Cytoskeleton Organization Is Aberrantly Rearranged in the Cells of B Chronic Lymphocytic Leukemia and Hairy Cell Leukemia

By Federico Caligaris-Cappio, Luciana Bergui, Luisa Tesio, Guelfa Corbascio, Franca Tousco, and Pier Carlo Marchisio

The organization of actin-containing microfilaments and vimentin-containing intermediate filaments has been investigated in B chronic lymphocytic leukemia (B-CLL), hairy cell leukemia (HCL), and normal B cells cultured in vitro under basal conditions and after induction with 12-O-tetradecanoyl-phorbol-13-acetate (TPA). In uninduced B-CLL cells, F-actin was predominantly associated with dot-shaped structures scattered over the ventral membrane representing34 spot12 close contact adhesion sites analogous to "podosomes" described in other cell types. On TPA induction, podsomes became clustered in sharply defined areas sitting in the cell center beneath the nucleus. In some cells, long actin-containing protrusions appeared. In HCL cells, F-actin was associated with thin microvilli responsible for the "hairy" appearance; occasional cells showed scattered podsomes. On TPA induction, HCL cells sprouted long dendritic processes rich in submembranous F-actin, which made intertangled networks. Therefore, in both B-CLL and HCL cells, adhesion structures were present and the capacity for adhesion in vitro was marked, which might explain some peculiar clinical features of the diseases. Adhesion structures and adhesive properties never appeared in normal B cells. These data further support the notion that B-CLL and HCL, although clinically different, may share common biological features and suggest that in these disorders, cytoskeleton modifications may represent a hallmark of transformation.

A NUMBER OF MAJOR lymphocyte functions mediated by surface receptors are controlled by the cytoskeleton. These functions include blood-lymph recirculation, cell-cell interactions, and secretion of antibodies and lymphokines (for review, see refs 1 and 2).

The malignant cells of B chronic lymphocytic leukemia (B-CLL) and hairy cell leukemia (HCL) are characterized by abnormalities that suggest defects of cytoskeletal functions. B-CLL cells have low mobility,3 show decreased capping by different ligands,44 and are unusually susceptible to microtubule disrupting drugs.5 HCL owes its name to the presence of many irregular projections protruding from the surface of cells, which are clearly visible in a phase contrast microscope and appear as irregular undulating ruffles or long villi when examined by the scanning electron microscope. HCL cells are able to cap surface immunoglobulins (sIg) and concanavalin A (ConA) receptors; the membrane redistribution of these structures is inhibited by cytochalasin B.6,10

In the in vitro stimulation of B-CLL and HCL cells with the differentiation-promoting agent 12-O-tetradecanoyl-phorbol-13-acetate (TPA) induces changes of morphology, cytoplasmic enzyme activity, and surface markers.8-11 B-CLL cells acquire an irregular hairy cell-like appearance with ruffles and protrusions.12,13 HCL cells become firmly attached to culture dishes and exhibit fibroblast-like projections and stellate features.14 Details of the action of TPA are not yet fully elucidated; recent evidence suggests that TPA permeates the plasma membrane and activates protein kinase C, mimicking the physiological activator diacylglycerol.15 By such mechanism, some protein targets are phosphorylated and this leads to reorganization of the subsurface microfilamentous network.15-21

These observations provide the rationale for the present study, in which we have investigated the organization of the cytoskeleton of normal B, B-CLL, and HCL cells in the uninjured state and after TPA induction in vitro. The experimental approach was adopted to answer the following questions: (1) Can TPA induce a rearrangement of cytoskeletal structures in malignant B cells? (2) Are the peculiar morphological features of HCL, also reproduced by TPA treatment of B-CLL cells, dependent on cytoskeleton rearrangements? (3) Is the cytoskeleton of normal B cells behaving differently compared with its malignant counterparts under the same experimental conditions?

MATERIALS AND METHODS

Preparation of cells. Peripheral blood lymphocytes (PBLs) were obtained from 26 patients. Twenty-three patients (14 males, nine females; aged 49 to 70) had B-CLL; ten were in stage O-I, six in stage II, six in stage III, and one in stage IV, according to Rai.21 In all cases, B lymphocytes expressed monoclonal Ig (k" or A") with low intensity, formed rosettes with mouse red blood cells (MRBC), and were Tr cells antigen positive as detected by the monoclonal antibody RFTI.22 None of the patients had received chemotherapy in the four months preceding the study. Three patients (all males aged 52 to 68) had HCL. The diagnosis was based on cell morphology, the presence of 50% to 75% tartrate-resistant acid phosphatase-positive (TRAP+) cells in peripheral blood, and the bone marrow histology on trephine biopsies.

Mononuclear cells were separated from patients' peripheral blood samples on Ficol-Hypaque (FH). Cells layered at the interface were washed twice with phosphate-buffered saline (PBS) and resuspended in RPMI medium supplemented with 10% fetal calf serum (FCS). The percentage of monocytes was 1% to 4% in B-CLL samples and less than 1% in HCL samples.

Tonsils (three samples) were obtained from children undergoing tonsillectomy after antibiotic treatment and were teased with blunt forceps. Cell suspensions were washed with PBS and allowed to adhere to plastic for one hour. The nonadherent cells were resuspended with sheep red blood cells (SRBC) and spun onto FH for 30 minutes at room temperature. The cells at the interphase were collected and resuspended in RPMI containing 10% FCS and the percentage of

From the Cattedra di Clinica Medica A, Istituto di Medicina Interna, and the Istituto di Istologia e Embriologia Generale, Facoltà di Medicina, Università di Torino, Torino, Italy. Supported by Progetto Finalizzato "Oncologia" of the Italian National Research Council (CNR) with Grants No. 84.00482.44 (F.C.C.) and 84.00638.44 (F.C.M.). L.B. is recipient of a fellowship of the Comitato Gigi Ghirotti.

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Address reprint requests to Dr Federico Caligaris-Cappio, Cattedra di Clinica Medica A, Via Genova 3, 10126 Torino, Italy.© 1986 by Grune & Stratton, Inc.

residual T cells was evaluated with Leu 4 (Becton Dickinson, Mountain View, Calif, cat. 7340). In one experiment, MRBC* cells were purified as previously detailed.12,13 Briefly, 5 × 10^6/mL tonsil lymphocytes were incubated with 10^6/mL MRBCs as a pellet for five minutes at 37 °C and then for 30 minutes at 4 °C. The cells, after gentle resuspension, were spun onto FH at 400 g for 25 minutes. The pelleted cells were treated with 0.1% Tris-NH4Cl for 15 minutes at 20 °C to lyse RBCs and spun through FCS.

In vitro cultures. B-CLL, HCL, and normal B cells were resuspended in RPMI 1640 supplemented with 10% FCS at 1 × 10^6 cells per milliliter. Cells were cultured in vitro in 5-mL cultures and incubated for 72 hours on glass coverslips in 60-mm tissue culture dishes (Falcon, Oxnard, Calif) at 37 °C in a water-saturated atmosphere containing 5% CO2. In each experiment, duplicate cultures were set up as controls and as TPA-stimulated samples. TPA was first dissolved in acetone (1.6 mmol/L) and then in absolute ethanol (0.016 mmol/L). The final TPA concentration in the cultures was 0.016 mmol/L, which previous experiments12 had shown to be optimal for inducing phenotypic changes without major decrease in viability.

After 72 hours, the percentage of control uninduced B-CLL and HCL cells that showed spontaneous adherence to coverslips was 50% to 80%. The percentage of adherent TPA-induced B-CLL and HCL cells was more than 80%. Of the nonadherent cells, 10% to 20% were nonviable as assessed by trypan blue dye exclusion. On the contrary, almost all normal B cells were nonadherent, and the percentage of dead cells was 5% to 10%. The free-floating cells were harvested and stained in a poly-L-lysine-treated glass coverslips for 30 minutes. The slides were then washed briefly with PBS, allowed to dry, fixed and stained as described.

Fluorescence microscopy. Coverslip-attached cells were processed both for the expression of surface markers and for the analysis of cytoskeletal structures.

The expression of surface markers was investigated on air-dried, acetone-fixed cells stained in double fluorochrome immunofluorescence using mouse monoclonal antibodies (Mo Abs), followed by goat antimouse IgGs tagged with fluorescein isothiocyanate (FITC) and goat antihuman Ig isotypes tagged with tetraethylyrhodamine isothiocyanate (TRITC). We used the B cell specific Mo Abs B1 (Coulter cat. No. 6602140), B4 (Coulter, Hialeah, Fla, cat. No. 6602683), RB4 (gift of Professor G. Janossy, London), the monoclonal reacting MOI (Coulter, cat. No. 6602147), MO2 (Coulter cat. No. 6602141), UCHM1 (gift of Dr P. Beverley, London), and rabbit antiserum to human Ig isotypes (μ, δ, γ, σ, ε, λ) labeled with TRITC from Dakopatt (Glostrup, Denmark, cat. No. R-152, R-148, R-151, R-153, R-154, R-155). MO1 and MO2 Abs are not monocyst-specific, since they react with granulocytes and bone marrow precursors. Nevertheless, they are unreactive with normal and malignant B cells while detecting both normal and malignant monocytes. Thus, MO1 and MO2 discriminate between B lymphocytes and cells of the monocyte-macrophage lineage.

The analysis of cytoskeletal structures was performed on coverslip-attached cells fixed in 3% formaldehyde (from paraformaldehyde) in PBS containing 2% sucrose directly layered on detergent-permeabilized cells and incubated for 30 minutes at 37 °C. After rinsing with PBS, coverslips were incubated with either FITC- or TRITC-tagged rabbit antimouse IgGs (Dakopatt, cat. No. R-270) used at 0.1 mg/mL for 30 minutes at 37 °C. In some experiments, F-actin was simultaneously decorated by adding R-PH (1 μg/mL) in the second antibody step.

Stained coverslips were mounted either in 50% glycerol in PBS or in Mowiol 4:80 ( Hoechst, Frankfurt, FRG) and viewed with the appropriate filters in a Leitz Dialux epifluorescence microscope equipped with a Zeiss planapochromat 63 x/1.4 oil immersion lens. For determining adhesion, PBS-glycerol–magnified coverslips were examined at 50 x power in a Leitz Diavert microscope equipped for interference-reflection contrast.

Fluorescence photographs were recorded on Kodak Tri x films rated at 1250 ISO and developed in Gradual ST20 developer (Ornano, Milan). Interference reflection pictures were recorded on Agfa Ortho 25 films.

Finally, in each experiment, coverslip-attached cells were checked for the presence of nonspecific acid esterase with standard cytochemical methods.

RESULTS

Characterization of cell populations. Normal tonsil B cells were strongly IgM* and did not form rosettes with MRBCs. The purified MRBC* cells were weakly IgM* and coexpressed the T1 antigen (RFT1*). Both cell populations reacted with B1, B4, and RB4 (95%) and did not stain with Mo1, Mo2, UCHM1 (1%). The phenotype was left essentially unchanged by TPA treatment.

B-CLL cells showed the typical features of weak surface Ig expression (80%) and MRBC positivity (60% ± 9%). In 21 of 23 cases, the monoclonal B cells were RFT1* (90%). All cases were also B1*, B4*, RB4* (85%) and failed to react with Mo1, Mo2, UCHM1 (1%). After TPA, all cases lost slg; B1, B4, and RB4 reactivity became faint or was lost. As already shown in previous studies,12,13 the T1 antigen expression was retained. Sixteen of 23 cases expressed cytoplasmic Ig of the same heavy and light chain expressed by fresh uninduced cells. In no instance was positivity with Mo1, Mo2, or UCHM1 recorded (less than 1%).

HCL cells were Ig*, reacted with B1, B4, RB4 (80%) but failed to stain with Mo1, Mo2, UCHM1 (5%). The phenotypic features were left essentially unchanged after TPA treatment. B-CLL and HCL cells, in both uninduced state and after TPA, never showed cytoplasmic nonspecific esterase enzyme activity.

Microfilaments in normal and TPA-induced human B lymphocytes. Substratum-attached human B lymphocytes appear as spherical cells provided with thin laminar slabs accounting for their attachment. The distribution of F-actin–containing microfilaments is difficult to document because of the rounded shape of cells, which is responsible for a uniform fluorescent signal associated with the plasma membrane (Fig 1A). After 72 hours’ exposure to TPA, B cells tend to flatten on a poly-L-lysine substrate, giving rise to a polymorphic population in which F-actin is mostly associated to short peripheral protrusions. Such extensions are in the form of slender (Fig 1B) or thicker (Fig 1C) microvilli, flattened ruffles (Fig 1C and D), and thorns (Fig 1E). Occasionally, arrowhead-shaped protrusions containing
large amounts of F-actin sprout from the cell periphery (Fig 1F).

After TPA treatment, the general pattern of microfilaments suggests that B cells have arranged their actin-containing structures and have probably acquired motility properties that are not expressed before treatment. The marked polymorphism of TPA-treated B cells suggests analogy with the cytoskeletal pattern expressed by control HCL cells (see later).

**Microfilaments in untreated B-CLL and HCL cells.** In cultured B-CLL cells, three phenotypically variant forms can be identified. In each case examined, a small proportion (about 10%) of cells are rounded, and their appearance and staining pattern with R-PHD are barely distinguishable from control B lymphocytes (eg, Fig 1A). This cell population probably represents a variant that cannot achieve adhesion with the culture substratum. The size of this subpopulation may be underestimated because staining manipulations may have removed a considerable proportion of these cells.

The majority of cells (about 80%) shows a characteristic staining pattern with R-PHD. The main feature is the concentration of F-actin in multiple, rather regularly distributed dots that, by careful focusing, appear to be associated with the ventral membrane sitting in contact with the adhesion substratum (Fig 2A). When examined in the interference reflection microscope, which measures the distance between cells and the adhesion substratum, these dots appear to correspond to close contact areas (Fig 2B). In these cells, the nucleus is mostly centrally located (Fig 2C). Further evidence that actin-containing dots are associated with punctate adhesion sites can be inferred from the fact that, when cells are gently streamed with a jet of buffer before fixation, dots remain attached to the substratum and leave a footprint of the cell ventral membrane.

In a third subpopulation, representing a minority of attached cells (about 10%), the dots are absent, but the general shape is more irregular because polymorphic protrusions sprout from the cell surface. Such patterns are similar to that exhibited by TPA-induced normal B lymphocytes (see Fig 1B through F) and represent the main phenotype of in vitro HCL cells (see later). B-CLL cells showing mixed features, ie, both dots and processes, are observed with low frequency (less than 5%).

As a rule, the majority of control HCL cells show a wealth of protrusions in the form of filopodia and ruffles, which are located both at the peripheral rim and over the dorsal surface (Fig 3A). Such protrusions are clearly visible on fluorescent PHD-stained cells on the basis of their rich F-actin content. A minor fraction of HCL cells (around 20%; Fig 3B) show dot-like accumulations of F-actin at the ventral membrane similar to those shown by the majority of B-CLL cells and some thin microfilament bundles (Fig 3B). Cells showing both dots and membrane protrusions (eg, Fig 3C) are infrequently observed (about 5%).
Changes of microfilament pattern induced by TPA treatment in B-CLL and HCL cells. TPA treatment induces marked changes in the microfilament distribution of B-CLL and HCL cells. The most remarkable effect in B-CLL cells is the constant clustering of the dot-like accumulations of F-actin observed in untreated cells, which comes along with considerable flattening and improvement of adhesion (Fig 2D). Clusters of dots are usually positioned in the area below the nucleus but may be eccentric or clearly displace the nucleus, which sometimes acquires a bean-like shape (Fig 2F). In the interference reflection microscope, a broad close contact area appears to be coincident with the cluster of dots (Fig 2E) and often extends beyond it. The clustering of dots is a regular event on TPA treatment and can be observed in most cells in the vast majority of cases.

In a number of B-CLL samples (about 30%), TPA treat-

Fig 2. R-PHD fluorescence staining of F-actin in uninduced (A) and TPA-induced B-CLL cells (D, G). Interference reflection and phase contrast pictures of the same cells are shown, respectively, in (B) and (E) and in (C), (F), and (H). In uninduced B-CLL cells (A), actin-containing podosomes are scattered all over the ventral membrane and give rise to a grayish pattern of close contact in the interference reflection microscope (B). The nucleus is apparently located in the cell center (C). After TPA induction, podosomes cluster in a sharply defined area (D) that corresponds to spotty close contacts (E). The nucleus is generally displaced outward by the cluster of podosomes, suggesting that these structures extend deeper in the cytoplasm of these cells (F). A nonadherent cell overlying the flattened one is out of focus and barely visible (lower right) in (D), (E), and (F). In some induced B-CLL cells, sprouting of long dendritic processes takes place (G and H). Such a pattern is identical to that induced by TPA in HCL cells (see Fig 3). (Original magnification ×3,500; current magnification ×2.275)

Fig 3. R-PHD fluorescence staining of uninduced (A through C) and TPA-induced (D) HCL cells. Uninduced HCL cells show a polymorphous pattern of ruffles and peripheral protrusions, justifying the "hairy" appearance. Some cells show obvious podosomes (B, lower left) and, occasionally, thin microfilament bundles (B, upper right). On TPA induction, HCL cells sprout long processes that make intertwined networks reminiscent of some tissue cultures of nerve cells (D). (Original magnification ×3,500; current magnification ×2.275)
ment induces also the appearance of polymorphic stellate cells whose long processes form a complex intertwined network (Fig 2G and H). Within such network, scattered cells with clustered dots are sometimes found together with intermediate forms, thus suggesting that cells showing either stellate appearance or clustered dots may represent a continuum of interchangeable forms (not shown).

In all three cases of HCL examined, TPA treatment induces the appearance of stellate cells forming an intricate dendritic network (Fig 3D). Microfilaments are associated with the membrane in polymorphic protrusions. The presence of clustered F-actin dots is never observed in TPA-treated HCL cells.

**DISCUSSION**

The close association between lymphocyte receptor function and the cellular contractile system has focused attention on lymphocyte cytoskeleton (for review, see refs 1 and 2). Both normal and malignant lymphoid cells have been analyzed, and several reports have detailed the biochemistry and function of cytoskeletal proteins in B-CLL and HCL. The present study was undertaken to evaluate the organization of some cytoskeletal structures in HCL and B-CLL cells, as well as in normal B cells, and the rearrangements that might occur on activation in vitro. Our approach has been to study cultured adherent cells at rest or after activation with TPA. The rationale of this approach is threefold. First, adherent cells allow a detailed analysis of intracellular filamentous networks. Second, TPA has been shown to influence both shape and motility of different cell systems. Finally, a close lineage relationship has recently been established between B-CLL and HCL by culturing the malignant cells in vitro in the presence of nanogram TPA concentrations and demonstrating that TPA-induced B-CLL cells acquire the typical morphology, enzyme activity, and surface markers of HCL cells.

Our first observation is that circulating uninduced HCL cells already have an altered organization of the microfilament pattern, since they show a wealth of peripheral microvilli, which account for their “hairy” appearance. These are absent in both normal and other neoplastic B cells. TPA stimulation emphasizes such pattern by producing long dendritic processes in which intermediate filaments also grow.

The peculiar organization of the cytoskeleton in HCL may explain the tendency of these cells to adhere spontaneously in vitro without any artificial “coating” of the substratum. The in vitro adhesive properties of HCL cells may provide an explanation for the peculiar clinical behavior of the disease, which, in its typical form, presents very low numbers of circulating malignant cells but a marked infiltration of both bone marrow (BM) and peripheral lymphoid organs, notably the spleen. B-CLL cells relentlessly accumulate in the BM and lymphoid organs and fail in large part to recirculate. These cells, when cultured in vitro, adhere spontaneously to glass coverslips and do show adhesion structures. Our in vitro data may represent the experimental counterpart of the B-CLL sluggish recirculation in vivo.

![Fig 4. Immunofluorescence localization of vimentin in uninduced (A) and TPA-induced B cells (B), B-CLL (C), and HCL cells (D). Individual vimentin filaments cannot be resolved in normal B cells (A), while they can be observed after induction (B) also in B-CLL cells (C). Vimentin filaments do not extend into thin cellular processes but do so in the long dendritic extensions of TPA-induced HCL cells (D). (Original magnification ×3,600; current magnification ×2,275)](attachment)
The second observation is that the substratum adhesion of B-CLL cells is not mediated by the conventional focal contacts (also called "adhesion plaques") described in other cell types, eg, in fibroblasts. Rather, adhesion is mediated by dot-shaped close contacts that have been earlier described in a variety of transformed cells and recently characterized and called podosomes. Podosomes concentrate the bulk of cellular actin and other cytoskeletal proteins in short protrusions of the ventral membrane identified by electron microscopy. In B-CLL cells, the major portion of cellular F-actin is localized in podosomes, but some is found also submembraneously in peripheral and dorsal ruffles. There is a substantial lack of bundled microfilaments, and therefore, stress fibers are not observed.

The expression of podosomes and the distribution of cytoskeletal proteins appear as a continuum in uninduced B-CLL, TPA-induced B-CLL, and HCL cells. This finding further supports the existence of a close link between B-CLL and HCL. However, some points still have to be clarified. In uninduced B-CLL cells, podosomes are scattered, but after induction, they consistently cluster in a sharply defined area of the ventral membrane sitting beneath the nucleus and sometimes displacing it. The mechanism(s) that governs this event is unknown. Unknown as well is the reason why some cases of TPA-induced B-CLL cells sprout long dendritic processes identical to those shown by HCL cells after TPA. Also, the relationship between podosomes and dendritic processes bringing about a full redistribution of intracellular actin is not yet understood. The mechanics of these events should be further investigated in the pleiotropic actions of TPA (for review, see refs 21 and 39) and in the largely unknown cascade of events following the activation of protein kinase C.

The third observation is that bona fide podosomes are not observed in normal lymphocytes at rest or after TPA induction. So far, the only nontransformed cells that have been shown to adhere by means of podosomes are osteoclasts and some monocyte-derived cells, including macrophages (ref 48 and P.C. Marchisio, F.G. Giancotti, A. Telf, A. Zambonin-Zallone, and G. Tarone, manuscript in preparation). However, the number of monocytes in our samples was almost negligible compared with the number of adherent cells. Also, monocyte markers could not be detected in B-CLL cells both uninduced or after TPA treatment. B-CLL cells express monoclonal IgS on their surface or within the cytoplasm and stain with the B cell-specific Mo Abs B1, B4, and RBF4. They fail to react with the monocyte-specific Mo Abs Mo1, Mo2, and UCHM1 and do not exhibit cytoplasmic nonspecific esterase activity. HCL cells have been shown by gene rearrangement studies to belong to the B cell lineage, although with some usual features, such as a strong adhesive tendency (but see earlier) and the ability to phagocytose latex particles. They are also nonspecific esterase negative.

In conclusion, podosomes are adhesion structures that appear associated with B-CLL and (partially with) HCL malignant B lymphoid cells but are absent in their normal counterparts. It is, therefore, important to point out that podosomes have been found also in mammalian and avian cells transformed by Rous sarcoma virus. In transformed cells, podosomes are peculiar adhesive structures that concentrate tyrosine-phosphorylated proteins, including the specific tyrosine kinase pp60src. Thus, it is tempting to suggest that these cytoskeletal structures may represent one hallmark of transformation also in B-CLL cells. The transformation of a normal B lymphocyte into a neoplastic B-CLL cell might be marked by a rearrangement of its membrane-microfilament interactions, leading to the appearance of novel adhesion properties. Whether the expression of cellular oncogenes is responsible for the whole pattern of cytoskeletal changes in these cells is a promising avenue for future investigations.

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F Caligaris-Cappio, L Bergui, L Tesio, G Corbascio, F Tousco and PC Marchisio