Syndecan-1 (CD138) Immunoreactivity in Bone Marrow Biopsies of Multiple Myeloma: Shed Syndecan-1 Accumulates in Fibrotic Regions

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Syndecan-1 (CD138) mediates myeloma cell adhesion, and loss of syndecan-1 from the cell surface may contribute to myeloma proliferation and dissemination. Flow cytometry analysis of myeloma cells in bone marrow specimens shows heterogeneity in cell surface syndecan-1 expression. It is not known whether weaker expression correlates with more aggressive disease. However, recent reports suggest that variations in syndecan-1 staining intensity on myeloma cells may be an artifact of specimen handling. In this study, we evaluate syndecan-1 expression in bone marrow biopsy sections from 28 multiple myeloma patients, to elucidate the heterogeneity of syndecan-1 expression *in situ*. Immunoreactivity for syndecan-1, using the antibody B-B4 (CD138), was found in more than 95% of multiple myeloma cells in 27 of 28 biopsies. However, one biopsy had more than 50% CD138-negative cells and cells with weak CD138 expression were identified in the majority of cases. Loss of syndecan-1 did not appear to relate to myeloma cell differentiation. In addition, syndecan-1 was detected on intravascular and intrasinusoidal myeloma cells suggesting that loss of syndecan-1 may not be required for extramedullary dissemination. Bone marrow biopsies from nine additional patients, with variable CD138 staining intensity on myeloma cells as determined by flow cytometry, were studied by immunohistochemistry. The heterogeneous CD138 expression was confirmed *in*

situ, with weakly positive cells concentrated in areas of reticulin fibrosis. These cells had a disrupted pattern of membrane staining in contrast to the strong linear membrane staining seen in the other multiple myeloma cells. In addition, the fibrotic stroma stained intensely for syndecan-1. Accumulation of syndecan-1 within the extracellular matrix of the marrow likely is derived by shedding of the molecule from the surface of myeloma cells. Because syndecan-1 can act to regulate the activity of heparan-binding growth factors, these reservoirs of syndecan-1 may play a critical role in promoting myeloma pathogenesis, or in regeneration of the tumor after chemotherapy.

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Syndecans are a family of cell surface proteoglycans of which syndecan-1 is the prototypical member. Syndecan-1 is expressed on cells from human myeloma cell lines, and mediates cell-cell adhesion, adhesion to type I collagen, and inhibition of invasion into type I collagen gels (1-3). Cells lacking syndecan-1 expression adhere poorly to one another, but after their transfection with the syndecan-1 cDNA, these cells form tight cell-cell adhesions (3). Additionally, cells that normally invade type I collagen gels are rendered noninvasive after their transfection with syndecan-1 cDNA, thus providing direct evidence that loss of syndecan-1 expression on the cell surface may be an essential element in the process of multiple myeloma (MM) cell invasion (2). This is supported by studies in *scid* mice, whereby syndecan-1 expression on the cell surface inhibits tumor growth and delays development of myeloma-related morbidity (4).

The cell surface monoclonal antibodies B-B4 and MI15, recognize epitopes of syndecan-1 within the cluster designation CD138 (5, 6). Evaluation of B-B4 and MI15 by immunohistochemistry (IHC) techniques in paraffin-embedded bone marrow (BM) biopsy sections shows staining in all cases of plasma cell dyscrasias and many Hodgkin's lymphomas with classic Reed-Sternberg cells (5, 7). Analysis of MM by flow cytometry (FCM), in both cell lines and inpatient specimens, shows heterogeneous B-B4 expression among myeloma cells within specimens (4, 6, 8–11). This heterogeneous expression may be due to syndecan-1 shedding from the cell surface, with loss of syndecan-1 being a possible requirement for intravascular invasion. Syndecan-1 is shed from myeloma cells and accumulates in the serum of myeloma patients (12). Shed syndecan-1 is composed of an intact ectodomain that contains the extracellular portion of the core protein with attached heparan sulfate chains, and thus remains biologically active. A recent study shows that soluble syndecan-1 from myeloma patients exists in a complex with hepatocyte growth factor and therefore may act to regulate the biologic activity of this and other heparin-binding cytokines (13). In addition, elevated levels of serum syndecan-1 correlate with increased tumor mass and decreased patient survival (12, 14).

The heterogeneous syndecan-1 expression by myeloma cells may relate to shedding of the molecule and this may result in differential syndecan-1 expression on myeloma cell surfaces. However, Jourdan *et al.* suggest that loss of CD138 seen by FCM analysis may be the result of myeloma cell apoptosis induced by prolonged specimen handling (15). Therefore, in the present study, syndecan-1 expression is examined in BM biopsies that were immediately fixed in Zenker's solution to ensure optimal epitope recovery. BM sections from 28 patients with progressive (evolutionary) MM are reviewed for specificity and distribution of CD138 staining. Variations in CD138 expression on myeloma cells evident by FCM analysis are also compared with IHC in nine additional patient specimens.

MATERIALS AND METHODS

Zenker-fixed paraffin embedded BM biopsies, from 28 patients with progressive MM and greater than 30% plasma cells, were obtained from the Department of Pathology tissue files at the University of Arkansas for Medical Sciences. The cases were graded based on the Bartl *et al.* classification of MM cell types as follows: grade I, low grade (Marschalko, small cell); grade II, intermediate grade (asynchronous, polymorphous, cleaved); and

grade III, high grade (plasmablastic) (16). Nine additional BM biopsies from patients with previous FCM analysis (described below) were also examined. These biopsies were stained for reticulin fibrosis, which was quantitated utilizing a scoring system described by Bauermeister (17).

Immunohistochemical Techniques

Three micron sections were cut and mounted on 3-aminopropyltrethoxy-silane-coated slides, dried, and deparaffinized before undergoing antigen retrieval by heat treatment in DAKO Target Retrieval solution (DAKO, Carpinteria, CA). Endogenous peroxidase activity was quenched with 0.3% peroxidase and nonspecific binding was quenched with horse serum block (Oncogene Research Products, Cambridge, MA). The DAKO Large Volume LSAB2 Alkaline Phosphatase Kit (DAKO) was used with 1:100 dilution of antibody to CD138 (clone B-B4, Serotec, Raleigh, NC), a mouse anti-human antibody, which recognizes an epitope of human syndecan-1 (7, 18). Sections were incubated with biotinylated secondary antibody for 30 minutes, and then with streptavidin alkaline phosphatase for 30 minutes. The signal was visualized with DAKO Fast Red Substrate System (DAKO) yielding a red end product at the site of the target antigen. Positive control tissue consisted of normal skin in which cell membrane immunoreactivity for CD138 was evident in the basal and granular layers of keratinocytes, as previously reported (19, 20). The negative control consisted of a 1:100 dilution of a nonspecific isotype-matched IgG₁ mouse antibody, MCA 928 (Serotec), which was substituted for CD138. The pattern of CD138 staining and percent MM cells expressing CD138 were evaluated by two authors (IBG, CSW). The nine biopsies with FCM analysis were also evaluated for the percent MM cells with strongly positive CD138 immunostaining, either more than 95%, 80 to 95%, or less than 80% and for stromal CD138 deposition (0 scant, 1+ mild, 2+ moderate/abundant). FCM and IHC analyses were examined independently in a blinded fashion.

Flow Cytometry Studies

Nine cases of MM with variable levels of CD138 staining intensity by FCM were identified. The BM aspirate specimens had been enriched for mononuclear cells by centrifugation over Ficoll-Plaque (Pharmacia, Piscataway, NJ). The cells (10^6 in 100 μ L) were then incubated with 20 μ L phycoerythrin-conjugated anti-CD138 (Becton Dickinson Immunocytometry Systems, San Jose, CA), 20 μ L PerCP-conjugated anti-CD45 (Becton Dickinson), and 10 μ L fluorescein isothiocyanate-conjugated CD138

(Serotec) for 30 minutes at 4°; then washed and resuspended in 1% paraformaldehyde fixative. FCM was performed using a FACScan (Becton Dickinson, Mountain View, CA) equipped with an air-cooled argon laser. Data were analyzed using Cell-Quest software (Becton Dickinson). Gates were set around the CD38⁺ CD45⁻CD45^{dim} quadrant to isolate the plasma cell population for evaluation of CD138 expression. Plasma cell clonality was confirmed by cytoplasmic light chain/DNA analysis (21). For this study, histograms of CD138 were divided at the log decade points of 10⁰ to 10² and 10³ to 10⁴ to quantitate the percent plasma cells showing weakly positive and strongly positive staining, respectively.

RESULTS

Immunohistochemistry

Syndecan-1 was expressed exclusively on plasma cells and was not associated with endothelial cells, stromal cells or other hematopoietic elements in BM sections (Fig. 1A). Of the 28 BM biopsies, only one biopsy that was extensively involved by moderately differentiated MM (Bartl grade II, asynchronous cell type) showed large areas with greater than 50% CD138-negative myeloma cells (Fig. 1B). This finding was confirmed on multiple repeat stainings of the biopsy. In addition, scattered positive MM cells

served as an internal control. This case had an otherwise characteristic myeloma cell immunophenotype with absence of CD20 and CD45 expression by IHC and FCM, and bright CD38 staining with monotypic cytoplasmic kappa light chain by FCM. Two additional biopsies initially had large clusters of MM cells with absence of CD138. However, repeat staining showed weak expression. Therefore, 27 of the 28 biopsies exhibited immunoreactivity for CD138 in more than 95% of MM cells, although rare cells (less than 5%) in many cases lacked cell surface CD138 expression. Among the CD138-positive MM cells, two populations with different staining qualities were observed. One population of cells had strong linear staining of the cytoplasmic membrane, but the other stained weakly with a disrupted pattern of membrane staining. MM cells with weak (or negative CD138) expression were cytologically indistinct from strongly positive cells in a given biopsy. In addition, the 27 cases with more than 95% CD138-positive plasma cells included both well differentiated (Bartl grade I) and moderately differentiated (Bartl grade II) MM, suggesting that loss of syndecan-1 was not directly related to tumor differentiation. Surprisingly, deposits of CD138-positive material were evident within the extracellular matrix in fibrotic areas that contained myeloma cells (Fig. 1C). An increased proportion of

FIGURE 1. CD138 stains the majority of myeloma cells in BM biopsy sections (A, original magnification 500×). A single patient exhibited absence of CD138 immunoreactivity in more than 50% of myeloma cells (B). CD138-negative cells were cytologically indistinct from positive cells (original magnification 1000×). Syndecan-1 deposition within the fibrotic extracellular matrix (C, original magnification 500×). Intrasinusoidal and intravascular myeloma cells are CD138-positive (D, original magnification 200×).

MM cells in the fibrotic areas had a weak and disrupted pattern of membrane staining. Fibrosis was found when large clusters or sheets of myeloma were present in a paratrabecular, nodular or diffuse pattern of infiltration. In contrast, interstitial infiltrates were not associated with fibrosis and syndecan-1 deposition. Circulating MM cells, located within the lumens of blood vessels and sinuses also showed CD138 positivity (Fig. 1D).

Flow Cytometry

Nine cases with variations in CD138 staining intensity as determined by FCM were also examined by IHC. The BM biopsy morphologic and immunohistochemical findings for these cases are listed in Table 1. On FCM, myeloma cells were identified by gating on the CD38⁺ CD45⁻CD45^{dim} cell population (Fig 2A). Three cases had ≥95% MM cells with strong CD138 staining intensity by FCM (Patients 1, 2, and 3). MM cells with weak CD138 expression ranged from 14% to 44% of the total plasma cells in the other six patients. Although CD138 weakly positive cells were identified by IHC evaluation, the percent was not as easily quantitated as by FCM. However, substantial deposition of CD138 in fibrotic areas of the BM stroma was associated with an increased number of MM cells showing weak CD138 expression by FCM. In the BM biopsy sections, a disproportionate number of MM cells in these fibrotic areas also had an irregular or disrupted pattern of weak CD138 membrane staining. FCM and IHC studies for Patient 1, who had a majority of MM cells exhibiting strong CD138 staining are illustrated in Figure 2, B-C. Similarly, the studies for Patient 7, who had a significant subpopulation of cells with weak CD138 staining by FCM, are shown in Figure 2, D-E. BM aspirate smears showed the MM cells from this patient (and Patient 9) had abundant cytoplasm with prominent

vacuolation and cytoplasmic fraying. Reticulin stains confirmed moderate to marked reticulin fibrosis in cases with moderate to marked stromal CD138 deposition (Table 1, Fig. 3). Four of five cases (Patients 5 through 9) with more than 15% weakly staining CD138 MM cells by FCM had moderate to marked marrow reticulin fibrosis, in contrast to none of the four cases (Patients 1 through 4) with less than 15% weakly staining cells ($P < .05$, Fisher's Exact Test). In addition, all myeloma of polymorphous cell type were associated with marked reticulin fibrosis.

DISCUSSION

Within the BM of myeloma patients, CD138 (syndecan-1) was found to be a specific marker for plasma cells and was not present in association with other hematopoietic cells or endothelial cells. In 27 of the 28 MM cases, greater than 95% of the plasma cells expressed CD138. The single case with a large population of CD138-negative cells had a BM biopsy packed with moderately differentiated MM cells (asynchronous cell type). Worsening cytologic differentiation among the MM cells in this case, and the others, which might predict for more aggressive potential was not associated with CD138 loss. In addition, *in vitro* studies have demonstrated that syndecan-1 mediates myeloma adhesion, and thus loss of syndecan-1 expression may precede intravascular invasion and extramedullary spread of MM (1, 2, 22). We find that CD138 is expressed on cells within the intrasinusoidal spaces and lumen of vessels. This suggests that loss of syndecan-1 is not required for cells to leave the marrow, or that expression is lost within the marrow and then regained as the cells circulate.

Rawstron *et al.* described differential expression of CD138 on MM cells, whereby circulating cells

TABLE 1. Evaluation of BM Histology in Nine Patients with MM—Correlation of CD138 Expression by Flow Cytometry Analysis and Immunohistochemistry

Patient Number	BM Biopsy			Flow Cytometry		Immunohistochemistry	
	Plasma Cells (%)	Bartl Grade (Cell Type)	Reticulin Score ^a	Moderate-Strong CD138 ^b (% MM Cells)	cIg, DNA Index	Strong CD138 Expression ^c	Stromal CD138 Deposition ^d
1	60	II (asynchronous)	2	97	Lambda 0.97	2+	0
2	95	II (asynchronous)	1	96	Kappa 1.15	2+	1+
3	40	I (marschalko)	2	95	Lambda 0.97	3+	0
4	60	II (asynchronous)	2	86	Kappa 1.0, 1.24	2+	1+
5	50	II (polymorphous)	3	84	Lambda 1.91	2+	2+
6	40	II (asynchronous)	1	79	Lambda 1.0, 1.12	2+	1+
7	70	II (asynchronous)	3	77	Kappa 0.94, 1.80	2+	2+
8	70	II (polymorphous)	3	75	Kappa 1.0, 1.92	2+	2+
9	95	II (polymorphous)	4	56	Kappa 1.0, 1.77	1+	2+

^a Reticulin fibrosis score: 0–1 normal, 2 mild, 3 moderate, 4 marked.

^b Moderate-strong positive CD138 population at log decade of 10³ to 10⁴. Remaining cells showed weakly positive staining at log decade of 10⁰ to 10² with <5% negative cells in all cases.

^c Strongly positive CD138 expression: 3+ >90%, 2+ 80–90%, 1+ <80% plasma cells.

^d Background stromal CD138: 0 scant, 1+ mild, 2+ moderate/abundant.

cIg, cytoplasmic immunoglobulin; BM, bone marrow; MM, multiple myeloma.

FIGURE 2. FCM analysis of BM shows the gate around the CD38⁺ CD45⁻ CD45^{dim} cells used to isolate the myeloma cell population (A). FCM histogram of BM from Patient 1 shows strong CD138 expression in the majority of myeloma cells (B). BM biopsy section from Patient 1 confirms strong CD138 immunoreactivity by myeloma cells (C, original magnification, 500 $\times$). FCM histogram of BM from Patient 7 shows two distinct peaks of CD138 staining intensity on myeloma cells, consistent with weak and strong CD138 expression (D). BM biopsy section from Patient 7 displays a significant subpopulation of myeloma cells with weak CD138 immunoreactivity in a background of fibrosis (E, original magnification 500 $\times$).

FIGURE 3. BM biopsy section of MM with stromal fibrosis from Patient 9 (A, original magnification, 200 $\times$). Inset shows CD138 immunoreactivity (B, original magnification 500 $\times$). A reticulin stain highlights the marked reticulin fibrosis (C, original magnification 200 $\times$).

expressed lower levels of CD138 than their BM counterparts (23). Wijdenes *et al.* found that CD138 recognized only about 70% of myeloma cells by FCM, possibly due to shedding of syndecan-1 before the analysis (24). Jourdan *et al.* confirmed that apoptotic primary myeloma cells lose CD138 after harvesting and storage at room temperature, whereas viable myeloma cells retain CD138 (15). Our finding of CD138 staining by a majority of MM cells, using both IHC and FCM, indicates technical issues may have been a problem in previous reports. However, when IHC was performed on biopsy sections from nine cases with varying intensities of CD138 staining by FCM, we confirmed heterogeneous CD138 expression on MM cells *in situ*. FCM was more sensitive than IHC in separating weakly from strongly positive cells. Nonetheless, sampling differences between BM biopsy and aspirate specimens most likely explains the differences observed between the IHC *versus* FCM results. Furthermore, increased stromal syndecan-1 deposition and reticulin fibrosis in biopsy sections was associated with a higher percentage of MM cells showing weak CD138 by FCM.

Explanations for a population of MM cells that weakly express CD138 include therapy effect, apoptosis, decreased syndecan-1 synthesis, and increased shedding of syndecan-1 from the cell surface. Jourdan *et al.* found dexamethasone-induced apoptosis of myeloma cells was associated with loss of CD138 (15). Although the majority of patients in our study had received previous therapy, often before coming to our institution, the myeloma cells appeared viable and showed strong CD138 staining. The single case with loss of CD138 in a majority of cells was associated with an aggressive disease course and the patient died shortly after the biopsy. In addition, a defect in plasma cell apoptosis has been described in MM, whereby decreased rather than increased apoptosis appears to be a factor in progression to overt myeloma and advancement of disease (25). Although decreased CD138 synthesis cannot be entirely excluded, the most likely explanation for a weak CD138 population of MM cells is shedding of the syndecan-1 ectodomain. Syndecan-1 shedding is usually highly regulated but can be accelerated in areas of tissue injury by release of growth factors or proteases (26). In the BM biopsy specimens, the weaker staining MM cells had a disrupted pattern of membrane staining and were found preferentially in areas of fibrosis.

Importantly, we demonstrate for the first time, that fibrotic stroma associated with MM shows increased CD138 immunoreactivity that is not associated with fibroblast or myeloma cell surfaces. The fact that CD138 was present as relatively large deposits, was associated with MM cells showing a weak and disrupted pattern of CD138 expression, and was isolated to the areas with marked reticulin fibrosis, suggests

that staining for syndecan-1 is not an artifact. Instead, it is likely that shed syndecan-1 is trapped within the extracellular matrix, similar to a report of syndecan-1 within stromal tissue of infiltrating ductal carcinoma of the breast (27). Although the fibrosis is most likely due to cytokine production, similar to the process that occurs in tissue injury, many of the same cytokines also promote angiogenesis, tumor growth, and invasion (28). It is likely that the shed syndecan-1 or its degradation products regulate heparin-binding growth factors in this process. For example, heparin-mediated FGF-2 mitogenesis is normally inhibited by soluble syndecan-1 ectodomains (29). When heparinases are released in sites of injury and inflammation, they can degrade syndecan-1 producing heparin-like heparan sulfated fragments that are potent mitogenic activators for FGF-2. The finding of higher levels of FGF-2 in plasma cell extracts from patients with active MM than in nonactive MM or monoclonal gammopathy of undetermined significance supports this theory (30). Furthermore, BM fibrosis is associated with increased microvessel density in biopsies of MM and both increased microvessel density and increased circulating syndecan-1 levels are associated with decreased patient survival (14, 31, 32). Thus, syndecan-1 shedding, fibrosis, and angiogenesis are likely intertwined. Moreover, the syndecan-1 within the fibrotic marrow may provide a reservoir of growth factors that drives re-emergence of the tumor after chemotherapy.

In conclusion, we find that syndecan-1 is present on the majority of MM cells within the BM and is not expressed by other benign hematopoietic elements or by endothelium. CD138 expression in MM is independent of cytologic differentiation or intravascular invasion. The identification of a single case with a large population of CD138-negative MM cells suggests that utilizing CD138 for purifying MM cells from clinical specimens, especially stem cell products, may be problematic in some patients. MM cells exhibiting weakly positive CD138 staining were present in many cases, often in association with tumor fibrosis. Lastly, the important observation that syndecan-1 accumulates within fibrotic areas of the BM, raises the possibility that these areas act to store and regulate the activity of heparin-binding growth factors, and perhaps contribute to the pathogenesis and persistence of the tumor.

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