Brief Definitive Report

ESTABLISHMENT OF TWO INTERLEUKIN 6 (B CELL STIMULATORY FACTOR 2/INTERFERON β_2)-DEPENDENT HUMAN BONE MARROW-DERIVED MYELOMA CELL LINES

By SHIRO SHIMIZU, RITSUKO YOSHIOKA, YUKO HIROSE, SUSUMU SUGAI, JUNKO TACHIBANA, and SUSUMU KONDA

From the Kanazawa Medical University, Division of Hematology and Immunology, Department of Internal Medicine, Ishikawa 920-02, Japan

It is extremely difficult to establish human multiple myeloma (MM) cell lines, especially from the bone marrow (1). Almost all myeloma cell lines reported have been derived from extramedullary invasions of MM (1–4). Macrophage (MΦ) feeder layers or MΦ-conditioned media were successfully used for the culture of murine plasmacytoma cells (5). Fibroblasts were also competent for long-term cultures of human myeloma cells (1, 4). These facts suggest that bone marrow microenvironments, especially MΦs or fibroblasts, may play an important role in the growth of myeloma cells through secreting growth factor(s).

It is now known that B cell stimulatory factor 2, originally described as a T cell-derived B cell differentiation factor, is identical to hybridoma plasmacytoma growth factor, IFN-β_2, 26-kd protein, and hepatocyte stimulating factor (6–10), and is now called IL-6. IL-6 is produced not only by T cells but also by MΦs (10–13) or fibroblasts (7–9). MΦs produce IL-6 in the absence of an apparent stimulus, although T cells require antigen or mitogen stimulation for IL-6 production (12, 13).

These facts suggest that IL-6 produced by MΦs or fibroblasts may support the growth of human myeloma cells. In this paper, we report that MΦ-derived IL-6 augments the growth of myeloma cell lines, and both cell lines, ILKM2 and ILKM3, can be maintained in vitro in RPMI 1640 with 8% FCS and rIL-6.

Materials and Methods

Human Myeloma Cell Lines (ILKM2 and ILKM3). The cultures were initiated on July 8, 1986 for ILKM2 and October 7, 1986 for ILKM3 from the bone marrow aspirates of patients with MM, IgG-K type. They are now being maintained in RPMI 1640 with 8% FCS, 100 μg/ml piperacillin, and 2 ng/ml rIL-6 with incomplete medium change every 4 d.

Reagents. The sources of rIL-1α, rIL-1β, rIL-2, rIL-3, rIL-4, rIFN-α, native IFN-β (nIFN-β), rIFN-γ, rTNF-α, recombinant granulocyte/macrophage CSF (rGM-CSF), rG-CSF, recombinant epidermal growth factor (rEGF), and mAbs are described elsewhere (13, 14). Polyclonal anti-IL-6 (α-IL-6) antibody and rIL-6 were generously provided by Drs. T. Hirano and T. Kishimoto of Osaka University (6, 15). The sp act of the rIL-6 is 5×10^6 U/mg of protein.

Production of MΦ-derived Factors (MDF). The procedures to obtain MΦs and MDF are described elsewhere (14). MDF used in this experiment has IL-6 and IL-1 activity but no IL-2

Address correspondence to Shiro Shimizu, Division of Hematology and Immunology, Department of Internal Medicine, Kanazawa Medical University, Daigaku 1-1, Uchinada-cho, Kahoku-gun, Ishikawa 920-02, Japan.

or IFN-γ (13, 14). Partially purified MDF (pMDF) was prepared by using Sephadex G-100 chromatography. Active fractions on myeloma cell growth, whose apparent mol wt was 26,000, were used as pMDF.

Proliferative Assays. Myeloma cells were washed and resuspended in RPMI 1640 with 8% FCS and 100 μg/ml piperacillin. A quantity of 10^4 cells was plated in 96-well flat-bottomed microplates and stimulated with MDF, various cytokines, or MΦs. For the inhibition assay using α-IL-6 antibody, MDF or recombinant cytokines were preincubated with α-IL-6 antibody (10 μg/ml) for 1 h at 37°C and then myeloma cells were added to the culture. The culture was incubated for 96 h and pulsed for a final 24 h with 0.5 μCi [^{3}H]thymidine. [^{3}H]Thymidine incorporation was determined by scintillation counting.

Characterization of Myeloma Cell Lines. The methods for morphological analysis and cell surface marker analysis are described elsewhere (14).

Results

Establishment and Characterization of Cell Lines. For the first 3 mo of the long-term cultures, myeloma cells proliferated very slowly on bone marrow-derived MΦs and/or fibroblasts. 3 mo later, these cells could be transferred to the wells that were feeder layered with allogeneic MΦs. They could also be maintained in the medium containing MDF and then in the medium containing 2 ng/ml rIL-6. Both cell lines, named ILKM2 and ILKM3, had the typical morphological features of myeloma cells as shown in Fig. 1. The surface phenotype of both cells was PCA-1$^+$, OKT10$^+$, CD10$^-$, CD19$^-$, CD20$^-$, CD21$^-$, and OKIa-1$^-$, showing a typical myeloma cell phenotype. Cytoplasmic Ig staining showed that IgG-K was positive in ILKM2 and only K chain was positive in ILKM3. EBV nuclear antigen (EBNA) was negative in both cell lines. The population doubling time was 120 h for ILKM2 and 96 h for ILKM3, when they were cultured in the medium containing 2 ng/ml rIL-6.

Proliferation of ILKM2 or ILKM3. ILKM2 and ILKM3 did not proliferate spontaneously. They proliferated vigorously in the presence of allogeneic MΦs (Fig. 2, *A* and *D*). MDF could substitute for the MΦ functions and induced the growth of myeloma cell lines in a dose-dependent manner (Fig. 2, *B* and *E*). Among the cytokines examined, IL-6 had the strongest activity on the DNA synthesis in both cell lines (Fig. 3). rIL-6, even at the dose of 0.05 ng/ml, had significant growth-enhancing

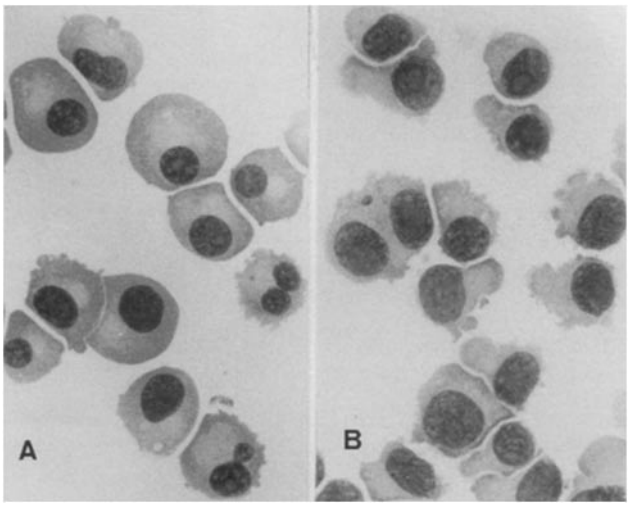

FIGURE 1. The morphology of two established cell lines. May-Giemsa staining shows the typical plasmacytoid features of both cell lines, ILKM2 (*A*) and ILKM3 (*B*).

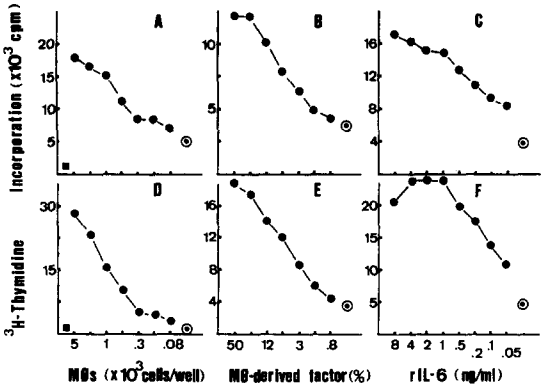

FIGURE 2. Proliferative responses of ILKM2 (*A, B,* and *C*) and ILKM3 (*D, E,* and *F*) to added macrophages (*A* and *D*), MDF (*B* and *E*), or rIL-6 (*C* and *F*). A quantity of 10^4 myeloma cells was cultured with various concentrations of macrophages, MDF, or rIL-6, and the DNA synthesis was measured as described in Materials and Methods. Medium control experiments (⊙); DNA synthesis in macrophages alone (■).

activity (Fig. 2, *C* and *F*). IL-1α, IL-1β, IFN-α and, to a lesser extent, TNF-α also had growth-enhancing activity on ILKM2, however, these active factors were not competent for the maintenance of ILKM2. ILKM3 proliferated only in response to IL-6 or MDF (Fig. 3). These results suggested that the myeloma cell growth activity in MDF was derived from IL-6 for the most part.

Inhibition of Myeloma Cell Growth by α-IL-6 Antibody. α-IL-6 antibody completely inhibited rIL-6-dependent growth of ILKM2 and ILKM3 (Table I). It also completely inhibited MDF-dependent growth of ILKM2. Anti-IL-6 antibody only partially inhibited MDF-induced proliferation of ILKM3, but almost completely inhibited

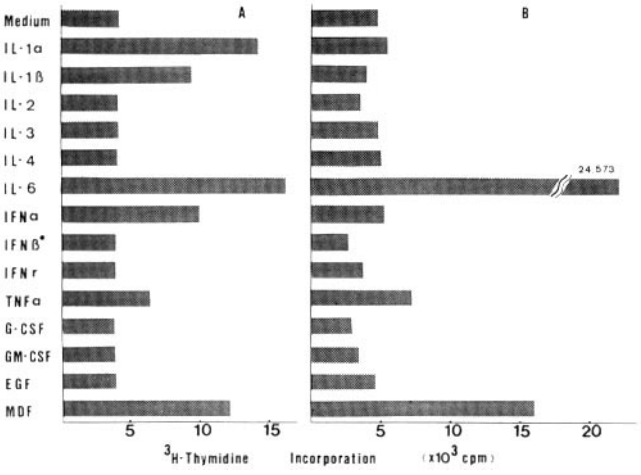

FIGURE 3. Proliferative responses of ILKM2 (*A*) and ILKM3 (*B*) to various recombinant cytokines. A quantity of 10^4 myeloma cells was cultured with various cytokines and the DNA synthesis was measured as described in Materials and Methods. The concentrations of cytokines used in these experiments were as follows: IL-1α, 2–20 ng/ml (20); IL-1β, 2–20 ng/ml (20); IL-2, 0.25–2.5 ng/ml (2.5); IL-3, 1–10 U/ml (10); IL-4, 2–20 U/ml (20); IL-6, 0.4–4 ng/ml (4); IFN-α, 100–1,000 U/ml (100); IFN-β, 100–1,000 U/ml (100); IFN-γ, 100–1,000 U/ml (100); G-CSF, 0.25–2.5 ng/ml (2.5); GM-CSF, 0.25–2.5 ng/ml (2.5); EGF, 10–100 ng/ml (100); and MDF, 5–50% (50). [³H]Thymidine uptake shows the maximum response in those dose ranges. The dose of each cytokine described in parentheses was used in the experiments shown in Fig. 3. (*) Native, fibroblast-derived IFN-β was used in this experiment.

TABLE I

Effect of Anti-IL-6 Antibody on DNA Synthesis in Myeloma Cell Lines Induced by rIL-6 MDF, or pMDF

Reagent added	Antibody added	[^{3}H]Thymidine uptake		
		ILKM2	ILKM3 (Exp. 1)	ILKM3 (Exp. 2)
		mean cpm ± SD		
Medium	-	4,941 ± 423	805 ± 203	5,270 ± 321
Medium	Anti-IL-6	4,160 ± 367	943 ± 142	5,826 ± 348
Medium	Control IgG	4,826 ± 235	1,005 ± 132	ND
IL-6 (0.4 ng/ml)	-	16,355 ± 451	8,317 ± 276	19,866 ± 820
IL-6 (0.4 ng/ml)	Anti-IL-6	4,320 ± 102	1,475 ± 121	3,363 ± 424
IL-6 (0.4 ng/ml)	Control IgG	15,788 ± 899	8,482 ± 761	ND
IL-6 (0.2 ng/ml)	-	9,575 ± 88	5,482 ± 467	8,160 ± 377
IL-6 (0.2 ng/ml)	Anti-IL-6	4,175 ± 367	1,246 ± 260	3,976 ± 274
IL-6 (0.2 ng/ml)	Control IgG	10,073 ± 434	7,135 ± 652	ND
MDF (25%)	-	13,043 ± 387	6,055 ± 421	17,088 ± 97
MDF (25%)	Anti-IL-6	4,659 ± 367	4,751 ± 214	13,749 ± 75
MDF (25%)	Control IgG	14,092 ± 583	6,984 ± 466	ND
pMDF (25%)	-	ND	10,373 ± 234	ND
pMDF (25%)	Anti-IL-6	ND	3,765 ± 223	ND
pMDF (25%)	Control IgG	ND	12,975 ± 1,687	ND

Anti-IL-6 antibody (10 μg/ml) or normal rabbit IgG (control IgG, 10 μg/ml) was preincubated with MDF, pMDF, or rIL-6 at 37°C for 1 h and the DNA synthesis in ILKM2 or ILKM3 was measured as described in Materials and Methods. MDF, macrophage culture supernatant; pMDF, partially purified MDF (active fractions on myeloma cell growth, 26 kD), separated by Sephadex G-100 chromatography.

pMDF-induced proliferation of ILKM3. These results indicated that IL-6 is the primary myeloma cell growth factor included in the MDF.

Discussion

In this report, we demonstrated that two bone marrow-derived myeloma cell lines proliferate dependently on MΦ feeder layers, MDF or rIL-6, and that both cell lines have been maintained in vitro in the medium containing rIL-6 for >1 yr.

Although autocrine mechanisms of myeloma cell growth have been documented by several authors (16, 17), the establishment of spontaneously proliferating myeloma cell lines in vitro is extremely difficult (1). In addition to autocrine mechanisms, paracrine mechanisms have also been suggested to play an important role in myeloma cell growth (1, 4, 18, 19). Our results agree with the paracrine mechanisms of myeloma cell growth; bone marrow-derived myeloma cell lines proliferate in the presence of MΦs, MDF is able to substitute for the MΦ functions, MΦs produce IL-6, myeloma cell lines most strongly proliferate in response to rIL-6, α-IL-6 antibody inhibits the rIL-6- or MDF-induced proliferation of myeloma cell lines, and myeloma cell lines have been maintained for >1 yr in the medium containing rIL-6. Furthermore, two other bone marrow-derived myeloma cell lines have now been maintained for >6 mo by using MΦ feeder layers (unpublished observation). This indicates that our method is reproducibly applicable for establishing myeloma cell lines. All this evidence, as well as the fact that MΦ is a constituent of bone marrow stromal cells, suggests that MΦs, through secreting IL-6, play a key role in the growth of myeloma cells in vivo. In accordance with our results, Nordan and Potter (19) demonstrated that murine plasmacytoma had developed exclusively from the mineral

oil-induced granulomatous tissue composed of MΦs and neutrophils and the MΦ-derived factor, which is now known to be identical to IL-6, was able to maintain mouse plasmacytoma cells in vitro. Since it was reported that IL-6 is an autocrine growth factor for human muliple myeloma (16), both autocrine and paracrine mechanims, mediated through IL-6, may be involved in the in vivo growth of myeloma cells.

The proliferative response of ILKM2 to IL-1α, IL-1β, and IFN-α is also interesting. Another myeloma cell line (KM1) and several fresh myeloma cells also proliferate in response to IL-1 (unpublished observation). Furthermore, in addition to IL-6, ILKM3 appears to proliferate in response to a monokine or monokines that are different from the known monokines examined (Fig. 3 and Table I). IL-1 and TNF-α are known to induce IL-6 secretion from fibroblasts (7–9). Although we could not detect IL-6 activity in the culture supernatant of both cell lines when they were cultured with various mitogens or cytokines, a possibility that IL-6 produced by myeloma cells binds to their own IL-6-Rs immediately after secretion is not excluded. Whether IL-1, IFN-α, or other unknown factors induce IL-6 secretion from myeloma cells, and whether another myeloma cell growth factor exists or not, remains to be elucidated in the future.

Our data imply that MΦs or IL-6 can be uesd to establish bone marrow-derived myeloma cell lines more efficiently and that myeloma cells established could be used for the analysis of myeloma cell growth regulation by cytokines or drugs. These studies may open up a new approach to the treatment of MM.

Summary

Two IL-6-dependent human multiple myeloma cell lines, ILKM2 and ILKM3, were established from the bone marrow of patients with IgG-K multiple myeloma. Both cell lines had the typical morphology and immunocytochemical features of myeloma cells. The surface phenotype of both cell lines was PCA-1$^+$, OKT10$^+$, CD10(J-5)$^-$, CD19(B4)$^-$, CD20(B1)$^-$, CD21(B2)$^-$, and OKIa-1$^-$. A monoclonal cytoplasmic Ig, IgG-K or K L chain, was positive in ILKM2 or ILKM3, respectively. EBV nuclear antigen was negative in both cell lines. They proliferated in the presence of macrophages or macrophage-derived factors (MDF). Among the recombinant cytokines examined, IL-6 most strongly augmented the growth of both cell lines. The anti-IL-6 antibody completely inhibited the IL-6-dependent growth and almost completely inhibited the MDF- or purified MDF-dependent growth of both cell lines. ILKM2 and ILKM3 are now being maintained in the culture medium containing 2 ng/ml rIL-6.

These results suggest that IL-6 produced by macrophages may play an important role in the growth of myeloma cells in vivo and that macrophages or IL-6 can be used for establishing human myeloma cell lines.

Received for publication 5 July 1988 and in revised form 23 September 1988.

References

1. Jernberg, H., K. Nilsson, L. Zech, D. Lutz, H. Nowotny, and W. Scheirer. 1987. Establishment and phenotypic characterization of three new human myeloma cell lines. *Blood.* 69:1605.
2. Gazdar, A. F., H. K. Oie, I. R. Kirsch, and G. F. Hollis. 1986. Establishment and char-

acterization of a human plasma cell myeloma culture having a rearranged cellular myc proto-oncogene. *Blood.* 67:1542.

3. Matsuoka, Y., G. E. Moore, Y. Yagi, and D. Pressman. 1967. Production of free light chains of immunoglobulin by a hematopoietic cell line derived from a patient with multiple myeloma. *Proc. Soc. Exp. Biol. Med.* 125:1246.
4. Jobin, M. E., J. L. Fahey, and Z. Price. 1974. Long-term establishment of a human plasmacyte cell line derived from a patient with IgD multiple myeloma. *J. Exp. Med.* 140:494.
5. Namba, Y., and M. Hanaoka. 1972. Immunocytology of cultured IgM-forming cells of mouse. I. Requirement of phagocytic cell factor for the growth of IgM-forming tumor cells in tissue culture. *J. Immunol.* 109:1193.
6. Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S. Kashiwahara, K. Nakajima, K. Koyama, A. Iwamatsu, S. Tsunasawa, F. Sakiyama, H. Matsui, Y. Takahara, T. Taniguchi, and T. Kishimoto. 1986. Complementary DNA for a novel human interleukin (BSF2) that induces B lymphocytes to produce immunoglobulin. *Nature (Lond.).* 324:73.
7. Zilberstein, A., R. Ruggieri, J. H. Korn, and M. Revel. 1986. Structure and expression of cDNA and genes for human interferon β_2, a distinct species inducible by growth stimulatory cytokines. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2529.
8. Kohase, M., L. T. May, I. Tamm, J. Vilcek, and P. B. Sehgal. 1987. A cytokine network in human diploid fibroblasts: interactions of β-interferons, tumor necrosis factor, platelet-derived growth factor, and interleukin 1. *Mol. Cell. Biol.* 7:273.
9. Van Damme, J., G. Opdenakker, R. J. Simpson, M. R. Rubira, S. Cayphas, A. Vink, A. Billiau, and J. Van Snick. 1987. Identification of the human 26-kD protein, interferon β_2, (IFN-β_2) as a B cell hybridoma/plasmacytoma growth factor induced by interleukin 1 and tumor necrosis factor. *J. Exp. Med.* 165:914.
10. Gauldie, J., C. Richard, D. Harnish, P. Lansdorp, and H. Baumann. 1987. Interferon β_2/BSF2 shares identity with monocyte derived hepatocyte stimulating factor and regulates the major response in liver cells. *Proc. Natl. Acad. Sci. USA.* 84:7251.
11. Tosato, G., K. B. Seamon, N. D. Goldman, P. B. Sehgal, L. T. May, G. C. Washington, K. D. Jones, and S. E. Pike. 1988. Monocyte-derived human B-cell growth factor identified as interferon β_2 (BSF2, IL-6). *Science (Wash. DC).* 239:502.
12. Horii, Y., A. Muraguchi, S. Suematsu, T. Matsuda, K. Yoshizaki, T. Hirano, and T. Kishimoto. 1988. Regulation of BSF-2/IL-6 production by human mononuclear cells. *J. Immunol.* 141:1529.
13. Shimizu, S., T. Hirano, R. Yoshioka, S. Sugai, T. Matsuda, T. Taga, T. Kishimoto, and S. Konda. 1988. Interleukin 6-dependent growth of a Lennert's lymphoma-derived T cell line (KT-3). *Blood.* 72:1826.
14. Shimizu, S., T. Takiguchi, S. Sugai, M. Matsuoka, and S. Konda. 1988. An established $CD4^+$ T lymphoma cell line derived from a patient with so called Lennert's lymphoma - possible roles of cytokines in histopathogenesis. *Blood.* 71:196.
15. Hirano, T., T. Matsuda, K. Hosoi, A. Okano, H. Matsui, and T. Kishimoto. 1988. Absence of anti-viral activity in recombinant B cell stimulatory factor 2. *Immunol. Lett.* 17:41.
16. Kawano, M., T. Hirano, T. Matsuda, T. Taga, Y. Horii, K. Iwato, H. Asauku, B. Tang, O. Tanabe, H. Tanaka, A. Kuramoto, and T. Kishimoto. 1988. Autocrine generation and requirement of BSF2/IL-6 for human multiple myelomas. *Nature (Lond.).* 332:83.
17. Klein, B., M. Jourdan, A. Vazquez, B. Dugas, and R. Bataille. 1987. Production of growth factors by human myeloma cells. *Cancer Res.* 47:4856.
18. Cancro, M., and M. Potter. 1976. The requirement of an adherent cell substratum for the growth of developing plasmacytoma cells in vivo. *J. Exp. Med.* 144:1554.
19. Nordan, R. P., and M. Potter. 1986. A macrophage-derived factor required by plasmacytomas for survival and proliferation in vitro. *Science (Wash. DC).* 233:566.