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Fas ligand expression in the bone marrow in myelodysplastic syndromes correlates with FAB subtype and anemia, and predicts survival

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Increased apoptosis in the bone marrow (BM) may contribute to the cytopenias that occur in myelodysplastic syndromes (MDS). The Fas receptor, Fas ligand (FasL) pathway is a major mechanism of apoptosis. Since hematopoietic progenitors can express the Fas receptor, they may be susceptible to apoptosis induced by FasL-expressing cells. We examined FasL expression in the BM of patients with MDS ($n = 50$), *de novo* acute myeloid leukemia (AML; $n = 10$), AML following prior MDS ($n = 6$), and normal controls ($n = 6$). Compared to controls, FasL expression was increased in MDS, and was highest in AML. In MDS, FasL expression was seen in myeloid blasts, erythroblasts, maturing myeloid cells, megakaryocytes and dysplastic cells, whereas in AML, intense expression was seen in the blasts. FasL expression correlated with the FAB subtype groups of MDS, and also correlated directly with the percentage of abnormal metaphases on cytogenetic analysis. The FasL expressed in MDS BM inhibited the growth of clonogenic hematopoietic progenitors. This inhibition could be blocked by a soluble recombinant FasFc protein. In MDS, FasL expression in the initial diagnostic BM was higher in patients who were more anemic, correlated directly with red cell transfusion requirements over the subsequent course of the disease, and was predictive of survival. These studies indicate that FasL expression in MDS is of prognostic significance, and suggest that pharmacological blockade of the Fas-FasL pathway may be of clinical benefit.

Keywords: myelodysplastic syndromes; acute myeloid leukemia; apoptosis; prognostic factors; Fas ligand

Introduction

The myelodysplastic syndromes (MDS) are a group of disorders characterized by cytopenias, often in the presence of adequate or increased numbers of cells in the bone marrow, and an increased propensity to develop acute leukemia.[1] Death occurs more frequently from the consequences of chronic cytopenias than from transformation to acute myeloid leukemia (AML).[2–4]

Several recent reports indicate that a high proportion of bone marrow (BM) cells are undergoing apoptosis in patients with MDS,[5–15] which may contribute to the cytopenias that occur in these disorders. A correlation between apoptosis and cytopenias in MDS has been observed in some studies but not in others.[6–14] There are several possible reasons for variability in correlation between the extent of apoptosis detected in the marrow and the clinical features of MDS. It is possible that *in situ* end labeling may not detect a proportion of apoptotic cells that can be identified by other methods.[16] Further, since apoptosis is a dynamic process, assays for apoptosis at one time point in complex tissues, such as the marrow, may not accurately reflect the total number of cells having undergone apoptosis over time. Certain assays may fail to detect cells at early stages of apoptosis, whereas cells at later stages of apoptosis may already have been rapidly removed by reticuloendothelial cells in the marrow. Moreover, the extent of apoptosis seen may depend on the proportions of residual normal hematopoietic cells and of blasts in the sample, since the former but not the latter may be apoptotic. Thus, paradoxically, a higher degree of apoptosis may be seen in marrows with low blast counts and a higher content of normal hematopoietic cells.[8]

One major mechanism for apoptosis in both hematopoietic and non-hematopoietic cells is the Fas pathway. Fas is a tumor necrosis factor-receptor (TNF-R) family cell surface receptor that induces apoptosis when ligated by Fas ligand (FasL), a cell surface TNF family molecule.[17] Since normal hematopoietic cells express Fas receptor,[18–24] they may be susceptible to apoptosis triggered by FasL-expressing cells. Mature peripheral blood cells including neutrophils express high levels of Fas and are susceptible to Fas-mediated apoptosis,[23] whereas normal progenitors express low levels of Fas and are not susceptible to Fas-mediated apoptosis.[25] However, on normal $CD34^+$ hematopoietic cells, Fas expression is synergistically induced by TNF-α and interferon-γ (IFN-γ), resulting in increased susceptibility of clonogenic, as well as primitive progenitors to Fas-mediated apoptosis.[18,19,26] In aplastic anemia, the increased expression of TNF-β and IFN-γ in the BM is accompanied by increased Fas on $CD34^+$ cells and increased susceptibility of clonogenic progenitors to Fas-mediated killing.[27] In addition, TNF-α expression is increased in the BM of patients with MDS, and correlates with the degree of apoptosis.[28]

A proportion of BM and peripheral blood cells are derived from normal progenitors in MDS,[29,30] indicating that residual normal hematopoiesis contributes to the maintenance of peripheral blood cells in patients with MDS. The finding that $CD34^+$ cells, differentiating hematopoietic cells and stromal cells undergo increased apoptosis[6,9,10] suggests that cells derived from both the abnormal clone and those derived from normal progenitors may be apoptotic in MDS. Recent studies indicate that in MDS, the Fas pathway is functional and capable of inducing apoptosis in clonogenic progenitors when activated. Fas is expressed on $CD34^+$, $CD33^+$ and glycophorin$^+$ cells in MDS, and both erythroid and granulocyte–macrophage colony-forming cells are susceptible to apoptosis induced by an agonist anti-Fas antibody.[7,8]

A variety of tumor cell types express FasL, the ligand for the Fas receptor.[31–35] Malignant cells appear to possess mechanisms to protect themselves against FasL-mediated apoptosis, while maintaining the ability to induce apoptosis in normal cells.[6,35,36] Recent reports indicate that FasL expression is increased in MDS.[15,37] We hypothesized that the expression of FasL (1) is increased in the bone marrow of patients with MDS; (2) contributes to the cytopenias observed in these dis-

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relates with cytopenia and prognosis. We demonstrate that FasL expression is significantly increased in MDS and AML, that FasL expressed by MDS marrow cells directly inhibits the growth of hematopoietic progenitors *in vitro*, and that FasL expression correlates with anemia and predicts transfusion requirements and survival in patients with MDS.

Methods

Patients

A cohort of 50 consecutive patients diagnosed with MDS and 10 with AML at the Minneapolis VA Medical Center from 1992–1997 were identified by retrospective review of BM pathology records. Clinical information, including features at presentation, clinical course and outcome, was obtained by chart review. The demographic characteristics of the patients and clinical features at presentation are shown in Table 1. All the patients were males, reflecting the patient population at the Minneapolis VA Medical Center. The French–American–British (FAB) classification of the 50 patients with MDS was: RA ($n=4$), RA with ringed sideroblasts (RARS; $n=6$), RA with excess blasts (RAEB; $n=10$), RAEB in transformation (RAEBT; $n=7$), chronic myelomonocytic leukemia (CMML; $n=13$), and unclassified MDS ($n=10$). Forty patients required blood product transfusions: RBC ($n=19$), platelets ($n=2$), or both products ($n=19$). To normalize RBC transfusion requirements over time, the total number of units of RBC transfused between the diagnosis of MDS and either death or transformation to AML were converted to the average number of units transfused per 6 months, as follows: Number of units transfused between diagnosis of MDS and death or transformation to AML/Time in months from diagnosis of MDS to death or transformation to AML × 6. Patients included in this study were treated with pyridoxine ($n=7$), androgens ($n=1$), prednisone ($n=1$), hydroxyurea ($n=2$), 6-thioguanine + cytosine arabinoside ($n=1$), 5-azacytidine ($n=2$), erythropoietin (EPO; $n=2$), and folic acid ($n=4$). The remaining patients received only supportive treatment. One patient underwent allogeneic bone marrow transplantation. Five of 50 patients (10%) have transformed to AML to date. Twenty-nine of 50 patients (58%) have died, largely of complications related directly to MDS (infection in 12, hemorrhage in 7, AML in 4).

Table 1 Clinical features of 50 patients with MDS

Age (years), median (range)	73 (26–87)
Hemoglobin (g/dl), median (range)	10.2 (7.5–13.8)
WBC (per mm^3), median (range)	4.3 (1.1–112.6)
Platelet count (per mm^3), median (range)	120 (6–693)
Absolute neutrophil count (per mm^3), median (range)	2.5 (0.3–44.3)
Absolute monocyte count (per mm^3), median (range)	0.4 (0.0–22.6)
Blasts in peripheral blood (%), median (range)	0.0 (0.0–9.0)
Reticulocytes (%), median (range)	2.0 (0.1–8.8)
LDH (number of patients)	
Elevated	13
Normal	19
BM cellularity (number of patients):	
hypercellular; normocellular; hypocellular	36; 7; 7
BM blasts (%), median (range)	2.6 (0.2–24)
Bournemouth prognostic score (number of patients)	11; 11; 11; 11; 6
0; 1; 2; 3; 4	
Cytogenetic analysis	
Abnormal	15
Normal	23

Immunohistochemical staining for FasL

FasL expression was determined on BM samples that had been obtained at diagnosis of MDS, prior to treatment, by modification of a previous method.[31] Sections (5 μm thick) were cut from bone marrow aspirate clots embedded in paraffin in B-5 fixative and mounted on poly-l-lysine-coated slides. The sections were deparaffinized in xylene, rehydrated through graded alcohols and demercurized in alcoholic iodine. Endogenous peroxidase activity was quenched by immersing the slides in 1.5% hydrogen peroxide for 10 min at room temperature, followed by rehydration through three changes of distilled water. To induce heat-induced epitope retrieval, slides were immersed in 10 mm citrate buffer (pH 6.0) in a pressure cooker which was heated to boiling at 12 psi for 10 min and then cooled to room temperature over 20 min. Nonspecific binding was blocked by incubating sections with 15 μg/ml horse serum for 15 min at room temperature. The sections were then incubated for 1 h with 0.6 μg/ml mouse MAb against residues 116–277 of human FasL (MAb clone 33; Transduction Laboratories, Lexington, KY, USA). Negative control sections were incubated with nonimmune mouse serum or ascites fluid diluted 1:400 in PBS. Following incubation for 30 min with horse anti-mouse secondary antiserum, sections were immunolabeled using an avidin-biotin-immunoperoxidase method (Vector Laboratories, Burlingame, CA, USA). The chromogen was a solution of 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.009% hydrogen peroxide. Color development was terminated after 5 min by immersion in water. To differentiate iron deposits in hematopoietic cells, sections were immersed in equal parts of 5% potassium ferrocyanide and 5% HCl for 5 min. Sections were finally counterstained with hematoxylin, dehydrated and coverslipped with permount. FasL expressing cells were identified by granular intracytoplasmic staining. In each section, 1000 cells were scored for FasL expression by a pathologist (GAN) who was blinded to the FAB subtype and clinical information regarding the patients.

Additional sections from some of the same BM samples were immunostained using another anti-FasL primary antibody (Q20, a rabbit polyclonal directed against N-terminal amino acid residues 2–21; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Heat-induced epitope retrieval was induced by incubation of the sections in 10 mm citrate buffer for 20 min at 100°C, at atmospheric pressure. Sections were incubated with 1 μg/ml of the primary antibody for 45 min, and developed with a multi-link secondary antibody using the Vector Elite kit (Vector). To further confirm the specificity of the staining for FasL, blocking experiments were performed by pre-incubation of the sections with a four-fold excess of a blocking peptide (amino acid residues 2–21 of FasL).

The immunohistochemistry technique described can be used on BM clot specimens which have been fixed in either B5 or formalin, but not in Zenker's or Bouin's fixatives. Also, the process used for decalcification of BM trephine specimens

Immunoblotting for FasL

BM was aspirated in preservative-free heparin from the posterior iliac crest of patients with MDS or healthy volunteers after obtaining written informed consent. The study was approved by the VA Medical Center Human Subjects Subcommittee. The BM aspirates were immediately subjected to Ficoll–Hypaque (sg 1.077) centrifugation. BM mononuclear cells (BMMNC) at the interface were aspirated, washed three times, counted and resuspended in Iscove's modified Dulbecco's medium (IMDM) + 20% fetal calf serum + 20% dimethylsulfoxide, and stored in liquid nitrogen following controlled rate freezing, until used in co-culture with normal $CD34^+$/HLA-DR^+ cells or for immunoblotting. For immunoblotting, the frozen cells were rapidly warmed to 37°C, diluted in warm IMDM, and immunoblotting performed as described.[31] Briefly, BMMNC were pelleted and lysed in 1 ml of 50 mm Tris-HCl (pH 7.5), 250 mm NaCl, 5 mm EDTA, 0.1% NP40, 50 mm NaF and 1 mm phenylmethylsulfonylfluoride. Approximately 30 μg of total protein from each sample was electrophoresed on 12.5% SDS-PAGE gel, and electroblotted on to a nitrocellulose membrane. The membranes were blocked using 5% dry milk + 1% BSA in PBS, incubated with 1:1000 dilution of murine anti-human FasL MAb (Transduction Laboratories) at 4°C overnight and then with a rabbit anti-murine IgG antibody (PharMingen, San Diego, CA, USA). Protein bands were visualized by enhanced chemiluminescence as recommended by the manufacturer (Amersham, Arlington Heights, IL, USA).

In parallel, membranes were incubated with 1.5 μg/ml of a different primary antibody (anti-FasL IgG MAb clone G-247-4; PharMingen) for 1 h at room temperature. Following incubation with a secondary antibody, FasL was detected using an enhanced chemiluminescence kit (Amersham). A pGEX2TK-FasL fusion protein (size approximately 60 kDa), possessing the full-length recombinant human FasL protein with a GST leader was run as a positive control, to confirm the capability of the antibody to recognize human FasL.

Effect of FasL expressed by MDS BM cells on growth of clonogenic progenitors

$CD34^+$/HLA-DR^+ (DR^+) cells were purified from bone marrow aspirates taken from normal adult donors, by sequential Ficoll–Hypaque (sg 1.077) centrifugation, Ceprate-LC (CellPro, Bothell, WA, USA) CD34-biotin immunoadsorption column selection and flow cytometry, as previously described.[38] To simulate the effect of increased levels of TNFα found in the marrow in MDS,[28] the DR^+ cells were suspended at a concentration of 100 000/ml in long-term bone marrow culture (LTBMC) medium (12.5% FCS, 12.5% horse serum, 2 mm l-glutamine, 1000 U/ml penicillin, 100 U/ml streptomycin and 10^{-6} m hydrocortisone) supplemented with 100 ng/ml TNFα (Sigma) and 10 ng/ml interleukin (IL)-3 (R&D Systems, Minneapolis, MN, USA) and incubated at 37°C in 5% CO_2 atmosphere for 48 h.

For co-cultures of DR^+ cells with cell lysates of MDS BMMNC, the frozen cells were rapidly warmed to 37°C and diluted in warm IMDM. The cells were pelleted gently, resuspended in IMDM at a concentration of 25×10^6/ml and lysed by snap-freezing and thawing three times. Lysates from 2×10^5 or 2×10^6 MDS BMMNC were mixed with 2000 DR^+ cells in IMDM + 10% FCS + 10 ng/ml TNFα + 10 ng/ml

In some cultures, the MDS cell lysates were incubated with either 10 μg/ml recombinant FasFc protein (R&D Systems), 50 μg/ml neutralizing anti-TNFα antibody (R&D Systems), or both, for 1 h at room temperature, to block the FasL[39] and TNFα present, prior to addition of the DR^+ cells to the lysates. For control cultures, DR^+ cells were incubated with the cytokines in the absence of cell lysates. Following the incubation, cells were washed once with IMDM + 20% FCS to remove cytokines, cell debris and FasFc, and plated in semisolid methylcellulose cultures supplemented with 3 U/ml EPO and 5 ng/ml IL-3. In cultures examining the effect of FasFc protein and anti-TNFα antibody, additional FasFc (0.5 μg/ml) and anti-TNFα antibody (2.5 μg/ml) were added directly to the methylcellulose medium. Colonies were scored after 14 days.[40]

Statistics

Data were entered on and analyzed using an SPSS software package. Mean differences between groups were compared using either the *t*-test or ANOVA. Correlations of interval scale variables were analyzed using linear regression. Overall survival by FasL expression (FasL $\leqslant$12% and FasL $>$12%) was analyzed by the Kaplan–Meier method[41] and compared using the log-rank statistic. In addition, the correlation between % FasL expression and overall survival was analyzed using Cox regression. Results were expressed as the mean ± s.e.m.

Results

FasL expression is increased in MDS and AML

We examined by immunohistochemistry (Figures 1 and 2) the expression of FasL in the BM of patients with MDS, AML developing in patients with MDS (secondary AML), *de novo* AML and age- and sex-matched normal controls. Immunohistochemistry was performed using two separate anti-FasL antibodies, directed against a C-terminal epitope (MAb clone 33; Figure 1) or an N-terminal epitope of FasL (polyclonal antibody Q20; Figure 2). There was a high degree of correlation ($r = 0.97$) between the percentage of FasL positive cells (normal controls, MDS and AML) detected by the two antibodies, MAb clone 33 and polyclonal Q20 (data not shown). In the BM of normal controls ($n = 6$), a uniformly low level of FasL expression was detected (6 ± 1% positive cells; range 3–9%) and was mostly confined to lymphocytes (Figures 1a and 3a). In contrast, a wide range of FasL expression was seen in the BM of patients with MDS ($n = 50$; 17 ± 2% positive cells; range 2–51%; Figures 1b, 1c, 2a, 2c and 3a), which was higher than the controls ($p = 0.02$). FasL expression in MDS was seen in erythroblasts, myeloblasts, megakaryocytes, maturing myeloid precursors and dysplastic cells, and was not confined to lymphocytes (Figures 1 and 2). Mature neutrophils and erythroid normoblasts did not express detectable FasL. The staining in both MDS and AML BM cells by the Q20 antibody was almost completely blocked by pre-incubation of the sections with a peptide (amino acids 2–12 of FasL) comprising the epitope for the Q20 antibody (Figures 2b, d and f).

FasL expression in MDS was further confirmed by immunoblot analysis of BMMNC. A protein of the expected size for FasL (37 kDa) was recognized by a murine anti-human FasL antibody (MAb clone 33) in lysates from MDS BMMNC

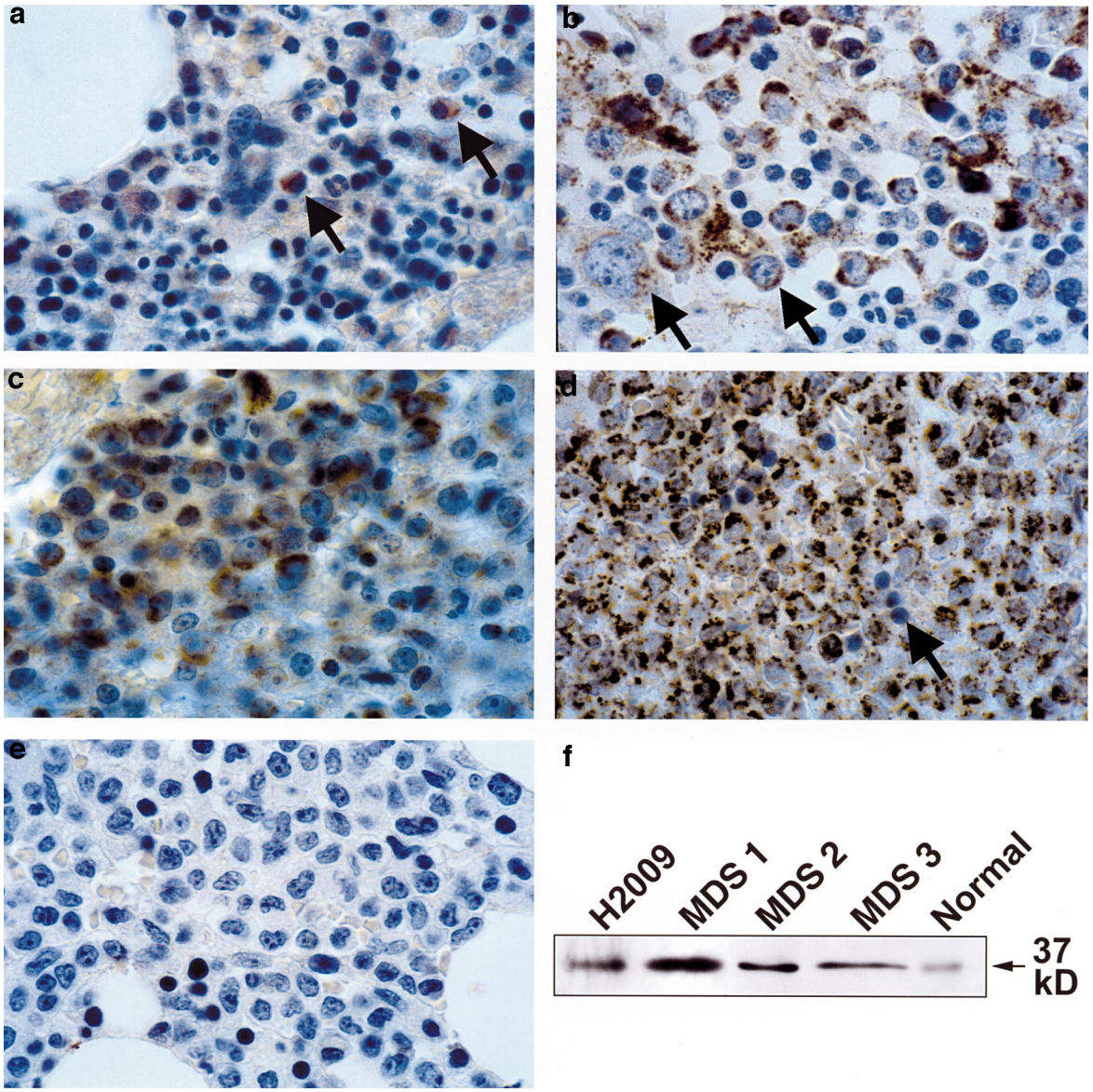

Figure 1 (a–e) Immunohistochemical detection of FasL expression in BM clot sections. (a) Normal control with occasional FasL+ lymphocytes (arrow); (b) MDS, unclassified, with 31% FasL+ cells. FasL in blast cells (arrow) and in a megakaryocyte (arrowhead); (c) MDS, RAEB, with 51% FasL+ cells; (d) AML M5 subtype, with intensely FasL$^+$ blasts and negative erythroblasts (arrow); (e) AML M5, negative control using preimmune serum in place of the anti-human FasL MAb (Transduction Laboratories MAb clone 33). Original magnification ×800. (f) Immunoblotting analysis of FasL expression in MDS. FasL expression was examined in cell lysates (30 μg total protein per lane) of BMMNC from MDS patients and from normal controls. The lung cancer cell line H2009 which expresses FasL[31] was used as a positive control. A 37 kDa protein was detected by an anti-human FasL MAb (Transduction Laboratories MAb clone 33).

cantly higher in MDS than in normal control BMMNC, using equivalent quantities of total protein from cell lysates. FasL (37 kDa) was also detected in MDS BMMNC by immunoblotting with another primary antibody (anti-FasL IgG MAb clone G-247-4, PharMingen), which also recognized the 60 kDa recombinant FasL fusion protein (Figure 2g). Taken together, these results indicate that FasL is expressed in BM cells in both MDS and AML.

In the BM of patients with *de novo* AML ($n = 10$), an extremely high proportion of cells showed FasL expression (56 ± 9%; range 10–97%; Figures 1d, 2e and 3a) which was higher than that found in controls ($P = 0.001$) and patients with MDS ($P < 0.001$). FasL expression in AML was largely seen in the blast cells, which showed intense positivity for the

expression increased at least three-fold upon transformation of MDS to secondary AML (Figure 3b). In three additional patients with secondary AML, FasL expression ranged from 56 to 86% (initial diagnostic MDS marrows were not available for FasL staining in these patients). As in *de novo* AML, expression of FasL was seen largely in the blasts in secondary AML. The overall FasL expression in secondary AML ($n = 6$; 48 ± 13%; range, 9–86%) was comparable to *de novo* AML.

FasL expression correlates with FAB subtypes of MDS

A significant correlation was observed between FasL expression and FAB subtype groups of MDS:RA/RARS (9 ± 3%

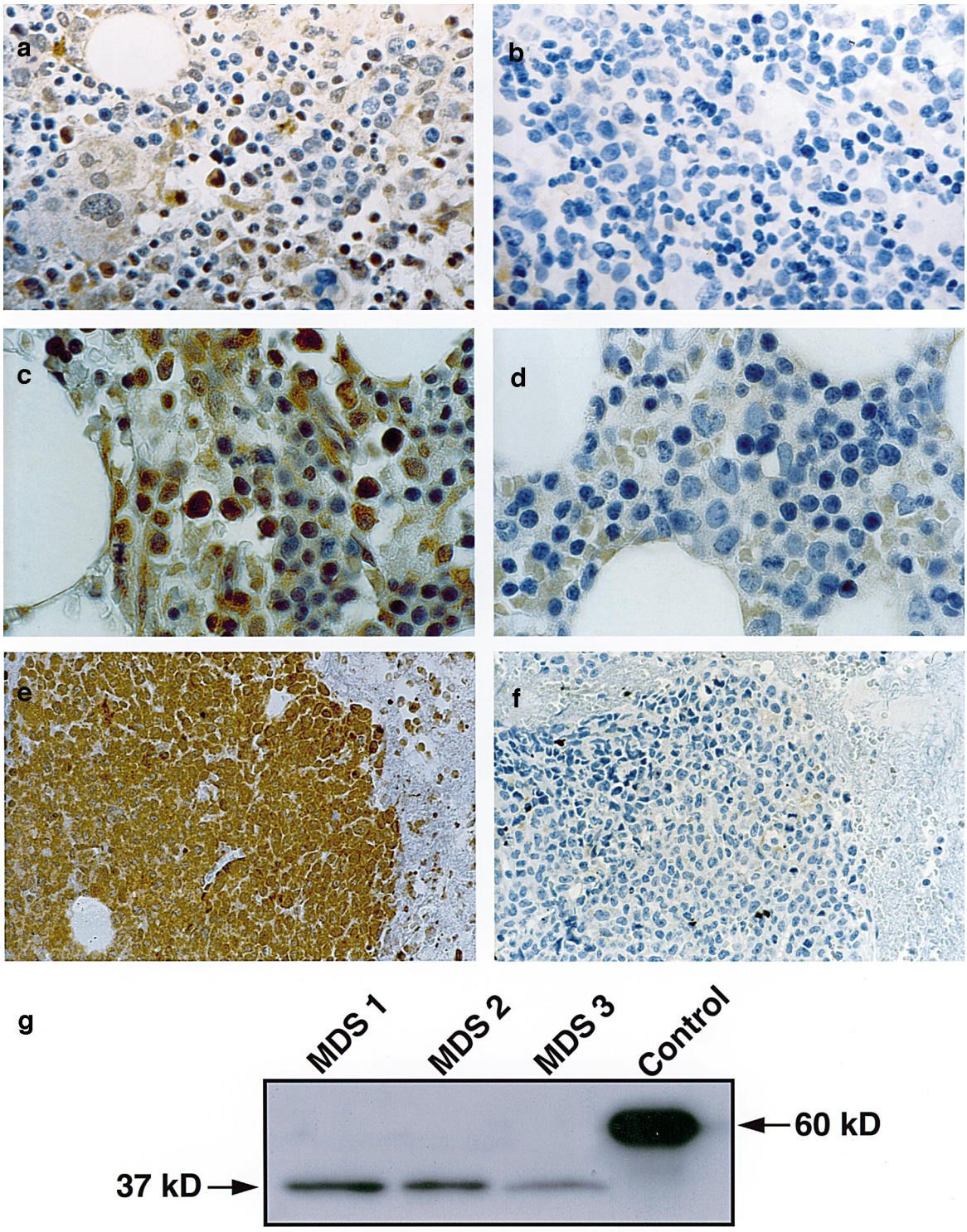

Figure 2 (a–f) Immunohistochemical detection of FasL expression in BM clot sections. Sections were incubated with Q20, a rabbit polyclonal directed against N-terminal amino acid residues 2–21 (Santa Cruz Biotechnology). (a) MDS, unclassified (c) MDS, RAEB-T (e) AML M5 subtype. Photomicrographs b, d and f are sections from the same marrows, which were pre-incubated with a blocking peptide (amino acid residues 2–21 of FasL) before staining with the antibody, demonstrating that the staining is blocked by the FasL peptide containing the epitope for the antibody. Original magnification ×320 (a,b,e,f) and ×800 (c,d). (g) Immunoblotting of FasL expression in MDS. FasL expression was examined in cell lysates (30 μg total protein per lane) of BMMNC from three MDS patients, using a third primary antibody (anti-FasL IgM MAb clone G-247-4, PharMingen). A pGEX2T/K-FasL fusion protein (60 kDa), with the full-length recombinant human FasL protein and a GST leader was used as a positive control. A 37 kDa protein corresponding to FasL was detected in all three MDS samples.

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