL), IL-6, or other myeloma growth and survival factors, and was stronger than apoptosis observed with chemotherapy drugs currently used to treat MM (e.g., dexamethasone). Although the expression of β_2M on normal hematopoietic cells is a potential safety concern, the mAbs were selective to tumor-transformed cells and did not induce apoptosis of normal cells, including T and B lymphocytes, plasma cells, and purified $CD34^+$ stem cells. Furthermore, the mAbs selectively and effectively killed myeloma cells without damaging osteoclasts (OCs) or blood mononuclear cells in their cocultures with myeloma cells. More importantly, anti- β_2M mAbs were shown to be therapeutic in vivo in xenograft SCID and SCID-hu mouse models, and in the HLA-A2-transgenic NOD-SCID (A2-NOD-SCID) model of myeloma, in which every mouse tissue expresses human MHC class I/ β_2M molecules and circulating human β_2M can reach the levels seen in most myeloma patients without causing damage to normal human hematopoiesis or murine organs. Interestingly, following our publication, others reported similar results using anti-MHC class single-chain Fv diabody or anti- β_2M antibodies, respectively, to induce apoptosis in human myeloma or renal cell carcinoma. These results indicate that anti- β_2M mAbs may have potential as a therapeutic agent to treat MM and other malignancies.

Finally, our recent studies have suggested that C-reactive protein (CRP) may also be a therapeutic target in MM. Elevated levels of CRP are present in many disease situations including malignancies, and may contribute to the pathogenesis of cardiovascular disorders. We have undertaken a study in a myeloma setting to determine whether CRP affects tumor cell growth and survival. We showed that CRP enhances myeloma cell proliferation under stressed conditions and protects myeloma cells from chemotherapy drug–induced apoptosis in vitro

and in vivo. CRP binds activating Fcγ receptors; activates PI3K/Akt, ERK, and NF-κB pathways; and inhibits caspase cascade activation induced by chemotherapy drugs. CRP also enhanced myeloma cell secretion of IL-6 and synergized with IL-6 to protect myeloma cells from chemotherapy drug–induced apoptosis. Thus, our results implicate CRP as a potential target for cancer treatment.

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Growth And Survival Signals In Myeloma: Roles For Baff and April.

Diane F. Jelinek, Ph.D.

Mayo Clinic, Rochester, MN USA

B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) are members of the tumor necrosis factor superfamily displaying significant sequence homology with each other. Curiously, three receptors have been identified: B cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI), and BAFF-R. Whereas BAFF binds to all three receptors, APRIL only binds to TACI and BCMA. Although all three receptors display a largely B lineage-restricted pattern of expression, there is clear evidence that the precise BAFF/APRIL binding profile is a dynamic and highly regulated process. Thus, we have shown that: 1) normal human naïve and memory B cells express BAFF-R; 2) TACI is expressed only by memory B cells and a subpopulation of activated $CD27^{neg}$ B cells and plasma cells (PCs) to a lesser degree; and 3) BCMA expression is restricted to PCs (1). Importantly, the significance of this regulation remains incompletely understood as does what signals drive receptor expression during B cell differentiation and biological consequences of expressing various combinations of receptors.

The differentiation stage specific pattern of receptors does however suggest specialized roles for each receptor. For example, BAFF appears essential for normal B cell development and homeostasis and is consistent with the expression pattern of BAFF-R (2). Moreover, BCMA appears critical for development of bone marrow resident long-lived PCs, also consistent with the largely PC-restricted pattern of BCMA (3). Recently, Noelle and colleagues reported the dependence of PCs and independence of memory B cells on BAFF and APRIL (4). In addition, a specialized role for APRIL in the biology of normal as well as malignant PCs is further suggested by the now appreciated ability of APRIL to also bind to heparan sulfate proteoglycans such as syndecan-1, a molecule characteristically expressed at high levels by PCs (5). TACI expression and its unique role is even more enigmatic but may be specialized to facilitate molecular changes induced by antigen in differentiating B cells such as somatic hypermutation and class switch recombination as suggested by its expression only on antigen experienced B cells (1).

Because tumor cells are notorious for their ability to "hijack" pathways utilized in other cells or normal counterpart cells to promote growth and/or extend survival, we have studied BAFF/APRIL and BAFF-R/TACI/BCMA expression in the chronic B cell malignancies, including multiple myeloma (MM) and were the first to demonstrate that some MM cells could acquire expression of a novel aberrant autocrine BAFF expression pathway that may fuel tumor progression (6). In this same study, we also showed that the BAFF/APRIL receptor profiles varied from patient to patient, although BCMA expression was consistently observed. The mechanisms underlying differential receptor expression remains unclear. Since these initial studies of BAFF and APRIL in MM, we and others have carried out additional experimentation with an end-goal of identifying agents that would interrupt this putative crucial pro-survival and perhaps growth-promoting cytokine axis. Relevant to new work that will be presented are prior studies by Klein and colleagues describing the ability of MM cell TACI expression to predict disease outcome. Thus, the $TACI^{LO}$ subset of MM patients was associated with a bad prognosis and gene expression profiling revealed that the $TACI^{HI}$ MM cells exhibit a signature suggestive of a dependence on the microenvironment (7). The association between TACI expression and low risk disease was confirmed by Yaccoby et al (8) who further demonstrated that a TACI-Ig fusion protein induced $TACI^{HI}$ MM cell death in a SCID-hu mouse model. Other strategies to target this pathway have also had some success, including antibodies to BAFF (9).

Therapeutic strategies designed to interrupt this pro-survival pathway have thus far primarily focused on blocking ligand binding. Therapeutic

modalities impacting receptor expression may be similarly effective. However, despite the apparent precise activation stage-dependent orchestration of B cell BAFF-R, TACI, and BCMA expression, the genetic mechanisms regulating expression of the three receptors remain undefined, and the question of whether each receptor governs expression of the other two remains unanswered. To address this, we have employed a panel of MM cell lines we have established and potential promoter constructs of each receptor are being used to identify critical factors responsible for the orderly expression of all three receptors. To this end, we have already identified the putative BAFF-R promoter, which is active in a range of non-MM human immature and mature B cell lines but is inactive in BAFF-R negative MM cells. These results suggest that B lineage cells at the plasma cell stage of differentiation no longer express a transcription factor(s) critical for BAFF-R expression. It also remains unclear as to whether loss of BAFF-R expression is coincidental with changes in transcription factor expression or alternatively, that extinction of BAFF-R expression is a requisite step in PC generation. Similar studies are in progress to map critical elements underlying TACI and BCMA expression.

In addition to using reporter promoter constructs, we have employed two recently established and characterized sister MM cell lines designated ALMC-1 (established from a patient diagnosed with primary amyloidosis; AL) and ALMC-2 (established from the same patient after autologous stem cell transplantation and relapse to symptomatic MM) that differ with respect to BAFF and APRIL responsiveness and TACI expression (10). Of interest, ALMC-1 cells directly respond to exogenous APRIL and BAFF and proliferative responses to IL-6 and IGF-I are significantly augmented by each cytokine as well. By contrast, ALMC-2 cells are only marginally responsive to either cytokine in this assay. We hypothesize these observations can be explained by the differential expression of TACI and BCMA in the two sister cell lines. Thus, ALMC-1 cells coexpress TACI and BCMA whereas ALMC-2 cells solely express BCMA. This receptor phenotype was also exhibited by the primary patient cells from which each cell line was derived. These results suggest that signals elicited downstream of TACI differ from those elicited downstream of BCMA and may explain why loss of TACI is associated with more aggressive disease. To our knowledge, this would be the first description of a AL/MM patient whose tumor cells evolved from expressing TACI into tumor cells lacking expression of this receptor. Because of matched genetic backgrounds, these fortuitous cell lines have permitted us to directly assess the gene expression signatures of $TACI^{HI}$ vs $TACI^{LO}$ MM cells. Study of these cell lines also provides new insight into mechanisms underlying generation of these two subsets of MM. Molecular analyses of the two cell lines using metaphase cytogenetics and array CGH fails to reveal structural abnormalities of chromosome 17 where the *TNFRSF13b* (TACI) gene resides. Ectopic expression of TACI into the ALMC-2 cell line will also permit an assessment of the biological impact of this receptor on tumorigenesis using a NOD/SCID model system. Collectively, our recent studies provide crucial new insight into mechanisms regulating BAFF/APRIL receptor expression in MM cells and a more accurate signature of $TACI^{HI}$ vs. $TACI^{LO}$ MM variants.

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Immune Microenvironment In Myeloma Pathobiology

Madhav V. Dhodapkar

MD; Yale University, New Haven, CT, USA

There is a growing body of evidence that clinical behavior of tumors depends not just on the genetic changes in tumor cells, but also their interaction with the microenvironment. This is particularly true in the case of myeloma (MM) and its precursor lesion, monoclonal gammopathy of undetermined significance (MGUS). Recent studies have shown that the great majority of cytogenetic changes initially found in the tumor cells in MM can also be detected in the tumor cells in MGUS. Therefore changes in the microenvironment may play a major role in regulating malignant transformation in MM. An important component of this microenvironment are the cells of the immune system. A growing body of data from several labs now suggests that these cells can play a dual role, both promoting and inhibiting tumor growth in MM. Some aspects of the immune response appear to be protective. For example, tumor bed in MGUS is enriched in preneoplasia specific Th1T cells that make IFNγ[1,2]. Progression to MM is associated with the loss of this effector response, although tumor reactive T cells can still be detected in the myeloma marrow. Several aspects of the tumor microenvironment may contribute to this, including immune suppressive cytokines (such as VEGF, TGFβ), T regulatory (Treg) cells, myeloid derived suppressor cells[3], and upregulation of coinhibitory molecules (such as PDL1)[4] or alteration in the expression of ligands for immune activation receptors (such as MHC class I related protein A; MICA)[5] on tumor cells.

An important aspect of the immune response in preneoplasia may be the nature of targets of spontaneous immunity. For example, recent studies suggest that the presence of immune responses against SOX2, a gene critical for the biology of embryonal stem cells (and also expressed on putative MM stem cells), is associated with long term stability of disease[6]. In addition to CD4 and CD8+ T cells, cells of the innate system may also play a major role in tumor regulation. For example, myeloma cells express CD1d and are sensitive to lysis by CD1d restricted natural killer T (NKT) cells[7]. Other innate cells including NK cells and γδ T cells can also mediate anti-myeloma effects and are therefore being targeted in the clinic. Certain components of the host response may in fact, contribute to worsening of tumor growth and have a potentially deleterious effect. A common emerging theme with the progression to MM appears to be an increase in inflammation. This includes an increase in inflammation associated cytokines (e.g. IL6, TNF), as well as immune cells associated with inflammation. For example, we have recently observed a marked increase in inflammation associated IL17 producing T cells (Th17 cells) in the tumor bed in MM[8]. Inflammation may also lead to increase in bioactive lipids that can be direct targets of CD1d restricted T cells (termed type II NKT cells) that secrete T helper 2 cytokines, and inhibit immunity[9]. In addition to lymphocytes, an important aspect of the tumor bed is the infiltrating myeloid cells, that may have major effects on tumor growth and biology. For example, MM tumors are commonly infiltrated with dendritic cells (DCs) and these may play a role in promoting growth, survival and clonogenicity of tumor cells[10]. Alternately, at least in mouse models, some macrophages mediate an important anti-tumor effect. It is likely that both DCs and macrophages in the tumor bed provide both growth promoting and anti-tumor effects depending on the signals they receive from tumor cells and the microenvironment. Interactions between tumor cells and the immune microenvironment have already taken center stage in MM therapeutics. For example, both thalidomide and its analogue lenalidomide can activate both innate and adaptive immunity. Induction of tumor cell death by Bortezomib, a proteasome inhibitor, leads to exposure of heat shock proteins, including hsp90 on the surface of dying cells. Uptake of these cells by DCs leads to DC activation and subsequent generation of anti-tumor immunity in culture. These studies have provided the rationale for combining these agents with other biologic therapies such as vaccines and

monoclonal antibodies. Understanding the balance between immunity and inflammation in MM may provide novel approaches to not only treat MM, but also to prevent the progression of this tumor.

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Monoclonal Antibody-Based Therapies in Human Multiple Myeloma

Yu-Tzu Tai, Mariateresa Fulciniti, Pierfancesco Tassone, Paola Neri, Constantine Mitsiades, Teru Hideshima, Dharminder Chauhan, Paul Richardson, Kenneth C Anderson, Nikhil C Munshi

Dana-Farber Cancer Institute, Boston VA Healthcare System, Harvard Medical School, Boston, MA

Monoclonal antibodies (mAb) are now established as an important therapeutic modality in various cancers. Since the first approval of Rituximab targeting CD20 by US FDA for the treatment of B-cell non-Hodgkin's lymphoma in 1997, at least one anticancer mAb has been approved each year. However, there is still no mAb-based therapy approved for the treatment of multiple myeloma (MM). By 2000, there were relatively few surface antigens on the plasma cells suitable for mAb-directed treatment. Possible molecules include CD20, HM1.24, CD38, ICAM (CD54), CD40, and syndecan-1(CD138). Studies in early 2000 showed that Rituximab and anti-CD38 Abs had minimal clinical activity due to only few MM cells expressing CD20 and nonspecific toxicities of anti-CD38 mAbs conjugated with immunotoxin. Despite disappointing beginning with these two mAbs, more than 10 potential mAb candidates targeting MM cells, i.e., CD40, IL-6, CD56, BAFF, IL-6R, CS1, IGF-1R, CD38, TRAIL-R1, osteoprotegerin (OPG), DKK, CD74, VEGF, have entered clinical development in recent years. This is mainly accredited to better understanding of MM biology and identification of antigen expression pattern by gene expression profiling and oncogenomics, as well as the advancement in mAbs engineering.

Many of these mAbs are now being tested as single agent or as conjugate targets to assess their efficacies in improving response to current treatments. Two anti-CD40 mAbs SGN-40 [1,2] and HCD122 [3,4] are currently under clinical investigations in MM. Unlike SGN-40, which is a weak agonist in stimulating normal B-cell proliferation, HCD122 is an fully human IgG_1 antagonistic mAb specifically blocked CD40L-induced adhesion, cytokine secretion, and survival of MM [3]. Both mAbs kill MM cells via antibody-dependent cellular cytotoxicity (ADCC) and blocks MM cell growth in mice [1,3]. Although SGN-40 did not prove to be highly effective in MM patients as a single agent [2], SGN-40 Phase Ib clinical trials in combination with lenalidomide and bortezomib were recently initiated based on enhanced SGN-40-induced cytotoxicity by lenalidomide in preclinical studies [5].

HuN901-DM1 (BB-10901), an anti-CD56 mAb conjugated with a potent antimicrotubular cytotoxic agent DM1, has significant in vitro and in vivo antimyeloma activity at doses that are well tolerated in a murine model [6]. Phase I study of BB-10901 has provided preliminary evidence of safety and clinical activity in MM patients [7].

Most recently, others and we have defined the cell surface glycoprotein CS1 (CD2 subset 1, CRACC, SLAMF7) to be a new MM antigen [8,9]. CS1 is differentially expressed on the cell surface of the majority of MM cell lines and patient MM cells (>95% tested samples) but not in normal organs or CD34+ stem cells. Importantly, a novel anti-CS1 elotuzumab (formally HuLuc63) triggered autologous ADCC against primary MM cells resistant to conventional or novel therapies and inhibits tumor growth in several MM xenografts [8]. Elotuzumab in clinical trials, either alone or in combination with bortezomib or lenalidomide, were currently ongoing [10].

B-B4-DM1, an anti-CD138 mAb conjugated with DM1, is a potent anti-MM agent that kills cells in an antigen-dependent manner in vitro and mediates in vivo antitumor activity at doses that are well tolerated, providing the rationale for clinical trials of this immunoconjugate in MM. B-cell-activating factor (BAFF), a recently defined myeloma survival factor, was mainly produced within BM microenvironment [10]. A clinical grade-neutralizing antibody to BAFF demonstrated an

in vivo anti-myeloma activity, providing the preclinical rationale for its evaluation in the treatment of MM [11]. Anti-CD38 HuMax-CD38 was effective in killing primary CD38+CD138+ patient MM cells and a range of MM/lymphoid cell lines by both ADCC and complement-dependent cyttoxicity (CDC). Clinical trials of the mapatumumab targeting TRAIL-R1, alone or in combination with bortezomib, were recently started. By binding to receptor activator of nuclear factor kappa B (RANK) ligand, OPG prevents the formation and activation of osteoclasts and helps keep the bone loss process in check. Denosumab (AMG 162) mimicking the effects of OPG, has showed early sign of clinical benefit [12] and a novel anti-DKK neutralizing mAb BHQ880 may also represent the next generation of therapeutic options for the enhancement of bone repair in MM [13]. Furthermore, a phase II study of bevacizumab, an anti-VEGF mAb, plus Thalomid in MM is ongoing. A better understanding of the immune defects that prevent MM patients from mounting a strong response against their tumor cells should also improve establishment of effective mAb-based immunotherapy strategies. We expect that, the use of potentially targeted therapies by mAbs, such as naked or immunoconjugate or bispecific, would soon claim defined therapeutic roles in patients with MM. The favorable toxicity profile of tumor-targeted therapy by mAbs, unlike other forms of therapy, would allow the maintenance of quality of life, while efficiently attacking the tumors.

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Defining Multiple Myeloma As A Target For DNA Fusion Gene Vaccines

Surinder S Sahota[1], Niklas Zojer[2], Gavin Babbage[1], Debora Joseph-Pietras[1], Nicola Weston-Bell[1], Karin Vanderkerken[3], Pieter Sonneveld[4], Bernard Klein[5], Karen Pulford[6], Jason Rice[1], Natalia Savelyeva[1] & Christian Ottensmeier[1]

[1]Cancer Sciences Division, School of Medicine, University of Southampton, UK, [2]Center for Oncology and Hematology, Wilhelminenspital, Vienna, Austria, [3]Department Hematology and Immunology, Vrije Universiteit Brussel, Belgium, [4]Department Hematology, Erasmus MC and Erasmus University, Rotterdam, The Netherlands, [5]Institute of Research in Biotherapy, INSERM Unit 847, CHU Montpellier, Hopital Saint Eloi, Montpellier, France, [6]Nuffield Department of Clinical Laboratory Sciences, John Radcliffe Hospital, University of Oxford, UK

For effective immunotherapy in cancer, there is an essential requirement for a precise knowledge of the tumour cell as a target. In multiple myeloma (MM), a tumour where fully differentiated malignant CD138+ plasma cells accumulate in the bone marrow, recent observations have raised questions as to the most appropriate target to ablate tumour. More specifically, reports of a CD138- tumour-related fraction, proposed to harbour putative MM stem cells (MSCs), have raised an important therapeutic issue, as it has been observed that these MSCs are drug resistant.[1] Furthermore, blood CD19+CD27+ cells from MM patients but not CD138+ cells engrafted immunodeficient NOD/SCID mice, suggesting a role for less-differentiated cells in propagating MM growth.[1] Such cells would potentially be more likely to survive during minimal residual disease (MRD), achieved by existing drug therapies. The MRD setting is also generally regarded as conducive to immunotherapeutic intervention.

The MSCNET was formed as a European network to examine the central question of the nature of the MSC in feeding MM growth, as a pre-requisite for delivering effective therapy. We have been evaluating a model where the MSC may be a B-cell (B-MSC) or a plasma cell (P-MSC), or a continuum between the two. In one study, we have focused on light-chain only MM (LC-MM) as a model to dissect the existence of a precursor cell, which should be identifiable by coupled expression of clonally-derived immunoglobulin (Ig) heavy and light chains. To examine this, we sought molecular evidence for rearranged Ig variable (V) genes which encode the Ig dimer. In 6 LC-MM cases, although V_L genes were readily found, transcripts for V_H could not be identified in the majority of cases (5/6). In 3 cases, where genomic residual VDJ segments provided robust probes for very sensitive analyses, no V_H transcripts were detected in 2/3 cases, but in a single case, low levels of clonal V_H transcripts were observed. Cells producing V_H transcripts appear to be rare in LC-MM. Genomic events yielding14q32 translocations have been proposed to cause deletion of the rearranged V_H gene in this form of MM,[2] and were also found in 4/6 of our cases. In this model, it appears that only MM cells that carry the full complement of genetic lesions resulting in a LC-MM phenotype actually feed tumour on relapse, as apparent from long term follow up in this type of disease. This argues against an 'early multipotent progenitor' in MM.[3] In the second study, we examined the 5T33 murine model of myeloma. Using anti-idiotype antibody, phenotypically heterogeneous tumour-related cells could be identified in the bone marrow cells used to passage tumour. Importantly, 5T myeloma offers a syngeneic model to assess tumour-related progeny, by-passing concerns with xenotransplantation, where human tumour-related cells are passaged in niche mismatched immunodeficient mice.[4] The striking finding from our study was that purified CD138+ plasma cells of 5T33 MM can readily seed tumour (Vanderkerken Laboratory). These observations suggest that a role for CD138+ cells in propagating MM cannot be overlooked.

The immunotherapeutic strategy that would offer widest scope however should focus on targets most likely to be retained by any putative

progenitor population and malignant plasma cells in MM. The tumour-derived idiotype (id) fulfils this criterion, and our previous work was based on developing a DNA fusion gene vaccine against this antigen (FK Stevenson Laboratory).[5] The principle of fusing a bacterial alert antigen, fragment C of tetanus toxin, to tumour associated antigen has been described previously.[6] Essentially, vaccine design provides linked T-cell help to potentiate immune responses against specific tumour-associated antigens. These pre-clinical studies have led to an on-going Phase I/II trial using DNA fusion gene vaccines to target id in MM (led by CH Ottensmeier).

Clues may also emerge from antigens which are retained by MM cells post-therapeutic relapse, following complete remission, as it suggests that persisting antigens are a necessary requisite for the re-seeding cell, and may mark any potential MSC. We had previously shown that in MM, of the important class of essentially tumour-specific cancer testis antigens (CTAs) in this disease, PASD1 is a new CTA and is indeed retained post-therapy.[7] To expand this analysis, MSCNET has systematically analyzed a large cohort of CTAs in MM pre- and post-therapy using gene expression profiles (Klein and Sonneveld Laboratories).[8] Interestingly, many of the CTAs regarded as important immunotherapeutic targets in MM are retained following therapy and could be targeted by vaccination during MRD. Another important category of antigens has also emerged during the MSCNET evaluation of the MSC. Reasoning that any putative MSC in this tumour may exhibit a core pattern of genes that are known to govern embryonal stem cell development, a comprehensive subtraction of genes expressed by embryonal related cell lines and MM cells at disease presentation has generated a bank of predominantly new embryonal cancer genes (ECGs) as potential targets for vaccination (Klein Laboratory). Importantly, expression of these ECGs was determined in CD138+ MM cells which had been highly purified for gene expression profiling. This bank also included SOX2, a gene previously described as relevant to clonal evolution in MGUS and MM.[9] Whereas SOX2 marked the clonogenic CD138- compartment in MGUS, it associated with a subpopulation of CD138+ tumour cells in MM, suggesting that in progressive disease, self-renewal becomes a property of a component of the fully differentiated tumour clone.[9] This again points to a role for CD138+ cells in MM growth.

To model vaccine design and intervention, we have used the pDOM[peptide] DNA vaccine format, shown to induce potent T-cell responses even in a challenging tolerised setting.[10] We have focused on PASD1, and in collaborative work had access to 2 HLA-A2 derived CD8+ cytotoxic T-cell (CTL) motifs known to be recognised by A2 positive lymphoma T-lymphocytes (Pulford Laboratory). We generated pDOM[PASD1-P1] and pDOM[PASD1-P2] vaccines against these 2 epitopes, and in the HHD A2 humanised transgenic murine model, were able to induce consistent peptide-specific T-cell responses as judged by *ex-vivo* IFN-□ ELISpot assays in all vaccinated mice. These T-cells mediated killing of specific targets. Boosting by delivery of DNA vaccine with electroporation enhanced killing via PASD1-P1. This vaccine design will also be applicable to A2 CTL motifs from ECGs, and we are currently evaluating anti-SOX2 responses. The relevance of this design for MM therapy is highlighted by a second on-going Phase II trial in a solid tumour setting, where a pDOM[peptide] vaccine design specific for a prostrate-specific membrane antigen (PSMA) epitope has been delivered in A2+ve prostrate cancer (led by CH Ottensmeier). Trial read-outs indicate induction of marked levels of epitope specific T-cells. Critically, the vaccine design induced high levels of CD4 T_H cells which can be readily detected *ex-vivo*, thereby recapitulating the principle of T-cell help established in pre-clinical models.[6] Those data also show long lasting CD8+ T-cell responses (over 1 year) at high levels in ca 70% of patients. These findings support the promise of DNA fusion gene vaccines in inducing T-cell control of malignancy.

Taken together, use of DNA fusion gene vaccines suggests that immune control of MM appears to be a real possibility, providing a flexibility of design in dealing with any of the guises that the MSC may adopt.

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p62 – A Potential Target For Blocking Microenvironmental Support of Myeloma

G. David Roodman[1,2], Yuko Hiruma[1] and Noriyoshi Kurihara[1]

[1]University of Pittsburgh, Medicine/Hem-Onc, Pittsburgh, PA; and [2]VA Pittsburgh Healthcare System, Medicine/Hem-Onc, Pittsburgh, PA. USA

Adhesive interaction between myeloma (MM) cells and stromal cells increase production of cytokines such as TNF-α, IL-6 and RANK ligand (RANKL) by the stromal cells, which induce MM cell growth and OCL formation.(1) These adhesive interactions activate multiple signaling pathways in stromal cells that play important roles in their enhancement of tumor growth and bone destruction. Ishitsuka and coworkers have shown that p38 MAPK is involved in the increased osteoclastogenesis in MM and that blocking p38 MAPK inhibited IL-6 secretion from long term cultures of bone marrow stromal cells derived from MM patients.(2) Further, inhibition of p38 MAPK decreased osteoclastogenesis in a murine model of human MM. Mitsiades et al have shown that NF-κB is also activated in bone marrow stromal cells when MM cells bind to marrow stromal cells, and that TNF-α increased adhesion of bone marrow stromal cells to MM cells and increased production of IL-6.(3) These results suggest that inhibition of multiple signaling pathways will be required to block the stimulatory effects of marrow stromal cells in MM. Therefore, we determined if targeting p62 could affect multiple signaling pathways in MM stromal cells. p62 is an adaptor protein that has no intrinsic enzymatic activity but is a critical component of multiple signaling pathways activated in MM stromal cells. p62 forms multiprotein complexes, which result in activation of ERK, p38, and NF-κB.(4,5) We previously reported that increased signaling through p62 in OCL precursors also increases OCL formation in vitro and in vivo.(6,7) Therefore, we determined if some of these signaling pathways are activated in primary marrow stromal cells from MM patients, their respective roles on MM cell growth and osteoclast formation, and the effects of knockdown or loss of p62 on tumor growth and OCL formation.

When primary marrow stromal cells from patients with MM were isolated and cultured for three weeks in Dexter-marrow cultures, the stromal cells from MM patients produced increased levels of IL-6 and VCAM-1, and demonstrated an increased capacity to support the growth of both IL-6 independent and IL-6 dependent human MM cell lines compared to primary normal marrow stromal cells. These differences in VCAM-1 and IL-6 production by marrow stromal cells from patients with MM and normals did not appear to result from loss or enrichment of endothelial cells or monocytic within the mixed-cell population of stromal cells present in these preparations, since FACS analysis demonstrated similar distributions of endothelial cells and monocytic cells in these preparations. These differences appear to be intrinsic to the MM-derived marrow stromal cells, since after passage for three weeks in the absence of any detectable CD138 expression in the stromal cell preparation, the stromal cells still expressed high levels of IL-6, VCAM-1, and TNF-α. These results suggest that an intrinsic change occurs in the marrow microenvironment of patients with MM that persists even after culture for more than three weeks. The basis for this change in the intrinsic properties of the stromal cells is currently unknown, but may reflect epigenetic changes that occur with chronic exposure of stromal cells to MM cells or cytokines that are upregulated in the marrow microenvironment in response to MM cells. Marrow stromal cells from patients with MM showed greater responses to TNF-α, a cytokine that is increased in the marrow microenvironment of patients with MM,(8) and treatment of primary stromal cells from MM patients significantly increased VCAM-1 expression compared to normal stromal cells. This increase responsivity to TNF-α was reflected by the increased NF-κB and p38 MAPK signaling. The increased expression of VCAM-1 induced by TNF-α was mediated by NF-κB while the increased IL-6 production by stromal cells was

mediated by p38 MAPK in both normal and MM cells.

Studies with siRNA knockdown of p62 demonstrated that p62 in marrow stromal cells play an important role in the activation of PKCζ, NF-κB, and p38 MAPK, which were necessary for increased expression of VCAM-1 and IL-6, and is an important contributor to the capacity of marrow stromal cells from both MM patients and normals to support the growth of MM cells. Taken together these results suggest that p62 is critical to the increased downstream signaling through NF-κB and p38 MAPK in the marrow microenvironment of patients with MM.

Importantly, loss of p62 in marrow stromal cells significantly decreased their capacity to produce RANKL and support osteoclast formation when co-cultured with human MM cells. The decreased RANKL production most likely resulted from the decreased p38 MAPK signaling with knockdown of p62 in these stromal cells. Xing and coworkers have previously reported that RANKL production was unaffected in stromal cells from p50 and p52 double knockout mice.[9] Consistent with these findings, marrow stromal cells lacking p62 produced very low basal levels of RANKL, and this was minimally increased when the cells were treated with TNF-α.

In summary, our results demonstrate that p62 plays an important role in the increased NF-κB and p38 MAPK signaling in marrow stromal cells from patients with MM that results in increase VCAM-1, IL-6, TNF-α and RANKL production. These data suggest that p62 is an attractive therapeutic target for treating MM and MM bone disease. However, any therapeutic target must be evaluated in terms of its hematologic toxicity. We found that CFU-GM, the granulocyte macrophage progenitors, are present in similar numbers in marrow and spleens from p62 knockout and p62 heterozygous mice. Similarly, p62 knockout mice have normal blood counts and do not display any hematologic abnormalities. These findings suggest that loss of p62 may only impact conditions in which NF-κB and p38 MAPK signaling is upregulated, as in MM. In support of this hypothesis, $p62^{-/-}$ mice have decreased OCL formation compared to normal mice in response to high levels of PTHrP but otherwise have normal basal OCL numbers.[10] These results support p62 as a potential therapeutic target in MM.

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Trafficking Of MM Cells: What Regulates Homing, Adhesion, And Mobilization Of Multiple Myeloma To And From The Bone Marrow Niches

Irene M. Ghobrial, MD, Aldo Roccaro, MD, PhD, Abdel Kareem Azab, PhD, Judith Runnels, PhD, and Xavier Leleu, MD, PhD

Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA

MM is characterized by the presence of multiple lytic lesions in most patients indicating continuous spread of MM cells into and out of the bone marrow (BM)[1]. Indeed, studies have demonstrated the presence of a small number of circulating plasma cells in over 70% of patients with MM[2], indicating that progression of MM occurs through the continuous trafficking of malignant cells to new sites of the BM. Therefore, adhesion of MM cells to the bone marrow niches is not a static process, but an active dynamic process that involves migration of the malignant cells to the specific bone marrow niches, adhesion to the microenvironment, and egress or mobilization of some of these cells into the peripheral circulation to home to other new sites in the bone marrow.

Migration of cells through the blood to the bone marrow niches, requires active navigation, a process termed homing. Homing is thought to be a coordinated, multistep process, which involves signaling by stromal derived factor-1 (SDF-1), and its chemokine receptor CXCR4, activation of adhesion receptors such as lymphocyte function–associated antigen 1 (LFA-1), very late antigen (VLA-4/5), cytoskeleton rearrangement, and activation of metalloproteases MMP2/9[13-15]. Other cytokines that also regulate migration of MM cells include VEGF and IGF-1. Studies of chemokine receptors in multiple myeloma have demonstrated that MM cell lines express high levels of CXCR3, CXCR4, CCR1 and CCR6. The ligands of these receptors are MIP-1α, MIP-1β, SDF-1, CXC, and RANTES. Of these, the CXCR4/SDF-1 axis plays a critical role in regulating migration and adhesion of MM cells. Studies to identify expression of chemokine receptors in MM have shown large variations in CXCR4 expression ranging from 10 to 100%[16]. SDF-1 induces migration of MM cells in vitro and homing into the bone marrow in vivo. In addition, SDF-1 induces modest proliferation of MM cells, as well phosphorylation of MEK1/2, p42/44 MAPK, and AKT in a time-dependent fashion in MM cell lines and primary MM cells[17]. We, and others have shown that CXCR4 is essential for the migration of MM cells in vitro and their homing in vivo. CXCR4 knockdown led to significant inhibition of migration to SDF-1 in MM cell lines and primary $CD138^+$ cells.

After migration, adhesion of MM cells to the bone marrow microenvironment occurs. The interaction of malignant cells with extracellular matrix (ECM) proteins and BM stromal cells (BMSCs), osteoblasts, osteoclasts, endothelial cells, as well as factors in the BM milieu play a crucial role in the pathogenesis of many malignancies and drug resistance [3-6]. Extensive studies have demonstrated that adhesion of malignant cells to the BM microenvironment confers resistance to apoptosis and increased proliferation[7-10]. At least 2 distinct niches supporting hematopoietic stem cells (HSCs) have been identified in BM: the osteoblastic niche and the vascular niche[11,12]. Osteoblasts and HSCs are closely associated in the bone marrow, suggesting a reciprocal relationship between the two. It was recently discovered that a subset of osteoblasts functions as a key component of the HSC niche (namely, the osteoblastic niche), controlling HSC numbers[12]. They also produce a variety of growth factors including the receptor activator of NF-kB ligand (RANKL). Sinusoidal endothelial cells in bone marrow have been revealed as an alternative HSC niche called the vascular niche[12]. Similar interactions of malignant cells in the BM occur, although they are less delineated.

There are four major families of cell adhesion molecules[18,19]. These are the immunoglobulin (Ig) superfamily cell adhesion molecules (CAMs), integrins, cadherins, and selectins[18,19]. The first

step of adhesion is rolling. Selectins are adhesion molecules expressed on the cell surface containing a lectin-like domain with selectivity to specific saccharide chains. There are three types of selectins E, L and P, with referral to the tissue that it was identified on (endothelium, lymphocytes and platelets, respectively). The role of selectins in MM is currently being explored. Integrins are non-covalently linked heterodimers of α and β subunits[20]. Members of the β1 integrins (also called VLA proteins) combine with the α subunits to form the different types of VLA proteins. MM cells lack VLA-2, 3 and 6[21]. In contrast, α4β1 integrin (VLA-4) and α5β1 integrin (VLA-5) are highly expressed in MM and mediate adhesion to fibronectin and VCAM[21]. SDF-1 has been reported to induce firm adhesion and migration by inducing activation of integrins LFA-1, VLA-4, and VLA-5 on stem cells[23-26]. Interestingly, adhesion molecules may trigger signals for both enhanced CXCR4 expression and increased function[27]. Recently, a new CXC receptor was discovered, namely CXCR7 (RDC-1)[32]. We recently showed that CXCR7 regulates SDF-1 dependent adhesion in MM.

Adhesion of MM cells to the bone marrow microenvironmental cells leads to proliferaton and resistance to therapy. These sequelae are due to cell-cell contact as well as NFκB- dependent transcription and secretion of IL-6. Cell-cell adhesion mediated by various adhesion molecules (e.g. VLA-4, ICAM-1) stimulates MM cell growth and survival both directly by induction of signaling cascades downstream of the surface molecules, as well as indirectly by autocrine and paracrine cytokine/growth factor production and secretion. Interaction of malignant cells with the BM microenvironment results in activation of several proliferative signaling cascades within the malignant cells including the PI3K, NF-kB, MAPK, JAK and STAT-3 pathways[7-10]. In addition, we recently demonstrated that play an important role in regulating adhesion and homing of MM cells. Rho-GTPAses . Rho-A, Rac-1 and CdC42 are guanosine triphosphatase (GTPases) and members of the Rho-GTPases family which is a subfamily of the Ras superfamily [33]. Rho GTPases have been implicated in many basic cellular processes that influence cell proliferation, motility, chemotaxis and adhesion [34].

Mobilization or egress of cells out of the bone marrow could be enhanced by disrupting the SDF-1/CXCR4 axis. This may occur by decreasing the concentration of endogenous SDF-1 (e.g., after infusion of G-CSF or cyclophosphamide) in BM as performed for stem cell transplant in MM[35], or by the cleavage and inactivation of SDF-1 by proteases in the BM[36]; or by upregulation of CXCR4 expression by hypoxia. Mobilization of stem cells from the BM may also occur if CXCR4 is inhibited[37]. Inhibitors of CXCR4 such as AMD3100 have been shown to induce mobilization of stem cells[38,39]. AMD3100 (Genzyme, MA) is a bicyclam molecule that reversibly blocks the binding of CXCR4 with SDF-1[40]. We recently showed that the CXCR4 inhibitor AMD3100 induced disruption of the interaction of MM cells with the bone marrow reflected by mobilization of MM cells into the circulation in vivo, with kinetics that differed from that of hematopoeitic stem cells. AMD3100 enhanced sensitivity to bortezomib in vitro by disrupting adhesion of MM cells to stromal cells. The combination of AMD3100 and bortezomib induced significant tumor reduction. In addition, the combination of AMD3100 and bortezomib significantly increased the ratio of apoptotic circulating cells compared to bortezomib treated groups. A phase I/II clinical trial of AMD3100 and bortezomib combination is being initiated at Dana-Farber Cancer Institute based on these studies.

In summary, understanding mechanisms of trafficking of MM cells into and out of the bone marrow may lead to significant advances in regulating dissemination, adhesion and resistance to therapy in this disease and translation of these studies into clinical trials in MM to prevent progression of the disease.

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Genes And Proteins Of Myeloma Endothelial Cells To Search Specific Targets Of The Tumor Vasculature

Angelo Vacca*, Roberto Ria*, Domenico Ribatti°, Franco Dammacco*

**Department of Internal Medicine and Clinical Oncology, and °Department of Human Anatomy, Histology and Embryology, University of Bari Medical School, I-70124 Bari, Italy*
a.vacca@dimo.uniba.it

General

Tumor blood vessels are irregular in size, shape, and branching pattern, do not have the normal vascular hierarchy and do not have recognizable features of arterioles, capillaries or venules (1). Endothelial cells (ECs) of tumors differ from those of normal vessels in some ways. Tumor ECs preferentially overexpress the cell-surface molecules integrin αvβ3 and αvβ5, E-selectin, CD105-endoglin, endosialin and VEGF receptors (VEGFRs) all of which stimulate adhesion and migration, while VE-cadherin is poorly expressed, resulting in vessel destabilization and abnormal remodeling (2). Also, ECs of tumors have multiple abnormalities in gene expression, since they overexpress specific transcripts as the result of qualitative differences in gene profiling compared with normal ECs (3). Here we wondered whether bone marrow ECs of patients with multiple myeloma (MMECs) differ from those of patients with MGUS (MGECs) and normal ECs (HUVECs) to search possible targets for antivascular therapy of MM.

Differential markers of MMECs compared to MGECs and HUVECs

MMECs expressed 5 to 70 times higher levels of several vascular markers, including Tie2/Tek, VEGFR2, FGFR2, CD105-endoglin, and VE-cadherin than HUVECs. MMECs produced 4 to 40 times more FGF-2 and VEGF, and 3 to 5 times more MMP-2 and MMP-9 than HUVECs. On a cDNA microarray, MMECs displayed up-regulation of angiogenic genes, including VEGF and FGF isoforms, HGF/SF, Tie2/Tek, TGF-β, Gro-α chemokine, fibronectin-1, HIF-1α, ETS-1, ID3, and osteopontin, compared to HUVECs. MMECs alone displayed a VEGF-dependent autocrine growth loop, due to high VEGF-A and VEGFR2 expression, constitutive auto-phosphorylation in both VEGFR2 and the associated kinase ERK-2, and inhibition of proliferation, angiogenesis and phosphorylation by neutralizing anti-VEGF-A and anti-VEGFR2 antibodies. MMECs expressed higher amounts of the CXC chemokines IL-8, I-TAC, SDF-1α, and MCP-1 than HUVECs. Paired plasma cells expressed cognate receptors of each chemokine. When MMECs were exposed to chemokines, IL-8 and SDF-1α stimulated their proliferation and all chemokines their chemotaxis. It is suggested that angiogenesis also favours MM progression through the release of CXC chemokines.

Expression of proteins of vasculogenesis

Vasculogenesis, i.e., formation of new vessels from hematopoietic stem and precursor cells (HSPCs), contributes to neovessel formation in MM (4). HSPCs from MM patients at diagnosis were seeded on fibronectin and exposed to VEGF, bFGF, and insulin-like growth factor (IGF): cells were able to differentiate into cells with an MMEC phenotype. HSCs gradually lost CD133 and acquired VEGFR2, FVIII-RA and VE-cadherin, indicative of a mature MMEC phenotype. At variance from MGUS and benign anemia (control) patients, in BM biopsies of the MM patients cells co-expressing FVIII-RA and CD133, VEGFR2, or VE-cadherin were involved in the formation of the microvessel wall. VEGF, bFGF, and IGF released by MM plasma cells and inflammatory cells during the active disease possibly induce the differentiation of CD133+ HSPCs into MMECs that contribute to the development of the MM vasculature through vasculogenesis.

The PDGF receptor beta (PDGFRβ)

Plasma cells and MMECs share symmetric receptor tyrosine kinases (RTKs), such as VEGFR and, which have been recently reported to be expressed on CD34+ CD133+ VEGFR2+ HSPCs "switching" them toward the EC differentiation (5). A combined targeting of both RTK's signaling cascades may therefore represent a more effective MM tumor/vessel-targeted approach *in vivo*. We observed that a constitutive and autocrine phospho-tyrosine activation of PDGFR was restricted to plasma cells of MM patients, correlating with higher levels of PDGF-BB compared to plasma cells of MGUS patients or peripheral blood mononuclear cells (PBMCs)

isolated from controls. Also, MMECs, but not MGECs or HUVECs, up-regulated PDGFRβ although they failed to express PDGF-BB. Conditioned media from MM plasma cells triggered PDGFRβ phospho-activation on MMECs, indicating that the PDGF/PDGFRβ kinase axis could be directly involved in the MM "angiogenic switch", hence into disease progression.

A molecular dissection of VEGF/PDGF-downstream signaling mediators in MM plasma cells and MMECs revealed that c-Src was preferentially activated in response to VEGF in both cells and sustained by a VEGF/VEGFR autocrine signaling in cell cultures (6). Down-regulation of c-Src expression by siRNA was sufficient to suppress MMEC growth, resulting in reduced cell migration, adhesion to fibronectin and angiogenic activity. Thus, a single protein, placed downstream of VEGFR2, may be crucial for MMEC survival *in vitro*. Moreover, the inhibitory effect elicited by silenced c-Src was partially rescued by exposure of siRNA-transfected MMECs to PDGF-BB, which can therefore represent an important paracrine mitogen *in vivo*. We investigated the effect of PDGF-BB on the transcription of *VEGF*, *bFGF*, *HGF*, *Ang-1* and *Ang-2* genes, that are primarily involved in the angiogenic cascade. RT-PCR and Western blotting analysis showed that MMECs, cultured in the presence of PDGF-BB, up-regulate the expression of these genes compared to serum-free control cultures, with the exception of *Ang-2* which was instead down-regulated. The PDGFRβ is an important target for therapy with dasatinib, an inhibitor of c-Src family.

Proteomic analysis of MMECs

Preliminary studies in global protein expression (7) of MMECs and MGECs vs. HUVECs has shown at least 20 proteins differentially expressed in MMECs. We identified a series of proteins that are shown to be important biomarkers to differentiate MMECs, and distinguish different stages in MM progression, i.e., diagnosis, relapse, and refractory disease. These proteins play an important role in cellular functions such as glycolysis, tumor suppression, apoptosis, angiogenesis and metastasis, and they might contribute to the adverse evolution of the disease. The increased expression of α-filamin in MMECs from active disease, refractory disease and stable disease, the upregulation of vimentin in MMECs from active disease, and down-regulation of annexin VI in MMECs from a refractory disease are related to the cell overangiogenic potential, and indicate a common machinery involved with the structural organization of the cytoskeleton and with the connection of matrix and cell–cell external signals with the intracellular signaling pathways. On the other hand, changes in the expression of some structural proteins, could account, at least in part, for the different morphology displayed by migrating MMECs. Because angiogenesis and lymphangiogenesis actively contribute to cancer progression, future studies to establish the role of these angiogenic proteins in disease may suggest potential new targets for tissue-specific therapies.

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Epigenetic Regulation Of Multiple Myeloma Within Its Bone Marrow Microenvironment

Karin Vanderkerken, Elke De Bruyne, Sarah Deleu, Miguel Lemaire, Eline Menu, Els Van Valckenborgh, Ivan Van Riet, Isabelle Vande Broek, Tomas Bos and Ben Van Camp

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Vrije Universiteit Brussel (VUB), Department Hematology and Immunology, Laarbeeklaan 103, B-1090 Brussels, Belgium

Multiple myeloma (MM) cells from a majority of patients harbor cytogenetic aberrations of which illegitimate rearrangements of the IgH gene locus on 14q32, (partial) deletion of chromosome 13 (del13q), del17p13 and del1p being the most important. In addition, recent evidence points to the importance of epigenetic mechanisms in MM resulting in perturbations of different signaling pathways.

In mammals, covalent epigenetic regulations are mainly determined by both DNA methylation and post-translational histone modifications. These epigenetic modifications are important both in early events in carcinogenesis and in tumor development. Genes affected by these alterations include those involved in DNA damage responses, regulation of apoptosis and cell cycle. Moreover, differential DNA methylation or histone acetylation status may also lead to differences in sensitivity to different types of chemotherapy.

DNA methylation occurs mainly in regions known as CpG islands. In most cases, promotor CpG are unmethylated and methylation is only observed in a small set of genes resulting in repressed transcription. Dedicated demethylation enzymes can restore the absence of methylation and reinduce gene expression. Histone acetyltransferases (HAT) and histone deacetylases (HDAC) determine the acetylation status of histones (among other proteins) and therefore also the chromatine structure and thus gene expression. Hypoacetylation leads to a dense and inaccessible chromatine, while hyperacetylation results in easier access for transciption factors associated with an increase in gene expression. In contrast to genetic aberrations, epigenetic alterations do not involve structural changes of the target genes. These alterations are therefore potentially reversible and represent interesting targets for therapy, especially in view of the multiple pharmacological inhibitors that were developed during the past years. HDAC inhibitors were moreover found to be far less toxic for normal cells than malignant cells.

In MM, both candidate gene approach and microarrays have been used to assess the effects of repressors of methylation such as 5-Aza-2'Deoxycitidine and analogues on gene expression and functions of MM cells. To date, approximately 20 cancer related genes have been identified that are frequently silenced by methylation, several of these contribute to the growth and survival of the MM cells (1-4).
Several broad spectrum HDACi used in MM have already demonstrated their ability to decrease proliferation, inhibit the production of cytokines such as IL-6 and VEGF and induce apoptosis *in vitro*.

As several studies underlined the importance of epigenetic regulation in the context of MM in relation to its local microenvironment (5-8) we assessed the effect of a HDACi (the hydroxamate based histon deacetylase inhibitor JNJ-26481585) on the development of MM disease *in vivo*, either when used as solo treatment or in combination with the proteasome inhibitor bortezomib. We focused on tumor development in the bone marrow, development of angiogenesis and induction of osteolytic bone disease. These studies were performed using the 5TMM models, spontaneously developed syngeneic models that mimic the MM disease closely (9). The 5T33MM model develops after 4 weeks while the 5T2MM model develops after 12 weeks. The first model was used in a prophylactic setting while the latter one was used in a therapeutic setting starting treatment from the onset of the MM disease. Both types of treatment with the

HDACi resulted in a dramatic decrease of tumor burden in the bone marrow, decrease in bone marrow microvessel density and decrease of bone disease in the 5T2MM. When used as a combination therapy with bortezomib, we observed a synergistic reduction in number of osteoclasts, trabecular bone volume and an increase in number of osteoblasts, suggesting that low dose of this HDACi can improve the anabolic bone properties of bortezomib (Deleu S, submitted).

We furthermore investigated the epigenetic regulation of the tetraspanin CD9. CD9 was found to be expressed in patients with non-active MM disease while patients with active disease showed no expression. This was confirmed in the murine 5T2 and 5T33MM models, showing a decreased expression during disease development. Treatment of the 5TMM cells with the HDACi LBH589 resulted in an increased CD9 expression, while treatment with 5-Aza-2'Deoxycitidine barely showed an increase. Combination of both inhibitors resulted in a strong synergistic reactivation of CD9 expression. Our study indicated that histone modifications and to a lesser extend CpG methylation are key epigenetic events involved in CD9 downregulation (10). Currently, we are investigating the epigenetic regulation of the pro-apoptotic gene Bim. In contrast to CD9, pyrosequencing analysis showed no DNA methylation of the Bim promoter. Both Western Blot and ChIP demonstrated that induced upregulation of Bim upon treatment with LBH589 and/or 5'Aza-2'Deoxycitidine was due to the acetylation and methylation of histones in the promoter region. These results are thus more or less in line with the results obtained for CD9.

Therefore, we can conclude that epigenetic phenomena represent an interesting target for treatment in MM and MM stem cells and warrants further studies on targeted genes and biological sequelae. Additional studies on new and less known epigenetic mechanisms (e.g. acetylation of proteins, miRNA, …) could furthermore provide new and improved insights in the development and progression of MM.

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Novel Anti-Myeloma Agents And Bone Metabolism: Implications Into The Management Of Myeloma Bone Disease

Evangelos Terpos, Dimitrios Christoulas, Efstathios Kastritis, Magda Migkou, Maria Gavriatopoulou, Meletios-Athanassios Dimopoulos

Department of Clinical Therapeutics, University of Athens School of Medicine, Athens, Greece

Multiple myeloma (MM) is characterized by the presence of osteolytic bone lesions that result in debilitating skeletal complications such as pathologic fractures, severe bone pain, and hypercalcemia. The development of lytic bone lesions is related to an uncoupled bone remodeling: the increased osteoclast-mediated bone resorption is accompanied by a reduction in new bone formation (Figure 1) [1]. Over the last decade, novel agents have been used in the management of MM. Immunomodulatory agents (IMiDs), such as thalidomide, lenalidomide and pomalidomide, and proteasome inhibitors, such as bortezomib have shown significant anti-myeloma activity in both newly-diagnosed and relapsed/refractory MM patients. Besides their potent efficacy against myeloma cells, these agents modify the interactions between malignant plasma cell and bone marrow microenvironment, and alter abnormal bone metabolism in MM (Figure 1).

Thalidomide and pomalidomide almost completely abrogated receptor activator of nuclear factor kappa B ligand (RANKL)-induced osteoclast formation *in vitro*. The inhibition of osteoclast formation was observed at concentrations of 1 μM of pomalidomide, which is similar or even lower than that achieved *in vivo* after the therapeutic administration of this agent [2]. Lenalidomide also inhibited osteoclast formation through similar mechanisms, such as the down-regulation of PU.1 and cathepsin K gene expression, and the reduction of RANKL secretion by bone marrow stromal cells derived from MM patients [3].

In relapsed/refractory MM patients, intermediate dose of thalidomide (200 mg/d) in combination with dexamethasone produced a significant reduction of serum markers of bone resorption, i.e. C-telopeptide of collagen type-I (CTX) and tartrate-resistant acid phosphatase isoform-5b (TRACP-5b) and also of sRANKL/osteoprotegerin (OPG) ratio. Furthermore, there was a strong correlation between changes of sRANKL/OPG ratio and changes of TRACP-5b and CTX, suggesting that the reduction of bone resorption by thalidomide is, at least partially, due to the reduction of RANKL levels [4]. Despite the reduction of bone resorption, intermediate doses of thalidomide and dexamethasone showed no effect on bone formation, as assessed by serum levels of bone-specific alkaline phosphatase (bALP) and osteocalcin (OC), and no healing of the observed lytic lesions [4]. Similar findings were reported in newly diagnosed patients who received the combination of thalidomide (100 mg/d for 2 weeks and then 200 mg/d), and dexamethasone. A significant reduction in both studied markers of bone resorption, i.e. urinary N- terminal cross-linking telopeptide of type-I collagen (NTX) and serum CTX, was observed, but only in responders. This reduction was accompanied by a reduction in bone pain in 60% of the patients. However, markers of bone formation (bALP and OC) were also reduced in all patients (responders and refractory), suggesting that the combined regimen may have a negative effect on the already exhausted osteoblasts of newly diagnosed patients, possibly due to the concomitant use of dexamethasone [5]. Lenalidomide reduced also the serum levels of sRANKL/OPG ratio in MM patients [3].

Bortezomib is a first-in-class proteasome inhibitor with known activity against myeloma. Bortezomib affects osteoclast differentiation and function in a dose-dependent manner, thus reducing subsequent bone resorption, while at the same time induces osteoblast differentiation and increase the size of osteoblastic colony forming units [6]. Bortezomib seems to act in both early and late phase of osteoclast differentiation, through the inhibition of p38

mitogen-activated protein kinase (MAPK) pathways (early phase), activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) signaling (late phase). The concentrations of bortezomib used in studies *in vitro* were typically less than that required to induce tumor cell apoptosis. Bortezomib also inhibited the secretion of BAFF and APRIL by osteoclasts [7].

In relapsed/refractory MM patients, bortezomib was found to stimulate bone formation, producing significant increases of bALP and OC, irrespective of response to therapy [8, 9]. These results are in accordance with those by Giuliani *et al* who found significant increases in the number of osteoblasts/mm^2 of bone tissue and Runx2/Cbfa1-positive osteoblasts in the trephine biopsies of responding patients to bortezomib, but not in those who did not respond [6]. Furthermore, bortezomib administration in relapsed/refractory patients resulted in a significant reduction of dickkopf-1 (Dkk-1), which is a Wingless (Wnt) type antagonist and inhibits osteoblast differentiation and function, but also reduces serum levels of sRANKL along with a concomitant reduction in osteoclast function and bone resorption, as assessed by TRACP-5b and CTX serum levels, respectively [9]. However, when bortezomib is combined with other anti-myeloma agents, such as melphalan, dexamethasone and intermittent thalidomide (VMDT regimen) the reduction of Dkk-1, osteoclast stimulators (sRANKL and macrophage inflammatory protrin-1alpha, MIP-1α] and markers of bone resorption (CTX) was not accompanied by an increase in markers of bone formation (bALP and OC) [10]. This observation may suggest that bortezomib in combination with other anti-myeloma agents may lose its beneficial effect on osteoblasts. Indeed, Heider *et al* found a lower increase in bALP in patients who received the combination of bortezomib with dexamethasone compared with patients who received bortezomib alone (8). The effect of different novel antimyeloma agents on bone metabolism of MM patients is described in Table 1.

At this point, it is crucial to mention that different effective anti-myeloma regimens in combination with bisphosphonates also reduce bone resorption through the reduction of tumor burden and the inhibition of osteoclast function. It is very difficult to distinguish in the previously mentioned clinical studies whether the reduction of bone resorption is due to a direct effect of bortezomib or IMiDs on osteoclast, to an indirect effect through reduction of tumor burden or to both. Thus, randomized trials are needed to explore whether bortezomib or an IMiD alone or in combination with other agents, including bisphosphonates, can inhibit bone resorption more effectively.

Recent studies, published mainly in abstract forms, suggest that several other novel agents with anti-myeloma activity have also an impact on bone metabolism in MM. Most of these agents inhibit osteoclast formation. SDX-308 (a novel and potent etodolac structural analog), AZD6244 (an ERK1/2-MAPK inhibitor), KD5170 (an HDAC inhibitor) can inhibit osteoclast formation. Other proteasome inhibitors, such as MG-132, and MG-262 reduce both osteoclast differentiation and activity of mature osteoclasts *in vitro*, and others, such as epoxomicin, PS-1 and lactacystin, induce osteoblast function and bone formation [7].

In conclusion, novel anti-myeloma agents, such as IMiDs, bortezomib and more recent ones, alter abnormal bone metabolism in myeloma patients. Most of them reduce bone resorption either directly through the inhibition of osteoclast formation or indirectly through the modification of interactions between malignant plasma cells and osteoclasts. In terms of the restoration of osteoblast function, based on available evidence, we can suppose that bortezomib may directly stimulate osteoblast differentiation. However, to date, evidence of the effect of bortezomib on clinical endpoints specific to bone, such as skeletal-related events and bone mineral density, is limited, possibly as a result of relatively short follow-up periods. It is therefore important to design prospective trials that investigate endpoints related to bone formation, the results of which will be eagerly anticipated. In this period of skepticism about the prolonged use of bisphosphonates due to side-effects, the administration of agents, such as bortezomib that alter bone metabolism by both reducing bone resorption and enhancing bone formation may alter our way of management myeloma bone disease in the near future.

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Table 1. Clinical studies for the effect of novel anti-myeloma agents on bone metabolism

Agent	MM study population	No of pts	Results	Subpopulation analysis
Thalidomide (+Dexa)				
Tosi et al (5)*	Newly diagnosed	40	↓ bone resorption markers (CTX & NTX)	in responders
			↓ bone formation markers (bALP & OC)	in all patients
Terpos et al (4)*	Refractory/ relapsed	35	↓ bone resorption markers (CTX & TRACP-5b)	in all patients
			↓ osteoclast stimulators (sRANKL, sRANKL/OPG ratio)	in all patients
Lenalidomide				
Breitkreutz et al (3)*	Refractory/ relapsed	20	↔ bone formation markers (bALP & OC)	in all patients
			↓ osteoclast stimulators (RANKL, RANKL/OPG ratio)	ND
Bortezomib (± Dexa)				
Heider et al (8)*	Refractory/ relapsed	58	↑ bone formation markers (bALP & OC)	in all patients
Terpos et al (9)*	Refractory/ relapsed	34	↓ bone resorption markers (CTX & TRACP-5b)	in all patients
			↓ osteoclast stimulators (sRANKL, sRANKL/OPG ratio)	in all patients
			↑ bone formation markers (βALP & OC)	in responders†
			↓ osteoblast inhibitors (Dkk-1)	in all patients
Giuliani et al (6)*	Refractory/ relapsed	21	↓ bone resorption markers (CTX)	in all patients‡
			↑ osteoblast numbers	in responders
Terpos et al (10)* (VMDT regimen)	Refractory/ relapsed	62	↓ bone resorption markers (CTX & TRACP-5b)	in all patients
			↓ osteoclast stimulators (sRANKL, sRANKL/OPG, MIP-1α)	in all patients
			↔ bone formation markers (bALP & OC)	in all patients
			↓ osteoblast inhibitors (Dkk-1)	in all patients

*concomitant bisphosphonates administration in the majority of patients
†bALP was increased only in responders while OC was elevated in all patients
‡this reduction was not reached statistical significance.

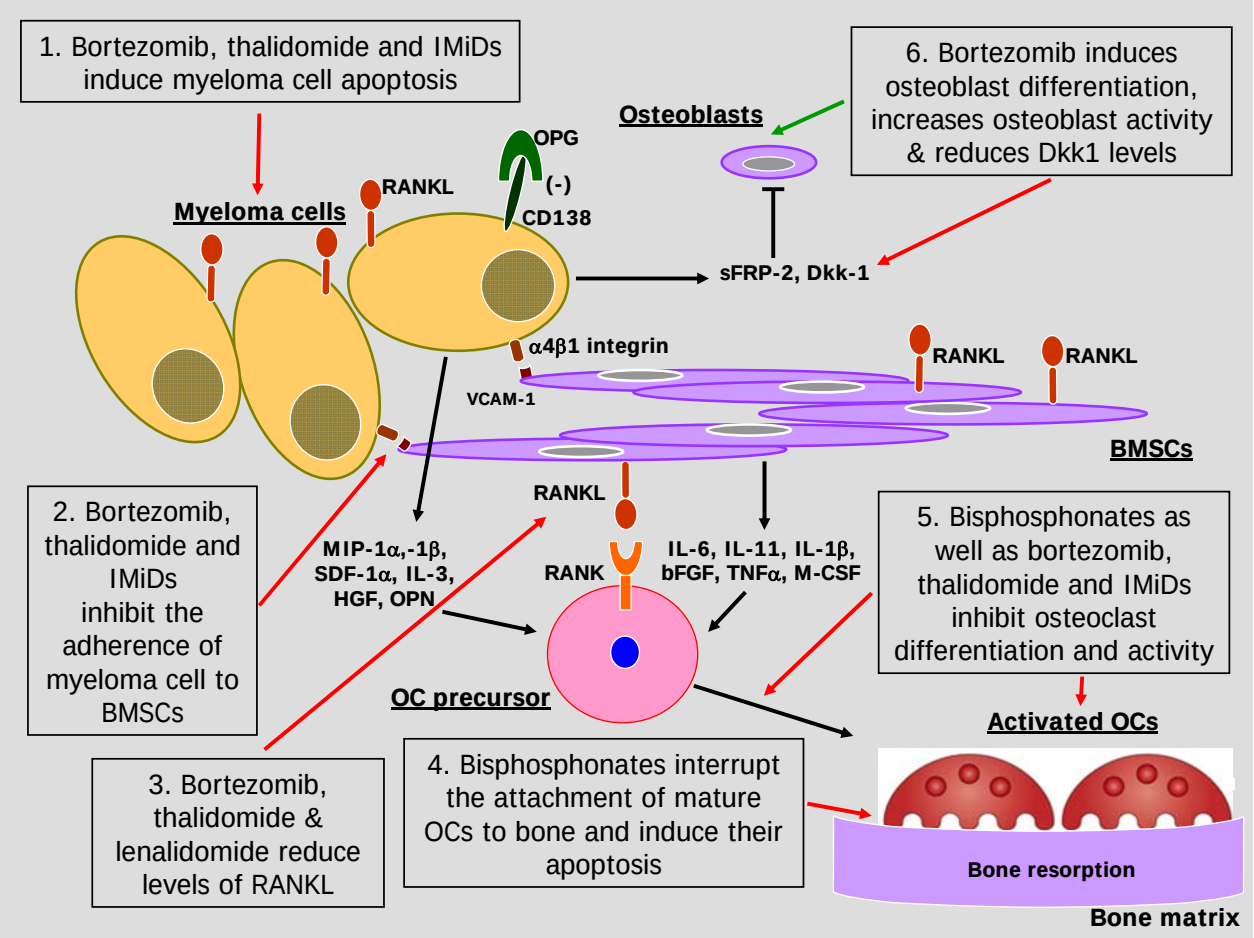

Figure 1. Abnormal interactions between myeloma cells and BMSCs lead to the overproduction of cytokines with osteoclast activation function (OAF): IL-6, IL-11, Il-1β, M-CSF that are mainly produced by BMScs and MIP-1α, MIP-1β, SDF-1α, IL-3, HGF, OPN that are mainly produced by myeloma cells. The most potent activator of osteoclasts is the RANKL, which is over-produced in the bone marrow milieu of MM. Furthermore, OPG, the decoy receptor of RANK is produced in lower amounts by BMSCs, while myeloma cells internalize and degrade OPG through CD138 (sydecan-1). Thus the ratio of RANKL/OPG is in favor of RANKL, which leads to increased osteoclastogenesis, and increased bone resorption, as assessed by bone resorption markers (NTX, CTX, ICTP, TRACP-5b). Further to osteoclast activation, myeloma cells produce molecules that can inhibit osteoblast differentiation and function, such as Dkk-1 and sFRP-2; thus osteoblasts are functionally exhausted in MM. The vicious cycle is completed by the survival benefit of myeloma cells due to osteoclast activation [1]. The effect of bisphosphonates, thalidomide, IMiDs and bortezomib on myeloma cells, and their interactions with BMSCs, osteoblasts and osteoclasts is also depicted in the figure [7].

Phase III Studies Published By the IFM (Intergroupe Francophone Du Myélome)

Philippe Moreau

University Hospital, Nantes, France.

The IFM was the first to conduct a randomized trial showing the superiority of high-dose therapy (HDT) with autologous stem cell transplantation (ASCT) compared to conventional chemotherapy in 200 patients with de novo multiple myeloma (MM) less than 65 years of age.[1] In this IFM 90 trial, HDT significantly improved the response rate (RR), event-free survival (EFS), and overall survival (OS). As a consequence of this study, ASCT became standard of care for frontline therapy in younger patients.

The IFM group then compared prospectively melphalan 200 mg/m^2 and melphalan 140 mg/m^2 plus 8 Gy total body irradiation (TBI) prior to ASCT (IFM95-02 trial), and showed that melphalan 200 mg/m^2 was at least as effective and better tolerated than the TBI containing regimen.[2] Moreover while EFS was identical in both group, OS was significantly longer with melphalan 200 mg/m^2 due to a longer OS after first relapse. Then melphalan 200 mg/m^2 became the preferred preparative regimen.

The IFM group was again the first to conduct a randomized trial comparing single and double ASCT in 599 patients up to 60 years of age.[3] On an intent-to-treat basis, the 7-year EFS and OS were significantly improved in the double ASCT arm. This study showed that achievement of CR (or at least a VGPR) had a favourable impact on EFS and/or OS, and confirmed the feasibility of double ASCT, since 75% of patients underwent the second ASCT, and the toxic death rate was less than 5%. The only parameter defining patients who did not benefit from double ASCT was response to the first ASCT. Patients with less than 90% reduction of their M-component after one ASCT had a longer OS in the double-ASCT arm, whereas patients experiencing CR of VGPR after the first ASCT had the same OS with or without the second.

In the IFM 9902 trial, 2 months after double ASCT, 597 patients with standard-risk MM (□2 microglobuline 3mg/L or less and/or no deletion 13 by FISH) were randomly assigned to receive no further treatment, pamidronate or thalidomide plus pamidronate.[4] Thalidomide increased the RR, the 3-year PFS (52% versus 36% and 37%, respectively) and the 4-year OS (87% versus 77% and 74%, respectively). The effect of thalidomide on EFS differed according to the response achieved after double ASCT. Patients who had at least a VGPR did not benefit from thalidomide while patients who failed to achieve at least VGPR had a significantly longer EFS in the thalidomide arm. This could mean that thalidomide mostly acts by further reducing the tumour mass after HDT, as a consolidation therapy, rather than by a pure maintenance effect.

In patients with high-risk disease, i.e. elevated □2-microglobulin plus chromosome 13 abnormalities, double ASCT (IFM99-04 trial) was prospectively compared to single ASCT followed by reduced-intensity conditioning regimen (RIC) allograft (IFM99-03 trial).[5,6] Our long-term results indicate that the tandem ASCT procedure is at least equivalent or even superior to a combination of autologous followed by RIC allogeneic SCT.

The standard induction therapy in patients candidate for ASCT was dexamethasone-based, either dexamethasone alone or VAD-like therapy. The primary objective of novel agents given in this context is to increase the CR rate, not only prior to but also after ASCT. Thus in 2005, the IFM initiated a randomized trial (IFM 2005-01) comparing 4 courses of induction treatment prior to ASCT with either VAD or Velcade-Dexamethasone (VD).[7] Compared to VAD, VD regimen increased not only the overall response rate (60% versus 13%) but also CR plus near-CR rate (21% versus 8%) and the CR plus VGPR rate (47% versus 19%). More importantly this higher pre-ASCT efficacy translated into a higher post-ASCT, CR plus near-CR rate (35% versus 24%) or CR plus VGPR (62% versus 42%) on an intent-to-treat analysis. Moreover when focusing on patients who actually underwent ASCT, the post-ASCT CR plus VGPR was 72% versus 51% in the VD

arms, which is quite comparable to the results achieved after a double ASCT and thalidomide maintenance in the IFM 9902 trial (despite the fact that, in this later trial, poor-risk patients were excluded). Therefore VD should now be considered as a standard induction treatment prior to ASCT, to which other more complex regimens should be compared.

In patients older than 65 years of age, the IFM prospectively compared dexamethasone and dexamethasone-based regimens with standard melphalan-prednisone (MP) in newly diagnosed MM patients ineligible for HDT. In the IFM95-01 trial, 488 patients aged 65 to 75 years were randomized between 4 regimens of treatment: MP, dexamethasone alone, melphalan-dexamethasone (M-dex), and dexamethasone-interferon alpha.[8] Response rates at 6 months were significantly higher among patients receiving M-dex, and PFS was significantly better among patients receiving melphalan, but there was no difference in OS between the 4 treatment groups. Moreover, the morbidity associated with dexamethasone-based regimens was significantly higher than with MP, especially for severe pyogenic infections in the M-dex arm and hemorrhage, severe diabetes, and gastrointestinal and psychiatric complications in the dexamethasone arms. Overall, these results indicated that dexamethasone should not be routinely recommended as first-line treatment in elderly patients with MM. In the context of the IFM95-01 trial, the standard MP remained the best treatment choice when efficacy and patient comfort were both considered.

In order to improve survival in patients older than 65 years, two options have been proposed, combination of MP with new agents, or dose-response effect of melphalan followed by ASCT. Thus, to compare these 3 strategies, the IFM initiated in 1999 a three-arm prospective randomized trial in 447 previously untreated patients with MM (IFM99-06 study), who were aged between 65 and 75 years.[9] Patients were randomly assigned to receive either MP (n=196), MP + thalidomide (MPT) (n=125), or reduced-intensity stem cell transplantation using melphalan 100 mg/m^2 (n=126). The primary endpoint of the study was overall survival. Best response rates at 12 months in the MPT group were similar to those achieved in the high-dose therapy group. Furthermore both groups were associated with RR as compared with MP group (at least PR in 76% and 65% of the MPT group and high-dose therapy group, respectively versus 35% in the MP group; at least VGPR in 47% of the MPT group and 43% of the high-dose therapy group, respectively versus 7% in the MP group). The PFS was significantly longer in the MPT group (27.5 months versus 19.4 in the high-dose therapy group and 17.8 in the MP group, respectively). With a median follow up time of 51.5 months, median OS times were 33.2 months for MP, 51.6 months for MPT and 38.3 months for high-dose therapy. The MPT regimen was associated with a significantly better OS than was the MP regimen or high-dose therapy regimen.This IFM99-06 trial clearly indicated that MPT was superior to MP in patients older than 65 years of age. This study was used as the pivotal trial for the approval of Thalidomide in combination with MP in patients with de novo MM older than 65 years of age, and this combination became standard of care in this population of patients. The efficacy of MPT compared with MP alone was recently confirmed in a very elderly population of 229 patients above 75 years of age in the randomized double-blind placebo-controlled IFM01-01 trial.[10]

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US Cooperative Group Trials

S Lonial, S Giralt, B Durie, B Barlogie, E Stadtmauer, K Anderson, V Rajkumar

The 4 major cooperative groups in the US, ECOG, CALGB, SWOG, and BMT CTN, have a number of ongoing and proposed clinical trials addressing various phases of myeloma therapy. The newest of these groups, BMT CTN, has become the predominant national cooperative group in which transplant trials are conducted.

Newly diagnosed multiple myeloma

Recently, 2 large phase III trials were completed evaluating different induction regimens with regards to response rate, and the BMT CTN trial recently also closed. In the ECOG 4A03 trial, 445 patients were randomized to receive either lenalidomide and high dose (12 days of 40 mg every 28 days) dexamethasone (RD) or lenalidomide and low dose (4 weekly pulses of 40mg every 28 days) dexamethasone (Rd). The primary endpoint of this trial was response rate following 4 cycles of induction therapy. Following an unprecedented speedy accrual, the first analysis demonstrated a survival decrement for the patients randomized to RD over Rd. This difference in overall survival was noted despite a 10% higher overall response rate favoring the use of high dose dexamethasone. While the detrimental outcome associated with the use of high dose dexamethasone was not as strong a factor for patients <65 years old, this trial has led to a massive rethinking in how we use dexamethasone in all phases of myeloma.

In the SWOG 0232 trial, patients were randomized to either lenalidomide and high dose dexamethasone (RD) or placebo and high dose dexamethasone (D). While the study had planned to enroll more patients, enrollment was halted at 198 by an NCI mandate after the early analysis of the ECOG trial demonstrated a survival decrement for the high dose dexamethasone group. The trial demonstrated an improvement in response rate, progression free survival, and overall survival that favored the use of LD over D alone. More recent and planned analysis will evaluate the impact of cytogenetics and FISH on response rate, and response duration.

The BMT CTN trial 0102 was a biologically randomized trial of tandem autologous transplant vs autologous transplant followed by mini-allogeneic transplant if the patient had an HLA matched sibling donor. For patients randomized to receive tandem autologous transplant, there was a secondary randomization to thalidomide/ dexamethasone or observation. The trial also rapidly completed accrual, and is due for its first interim analysis in 2010.

Planned and Ongoing Trials for Newly Diagnosed Multiple Myeloma

Currently there are 4 trials ongoing or planned for the near future. The ECOG 1A05 and 1A06 trials are for induction/consolidation therapy for transplant eligible patients and induction therapy of older patients. The E1A05 trial is a randomized trial of VRD vs RD which was initially planned as a novel consolidation trial, but was recently amended to serve as an induction trial where patients may have received between 1 and 6 prior cycles of induction therapy. The E1A06 trial is a randomized trial of MPT vs MPR for patients not eligible or suitable for high dose therapy. Both of these trials are currently open for enrollment.

The current SWOG induction trial, S0777, is a randomized trial evaluating combination therapy vs the potential for sequential therapy by randomizing patients to receive either RVD or Rd as induction therapy.

Finally, the new BMT CTN trial will randomize all patients following a single autologous transplant to receive either a)a second autologous transplant followed by lenalidomide maintenance b)VRD consolidation followed by lenalidomide maintenance or c) lenalidomide maintenance. This trial is slated to open in the summer of 2009.

Maintenance therapy

The CALGB is conducting a lenalidomide

maintenance trial that is nearing completion. In this trial, patients are randomized to receive either low dose lenalidomide (10 mg) or placebo, and both are continued until progression. Following completion of this trial, the CALGB is planning a lenalidomide maintenance trial following allogeneic transplant.

Induction		
ECOG	VRD vs VD	May have received 2-6 cycles of therapy
ECOG	MPT vs MPR	Non-transplant eligible
SWOG	RVd vs Rd	Newly Diagnosed
BMT CTN	Single Transplant → Auto vs RVD vs Len Maint	To open Mid 2009
Relapsed Ds		
ECOG	Carfilzomib/RD vs RD	In development
ECOG	Vel/Dex/Doxil/Cyctoxan	In development (High risk)
Maintenance		
CALGB	Lenalidomide vs Obs	To complete mid 2009
Asymptomatic MM		
ECOG/SWOG	Lenalidomide vs Obs	High risk AMM only

Canadian Collaborative Trials

Donna E. Reece, M.D

Princess Margaret Hospital, University Health Network, Toronto, Canada

Approximately 2000 new cases of multiple myeloma (MM) are diagnosed each year in Canada, while the prevalence of MM is 6500 patients per annum. The National Institute of Canada (NCIC) is the main body conducting national trials in Canada, although a national investigator-initiated trial is also in progress. Recent Canadian MM trials can be summarized as follows:

Induction therapy in patients not eligible for autologous stem cell transplantation (ASCT)

MY-7: A randomized comparison of melphalan with prednisone or dexamethasone as induction therapy, and dexamethasone or observation as maintenance therapy, in multiple myeloma

Between 06/1995 and 07/2003, 585 patients were randomized to one of four therapeutic regimens: monthly melphalan + prednisone (MP) for 12 cycles followed by observation (OBS), MP followed by monthly dexamethasone maintenance (40 mg/day x 4 days) until disease progression (Dex maint), monthly melphalan + dexamethasone (M-Dex) for 12 cycles followed by OBS, or M-Dex followed by Dex maint. Entry into the M-Dex induction arm was stopped after analysis met a predetermined event-related criterion. No differences in median progression-free survival (PFS) or overall survival (OS) were observed in among the 466 patients randomized to either MP or M-Dex (median PFS 1.8 years for MP and 1.9 years for M-Dex; HR=0.01, p=0.2). The median OS was 2.5 versus 2.1 years, respectively (HR=0.61, p=0.3). Although Dex maint produced a significantly longer median PFS compared with OBS in the 292 evaluable patients (4.1 versus 3.8 years; HR 0.61; p=0.0002), this did not confer an OS advantage (4.1 versus 3.8 years; HR 0.88; p=0.4). [1]

MY 11: Phase II testing of lenalidomide plus melphalan for previously untreated older patients with multiple myeloma

This trial was originally designed as a randomized phase II evaluation of melphalan (M) 9 mg/m^2 days 1-4 and lenalidomide (L) at a dose of either 10 mg (L10) or 20 mg (L20) days 1-21 of a 28 day cycle (M9L10 versus M9L20). Corticosteroids were not included to avoid duplication with trials in other countries evaluating these agents. Importantly, although G-CSF was permitted for documented neutropenia, it was not utilized prophylactically, as one goal of the trial was to identify doses that did not mandate routine growth factor support. Hematologic dose-limiting toxicity (DLT) was defined as the requirement for 2 dose reductions of L or 1 dose reduction of M . Before trial commencement, a safety run-in of 6 patients treated with M9L10 was performed; this was stopped after the first 4 patients experienced DLT, including one septic fatality. The first study amendment called for randomization of M 4 mg/m^2 plus L 15 mg (M4L15) and M 6 mg/m^2 plus L 10 mg (M6L10). Again, a 6 patient run-in was conducted for each arm. Four of 6 patients treated with M4L15 developed hematologic DLT and a fifth experienced other SAEs. Of the 6 patients treated with M6L10, 3 developed grade 3-4 neutropenias with 2 requiring hospitalization. Therefore, both of these arms were closed, and the trial was amended a second time to proceed as a single-arm phase II evaluation of M5L10.[2] The trial has now been closed prematurely, and the results will be presented at the ASH meeting in December 2008.

Maintenance after ASCT

MY10 (ECOG-NCIC-JMY10); A randomized phase II study of thalidomide and prednisone as maintenance therapy following ASCT

Following the MY9 NCIC randomized phase II study to establish the dose of thalidomide and prednisone maintenance therapy to be used for phase III testing[3], the MY10 trial randomizes patients who have undergone ASCT using

melphalan 200 mg/m^2 within 1 year of diagnosis to either thalidomide 200 mg per day plus prednisone 50 mg every second day, or OBS only, for 4 years. The primary endpoint is OS, with time to progression (TTP), toxicity and quality of life as secondary outcome measures. The trial is nearing completion, as the target number of 324 patients has almost been reached. The trial has been open since 09/2002, and the improved survival of MM patients after disease progression has likely contributed to the relatively prolonged time to reach the primary survival endpoint.

National investigator-initiated trial high-risk MM

Trial of bortezomib-based therapy for newly diagnosed patients with t(4;14)

About 15% of MM patients have t(4;14), and compared to other MM patients, those with t(4;14) have a shorter median TTP -- approximately 8-9 months--and OS-- approximately 18 months--following a single ASCT.[4] On the other hand, bortezomib has shown promising activity in this subtype. Therefore, a Canadian multicenter phase II trial was initiated last year in which newly diagnosed MM patients with t(4;14) disease receive induction with DBd (pegylated liposomal doxorubicin + bortezomib + dexamethasone) for 4 cycles followed by 8 cycles of CYBOR-P (weekly cyclophosphamide + bortezomib + prednisone) as post-induction therapy; each of these regimens has been evaluated in previous Canadian phase I-II trials.[5,6] Although stem cells are collected between these phases, ASCT is reserved for progressive disease. Maintenance dex is included. The primary endpoint of the study is TTP, with a goal to extend the TTP from initial therapy from 12 to 21 months; the trial will enter 36 patients. To date, 59 newly diagnosed Canadian patients have been screened for t(4;14), and 5 positive patients have been entered into study.

Future/proposed trials

One proposed NCIC trial would evaluate first-line therapy with ASCT versus therapy with novel agents, with ASCT reserved for recurrent disease. However, the number of patients required will likely limit the feasibility of such a study in Canada, and alternative approaches for initial therapy, based on biomarkers to guide therapy, are under discussion.

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The activity of Spanish and Italian Myeloma Groups

Boccadoro M, Palumbo A, Cavo M, Di Raimondo F, Bringhen S, Magarotto V, Larocca A, Mateos MV, Lahuerta JJ, Cibeira MT, Martínez J, Rosiñol L, Bladé J and San Miguel J

GIMEMA (Italian Group for Adult Hematologic Diseases /Multiple Myeloma Working Party), and PETHEMA (Programa para el Estudio de la Terapéutica en Hemopatía Maligna)

The activity of Spanish and Italian Myeloma Groups since 2000 was shown in Table 1. Here we will focus on the results of the currently ongoing trials.

Spanish Myeloma Group

In untreated patients 65 years-old or younger, the randomized phase III trial compares three induction regimens: thalidomide plus dexamethasone (TD, thalidomide 200 mg daily and dexamethasone 40 mg on days 1-4 and 9-12 at 4-week intervals for 6 cycles.) vs. bortezomib plus thalidomide and dexamethasone (VTD, identical to TD plus the addition of Velcade 1.3 mg/m2 on days 1,4,8,11 of each cycle) vs. VBMCP/VBAD/Velcade (4 cycles of VBMCP/VBAD, on an alternating basis, followed by 2 cycles of Velcade 1.3 mg/m2 on days 1,4,8, and 11 every 3 weeks). All patients will subsequently receive ASCT with MEL-200. 173 patients were already evaluable for response and toxicity to induction therapy. The ≥PR rate was 62%, 77% and 70% with TD, VTD and VBMCP/VBAD/Velcade, respectively (p=NS). The IF negative CR rate was significantly higher with VTD (31%) and with VBMCP/VBAD/Velcade (22%) as compared to TD (6%) (p<0,01). The post-ASCT CR rate were higher with VTD (50%) and with VBMCP/VBAD/Velcade (39%) as compared to the TD arm (26%). The incidence of grade 3 /4 AEs was 38%, 54% and 50% with TD, VTD, and VBMCP/VBAD/Velcade, respectively. 13% of patients receiving TD developed ≥ grade 3 thrombotic events while 16% of patients in the VTD arm had grade ≥3 peripheral neuropathy. Longer follow-up is needed to estabilish whether or not this higher tumor reduction is translated into a significant better long-term outcome[1].

In elderly untreated patients older than 65 years, the Pethema/GEM phase III trial compares bortezomib and melphalan plus prednisone (VMP) versus bortezomib and thalidomide plus prednisone (VTP) in order to answer the question which agent is the optimal partner for bortezomib: an alkylating or an immunomodulatory drug. Patients in the VMP arm received intravenous bortezomib 1.3 mg/m^2 twice weekly (days 1, 4, 8, 11; 22, 25, 29 and 32) for one 6-week cycle (8 doses per cycle), followed by once weekly (days 1, 8, 15 y 22) for five 5-week cycles (4 doses per cycle) in combination with oral melphalan 9 mg/m^2 and prednisone 60 mg/m^2 once daily on days 1–4 of each cycle. Patients in the VTP arm received the same bortezomib and prednisone regimen, plus continuous thalidomide at dose of 100 mg daily. 167 patients are evaluables for response to induction therapy and adverse events (AEs). 80 pts were randomly assigned to receive VMP and 87 to VTP. No significant differences were observed in RR: ≥ PR in 78% of patients in VMP and VTP groups, with a CR rate of 18% vs 23% (p=NS). Regarding haematological toxicity, VMP resulted in higher incidence of ≥G3 neutropenia (34% vs 19%; p=0,009) and thrombocytopenia (21% vs 9%; p=0,01). The most relevant toxicities were: ≥G3 cardiac toxicity (7% in VTP vs 0% in VMP); ≥G3 thromboembolic events (3,4% in VTP vs <1% in VMP; ≥G3 PN (15% in VTP vs 9% in VMP). Five pts in VMP and 7 in VTP died due to AEs. In summary this initial analysis indicates that there are no significant differences in terms of efficacy between VMP and VTP, while the incidence of non-hematological AEs, specially cardiac events, was higher in the VTP arm, resulting in more serious AEs and treatment discontinuations. These data suggest that thalidomide may not be the partner of choice for combination with bortezomib and other IMIDs such as lenalidomide should be explored. In addition, our data indicate that the modified VMP regimen used in this trial (weekly schedule after cycle 1 and only six cycles) is well tolerated although the CR rate is lower than in the VISTA trial[2].

Finally, the group has recently activated a phase III trial in smouldering MM patients with high

risk of progression to symptomatic MM patients in order to evaluate if the use of lenalidomide plus low dose dexamethasone as induction therapy followed by maintenance therapy with lenalidomide alone prolongs the time to progression to symptomatic MM. For this purpose, 120 smouldering MM patients will be randomized to receive either the proposed treatment or therapeutic abstention. As of October 30, 2008, 50 patients have been included and the first interim analysis, when 10 patients have completed the first three cycles of treatment, have shown that treatment is well tolerated without significant toxicity. A comprehensive series of biological studies complements this trial (personal communication).

Italian Myeloma Group

In untreated patients 65 years-old or younger, the randomized phase III trial compares TD (thalidomide 200 mg daily and dexamethasone 40 mg on days 1-4 and 9-12 at 3-week intervals for 4 cycles) vs. VTD (thalidomide 200 mg daily, dexamethasone 40 mg on days 1,2; 4,5; 8,9; 11,12 and Velcade 1.3 mg/m2 on days 1,4,8,11 at 3-week intervals for 4 cycles). All patients will subsequently receive ASCT with MEL-200. 187 patients were evaluable for response and toxicity to induction therapy. The IF negative CR+nCR rate was significantly higher with VTD (36%) as compared to TD (9%) ($p<0.001$). The post-ASCT CR rate were higher with VTD then with TD. Grade 3-4 peripheral neuropathy was reported in 8% of VTD patients and in 2% of TD patients. Longer follow-up is needed to evaluate long-term outcome[3].

In elderly untreated patients older than 65 years, the phase III trial compares bortezomib, melphalan and prednisone (VMP) versus bortezomib, melpahalan, prednisone and thalidomide (VMPT). Initially, patients were treated with nine 6-week cycles of VMPT (bortezomib 1.3 mg/m^2 days 1,4,8,11,22,25,29,32 in cycles 1-4 and days 1,8,22,29 in cycles 5-9; melphalan 9 mg/m^2 days 1-4; prednisone 60 mg/m^2 days 1-4 and thalidomide 50 mg days 1-42, followed by bortezomib 1.3 mg/m^2 every 15 days and thalidomide 50 mg/day as maintenance) or VMP (bortezomib, melphalan and prednisone at the same doses and schedules previously described without maintenance). In March 2007, the protocol was amended: both VMPT and VMP schedules were changed to nine 5-week cycles and bortezomib schedule was modified to weekly administration (bortezomib 1.3 mg/m^2 days 1,8,15,22 in cycles 1-9). 304 patients are evaluable for response to induction therapy and adverse events (AEs). The very good partial response (VGPR) rate was higher in the VMPT group (55% versus 42%, $p=0.02$), including a CR rate of 31% in the VMPT group and 16% in the VMP group ($p=0.003$). In the subgroup treated with weekly infusion of bortezomib, VGPR was 59% for VMPT and 37% for VMP ($p=0.004$). After a median follow-up of 13.6 months, the 2-year PFS was 83.9% in the VMPT group and 75.7% in the VMP group. The 3-year overall survival (OS) was 89.5% in the VMPT group and 88.7% in the VMP group. The incidence of grade 3-4 adverse events was similar in both groups. In the VMPT patients and in the VMP patients, the more frequent AEs were neutropenia (36% vs 31%), thrombocytopenia (20% vs 19%), peripheral neuropathy (18% vs 12%), infections (14% vs 10%), and gastrointestinal complications (7% vs 8%), respectively. The weekly infusion of bortezomib significantly decreased the incidence of grade 3-4 peripheral neuropathy (9% for VMPT and 3% for VMP)[4].

Newly diagnosed patients enrolled in both above studies were randomly assigned to receive LMWH (Enoxaparin 40 mg/d) or ASA (Aspirin 100 mg/d) or WAR (Warfarin 1.25 mg/d) for the duration of the induction therapy, as prophylaxis for thrombosis. Patients treated with VMP did not receive any prophylaxis and were used as controls. The incidence of thrombosis was 3.9% in the LMWH group, 5.0% in the ASA group and 3.3% in the WAR group (p ns). Thrombosis were 1.8% in the VMP group. The cumulative incidence of thrombosis was 3.0% in patients treated with Velcade plus Thalidomide, and 5.8% in those treated with TD. The overall incidence of VTE was less than 10% in all groups; patients who received Velcade had lower frequency of VTE[5].

Finally, untreated patients aged 65-75 years were enrolled in a phase II study with an induction therapy consisting of 4 21-day PAD cycles (Bortezomib 1.3 mg/m^2 days 1, 4, 8, 11, Pegylated-lyposomal-doxorubicin 30 mg/m^2 day 4 and Dexamethasone 40 mg days 1-4, 8-11, 15-18). Two cycles of Cyclophosphamide 3 g/m^2 plus Granulocyte-Colony Stimulating Factor were used to harvest stem cells. Patients were conditioned with tandem Melphalan 100

mg/m^2 (MEL100) followed by stem cell support. After ASCT patients received consolidation with 4 28-day LP cycles (Lenalidomide 25 mg days 1-21 plus, Prednisone 50 mg every other day) followed by Lenalidomide alone maintenance (10 mg days 1-21 every 28 day). 102 patients were enrolled. After PAD cycles at least partial response (PR) rate was 94%, at least very good partial response (VGPR) was 59% including 13% CR. After tandem MEL100, 88% of patients achieved at least VGPR and 41% CR. After LP consolidation all patients obtained PR, 88% at least VGPR and 53% immunofixation negative CR. After a median follow-up of 14 months, 1-year progression free survival (PFS) was 92%, 1 year time to progression was 97% and 1 year overall survival was 92%. During PAD, grade 3-4 adverse events included thrombocitopenia (13%), neutropenia (11%), infections (18%), gastrointestinal toxicities (12%), peripheral neuropathy (11%) and deep vein thrombosis (6%). During LP consolidation, grade 3-4 toxicities included neutropenia (18%), thrombocytopenia (6%), infections (6%) and deep vein thrombosis (6%). The other grade 3-4 toxicities occurred in less than 5% of patients[6].

Table 1. Summary of main Spanish and Italian studies.

Name of protocol	Phase	No. of patients	Median age	ABMT	CR (%)	PR (%)	PFS	OS	REF
VMP	I/II	60	75	N	32	45	27 months	85% @ 3 years	7
Vdex in alternated regimen	II	40	54	Y	12,5	42,5	n.a	n.a	8
VBCMP/VBAD+V vs TD vs VTD	III	173	57	y	22 vs 6 vs 31	70 vs 62 vs 77	n.a	n.a	1
VMP vs VTP	III	167	74	N	18 vs 23	78 vs 78	n.a.	n.a	2
VTD vs TD	III	187	58	Y	36 vs 9*	n.a.	n.a.	n.a.	3
Mel200 vs Mel100	III	298	57	Y	15 vs 8	72 vs 78	18 vs 44 @ 4 years	45 vs 59 @ 5 years	9
Pad-Mel100-RP-R	III	102	67	Y	53	100	92 @ 1 year	92 @ 1 year	6
MP vs MPT	III	331	72	N	4 vs 16	48 vs 69	median 14 vs 22	median 48 vs 45	10
MPR	I/II	53	71	N	23,8°	81°	92 @ 1 year	100 @ 1 year	11
VMP vs VMP-T	III	354	72	N	21 vs 39	80 vs 87	70 vs 74 @ 3 years	87 vs 88 @ 3 years	4

* CR+nCR

° in the subgroup of patients who received the MTD

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Phase III Studies by Northern European Groups in Multiple Myeloma

P. Sonneveld

Erasmus MC, Rotterdam, the Netherlands

The prognosis of patients with Multiple Myeloma (MM) has improved significantly due to introduction of high-dose therapy (HDT), novel agents, and supportive care. Randomized clinical trials are vital for establishing the right place and dosing of these treatment modalities. Phase II trials in Europe looked at Thalidomide, Lenalidomide and Bortezomib in 1st relapse in combinations such as Thalidomide/ Dexamethason (TD), Bortezomib/ Doxorubicin/ Dexamethason (PAD), Lenalidomide/Doxorubicin /Dexamethason (RAD), Cyclophosphamide with Bortezomib and /or Dexamethason and VRD[1-4]. Based on these and other phase II trials the large prospective phase III trials with Thalidomide or Bortezomib based induction treatment could be initiated.

In North-western Europe several cooperative clinical trial groups have performed prospective phase III trials addressing the role HDT and stem cell transplantation in MM[5-8] . These trial groups include the British UK Myeloma Forum (UKMF), the Scandinavian Nordic Myeloma Study Group (NMSG), the Deutsche Sprache MM group (DSMM) and the German Multiple Myeloma Group (GMMG), the central European CEMSG and Dutch-Belgian HOVON. More recently, the potential benefit of Thalidomide and Bortezomib was assessed by these groups in the context of front-line treatment or with HDT or in an international context at relapse treatment[7,9-16]. In these trials the role of Thalidomide and Bortezomib in pre-transplant induction treatment was investigated as well as the use of these agents in post-transplant consolidation and maintenance therapy.

The prospective phase III HOVON trial H50 (550 pts ≤ 65 yr) demonstrated that Thalidomide/ Doxorubicin/Dexamethason (TAD) induction and Thalidomide maintenance increased the VGPR from 54 % to 65 % ($p< 0.01$) and CR from 21 % to 30 % ($p<0.03$) after HDT compared with VAD induction. Progression-free survival (PFS) increased from 25 to 33 months ($p<0.01$) with no difference in overall survival (OS), 62 vs 59 months, respectively (H. Lokhorst et al., ASH 2008, # 157). A similar trial performed by the GMMG group (600 pts) using the same protocol still awaits final analysis.

The issue of Thalidomide maintenance was also addressed by the UK group in the Myeloma IX maintenance randomization after HDT or standard chemotherapy. There was no significant improvement of PFS across the whole group, except for patients who did not achieve a VGPR prior to HDT, and no benefit in OS. Importantly, patients with 17p deletion by FISH had a worse PFS when treated with Thalidomide maintenance (G. Morgan et al., ASH 2008 # 656). Taken these data together, the use of Thalidomide in various combinations appears to increase response and CR rate and possibly PFS, however as yet not OS.

The prospective phase III HOVON/GMMG trial H65/HD-4 (825 pts ≤ 65 yr) investigated the effect of Bortezomib/Doxorubicin/Dexamethason (PAD) induction as compared with VAD prior to HDT and Bortezomib vs Thalidomide maintenance. In an interim analysis for response in 300 patients after closure of the trial it was shown that VGPR was significantly better with Bortezomib induction prior to HDT (42 % vs 15 %, $p< 0.0001$)and after HDT (80 % vs 50 %, $p<0.0001$). In addition, the CR/nCR rate increased from 25 % after HDT to 37 % during maintenance (P. Sonneveld et al., ASH 2008 # 653). As in the French IFM trial Bortezomib clearly improves the quality and percentage of response, with a possible beneficial role in maintenance.

GMMG, DSMM and HOVON also addressed the role of consolidation therapy with allogeneic transplantation in newly diagnosed MM in the context of phase III trials. A donor vs no donor analysis of patients included in H50 showed no

benefit of tandem auto-allo for response (94 % vs 96 %), CR (35 % vs 40 %) nor of PFS (32 vs 28 months) or OS (61 vs 54 months) (H. Lokhorst et al., ASH 2008, # 461). These results do not support a role for standard allogeneic transplantation in newly diagnosed MM.

Based on the encouraging results with Bortezomib induction and following the excellent results obtained in the VISTA trial, the Northern European groups have decided to investigate whether a Bortezomib based regimen is equivalent to HDT. For this question, HOVON, DSMM, NMSG, CEMSG together with the Italian GIMEMA group are preparing an European Intergroup trial to evaluate induction with a Bortezomib based regimen followed by a comparison of intensification with standard HDM plus stem cell support or with MP-V.

Several trials were performed in patients who are not candidates for HDT. In analogy with trials performed by the Italian and French groups, 2 trials were preformed comparing MP vs MPT by HOVON (P. Wijermans et al., ASH 2008 # 649) and by NMSG (EHA 2008). The results of these trials were presented at recent meetings as shown in the table.

MP-Thalidomide versus MP in patients not eligible for HDT

Trial	# pts	Median age	ORR MPT / MP	PFS MPT / MP	OS MPT / MP
Italian	254	72	76 / 48	26 / 14	>36 / >36
French	447	70	76 / 35	27 / 18	52 / 33
NMSG	357	74	58 / 40	15 / 14	31 / 28
HOVON	344	73	62 / 47	14 / 14	37 / 30

The observed benefit of MPT for response did not translate into a better PFS in the HOVON and NMSG trials, partly due to higher dose of Thalidomide in the NMSG trial and higher melphalan dose in HOVON, with associated toxicities. A meta-analysis of these trials is currently being prepared.

In another trial by the CEMSG, Thalidomide/Dexamethasone (TD) was compared with standard MP in non-transplant candidates (H. Ludwig et al, Blood Epub 2008). This important trial demonstrated that the response rate with TD was higher, however PFS and OS were worse due to early mortality from infections especially in patients > 75 years of age. As mentioned above, the Thalidomide maintenance trial in the UK (MRC IX) did not show a survival benefit.

Based on encouraging results with MP-Lenalidomide (MP-R), Lenalidomide was introduced in several randomized trials in newly diagnosed and relapse patients. In 2008 a prospective phase III trial, comparing MP-Thalidomide with MP-Lenalidomide in patients not eligible for HDT was initiated by HOVON and NMSG. This trial will also evaluate prospectively the prognostic value of gene expression profiles in both treatments, as well as the Quality of Life QoL of the patients.

In addition to randomized trials for different treatment regimens, several groups also performed correlative studies focusing on treatment related complications[17-19] and on demographic aspects[20-22] and Quality of Life aspects. The UK group performed an open, randomized, controlled, phase II, single centre, two-period cross-over study to compare the quality of life and toxicity experienced on PEG interferon with interferon-alpha2b in patients with MM maintained on a steady dose of interferon-alpha2b, showing no difference of efficacy, but better QoL with PEG interferon[23].

The NMSG compared 30 or 90 mg monthly dose of bisphosphonates (P. Gimsing et al., ASH 2007). While still unpublished, the results indicate that these regimens are equivalent for the development and healing of bone lesions.

The results of completed trials and interim analyses will be discussed and the design of

ongoing and future trials will be presented.

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Novel Combination Therapies For The Treatment Of Relapsed/Refractory Multiple Myeloma: Current Phase I/II Combinations.

Paul Richardson MD[1], Jacob Laubach MD[1], Robert Schlossman MD[1], Irene Ghobrial MD[1], Constantine Mitsiades MD PhD[1], Teru Hideshima MD[1], Dharminder Chauhan PhD[1], Noopur Raje MD[1,2], Nikhil Munshi MD[1], Kenneth C. Anderson MD[1]

[1]Jerome Lipper Multiple Myeloma Center, Dana-Farber Cancer Institute and [2]Massachusetts General Hospital Cancer Center, Harvard Medical School, Boston, MA, USA

Introduction

Multiple myeloma (MM) is an incurable hematological malignancy with a median survival of 5–7 years, with approximately a third of patients still alive at 5 years.[1] Recently, treatment with lenalidomide, thalidomide, and bortezomib has transformed the clinical management of MM, resulting in improved overall survival, even in heavily pre-treated patients.[2] Novel combinations of these compounds offer an exciting platform for partnerships between the newer small molecule inhibitors, monoclonal antibodies, and other immunological strategies, including vaccination (Figure 1).[3-5] Here, we will outline selected current phase I/II trials exploring these novel combinations in the relapsed/refractory MM setting (Table 1).[6-16]

Lenalidomide and bortezomib as a therapeutic backbone

Following pre-clinical studies which showed a potential synergy between lenalidomide and bortezomib, a phase I dose-escalation study of this combination yielded an overall response rate (ORR; defined as partial response [PR] or better) of 58%, with 6% of patients achieving near-complete remission (nCR) or better.[6] Median duration of response at early follow-up was 6 months, with 30% of patients remaining on therapy for more than 1 year. Dexamethasone was added to the combination in 14 patients who had disease progression, and a subsequent objective response was reported in 10 (71%) patients. Favorable tolerability of the three-drug regimen was seen.

The combination of lenalidomide and bortezomib plus dexamethasone was further assessed in 64 patients in a phase II trial. Patients received up to eight 21-day cycles of bortezomib 1.0 mg/m^2 (days 1, 4, 8, and 11), lenalidomide 15 mg/day (days 1–14), and dexamethasone 40/20 mg/day (cycles 1–4/5–8, respectively). After cycle 8, patients with stable/responding disease received maintenance treatment (bortezomib 1.0 mg/m^2 days 1 and 8; lenalidomide 15 mg/day days 1–14 [doses per end of cycle 8]; and dexamethasone 10 mg/day, days 1, 2, 8, and 9) until progression or unacceptable toxicity. Patients with grade ≥ 2 peripheral neuropathy were excluded. The ORR was 73%, including 36% of patients with a very good PR (VGPR) or better.[7] Toxicities were manageable, consisting mainly of grade 1/2 myelosuppression. Non-hematological toxicities included deep vein thrombosis (DVT) in 2 patients, grade 3 peripheral neuropathy (1 patient), and grade 3 atrial fibrillation (2 patients) which resolved with dose reduction of dexamethasone. As a result of therapy-related side effects, lenalidomide dose reduction was required in nine patients, bortezomib reduction in five, and dexamethasone reduction in fourteen.

Several other phase I/II trials in relapsed/refractory MM have explored lenalidomide in combination with both conventional and newer agents. Treatment with liposomal doxorubicin, vincristine, dexamethasone, and lenalidomide (DVd-R) achieved an ORR of 75% in relapsed patients; a complete response (CR) or nCR was achieved in 29%, but peripheral neuropathy from vincristine is a concern with this combination.[8] Two other studies in which lenalidomide was combined with dexamethasone and either adriamycin (RAD) or cyclophosphamide (RCD) have shown high response rates and no significant neurotoxicity.[9,10] In the RAD study the ORR was 85%, with 24% of patients achieving a CR and 59% a VGPR; adverse events were generally manageable.[9] In the RCD study, the ORR was 65%, and the median time to response was rapid at 31 days.[10]

Palumbo et al. investigated the addition of lenalidomide to the melphalan/ prednisone/thalidomide regimen.[11] In this phase II study, 43 patients received 6 cycles of lenalidomide (10 mg/day, days 1–21 of each 28-day cycle), melphalan (0.18 mg/kg, days 1–4), prednisone (2 mg/kg, days 1–4), and thalidomide (50–100 mg/day, days 1–28), followed by maintenance treatment with 10 mg/day lenalidomide. After a median of 4 cycles, a PR or better was achieved in 91% of patients. Adverse events were mainly hematological, with 48% experiencing grade 3 neutropenia and 16% grade 4 neutropenia. No thromboembolic events were described. This regimen, therefore, may have utility in relapsed and refractory MM, but caution regarding myelosuppression as well as peripheral neuropathy is warranted.

Bortezomib, which has established efficacy in combination with conventional therapies (such as liposomal doxorubicin and alkylating agents), has been studied in combination with numerous novel agents and extensively assessed in several phase I/II trials, including tanespimycin (an inhibitor of heat-shock protein 90), perifosine (an AKT inhibitor), as well as oral vorinostat and related histone deacetylase inhibitors.[12-15] The bortezomib plus tanespimycin combination yielded an ORR of approximately 50%. The most common adverse events were fatigue, thrombocytopenia, and mild/moderate gastrointestinal toxicity that was manageable with supportive care. Interestingly, significant peripheral neuropathy was not reported and may reflect a putative neuroprotective effect of tanespimycin. A working hypothesis is that such an effect could be mediated by upregulation of HSP-70, an important neuroprotectant.[12] Based upon these promising results, a phase III trial is underway.

In a phase I/II trial, the combination of perifosine plus bortezomib resulted in an ORR (CR + PR + minor response) of 56% (n = 16).[13] Grade 3/4 toxicities were primarily thrombocytopenia, anemia, and fatigue. No cases of DVT and/or significant peripheral neuropathy were reported. Patients had received a median of 5 prior lines of therapy, and all had received prior bortezomib; 83% of patients had had relapsed and refractory MM. The activity of this combination regimen was thus notable, and further benefit was seen with the addition of dexamethasone. The dose-escalation part of this trial has been completed, and accrual has continued at a dose level of perifosine 50 mg/day and bortezomib 1.3 mg/m^2 on days 1, 4, 8, and 11, given in 21-day cycles.[13] Phase III studies are planned.

Two phase I studies of vorinostat given in combination with bortezomib showed promising activity in about half the patients treated.[14,15] In the study performed by Badros and colleagues, most dose levels used bortezomib 1.3 mg/m^2 on days 1, 4, 8 and 11, with escalating doses of vorinostat given once- or twice-daily on days 4–11 (100–200 mg twice daily, for a total of 200–500 mg/day).[14] Patients were heavily pretreated, with a median of 6 prior lines of therapy, and the majority of patients had received a median of 2 prior bortezomib-based regimens. In the Weber et al. study, dose levels of bortezomib from 0.7–1.3 mg/m^2 were studied in combination with vorinostat 200 or 400 mg/day.[15] In a 21-day cycle, vorinostat was given on days 1–14 and bortezomib on days 1, 4, 8, and 11. The median number of prior therapies was 3, and relatively few patients had received prior treatment with bortezomib.[15] Interestingly, more activity was seen in the Badros et al. study. This population had received more prior therapies and more prior bortezomib-based therapies, although they also received maximal doses of bortezomib (1.3. mg/m^2). A total of 10 of 23 (43%) patients achieved a PR or better. Hematological toxicity in this study was cumulative, but QTc prolongation noted in the first cycle was not seen in subsequent cycles. Phase III studies are now in progress.

Finally, a phase I/II trial of combination bortezomib, melphalan, prednisone, and thalidomide yielded an ORR (PR or better) of 67%, including 43% of patients with a VGPR. Patients who had previously been treated with bortezomib and thalidomide showed favorable tolerability, suggesting that this four-drug combination incorporating a proteasome inhibitor (such as bortezomib) and immune-modulatory drugs may be especially active.[1]

Conclusions

The results yielded to date using novel combination-based therapies have been encouraging, with high response rates (including CR) and manageable toxicity. Further studies are ongoing incorporating combinations

of conventional treatments, novel agents, and other small molecules, with the goal of further optimizing the regimens to ensure that patients receive more effective and well-tolerated treatment.

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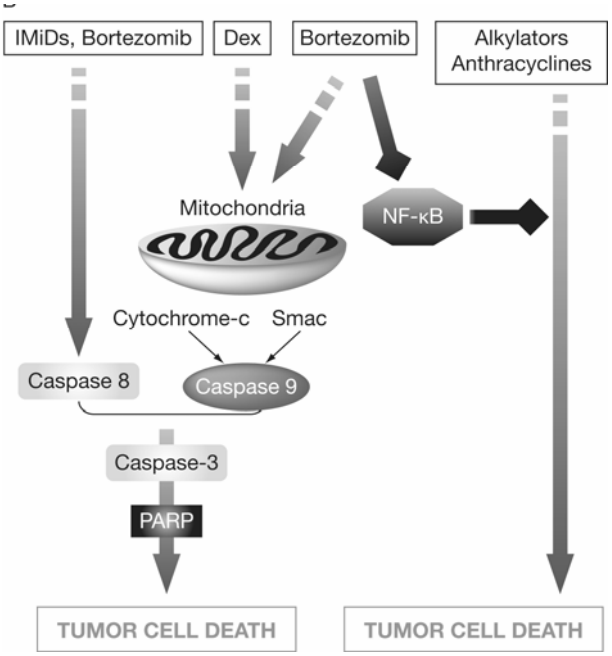
Table 1. Current phase I/II combinations for the treatment of relapsed/refractory multiple myeloma.

Reference	Combination	ORR, %
Richardson PG, et al. 2006[6]	Lenalidomide + bortezomib	58
Anderson KC, et al. 2008[7]	Lenalidomide + bortezomib + dexamethasone	73
Baz R, et al. 2006[8]	Doxorubicin + vincristine + dexamethasone + lenalidomide	75
Knop S, et al. 2008[9]	Lenalidomide + dexamethasone + adriamycin	85
Morgan GJ, et al. 2006[10]	Lenalidomide + dexamethasone + cyclophosphamide	65
Palumbo A, et al. 2008[11]	Lenalidomide + melphalan + prednisone + thalidomide	91
Richardson PG, et al. 2007[12]	Bortezomib + tanespimycin	50
Richardson P, et al. 2007[13]	Bortezomib + perifosine	56*
Badros AZ, et al. 2008[14]	Bortezomib + vorinostat	43
Weber DM, et al. 2008[15]	Bortezomib + vorinostat	24
Palumbo AP, et al. 2007[16]	Bortezomib + melphalan + prednisone + thalidomide	67

*ORR included minor response.
ORR, overall response rate (partial response or better).

Figure 1. Rationale for combination therapy in multiple myeloma.[5]



From Richardson PG, et al. *Expert Rev Anticancer Ther* 2008;8:1053-72.[5]

New Combination Approaches For Multiple Myeloma

Palumbo Antonio[1], Bringhen Sara[1], Gay Francesca[1], La Rocca Alessandra[1], Cavallo Federica[1], Offidani Massimo[2], Di Raimondo Francesco[3], Boccadoro Mario[1]

[1]Divisione di Ematologia Dell'Università di Torino, Azienda Ospedaliera San Giovanni Battista, Torino, Italy; [2]Clinica di Ematologia Azienda Ospedaliero-Universitaria, Ospedali Riuniti Ancona, Italy; [3] Cattedra di Ematologia, Ospedale Ferrarotto, Catania, Italy.

Correspondence: *Dr. Antonio Palumbo, Divisione di Ematologia Dell'Università di Torino, Azienda Ospedaliera San Giovanni Battista, Via Genova 3, 10126 Torino, Italy. E mail: appalumbo@yahoo.com. Tel.: +39 01166 35814; Fax: +39 01169 63737*

In patients with Multiple Myeloma (MM) the best choice of treatment is the combination of the old chemotherapy with new drugs. These drugs have been mainly associated with Dexamethasone (Dex), pegylated liposomal doxorubicin (PLD), and alkylating agents.

In a study comparing Thalidomide(Thal)-Dex vs Dex the association Thal-Dex has been more effective than Dex alone with improvements in terms of partial response (PR) rates and median time to progression. Similar results have been reported when Lenalidomide(Len)-Dex has ben compared with high-dose Dex. In order to reduce Dex-associated adverse events, *Len*-high-dose Dex (480 mg/month) has been compared with *Len*-low-dose Dex (160 mg/month) in newly diagnosed patients. This study has been prematurely stopped when patients on Len-low-dose Dex had improved 1-year overall survival (OS) compared to those receiving Len plus standard dose Dex (96% vs 88%; p<0·001). The arm Len-high-dose Dex showed more frequent grade 3–4 adverse events, including deep venous thrombosis (DVTs) and infections. In a non-randomized study, 32 previously untreated patients were treated with single-agent Bortezomib (Bor), with Dex added for patients who obtained ≤PR after 2 cycles or ≤ complete response (CR) after 4 cycles. Ten patients received single-agent Bor, and Dex was added in the remaining 22, leading to 15 improved responses. The association Bor-Dex as induction has been also demonstrated in two other phase II studies, with CR+near CR rates of 21–59%.

Offidani et al. treated 50 patients >65 years with Thal-Dex-PLD and reported an 88% ≥PR rate with a median follow-up of 18 months, a 57% of event free survival (EFS) and 74% of OS at 3 years.[1] In a phase II study the association Bor-PLD-Dex has been tested in untreated patients eligible for autologous stem cell transplantion (ASCT) showing a positive impact on quality of life (QoL) and a convincing activity as induction therapy in frontline treatment. A US phase II study demonstrated similar results with CR+nCR rates of 37%.

The MRC Myeloma IX trial is comparing the association of Cyclophosphamide(Cy)-Thal-Dex *vs* melphalan-prednisone (MP) in older patients and Cy-Thal-Dex *vs* Cy-vincristine-Doxo-Dex in younger patients showing improvements in terms of PR rates.[2] Among younger patients, post-induction ≥PR rate was 95·7% (with 20·3% CR) for Cy-Thal-Dex compared with 83·4% (11·7% CR) for Cy-vincristine-Doxo-Dex. The Cy-Thal-Dex treatment arm maintained the advantage in CR rates (58·2% *vs* 41%) 100 days post-high dose therapy (HDT).[2] In newly diagnosed patients, 4 cycles of Bor-Dex-Cy induced a PR rate of 100% prior to ASCT and a 85% of very good partial response (VGPR). This association has been effective in newly diagnosed MM patients producing responses exceeding those seen with other induction regimens. A study with 3 cycles of Bor-Dex-Cy followed by 3 cycles of Bor-Thal-Dex as first-line therapy has found a PR rate of 100% and a VGPR rate of 55%.[3] The administration of Bor-Thal-Dex after Bor-Dex-Cy increased the CR+nCR rate from 19% to 42%, with a marginal increase in ≥VGPR rate.

In three phase III studies, one from Italy[4] and two from French,[5] the MP-Thal regimen was superior

to MP in terms of ≥PR rate and progression free survival (PFS) in newly diagnosed MM patients while in the trial conducted by the Nordic Group there was a significant difference in time to progression (p<0·03). In a recent study by the Dutch cooperative group MP has been compared with MP-Thal with an improvement in ≥PR rate and EFS (p<0·001) in favour of MP-Thal. In conclusion, all 5 studies have shown better remission durations, but only the 2 French studies also reported better OS with MP-Thal. In the Italian phase I/II study of MP-Len the PR rate of 81% included 48% VGPR and 24% immunofixation-negative CR.[6] At 1 year, the EFS was 92% and the OS was 100%. This study formed the basis for the ongoing international phase III study, comparing MP *vs* MP-Len with or without Len maintenance. An international phase III randomized trial compared the MP regimen with MP-Bor in 682 untreated patients showed that the addition of Bor to MP improved the response rates and the median duration of the response that was 19·9 months in the bortezomib group and 13·1 months in the control group. In the bortezomib group, OS was improved of approximately 40%, the hazard ratio (HR) for OS was 0·61 (p=0·008).

A phase II study, evaluated the association Bor-Doxo-Dex as induction before ASCT in newly diagnosed patients, using Bor at the dose of 1·3 mg/m^2 (PAD1) or 1·0 mg/m^2 (PAD2) with VGPR rates with PAD1/PAD2 of at least 62%/42% post-induction and 81%/53% post-transplant while the PFS and the OS were similar. Both regimens were highly active as front-line induction and toxicity was lower in PAD2.[7] In an ongoing phase III randomized trial, the GIMEMA group compared Bor-Thal-Dex with Thal-Dex as induction therapy and consolidation therapy following double ASCT with Mel 200 mg/m^2.[8] Preliminary results indicate that patients on Bor-Thal-Dex had a higher ≥PR rate after induction (60% ≥VGPR *vs* 25% ≥VGPR) and after the first transplantation (77% ≥VGPR *vs* 54% ≥VGPR). A phase I/II study has been conducted to test the combination Bor-Len-Dex in patients newly diagnosed; the overall PR rate was 98%, including 71% ≥VGPR and 29% CR+nCR with no treatment-related mortality, no significant peripheral neuropathy, and only rare cases of deep-vein thrombosis.[9]

New agents have been tested as induction treatment prior to ASCT, and as consolidation or maintenance in a sequential approach. The association Bor-PLD-Dex as induction prior to reduced intensity ASCT followed by Len-prednisone as consolidation and Len alone as maintenance has been recently tested in 94 newly diagnosed patients aged 65–75 years.[10] After 4 induction cycles with Bor-PLD-Dex, 60% of patients achieved at least a VGPR. After tandem Mel 100 mg/m^2, 80% of patients showed at least VGPR, including 33% of CR while after the consolidation regimen with Len-prednisone 89% of patients achieved at least VGPR, including 56% CR.

The new 3 drug combinations (including chemotherapy, Dex and new drugs) are improving the response rate obtained with the 2 drug combination (including Dex and new drugs). If this associations will also improve clinical outcome needs to be elucidate in prospective controlled clinical trials.

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New Proteasome Inhibitors

Robert Z. Orlowski, M.D., Ph.D.

The University of Texas M. D. Anderson Cancer Center Houston, Texas, U.S.A.

The ubiquitin-proteasome pathway has been validated as a rational target for multiple myeloma therapy (1) by the approval of the proteasome inhibitor bortezomib (VELCADE®). Initially,encouraging pre-clinical (2) and phase I clinical activity (3) for this agent was described, and based on a phase II trial (4), bortezomib was approved as a single agent for patients who had received at least two prior lines of therapy, and who had relapsed/refractory disease after their most recent treatment. Later, based on positive results from a randomized phase III trial (5), this approval was expanded to allow patients with relapsed and/or refractory disease after one or more prior lines of therapy to benefit from this novel therapeutic. Since modulation of proteasome function is also a rational approach to overcome chemoresistance and achieve chemosensitization (1), a number of combination regimens incorporating bortezomib have shown encouraging activity. Indeed, one such combination with pegylated liposomal doxorubicin (Doxil®), which pre-clinically (6) and in a phase I study (7) showed signs of enhanced activity, was later found in a phase III trial to be superior to single-agent bortezomib in a number of clinically relevant endpoints, including overall survival (8), for relapsed and/orrefractory multiple myeloma. Most recently, bortezomib with melphalan and prednisone has been found to significantly improve upon the benefits of induction therapy with melphalan and prednisone alone (9), leading to the first approval of a proteasome inhibitor as part of initial myeloma therapy.

Bortezomib is a slowly reversible inhibitor of the proteasome which targets the chymotrypsin-like and post-glutamyl peptide hydrolyzing, or caspase-like, activities (1). Its success suggested the possibility that other proteasome inhibitors with different properties could be of benefit as well, and the two that are furthest along in development are NPI-0052 and carfilzomib (formerly PR-171). NPI-0052 irreversibly binds to the proteasome with greatest affinity for the chymotrypsinlikeand trypsin-like activities (10), while carfilzomib, which also binds irreversibly, targetspredominantly the chymotrypsin-like activity in myeloma models (11). Preclinically, both ofthese agents showed enhanced potency compared to bortezomib, and were able to overcomebortezomib resistance. The former activated programmed cell death predominantly throughcaspase-8 (10), while carfilzomib proved to have a mechanism of action more like bortezomib inits ability to induce dual apoptotic signaling through both caspases-8 and -9 (11).

NPI-0052 and carfilzomib have been evaluated in phase I clinical trials, the results of which have been presented only in abstract form to date. These agents have demonstrated good clinical tolerability profiles despite their irreversible inhibition of the proteasome, which might have been expected to increase toxicity compared to the known profile of bortezomib. In the case of carfilzomib, daily dosing for either two or five consecutive days was found to be welltolerated, in contrast with bortezomib, for which the approved regimen recommends dosing no more frequently than every 72 hours. Both novel drugs have demonstrated a pharmacokinetic profile similar to bortezomib, with rapid clearance of the agents from the blood, and a dosedependent inhibition of the proteasome in studies of their respective pharmacodynamics. One study of NPI-0052 in patients with solid tumors and lymphoma has shown signs of clinical activity, including stable disease in a total of six patients, including one with melanoma, one with colorectal carcinoma, one with hepatocellular carcinoma, two with adenoid cystic carcinoma, and one with cervical carcinoma (Aghajanian, CA et al. 2008 ASCO Abstract 3574). Two phase I studies have been performed with carfilzomib, both focusing on patients with B-cell hematologic malignancies including multiple myeloma. These also have shown evidence of anti-tumor activity in the

form of partial responses in patients with multiple myeloma, including some who had not previously responded to bortezomib or a bortezomib-based regimen (Orlowski, RZ et al. 2007 ASH Abstract 409; Alsina, M et al. 2007 ASH Abstract 411). Interestingly, this second generation of proteasome inhibitors may have a lesser risk of peripheral neuropathy, though some renal effects have been seen with both agents that will need further study.

Based on these encouraging signs of activity, carfilzomib is being evaluated in two phase II trials targeting patients with relapsed, and relapsed/refractory multiple myeloma, respectively. The schedule for both of these studies calls for drug administration as an intravenous bolus dose on days 1, 2, 8, 9, 15, and 16 of every 28-day cycle. Early results from these trials, and further updates on the progress of NPI-0052, will be presented for the first time at the December, 2008 meeting of the American Society of Hematology in San Francisco. These datawill be presented and updated at the 12th International Myeloma Workshop, in Delhi.

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Anti-Angiogenic Agents In Multiple Myeloma

Shaji Kumar, M.D.

Mayo Clinic, Rochester, MN

The phenomenon of increased tumor vascularity was described decades ago, but its potential role as a therapeutic target has been the subject of intense investigation only recently. Studies during the past decade have highlighted the presence of aberrantly increased tumor vasculature in hematological malignancies, similar to what had been described in solid tumors. In the setting of plasma cell disorders, increased angiogenesis has been described in the bone marrow of patients with multiple myeloma (Figure) as well as within plasmacytomas. BM angiogenesis increases with disease progression from monoclonal gammopathy of undetermined significance to active myeloma(1). Multiple studies have confirmed BM MVD to be prognostic in patients with MM, including patients undergoing high dose therapy, and can also predict progression in those with solitary plasmacytoma. (1-5) Increased angiogenesis in the bone marrow correlate with other prognostic factors in myeloma, such as plasma cell labeling index, cytogenetic abnormalities and presence of circulating plasma cells. In vitro experiments have shown that endothelial cell lines as well as primary marrow derive endothelial cells are capable of inducing myeloma cell proliferation, as well as protecting them from effect of commonly used anti-myeloma drugs. These findings certainly form a convincing argument to target the aberrant vasculature and the endothelial cells as a potential therapeutic approach.

'Anti-angiogenic' approaches to treatment of myeloma can be broadly grouped into two, those utilizing agents also known to have anti-angiogenic activity and those drugs there designed as specific anti-angiogenic agents. The novel agents currently approved for use in myeloma belong to the first category while many of the drugs in the second category are currently undergoing evaluation in phase I and II clinical trials. Thalidomide was initially introduced for treatment of myeloma, nearly a decade ago, because of its potential anti-angiogenic activity.(6) The success seen with this drug led to a series of clinical trials that evaluated various combinations that incorporated thalidomide and conventional therapies resulting in its approval for use in newly diagnosed and relapse disease. However, it remains unclear how much of the anti-myeloma activity of thalidomide can be ascribed to the anti-angiogenic properties. Correlative studies performed alongside some of the thalidomide clinical trials have provided conflicting evidence.(6-8) While baseline bone marrow angiogenesis does not seem to predict the likelihood of response to thalidomide, some have reported decrease in the bone marrow microvessel density following thalidomide therapy in responding patients. Similar decrease can also be seen with non-thalidomide therapy and hence may just reflect disease control and may not be specific for thalidomide. Other studies have failed to sow any significant changes in the angiogenic cytokine profile among patients undergoing thalidomide therapy. Similarly, the thalidomide analogue lenalidomide has been shown to have anti-angiogenic activity in vitro, but definite proof of the role of this anti-angiogenic property in its clinical effects remains unproven. Finally, recent studies have also shown that the proteasome inhibitor bortezomib has potent anti-endothelial cell activity in vitro and raises the possibility that at least some of its activity can be explained on the basis of its effect on the endothelial cells.(9)

During the past few years a multitude of targeted 'anti-angiogenic' agents have been introduced as potential therapies for cancers, primarily in the setting of solid tumors. These have included predominantly agents targeted towards blocking the effects of vascular endothelial growth factor, while others have targeted platelet derived growth factor, fibroblast growth factor on angiopoietin among others.(10) The most prominent of these have been the anti-VEGF antibody, bevacizumab, which is currently approved for use in a wide spectrum of

malignancies. Bevacizumab has been studied in the setting of myeloma without any significant activity and ongoing trials are evaluating the antibody in combination with other drugs. Other anti-VEGF strategies have included small molecule tyrosine kinase inhibitors that selectively block the activity of VEGF receptors and the downstream signaling pathways. In vitro experiments suggest significant activity in the setting of myeloma and many of these drugs such as sorafenib and sunitinib are currently being evaluated in phase II clinical trials. However, based on the current understanding of the myeloma biology, it is reasonable to speculate that these drugs by them selves may not have significant activity in myeloma. The redundancy of survival factors and their downstream signaling pathways is well known and it is likely that these agents may work better when used in combination with other drugs. Ongoing clinical trials are evaluating combinations of anti-VEGF agents with currently used drugs such as thalidomide and bortezomib.

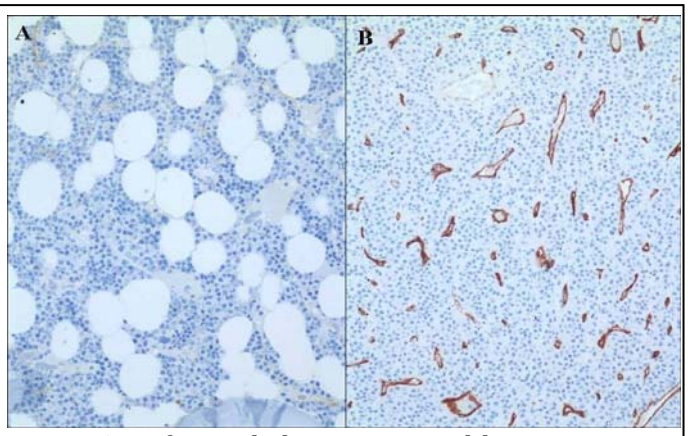

Figure 1: Left panel shows a normal bone marrow; right panel shows bone marrow from a patient with newly diagnosed myeloma. Microvessels are stained using an antibody to CD34 antigen.

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Novel Bone Targeting Agents In Multiple Myeloma (MM)

S. Vallet,[1] N. Raje.[1]

[1]Massachusetts General Hospital Cancer Center and Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA.

Eighty percent of MM patients present with bone disease, often complicated by severe pain, pathologic fractures, spinal cord compression and hypercalcemia [1]. These skeletal-related events (SRE) negatively impact patients' quality of life and are managed by a combination of surgical, radiotherapeutic, and medical approaches. Bisphosphonates (BP), pamidronate and zoledronic acid in particular, are currently considered the standard of care for cancer-related bone disease. They exert an anti-catabolic effect via inhibition of osteoclast (OC) activity and induction of OC apoptosis. Treatment of cancer patients with BP has resulted in the reduction of the rate of SRE by about 20% compared to placebo. Although of significant benefit BP therapy has most recently been associated with complications such as osteonecrosis of the jaw. Ongoing studies are focused on understanding the underlying patho-physiolgy and testing alternate dosing schedules of BP in the treatment of MM bone disease.

The pathogenesis of MM bone disease relies on the interaction between malignant plasma cells and their bone microenvironment. MM cells release cytokines that recruit OC precursor cells and enhance OC activity, while inhibiting bone marrow stromal cells (BMSC) differentiation to osteoblasts (OB)[1]. Importantly, OC and BMSC sustain MM cell growth in contrast to OBs. Therefore, MM cells shape their microenvironment and create a niche suitable for their survival resulting in disruption of the OC/OB balance and bone remodeling. With the aim of restoring physiologic bone remodeling, several new agents with anti-catabolic and anabolic properties have been developed and studied in MM and will be discussed here.

The disruption of the receptor activator of NF-kB ligand (RANKL)/osteoprotegerin (OPG) balance is an important feature of MM related bone disease. The RANKL/OPG ratio plays a key role in regulating OC development. Indeed, RANKL stimulates OC differentiation and promotes OC survival, while OPG is a soluble receptor secreted by OB that inhibits RANKL effects by specific binding. Both BMSC and MM cells secrete RANKL resulting in high bone marrow plasma levels in MM patients. Conversely, OPG levels are significantly reduced, thus promoting OC differentiation. Based on these data, both OPG recombinant protein and neutralizing antibody anti-RANKL have been studied in MM patients with bone disease. Although both compounds effectively reduced bone resorption parameters, only neutralizing antibody anti-RANKL, Denosumab (AMG162), was further developed. A single subcutaneous infusion of Denosumab had a safe toxicity profile and effectively reduced bone resorption, assessed by N-telopeptide levels. Interestingly, the suppressive effects of Denosumab on bone resorption were persistent for 12 weeks compared to the 3-4 weeks of BP [2]. Since OC support MM cell growth, current trials are evaluating the effects of Denosumab on tumor progression.

The chemokine CCL3 is another promising target in MM bone disease. CCL3 levels correlate with bone disease in MM patients and it is both a promoter of osteoclastogenesis and MM cell growth. Two receptors mediate its effects, CCR1 and CCR5. CCR1 inhibitors, in particular, have been studied for their anti-OC activity via inhibition of OC differentiation. Importantly, CCR1 inhibition blocks the proliferative advantage conferred by OC on MM cells, therefore suggesting a dual effect on bone resorption and tumor burden [3]. Preliminary data confirmed the anti-OC effects in in-vivo models of MM bone disease supporting future clinical trials in MM patients. Both MM cells and OC promote each others' proliferation and survival and the B-cell Activating Factor (BAFF) is a key player in this loop. BAFF is a tumor necrosis factor (TNF)-related ligand secreted by OC and BMSC that

supports MM cell growth and stromal cell adhesion. In vivo, an anti-BAFF neutralizing antibody inhibited MM cell growth leading to prolonged survival. Importantly, it reduced OC number as well as osteolytic lesions in a mouse model of humanized MM bone disease [4]. Therefore, BAFF is a promising target in MM bone disease and clinical trials are ongoing to evaluate the effects of neutralizing BAFF antibody in MM patients. Finally, also the immunomodulatory compound Lenalidomide has been studied for its anti-OC effect mediated by inhibition of PU.1, a critical transcription factor in OC differentiation [5].

The focus of research until recently has been on the OC axis, with the OB axis remaining largely underexploited. Recent studies have demonstrated that stimulation of OB differentiation results in a hostile environment for MM cells that led to reduced plasma cell growth. Indeed, in vitro and in vivo assays showed that OBs do not support MM cell proliferation when compared to either OC or BMSC. The proteasome inhibitor, Bortezomib is a widely used anti-MM drug. It inhibits MM cells directly as well as indirectly by modifying their microenvironment. By inhibition of protein degradation and consequent restoration of beta-catenin levels, low doses of Bortezomib potently stimulate OB differentiation from BMSCs and overcome the inhibitory effect of MM cells on OB [6,7]. Importantly, alkaline phosphatase (ALP) levels, a parameter of OB differentiation, correlated with response to Bortezomib, suggesting that modifications of the bone microenvironment could lead to reduction in tumor burden.

A critical player in MM-mediated bone disease is dickoppf1 (DKK1), an inhibitor of WNT signaling pathway that prevents OB differentiation. High levels of DKK1 are observed in bone marrow plasma of MM patients with active bone disease compared to monoclonal gammopathy of unknown significance (MGUS) and normal donors. DKK1 is secreted mainly by primary MM cells and it promotes interleukin-6 (IL6) secretion by inhibiting BMSC differentiation in OB. Neutralizing antibody anti-DKK1 stimulates OB differentiation both in vitro and in vivo in the presence of MM[8,9], rescuing bone disease in an in-vivo model of MM. DKK1 inhibitors are currently undergoing phase I clinical trials in MM patients with bone disease.

We have recently identified activin A as another promising target in MM bone disease. Activin A is a transforming growth factor (TGF)-β super-family member involved in bone catabolism with both pro-OC and anti-OB effects. Its levels are increased in BM plasma of MM patients with osteolytic lesions, and in contrast to DKK1 it is synthesized and secreted mainly by BMSC and OC cells. Importantly, adhesion of MM cells to BMSC further enhanced its secretion. Inhibition of activin A by a soluble receptor promotes OB differentiation and overcomes myeloma-induced OB inhibition. In vivo, it translates in improved bone density and decreased osteolytic lesions in a mouse model of humanized MM bone disease. Importantly, activin A inhibition reduces MM growth in the context of the microenvironment both in vitro and in vivo [10].

Since unbalanced OC/OB axis characterizes MM, new treatment strategies should focus on promoting OB differentiation while inhibiting OC activity. Agents such as activin A inhibitors with dual effects on OC and OB differentiation are particularly promising for restoring bone homeostasis in MM. Other approaches rely on the combination of anabolic and anti-catabolic agents. Indeed, ongoing trials are combining anti-BAFF neutralizing antibodies with Bortezomib, with the purpose of restoring physiologic bone remodeling in MM patients. These combined approaches along with optimal use of BP will result not only in alleviating SRE but more importantly may also contribute to improved anti-tumor activity in future clinical trials.

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Pomalidomide (CC4047) Plus Low-Dose Dexamethasone (Pom/Dex) As Therapy For Relapsed Multiple Myeloma

Martha Q. Lacy, MD, Suzanne R. Hayman, MD, Morie A. Gertz, MD, , Angela Dispenzieri, MD, Stephen R. Zeldenrust, MD, PhD, Shaji Kumar, MD, Philip R. Greipp, MD, John A. Lust, MD, PhD, Stephen J Russell, MD, PhD, Francis Buadi, MD, Robert A. Kyle, MD, Rafael Fonseca, MD, P. Leif Bergsagel, MD, Vivek Roy, MD, Joseph R. Mikhael, MD, Keith Stewart, MD, Jacob B. Allred, MS., Kristina Laumann, Melanie Thompson, Sumithra J. Mandrekar, Ph.D., S. Vincent Rajkumar, MD

Mayo Clinic, Rochester, MN, Mayo Clinic, Scottsdale, AZ, Mayo Clinic, Jacksonville, FL

Introduction

The introduction of thalidomide represented a major milestone in the treatment of multiple myeloma. Thalidomide was initially used for treatment of multiple myeloma because of its antiangiogenic properties[1]. Promising clinical results lead to the development of a class of thalidomide analogues termed immune-modulatory drugs (IMiDs). In relapsed myeloma, single thalidomide has response rates of 30-35%[1,2]. The addition of dexamethasone to thalidomide improves responses to 40-50%[3]. Lenalidomide and pulse dexamethasone has response rates of 55-60%[4,5] in relapsed myeloma and 90% in newly diagnosed myeloma[6,7]. CC4047 (pomalidomide) is the newest IMiD. Phase I trials established the agent is well tolerated in doses ranging from 1-5 mg/day[8]. We report on the first Phase 2 trial of pomalidomide combined with low dose dexamethasone (Pom/dex) in patients with relapsed or refractory multiple myeloma.

Methods

37 patients (21 male and 16 female) were enrolled. Pomalidomide was given orally 2 mg daily on days 1–28 of a 28-day cycle. Dexamethasone was given orally at a dose of 40 mg daily on days 1, 8, 15 and 22 of each cycle. The primary endpoint of this trial is the proportion of confirmed responses. A confirmed response is defined to be a CR, PR or VGPR as assessed by the International Myeloma Working Group Uniform Response criteria. The regimen would be declared effective if at least 11 responses were observed out of 37 patients using a single stage phase II design. All patients received aspirin 325 mg daily as prophylaxis against DVT. After the first 37 patients were enrolled a second cohort of 23 patients were added to gain additional information about toxicity and to gain insight as to whether increasing the dose from 2 mg/day to 4 mg/day in non-responders is effective.

Patient Population

Among all 60 patients enrolled, the median age was 66 years (range, 35 - 88). All patients were evaluable for response and toxicity, and all analysis were done on intent to treat basis. All patients had received prior therapy; 35% had 3 prior regimens; 37% had 2 prior regimens and 28% had one prior regimen. 65% had previous autologous stem cell transplant (ASCT) including 30 patients who had one previous ASCT and 9 who had two previous ASCT. 36 patients (60%) had previous IMiD therapy. The median time from diagnosis to enrollment on study was 44 months.

Toxicity

Toxic effects were graded according to the National Cancer Institute's Common Toxicity Criteria, version 3. Toxicity consisted primarily of myelosuppression. Grade 3 or 4 hematologic toxicity included: neutropenia in 18 patients (30%), anemia in two patients (3%), and thrombocytopenia in one patient (1.6%). The most common Grade 3/4 non-hematologic toxicity consisted of fatigue (12%) and pneumonia (7%). Other grade 3/4 non-hematologic toxicities seen less than 5% included: diarrhea, atrial fibrillation, dehydration and renal insufficiency, constipation, hyperglycemia and dizziness. 25 % had neuropathy including 12 patients with grade 1 (20%) neuropathy and 3 (5%) with grade 2 neuropathy. There was grade 3 or 4 neuropathy. No patients have had thromboembolic events. Two patients died on study. One patient, an 88

year old female, died 3.3 months after initiating study treatment. This patient began study treatment 86 months after diagnosis and had received one previous chemotherapy regimen. Lytic lesions, high bone marrow labeling index and high beta-2 microglobulin were observed at baseline. This patient maintained stable disease for two cycles before passing due to valvular heart disease not related to study treatment. The second patient, an 82 year old female, developed neutropenic fever and pneumonia requiring mechanical ventilation during cycle 1. Her death from infection was attributed to her treatment.

Efficacy

Among the first 37 patients enrolled objective responses were seen in 23 patients (62%) including 9 (24%) with VGPR; 14 patients (38%) with PR; 6 (16%) with stable disease. Objective responses were seen in 4 of 13 patients (29%) who were refractory to lenalidomide. Based on this, the trial was expanded to include 23 additional patients for a total of 60. With a median follow-up of 3.6 months (range, 0-9.6) objective responses were seen in 35 patients (58%) including PR 20 patients (33%), VGPR 14 patients (23%) and CR one patient (2%). Eleven patients are stable and one patient is inevaluable for response. 43 patients continue to receive treatment. Thirteen have progressed.

Discussion

The combination of pomalidomide and low dose dexamethasone is highly active and well tolerated in the treatment of relapsed/refractory multiple myeloma. Lenalidomide was approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) based on the results of two randomized phase three trials that showed the combination lenalidomide and dexamethasone (len/dex) was superior to dexamethasone alone[4,5]. Much of the toxicity of the len/dex regimen was due to the high dose pulse dexamethasone. The Eastern Cooperative Oncology Group has recently reported results of a randomized phase three trial that shows lenalidomide with weekly dexamethasone is safer and associated with improved survival in patients with newly diagnosed multiple myeloma[9]. The choice to use low dose weekly dexamethasone in our trial was based on these results. Here we report objective response rates that are similar to what has been seen in the trials of lenalidomide with high dose pulse dexamethasone. Toxicity in our trial has been mild and consists primarily of myelosuppression with neutropenia. Importantly, we are seeing responses in patients who have been shown to lenalidomide-refractory. Based on this, we plan to open a phase II trial of pomalidomide and dexamethasone for lenalidomide-refractory patients.

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Current Status And Future Directions Of HCT In Myeloma: Moving Beyond High Dose Melphalan

Muzaffar H. Qazilbash, MD; Sergio A. Giralt, MD.

Department of Stem Cell Transplantation and Cellular Therapy ,University of Texas-MD Anderson Cancer Center .1515 Holcombe Boulevard-423 ,Houston, TX, 77030 ,E-mail: mqazilba@mdanderson.org

Introduction

Multiple myeloma is currently the most common indication for high dose therapy with autologous HCT in North America and Europe (1). In North America alone more than 2000 transplantations are performed each year. **Table 1** summarizes the patient characteristics of patients undergoing autologous HCT for myeloma as reported to the Center of International Blood and Marrow Transplant Research (CIBMTR). High dose melphalan is used in over 90% of patients with the most common regimen used being single agent melphalan at a median dose of 180-200 mg/m^2 performed as a single transplantation (CIBMTR unpublished observations).

Improving High Dose Melphalan Therapy: Dose Intensification

Relapse after autologous HCT remains the single most important cause of treatment failure in patients. Strategies aimed at improving the conditioning regimen have usually focused on intensifying the conditioning regimen either by increasing the dose of melphalan or by adding other alkylating agents as summarized in **Table 2**. Of these only tandem high dose therapy has been reported to improve outcomes in randomized trials.(2,3,4,5) These trials were performed prior to the advent of lenalidomide or bortezomib, and included ineffective maintenance therapy. Thus the role of tandem transplantation in the era of novel therapies needs to be reconsidered. Much has been made about the potential lack of benefit of tandem transplantation in patients achieving a 90% or more reduction in tumor burden. This data emerges from a post hoc analysis of both the French and Italian randomized trials and is limited by the post-hoc nature of the analysis and the relatively small number of patients.

Increasing the dose of melphalan with or without cytoprotectors such as amifostine has been explored. Melphalan doses of up to 280 mg/m^2 of melphalan with amifostine have been administered. Significant mucosal toxicities were observed, and at the higher doses cardiotoxicity was dose limiting (6).

Moreau et al. have explored using melphalan at a dose of 220 mg/m^2 as part of a tandem transplantation strategy for patients with high risk myeloma as defined by cytogenetics and beta 2 microglobulin. Complete remissions were seen in 30% of patients with another 18% achieving at least a 90% reduction in tumor burden (7).

Improving High Dose Melphalan Therapy: Novel Conditioning Regimens

The recent expansion of therapeutic options for MM can be partly attributed to a better understanding of the interactions between malignant plasma cells and bone marrow microenvironment that includes stromal cells, extracellular adhesion molecules and secreted cytokines. Novel therapies targeting these interactions have shown promising responses and outcomes.(8)

Although intensification using more alkylating agents has not resulted in improved outcomes, the use of novel agents in combinations with high dose melphalan may provide more promising results (9,10). Arsenic trioxide, bortezomib and targeted skeletal radiotherapy with either Holmium-DOTMP or Samarium EDMTP have been studied as part of the conditioning regimen with encouraging phase II results that need to be confirmed in larger phase III trials. (11,12,13,14)

Optimizing High Dose Melphalan Therapy

Tolerance and response to melphalan at a dose of 200 mg/m^2 is extremely heterogeneous. To a certain degree this variability is probably due to

genetic polymorphisms among enzymes such as Glutathione-S-Transferase (GST) (15). However, the practice of dosing melphalan according to body surface area, with arbitrary modifications for overweight patients also suggests that melphalan dosing may be implicated as reported by Grazziuti et al.(16) Lastly, Dimopoulos et al. have demonstrated that the extent of damage and repair seen in the *p53* tumor-suppressor gene of peripheral blood lymphocytes after exposure to melphalan is an important predictor of outcome and could serve as a tool for developing a personalized or individually tailored conditioning regimen.(17)

Summary

The advent of new agents such as bortezomib, thalidomide, and lenalidomide and the improved outcomes after induction therapy should move the field to re-explore the role of high dose melphalan as consolidation therapy for all patients with multiple myeloma. Likewise, exploring strategies that could improve on the outcomes of high dose melphalan are necessary. Post transplantation therapy is also becoming an important adjuvant to improve outcomes.

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Table 1: Characteristics Of Patients Who Underwent Autologous HCT For Multiple Myeloma For 1st Remission Consolidation Between 2000 And 2004 In North America: Reported To The CIBMTR

Characteristics of patients	N (Eval)	Median (range)[a] N(%)[b]
Number of patients	1371	
Age, (median, range), years		58 (22-80)
Time from Dx to SCT (months)		7 (1-103)
Conditioning regimen	1367	
Lpam alone		1133 (83)
Lpam+TBI ± other		44 (3)
Lpam ± other (not TBI)		130 (10)
Others		60 (<1)
Dose of Melphalan 200, median (range), mg/m^2	1259	187 (0.9-278)

Lpam=melphalan; TBI=total body irradiation; Dx=diagnosis; SCT=stem cell transplant

Table 2: Outcomes Using Intensified Conditioning Regimens Compared to Melphalan 200 mg/m^2- Single Autologous HCT Trials

Strategy	EFS Median (months)	%CR / %VGPR	Reference & Comments
Melphalan 200 vs. Melphalan 140 + TBI	20 21	35/20 29/14	Randomized Trial
Melphalan 200 vs. Busulfan-Melphalan vs. Melphalan + TBI	22 32 20	43/NR 49/NR 31/NR	Registry Analysis
Melphalan 200 vs. Melphalan + Holmium-DOTMP (<2400 cGy)	19 30	32/NR 55/NR	Retrospective Analysis
Melphalan 200 vs. Thiotepa busulfan cyclophosphamide	20 21	16/NR 27/NR	Retrospective Analysis

Timing Of Stem Cell Transplant In The Era Of Novel Agents

Donna M Weber, MD

M.D.Anderson Cancer Center, Houston TX U.S.A.

Multiple myeloma has generally been considered an incurable disorder of malignant plasma cells. The longest survivals have historically been achieved after treatment with myeloablative therapy and autologous stem cell support (AuSCT). Evidence for this is largely based on 2 phase III trials [Intergroupe Francophone du Myelome (IFM-90) and the Medical Research Council Myeloma VII Trials] that demonstrated superior survival for patients randomized to treatment with AuSCT compared with conventional, alkylating agent or anthracycline-based chemotherapy.[1,2] These survival benefits were likely attributable to the higher rate of complete remission (CR) attainable in approximately 1/4 -1/2 of patients with myeloablative therapy. Based on these results myeloablative therapy with stem cell support emerged as a relative standard of care for early treatment of myeloma. After even higher rates of CR, very good partial remission (VGPR), and ultimately, improved survival, were demonstrated with myeloablative therapy and tandem AuSCT, tandem transplant became the standard for most patients not achieving at least VGPR after a single AuSCT.[3,4]

Subsequently during the past decade, 3 agents with novel mechanisms of action (thalidomide, bortezomib, and lenalidomide have proven to be highly effective for myeloma, resulting in improved survival of patients with this disorder, particularly in combination with steroids, other novel agents and/or conventional chemotherapeutics (alkylating agents, anthracyclines, etc). For the first time CR and VGPR rates equivalent or superior to those seen after myeloablative therapy and AuSCT (25-65%) are attainable without myeloablative therapy, raising the question of the timing, and/or necessity of intensive therapy (See Table). Additionally, data from Fermand et al previously revealed no significant difference in overall survival of patients randomized to receive AuSCT after induction therapy or at first relapse; subsequent studies have included subanalyses that appear to confirm these initial observations.[5,6] While these studies suggest AuSCT may not be necessary for consolidation of induction response and that it may be reserved for use as consolidation of salvage therapy for relapsing disease, others have suggested no benefit of AuSCT supported therapy over chemotherapy alone or that benefits are limited to narrow patient subgroups, such as those with primary refractory disease .[7]

Although over the past few decades myeloablative therapy with AuSCT has often been the focus of much debate, recently the superiority of thalidomide-melphalan-prednisone compared with not only melphalan-prednisone, but also melphalan 100 mg/m^2 and AuSCT for survival of patients $\geq$ 65 years old, has raised doubt regarding the necessity of AuSCT supported therapy for not only elderly, but also patients of all ages in the era of novel agents.[8] These results coupled with the high rates of CR, near CR and VGPR seen particularly with 3 drug regimens including novel agents (See Table) have increased the speculation that AuSCT may not be necessary for consolidation of induction, or at all, for treatment of myeloma patients. However, many of the best results in terms of response have been noted after induction therapy with novel agents followed by consolidation with AuSCT supported therapy (See Table). The difficulty with trying to decide on the timing or necessity of transplant supported therapy lies in trying to determine superiority in the absence of actual phase III data demonstrating a survival benefit for novel agent combinations alone compared with novel agent induction regimens followed by myeloablative therapy with AuSCT. This lack of data is compounded by many other questions that are frequently debated including: 1. Does CR improves survival? 2. Is the equality of a response better than near CR or even VGPR to CR? 3. Is it most useful to use 3 or 4 drug regimens for induction or to reserve naïveté of some agents for use in relapse? 4. Is the superiority of novel agent combination therapy compared with reduced

intensity myeloablative AuSCT valid when considered in comparison to full intensity myeloablative therapy/AuSCT in patients ≥ 65 years? 5. Is phase III data showing superiority of AuSCT performed prior to novel agent induction therapy valid in the era of novel agents? 6. Is the quality of life better after prolonged therapy with novel agents or after induction therapy followed by AuSCT.

Recently, LaHuerta et al published results of a subanalysis of 632 patients from the prospective Grupo Espanol de Mieloma 2000 protocol during which patients received induction therapy with alkylating agent-anthracycine-steroid-based therapy consolidated by melphalan-based myeloablative regimens/AuSCT, followed by either tandem AuSCT or reduced-intensity conditioning allogeneic SCT with a matched sibling donor for patients not achieving near-CR or better.[9] All patients received interferon/prednisone maintenance therapy. Patients whose disease was in CR had a significant survival advantage over all other groups including near CR, regardless of when CR was achieved. Additionally, failure to convert to CR from near-CR post-AuSCT was associated with a worse outcome even when compared with patients who upgraded to near CR post-AuSCT from a lesser response pre-AuSCT. Trends (but not significance) suggested similar, but less evident differences between nCR and PR favoring nCR. These data raise doubt of the validity of the current practice of pooling patients with VGPR or better together, and may confirm observations made by others that CR, the ability to convert to CR (defining continued sensitivity to alkylating agents) post-AuSCT, and duration of CR are the most important predictors of survival. Even though initial scrutiny of these data support early intensification/consolidation with AuSCT to increase early attainment/conversion to CR, the lack of an induction regimen inclusive of novel agent combinations, that generally offer higher pre-AuSCT CR rates makes clear interpretation of these data and survival benefits of AuSCT less than clear in the era of novel agents. Furthermore, this report adds to the confusion of how to proceed with respect to patients ≥ 65 years, since among the 55% of patients within this age group that were fit enough to proceed to AuSCT, similar benefits in survival as described for younger counterparts were noted. These data seem similar to previously reported data at our own center and a recent meta-analysis as well.[10]

New encouraging data for response, event-free and overall survival for myeloma patients continues to accumulate rapidly. It is clear that well designed phase III trials are imperative to answer questions of whether induction therapy with novel agents should be immediately consolidated by AuSCT, reserved for first relapse, or whether AuSCT is necessary in this era. Although the debate regarding the necessity of CR remains unanswered, it is clear that CR is necessary for cure and as better methods of defining CR continue to be developed, CR needs to remain a goal of therapy. The quest for CR needs to be balanced by quality of life in the absence of significant cure rates and thus, well-designed quality of life studies are also essential to evaluate the impact of side effects like neuropathy, thrombosis, myelosuppression and fatigue for both AuSCT and non- AuSCT supported therapies in order to make rational decisions regarding the timing of treatments for myeloma patients. In the absence of this data, it seems appropriate to utilize induction therapy with novel agents to maximize potential for early CR and to consider early consolidation therapy with AuSCT. Current phase III data still supports the role of AuSCT supported therapy and thus, if not initially performed for consolidation of first remission, second remission consolidation should remain an option for patients with myeloma.

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AuSCT Study	**CR (%)**	**CR/nCR (%)**	**VGPR (%)**	**PR (%)**	**EFS/PFS/TTP**	**OS**
IFM 90 (AuSCT arm) Attal et Al	22		16	63	27med mos/-/-	NR (52%5yr)
IFM 94 (tandem) Attal et al			≥VGPR 50	38	25med mos/-/-	48mos med
IFM 2005 Harrouseau et al		40.8	31.8	21	5yr EFS 52%/-/-	NR
Novel Agent Study						
Vista San Miguel et al	35		10	46	-/-/24 med mos	2 yr 83%
CyBorD Reeder et al		64	21	15		
Bor-Len-Dex Richardson et al	21	7 (nCR)	24	45	NR	NR
Len-Mel-Pred Palumbo et al	48		24	9	1yr EFS 100 (≥ VGPR) 88% (≤ PR)	
BiRD Niesvizky et al	28	38	25	12		
MPT (IFM99-06) Facon et al	13	34	29		-/27.5/-	51.6 med mos

Table 1: Efficacy of Selected Induction Regimens with Novel Agents or with Induction Therapy and Consolidation with AuSCT

Single Or Double Autologous Stem-Cell Transplantation Before And After The Era Of Novel Agents

Michele Cavo, Elena Zamagni, Paola Tacchetti, Michela Ceccolini, Nicoletta Testoni, Carolina Terragna, Annamaria Brioli, Caterina Pallotti, Lucia Pantani, Giulia Marzocchi, Sandra Durante, Alessandro Petrucci, Patrizia Tosi, Michele Baccarani

"Seràgnoli" Institute of Hematology and Medical Oncology,
Bologna University School of Medicine, Italy

Because of its potential of significantly increasing the rate of complete response (CR) up to the 30% range and prolonging the duration of event-free survival (EFS) and overall survival (OS) by approximately one year when compared with conventional chemotherapy[1], since the mid 1990s autologous stem-cell transplantation (ASCT) has been considered the standard of care for younger patients with newly diagnosed multiple myeloma (MM). Possible selection bias in study design, such as inclusion of patients with chemosensitive disease or possibility to receive ASCT after failure of conventional chemotherapy, may explain the lack of a survival gain with ASCT reported in several studies. Similar results were found in other studies and were more likely to be related, on the one hand, to the high CR rate effected by traditional therapy and, on the other hand, to the low CR rate with suboptimal regimens in preparation for ASCT. In most of the studies published so far, achievement of CR or at least very good partial response (VGPR) were closely associated with improved clinical outcome[2]. On this basis, in subsequent studies efforts to increase the CR rate as a way to furtherly improve the duration of OS have focused on cytotoxic dose intensification, as delivered by administering two sequential courses of myeloablative therapy with double – or tandem – ASCT and, more recently, on incorporation of novel agents into ASCT. Long-term outcome results of Total Therapy 1, the first double ASCT trial, showed that 33% and 15% of patients were alive and event-free, respectively, after 10 years, while 18% remained in continuous CR[3]. Following this phase II trial, several phase III studies were designed to prospectively compare a single versus double ASCT as up-front therapy for MM[1]. In two published studies conducted in France and Italy, superior EFS and/or OS were seen with double autotransplantation. In particular, in Bologna 96 study random assignment to receive double ASCT significantly increased the rate of at least near CR (nCR) from 33% with a single ASCT to 47% following the second ASCT, regardless of whether it was actually received, and prolonged EFS from 23 to 35 months, respectively[4]. In this study, as in the French IFM 94 trial, the greatest benefit with the second ASCT was observed among patients who failed at least nCR following the first autotransplantation. At the opposite, patients who achieved ≥nCR or ≥VGPR with the first ASCT did not significantly benefit with tandem transplants. Based on these results, the National Comprehensive Cancer Network Multiple Myeloma guidelines, version 2.2009, indicate that a tandem transplant within six months of the initial transplant is an option for patients with partial response or stable disease to the first ASCT.

More recently, introduction into the clinical practice of novel agents targeting the myeloma clone in its bone marrow microenvironment has changed the treatment paradigm for younger patients who are candidates for ASCT. In particular, recognition that thalidomide and bortezomib exibit remarkable activity in advanced and refractory/relapsed MM has stimulated their testing in different clinical scenarios, such as induction therapy in preparation for ASCT[5]. Studies of thalidomide-dexamethasone (TD), eventually combined with a third drug such as doxorubicin (TAD) or cyclophosphamide (CTD), or of thalidomide added to VAD, provided demonstration of increased rates of at least partial response, in the range between 63% and 87%, with thalidomide-based regimens in comparison with traditional therapy given before ASCT[5,6]. Although in most of these studies the rate of CR did not exceed 10%, in three of them thalidomide-based regimens were superior to VAD in terms of ≥VGPR, although it remains unclear whether benefit with thalidomide up-

front translated into improved ≥VGPR rate also after the first ASCT[5]. Since these studies were intended to explore pre-transplantation induction therapy, in none of them EFS and OS comparisons were possible. A single trial addressed this issue by randomizing patients to receive or not thalidomide up-front incorporated into melphalan-based double ASCT. In comparison with the no thalidomide arm of the study, addition of thalidomide to double ASCT significantly increased the CR rate, up to 62%, and prolonged EFS, whose 5-year estimate was 56%. After a median follow-up of 8 years, a survival advantage has recently become apparent among the one third of patients with metaphase cytogenetic abnormalities, a finding initially not reported[7]. Similarly to thalidomide, also bortezomib has been incorporated into newer induction regimens in an attempt to increase the CR rate before ASCT and, hopefully, to ultimately improve post-autotransplantation outcome[8]. Two large phase III studies conducted by IFM and the Italian Myeloma Network GIMEMA have explored the role of bortezomib-dexamethasone (VD)[9] and combined bortezomib- thalidomide- dexamethasone (VTD)[10] in comparison with traditional VAD and TD regimens, respectively, as induction therapy for younger patients who are candidates to receive ASCT. In both of them, VD and VTD were superior to the control group in terms of CR+nCR (19% vith VD and 36% with VTD) and ≥VGPR (47% vith VD and 60% with VTD), a finding confirmed also in high-risk patients carrying chromosome 13 deletion and translocation (4;14)[9,10]. Importantly, high-quality responses effected by bortezomib-based regimens were furtherly increased following the first ASCT, up to ≥nCR (≥VGPR) values of 35% (62%) with VD and 57% (77%) with VTD[9,10], a goal which resulted in a decreased need to receive a second ASCT for patients randomized to VD in comparison with those assigned to VAD. Prolonged follow-up is warranted to assess the impact, if any, of increased post-transplant CR or ≥VGPR on PFS and OS.

Although data so far available suggest that novel agents and ASCT are complementary, a key issue is whether treatment with novel agents delays or eliminates the need for autotransplantation. Randomized clinical trials comparing these two approaches will be conducted in Europe and US within the next few years and will definitely answer this still unresolved question.

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Curing Multiple Myeloma – Lessons From Total Therapy Protocols

Bart Barlogie, Elias Anaissie, Frits van Rhee, John Crowley and John Shaughnessy Jr

Myeloma Institute for Research and Therapy, university of Arkansas for Medical Sciences, Little Rock, AR, USA; Cancer Research and Biostatistics, Seattle, WA, USA.

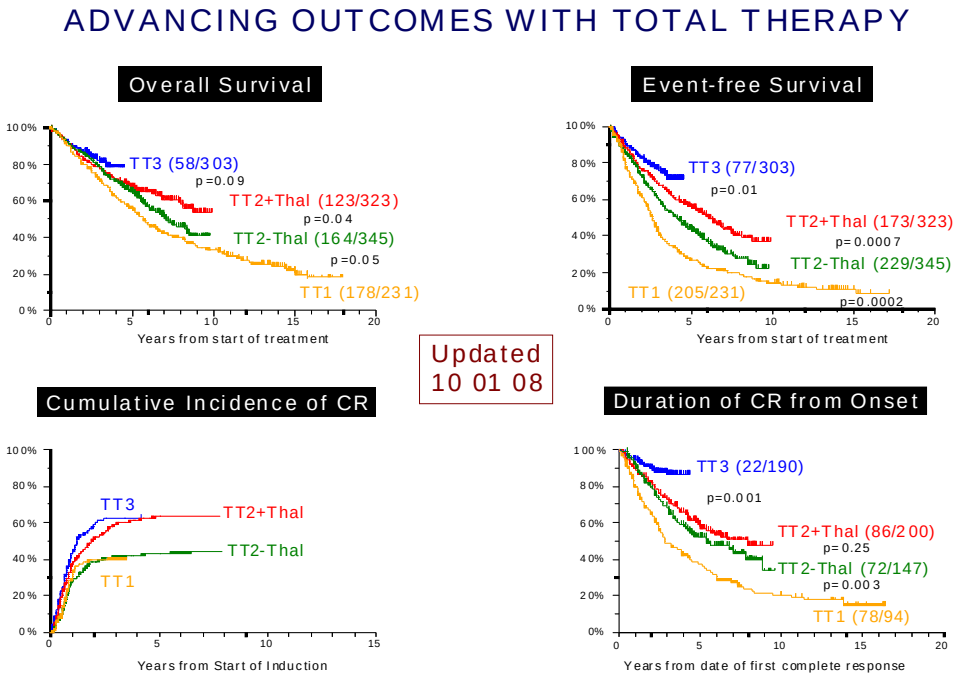
Total Therapy (TT) programs performed at the University of Arkansas for Medical Sciences since 1989 will be reviewed and the contributions on clinical outcomes examined of 2nd high-dose therapy cycles, post-transplant consolidation therapy as well as upfront use of thalidomide and bortezomib in TT2 and TT3. While enhancing the frequency of complete remission (CR) by melphalan dose escalation facilitated by peripheral blood progenitor support was crucial to extending both event-free survival (EFS) and overall survival (OS), we also generated data to indicate that in MGUS/SMM-evolved MM long-term OS was observed in the absence of ever having achieved CR. Together with gene expression profiling (GEP)-derived data distinguishing MGUS-like and non-MGUS-like MM with significantly longer survival in the former scenario, these results collectively support a reversal to a MGUS-like condition with persistent M-protein in such patients. Prior to the utilization of GEP analysis of CD138-purified plasma cells (PC), the presence of cytogenetic abnormalities (CA), especially of the hypodiploid variety with amplification of chromosome 1q and deletion of chromosome 1p regions, was the dominant adverse prognostic variable followed in clinical significance by elevated serum levels of LDH, reflecting often the transition to a bone marrow-independent, extramedullary disease (EMD) growth, now best recognized by FDG-avidity on PET-CT scan examination.

The use of GEP has led to a fundamental understanding of molecularly discrete MM entities with distinct clinical disease manifestations and prognoses. Through supervised hierarchical cluster analyses, a 70-gene-derived risk model was established in TT2 that was readily validated in TT3, although the previously prognostically adverse FGFR3-type MM was no longer a high-risk feature with TT3. When analyzed together with all standard prognostic variables, GEP-based risk provided unprecedented prognostic discrimination as reflected in high hazard ratio (HR) values and R2 statistics denoting greater accountability potential of clinical outcome heterogeneity. In the GEP-defined risk context, we observed that attaining and sustaining CR status was only critical for high-risk MM, where timely applications of all protocol phases independently enhanced survival prospects.

Long-term outcome analyses revealed that the addition of thalidomide in TT2 also extended OS and was particularly evident in patients with CA-type MM. With bortezomib added to thalidomide upfront in TT3, we now have observed sustained 4-yr CR rates in low-risk disease of 90%, a true quantum leap in comparison with predecessor trials TT2 and TT1. Yet, as gleaned from 20% continuous CR beyond 10yr in TT1 and 40% estimates with TT2 at almost 10yr, cure plateaus have been established that will be raised to an anticipated level of at least 60% in TT3 in GEP-defined low-risk MM.

Based on these observations, we have begun a risk-based protocol assignment era, whereby the ~80% of patients with low-risk are offered a randomized trial of TT4 that compares standard TT3 with TT3-lite in an effort to reduce morbidity and eliminate treatment-related mortality, while sustaining/enhancing efficacy by adding both thalidomide and bortezomib to a 4-day fractionated melphalan schedule. TT5 for high-risk MM emphasizes timely dose-dense but less dose-intense 8-drug melphalan-VTD-PACE hybrid combinations, followed by alternating combinations of melphalan or lenalidomide with bortezomib plus dexamethasone. In this fashion, we hope to sustain CR status through quasi-continuous exposure to highly synergistic combination therapy without host exhaustion. Both TT4 and TT5 protocols will continue to address short-term bortezomib pharmaco-genomic analyses having revealed, in TT3, that

failure to up-regulate proteasome genes in MM-PC and down-regulation of micro-environment-based genes both were associated with highly favorable outcomes, independent of baseline risk status. Similar GEP studies are in progress to investigate whether, similarly, a test-dose application of melphalan can reveal a benefit signature for this valuable old agent that may target MM stem-cells and also components of the bone marrow micro-environment.



Allogeneic Stem Cell Transplantation For Multiple Myeloma: Maxi, Mini Or In Between

William Bensinger, MD, Marcello Rotta, and David Maloney

University of Washington, Fred Hutchinson Cancer Research Center, Seattle, WA

Despite the abundance of new therapeutic agents for multiple myeloma (MM), cure is almost never achieved with conventional chemotherapy. Success in the management of refractory hematologic malignancies with stem cell transplantation (SCT) led to the exploration of this treatment for patients with MM. [1-3]. SCT from autologous or syngeneic donors allows the intensive use of chemotherapy +/- radiation to eradicate disease in the patient, since the most common dose limiting toxicity, marrow ablation, can be overcome by infusing stem and progenitor cells which accelerate marrow recovery. While autologous SCT has been established as an important therapeutic option for patients with MM, long-term disease free survival is only rarely achieved due to relapse rates exceeding 90%. SCT from an allogeneic donor can similarly allow the use of intensive cytoreductive therapy but provides an additional immunologic "graft versus myeloma" (GVM) effect resulting in more frequent and durable responses. Patients with MM are however, more prone to transplant related mortality (TRM) after allografting with death rates of 30-50%, depending on performance status. [4-7] The US intergroup trial of early v. delayed autologus SCT had an allogenic arm for patients with matched, related donors that was closed after only 36 patients were enrolled due to a TRM exceeding 50%. [8] At a 7 year follow-up, however, the overall survivals are identical at 39% for both autologous and allogeneic recipients, while the progression-free survivals are 15% for autologous recipients compared to 22% for allogeneic recipients. Additonally, while the risk of relapse continues in the group that received autologous SCT, the overall survival curve for the allogeneic SCT group is flat with follow-up extending to 10 years.

Due to the high TRM associated with an ablative allograft recent efforts have been directed to application of reduced intensity conditioning, designed primarily for immunosuppression rather than cytoreduction. These regimens collectively referred to as "non-myeloabalative", utilize varying degrees of immunosuppressive drugs or radiation, sometimes combined with lower doses of chemotherapeutic drugs to achieve the desired degree of tumor reduction and immune ablation. This approach relies heavily on the GVM effect of the allograft rather than cytoreduction. One widely used non-ablative regimen was developed in Seattle based on canine transplant studies where it was shown that reliable allogeneic donor peripheral blood stem cell engraftment could be achieved with a very low dose of total body irradiation of 200 cGy and a combination of 2 potent immune-suppressive drugs including mycophenolic acid and cyclosporine.[9] This strategy was applied to 18 patients undergoing allogeneic transplant for multiple myeloma. Seven patients had refractory disease and 6 had failed a prior autograft. Two patients of the first 4 rejected the donor graft leading to the addition of fludarabine, which provided additional immunosuppression. [10] There were no further occurrences of rejection following the addition of fludarabine to the regimen. Although only 1 of 18 died of transplant related toxicities, complete responses occurred in only 2 patients and only 3 others achieved partial responses. None of the responses were durable.

The European Bone marrow Transplant Group (EBMT) has compared reduced intensity conditioning with standard ablative conditioning for allografting in multiple myeloma. [11] Between 1998-2002 196 patients conditioned with ablative regimens were compared with 321 patients undergoing reduced intensity conditioning. Transplant related mortality was significantly lower for the reduced intensity group, p=0.001. There was, however, no statistical difference in overall survivals between the 2 groups and progression free survivals were inferior for patients receiving reduced intensity regimens, p=0.009. This was due to a rate of

relapse for the reduced intensity group that was more than double the rate for standard conditioning patients, p=0.0001. These results suggested that in multiple myeloma, the GVM effects are relatively modest and that additional cytoreduction would be required to improve the responses after a reduced intensity allograft. In some trials intermediate dose cytoreductive therapy has been performed prior to allografting in an attempt to reduce recurrence rates but not increase TRM [12]

After these results, a strategy was adopted to perform the non-ablative allograft 2-4 months following an autologous SCT designed to achieve maximum cytoreduction with a period of time to allow the patient to recover from the side effects of the high dose melphalan conditioning regimen. Fifty-four patients ages 29-71 years, median age 52 years, received this tandem autologous, allogeneic transplant strategy. All patients were stage II or III and 48% had refractory or relapsed disease. One patient died of cytomegalovirus pneumonia after the initial autologous stem cell transplant, 1 patient progressed after the autograft and 52 proceeded to Allogeneic stem cell transplant. All 52 except 1 achieved full donor chimerism with a single patient requiring donor lymphocyte infusions on day 84 for partial chimerism. The overall transplant mortality was 22% and the complete remission rate was 57%. Four patients developed severe acute GVHD (grades 3-4) and chronic GVHD developed in 60%. [13] With a median follow-up of 60 months after allograft, the survival at 60 months was 69%, and the progression-free survival 38%. [14] Two trials which have compared tandem autologous SCT with tandem autologous, reduced intensity allogeneic SCT have reported conflicting results. [15,16] One trial performed in high risk patients demonstrated no advantage over tandem autologus SCT while a second trial demonstrated significant benefit.

We reviewed our data of donor stem cell transplant recipients with a diagnosis of MM going back to 1975. There were 293 patients who received marrow or PBSC from allogeneic (n=276) or syngeneic (n=17) donors. The allogeneic donors were related (n=211) or unrelated (n=65). Eight donors were children or patients and 2 were parents. Conditioning regimens were quite varied and included cyclophosphamide (CY) + fractionated total body irradiation (TBI) 12-13.2 Gy, (n=24), busulfan (BU) + CY (n=70) or with additional modified TBI (7.5-10.5 Gy) (n=45), BU + modified TBI (n=8), BU + melphalan (L-PAM) (n=3), BU + L-PAM + thiotepa (n=4), L-PAM + fludarabine (FLU) (n=1), L-PAM, FLU, TBI (2Gy) (n=11), TBI (2Gy) (n=63) + FLU (n=56), + CY (n=2) or dimethyl busulfan + TBI 10 Gy The regimens were broadly classified into myeloablative (n=160), intermediate intensity (n=12) or low intensity (n=121). Fifty-three patients received reduced intensity transplants from matched, related donors following an autologous transplant performed 2-4 months before.

Analysis of overall survivals, relapse or progression and event-free survivals for all patients were at 5 years 31%, 27%, and 22% and for 10 years 28%, 30% and 19%. Tandem autologous, reduced intensity SCT was associated with improved early outcomes; the 5 year overall survivals, and event free survivals were 68% and 37%. The rates of relapse of progression were 50% at 5 years. There is not yet evidence of a clear disease free plateau in survival with reduced intensity regimens. Further comparisons are ongoing with respect to intensity of conditioning regimens, donor type, disease status, decade of transplant and high risk features.

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Post-Transplantation Maintenance In Patients With Multiple Myeloma

Thierry Facon, M

Lille University Hospital, France.

Despite therapeutic advances, multiple myeloma (MM) remains largely incurable with current therapies. High-dose therapy followed by autologous stem cell transplantation (ASCT) is considered the standard of care for younger patients (usually less than 65 years) at diagnosis. Unfortunately, almost all patients will ultimately relapse and salvage treatment options are often limited. Maintenance strategies could extend the duration of response. In the past, maintenance therapy with alkylating agents failed to demonstrate any benefit.[1,2] Most randomized studies and meta-analyses evaluating maintenance interferon alpha (IFNα) showed a modest increase in progression-free survival without any, or with minimal, survival benefit after conventional or high-dose therapy.[3-5] Corticosteroid maintenance was found to prolong the duration of response but the effect on survival was controversial.[6,7] More recently, thalidomide, bortezomib, and lenalidomide are being studied in new maintenance protocols that show promise for increasing response duration and prolonging survival along with decreased toxicity.

Post-ASCT thalidomide maintenance

Several completed and ongoing studies have addressed the role of thalidomide as post-ASCT maintenance.[8-16] In the early 2000s, retrospective or phase II studies suggested that thalidomide was feasible and might improve outcomes after ASCT.[8,9] However, trial data also demonstrated that higher doses of thalidomide can lead to intolerable toxicities and high discontinuation rates.[10] In the IFM 99-02 trial, patients were randomly assigned to receive no maintenance (group A), pamidronate only (group B), or pamidronate + thalidomide (group C; 400 mg/d, with dose reduction to 50 mg/d allowed for treatment-related toxicity).[11] A very good partial response or better was achieved in 55% of patients in group A, 57% in group B, and 67% in group C*; P* =0.03, with 3-year event-free survival (EFS) of 36%, 37%, and 52%, respectively, *P* <0.009) and 4-year overall survival (OS) of 77%, 74%, and 87%, *P* <0.04). Patients received thalidomide for a median of 15 months (range, 0.1-50 months). Drug-related adverse events led to discontinuation of thalidomide in 39% of patients, mainly due to peripheral neuropathy. The mean dose of thalidomide was 200 mg/day. The most frequent toxic effects associated with thalidomide included neuropathy (68%), fatigue (34%), constipation (20%), neutropenia (7%), and cardiac events (4%). The DVT incidence did not differ significantly in the 3 groups.

In the Australian randomized study, thalidomide (200 mg/day for a maximum of 12 months) combined with prednisolone (50 mg on alternate days) was compared with prednisolone alone as maintenance. At 12 months post randomization, the thalidomide group demonstrated a higher partial response (PR) rate (83% vs 52%; *P* <0.01), better 2-year progression-free survival (PFS) (63% vs 36%; *P* <.001), and better 3-year OS (86% vs 75%; P =0.02).[12] Similar positive survival findings were observed in the Tunisian study. Patients were randomly assigned to receive either a single ASCT followed by thalidomide maintenance or tandem ASCT. Single ASCT followed by maintenance with thalidomide proved better than tandem ASCT for both 3-year PFS (85% vs 57%; P =0.02) and 3-year OS (85% vs 65%; P = 0.04).[13]

The Total Therapy 2 program addressed the role of thalidomide in the up-front management of patients with MM undergoing melphalan-based tandem transplants. Patients were randomly

allocated to a control arm or an experimental arm that included thalidomide during all phases of treatment (4 induction cycles, tandem ASCT, 4 consolidation cycles, and maintenance with IFNα ± dexamethasone). The initial report found that complete response rates and EFS were significantly superior among patients randomized to thalidomide without any OS advantage. With longer follow-up (median 72 months), however, thalidomide demonstrated a survival benefit, reaching statistical significance for the one-third of patients exhibiting cytogenetic abnormalities.[14]

Thalidomide maintenance was also investigated in an UK myeloma forum phase 2 study (which was a pilot study to the UK Medical Research Council (MRC) Myeloma IX trial). In this study, maintenance doses greater than 200 mg/d were largely unachievable with peripheral neuropathy being the main toxicity.[15] The MRC Myeloma IX trial incorporated intensive (CTD vs CVAD followed by high-dose melphalan [HDM] before being randomized to either thalidomide or no maintenance) and non-intensive (MP vs attenuated CTD prior to maintenance randomization) pathways selected according to performance status and age. The authors concluded that thalidomide maintenance may improve PFS, without any benefit on OS. They also suggested that thalidomide maintenance might be detrimental in 17p- patients.[16]

Ongoing Post-ASCT maintenance studies with bortezomib or lenalidomide

The HOVON-65/GMMG-HD4 trial was designed to evaluate the efficacy of bortezomib prior to HDM and bortezomib maintenance (VAD vs PAD followed by HDM before being randomized to 2 years of maintenance treatment with either thalidomide [50 mg/d] or bortezomib 1.3 mg/m^2 [every 2 weeks]).

The CALGB 100104 study addressed the role of lenalidomide maintenance (lenalidomide 10 mg/d increasing to 15 mg/d if tolerated vs placebo). In the IFM 2005-02 study, all responding patients received lenalidomide consolidation (lenalidomide 25 mg/d, days 1-4 of every 28 days for 2 months) before being randomized to either lenalidomide (10-15 mg/d continuously until relapse) or placebo.

Conclusion

The role of post-ASCT maintenance for the long term control of the plasma cell clone is an important outstanding question. So far results with thalidomide maintenance have been more consistent with a consolidation rather than a maintenance effect. The impact on survival and in different cytogenetic subgroups is still unclear. Results from the ongoing HOVON-65/GMMG-HD4, CALGB 100104 and IFM 2005-02 with thalidomide, bortezomib and lenalidomide are eagerly anticipated.

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Statistical Issues In The Design And Analysis Of Clinical Studies In Myeloma

John Crowley

Cancer Research And Biostatistics

In this talk I will cover several principles and pitfalls in the design and analysis of clinical studies. These include:

When to randomize. This is illustrated using studies of autotransplant, both single transplant vs standard therapy and tandem vs single transplant.

Assessing the value of response in prolonging survival. Analyses of survival by response should be done using landmark analyses, time-dependent Cox regression or similar methods to minimize bias. This will be illustrated in the context of trials utilizing “Total Therapy”.

Subset analyses, planned and unplanned. How to control the false positive rate.

The value of long term follow-up. As in Total Therapy II, you don’t know what will happen until it happens.

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