Myeloma Cell Growth Arrest, Apoptosis, and Interleukin-6 Receptor Modulation Induced by EB1089, a Vitamin D_3 Derivative, Alone or in Association With Dexamethasone

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We have previously shown that malignant plasma cells expressed the specific receptor for 1,25-dihydroxyvitamin D_3 and that this derivative could significantly inhibit the proliferation of such malignant cells. More recently, new vitamin D_3 derivatives have been generated with extraordinarily potent inhibitory effects on leukemic cell growth in vitro. These new data prompted us to (re)investigate the capacity of such new vitamin D_3 derivatives to inhibit myeloma cell growth in comparison with that of dexamethasone, a potent antitumoral agent in multiple myeloma. In the current study, we show that EB1089, a new vitamin D_3 derivative, (1) induces G1 growth arrest of human myeloma cells, which is only partially reversed by interleukin-6 (IL-6); (2) induces apoptosis in synergy with dexamethasone, IL-6, leukemia-inhibitory factor, and Oncostatin M, with an agonistic anti-gp130 monoclonal antibody being unable to prevent this apoptosis; (3) downregulates both the gp80 (ie, the α chain of the IL-6 receptor [IL-6Rα]) expression on malignant plasma cells and the production of soluble IL-6Rα, and finally (4) inhibits the deleterious upregulation of gp80 expression induced by dexamethasone while limiting the dexamethasone-induced upregulation of gp130 expression. Considering that these in vitro effects of EB1089 have been observed at doses obtainable in vivo (without hypercalcemic effects), our present data strongly suggest that EB1089 could have a true interest in the treatment of multiple myeloma, especially in association with dexamethasone.

MULTIPLE MYELOMA (MM) is a B-cell malignancy characterized by the slow proliferation of malignant plasma cells within the bone marrow (BM). We and others have shown that interleukin-6 (IL-6) was an essential survival and growth factor for malignant plasma cells through the expression of a specific IL-6 receptor (IL-6R), the α chain gp80, and the transducer element, the β chain gp130.[1-4] IL-6 is overproduced in patients with MM and stimulates the proliferation of myeloma cells in a paracrine[2] rather than autocrine[1] fashion. Myeloma cell proliferation is the result of an activation signal by the IL-6/IL-6Rα complex through the transducer chain gp130.[3] It is of major interest that the soluble form of the IL-6Rα is an agonist of IL-6 and is able to amplify the response of myeloma cells to IL-6.[3,4] In vivo neutralizing murine anti–IL-6 monoclonal antibodies (MoAbs) can limit disease progression in patients with advanced MM.[5,6] Thus, antagonizing IL-6 and/or the IL-6R complex represents a very attractive and new therapeutic approach in MM.[7]

Several years ago, we showed for the first time that malignant plasma cells expressed the specific receptor for 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and that this derivative could inhibit the proliferation of the human myeloma cell line (HMCL) RPMI 8226.[8] More recently, new vitamin D_3 derivatives have been generated with extraordinarily potent inhibitory effects on leukemic cell growth in vitro.[9] These new data prompted us to (re)investigate the capacity of vitamin D_3 derivatives to inhibit myeloma cell growth. Dexamethasone (Dex) is a potent antitumoral agent in the treatment of MM, in vitro[10] and in vivo.[11] It represents the steroid of reference in the treatment of MM. For this reason, we have compared its effects on myeloma cells with those of vitamin D_3. Finally, we have tested the effects of both drugs on MM. In the current study, we show that EB1089, a new vitamin D_3 derivative, has potent cytostatic effects by itself and induces apoptosis on human myeloma cells in synergy with Dex. Furthermore, we show that EB1089 downregulates both gp80 expression and production of its soluble form and counteracts the deleterious effects of Dex on myeloma cells, ie, upregulation of both gp80 and gp130 expression. Its association with Dex could be very helpful in the treatment of MM.

MATERIALS AND METHODS

Reagents. Dex was obtained from Merck Sharp USA (Paris, France). 1,25$(OH)_2D_3$ and the derivative EB1089 were obtained from Leo Pharmaceuticals (Ballerup, Denmark). Anti-gp80 MoAb (MT18) and anti-gp130 MoAb (AM64) were provided by Dr Taga (Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan). The agonistic anti-gp130 MoAb (BS12) was kindly provided by Dr J. Wijdenes (Diaclone Research, Besançon, France). Human recombinant IL-6 and Oncostatin M (OSM) were obtained from Sandoz (East Hanover, NJ) and Dako (Glostrup, Denmark), respectively. Leukemia-inhibitory factor (LIF) was kindly provided by Dr A. Godard (U211, Nantes, France).

HMCL and culture conditions. The HMCL LP1 and NCI H929 were purchased from DSM (Braunschweig, Germany), and RPMI 8226 was from American Type Culture Collection (Rockville, MD). They were maintained in RPMI-1640 supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 100 mg/mL streptomycin, 100 U/mL penicillin, 5 $\times 10^{-5}$ mol/L 2-β mercaptoethanol, as previously described.[12] Freshly-expanded, pure and slowly growing, 6-month-old myeloma cells from a malignant pleural effusion (SBN1) were maintained in similar conditions of culture. SBN1 cell growth was totally dependent on conditioned medium (ie, pleural effusion) or recombinant IL-6 (rIL-6). Freshly explanted myeloma cells (purity, >80%) were obtained

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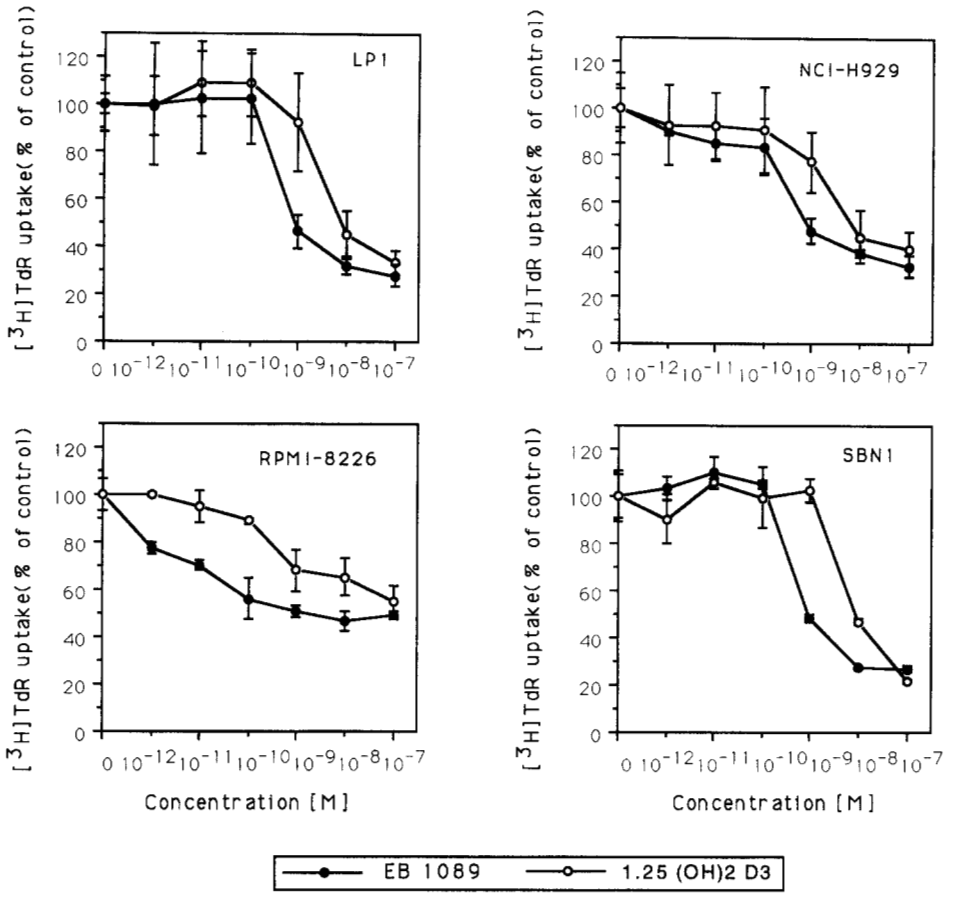

Fig 1. Comparative dose-dependent effect of $1,25(OH)_2D_3$ and EB1089 on the growth of LP1, NCI-H929, RPMI 8226, and SBN1. Cells (10^4/well) were cultured with varying concentrations of $1,25(OH)_2D_3$ or EB1089 (10^{-7} to 10^{-12} mol/L). DNA synthesis was measured by [^{3}H]-TdR incorporation after a 3-day culture.

after Ficoll-Hypaque separation from the peripheral blood of a patient with MM during a terminal leukemic phase.

Proliferation assays. Proliferation assays were performed in 96-well round-bottom microtiter plates at a cell density of 10^5 cells/mL. As previously described, 12 cell cultures were incubated for 3 days at 37°C in a 5% CO_2 humidified atmosphere. Then, 1 mCi of [^{3}H]-thymidine was added during the last 18 hours. [^{3}H]-thymidine incorporation was quantified by liquid scintillation spectroscopy.

BM stromal cell culture. BM mononuclear cells were isolated by Ficoll-Hypaque density sedimentation. The cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum. After 2 or 3 weeks, a confluent adherent cell monolayer was obtained. Then, after 2 passages using trypsin/EDTA solution, they were used for cytokine production, with 15×10^3 cells being seeded in 96-well plates and incubated for 48 hours with different reagents. Finally, the supernatants were collected for IL-6 determination.

IL-6 and soluble IL-6Rα determinations. Supernatants were tested by enzyme-linked immunoadsorbent assay (ELISA) using a commercially available kit for IL-6 and soluble IL-6Rα (Diaclone Research). These assays measured IL-6 and soluble IL-6Rα concentrations with a sensitivity of 1 and 5 pg/mL, respectively. For IL-6Rα determination, cells were seeded at 1×10^6 cells/mL and incubated with the different drugs for 48 hours, and then the supernatants were collected.

Flow cytometry analysis. For immunofluorescence staining of HMCL,[13] 5×10^5 cells were incubated with different MoAbs for 30 minutes at 4°C, then incubated with a goat antimouse fluorescein isothiocyanate conjugate (Immunotech, Marseilles, France). Cells were fixed in 4% formaldehyde and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). To determine the level of positivity for each marker, we divided the mean fluorescence intensity of each marker by that of the control staining, thus, defining the intensity fluorescence ratio.

Northern blotting. Total RNA was extracted from myeloma cells using the isothiocyanate phenol method. Total RNA (25 μg) was separated on a 1% agarose gel after denaturation with glycoxal and dimethyl sulphoxide. Blotting onto nylon membranes, hybridization, washing, and developing were performed using standard methods. Membranes were hybridized with the gp80 and gp130 cDNA probes. After developing, the membranes were stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

Cell cycle distribution. For cell cycle analysis LP1 cells (1×10^6) were fixed in 70% cold ethanol for 30 minutes at 4°C, washed twice in phosphate-buffered saline, and stained with 40 μg/mL propidium iodide for 10 minutes at room temperature. Flow cytometry analysis was performed on a FACSCalibur using CELLQuest program (Becton Dickinson). Data were gated on the Fl2-Area versus Fl2-Width cytogram to exclude doublets and aggregates, and a minimum of 1.5×10^4 gated cells were collected per sample. Analysis of the cell cycle was performed using the Modfit LT for Mac V1.01 program (Verity Software House, Inc). Apoptotic cells were detected as a subdiploid peak as described.[14]

Statistical methods. For statistical analyses, we used the Wilcoxon rank sum test.

RESULTS

Inhibition of myeloma cell growth by $1,25(OH)_2D_3$: Comparison with the new vitamin D_3 derivative EB1089.

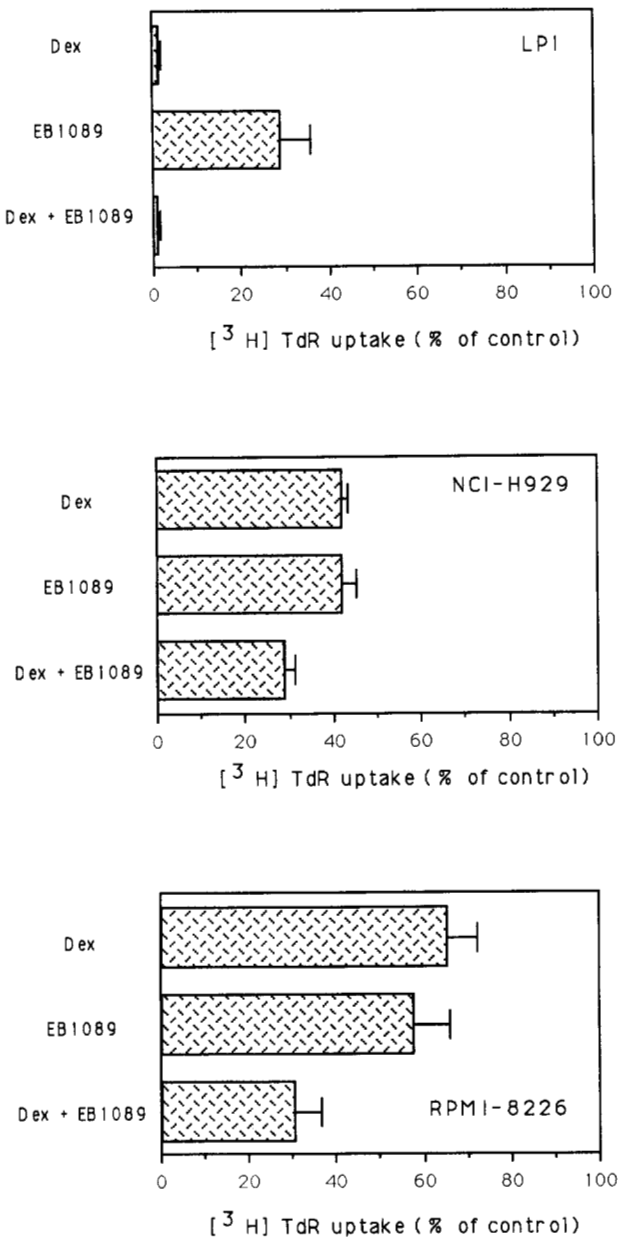

Fig 2. Comparative effect of Dex and EB1089 on growth inhibition of the myeloma cell lines. Dex was used at a concentration of 10^{-7} mol/L, and EB1089 was used at a concentration of 10^{-8} mol/L. DNA synthesis was measured by [^{3}H]-TdR incorporation after a 3-day culture.

EB1089 is a new vitamin D_3 derivative with potent inhibitory effects on leukemic cell growth in vitro.[9] Thus, it was tested on the three HMCLs and the fresh IL-6–dependent myeloma cells SBN1 and compared with 1,25-$(OH)_2D_3$. As previously emphasized by ourselves on RPMI 8226[8] and as outlined in Fig 1, 1,25-$(OH)_2D_3$ inhibits not only the growth of the three HMCLs but also that of the IL-6–dependent SBN1 cells in a dose-dependent way. However, whereas a progressive inhibition is observed with RPMI 8226 with significant effects at doses as low as 10^{-12} mol/L, no effect is observed with LP1, NCI H929, and SBN1 cells until a dose of 10^{-10} mol/L is used, with significant effects beginning at 10^{-9} mol/L. Of note, EB1089 gives similar response curves but turns out to be more efficient than 1,25-$(OH)_2D_3$ on the three HMCLs and SBN1 cells at least at 10^{-8} and 10^{-9} mol/L (Fig 1). This is especially true for LP1 and SBN1 cells (54% and 51% inhibition, respectively, with EB1089 *v* 7% and 3%, respectively, with vitamin D_3 at 10^{-9} mol/L). Of major interest and as previously emphasized, the inhibition of SBN1 is observed in the presence of rIL-6 because the growth of these cells (as opposed to that of the other HMCLs) is totally dependent on IL-6. The concentration of 10^{-8} mol/L was chosen for the next experiments performed with EB1089 because a maximum of inhibitory effects is observed at this concentration (71% of inhibition for LP1, 58% for NCI H929, 42% for RPMI 8226, and 73% for SBN1 cells).

Inhibition of myeloma cell growth by EB1089: Comparison with Dex. As previously emphasized[10] and as recently reassessed by ourselves,[12] Dex is a potent inhibitor of myeloma cell growth in vitro. In our experience, a ≥20% inhibition of growth is observed in six of six HMCLs independent of exogenous IL-6 to grow (the current study and Juge-Morineau et al[12]). For treatment in vivo, Dex is used at a concentration of 10^{-7} mol/L[15]; this concentration also has been chosen for the following experiments. As shown in Fig 2, Dex significantly inhibits the growth of LP1 and NCI H929 (99% and 58% inhibition, respectively) but has a less significant effect on RPMI 8226 (35% inhibition). Again, EB1089 at 10^{-8} mol/L has inhibitory effects on the three cell lines. Clearly, EB1089 alone is less inhibitory than Dex on LP1 (71% *v* 99%). However, when compared with Dex, it is as efficient on NCI H929 and more efficient on RPMI 8226. Of note, the effect of the combination of EB1089 and Dex is always superior to the effect of each one alone, and, only on RPMI 8226, does EB1089 have additive effects to those of Dex. With respect to the SBN1 cells, which are totally dependent on IL-6 to grow, it is impossible to inhibit their growth with Dex in the presence of IL-6. On the other hand, and as indicated in the previous section (Fig 1), a significant inhibition of these cells is observed with EB1089, despite the presence of IL-6. Finally, the combination of EB1089 and Dex has no greater effect than that of EB1089 alone (data not shown).

Table 1. Effects of Dex and EB1089 on the Cell Cycle Distribution of LP1 Cells

	Cell Cycle Analysis (% cells in)†		
Drug Exposure*	G1	S	G2
Control	43.3 ± 2.2	40.6 ± 3.5	15.3 ± 2.3
Dex	90.2 ± 1.9	6.0 ± 1.3	3.7 ± 0.8
EB1089	73.91 ± 5.7	15.3 ± 4.2	11.47 ± 2.0
Dex + EB1089	94.0 ± 2.1	3.5 ± 1.3	2.7 ± 0.7

Data are expressed as the mean ± SD of five experiments.

* Two-day drug exposure. Dex and EB1089 were used at 10^{-7} mol/L and 10^{-8} mol/L, respectively.

† The percentage of cells in different phases of the cycle (among nonapoptotic cell population) after treatment with the different drugs is presented.

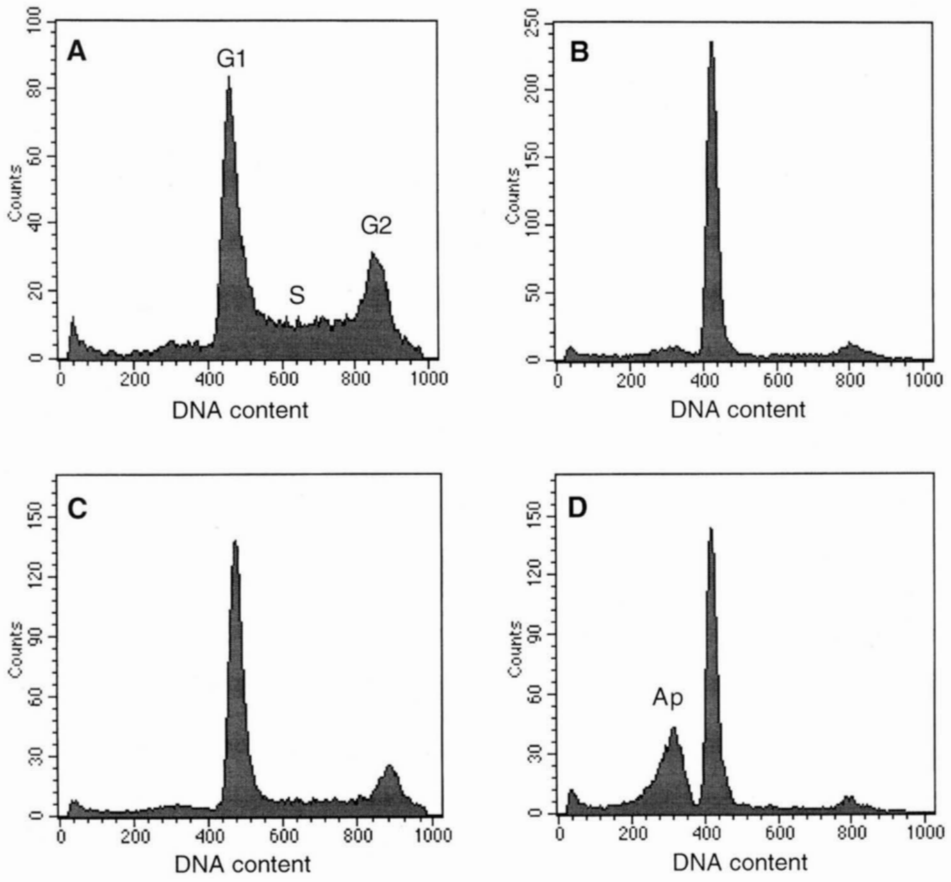

Fig 3. DNA fluorescence histograms of propidium iodide-stained LP1 cells after 48 hours of incubation with medium alone (A), Dex (10^{-7} mol/L; B), EB1089 (10^{-8} mol/L; C), and EB1089 (10^{-8} mol/L) plus Dex (10^{-7} mol/L; D). Drug-treated cells show a reduced proportion of S and G2 phase (B,C, and D). In (D), a subdiploid peak that identified apoptotic cells is indicated as Ap.

EB1089 induces G1 growth arrest by itself but induces apoptosis in synergy with Dex. When the cell cycle was analyzed, EB1089 turned out to have potent cytostatic effects without inducing apoptosis in our experimental conditions at 3 days. Indeed, in five experiments, a significant reduction of the percentages of myeloma cells in S phase was observed (40.6% ± 3.5% *v* 15.3% ± 4.2%; $P = .01$), with a significant increase of the percentages of myeloma cells in the G1 phase (43.3% ± 2.2% *v* 73.9% ± 5.7%; $P = .01$; see Table 1). Of note, these effects are similar to although less marked than those of Dex as shown in the Table 1.

Flow cytometry analysis of the DNA content indicates that EB1089 or Dex used alone cannot trigger apoptosis of myeloma cells in our current experimental conditions within 3 days. On the contrary, treatment of LP1 cells with a combination of the two drugs leads to a strong induction of apoptosis that is shown after 2 days by the appearance of a hypodiploid DNA peak that identified the apoptotic cell population (29%), as shown in the typical experiment represented in Fig 3.

IL-6, LIF, OSM, and an agonistic anti-gp130 MoAb cannot prevent the apoptotic effects of EB1089 with Dex. We[12] and others[15] have recently shown that IL-6, even at low doses (10 pg/mL), and some other cytokines belonging to the gp130 family such as LIF and OSM could be the major factors responsible for the resistance of myeloma cells to Dex. Because EB1089 has cytotastic effects on myeloma cells too, we have examined the capacity of IL-6, LIF, and OSM to reverse these effects in comparison with those of Dex. As shown in Fig 4A, on LP1 cells, IL-6 only partially reverses the inhibitory effects of EB1089, whereas it reverses totally those of Dex. On the other hand, LIF and OSM totally reverse the inhibitory effects of both EB1089 and Dex. However, because EB1089 and Dex have potent synergistic effects to induce apoptosis of myeloma cells, we have examined the capacity of IL-6, LIF, and OSM to prevent these effects. As shown in Fig 4A, IL-6, LIF, and OSM have only minimal effects to prevent those of EB1089 in combination with Dex. Of note, IL-6 at doses as high as 20 ng/mL (which are generally encountered in the myeloma intermediate milieu) are unable to prevent the effects of both EB1089 and Dex (data not shown). BS12 has been described as an agonistic anti-gp130 MoAb with IL-6–like effects on HMCLs.[16] In the current study, such agonistic effects have been reproduced on LP1 (Fig 4B), with the BS12 MoAb being able to reverse the inhibitory effect of Dex better than IL-6. Of major interest, BS12 is unable to prevent the effects induced by the combination of EB1089 and Dex, although it partially reverses those of EB1089 alone (Fig 4B).

EB1089 downregulates the expression of the gp80 on myeloma cells and reduces the production of soluble IL-6Rα. Considering that IL-6 and BS12 only partially reverse the inhibitory effects of EB1089, we have investigated the effect of EB1089 on the expression of both gp80 and gp130 in

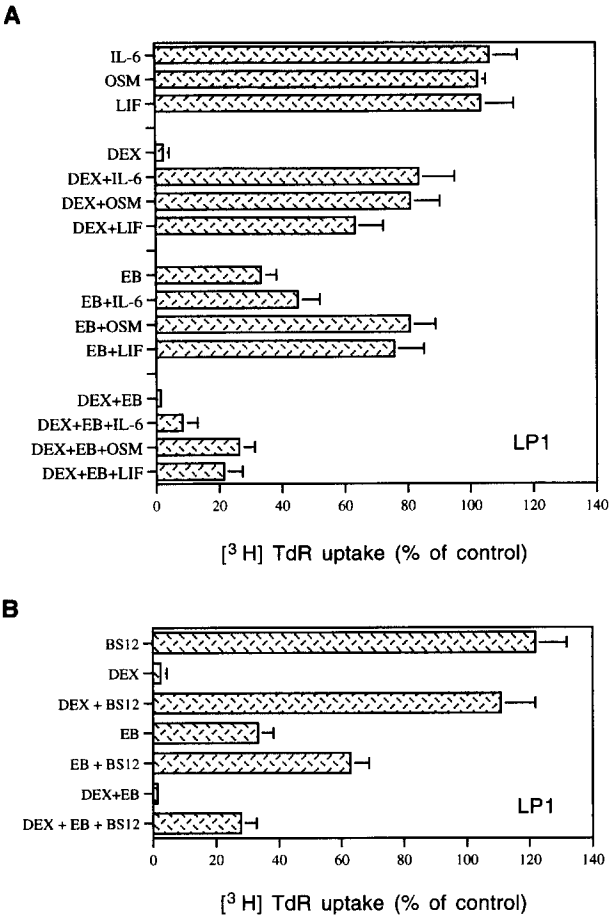

Fig 4. (A) Effect of IL-6, LIF, and OSM on the inhibitory effects induced by Dex, EB1089, and Dex plus EB1089. Cells (10^4/well) were cultured for 72 hours in the different conditions as indicated. Dex and EB1089 were used at the concentration of 10^{-7} and 10^{-8} mol/L, respectively. IL-6 was added at 125 pg/mL and LIF and OSM were added at 10 ng/mL. (B) Effects of BS12, an agonist anti-gp130 MoAb, on the inhibitory effects induced by Dex, EB1089, and Dex plus EB1089. Dex and EB1089 were used at the concentrations of 10^{-7} and 10^{-8} mol/L, respectively. BS12 was added at 0.1 μg/mL.

comparison with that of Dex. In contrast to Dex, which clearly upregulates both gp80 and gp130 expression, on LP1, EB1089 significantly downregulates the gp80 expression (P = .05), having either no effect or weak effects (ie, downregulation) on gp130 expression (Fig 5 and Table 2). Of major interest, this Dex-induced upregulation of the IL-6R (mainly that of the gp80 expression) is significantly reduced by the addition of EB1089. The same interesting regulation of gp80 and gp130 expression (mainly that of the gp80) has been observed on fresh myeloma cells obtained from one patient with MM in leukemic phase (Table 2). Analysis of the gp80 transcripts in LP1 show that EB1089 significantly reduces the gp80 RNA (Fig 6), which is consistent with the previous observation on gp80 regulation. Considering the effects of EB1089 on the gp80 expression at the surface of the myeloma cells and the importance of the soluble form of the IL-6Rα as an agonist of IL-6 on myeloma cell growth, we looked at the effects of EB1089 and Dex on the production of the soluble IL-6Rα by LP1. In five experiments, significant reduction (63% ± 10%; P = .01) of the IL6-Rα production was observed after EB1089 treatment, as shown in Table 3 for 3 of them. Of note and as observed for gp80, Dex increased IL-6Rα production (28% ± 12%; P = .01), but the addition of EB1089 to Dex gave a decrease (59% ± 8%; P = .01) similar to the one obtained in the presence of EB1089 alone.

Comparative effects of EB1089 and Dex on the paracrine production of IL-6. Considering the importance of the paracrine production of IL-6 in MM,[2] we studied the effects of EB1089 on the production of IL-6 by stromal cells from the BM of patients with MM. The effects of Dex were tested in parallel. As shown in Fig 7, which shows the results of 2 of 7 experiments, a constitutive production of IL-6 is found, which is significantly increased by EB1089. On the other hand, Dex almost completely inhibits this production. Of major interest, this inhibition is maintained in the presence of EB1089.

The fact that EB1089 was able to stimulate the production of IL-6 by stromal cells prompted us to test the possibility that it could activate the IL-6 gene in the human myeloma cells themselves. However, this was unlikely considering its inhibitory effects on the HMCL growth. Actually, we have found that these HCMLs did not produce IL-6 constitutively and that their treatment by EB1089 never induced detectable IL-6 levels (ELISA sensitivity, <1 pg).

DISCUSSION

The generation of new vitamin D_3 derivatives such as EB1089 with potent antileukemic effects in vitro[9] at least prompted us to investigate them on human myeloma cells, considering the presence of specific receptors for vitamin D_3 on such tumor cells.[8] In the current study, we have shown that $1,25(OH)_2D_3$ and EB1089, a new vitamin D_3 derivative, inhibit the growth of all myeloma cells tested, even those growing in the presence of IL-6, a major survival and growth factor for myeloma cells.[1,2] EB1089 is clearly more potent than $1,25(OH)_2D_3$ and is significantly efficient at 10^{-9} and 10^{-8} mol/L on the three HMCLs (even at 10^{-12} mol/L on RPMI 8226) and on fresh proliferative myeloma cells. The efficacy of EB1089 was compared with that of Dex, first, because Dex is well known to exert an inhibitory effect on the proliferation of myeloma cells in vitro[10,12,14,17-19] and, second, because Dex is a steroid of reference in the treatment of MM.[11] In the current study, on three HMCLs independent of exogenous IL-6 administration, EB1089 was clearly less efficient than Dex on LP1, the HMCL most sensitive to Dex, but was as efficient on NCI H929 and was more efficient on RPMI 8226. On the other hand, Dex lacked any inhibitory effect on SBN1 cells, which are myeloma cells totally dependent on IL-6 for growth, whereas EB1089 had significant inhibitory effects. The lack of correlation between the effects of EB1089 and those of Dex suggests that both drugs have different mechanisms of action. This is highly likely because it has been shown on a human breast cancer cell line that the antiproliferative effects of EB1089 might be related to promoter selectivity and were not mediated by inhibition of

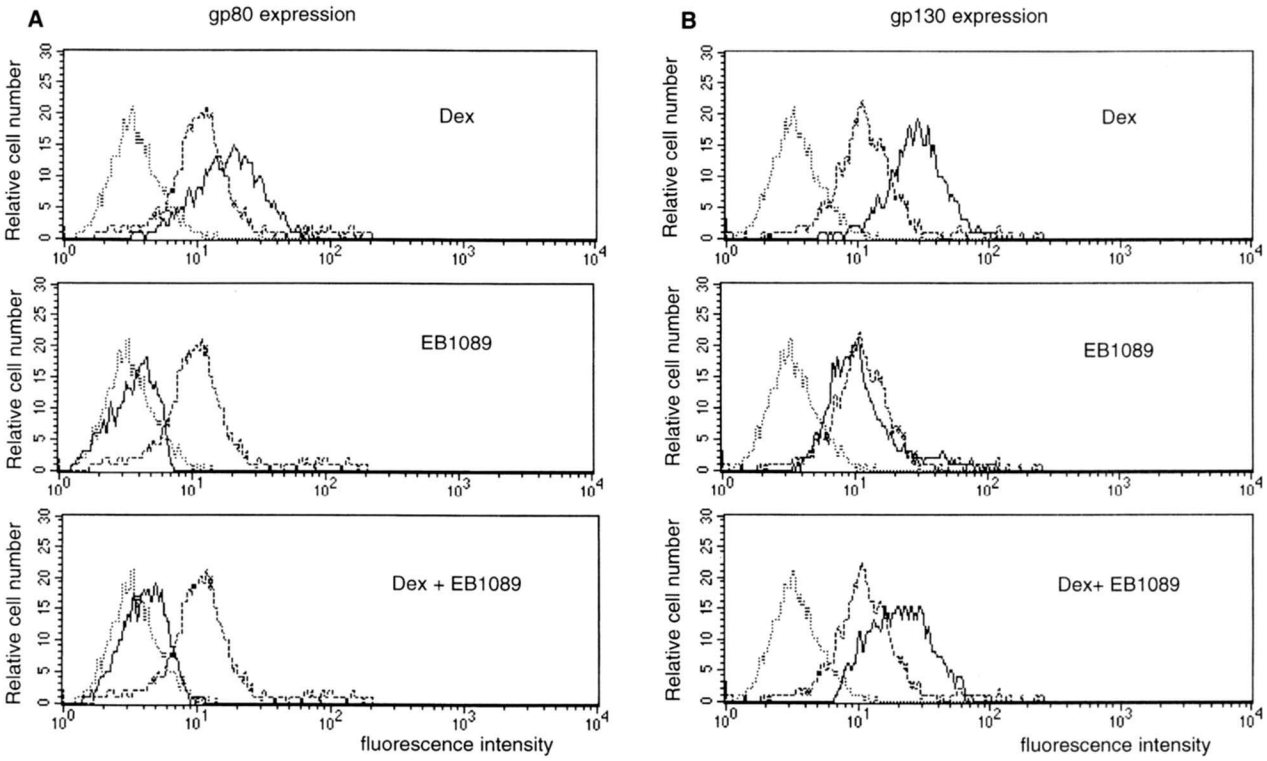

Fig 5. (A) Regulation of gp80 on LP1 after treatment with EB1089 or Dex or EB1089 plus Dex for 24 hours: (· · ·), staining with an isotype control antibody; (---), staining with MT18 MoAb without treatment; and (—), staining with MT18 MoAb after the indicated treatment. (B) Regulation of gp130 on LP1 after treatment with EB1089 or Dex or EB1089 plus Dex for 24 hours: (· · ·), staining with an isotype control antibody; (---), staining with AM64 MoAb without treatment; and (—), staining with AM64 MoAb after the indicated treatment.

the AP-1 pathway, as opposed to those of Dex.[20] The density of vitamin D_3 receptors and their upregulation by Dex[21] could also be critical to the explanation of some of the differences of sensitivity we observed between the HMCLs. These critical points deserve further investigation.

Our cell cycle analysis has shown that EB1089, similar to Dex, was able to induce G1 growth arrest by itself, as recently published for $1,25(OH)_2D_3$.[22] Of note, whereas the Dex-induced G1 growth arrest was reversed by low doses of IL-6, IL-6 only partially reversed the G1 growth-arrest induced by EB1089. We must emphasize that, in our experimental conditions, within 3 days, neither EB1089 or Dex alone was able to induce apoptosis. When we tried to understand why IL-6 only partially reversed the G1 growth arrest induced by EB1089 while it easily reversed the growth arrest induced by Dex, we discovered that EB1089 downregulated both gp80 expression and the production of the soluble IL-6Rα, which is a potent agonist of IL-6. These data are remi-

Table 2. Effects of Dex and EB1089 on the IL-6Rα (gp80) and β (gp130) Expression Analyzed by Flow Cytometry

24-h Drug Exposure	LP1		Patient 1	
	gp80	gp130	gp80	gp130
Control	2.15 ± 0.15	2.58 ± 0.26	2.49 ± 0.02	1.48 ± 0.07
Dex	3.75 ± 0.27	6.96 ± 0.29	4.25 ± 0.04	2.1 ± 0.12
EB1089	1.38 ± 0.01	2.39 ± 0.19	1.96 ± 0.04	1.21 ± 0.12
Dex + EB1089	1.55 ± 0.24	5.37 ± 0.14	3.47 ± 0.13	1.77 ± 0.15

Values shown are the mean fluorescence ratio ± SD.

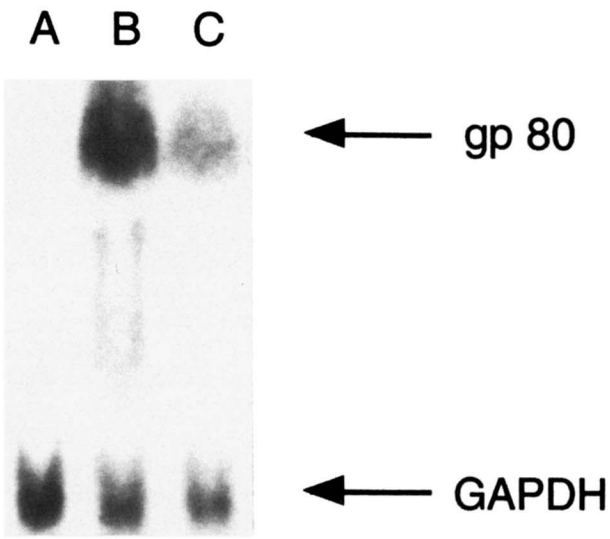

Fig 6. Northern blot analysis of gp80 expression on LP1 cells. Lane A, EB1089 treatment; lane B, Dex treatment; lane C, control.

niscent of those showing the downregulation of epidermal growth factor receptor levels by $1,25(OH)_2D_3$ in human breast cancer cells.[23] The regulation of some critical growth and differentiation factor receptors could be a key physiological role of this hormone. In parallel, we also found that Dex could strongly upregulate both gp80 (and the production of soluble IL-6Rα) and gp130 expression, as previously observed on epithelial cells,[24] hepatocytes,[25] and osteoblasts[26] and as recently shown on myeloma cells by Chen et al.[27] However, we found that EB1089 could inhibit the upregulation of gp80 expression by Dex while significantly limiting that of gp130. Whereas Dex significantly inhibited the IL-6 production by stromal cells, EB1089 had a weak but significant stimulatory effect. However this effect is always annihilated in the presence of Dex. The fact that EB1089 could activate the IL-6 gene in stromal cells contrary to Dex is reminiscent of the IL-6 gene induction by $1,25(OH)_2D_3$ in M1 cells.[28] However, no activation was observed in the HMCLs. Whereas neither EB1089 or Dex alone was able to induce apoptosis, we discovered that EB1089 could induce apoptosis in about one third of myeloma cells in synergy with Dex. A striking observation is that IL-6 (at doses as high as 20 ng/mL), LIF, OSM, and even an agonistic anti-gp130 MoAb were unable to prevent it.

From a therapeutic point of view, all these observations are of major importance. Higher doses of EB1089 than those we have used in vitro can be achieved in vivo without any hypercalcemic effects.[29] Although an excessive osteoclastic resorption is generally observed in patients with MM, the efficacy of EB1089 at 10^{-9} mol/L or even at 10^{-12} mol/L suggests that this drug could be used in vivo, especially if agents able to limit calcium uptake (glucocorticoids) and bone resorption (bisphosphonates) are associated. Although Dex has a clear-cut antitumoral effect in MM, some patients do not respond to this treatment, and finally all become resistant. There is more and more evidence that Dex inhibits the IL-6 gene transcription, at least in stromal cells and myeloma cells, by interacting directly with the autocrine/paracrine IL-6 network in MM.[19] As a consequence, we and others have shown that low doses of IL-6 could counteract the antiproliferative effects of Dex on myeloma cells in vitro.[15] Thus, in vivo, the overproduction of IL-6 observed in the microenvironment of patients with MM (ie, several nanograms per milliliter) could be a major cause for the resistance of myeloma cells to the inhibitory effects of Dex. Furthermore, its newly described effects on the IL-6R$\alpha\beta$ expression (ie, upregulation) and production (ie, increase) of soluble IL-6Rα could lead to the selection of myeloma clones sensitive to low amounts of IL-6 to survive and to grow. In this context, the effects of EB1089, if observed in vivo could be of major therapeutic interest as a maintenance treatment. Because EB1089 (1) has potent cytostatic effects on human myeloma cells by itself even in presence of IL-6, (2) acts in synergy with Dex to induce unescapable apoptosis, and (3) inhibits both gp80 expression and production of the soluble IL-6Rα while counteracting the deleterious effects of Dex (ie, upregulation of both gp80 and gp130 expression), its association with Dex could be very helpful in the treatment of MM. Finally, it has been recently shown that the sensitivity of human myeloma cells to the cytotoxicity of immune cells inversely correlates with the degree of gp80 expression.[30] In this context, EB1089 could facilitate the cytotoxicity of immune cells against myeloma cells.

Table 3. Effects of Dex and EB1089 on the Production of the Soluble IL-6Rα Determined by ELISA

48-h Drug Exposure	Soluble IL-6Rα (ng/mL)		
	Exp 1	Exp 2	Exp 3
Control	4.6	5.6	11.5
Dex	5.3	7.3	16.9
EB1089	1.5	1.6	6.2
Dex + EB1089	1.6	1.9	6.3

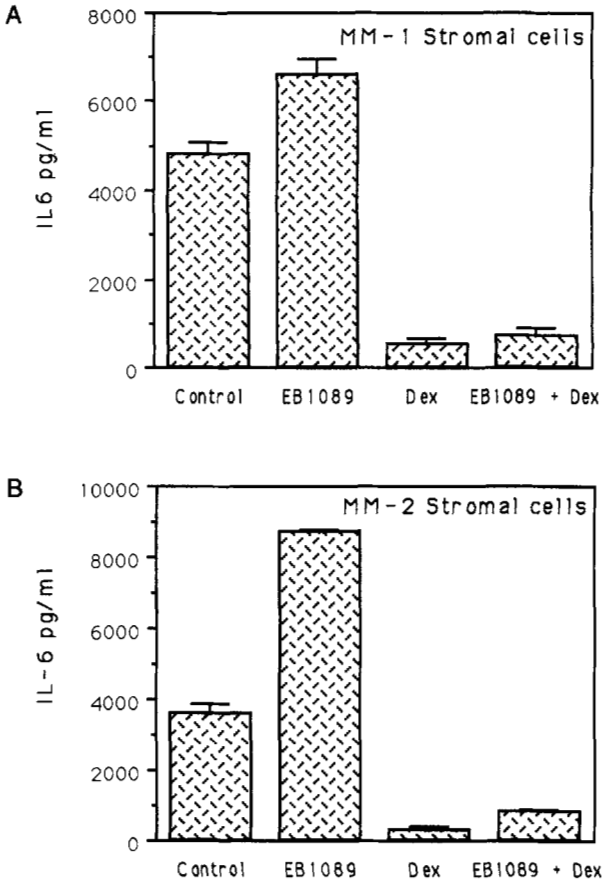

Fig 7. IL-6 production by stromal cells derived from two myeloma patients. Stromal cells were cultured for 48 hours with EB1089, Dex, or a combination of Dex plus EB1089; then, the supernatant was collected for IL-6 determination by ELISA.

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