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Cytokines involved in the progression of multiple myeloma

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SUMMARY

We have investigated which of the cytokines that are relevant in the *in vitro* growth of multiple myeloma (MM) malignant plasma cells are actually produced *in vivo* by MM patients. To this end, we have measured the levels of IL-1β, IL-3, IL-4, IL-6, IL-7, IL-8 and tumour necrosis factor-alpha (TNF-α) both in sera and in the supernatant of bone marrow (BM) stromal cell cultures from patients with MM and monoclonal gammopathy of undetermined significance (MGUS). The significance of our findings is three-fold. First, IL-6 and IL-8 are produced by MM BM stromal cells, while IL-1β, TNF-α, IL-4 and IL-7 are not. Second, IL-3 is the only cytokine consistently raised in serum samples; we have also detected low levels of serum IL-6 in a minority of cases, usually in advanced stage of the disease. Third, MM BM stromal cells are active IL-6 and IL-8 producers, while both normal and MGUS BM stromal cells are low producers, thus suggesting that in the BM of MM a number of environmental cells, that would normally be quiescent, are instead activated and that, in MM, activated BM stromal cells play an active role in supporting the progressive expansion of the B cell clone.

Keywords multiple myeloma cytokines stromal cells

INTRODUCTION

Evidence is growing that a network of cytokines are operating in the growth, progression and dissemination of human multiple myeloma (MM; [1]). Some cytokines are actually produced by malignant plasma cells. These include IL-1β [2], tumour necrosis factor-beta (TNF-β) [3] and a functionally active truncated version of macrophage colony-stimulating factor (M-CSF) [4]. Though a minority of human MM cell lines secrete small amounts of IL-6 [5], it appears unlikely that fresh MM plasma cells may be able to produce IL-6 [6].

A number of cytokines increase the *in vitro* proliferation of MM cell lines and sometimes also of freshly purified malignant plasma cells. IL-6, a potent growth factor for murine hybridomas and plasmacytomas [7], is presently considered the most relevant growth factor for human MM as well [8,9]. MM plasma cells express the IL-6 receptor (R) and, *in vitro*, the growth of human MM cell lines can be facilitated by supplementing the culture medium with IL-6 or is dependent on IL-6 producing feeder cells [5]. Other cytokines, like granulocyte macrophage (GM)-CSF, IL-1, IL-3 and IL-5, may increase, frequently in synergy with IL-6, the ^{3}H-thymidine (TdR) incorporation of purified MM plasma cells cultured *in vitro* [10–12]. Finally, IL-1β [3,13] and TNF-β [14] cause osteoclast activation and bone reabsorption and are defined osteoclast activating factors (OAF; [15]); also M-CSF [16], IL-3 [17] and IL-6 [18] have an OAF activity.

We have asked which of the *in vitro* relevant cytokines are actually produced *in vivo* by MM patients. Central to this question is the fact that MM plasma cells grow specifically within the bone marrow (BM) microenvironment [19]. We have thus investigated which cytokines are produced by the BM stromal cells which, in MM patients, form a microenvironment favourable to the expansion of plasma cells. We have taken advantage of an experimental system that allows the culture from MM BM of stromal cells which are able to support the growth and final differentiation of peripheral blood-borne precursors of malignant plasma cells [20]. The levels of IL-1β, IL-3, IL-4, IL-6, IL-7, IL-8 and TNF-α have been measured both in sera and in the supernatant of BM stromal cell cultures from patients with MM and monoclonal gammopathy of undetermined significance (MGUS).

PATIENTS AND METHODS

The study included 30 patients with MM, 16 females and 14 males, aged 43–77 years and six patients with MGUS, four females and two males, aged 50–71 years. MM patients were 12 stage I, three stage II, and 15 stage III according to Durie &

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Salmon [21] and were studied either at diagnosis before the initiation of treatment ($n=19$) or at relapse ($n=11$). MGUS patients had a mean follow up of 5 years. Twenty-four normal donors matched for age and sex were analysed as controls. Serum samples were harvested from all patients.

In 18 MM patients (seven stage I, two stage II, nine stage III) and six MGUS patients a BM aspirate and peripheral blood (PB) samples were concomitantly obtained after informed consent and utilized to perform stromal cell cultures as described [20]. The normal controls for BM stromal cell cultures were BM aspirates obtained from five young subjects (<25 years of age), undergoing BM biopsy as a part of clinical staging for Hodgkin's disease and who had no histological evidence of BM localizations.

Serum samples

Immediately after venipuncture, blood samples (both patients' and controls') were allowed to clot for 1 h. Serum samples were separated from the cells plus clot, aliquoted and stored at −80°C until cytokine measurement was performed.

Cells

BM nucleated cells were separated on methylcellulose, washed twice with PBS and cultured immediately.

PB mononuclear cells (PBMC) were separated on Ficoll–Hypaque and frozen in liquid nitrogen.

Cell cultures

The cell culture protocol is detailed elsewhere [20]. Briefly, BM cells resuspended in Dulbecco medium and 5% horse serum (HS) were layered onto coverslips in 24-well culture plates at a concentration of 2×10^6 cells/ml and cultured at 37°C in 5% CO_2, replacing half of the spent medium with an equal amount of fresh every 4 days. After 7–8 weeks of culture, when no residual lymphocytes or plasma cells could be identified and a confluent layer of adherent fibroblast-like elements interspersed with a small proportion of macrophages and few osteoclasts was observed, aliquots of culture supernatants were harvested and stored at −80°C. Autologous PBMC (2×10^6/ml) were then thawed, washed twice with PBS and seeded over the stromal cell layer. The BM-PBMC co-cultures were performed for 3 weeks at 37°C in 5% CO_2, supplementing the medium with 20% fetal calf serum (FCS) and replacing half of the spent medium with an equal amount of fresh one every 4 days. After 3 weeks of co-culture, when variable proportions of monoclonal lymphoid cells and plasma cells were observed tightly adherent to the stromal cell layer and the number of osteoclasts had significantly increased [20], aliquots of culture supernatants were harvested and stored at −80°C.

Cytokine evaluation

Cytokines were measured in serum and culture supernatant samples. Supernatants were from 7–8-week-old BM stromal cell cultures and from 3-week-old BM/PBMC cultures.

ELISA following the manufacturer's instructions was used to measure IL-1β (Cistron Biotechnology, Pine Brook, NJ), IL-4 (Genzyme, Boston, MA), IL-6 (Genzyme), IL-7 (Amersham International, Amersham, UK) and TNF-α (T Cell Science, Cambridge, MA): the measurable threshold levels were respectively 20 pg/ml for IL-1β, 45 pg/ml for IL-4, 0·1 ng/ml for IL-6, 4·4 pg/ml for IL-7 and 10 pg/ml for TNF-α.

IL-8 levels were measured using material kindly provided by Dr M. Ceska, Sandoz Forschungsinstitut, Vienna, Austria. Ninety-six-well microplates with a non-cross-reacting murine MoAb specific for human IL-8 attached to the wells were used with the aim of capturing IL-8 present in the samples by the solid phase MoAb. A goat polyclonal anti-human IL-8 antibody conjugated with alkaline phosphatase was added and the absorbance was increased by adding *p*-nitrophenylphosphate. The absorbance measured by an ELISA reader at 405 nm was proportional to the concentration of IL-8 present in the sample [22]. A reference curve was obtained by plotting the IL-8 concentration of several dilutions of standard samples *versus* absorbance. The IL-8 levels of the samples tested were determined by comparing their absorbance with the absorbance of the known amounts of IL-8 provided with the standards. The lowest concentration of IL-8 reproducibly detectable was 0·1 ng/ml.

IL-3 levels were measured with a biological assay using the megakarioblastic cell line M-07 whose proliferation is IL-3- or GM-CSF-dependent [23]. In the absence of IL-3 or GM-CSF, cells stop growing within 28 h; after readdition of the growth factor, DNA synthesis is restored and the peak of proliferative activity is reached 24 h thereafter. Stock M-07 cells grown in 1–5 ng/ml IL-3 were thoroughly washed to remove growth factors and grown at a concentration of $1{\cdot}5\times10^6$/ml in Iscove's modified Dulbecco's medium (IMDM; Gibco Europe, Paisley, UK) with 5% FCS at 37°C and 5% CO_2. After 28 h, 5×10^4 cells were resuspended in IMDM medium + 5% FCS and seeded in 200-μl wells in presence or absence of IL-3 (Genzyme). After 24 h incubation, 1 μCi of ^{3}H-TdR (specific activity 5 Ci/mM; Amersham) was added to each well for an additional 4 h. The growth response of M-07 cells was used to design an IL-3 titration curve: the smallest amount of IL-3 which induced proliferation was 0·3 pg/ml and the plateau was reached with 6·6 pg/ml.

Each serum sample to be tested for the presence of IL-3 was divided into two aliquots. The first aliquot was added to the cultures instead of IL-3 at 10% concentration; the second aliquot was added after 2 h preincubation at 37°C with 10 μg/ml anti-IL-3 neutralizing antibody (British Bio-technology Ltd, Cowley, UK). The growth response of M-07 plus 10% patient's serum in presence or absence of anti-IL-3 antibodies was compared with IL-3 titration curve and used to measure in pg/ml the amount of IL-3 present in each sample.

RESULTS

Cytokine levels in BM stromal cell culture supernatants

Only IL-6 and IL-8 were consistently found in BM stromal cell culture supernatants. IL-6 levels (threshold detection level 0·1 ng/ml) were measured in 13 MM cases (five stage I, two stage II, six stage III), in five MGUS and in five normal donor specimens. As shown in Fig. 1, all MM cases were positive with a mean value of $2{\cdot}1+1{\cdot}6$ (range 0·5–3·7 ng/ml) and 4/5 MGUS cases were likewise positive with a mean value of $1{\cdot}1+1{\cdot}2$ (range 0·15–2·3). No apparent relationship was observed between IL-6 levels and the patient's clinical stage; likewise no difference was observed between patients at diagnosis or in relapse. IL-6 levels were undetectable in the supernatants of BM stromal cell cultures from normal donors.

IL-8 levels (threshold detection level 0·1 ng/ml) were meas-

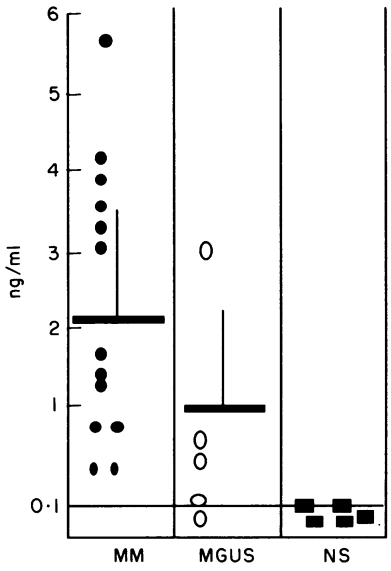

Fig. 1. IL-6 levels in the culture supernatant of bone marrow (BM) stromal cells. MM, Multiple myeloma; MGUS, monoclonal gammopathies of undetermined significance; NS, normal subjects.

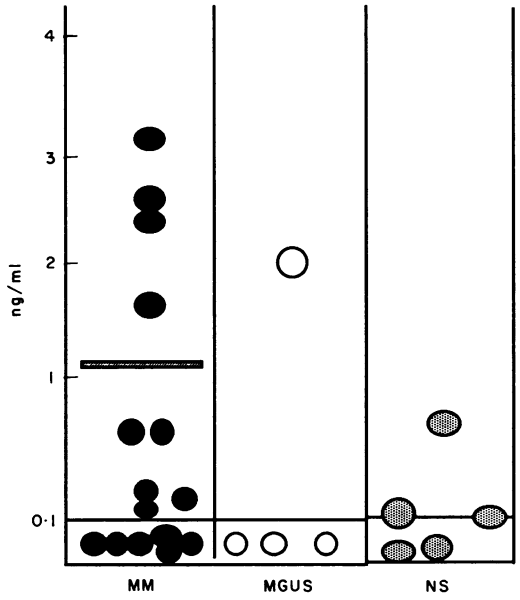

Fig. 2. IL-8 levels in the culture supernatant of bone marrow (BM) stromal cells. MM, Multiple myeloma; MGUS, monoclonal gammopathies of undetermined significance; NS, normal subjects.

ured in 15 MM (six stage I, two stage II, seven stage III), in four MGUS and in five normal donor specimens. As shown in Fig. 2, 9/15 MM cases (four stage I, one stage II, four stage III) were positive with a mean value of 1·16 + 1·1 ng/ml (range 0·13–2·26) and only 1/4 MGUS patient had detectable IL-8 levels (2·19 ng/ml). This case was undoubtedly a MGUS on clinical grounds; still, she had high levels of β_2 microglobulin and of C-reactive protein. As for normal donors, IL-8 levels were detected in only one subject (0·89 ng/ml).

TNF-α levels were below the threshold detection level (10 pg/ml) in 18/18 MM, in 5/5 MGUS and 3/3 normal donor specimens.

Table 1. Growth response of MO-7 cells in presence of patient's serum and patient's serum preincubated with anti-IL-3 neutralizing antibody

Patient	IMDM + 5% FCS (ct/min)	Patient serum (10%) (ct/min)	10 μg/ml anti-IL-3 antibody (ct/min)
1	2330	6253	1987
2	2345	10522	3247
3	2335	14693	6781
4	2300	12602	3320
5	2350	12660	4811
6	2842	7343	4056
7	2337	7114	3004
8	2490	5389	3745

The data are the mean of each experiment done in triplicate.

IMDM, Iscove's modified Dulbecco's medium; FCS, fetal calf serum.

IL-1β levels measured in seven MM (two stage I, one stage II, four stage III), in three MGUS and in four normal donor specimens were below the threshold of 20 pg/ml in all the samples tested.

IL-4 levels, measured in 14 MM (six stage I, two stage II, six stage III) and in four normal donor specimens, were below the threshold detection level (0·045 ng/ml).

IL-7 levels were below the threshold detection level (4·4 pg/ml) in 18 MM (seven stage I, two stage II, nine stage III), in 6/6 MGUS and in five normal donor specimens.

Cytokine levels in serum samples

TNF-α was above the threshold detection level (10 pg/ml) in only 1/30 MM cases and was likewise undetectable in the four MGUS studied and in 24 normal donors. The only positive MM sample was a stage III patient with 12 pg/ml TNF-α.

IL-1β was above the threshold detection level (20 pg/ml) and was measurable in only 1/30 MM samples and could not be detected in the three MGUS studied as well as in 24 normal donors. The only positive MM patient (who was TNF-α-negative) was a stage III patient with 33 pg/ml IL-1β.

IL-3 levels (threshold detection level 0·3 pg/ml) were measured in 20 cases. In eight patients (three stage I, five stage III) serum preincubation with anti-IL-3 neutralizing antibody drastically reduced the growth response of M-07 cell line (Table 1), thus indicating the presence of IL-3. As shown in Fig. 3, the mean value of IL-3 in these patients' sera was 1·73 + 0·57 (range 1·16–2·3 pg/ml). IL-3 levels were undetectable in the four MGUS studied and in 24 normal donors.

IL-4 was evaluated in 14 MM and in 19 normal donor samples: the levels were always below the threshold detection level.

IL-6 levels were detected at very low levels (0·12 ng/ml; theshold detection levels 0·10 ng/ml) in 4/30 MM samples (one stage I and three stage III) and were undetectable in the four MGUS studied as well as in 24 normal donors.

IL-8 levels were measured in 29 MM samples (10 stage I, three stage II, 16 stage III). Positive results were observed in 13 cases: six stage I (mean 0·2 + 0·1 ng/ml; range 0·1–0·3), one stage II (1·07 ng/ml), seven stage III (mean value 1·53 + 1·4; range 0·13–1·93); 1/4 patients with MGUS produced IL-8 in the serum

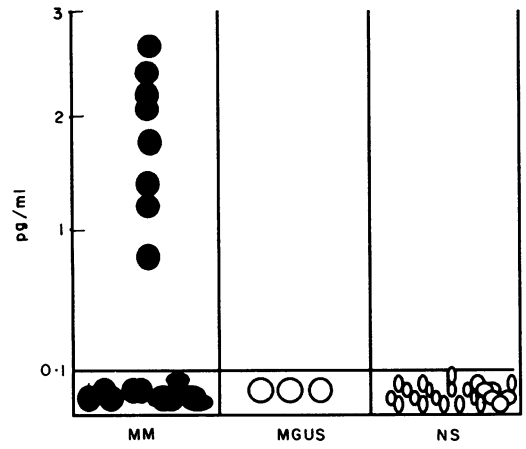

Fig. 3. IL-3 levels in serum samples. MM, Multiple myeloma; MGUS, monoclonal gammopathies of undetermined significance; NS, normal subjects.

(0·33 ng/ml). Very low levels were detected in 5/15 normal samples (0·22+0·05 ng/ml; range 0·17–0·27).

DISCUSSION

In vitro investigations, reviewed by Caligaris-Cappio *et al.* [24] suggest that the natural history of MM is influenced by several cytokines. In this study we have investigated which, among the cytokines active *in vitro*, are actually produced *in vivo* by MM patients and, even more specifically, which cytokines are produced by the stromal cells that form the BM microenvironment which hosts and favours the growth of malignant plasma cells.

The significance of our findings is three-fold. First, IL-6 and IL-8 are produced by MM BM stromal cells, while IL-1β, TNF-α, IL-4 and IL-7 are not. Due to the pivotal role played by IL-6 *in vitro* [1,8,10,12], recently confirmed *in vivo* by treating plasma cell leukaemia patients with anti-IL-6 MoAbs [25], it is not surprising that BM stromal cells produce high levels of IL-6. This observation lends credit to the studies that indicate a paracrine role for IL-6 [1]. In accordance with other series recently reported [26,27], we have also detected low levels of serum IL-6 in a minority of cases, usually in advanced stages of the disease. This finding would suggest that MM IL-6 is produced locally within the BM and occasionally may leak into the circulation.

IL-8 production by BM stromal cells indicates that even macrophages and fibroblasts from the BM (as from other sources [28]) are IL-8 producers. Interestingly, MM stromal cultures are definitely more active in producing IL-8 than stromal cultures from MGUS and normal donors. As IL-8 is chemotactic for different cell populations, including neutrophils [28] and T lymphocytes [29], we are now verifying the hypothesis that IL-8 may be able to attract circulating malignant plasma cell precursors into the IL-6-rich BM environment which is suitable for their proliferation and differentiation.

In agreement with recently published data [30], we have observed that in the supernatant of BM stromal cells there are no detectable levels of IL-7. This surprisingly negative finding may be due to one of the following possibilities: (i) stromal cells might produce IL-7 at levels too low to be revealed by our detection system; (ii) stromal cells might produce IL-7 only when they come into contact with B cell precursors and directly feed these cells, but do not excrete the molecule in the surroundings. To investigate further the relationship between BM stromal cells and IL-7 we are presently performing *in situ* hybridization experiments on stromal cells from MM and normal donors.

Next, we have observed that sera from a proportion of patients are able to induce the proliferation of M-07 cells and that this proliferation is drastically reduced by the addition of anti-IL-3 neutralizing antibody, thereby indicating the presence of IL-3. In these cases the proliferative activity of M-07 cells is usually not abrogated. As M-07 cell line may proliferate in presence of several cytokines, including GM-CSF and stem cell factor [31], it is highly likely that other cytokines may be identified in the sera of MM patients. High IL-3 levels may simply reflect the activation state of the T cell compartment in this malignancy [32]. Our small series of patients does not allow us to determine whether serum IL-3 measurement may become a parameter of prognostic significance. However, as IL-3 has been shown *in vitro* to have a proliferative activity on MM plasma cells frequently in synergy with IL-6 [10,11], it is not unreasonable to suggest that *in vivo* high IL-3 levels might be implicated in the progression of the disease.

Finally, MM BM stromal cells are active IL-6 and IL-8 producers, while both normal and MGUS BM stromal cells are low producers. This finding is consistent with the observation that normal BM stromal cells secrete IL-6 only after activation by inflammatory mediators [33] and leads to the concept that in the BM of MM a number of environmental cells, that normally would be quiescent, are instead activated. As MM BM stromal cells are exposed to the activity of the large array of cytokines produced by malignant plasma cells [24], it is tempting to consider the activated state of stromal cells in MM as a direct consequence of the host of accessory cell-activating cytokines produced by the expanding monoclonal B cell population. The low cytokine production by MGUS BM stromal cells as well as their inability to support B cell growth [24] are consistent with this conclusion. The implication is that, in MM, activated BM stromal cells play an active role in supporting the progressive expansion of the B cell clone.

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