Tubulin as a Major Cell Surface Protein in Human Lymphoid Cells of Leukemic Origin

Robert W. Rubin,2 Marijane Quillen, James J. Corcoran, Ram Ganapathi, and Awtar Krishan

Departments of Anatomy [R. W. R., A. K., M. O.], Pharmacology [J. C.], and Oncology [R. G., A. K.], University of Miami Medical School and Comprehensive Cancer Center for the State of Florida, Miami, Florida 33101

ABSTRACT

Surface-exposed proteins of vinblastine-sensitive human lymphoid cell line of leukemic origin (CCRF-CEM) were examined by the lactoperoxidase-catalyzed iodination and two-dimensional polyacrylamide gel electrophoresis methods. Spots which comigrated with bovine brain tubulin and rabbit muscle actin were prominently labeled in the whole membrane but not in the high-speed supernatant fraction of the disrupted cells. Mild trypsinization of labeled cells removed the iodinated tubulin and actin without significantly affecting the protein staining pattern. Iodination of normal human lymphocytes resulted in no labeling of the tubulin or actin. The presence of surface-exposed tubulin in this leukemic cell line suggests a possible mechanism for their enhanced sensitivity to the cytotoxic action of vinblastine.

INTRODUCTION

The Vinca alkaloids, vinblastine and vincristine sulfate, are important cancer chemotherapeutic agents with clinical activity against a variety of human cancers (8). It is generally assumed that the mechanism for their cytolytic and cytostatic activity is related to their binding to microtubular components of the mitotic spindle. We propose that a fraction of tubulin in these cells exists in the plasma membrane and perhaps in association with other membrane systems and may not have any cytoskeletal function.

MATERIALS AND METHODS

Human leukemic lymphoblasts of the CCRF-CEM cell line (T-cell origin) were obtained from Dr. Herbert Lazarus of Sidney Farber Cancer Center, Boston, Mass. (10, 11). Log-phase cultures were propagated in Eagle's minimal essential medium supplemented with 10% fetal calf serum and the antibiotics, penicillin and streptomycin. Human peripheral blood was collected in heparinized tubes, and mononuclear cells were isolated on a Ficoll-Hypaque density gradient (6).

Cells were washed in HBSS2 and recovered by centrifugation at 100,000 × g for 7 min. The cell pellets were resuspended in 2 ml of HBSS and iodinated with 400 μCi of 125I. The iodination procedure involved the addition of LPO and H2O2, according to the method of Phillips and Morrison (21). Cell viability was monitored prior to and after iodination by trypan blue exclusion. A 100-μl aliquot of the cell suspension was diluted up to 1 ml with HBSS, and 1 drop of trypan blue was added. Counts of dye-excluding and nonexcluding cells were made on a hemocytometer.

Counts of dye-excluding and nonexcluding cells were made on a hemocytometer. The reaction was stopped by adjusting the mixture to 5 mM with cold NaCl. The cells were then diluted with HBSS, divided into 2 equal aliquots, and washed 3 times as above. Labeling was also accomplished by 2 additional methods: (a) iodination with 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (lodox) (12) and (b) 125Iodo sulfuric acid as described previously (26). For the trypsin experiments, the cell pellet was resuspended in 5 ml of HBSS containing trypsin, 200 μg/ml. The control pellet was also resuspended in 5 ml of HBSS. The 2 cell suspensions were incubated at room temperature for 5 min, diluted 10 times with cold HBSS, and immediately washed 3 times. The final pellets were then resuspended in 5 ml of 10 mM Tris buffer, pH 7.4, and allowed to stand on ice for 5 min to induce swelling. The samples were then disrupted with a Polytron tissue homogenizer (20 sec at a setting of 7) and centrifuged at 100,000 × g for 1 hr in an SW50 rotor (Beckman). Both the supernatants and resulting pellets were saved for 2-dimensional PAGE. The frozen supernatants were concentrated by lyophilization, and all samples were stored at −80° until the electrophoretic run.

The lyophilized and frozen samples were thawed at room temperature and were solubilized for 2-dimensional PAGE as described previously (25). After the 2-dimensional gel run was completed, the gels were stained with Coomassie blue, photographed, and then dried or filter paper. This was followed by autoradiography of the dried gels using Dupont Cronex film and rare earth screens. For most experiments, 1 to 3 days of exposure were sufficient to detect as many as 25 individual spots. Tubulin was identified by comigration with 3-times-cycled bovine brain tubulin, which was stored at −80° prior to solubilization.

1 This work was supported by USPHS Grants CA-14395 and CA-23688.
2 To whom requests for reprints should be addressed, at Department of Anatomy (R-124), University of Miami Medical School, P. O. Box 016960, Miami, Fla. 33101.

Received October 28, 1980; accepted December 22, 1981.

Downloaded from cancerres.aacrjournals.org on July 21, 2017. © 1982 American Association for Cancer Research.
RESULTS

Our preliminary results indicate that peroxidase-catalyzed iodination of CCRF-CEM cells in suspension produced a pattern of 15 to 25 spots depending on the length of autoradiographic exposure. In all cases, the most prominently labeled proteins comigrated with bovine tubulin purified by 3 cycles of polymerization. Also labeled was a protein we have tentatively identified as actin, as it comigrates with rabbit muscle actin in the 2-dimensional gel electrophoretogram. In our best preparation, the CCRF-CEM tubulin clearly resolves into a faster running β component and 2 α spots. This is typical of bovine brain tubulin and can be taken as a diagnostic characteristic for this protein. An examination of Figs. 1 and 2 indicates the following. (a) It is apparent that the radioactivity pattern is quite different from the staining pattern, which would be expected if only cell surface-exposed proteins were being iodinated. (b) A protein tentatively identified as CCRF-CEM intermediate filament protein was never labeled in our experiments. This spot on our 2-dimensional gels comigrates with intermediate filament protein isolated from acetic acid extracts of normal rat kidney epithelial cells (24). The lightly labeled triplet spots underneath α tubulin were shown to partially comigrate with intermediate filament protein but to possess an entirely different shape than the staining pattern of intermediate filament protein (compare Fig. 1, A, C, and G). Furthermore, we never observed trichloroacetic acid-precipitable radioactivity in the high-speed supernatants from the CCRF-CEM cells labeled as intact live cells and then lysed by Polytron treatment. Thus, it would appear that none of the soluble proteins were being radioiodinated. (c) Mild trypsin treatment at room temperature after iodination greatly reduces the amount of bound radioactivity and completely eliminates it over the tubulin spots. This is under conditions where the trypsinization does not appear to significantly affect the staining pattern. Thus, we interpret these results to suggest that the trypsin only cleaved proteins exposed on the surface, and these proteins are in fact the ones that are labeled by the iodination procedure. Tubulin and actin therefore appear to be surface-exposed proteins in these cells. To further reduce the possibility that we were looking at internalized iodine, a whole-cell homogenate was made by swelling the cells in 10 mM Tris buffer followed by homogenization with a Polytron. This preparation was iodinated in a manner identical to the iodination of the intact cells. Fig. 1 shows that the iodination pattern now corresponds more closely to the staining pattern of the protein we tentatively identified as intermediate filament protein.

We have performed similar experiments on other cell types and on the mononuclear cell fraction isolated from the peripheral blood of normal donors. In all cases, except for the mononuclear population from peripheral blood, some radiolabeled tubulin could be detected. No radioactivity was detected over the tubulin spots in this population (see Fig. 2). No detectable tubulin staining or radioactivity after iodination could be detected in the tubulin region of 2-dimensional gels of high-speed pellets from these cells. The other cells labeled in this manner were from cultures of bovine chromaffin cells and from rat prostate tumor cells. The amount of labeling of tubulin varies dramatically from cell type to cell type with the CCRF-CEM line showing the greatest label over the tubulin. Actin also is labeled in the CCRF-CEM line although its labeling is variable in other cell lines studied. It is of interest that high-speed pellets from homogenized normal mononuclear cells show little or no tubulin staining with Coomassie blue, whereas the same preparation made from the CCRF-CEM line shows prominent tubulin spots (see Figs. 1 and 2).

To further eliminate the possibility that the radioiodination procedure causes the release of some intracellular tubulin, we examined 10 different iodinated preparations of CEM cells for trypan blue dye exclusion. The results were highly variable depending on the age of the culture and the number of centrifugations used. The values ranged from less than 5% nonexcluding cells (equal to untreated controls) to greater than 50%. However, these values had no effect on whether labeled tubulin was observed on 2-dimensional gels. In a number of cases, we observed a large amount of labeled tubulin on cells which showed control levels of dye exclusion. Surprisingly, for those preparations showing high numbers of nonexcluding cells, the LPO treatment alone (prior to H2O2 addition) produced the bulk of the nonexcluding cells. Therefore, in order to exclude the potential damaging effects of LPO, we have repeated these experiments using 2 completely different iodination methods. When cells were radioiodinated using the iodogen catalyst, tubulin and actin were labeled similarly to the LPO-catalyzed reaction. Labeling with [125I]iodosulfanilic acid produced a different autoradiographic pattern while still showing strong radioactivity associated with the tubulin and actin. The radioactivity was shifted toward the acidic side of these protein spots. At no time have we ever observed more than 5% nonexcluding cells after this nonenzymatic iodination or after the iodogen procedure.

DISCUSSION

Our results suggest that a protein containing the isoelectric point and molecular weight characteristics of brain tubulin is a major component of the cell surface of human leukemic lymphoblasts of CCRF-CEM cell line and may represent a somewhat less prominent plasma membrane constituent in a number of other cell types. Its absence in the plasma membrane of normal blood mononuclear cells suggests the possibility that cells that either came from leukemic blood or became established as long-term in vitro lymphoid cell lines have differential sensitivity to the cytolytic activity of Vinca alkaloids, possibly due to the insertion of microtubule protein into their plasma membrane.

There are possible pitfalls concerning the interpretation of LPO-catalyzed iodination of cell surface experiments. It is conceivable that the enzyme along with the 125I and hydrogen peroxide is internalized by cells, thereby allowing some iodination of internal proteins. This would affect our results since tubulin is a major cytoplasmic protein. To eliminate this possibility, we compared the iodination patterns of intact and homogenized CCRF-CEM cells and found the patterns to be quite different. Furthermore, should our results be due to nonspecific internalization of the label, one would expect that all the proteins (and not only a subset) resolved by the 2-dimensional PAGE would be labeled. Also of interest in these controls was the absence of bound radioactivity in high-speed supernatants.
from CCRF-CEM cells labeled intact. These observations further suggest that the label did not enter the cell, as otherwise the first proteins one would expect to see labeled under these conditions would be the internal soluble proteins. The trypsin experiments further confirm the cell surface specificity of the iodination procedure. Proteases are known to selectively cleave cell surface components (3, 22) without affecting the internal protein constituents. This appears to have been the case in our trypsin treatment experiments which were done at nonoptimal temperature (21 °). The staining pattern was unaffected, although the bulk of the iodinated proteins was removed from the trypsinized preparation (see Fig. 1).

Another possible explanation for our results could be that cellular material is left behind in the culture flask during normal cell growth and movement. We have seen such material adhering to the surface of plastic flasks in cultured normal rat kidney cells, and this could explain our results with monolayer cultures. However, in the CCRF-CEM line, the cells are grown in suspension, and throughout the course of the experiment, numerous washes were carried out to remove any adhering cell lysate material. In spite of this, it is conceivable that some nonspecific cellular proteins have adsorbed to the outside of the cell possibly during the preparation and labeling procedure. Under these conditions, one would expect the internal cell proteins also to be labeled. If this assumption were true, one would expect the CCRF-CEM cells to contain, under normal conditions, a relatively high percentage of cells which are in the process of dying in order for enough lysate material to adsorb to the outside of the living cells. This possibility can be ruled out since, on a number of occasions, CCRF-CEM cells with a viability (determined by trypan blue dye exclusion) of greater than 95% produced heavy tubulin labeling. This is further confirmed from our lodogen and [125I]iodosulfanilic acid labeling which never affected viability. An additional advantage of the lodogen catalyst is that the compound is coated on the reaction tube. Since it is insoluble in water, it is not free to enter any cells which may have become “leaky.” Also, the interesting acidic shift of the radioactivity associated with tubulin and actin labeled with [125I]iodosulfanilic acid would be expected if a portion of these proteins were having additional negative charge added to them by the iodosulfanilic acid compound. It appears that only a small portion of these proteins (cell surface exposed) exhibit the charge shift, as most of the protein spot, which represents the whole-cell tubulin and actin, remains unlabeled.

Finally, it should be mentioned that we have not unequivocally proven that the polypeptides we observe as spots are tubulin, actin, and intermediate filament proteins. However, the case for tubulin is especially strong for the following reasons. We consider it highly unlikely that a different cell surface protein(s) would: (a) possess the same isoelectric point and molecular weight as does purified tubulin; (b) resolve into 2-α components and one β component; (c) be present at the high concentrations we observe for these and many other cell types we and others have studied; and (d) display apparent 1:1 α:β ratios as is known for purified tubulin.

Our data therefore support the contention that CCRF-CEM leukemic lymphoblasts contain relatively large amounts of surface exposed tubulin, and this tubulin could be the binding site for the cytolicial action of these alkaloids in vitro and in vivo. If this were the case, the mechanism for the cytolytic drug action would be independent of the drug-induced stathmokinetic and cytostatic effects (due to the disruption of spindle microtubules). This mechanism could bring about cell lysis through alteration in the structure of the polypeptides of tubulin on the cell surface, thereby rupturing or disordering the cell membrane. This has been clearly suggested from our earlier time-lapse and electron microscope studies (17). Alternatively, the cell surface binding of the Vinca alkaloids might change the surface to the extent that these cells are now recognized by the immune system. Other explanations are clearly possible.

That tubulin is a normal membrane constituent has been suggested in a number of other reports (4, 5, 13–15, 24, 27, 28). The earliest report was one in which nucleated erythrocytes from chickens bound to a colchicine affinity column (27). This binding was inhibited by the addition of excess colchicine not bound to the column and was unaffected by the addition of lumicolchicine. More recently, it has been shown that liposomes can be centrifuged through high-speed supernatants of brain homogenates, and tubulin will bind to these liposomes in a very specific manner (7). The liposomes are then washed and run on sodium dodecyl sulfate gels. Tubulin is the only protein component resolved in these complexes.

Tubulin is also the major protein component of colchicine-treated membrane fractions from brain homogenate (9). In 2 other recent studies, tubulin was reported to represent a major fraction of the total protein present in flagella membranes (1, 27). These results along with our observations suggest that plasma membrane-bound tubulin may be a general feature of most of the cells, especially of those showing extreme sensitivity to the cytolytic effects of tubulin-binding alkaloids. We are currently attempting to confirm these results using immunofluorescence and flow cytometry with anti-tubulin antibody applied to vinblastine-sensitive and -resistant cell lines.

After completion of this work and preparation of this manuscript, we read the report of Bachvaroff et al. (2) which confirms our basic conclusions described above. Independently, these workers used procedures similar to ours to examine the cell surface proteins in various normal and transformed lymphocytic lines. Most of our work has centered upon our attempts to eliminate certain potential pitfalls in the interpretation of cell surface radiiodination experiments. In particular, we feel we have eliminated the possibility that we are examining internal proteins due to internalization of label or cytosis. In the report of Bachvaroff et al. (2), this problem was not addressed directly, although 2 very important experiments were performed which greatly add to the significance of the present work. In the first experiment, they showed that the apparent cell surface iodination phenomenon that they have been studying is not due to secretion of cytoskeletal proteins into the medium followed by adsorption onto the cell surface. They also demonstrated that normal lymphocytes when transformed by a mitogen can thereby be induced to express the cell surface cytoskeletal components. Thus, their study and our observations strongly suggest that leukemic transformation brings about a dramatic cell surface change whereby the majority of the total cell surface-exposed protein (or at least that protein available for iodination) is altered by the addition or insertion of actin and tubulin into the plasma membrane.

It is of interest that LPO alone can cause a significant increase in the percentage of cells which take up trypan blue. LPO is known to bind and copolymerize specifically with tubulin.
(23). Thus, its effect on our cells may be a result of binding to cell surface tubulin.

REFERENCES


Fig. 2. Shown are the staining and autoradiographic patterns of 2-dimensional gels of normal mononuclear cells after iodination and high-speed centrifugation (100-kg pellet). Note complete absence of staining or label of the tubulin region (arrows). These are enlargements of the central region of the gel.
Tubulin as a Major Cell Surface Protein in Human Lymphoid Cells of Leukemic Origin

Robert W. Rubin, Marijane Quillen, James J. Corcoran, et al.