Distinctive Phorbol Ester-induced Morphological and Surface Antigen Changes in Mycosis Fungoides, the Sézary Syndrome, and Adult T-Cell Leukemia

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ABSTRACT

Clinical and pathological studies of cutaneous T-cell lymphomas (CTCL) reveal variations in tumor cell morphology and surface membrane phenotype that are of diagnostic and prognostic importance. Our study investigates blastic transformation and surface antigen change on CTCL cells in vitro under the influence of tumor-promoting phorbol ester (TPA) and phytohemagglutinin. Both agents transformed tumor cells with cerebriform nuclei into blast cells within 5 days; however, Sézary cells were somewhat resistant to transformation with phytohemagglutinin. Multinucleated cells with prominent nucleoli resembled Reed-Sternberg cells of Hodgkin’s disease. These morphological changes simulated the appearance of the aggressive tumor stage of mycosis fungoides. During blast transformation, the erythrocyte rosette receptor was induced by TPA on sheep erythrocyte-rosette-negative Sézary cells from one patient. During the first 24 hr in vitro, Sézary and MF cells stimulated by TPA lost Leu 3a (T4) antigen while maintaining original high levels of Leu 1 antigen. In contrast, leukemia cells from patients with adult T-cell leukemia (ATL) were resistant to modulation of Leu 3a antigen by TPA; 3A1 antigen on CTCL and ATL cells was unaffected by TPA. Blastic transformation of CTCL cells was observed with both TPA and phytohemagglutinin, but helper T-cell antigen Leu 3a (T4) and erythrocyte rosette changes occurred only with TPA. Thus, blast transformation and surface differentiation were not directly related. These results provide a possible model for the study of blast transformation and surface antigen/receptor variation in CTCL. They also may provide an independent test for the distinction of CTCL and ATL in vitro. Finally, they illustrate the relative resistance of ATL to surface antigen modulation as previously shown for Tac antigen modulation by anti-Tac antibody.

INTRODUCTION

Initial studies of the immunological phenotype of circulating Sézary cells in CTCL have indicated that the neoplastic cells are derived from a restricted subset of well-differentiated post-thymic helper-inducer T-cells which are E-rosette positive, T11+, T4+, T8−, and 3A1− (4, 17). Further immunological studies of CTCL have revealed an unexpected heterogeneity of tumor cell phenotype that may be related to prognosis (38, 39).

Variation in the expression of the E-rosette receptor, other T-cell antigens (T4, T8, T9, Leu 1, 3A1), and la-antigens all have been described (5, 6, 10, 15, 16, 21, 38, 39). It has been suggested that expression of 3A1 antigen accompanies tissue infiltration of CTCL cells (16). Loss of Leu 1 and/or T4 antigens may occur during development of the tumor stage of disease (38, 39). Expression of la-antigens seem to forecast a short survival (39). Typical Sézary cells have irregular, cerebriform nuclei and most often small or inconspicuous nucleoli (6, 8, 24). However, in advanced stages of CTCL, the neoplastic cells sometimes appear less well-differentiated having blast-like nuclei and prominent nucleoli (13, 22, 31, 36, 38). In this study, we sought to develop an in vitro model for blast transformation of Sézary cells, and to determine whether phenotypic change of Sézary cells occurred during this process. We found that Sézary cells undergo blast transformation in vitro under the influence of TPA or PHA, but immunophenotypic change was observed only with TPA. We discuss the possible significance of blastic transformation and phenotypic change in CTCL.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells from patients with the Sézary syndrome (2 patients), mycosis fungoides (1 patient), and ATL (2 patients) were obtained by discontinuous density gradient centrifugation with Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). In each case, there was greater than 90% viability of the separated cells as determined by exclusion of trypan blue dye. The distinctive morphology of the leukemic cells was confirmed in Wright’s-Giemsa-stained peripheral blood smears and cytocentrifuge preparations. Peripheral blood mononuclear cells from normal healthy volunteers were used as controls.

Culture Medium. Cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 2% NaHCO3, antibiotics (streptomycin, 100 μg/ml; and penicillin, 100 units/ml), and 15% fetal calf serum (all obtained from Grand Island Biological Co. Laboratories, Grand Island, NY).

Inducing Agents. Cells were incubated in the presence of 1.6 × 10−7 M 4-β-phorbol 12,13-dimyristate (TPA) from Sigma Chemical, St. Louis, MO, and/or 1% PHA (Grand Island Biological Co.) in a humidified atmosphere with 5% CO2 at 37°. Since limited numbers of leukemic cells were available for our studies, an optimal concentration of TPA was selected from other studies of T-cell lines (7). Cells were harvested after 4 hr and at 24-hr intervals on Days 1, 2, and 5. TPA- and PHA-stimulated cultures were always at least 70% viable at the end of experiments. Cells were then washed 3 times in phosphate-buffered saline (pH 7.4) and used for morphological and immunological phenotypic study.

Surface Marker Analysis. The number of E-rosette-forming cells was determined by use of neuraminidase-treated sheep erythrocytes as described previously (19).

Cell surface antigens were examined by staining cells with a panel of monoclonal antibodies and examining them with a phase-contrast im-
Table 1. TPA induction of the E-rosette receptor on E-rosette-negative Sézary cells. PHA stimulated growth of E-rosette-positive cells.

<table>
<thead>
<tr>
<th>Antibody clone(s)</th>
<th>Antigen(s) recognized</th>
<th>Reactive cells</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.8 (T11)</td>
<td>E-rosette receptor p55*</td>
<td>All E-rosetting T-cells</td>
<td>Kamoun et al. (20)</td>
</tr>
<tr>
<td>10.2 (Leu 1)</td>
<td>p55-67</td>
<td>Thymocytes, all T-cells except some cytotoxic-suppressor T-cells</td>
<td>Martin et al. (25)</td>
</tr>
<tr>
<td>3A1</td>
<td>p40</td>
<td>Thymocytes, all T-cells except some helper T-cells</td>
<td>Haynes et al. (14)</td>
</tr>
<tr>
<td>T4 (Leu 3a)</td>
<td>p62</td>
<td>Thymocyte subpopulation, helper T-cells</td>
<td>Ledbetter et al. (23)</td>
</tr>
<tr>
<td>T8 (Leu 2a)</td>
<td>p32-33(reduced), p70(unreduced)</td>
<td>Thymocyte subpopulations, cytotoxic-suppressor T-cells</td>
<td>Evans et al. (9)</td>
</tr>
<tr>
<td>T6</td>
<td>p44-12</td>
<td>Cortical thymocytes, Langerhans cells</td>
<td>McMichael et al. (26)</td>
</tr>
<tr>
<td>T3</td>
<td>p19</td>
<td>Mature thymocytes, peripheral T-cells</td>
<td>Reinherz et al. (27)</td>
</tr>
<tr>
<td>7.2</td>
<td>Framework region of la p29-34</td>
<td>B-cells, dendritic cells, macrophages, activated T-cells</td>
<td>Hansen et al. (12)</td>
</tr>
<tr>
<td>HNK (Leu 7)</td>
<td></td>
<td>Human natural killer cells</td>
<td>Abo and Balch (1)</td>
</tr>
</tbody>
</table>

* p55, M, 55,000 protein. Other proteins have like designations.

For immunofluorescence, cells were incubated with the first-stage mouse monoclonal antibody at optimal dilution for 30 min at 4°C and washed 3 times with phosphate-buffered saline containing 0.1% sodium azide and 1% bovine serum albumin. This was followed by incubation with the second-stage goat anti-mouse IgG fluorescein isothiocyanate-coupled F(ab')2 fragments (Cappel Laboratories, West Chester, PA). After 3 washings, at least 100 cells were examined by immunofluorescence in a Zeiss photomicroscope III equipped with epifluorescence, a phase-contrast condenser, and ×63 phase-contrast objective.

RESULTS

Prior to stimulation with TPA or PHA, CTCL cells had convoluted nuclei, small nucleoli, and a small amount of pale blue cytoplasm (Figs. 1A and 2A). CTCL cells incubated with TPA or PHA underwent blastic transformation that was most marked on Day 5. At this time, the cell diameter increased from 2 to 3 times, the cytoplasm became more basophilic and often vacuolated, and nucleoli became more prominent (Fig. 1, B and C, and Fig. 2C). Mitoses were frequent. Many cells retained a convoluted or cerebiform nuclear outline (Figs. 1B and 2B) and in some instances, appeared to have a large multilobed nucleus (Fig. 1C). Binucleated or multinucleated cells with prominent nucleoli resembled Reed-Sternberg cells (Fig. 2C). ATL cells showed comparatively little morphological change following incubation with TPA or PHA.

At the beginning of culture, leukemic cells from patients with CTCL had a mature helper T-cell phenotype (Leu 1+, T3+, T4+) and did not express 3A1 antigen. The cells of Patient 1 with the Sézary syndrome lacked the E-rosette receptor detected by antibody 9.6 and did not form E-rosettes with sheep erythrocytes. During blastic transformation of the leukemic cells with TPA, there was induction of the E-rosette receptor and the E-rosette receptor-associated antigen, 9.6. The TPA-stimulated cells then formed rosettes with sheep erythrocytes (Fig. 2, B and C). As a result of incubation with PHA, TPA, and PHA plus TPA, the number of E-rosette-forming cells increased from 10% to 22, 40, and 62%, respectively, on Day 5 (Chart 1). In the cultures containing TPA, many of the E-rosette-forming cells retained the nuclear convolutions characteristic of Sézary cells and remained 3A1-negative (3A1-positive cells, presumably normal T-cells, were only 2%). In the cultures containing PHA alone, the number of E-rosette-forming cells was nearly the same as the number of 3A1-positive cells (20%). These E+, 3A1+ cells were thought to comprise an expansion of the patients' residual normal T-cells. Cultures containing both PHA and TPA showed the combined effect of the 2 reagents including an expanded population of 3A1+, E+ nonmalignant T-cells, and a 3A1−E+ leukemic cell population which acquired the ability to form E-rosettes as a result of TPA stimulation. Therefore, it appears that TPA, but not PHA, was able to induce an E-rosette-forming capacity in Sézary cells from Patient 1.

A striking finding was the complete loss of Leu 3a (OKT4) antigen from tumor cells in CTCL cases (Patients 1 and 2) within 4 to 48 hr after TPA, but not PHA, administration (Chart 2). Similar results were obtained with cells from a third patient with CTCL. Normal T-cells also lost Leu 3a (T4) antigen during this time. In contrast, expression of Leu 3a (T4) antigen by tumor cells persisted in 2 other ATL cases, including one case with leukemic cells infiltrating the skin. The number of Leu 3a-positive cells from ATL patients decreased from 82 to 28% (Patient 4) and from 88 to 38% (Patient 5) during the first 24 hr, but never reached the low level found in cultures from CTCL patients, and recovered to original levels by Day 3. At least 25% of cells from Patient 4 continued to express Leu 3a antigen even when the concentration of TPA was increased from 2- to 10-fold. However, normal T-cells and leukemic cells from CTCL patients showed only slight recovery of Leu 3a antigen expression after 5 days of culture with TPA.

Expression of other T-cell-specific antigens, 10.2 (Leu 1), 3A1,
and T8, remained unaltered during the culture of normal and tumor cells with TPA. Each patient’s leukemic cells continued to express high levels of 10.2 (Leu 1) antigen and remained negative for OKT8 antigen. Expression of 3A1 antigen remained at high levels in cultures of Patient 5 with ATL and low in cultures of all other patients. These results show a selective effect of TPA on surface antigen phenotype in T-cell lymphomas affecting the skin.

**DISCUSSION**

This study was undertaken to investigate the observations of blastic transformation and phenotypic change in cutaneous T-cell lymphomas. The appearance of blastic transformation is a sign of poor prognosis associated with leukemic dissemination and/or rapid growth of skin tumors (31, 38). In a separate report, we will describe an illustrative patient who had an original diagnosis of mycosis fungoides established by biopsies of skin and lymph nodes and then had rapid progression of his disease with morphological transformation to an immunoblastic lymphoma. His tumor cells had both cerebriform and immunoblastic features.

In our present study, the blastic transformation of CTCL was reproduced in vitro by TPA or PHA treatment of leukemic cells from several other patients with cutaneous T-cell lymphoma. Morphological examination confirmed the persistence of nuclear convolutions in the transformed leukemic cells. T-cells from normal volunteers also underwent blastic transformation when stimulated by TPA or PHA. However, as shown by others (40), only a small percentage of these transformed cells developed nuclear irregularities that began to approach the cerebriform characteristics of Sézary cells. Our transformed cultures of CTCL also contained binucleated and multinucleated cells resembling Reed-Sternberg cells that can sometimes be found in mycosis fungoides (30) and other T-cell lymphomas that may be confused with Hodgkin’s disease (37).

A consistent surface membrane phenotypic change was observed preceding the blastic transformation of tumor cells from patients with CTCL. There was complete loss of the mature helper T-cell antigen T4 or Leu 3a, while antigen Leu 1 (10.2) remained unchanged. Interestingly, this change occurred only from stimulation of CTCL cells with TPA, but not with PHA. Our experimental results are in agreement with those of Solbach (33) who described the selective loss of T4 (Leu 3a) antigen on normal T-lymphocytes and malignant cells from one patient of the Sézary syndrome. He did not describe the morphological features of these cells or their possible relationship to the histopathology of cutaneous T-cell lymphoma. We and others have noted that blastic transformation and selective loss of T4 and/or Leu 1 antigens can occur in cutaneous infiltrates of patients with advanced stages of CTCL (38). The histology of these lesions reveals a progression from mycosis fungoides to immunoblastic lymphoma.

The antigen 3A1 that is expressed by most mature T-cells is not detected on leukemic cells from most patients with CTCL (17). Thus, the absence of 3A1 antigen served as a convenient marker for CTCL cells during our studies of blastic transformation in vitro; 3A1 was detected only on the small population of residual nonmalignant T-cells persisting in these cultures. The expanding number of 3A1-negative cells correlated closely with the number of transformed tumor cells retaining cerebriform nuclei and lacking both T4 and T8 antigens. Studies of skin-infiltrating tumors in CTCL often reveal a mixture of 3A1− and 3A1+ cells. A major limitation of these studies thusfar has been the inability to differentiate on a cell-to-cell basis between malignant CTCL cells and benign reactive T-cells; the latter commonly express 3A1 antigen (15).

Patient 1 had typical clinical and morphological features of the Sézary syndrome, yet his leukemic cells did not express the E-rosette receptor usually found on Sézary cells. Infrequent cases with E-rosette-negative Sézary cells have been reported (5, 6, 10, 21) but no particular clinical significance of this finding has been demonstrated. Since E-rosette formation by Sézary cells is expected, absence of this characteristic may cause difficulty in the diagnosis of this disorder. Our study showed that the E-rosette receptor may be induced in vitro with TPA and may therefore clarify the diagnosis in such unusual E-rosette-negative cases.

ATL is another postthymic mature T-cell cancer that frequently affects the skin (11, 18, 32, 34, 35). The response to therapy usually is much worse, and survival much shorter for patients with ATL than for patients with CTCL (32). Because of overlapping clinical, pathological, and epidemiological features, it is not always possible to distinguish between patients with ATL and those with CTCL (2, 3, 11, 18, 28, 29). Immunological distinction of ATL from CTCL is also difficult, since leukemic cells from both ATL and CTCL patients express the same helper T-cell phenotype T3+ T4+ (4, 17, 34). Our study, however, indicates a possible difference in behavior between ATL and CTCL cells when stimulated in vitro by TPA. ATL cells were more resistant to blastic transformation and to the loss of T4 antigen. Recovery of T4 antigen expression by ATL cells was also more rapid. These
preliminary results suggest that TPA administration may become a useful in vitro test for the important distinction between ATL and CTCL.

REFERENCES

Fig. 1. Leukemic cells from Patient 3 with mycosis fungoides forming E-rosettes with sheep erythrocytes. A, prior to stimulation with TPA; B, blastic transformation with TPA; C, Reed-Sternberg-like cell after stimulation with TPA.

Fig. 2. Leukemic cells from Patient 1 with the Sézary's syndrome. A, cells do not form E-rosettes prior to stimulation with TPA; B, E-rosette-forming Sézary cell with convoluted nucleus, after stimulation with TPA; C, binucleate Reed-Sternberg-like cell after stimulation with TPA. All figures were made from cytocentrifuge preparations stained with Wright's-Giemsa stain and magnified x 1080.
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