Immunophenotypic and genotypic characterisation of multiple myelomas with adverse prognosis characterised by immunohistological expression of the T cell related antigen CD45RO (UCHL-1)

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Abstract

Aims—To investigate whether plasma cell expression of early B cell, late B cell/pre-plasma cell, T cell, and myelomonocytic antigens or myeloma associated lymphocytic infiltrates correlated with prognosis in bone marrow biopsy specimens of patients with multiple myeloma.

Methods—Bone marrow biopsy specimens of 23 patients with multiple myeloma were investigated for plasma cell expression and interstitial lymphocyte expression of T cell related antigen CD45RO (UCHL-1).

Results—Eight patients showed plasma cell expression of CD45RO and 16 showed increased tumour infiltrating CD45RO positive lymphocytes, which were correlated with poor survival by multivariate analyses ($p = 0.005$ and $p = 0.04$, respectively). B cell antigens (MB2, CD20) but no T cell specific antigens (CD3) or T cell receptor gene rearrangements were expressed by plasma cells in CD45RO positive myelomas. Of 16 patients with myeloma who had increased tumour infiltrating CD45RO positive lymphocytes, four had interstitial lymphocyte expression of B cell antigens and two had interstitial lymphocyte expression of the T cell specific antigen CD3.

Conclusions—The recognition of plasma cell expression of CD45RO and increased interstitial CD45RO lymphocytes in bone marrow biopsy specimens of patients with multiple myeloma is an adverse prognostic finding not indicative of an aberrant T cell phenotype or genotype; it is consistent with B cell/pre-plasma cell antigen expression by myeloma cells and their lymphocytic precursors.

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Multiple myeloma is characterised by circulating morphologically and immunophenotypically heterogeneous plasma cell precursors that by idiotypic,¹ genotypic,² and karyotypic³ analyses are progenitors of malignant plasma cells found in bone marrow. These peripheral blood progenitor cells usually express a late B

cell/pre-plasma cell phenotype characterised by CD45RO expression,⁴ adhesion molecules necessary for adhering to vascular endothelium of bone marrow,⁵ and interleukin receptors necessary for the induction of plasma cell differentiation by bone marrow stromal cells.⁶ The proliferation of plasma cell progenitor cells in peripheral blood in myeloma is associated with a hyperactive cellular immune response^{7,8} and with circulating cytotoxic T lymphocytes⁹ that express related anti-idiotypic receptors.¹⁰

An increase in circulating pre-plasma cell precursors^{11,12} and cytotoxic T lymphocytes¹³ has been associated with a poor prognosis in patients with multiple myeloma. A poor prognosis has also been associated with plasma cell expression of more immature B cell phenotypes¹⁴ and aberrant expression of myelomonocytic¹⁵ and T lymphocytic antigens.¹⁶ Because these prognostic factors have been established by flow cytometry, we investigated whether plasma cell expression of early B cell, late B cell/pre-plasma cell, T cell, and myelomonocytic antigens or myeloma associated lymphocytic infiltrates correlated with prognosis in paraffin embedded bone marrow biopsy specimens of patients with multiple myeloma.

Methods

Biopsy specimens of patients with multiple myeloma were collated at the University of Tübingen; these had previously been immunohistochemically characterised.¹⁷ Control cases consisted of plasmacytomas retrieved from the clinical archives of Mayo Clinic and of reactive plasmacytoses obtained from the clinical archives of the University of Tübingen. Extramedullary plasmacytomas without evidence of multiple myeloma at diagnosis were selected as neoplastic plasma cell controls, rather than bone marrow biopsy specimens from patients with monoclonal gammopathy of undetermined significance, in order to obtain plasma cell densities that were sufficient for immunohistochemical and image analyses. The following were recorded at diagnosis: date of histological diagnosis, the age and sex of the patient, site and stage of plasma cell malignancy, previous history of haematological or non-haematological neoplasm, automated blood cell counts, serum calcium value, presence of lytic skeletal lesions, percentage of marrow plasma cells, and treatment. The

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Table 1 Clusters of differentiation (CD), main reactivity, and source of antibodies

Antibody	Antigen cluster designation	Main reactivity	Source
Anti- δ		Ig heavy chain γ	Dakopatts
Anti- α		Ig heavy chain α	Dakopatts
Anti- μ		Ig heavy chain μ	Dakopatts
Anti- δ		Ig heavy chain δ	Dakopatts
Anti- ϵ		Ig heavy chain ϵ	Dakopatts
Anti- κ		Ig heavy chain κ	Dakopatts
Anti- λ		Ig heavy chain λ	Dakopatts
VS38c		Plasma cells	Dakopatts
4KB5	45RA	Early pre-B cells	Dakopatts
UCHL-1	45RO	Late pre-B/pre-plasma cell precursors	Dakopatts
TdT (HT-1, HT-3, HT-4)		Lymphoblasts	Dakopatts
L26	20	B cells	Dakopatts
MB2		B cells	Biotest, Dreieich, Germany
Anti-CD3	3	T cells	Dakopatts
MT2		T cells	Biotest
DF-T1	43	T cells	Dakopatts
OPD4	CD45RO	Different epitope of CD45RO than UCHL-1 ²¹	Dakopatts
Leu-7	57	NK cell subset	Becton Dickinson, Heidelberg, Germany
Ber-H2	30	B and T cell activation, Hodgkin's cells	Behringwerke, Marburg, Germany
KP1	68	Macrophages	Dakopatts
Leu-M1	15	Mature neutrophils, Hodgkin's cells	Dakopatts
Anti-LCA	45	Leucocytes	Dakopatts

patients were followed for detection of subsequent haematological neoplasia, lytic skeletal lesions, and survival.

Identical immunohistochemical investigations were standardised by having all immunohistochemistry studies performed at the University of Tübingen according to previously published methods.¹⁸ All tissues were processed using identical fixation and similar processing protocols, and the bone marrow specimens were decalcified in ethylenediamine-tetra-acetic acid. Tissue blocks were embedded in paraffin, and 4 μ m sections were mounted on silanised slides for morphological staining (haematoxylin-eosin and Giemsa), and immunohistochemical staining (cell markers and clonality). Plasma cells were graded as mature, immature, and plasmablastic according to nuclear characteristics of size, density of nuclear parachromatin, and prominence of nucleoli.¹⁹ Immunostaining was performed according to avidin-biotin-complex (ABC) techniques as previously published by Hsu and colleagues¹⁸ for detection of haematopoietic antigens.²⁰ Immunohistochemistry studies included antibodies against a broad variety of haematopoietic antigens (table 1).

Visual interpretation of immunohistochemical staining was semiquantitatively assessed jointly by two expert haematopathologists (DMM and H-PH): 0 = no plasma cell staining, 1 = > 0% to < 10% plasma cell staining, 2 = $\geq$ 10% to $\leq$ 50% plasma cell staining, and 3 = > 50% plasma cell staining. Plasma cell myeloma and plasmacytoma associated CD45RO lymphocytes, T (CD3 positive) and B (CD20 positive) lymphocytes, and macrophages (CD68 positive) were semiquantitatively assessed: 0 = no lymphocytes or macrophages, 1 = 1 to 2 cells per high power field, 2 = 3 to 5 cells per high power field, and 3 = > 5 cells per high power field.

Detection of polymerase chain reaction (PCR) amplified immunoglobulin heavy chain (IgH) and T cell receptor γ (TCR γ) gene rearrangements were used to assess clonality in cases of multiple myeloma and plasmacytoma which were not clonal by immunoglobulin light chain staining. TCR γ gene rearrangement and CD3 staining were used to define T cell differentiation in multiple myeloma cases that expressed the T cell related antigen CD45RO. DNA for gene rearrangement analysis was harvested from 10 μ m sections of paraffin embedded material. Primers and conditions for amplification were as previously described.^{22 23} One unit of Taq polymerase was added to the reaction at 80°C, and the final extension was done for 15 minutes at 72°C. Human placental DNA, DNA from a lymph node with non-specific lymph node hyperplasia, DNA from the T cell line Jurkat, DNA from a lymph node infiltrated by B cell chronic lymphocytic leukaemia, and DNA from five cases of myeloma without CD45RO expression served as negative, polyclonal, and monoclonal T cell, B cell, and myeloma controls. A second round of amplification was done on the first round IgH PCR products with semi-nested primers (VLH-F3A). PCR amplification products were separated on 2.5% (for TCR γ) and 3.5% (for IgH) agarose gels and visualised under ultraviolet light after staining with ethidium bromide.

STATISTICS

Statistical analyses were performed at Mayo Clinic. Comparison of discrete variables used the χ^2 test. Continuous variables were compared by group with the Wilcoxon rank-sum test. The Spearman rank correlation was used to measure the relation between continuous variables. Survival after diagnosis was estimated with Kaplan-Meier life table methods. The estimated mortality rates associated with discrete variables were compared with the log-rank test. Associations with survival for quantitative variables and for multiple explanatory variables considered simultaneously were evaluated with the Cox regression model.

Results

Review of the necropsy archives of the University of Tübingen from 1987 to 1990 yielded 23 cases of multiple myeloma. The average age of the patients at diagnosis was 59 years (range 29 to 82 years). Of the 23 cases, 22 were staged. Nineteen patients had lytic skeletal lesions (> 3 in nine), one had osteopenia without lytic lesions, and two had no skeletal surveys at diagnosis. Nine patients had increased serum calcium values (> 10.1 mg/dl; in five patients, > 12 mg/dl). Fourteen patients were anaemic (haemoglobin value < 12 g/dl; in four patients, < 8.5 g/dl). Serum immunoglobulin values were measured, but immunoglobulin peaks were not quantitated. Fourteen patients had Durie-Salmon stage III disease, four had stage II, and four had stage I at diagnosis. Only one patient had β_2 microglobulin measured (6.3 μ g/ml). No plasma cell labelling indices were measured.

Figure 1 CD45RO (UCHL1) staining of plasma cells in multiple myeloma ($\times 560$).

In 21 cases the myelomas were clonal by immunoglobulin light chain staining (11 κ and 10 λ), and two expressed neither light chain but showed IgH rearrangement. Immunoglobulin heavy chains were expressed in 14 of the 23 cases (11 γ and three α). All patients had bone marrow plasmacytosis of $> 10\%$ (mean 51%, range 10% to 90%). The plasma cells were morphologically immature (16 cases), plasma-

blastic (six cases), or rarely mature (one case). The plasma cells expressed the following antigens ($\geq 10\%$ plasma cell staining): VS38c, 89%; MB2, 61%; CD45RO, 35% (fig 1); CD45, 30%; and CD20, 22%. No cases expressed CD45RA, CD68, CD3, OPD4, or TdT (table 2). Plasma cell expression of CD45RO was not accompanied by expression of CD3 or by T cell receptor gene rearrangements. Multiple myeloma associated interstitial CD45RO lymphocytes (more than three lymphocytes per high power field) were identified in 16 (76%) of the cases, interstitial CD68 positive monocytes (more than three monocytes per high power field) in 60% of cases, increased CD20 positive (B) lymphocytes (more than three lymphocytes per high power field) in 21% of cases, and increased CD3 positive (T) lymphocytes (more than three per high power field) in 12% of cases. Of the 16 cases of myeloma with more than three CD45RO lymphocytes per high power field, in two cases the lymphocytes were reactive T lymphocytes (expressed CD3), and in three cases the lymphocytes were B lymphocytes.

Of the eight cases of multiple myeloma in which the plasma cells expressed CD45RO antigen, five expressed MB2 and four expressed CD20. No cases of myeloma with plasma cell expression of CD45RO expressed CD3 or CD43. All eight cases of myeloma in which plasma cells expressed CD45RO showed more than three CD45RO positive interstitial lymphocytes per high power field. Of 16 myeloma cases with CD45RO positive lymphocytes, eight showed plasma cell expression of CD45RO. The average age (59 years) and Durie-Salmon stage at diagnosis of patients with plasma cell expression of CD45RO were similar to those of patients with myeloma not expressing CD45RO (table 3). The percentage of marrow plasma cells at diagnosis (65%) was greater in patients with plasma cell expression of CD45RO.

The clinical archives of the Mayo Clinic from 1960 to 1991 yielded 24 extramedullary plasmacytomas, which served as neoplastic controls: 14 lymph node plasmacytomas and 10 extranodal plasmacytomas. No patients with plasmacytomas had lytic skeletal lesions at diagnosis. Bone marrow specimens were obtained in 13 of the 14 lymph node plasmacytomas and in seven of the 10 extranodal plasmacytomas: all showed normal or slightly increased mature plasma cells, which constituted $< 5\%$ of marrow cells.

Twelve lymph node plasmacytomas and all extranodal plasmacytomas had paraffin block material available for immunohistological and gene rearrangement analyses. Eleven of these 12 lymph node plasmacytomas showed immunoglobulin light chain restriction (10 κ and one λ); one case expressed neither light chain but showed IgH gene rearrangement. Seven of the 10 extranodal plasmacytomas showed immunoglobulin light chain restriction (five κ and two λ); three cases expressed neither light chain but showed IgH gene rearrangement. Immunoglobulin heavy chains were expressed in 10 of 12 lymph node plasmacytomas (eight γ and

Table 2 Immunohistochemical staining and morphology of multiple myeloma, plasmacytomas, and reactive plasmacytoses

Factor*	Multiple myeloma (n = 23)	Lymph node plasmacytomas (n = 12)	Extranodal plasmacytomas (n = 10)	Reactive plasmacytoses (n = 36)	p Value†
CD45	7 (30)	1 (8)	2 (20)	24 (67)	<0.001
CD45RA	0	1 (8)	Not tested	0	—
CD45RO-UCHL1	8 (35)	0	0	1 (3)	0.001
CD20	5 (22)	0	2 (20)	1 (3)	0.032
MB2	14 (61)	1 (8)	2 (20)	14 (40)	0.011
MT2	0‡	0	0	0	NS
CD43	7 (30)	8	0	2 (6)	0.037
CD57	0	0	0	2 (6)	NS
CD68	0	0	1 (10)	8 (22)	<0.001
CD15	0	0	0	0	NS
CD30	3 (13)	5 (42)	0	24 (67)	<0.001
Plasmablastic	6 (26)	1 (8)	0	0	NS
TIL‡ CD45RO	16 (76), n = 21	8 (67)	2 (20)	—	0.023
TIL CD20	5 (21)	0	0	—	NS
TIL CD3	0, n = 17	—	—	—	—
TIM‡ CD68	14 (60)	12 (100)	5 (50)	—	0.021

*Values are n (% showing 2+ staining).

†p value refers to significant differences between groups.

‡CD3 also negative in myeloma cases (n = 17).

§TIL, tumour infiltrating lymphocytes.

¶TIM, tumour infiltrating macrophages.

Table 3 Relation of CD45RO positive and CD45RO negative myeloma with conventional prognostic factors

Factor	CD45RO+ myeloma (n = 8)	CD45RO- myeloma (n = 15)	Significance between groups
Sex	4 F, 4 M	4 F, 11 M	NS
Age (years)			NS
Average	59.5	58.5	
Range	44 to 74	29 to 82	
Durie-Salmon stage (n)			NS
Stage 1	2	2 (n = 14)	
Stage 2	1	3	
Stage 3	5	9	
Bone marrow plasmacytosis (%)			NS
Average	65 (n = 4)	45 (n = 11)	
Range	28 to 89	10 to 90	
Plasma cell morphology (n)			NS
Mature	1	0	
Immature	5	11	
Plasmablastic	2	4

Figure 2 Survival in multiple myeloma, by plasma cell expression of CD45RO (UCHL1).

Figure 3 Survival in multiple myeloma, by number of interstitial CD45RO (UCHL1) lymphocytes.

Figure 4 Survival of patients with multiple myeloma and of patients with plasmacytomas.

two μ) and in six of 10 extranodal plasmacytomas (three γ , two α , one μ). The plasma cells in plasmacytomas expressed MB2 and CD45 less often than in multiple myeloma. No plasmacytoma cases expressed CD45RO, and no lymph node plasmacytoma cases expressed CD20. Plasmacytoma associated interstitial CD45RO positive lymphocytes were identified less often in plasmacytomas than in multiple myeloma (66% of lymph node and 20% of extranodal plasmacytomas). Interstitial CD68 positive monocytes were present in all lymph node plasmacytomas and were less often found in extranodal plasmacytomas (50%). Interstitial B lymphocytes were not identified in any plasmacytomas.

The pathology archives of the University of Tübingen from 1989 to 1991 yielded 36 cases of reactive plasmacytoses. These served as non-neoplastic plasma cell controls. The average age of patients was 50 years (range less than one to 80 years). The plasma cells in all reactive

cases expressed κ and λ immunoglobulin light chains. Immunoglobulin heavy chains were expressed in all cases (30 γ and α , and six γ , α , and μ). The reactive plasmacytoses were typically a component of a non-specific inflammation (24 cases) or were plasmacytoses associated with carcinoma (seven cases). The plasma cells in reactive plasmacytoses expressed MB2 less often and CD45 more often than in multiple myeloma. Only rare reactive plasmacytoses cases (3%) expressed CD45RO or CD20.

Patients with myeloma received chemotherapy (melphalan and prednisone). After an average follow up of three years (range less than one to nine years), 20 of the 23 patients died of disease. Patients with myeloma who had plasma cell expression of CD45RO survived for a median interval of one year after diagnosis, whereas those without plasma cell expression of CD45RO survived a median interval of four years after diagnosis. Plasma cell expression of CD45RO (fig 2) ($p = 0.005$) and the identification of interstitial CD45RO lymphocytes (fig 3) ($p = 0.05$) correlated with poor survival within the group of multiple myeloma by multivariate analyses, unlike age, Durie-Salmon stage, plasmablastic morphology, expression of any other haematopoietic antigens, or measures of other interstitial cells.

The ages of the patients with lymph node plasmacytomas ranged from 31 to 52 years (average 62), and the ages of those with extranodal plasmacytomas ranged from 57 to 81 years (average 68), and these patients had resection or radiation treatment. Patients with lymph node plasmacytoma were followed for an average of seven years (range one to 22 years): 12 were alive, 11 were without evidence of disease, and no patient developed multiple myeloma. Patients with extranodal plasmacytomas were followed for an average of six years (range two to 12 years): five were alive, four were without evidence of disease, and one developed myeloma and died. The survival of patients with plasmacytomas was significantly different from the survival of patients with multiple myeloma (fig 4) ($p < 0.001$) (median survival was 10.5 years with lymph node plasmacytomas, 9.1 years with extranodal plasmacytomas, and 2.7 years with multiple myeloma).

Discussion

Plasma cell expression of CD45RO and increased numbers of CD45RO positive, CD3 negative lymphocytes in bone marrow biopsy specimens defined a subset of multiple myeloma patients with a poor prognosis. The immunohistological identification of CD45RO expression in plasma cells and adjacent CD3 negative lymphocytes in patients with multiple myeloma suggested that plasma cells expressing pre-plasma cell antigens²⁴ and lymphoid cells expressing plasma cell markers²⁵ described in bone marrow aspirates of patients with myeloma may be immunohistologically identifiable in bone marrow biopsy specimens. Because immunohistological identification of plasma cells and interstitial lymphocytes that

express a pre-plasma cell phenotype in bone marrow biopsy specimens of patients with multiple myeloma was associated with a poor prognosis, immunostaining in a prospective series of patients with myeloma may be warranted for assessment of prognosis and comparison with other survival factors.

Previous published findings of the adverse prognosis of patients with multiple myeloma have been associated with circulating CD38 positive lymphoid pre-plasma cell precursors.²⁶⁻²⁸ The poor prognosis of patients with multiple myeloma associated with increased interstitial lymphocytes or plasma cells that express a pre-plasma cell phenotype has not been reported previously in bone marrow biopsy specimens of patients with myeloma. Although lymphoid pre-plasma cell precursors in peripheral blood²⁹ can be identified in as many as 21% of patients with myeloma,³⁰⁻³² they have not been identified before in bone marrow biopsy sections. Further investigations of CD45RO positive interstitial lymphocytes in bone marrow biopsies of patients with multiple myeloma should include a larger series of myeloma patients who are clinically well characterised, have plasma cell labelling indices, and have ploidy and proliferative analysis by image analyses techniques to determine whether lymphoid plasma cell precursors are more often aneuploid or proliferative than their plasma cell progeny, as suggested by flow cytometry of bone marrow aspirates.¹¹ Because serum thymidine kinase^{26, 27} and serum lactate dehydrogenase²⁸ correlate with increased peripheral blood CD38 positive lymphocytes as adverse prognostic factors in patients with myeloma, future clinical correlation is needed to determine whether serum thymidine kinase and serum lactate dehydrogenase correlate with increased interstitial CD45RO positive lymphocytes in bone marrow biopsy specimens of patients with multiple myeloma.

Plasma cell expression of CD45RO in bone marrow biopsy specimens distinguished multiple myeloma from more indolent plasma cell neoplasms and reactive plasmacytoses. Jensen *et al* also reported that extramedullary plasmacytomas did not express CD45RO.³³ Gloghini *et al* only recently reported that a minority of extramedullary plasmacytomas may express CD45RO.³⁴ As with plasma cell expression of CD45RO, plasma cell expression of the B cell marker MB2 was more prominent in multiple myeloma than in plasmacytomas and reactive plasmacytoses. Expression of B cell antigen has been shown in multiple myeloma by Leo *et al*,³⁵ who reported a 20% rate of expression of the B cell antigen CD20 in multiple myeloma, similar to the 22% rate of CD20 expression in multiple myeloma reported by us. Plasma cell expression of the B cell antigen MB2 in most of our myeloma cases (61%) was similar to the rate reported by Dehou *et al*,³⁶ although our control cases of reactive plasmacytoses expressed MB2, in contrast to the findings of Dehou *et al*. Although it has been reported that plasma cell expression of MB2 in bone marrow biopsy specimens is prognostically useful in monoclonal gammopathies of undetermined

significance,³⁶ we found no prognostic correlation between plasma cell MB2 expression in bone marrow biopsy specimens and prognosis in patients with multiple myeloma.

Plasma cell expression of CD45RO and MB2 and less often of CD45 and CD20, without expression of CD45RA, are consistent with the hypothesis that multiple myeloma may be derived from late (memory) B cell/pre-plasma cell precursors rather than earlier B cell or more primitive haematopoietic precursors, which may be expected to show CD45RA, TdT, T cell, monocytic, myeloid, or natural killer cell antigen expression or T cell receptor gene rearrangements. This hypothesis of a late B cell/pre-plasma cell derivation of multiple myeloma is also supported by the lack of acute leukaemias complicating the clinical course of our cases of multiple myeloma and the uniform VDJ rearrangements³⁷ with somatic mutations²⁷ in plasma cells of patients with myeloma reported by others.

We anticipated that the interstitial lymphocytes in bone marrow biopsy specimens of multiple myeloma would be of T cell phenotype because flow cytometric investigations have shown activated cytotoxic T lymphocytes in peripheral blood of patients with multiple myeloma, consistent with a hyperactive cellular immune response. We were surprised to find that the CD45RO lymphocytes usually failed to express T cell restricted antigens, a finding that indicated they are plasma cell precursors and not cytotoxic T cells. If these plasma cell precursors can be identified in larger prospective series of myeloma patients and are associated with a poor prognosis, they may be worthy of further investigation as targets for chemotherapy, differentiation treatment (interleukins), and anti-idiotypic (immune) treatment³⁸ and removed from peripheral blood stem cell collections before autologous bone marrow transplantation.

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