Modulation of Spontaneous B-Cell Differentiation in Macroglobulinemia by Retinoic Acid

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We previously showed that clonal blood B cells from patients with macroglobulinemia spontaneously differentiate in vitro to plasma cells. This process is dependent on an interleukin (IL)-6 autocrine pathway. We investigate here whether all-trans-retinoic acid (RA) interferes with B-cell differentiation either in patients with IgM gammopathy of undetermined significance (MGUS) or Waldenström's macroglobulinemia (WM). RA at a concentration of 10^{-5} to 10^{-9} mol/L inhibited by 50% to 80% the in vitro differentiation of purified B cells from four of five patients with MGUS and from one of five patients with WM as assessed by the IgM content of day 7 culture supernatants. We next determined whether this effect could be related to an inhibition of IL-6 secretion by cultured B cells and/or a downregulation of the IL-6 receptor (IL-6R), which is constitutively expressed on patients' blood B cells. A 50% to 100% (mean, 80%) inhibition of IL-6 production was found in seven of 10 patients (five with MGUS and two with WM). The IL-6R was no more detectable on cells from patients with MGUS after 2 days of treatment with RA and slightly downregulated in patients with WM. It was of interest that B cells susceptible to the action of RA belonged mostly to patients with IgM MGUS, which reinforces our previous data showing distinct requirements for IL-6-dependent differentiation of blood B cells from patients with WM or IgM MGUS.

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MATERIALS AND METHODS

Patients. The 10 patients studied had a serum monoclonal IgM. Five presented with bone marrow malignant lymphoplasmacytic infiltration typical of WM. The five others showed no evidence of overt lymphoid neoplasia on a bone marrow biopsy and were therefore considered to have MGUS.

Purification of blood B cells. Peripheral mononuclear cells (PBMC) were isolated from peripheral blood by Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. Purified B cells were obtained after initial monocytes elimination in the presence of 5 mmol/L of 1-leucine methyl ester (Sigma Chimie, La Verpilleire, France) or by 3-hour adherence on plastic dishes at 37°C and T-cell depletion by rosetting with 5-(2-amino ethyl)isothioammonium bromide–treated sheep erythrocytes. These preparations contained less than 1% monocytes and T cells.

Cell culture. Purified B cells were cultured in tissue culture tubes at a concentration of 0.25 × 10^9 to 1 × 10^9 cells/mL in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, and antibiotics. RA was obtained from Roche (Neuilly sur Seine, France). A stock solution was prepared in dimethyl sulfoxide at a concentration of 10^{-7} mol/L and stored at −80°C. RA was used at 10^{-5} or 10^{-12} mol/L after dilution of the stock solution in fresh complete medium. Culture supernatants collected at day 2 or day 7 were stored at −20°C.

Evaluation of B-cell differentiation. Cytoplasmic immunoglobulin (cIg)-containing cells were detected by direct immunofluorescence on fixed cells according to published methods. IgM in cell culture supernatants was measured by an immunocapture enzyme-linked immunosorbent assay (ELISA) as described previously.

IL-6 bioassay. The IL-6 content of culture supernatants was estimated by a bioassay using the IL-6–dependent B9 hybridoma cell line; 5 × 10^4 B9 cells were cultured for 72 hours in 200 μL of culture medium in 96-well microplates in the presence of triplicate serial dilutions of B-cell culture supernatants. B9-cell proliferation was measured by [3H]Thymidine incorporation (CEA, Saclay, France).

Human recombinant (rIL-6) IL-6 was used as an internal standard in all assays. One reference unit of IL-6 was defined by the half-
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Fig 1. Effect of RA on IgM secretion of blood B cells from patients with macroglobulinemia. Purified B cells from (A) five patients with MGUS and (B) five patients with WM were cultured for 7 days in medium alone or in the presence of RA (10^{-8} mol/L). IgM content of culture supernatants was measured by ELISA. Bars represent for each patient the percentage of RA-induced inhibition of IgM secretion.

maximal proliferation of B9 cells, and corresponded reproducively to 1 pg of rIL-6.

Analysis of cell-surface IL-6R expression. Staining with anti-IL-6R monoclonal antibody MT18 (kindly provided by T. Kishimoto) was accomplished by incubating 0.5 x 10^6 cells with 3 μg/mL of MT18 monoclonal antibody for 30 minutes at 4°C. After three washings in 3% bovine serum albumin (BSA)-phosphate buffered saline (PBS), phycoerythrin-labeled goat antibodies to mouse immunoglobulin or biotinylated anti-mouse IgG followed by fluoresceinated Streptavidin (Amersham, Les Ulis, France) were used as a second layer. Fluorescence intensity was analyzed on a cytofluorograph 50 HH (Ortho Pharmaceutical, Raritan, NJ). Results were displayed as frequency distribution histograms of log fluorescence of at least 10,000 cells. Irrelevant, isotype-matched monoclonal antibodies were used in each experiment as negative controls.

RESULTS

Effects of RA on blood B-cell differentiation. Highly purified B cells were obtained from PMBC after monocyte elimination by poisoning with L-leucine methyl ester or adherence to plastic dishes and depletion of T cell by E rosetting. B cells were thereafter cultured for 7 days in culture medium alone or in the presence of RA (10^{-8} to 10^{-12} mol/L). In the presence of culture medium alone, B cells enriched from PMBC of normal individuals did not differentiate (data not shown). In contrast, at the end of the culture period, B cells from the 10 patients studied differentiated to morphologically typical plasma cells, as previously observed. The generation of plasma cells varied from patient to patient (mean, 15%; range, 5% to 33%), but remained similar in a given patient. Measurement of IgM in culture supernatants confirmed these data, with a significant rate of IgM secretion being noted in all cases (mean, 2,120 ng/mL; range, 120 to 8,700 ng/mL).

As shown in Fig 1, B-cell differentiation was inhibited by 50% to 80% in five of 10 patients. Dose-response curves indicated that the maximal inhibition was observed for RA concentrations as low as 10^{-8} mol/L (data not shown). Four of five patients whose B cells no longer differentiated had IgM MGUS (Fig 1A). Less than 25% inhibition in IgM secretion was noted in the other patients (Fig 1B). Reproducible results were obtained in two to five experiments for each patient. There was no change in the percentage of viable cells in RA-treated cultures as compared with that of untreated cultures. In addition, the absence of inhibition of B-cell differentiation in some cases argues against nonspecific toxic effects of RA. As shown in our initial study, no cell proliferation was detected during the culture period.

Modulation of IL-6 secretion and IL-6R expression under RA. Bioactive IL-6 was measured in culture supernatants collected on day 2. In all cases, a significant amount of IL-6 was detected (mean, 127 pg/mL; range, 5 to 505 pg/mL) (Fig 2). In the presence of RA, IL-6 secretion was decreased by 50% to 100% (mean, 80%) in all patients with IgM MGUS (Fig 2A) and in two patients with WM (Fig 2B). RA alone did not interfere with the IL-6 bioassay as exemplified by the similar IL-6 content found in supernatants from three patients collected from cultures with or without RA (Fig 2B).

IL-6R expression on B cells from four patients was studied on day 2 of culture. We used the MT18 monoclonal antibody, the binding of which is unaffected when the IL-6R is saturated by its ligand. A varying density of IL-6R was constitutively expressed on blood B cells from patients with IgM MGUS and WM (Fig 3). As judged from fluorescence intensity, it appeared generally higher in patients with WM (Fig 3E and F). No appreciable reactivity was noted on B-cell preparations from healthy individuals. In the presence of 10^{-3} or 10^{-6} mol/L of RA, the expression of IL-6R was reduced to a variable extent. It was no longer detectable in two patients with MGUS (Fig 3B and D), whereas the percentage of positive cells, as well as the density of receptors, was moderately decreased in two patients with WM (Fig 3E and F).
DISCUSSION

IL-6 is believed to play a key role in the growth of some human lymphoid malignancies. Its biological activity as a plasmacytoma growth factor prompted studies in human multiple myeloma, which pointed to its role as an autocrine or paracrine factor in the proliferation of human neoplastic plasma cells. IL-6 is also a late differentiation factor for human B cells; this latter activity is implicated in the differentiation of B cells in WM. This lymphoid malignancy is characterized by a pleomorphic lymphoplasmacytic proliferation, as B cells at all stages of maturation to plasma cells are present in bone marrow and in peripheral lymphoid organs. We showed that blood clonal B cells from these patients could differentiate spontaneously in vitro without any cell proliferation, and that this process was likely dependent on an IL-6 autocrine mechanism. The latter was clearly demonstrated in patients with MGUS, since neutralizing antibodies to IL-6 inhibited the differentiation process. In contrast, differentiation of B cells from patients with WM was unaffected by the same anti-IL-6 antibodies. However, preliminary data indicate that inhibition of IL-6 synthesis by antisense oligonucleotides does abrogate the differentiation of B cells from patients with WM, indicating that this lymphokine is implicated in the differentiation process of both IgM MGUS and WM.

RA has been shown to influence the proliferation and differentiation of a variety of cell types. The biochemical systems through which retinoids appear to mediate their effects are still unclear. RA binds to specific nuclear receptors, and these complexes are thought to regulate the transcription of target genes. It has been shown that RA was able to downregulate IL-6R expression on cells from myeloma lines and Epstein-Barr virus–induced lymphoblastoid cells. In addition, RA represses the AP-1 (a protein complex of the c-jun and c-fos proto-oncogene products)–mediated induction of gene expression, and AP-1–responsive elements are present on the IL-6 gene. It was therefore tempting to investigate whether RA could interrupt the IL-6–dependent spontaneous differentiation of blood cells from patients with macroglobulinemia.

We purposely selected five patients with classical WM and five patients with IgM MGUS to investigate whether all-trans-RA would interfere with the in vitro B-cell differentiation. A 50% to 80% decrease of IgM secretion on day 7 of cultures of purified B cells was noted in five cases, whereas the differentiation process was not significantly affected in the other patients. Interestingly, RA-induced inhibition of blood B-cell differentiation was observed in four patients with IgM MGUS and in only one patient with WM. These results reinforce our hypothesis that a two-step neoplastic process occurs in patients with IgM gammopathies.

Further experiments suggested that RA could mediate its effects through a modification of the IL-6 triggering loop. We found that a varying percentage of purified B cells from patients with either WM or IgM MGUS expressed constitutively IL-6 Rα, giving credit to the autocrine differentiation pathway we described previously. In the presence of 10^-5 to 10^-8 mol/L of RA, there was a downregulation of this receptor in all patients studied, regardless of degree of lymphoplasmacytic proliferation. Of note, no IL-6R expression was detectable on RA-treated cells from three patients so studied (two MGUS and one WM) whose cells no longer fully differentiated under RA. In contrast, cells from two patients with WM displayed only a moderate decrease in both IL-6R density and number of IL-6R–positive cells. The known effect of RA on IL-6R expression on B cells was therefore again demonstrated and could explain the decrease of B-cell differentiation to plasma cells we observed in some patients. In addition, we noted a decrease in IL-6 secretion in seven patients (five with MGUS and two with WM), which could also play a role in the RA-induced arrest of differentiation that occurred in five of these cases. Here again, inhibition of IgM secretion was, in each case, associated with a significant decrease in IL-6 synthesis by RA-treated B cells. In particular, IL-6 secretion in the single patient with WM whose B cells no longer differentiated under RA was also decreased. Since inhibition of IL-6 secretion was noted in the first 48 hours of culture, it appears likely that the effect of RA was targeted on B cells, since plasma cell generation occurs later in these in vitro cultures.
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Fig 3. Modulation of IL-6R expression on patient's B cells by RA. B cells were cultured in medium alone or in the presence of RA (10^{-6} mol/L) for 48 hours and stained with monoclonal antibody to IL-6R. Patients with MGUS: (A and C) medium and (B and D) RA. Patients with WM (E and F): (- - -) IL-6R expression in medium alone; (---) IL-6R expression with RA. Shade histograms represent staining with control monoclonal antibody.

For instance, it is well known that an overt lymphoplasmocytic malignant lymphoma may develop, sometimes after 10 to 20 years of follow-up, in patients initially classified as having IgM MGUS. This possibility is exemplified by the natural course of chronic cold agglutinin disease or of peripheral neuropathies with antimyelin-associated glycoprotein monoclonal IgM, as was the case in our patients with IgM MGUS. It is therefore striking that the effect of RA distinguished almost perfectly patients with MGUS or WM with a single exception in each group. Indeed, RA did not inhibit the differentiation of B cells in four patients with WM, regardless of its effect on IL-6 secretion or IL-6R expression. Whether the absence of RA-mediated regulation of B-cell differentiation represents a step toward malignancy in WM remains speculative. Hopefully, a better knowledge of the mechanism(s) leading to autonomous IL-6 secretion in macroglobulinemia might have therapeutic implications.

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REFERENCES


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