High Levels of Interleukin-6 Are Associated With Low Tumor Burden and Low Growth Fraction in Multiple Myeloma

By Oscar F. Ballester, Lynn C. Moscinski, Gary H. Lyman, J. Victor Chaney, H.I. Saba, A.S.D. Spiers, and C. Klein

Interleukin-6 (IL-6) is a multifunctional cytokine postulated to play a central role as a growth factor for multiple myeloma (MM). We evaluated the spontaneous secretion of IL-6 in supernatants of Ficoll-Hypaque–enriched bone marrow (BM) cultures from 35 patients with MM. The levels of IL-6 were correlated with biological and clinical characteristics of the disease. High levels of IL-6 production defined a subgroup of patients with low tumor burden as determined by lower serum β_2-microglobulin (B2M) (P = .02) and lower percentage of myeloma cells infiltrating the bone marrow (P = .003), higher synthetic rates of monoclonal protein (P = .006), and low proliferative compartments as measured by the percentage of Ki-67–positive myeloma cells. Patients with high proliferative fractions (Ki-67–positive myeloma cells >20%) had significantly lower levels of IL-6 when compared with patients with low proliferative fractions (P = .005). Our findings do not support IL-6 as a major growth factor for MM, but demonstrate an association of high levels of IL-6 secretion with low tumor cell burden and low proliferative fraction.

MULTIPLE MYELOMA (MM) represents the malignant transformation of a B-lymphocyte clone, with terminal differentiation of the tumor cells into immunoglobulin-producing plasma cells. Delineation of pre-B, B-lymphocyte, and mature plasma cell compartments within the tumor cell mass has been possible on the basis of phenotypic, clonogenic, proliferative, and functional analyses.[1-4] Several clinical studies have associated features of incomplete differentiation, such as CD10 positivity,[5] high proliferative fraction,[6,7] low RNA content,[8] and increased in vitro colony formation,[9] with an aggressive clinical course and shortened patient survival. Thus, the heterogeneous biological behavior of this malignancy can be related, at least in part, to the degree of tumor differentiation. Within this context, factors that stimulate myeloma cell growth and/or differentiation are of interest. Interleukin-6 (IL-6), a multifunctional cytokine initially described for its ability to induce differentiation of normal B cells, has become the focus of intensive research for its effects on myeloma cells.[10-14] In this study, we report the correlation of spontaneously secreted levels of IL-6 in bone marrow (BM) cultures from MM patients with biological and clinical features of the disease. Our findings disagree with previous reports that suggest IL-6 to be a major growth factor for MM. We propose, instead, a primary role for IL-6 as a differentiation factor.

MATERIALS AND METHODS

From January 1991 to March 1993, 35 patients with MM who met standard diagnostic criteria[15] were admitted to the study. The protocol was approved by the Institutional Review Boards of the H. Lee Moffitt Cancer Center and the University of South Florida. All patients gave informed, written consent. Their mean age was 66.4 years (range, 50 to 84). There were 20 women and 15 men. Twelve were previously untreated, while the remaining 23 were studied during induction chemotherapy or at relapse. The Durie-Salmon Staging system[16] was used to assess tumor mass in newly diagnosed patients. All patients had serum β_2-microglobulin (B2M) measurements, as well as routine blood counts, chemistries, skeletal surveys, and urine protein studies. Serum samples for IL-6 measurements were obtained from the first 27 patients entered into the study. Twenty morphologically normal BM samples from individuals undergoing biopsies for various reasons were used as controls.

BM cultures. BM mononuclear cells (MNCs) were isolated from aspirates by Ficoll-Hypaque (density = 1.065) gradient centrifugation to remove erythrocytes and mature myeloid cells. Cells were counted in the presence of Trypan blue to assess viability. Without further attempts at myeloma cell purification, BM MNCs were cultured in marrow medium (alpha modified Eagle's medium containing 20% [vol/vol] heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin) at a concentration of 1×10^6 cells/mL. Triplicate cultures of 2.5×10^6 cells and a media control were analyzed for each time point. Cells were harvested at day 1 and day 4 following initiation of culture by gently pipetting into sterile polypropylene tubes, pelleting in a standard centrifuge, and carefully removing the supernatant. Aliquots of 500 μL of supernatant or media control were frozen at −70°C until analyzed. Cytospins were prepared from the cell pellets at both time points, as well as from the post-Ficoll (preculture) preparation to evaluate cell morphology and allow enumeration of plasma cells contained within the cultures.

IL-6 levels. IL-6 was measured in serum and culture supernatants using an enzyme-linked immunoabsorbent assay (ELISA; T Cell Sciences, Cambridge, MA).

IgG synthetic rate. Supernatants from cultures of 11 patients with IgG myeloma were assayed for IgG subtype levels using a sensitive ELISA assay (Janssen Biochimica, Flanders, NJ). The rate of myeloma protein synthesis was expressed as nanograms per milliliter of subtype specific IgG per 1×10^3 myeloma cells in culture at day 4.

Ki-67 proliferation index. Deparaffinized, 5-μm BM sections were reacted with the MIB-1 antibody (AMAC, Westbrook, ME) using a standard biotin-streptavidin immunoperoxidase technique (StrAviGen; BioGenex, San Ramon, CA). The MIB-1 antibody reacts with the Ki-67 nuclear antigen, which is associated with cell proliferation and found throughout the cell cycle (G1, S, G2, and M phases), but absent in resting (G0) cells. The slides were developed using 3-3′-diaminobenzidine tetrahydrochloride chromagen producing discrete dark brown nuclear staining. The slides were counterstained with hematoxylin and eosin for morphologic evalua-

From the Divisions of Bone Marrow Transplantation and Medical Oncology/Hematology Leukemia/Lymphoma Center, H. Lee Moffitt Cancer Center and Research Institute, Departments of Medicine and Pathology University of South Florida College of Medicine, Tampa, FL.

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Supported by a grant from the H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL.

Address reprint requests to Oscar F. Ballester, MD, H. Lee Moffitt Cancer Center, 12902 Magnolia Dr, Tampa, FL 33612-9497.

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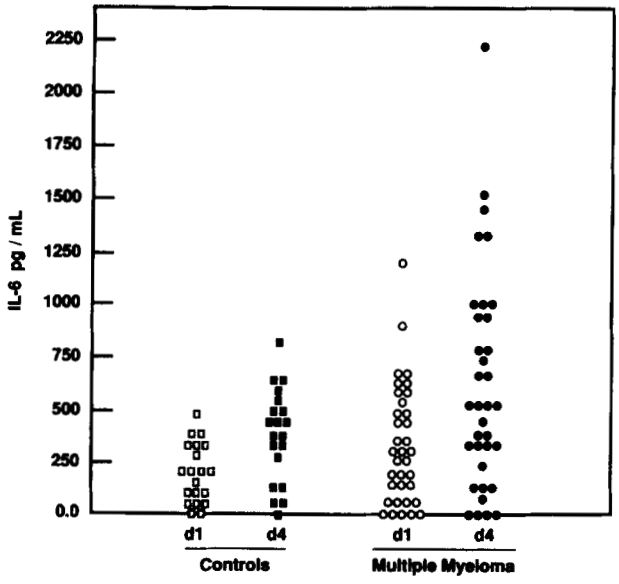

Fig 1. Levels of spontaneously secreted IL-6 in BM culture supernatants on day 1 (□, controls; ○, myeloma patients) and day 4 (■, controls; ●, myeloma patients).

tion. A proliferation index (PI; number of positively stained malignant plasma cell nuclei per total number of malignant plasma cell nuclei × 100) was obtained by microscopically counting at least 400 myeloma cells in duplicate. Enumeration of PI was independently performed by two morphologists (L.C.M. and J.V.C.), and the results averaged. Ki-67 PI was assessable in 31 of 35 patients; the remaining four patients had too few myeloma cells in the samples for adequate quantitation.

Patients were treated, when indicated, with standard protocols including melphalan and prednisone for newly diagnosed patients and vincristine, doxorubicin, and dexamethasone (VAD) in refractory or relapsed patients.

Statistical methods. Measurement point estimates and variances were expressed as means ± 1 SD. Where appropriate, both parametric and nonparametric tests of the null hypothesis of no measurement difference were performed. Group means were compared using Student's *t* test for two independent samples based on pooled estimates of the common variance. Group distributions were compared nonparametrically using the Wilcoxon rank-sum test (Mann-Whitney *U* test). A two-tailed test of the null hypothesis of no difference between the means was used throughout. The association between measurements was evaluated by the product moment correlation or Pearson's coefficient of correlation. Inference on the correlation coefficient was based on the *t* ratio test of the null hypothesis of no correlation in the underlying population. Proportional outcomes were compared for independent samples based on the Fisher's exact test of the null hypothesis of no difference in the population proportions. Survival analysis was based on Kaplan-Meier estimates of the survival function for censored data. Survival functions for groups were compared using the log-rank (Mantel-Haenzel) test of the null hypothesis of equal survival.

RESULTS

IL-6 secretion, myeloma cells in culture, and rate of monoclonal protein production. BM samples from myeloma patients secreted higher amounts of IL-6 than controls, both on day 1 and on day 4 supernatants (Fig 1). The levels of IL-6 on day 4 MM cultures varied greatly, with values ranging from 0 to 2,200 pg/mL. Fifteen patients had values greater than, and eight had values less than, the control's mean ± 1 SD (374 ± 215 pg/mL). No significant differences were found in the levels of secreted IL-6 when newly diagnosed and previously treated patients were compared. The percentage of myeloma cells in culture also varied greatly, ranging from 2% to 99% (mean, 29% ± 22%). IL-6 levels on day 4 samples were inversely correlated with the percentage of myeloma cells in culture ($r = -.4455$, $P < .008$) (Fig 2A). The rate of IgG secretion (ng/mL/1 × 10^3 myeloma cells) in the 11 patients with IgG myeloma showed a significant positive correlation with the spontaneous levels of secreted IL-6 ($r = .7494$, $P < .008$) (Fig 2B).

Clinical correlations: tumor mass and growth fraction. For clinical comparisons, the mean day 4 IL-6 value was

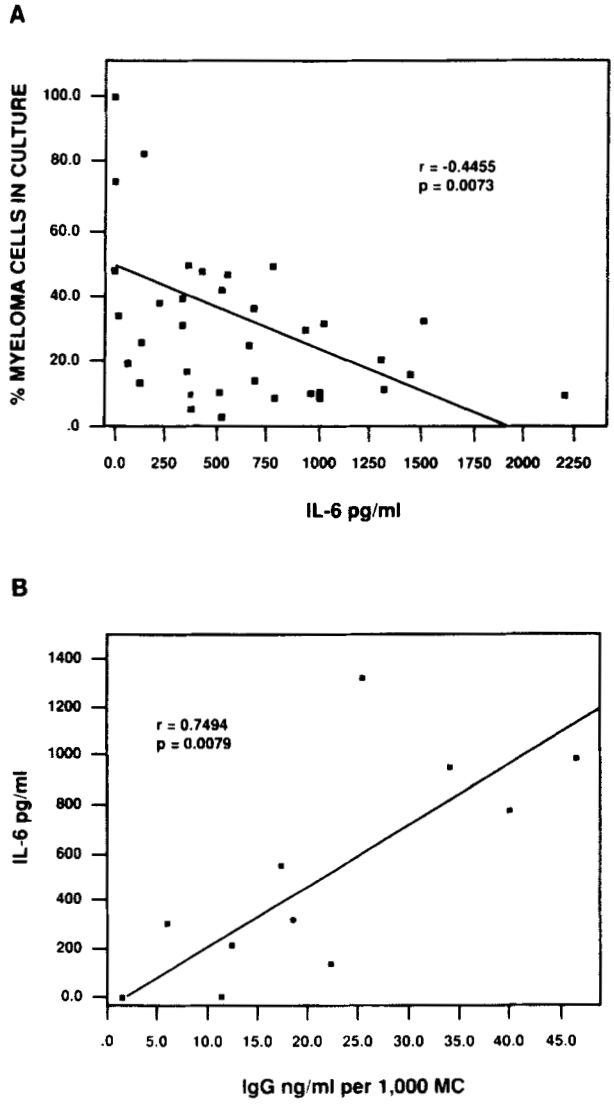

Fig 2. Correlation of spontaneously secreted IL-6 levels with the percentage of myeloma cells in culture (A), and with the rate of IgG secretion in 11 patients with IgG myeloma (B).

Table 1. Clinical and Biologic Characteristics Associated With BM IL-6 Secretion

Characteristic*	High IL-6 (>618 pg/mL, n = 15)	Low IL-6 (<618 pg/mL, n = 20)	P Value
Rate IgG secretion ng/mL/1 × 10^3 MC (n = 11)	29.7	12.4	.006
% BM MC (n = 35)	23.5	66.0	.003
Serum B2M (n = 35)	2.5	5.2	.02
Ki-67 PI (n = 31)	9	20	NS

Abbreviation: MC, myeloma cells.
* Median values.

used to group individuals with high (IL-6 > 618 pg/mL, n = 15) or low IL-6 levels (IL-6 < 618 pg/mL, n = 20) (Table 1). Tumor mass was assessed by serum B2M levels and the percentage of BM myeloma cells in all 35 patients, and by the Durie-Salmon staging system in the subgroup of 12 newly diagnosed patients. Patients with high IL-6 showed significantly lower levels of serum B2M (median, 2.5 mg/L) than those with low IL-6 (median, 5.2 mg/L) (P = .02). Similarly, patients with high IL-6 had lower percentages of myeloma cells infiltrating their BM (median, 23.5%) than patients with low IL-6 (median, 66.0%) (P = .003). Among newly diagnosed cases, three of four patients with high IL-6 were stage I, while six of eight patients with low IL-6 were stage II or III. As expected, stage I patients had significantly lower percentages of myeloma cells in BM (P = .01) and levels of serum B2M (P = .006) than patients with stage II or III disease. Serum IL-6 levels were undetectable in all 12 newly diagnosed patients, while three among 15 previously treated patients had detectable IL-6 levels (80, 380, and 3,360 pg/mL). BM day 4 IL-6 levels in these three patients were 960, 140, and 520 pg/mL, respectively. Ki-67 PIs ranged from 2% to 62%, with a mean value of 13.7%. A negative correlation was found between IL-6 levels in culture supernatants and Ki-67 PI (r = −.3491, P = .05). A strong correlation was found between Ki-67 PI and serum B2M (r = .6490, P = .00007). A Ki-67 PI greater than 20% (n = 7) defined a subgroup of patients with significantly lower levels of IL-6 on day 4 compared with patients with Ki-67 PIs of less than 20% (n = 24) (P = .0006). Patients with high Ki-67 PI had higher percentages of BM myeloma cells (P = .02) and higher serum B2M levels (P = .03) (Table 2). Among the 15 patients with high IL-6, Ki-67 PIs were uniformly low, with no values greater than 20%. The group of 20 patients with low IL-6 was heterogeneous, and included patients with both high and low Ki-67 PIs.

IL-6 and patient survival. With a median follow-up duration of 20 months, 19 of 35 patients have died. No significant differences in survival are evident comparing patients with low and high IL-6 levels, either for the entire group (Fig 3) or examining newly diagnosed patients separately. Six deaths have been documented among the seven patients with high Ki-67 PIs (median survival, 9 months), compared with 12 in the 24 patients with low PIs (median survival, 15 months). Significant differences between patients with high and low IL-6 levels were observed in the patterns of causes of death. Nine of 10 deaths in the subset of low IL-6 patients, but only three of nine deaths in the high IL-6 group, were due to disease progression, refractory to standard therapies (P = .02). The most common cause of death in patients with high IL-6 was postchemotherapy sepsis at the time of documented tumor response (four cases, including one patient after BM transplantation); other causes included progression to acute myelogenous leukemia (one case) and nephrotic syndrome (one case). Among the three patients with detectable serum IL-6 levels, one died with progressive unresponsive disease and one died of sepsis at the time of a documented clinical response after two courses of VAD. The third patient achieved a near complete remission, which has been maintained for more than 1 year. Interestingly, his serum IL-6 level has remained persistently elevated.

DISCUSSION

Our primary objective was to correlate the degree of spontaneous IL-6 myeloma cell stimulation with clinical and biological features of the disease to define more precisely the role of IL-6 in the growth, proliferation, and differentiation of MM. We have previously examined serum IL-6 levels in 60 patients with MM, but found detectable levels in only six, precluding further analysis of the data.[17] Serum IL-6 levels may not be reflective of BM IL-6 levels, as peripheral blood monocytes appear to be the body's major source of IL-6.[18] High serum IL-6 levels have been reported in patients following marrow transplantation at the time of marrow aplasia.[19] In our study, none of the controls had detectable serum IL-6, while their BMs produced IL-6 at rates slightly greater than 50% of those of myeloma patients. Two of three patients with detectable serum IL-6 had BM IL-6 levels below the group mean. The clinical behavior of these three patients could not be distinguished from the rest of the patient population.

Therefore, we studied the IL-6 production of unsorted myeloma BM samples, under the assumption that these measurements are more relevant to the levels of in vivo myeloma cell IL-6 stimulation. Myeloma cells proliferate in the BM microenvironment. Most investigations support a paracrine mechanism,[1] rather than the autocrine secretion of IL-6 by myeloma cells.[10] A recent report documents the expression of IL-6 mRNA in highly purified myeloma cells from 10 of 22 patients studied. Five of these patients secreted IL-6 in the culture media at concentrations ranging from 0.5 to 12.8

Table 2. Tumor Load and BM IL-6 Production in Myeloma Patients With High and Low Growth Fractions

Characteristic*	Ki-67 PI > 20 (n = 7)	Ki-67 PI < 20 (n = 24)	P Value
Serum B2M (mg/L)	7.4	3.1	.03
% BM myeloma cells	63	25	.01
IL-6 day 4 (pg/mL)	135	665	.005

* Median values.

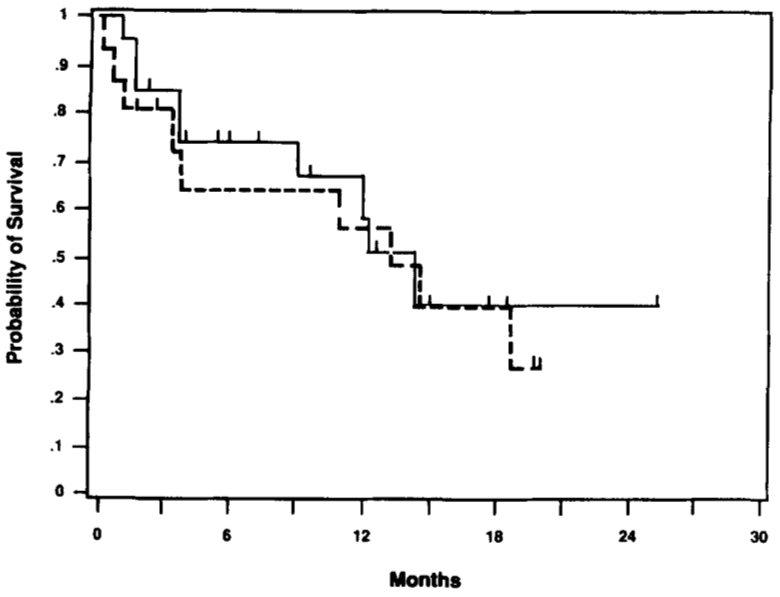

Fig 3. Survival probability for patients with high IL-6 (——) and low IL-6 (----) measurement from the time of entry into the study.

pg/mL.[20] Autocrine IL-6 secretion may, therefore, make a contribution to the IL-6 available in BM in a small proportion of myeloma patients. Quantitatively, macrophages, fibroblasts, and endothelial cells appear to be the major sources of IL-6 in the marrow environment.[21] Further evidence in support of this issue is our finding of an inverse correlation between the proportion of myeloma cells in culture and the expression of IL-6.

The levels of IL-6 detected in our cultures are comparable to the ones found in previous studies,[10,11] as well as those reported in BM plasma.[22] However, high levels of IL-6 expression in our study were not associated with adverse prognostic features, as previously reported.[11] We found that high levels of IL-6 expression were correlated with high rates of myeloma protein secretion, low proliferative compartment, and low tumor mass. The concept of IL-6 as the major growth factor for myeloma cells is supported primarily by the observation of enhanced proliferative activity induced by IL-6 in short-term culture. A significant increase in the proliferative index was demonstrated in 41 of 70 partially purified samples reported in four separate series.[10-12,14] However, in a recent report, none of 22 highly purified myeloma samples were stimulated to proliferate with exogenous IL-6.[20] This suggests that, in previous studies, cells responding to exogenous IL-6 may have been contaminating hematopoietic cells. A short burst of proliferative activity is not incompatible with the induction of terminal B-cell differentiation. IL-6 has been shown to be an important cofactor in the induction of high-rate immunoglobulin-producing plasma cells from peripheral blood B lymphocytes of normal individuals.[23] Similarly, IL-6 and interleukin-3 synergistically stimulate peripheral blood lymphocytes from myeloma patients.[24] An initial increase in proliferative activity is noted during the first 3 days of culture, associated with the appearance of CD10-positive immunoblasts. This is followed by the development of morphologically and functionally mature plasma cells expressing the same light chain as the myeloma clone and a return to a low proliferative activity on day 6. In our study, high levels of IL-6 expression were associated with uniformly low proliferative compartments, as assessed by the Ki-67 PI.[25,26] As expected, a high Ki-67 PI was associated with high tumor mass and shortened survival. However, these patients had significantly lower levels of IL-6 expression, including undetectable levels in two of seven patients with Ki-67 PIs greater than 20%.

Clonogenic myeloma cells do not appear to be stimulated by IL-6. In two separate reports including 51 patients, the ability of freshly isolated myeloma cells to form colonies was not affected or was inhibited by IL-6.[27,28] No correlation was found between IL-6 levels in BM plasma and in vitro myeloma colony formation.[22]

While it has been demonstrated that IL-6 stimulates immunoglobulin secretion by normal B cells,[29] two previous studies failed to show enhancement of paraprotein secretion by myeloma cells with exogenous IL-6.[30,31] We found a strong correlation between the levels of IL-6 in culture supernatants and the rate of IgG secretion in patients with IgG myeloma, consistent with a relationship between the levels of IL-6 secreted and the proportion of tumor cells committed to terminal differentiation. A high rate of immunoglobulin synthesis is characteristic of terminally differentiated B cells and is associated with loss of proliferative capacity and commitment to programmed cell death.[32] The association of IL-6 expression and the rate of IgG synthesis may be helpful in understanding the previously reported lack of correlation between serum myeloma protein concentration, myeloma protein synthetic rate, and tumor mass.[33] In particular, this may explain the subset of patients with small concentrations of serum myeloma protein and low synthetic rates but, yet, extensive disease.

The association of high levels of IL-6 expression with good prognostic indicators (low growth fraction, low tumor burden) would predict a survival advantage for this subset of patients. However, no significant differences in survival

were evident when we compared patients with high and low IL-6 levels. As our study was not primarily designed to assess responses to therapy and survival, these results are only tentative. An appropriately designed prospective study will be required to address this issue.

Our observations support a model where IL-6 induces an undifferentiated myeloma tumor cell compartment into terminal differentiation (high rate of immunoglobulin production, low proliferative fraction) and eventual tumor cell death (low tumor mass). This model is consistent with the role of IL-6 in normal B-cell physiology.[21,29]

On the basis of these findings, the role of ongoing preclinical[34] and clinical[35] efforts to neutralize IL-6 as a therapy for MM may need to be reexamined. Similarly, the use of human recombinant IL-6, currently undergoing clinical trials as a hematopoietic growth factor,[36,37] should not necessarily exclude patients with MM.

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