Clonal Membrane-bound IgM

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is characterized by an accumulation of monoclonal B lymphocytes in the hematopoietic organs. Rarely, CLL cells accumulate in a single atypical site. The mechanism underlying this unusual distribution of CLL cells has not been studied previously. We obtained peripheral blood from five patients having early stage CLL with heavy prostate infiltration. These patients' circulating CLL cells bound strongly in vitro to cultured prostate cell lines PC3, LNCaP, and DU145 and to short-term cultures of fresh prostate cells but not to colon, breast, or bladder cells. CLL cells from patients without prostate infiltration did not bind in vitro to any cell line. Peripheral blood CLL cells from one patient with CLL with heavy prostate infiltration were fused with a mouse–human heteromyeloma line to make hybridomas expressing the patient's CLL-PI cells. The hybridoma cells bound specifically to prostate cells. IgM secreted by the hybridoma blocked binding of the patient's CLL cells to prostate cells. Flow cytometry and immunohistochemistry demonstrated that the secreted IgM bound specifically to prostate cells. These results indicate that CLL with atypical prostate infiltration can be mediated by specific surface-bound IgM against an antigen expressed specifically by prostate cells and suggest that a similar mechanism might also apply to cases of CLL with atypical infiltration into other organs.

INTRODUCTION

In CLL, monoclonal, mature-appearing cells of B lymphocyte lineage accumulate in the blood, bone marrow, lymph nodes, and spleen. Except in end-stage CLL, infiltration of other organs is typically minimal or absent. Unusual cases have been reported, however, with early stage CLL with heavy infiltration into a single atypical site, such as the prostate, breast, heart, or lung. At least 73 cases of CLL with localized prostate infiltration (CLL-PI) in the absence of widespread disease or infiltration into another atypical site have been reported (1–39), but the pathogenesis of organ-specific infiltration in CLL-PI has remained obscure.

CLL cells express surface mAb. The predominant Ig is usually IgM, less often IgG or IgA (40–42). In the related malignancy multiple myeloma, the mAb is secreted into the serum and may be structurally normal and able to bind in vitro to a biological antigen, such as red cell antigens, insulin, myelin, or coagulation factors (causing hemolysis, hypoglycemia, neuropathy, and coagulopathy, respectively; Refs. 43 and 44). Similarly, one might predict that the mAb in CLL could recognize a biological antigen. We investigated the hypothesis that the surface-bound clonal Ig in CLL with specificity for an antigen expressed by prostate cells could explain cases of CLL-PI; this has not been investigated previously.

We first investigated in vitro binding of peripheral blood CLL cells, from five patients, to prostate cell lines. We then studied one case in detail, to prove that binding was mediated by specific surface-bound IgM.

MATERIALS AND METHODS

Identification of CLL-PI Cases. We identified 8 cases of CLL-PI seen at this medical center. In all cases, prostate resection was performed because of urinary obstruction, and the resected prostate contained a heavy lymphocytic infiltrate. All 8 patients were subsequently found to have an elevated white cell count, containing mainly mature-appearing monoclonal B cells, i.e., CLL. None of the patients was known previously to have CLL, and none had end-stage disease or evidence of other atypical organ involvement. As controls, we identified 10 other patients with CLL who underwent prostate resection, in whom the pathology report did not mention lymphocytic infiltrate. Reexamination of these patients’ slides confirmed the absence of a lymphocytic infiltrate.

Sources and Immunophenotypic Characterization of PBMCs. Immunophenotyping by flow cytometry, performed on PBMC in 5 cases, revealed that the CLL cells of all 5 expressed low-density surface IgM, were monoclonal by light-chain isotyping, and expressed CD5, CD20, and CD23. PBMCs (consisting of 78–92% lymphocytes) were isolated by Histopaque-1077 density centrifugation (46) and stored at −70°C. As controls, PBMCs were also isolated from 5 CLL patients without prostate infiltration. Control PBMC were also obtained from 1 patient with end-stage CLL and 1 with end-stage follicular cell lymphoma, both with a high tumor burden, who had undergone prostate resection. In these 2 patients, the biopsy revealed typical benign prostatic hypertrophy accompanied by moderate lymphocytic infiltration, consistent with the nonspecific lymphocytic infiltration of many organs often seen with end-stage CLL or lymphoma (47), which is distinct from the heavy infiltration seen in our CLL-PI patients and those in the literature.

Generation of Hybridomas. To create a large amount of soluble, monoclonal IgM of the same antigen specificity as the membrane-bound Ig on 1 patient’s CLL-PI cells, hybridomas were generated from his PBMCs. Control hybridomas were made from the PBMCs of a patient without prostate infiltration ("1641D") and from polyclonal PBMCs of a normal donor. PBMCs were fused with the mouse–human heteromyeloma cell line SHM-D33, as described (46), except that the CLL cells were not EBV transformed before fusion, because CLL cells are resistant to such transformation (48–50). Fusion CLL-PI/SHM-D33 cells were cultured in 96-well plates. At 4 weeks, supernatants were screened for human Ig production by ELISA. Monoclonality was established by progressive subcloning at 100, 10, 1, and 0.5 cells/well. The clone with the highest IgM production ("15160"; 12.5 μg/mL) was used in subsequent experiments.

ELISA and Ig Quantitation. ELISAs for Ig screening and quantitation were performed as described (51), using pooled human IgM as a standard. Briefly, plates were coated for 24 h at 4°C with rabbit IgG against human IgG, IgA, and IgM (μ-chain specific), and hybridoma supernatants were incubated (90 min, 37°C) in the wells. Alkaline phosphatase-conjugated rabbit Ig against human μ, λ, κ, γ, or α chains was added and incubated (90 min, 37°C).
RESULTS

Histology. The prostate biopsies of the patients with CLL-PI revealed diffuse infiltration with small lymphocytes, suggesting CLL (Fig. 1).

Hybridoma Generation. Four weeks after fusion of cells from CLL-PI patient 1 with SHM-D33 cells, 98% of the wells contained heterohybridoma cells secreting IgM/κ antibody, the same isotype as on his CLL cells. There was no γ or α chain secretion in any well, indicating that the hetero-hybridomas were derived from the malignant clone, rather than from polyclonal B cells, which may have been present as a minor population in his PBMCs. Several clones were serially subcloned and used for in vitro binding studies; the clone with the highest IgM production ("1516D"); 12.5 μg/ml) was studied by flow cytometry and immunohistochemistry. Hybridomas secreting IgM/κ from control CLL patients were also obtained. Control hybridoma 1641D also secreted IgM.

Binding Studies. Lymphocytes from all CLL-PI patients, but no control lymphocytes, bound to PC3, LNCaP, and DU145 prostate cells (Fig. 2). CLL-PI cells did not bind to bladder, breast, or colon cell lines (Fig. 2D). Thus, in all tested combinations, in vitro binding Extinction was read at 410 nm and compared with positive and negative controls.

In Vitro Binding of CLL and Hybridoma Cells to Cultured Cell Lines. Prostate cancer cell lines PC3, LNCaP, and DU-145 (52), bladder line RT-4, breast line MCF7, and colon lines HT-29 and HCT-116 were cultured (53) to confluent monolayers in 24-well plates. We also tested binding to short-term cultures (54) of fresh normal prostate cells. Cultured cells were washed three times in PBS; CLL-PI or hybridoma cells (10^6 cells in 0.5 ml) were added, and the plates were incubated (37°C, 5% CO2 × 30 min) and washed three times in PBS. Formaldehyde (3.7% in 10 mM HEPES buffer) was added to fix the cells, which were examined by microscopy.

Blocking Experiments. Three blocking experiments were performed: (a) PC3 cells were incubated with mAb 1516D (5 μg/well) or irrelevant mAb 1418-1 (IgG specific for parvovirus B19; Ref. 55) for 30 min before adding CLL-PI or hybridoma cells; (b) supernatant from day 3 PC3 cultures was incubated for 30 min with hybridoma cells producing mAb 1516D, before binding studies; and (c) purified PSA or BSA as a control was incubated for 1 h at concentrations from 1 to 100 ng/ml with CLL-PI cells or 1516D-producing cells before their addition to cultured prostate cells. Blocking was assessed by counting bound cells per high-power field.

Flow Cytometry. Binding of monoclonal IgM to prostate, colon, and breast cells was determined by flow cytometry as described (56). Briefly, single cell suspensions of 1–2 × 10^6 cells were washed in RPMI 1640 and incubated with 1 μg of mAb 1516D in 100 μl at 37°C for 45 min. An irrelevant human monoclonal IgM (RPMI 1788; American Type Culture Collection), polyclonal human IgM, or 15% FCS served as controls. The cells were washed in RPMI 1640, and the pellet was incubated with 100 μl of 1:5 or 1:50 dilution of goat antihuman IgM μ FITC conjugate (Caltag) for 30 min at 4°C, washed, and resuspended in PBS. Live cancer cells were selected for analysis by gating with forward and 90° scatter (FACScan flow cytometer; Becton Dickinson).

Immunohistochemistry. Supernatants from hybridomas made from CLL cells of patients with (1516D) or without (1641D) prostate infiltration were dialyzed against PBS to precipitate mAb. Biotin was bound to purified IgM for 2 h, glycine was added to stop the reaction, free biotin was removed by dialysis, and immunostaining was performed as described (57).

Fig. 1. Microscopy of the prostate biopsy on the patient whose PBMCs were used to make mAb 1516D. The prostate is massively infiltrated by small, mature-looking lymphocytes. The normal prostate architecture is obliterated. The lymphocytes were CD5, CD20, and CD23 positive on immunostaining, indicating a diagnosis of CLL. Staining was also attempted using anti-κ and -λ antibodies, but these antibodies rarely provide definitive results on paraffinized tissue and were inconclusive. Insufficient material was available for extraction of DNA from paraffin for DNA-based demonstration of monoclonality.

Fig. 2. Binding studies of CLL-PI cells to cultured prostate cancer cell lines PC3 and LNCaP. A, PBMCs (CLL cells) from a patient with CLL-PI show in vitro binding to PC-3 cells. B, control CLL cells, from a patient without prostate infiltration, added to PC3 cells (no binding). Lymphocytes from 5 additional early-stage CLL patients without prostate infiltration, and the 2 patients with end-stage lymphoproliferative diseases (1 CLL, follicular lymphoma) with moderate prostate infiltration (and likely widespread infiltration into other organs as well), similarly failed to bind in vitro to prostate cells (data not shown). C, patient 1 CLL and LNCaP cells (binding). D, patient 1 cells and colon cancer cell line HCT116 (no binding). Patient 1 CLL cells also bound to DU-145 cells (data not shown). CLL cells from all tested CLL-PI patients gave similar results, with binding only to prostate cells PC3, LNCaP, and DU145 and not to colon, breast, or bladder lines. Fresh PBMCs or frozen thawed PBMCs bound equally well.
was 100% sensitive and specific for the combination of CLL-PI lymphocytes with cultured prostate cells. To test whether the same mAb secreted by the hybridoma cells was also present on their surface, we studied in vitro binding to prostate cells of 1516D-secreting cells and of several other subclones from the same patient. The CLL-PI-derived hybridomas bound even more extensively to the prostate cell lines than did the parent PBMCs (Fig. 3A). The two control hybridomas, secreting mAbs 1418-1 and 1641D, did not bind to prostate lines (Fig. 3B). Cells making mAb 1516D did not bind to colon cells, confirming prostate specificity (Fig. 3C).

**Blocking Experiments.** We hypothesized that if the observed binding was mediated by membrane-bound IgM, then soluble mAb 1516D might block binding. As predicted, mAb 1516D, but not mAb 1418-1, decreased binding of CLL-PI and 1516D-secreting cells to PC3 cells by an average of 75% (data and pictures not shown). Incubation of the 1516D-secreting hybridoma or CLL-PI cells with PC3 cultured media completely blocked subsequent binding of these cells to PC3, LNCaP, and DU-145 cells, whereas incubation with cultured media from colon cells had no effect, suggesting that PC3 cells secrete or shed a large amount of the 1516D target antigen (data not shown). PSA incubated with 3 patients’ CLL-PI cells before their addition to PC3 or LNCaP failed to inhibit binding (data not shown).

**Flow Cytometry.** MAb 1516D strongly bound to PC3 cells, but not to nonprostate cell lines, indicating its specificity for a prostate-expressed antigen (Fig. 4). PSA incubated with 3 patients’ CLL-PI cells before their addition to PC3 or LNCaP failed to inhibit binding (data not shown).

**Immunohistochemistry.** MAb 1516D demonstrated intense prostate tissue staining, confirming its specificity (Fig. 5).

**DISCUSSION**

CLL cells usually traffic to the organs of the hematopoietic system, i.e., blood, bone marrow, lymph nodes, and spleen but not, except in end-stage disease, to nonhematopoietic organs (8, 9, 11, 47, 58, 59). Unusual cases have been reported, however, of early stage CLL with infiltration into prostate (1–33, 35, 36, 60, 61) or other individual organs, e.g., pituitary, myocardium, thyroid, lung, and orbit (47, 62–72).

Our observations of CLL-PI patients, plus those in the literature, suggested that CLL-PI could be mediated by membrane-bound mAb specific for one or more prostate-expressed antigens. In all of our
cases and most reported cases, the patient was not known to have CLL before developing urinary obstruction; rather, CLL was diagnosed after leukocytosis was discovered during the preoperative evaluation, or examination of the prostate biopsy provided the first evidence of a lymphoproliferative disease. In our patients and most reported cases, prostate gland hypertrophy was absent; urinary obstruction appeared to result instead from unusual heavy infiltration of CLL cells.

In vitro binding of CLL cells and hybrids derived from CLL cells to cultured fresh prostate cells and cancer cell lines correlated perfectly with the histological finding of heavy organ-specific in vivo infiltration, supporting the hypothesis that this binding is mediated by specific surface-bound Ig, targeting one or more prostate-expressed antigens. For our studies, prostate cancer cell lines were used not because we suspected binding to cancer-specific antigens but because these cells should also express normal prostate antigens. CLL cells also express adhesion molecules that could play a role in binding (73) but not in a tissue-specific manner. Flow cytometry and immuno-histochemical studies on 1 patient further confirmed that the binding of CLL cells to prostate is mediated by IgM with specificity for a prostate-expressed antigen.

We do not yet know if the same antigen is targeted in all CLL-PI cases. We can conclude, however, that neither PSA nor PSMA, another antigen expressed in the prostate, is targeted in our cases. PSA is ruled out in the 3 cases tested by its inability to block binding of CLL-PI cells to prostate cells, and PSMA is ruled out because all tested CLL-PI cells bound to PC3 cells, which do not express PSMA (74).

We have demonstrated that the monoclonal membrane-bound Ig in CLL can lead to the accumulation of CLL cells in a single organ. Although these studies apply directly only in cases where the prostate is the targeted organ, we predict that a similar mechanism also explains cases of CLL with unusual infiltration into other organs as well.

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REFERENCES

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