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Altered Actin Polymerization Dynamics in Various Malignant Cell Types: Evidence for Differential Sensitivity to Cytochalasin B

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ABSTRACT. Using the DNase I inhibition assay, fluorimetric measurements, and immunoblot analysis, we studied quantitatively changes in the actin polymerization dynamics in primary cultures of normal and malignant human lymphocytes, normal human endometrial cells, and in various leukemic and endometrial adenocarcinoma cell lines. The G/total-actin ratio of malignant cells was found to be 1.37 to 1.81-fold higher compared to normal cells, indicating that malignant cells express reduced amounts of polymerized actin. The above findings were corroborated by fluorescence measurements of the amounts of rhodamine-phalloidin-labeled F-actin in normal and neoplastic cells, which showed significantly lower F-actin content in malignant cell preparations. Moreover, the total actin content, as quantitated by the DNase I inhibition assay and by immunoblot analysis, was found to be significantly decreased in the primary cultures of malignant human lymphocytes and endometrial cells when compared to the total actin levels in corresponding normal cells. Proliferation and viability measurements of normal and neoplastic cells in culture, treated equally with cytochalasin B (CB), revealed an increased susceptibility of malignant cells to this anticytoskeletal agent. This was not due to increased CB incorporation in neoplastic cells, as indicated by ^{3}H-CB uptake experiments. In addition, fluorescence microscopy, in the presence of graded concentrations of CB, showed destabilization of microfilaments in the poorly differentiated endometrial adenocarcinoma HEC-50 cells, compared to the well-differentiated Ishikawa cells. In conclusion, all investigated malignant cells are characterized by: (a) higher G/total-actin ratio; (b) decreased F- and total-actin content; and (c) lower resistance to CB treatment. These quantitatively determined parameters may represent potential biochemical indicators reflecting malignant transformation. Moreover, it seems worthwhile to explore whether or not the differential sensitivity of malignant cells to anticytoskeletal drugs may provide a valuable approach to the manipulation of malignant cells. BIOCHEM PHARMACOL 52;9:1339–1346, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. G/total-actin ratio; F-actin ratio; microfilament destabilization; malignant transformation; lymphocytes; endometrial cells

Studies performed in various cell types showed a fast turnover between the actin monomer pool and the microfilaments [1–5]. Although the mechanisms underlying these phenomena remain incompletely understood [6], various aspects of cell behavior, such as changes in volume, locomotion, spreading, and cell-to-cell contact, seem to be dependent on the dynamic equilibrium between monomeric and polymerized actin [4, 7, 8]. These cellular functions are probably tightly controlled in nonneoplastic cells, whereas the uncontrolled growth of malignant cells seems to be related to microfilament disorganization [9]. Indeed, previous morphological studies have shown differences in the actin organization between normal and transformed cells. Thus, a loss of actin filaments was observed in SV40- or Rous sarcoma virus-transformed cells [10, 11], and in cells transformed with RNA or DNA tumor viruses [12]. Similarly, *Ha-ras-1* oncogene-transformed cells exhibit altered microfilament organization and stability [13]. Microfilament alterations, including F-actin fragmentation, were observed in human epithelial HBL-100 cells during their malignant conversion [14]. Moreover, altered actin cytoskeletal organization and loss of actin filaments were reported in human skin fibroblasts from patients at high risk for colon cancer and in cultures from human colonic epithelial cells [15–18].

However, disorganization of actin microfilaments studied by morphological approaches, such as immunofluorescent microscopy, can be estimated only semiquantitatively. Thus, searching for an alternative procedure to quantita-

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‖ *Abbreviations:* CB, cytochalasin B; PBL, peripheral blood lymphocytes; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia.

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tively study intracellular modifications in the dynamic equilibrium between monomeric and polymeric actin forms in malignant cells, in the present study, we used the DNase I inhibition assay, immunoblot analysis, and fluorescence measurements. We measured the G/total-actin ratio and the F- and total-actin content in primary cultures of normal and malignant human lymphocytes, normal human endometrial cells, and in several human leukemic or endometrial adenocarcinoma cell lines. In addition to the above experiments, we evaluated the distribution and stability of the microfilaments in two endometrial adenocarcinoma cell lines by fluorescence microscopy, in the absence or presence of CB,[||] a drug known to induce microfilament depolymerization. Finally, we compared the effect of CB on cellular viability and proliferation of normal and malignant lymphocytes and endometrial cells. The results reported here provide strong evidence that quantitatively assessed alterations in the polymerization dynamics of actin cytoskeleton may characterize malignant transformation.

MATERIALS AND METHODS

Materials

Culture media, DMEM-Ham's F-12, fetal calf serum (FCS), Minimum Essential Medium (MEM) and EDTA solutions were from Flow Laboratories (Irvine, Scotland). Porcine insulin was from Nordisk-USA (Bethesda, MD, U.S.A.). Hank's Balanced Salt Solution (HBSS) and the antibiotic-antimycotic mixtures were from GIBCO (Life Technologies Inc. Gaithersburg, MD, U.S.A.). Ficoll-Paque was from Pharmacia Biotech AB (Uppsala, Sweden). Bovine serum albumin, DNase I, DNA, PMSF, CB, and type I collagenase were obtained from Sigma (St. Louis, MO, U.S.A.). Rhodamine-phalloidin was from Molecular Probes Inc. (Eugene, OR, U.S.A.). [Methyl-^{3}H]-thymidine and [^{3}H]-CB were from New England Nuclear (Boston, MA, U.S.A.). Monoclonal antibody against β-actin and the ECL Western blotting kit were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). All other chemicals were obtained from usual commercial sources at the purest grade available.

Human Endometrial Cells

Primary cultures of human endometrial cells were obtained from endometrial specimens from patients undergoing biopsy for fertility evaluation or hysterectomy. The tissues were trimmed and minced under a laminar flow hood in MEM containing 1% of an antibiotic-antimycotic mixture. Cell dispersion and isolation was carried out in MEM supplemented with 10 μg/mL porcine insulin, 1% antibiotic-antimycotic mixture, and 10% FCS. As discussed in more detail elsewhere [19], it involves: (1) digestion of the minced tissue for 90 min at 37°C using 0.25% of type I collagenase; (2) separation of glands and stroma by filtration through a 45 μ stainless-steel sieve; (3) backwashing the glands from the sieve, followed by pelleting by centrifugation; and (4) separation of epithelial cells from stromal cells in the filtrate by taking advantage of the more rapid adhesion of the stromal cells to tissue culture plastic at 37°C.

Ishikawa and HEC-50 cells were established as permanent cell lines from a well-differentiated or a poorly differentiated endometrial adenocarcinoma, respectively [20, 21]. The cells were cultured in 25-cm^2 flasks in MEM containing 15% FCS in a 5% CO_2–95% air atmosphere at 37°C. Underconfluent cells were washed twice and cultured for 12 hr in DMEM/Ham's F-12 in the absence of serum, but supplemented with 10 mM L-glutamine, 15 mM Hepes, and 1% antibiotic-antimycotic solution to final concentrations of 100 U/mL penicillin and 100 μg/mL streptomycin. For determinations of the cellular actin content and the actin dynamic equilibrium, cells were scraped from the flasks with a rubber "policeman."

Human Lymphocytes

PBL were isolated from freshly obtained heparinized venous blood of healthy volunteers, from patients with chronic lymphocytic leukemia (B-CLL), or from children with acute lymphoblastic leukemia (ALL, cALLA+). Mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation. Interface cells, washed twice in HBSS, and cells from the established lymphoblastic leukemic cell lines Reh-6 and KM were resuspended (10^6 cells per mL) in complete medium RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 g/L sodium bicarbonate, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cell cultures were performed in a 5% CO_2–95% air atmosphere at 37°C in the absence or presence of various concentrations of CB for 24 hr. Cell viability was determined by counting intact cells in the presence of Trypan Blue dye.

DNase I Inhibition Assay for Actin Quantitations

The monomeric (G-) and total actin content was measured in cells by the G-actin-dependent DNase I inhibition assay [22], with minor modifications, as previously described [4, 5]. The intracellular actin content was quantified by reference to a standard curve for the inhibition of DNase I activity, prepared from rabbit muscle actin, isolated as previously described [23]. The G- and total-actin content in Ishikawa cells was related to the total protein content. Protein concentrations were measured with the Bio-Rad protein kit (Bio-Rad Laboratories, Palo Alto, CA, U.S.A.).

Immunoblot Analysis

For total cellular actin determination by immunoblot analysis, cells were rinsed with 1 mL distilled H_2O, followed by ultrasound treatment (3 × 10 sec). Equal amounts of protein (10 μg) of the cell extracts were subjected to SDS electrophoresis, and the resulting protein-bands transferred

to nitrocellulose membranes, using an LKB electroblot apparatus (LKB, Bromma, Sweden). Nitrocellulose blots were incubated with monoclonal mouse antiactin antibodies, followed by incubation with the appropriate labeled second antibody, using the ECL Western blotting kit. Nitrocellulose blots were exposed to Kodak X-omat AR films for variable periods of time. Band intensities were quantitated by PC-based Image Analysis (Image Analysis, Inc., St. Catharines, Ontario, Canada). Actin content in the sample was calculated by reference to a standard curve, prepared with graded concentrations (0.2 to 1 μg) of purified rabbit muscle actin.

Fluorescence Measurements of F-Actin

F-actin content was determined in normal and malignant human lymphocytes and endometrial cells by fluorescence measurements of rhodamine-phalloidin labeled samples, according to the method described by Cable *et al.* and Wu *et al.* [24, 25], with some minor modifications: Briefly, cells were fixed by addition of 0.3 mL of 3.7% formaldehyde in PBS, followed by 15-min incubation at room temperature. The cells were then permeabilized by adding 0.3 mL Triton X-100 (0.2% in PBS); 0.3 mL of rhodamine-phalloidin (1.5 μM in PBS) were added, and samples were left for 30 min. The cells were then washed with PBS (3 × 1 mL) and dissolved in 0.5 mL of 0.1 M NaOH. Fluorescence of the samples was measured in a Perkin-Elmer LS 3B fluorimeter using excitation and emission wavelengths of 550 and 580 nm, respectively.

Fluorescence Microscopy

Ishikawa and HEC-50 cells, grown on 22 × 22 mm cover slips in MEM medium containing 15% FCS, were incubated for 3 hr with different concentrations of CB (10^{-6} to 6×10^{-6} M) dissolved in culture medium. The procedure of cell fixation and the direct fluorescence staining of microfilaments by rhodamine-phalloidin were performed by immersing slides in acetone at −20°C for 3–5 min, with prior incubation in 3.7% formaldehyde for 20 min. Cells were then washed twice in DPBS, incubated for 20 min at room temperature with rhodamine-phalloidin at a dilution 1:20 in PBS, and washed 3 times for 3 min in PBS. All specimens were analyzed in a Leitz Dialux 2 OEB microscope (Wetzlar, F.R. Germany), equipped with epifluorescent illumination. Micrographs were obtained with a 35 mm (C-35AD-4) camera on Kodak P3200 black and white film.

Thymidine Incorporation Assay

Thymidine incorporation in endometrial cells was determined essentially as described in [26]. Briefly, 0.2 mL of final cell suspension was added to flat-bottomed microtiter dishes and incubated at 37°C in a 5% CO_2–95% air atmosphere for 24 hr in the absence and presence of various concentrations of CB. Cells were pulsed with 1 μCi of [methyl-^{3}H]thymidine (specific activity 20 Ci/mmol) for 24 hr. Cell-associated radioactivity, determined by liquid scintillation, was normalized to the total protein content.

^{3}H-CB Uptake Experiments

The intracellular uptake of CB was studied in normal and malignant cells, incubated for different time periods (3 or 24 hr) with 1 μCi [^{3}H]-CB (specific activity 12.5 Ci/mmol), exactly as described previously [13]. Experiments were performed in quadruplicate, and the results expressed as cpm per 10^6 cells and/or cpm per mg of total protein.

Statistical Analysis

Statistical analysis of the total- and F-actin content and the G/total-actin ratio in normal and malignant cells was performed by one-way ANOVA and by unpaired Student's *t* test. In the cell viability and thymidine incorporation experiments, values were normalized as percentage of control. Thus, these data were statistically treated with two nonparametric methods (Kruskal–Wallis and Mann–Whitney tests). In all experiments, N represents the number of patients from whom cells were isolated or the number of cell preparations from the transformed cell lines used in this study.

RESULTS

Malignant Cells and Total Actin Amounts

Total actin amounts were determined in various human normal and malignant cell types by the DNase I inhibition assay. Figure 1A demonstrates that the total actin content in normal human PBL was 108.7 ± 10.1 (mean ± SE, N = 10) μg per mg of total cellular protein, and in primary cultures of CLL, ALL, and in Reh-6 and KM leukemic cells, the corresponding total actin values were significantly lower: 52.5 ± 7.5 (N = 6, $P < 0.01$), 63.1 ± 8.2 (N = 4, $P < 0.01$), 39.2 ± 1.9 (N = 6, $P < 0.01$) and 30.1 ± 2.4 (N = 4, $P < 0.01$) μg actin per mg protein, respectively. Similar results were obtained in endometrial cells. Normal human endometrial cells contained 83.9 ± 11.4 (N = 5) μg total actin per mg protein, and in both Ishikawa and HEC-50 endometrial adenocarcinoma cells, the total actin levels were significantly reduced: 52.4 ± 2.4 (N = 24, $P < 0.01$) and 41.0 ± 0.7 (N = 12, $P < 0.01$) μg actin per mg protein, respectively (Fig. 1B). From these results, we concluded that in all tested malignant cells, the total actin levels were significantly reduced.

These findings were supported by control experiments using quantitative immunoblot analysis. For this, equal aliquots of cellular protein preparations of PBL, CLL, and Reh-6 leukemic cells, as well as Ishikawa and HEC-50 cells, were subjected to SDS electrophoresis and Western blot analysis. As shown in Fig. 2, leukemic cells expressed decreased amounts of total actin compared to normal human PBL. Indeed, by reference to a standard curve prepared with

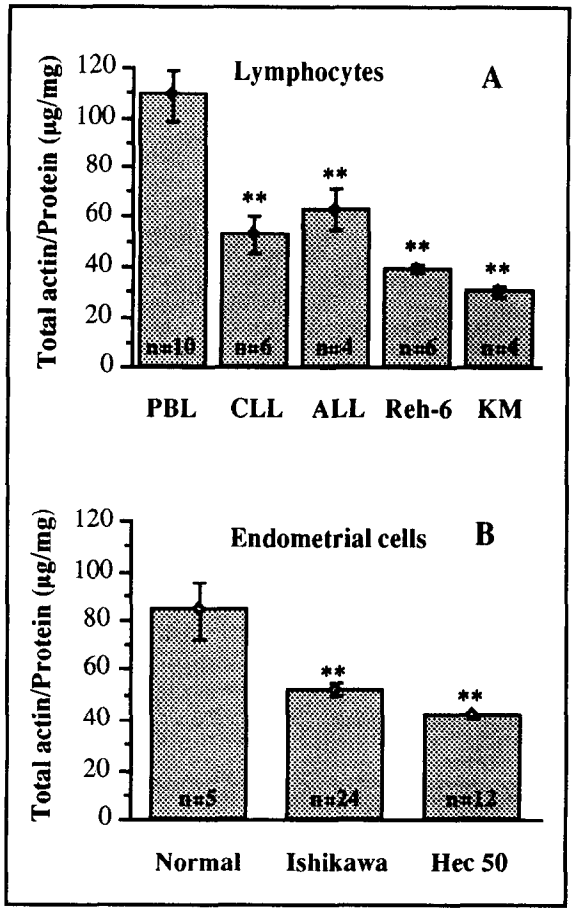

FIG. 1. Amounts of total actin (µg actin per mg of total cellular protein) determined by the DNase I inhibition assay in (A) normal and leukemic human lymphocytes, and in (B) normal human endometrial cells and human adenocarcinoma cell lines. n = number of patients or number of cell preparations from malignant cell lines. **denotes $P < 0.01$.

isolated pure rabbit muscle actin (Fig. 2, lanes 1–5, r = 0.989), total actin content amounted to 125.6 ± 13.4 µg/mg (N = 3) for PBL, 43.7 ± 7.1 µg/mg (N = 3, $P < 0.01$) for Reh-6 and 75.2 ± 6.8 µg/mg (N = 3, $P < 0.01$) for CLL. Similarly, HEC-50 cells expressed lower total actin content (36.7 ± 5.9 µg/mg, N = 3, $P < 0.01$) compared to Ishikawa cells (48.5 ± 4.1 µg/mg, N = 3) (Fig. 2). These values, which are in good agreement with the total actin amounts determined by the DNase I assay (Fig. 1), fully support the conclusion that malignant cells express lower total actin levels.

The Monomer-Polymer Actin Ratio in Malignant Cells

In a second step, in all the above cell preparations, we measured the G/total-actin ratio, which reflects the actin polymerization state [4, 5]. Table 1 shows the proportion of monomeric to filamentous actin, and the G/total-actin ratio in normal PBL preparations and in cell preparations of CLL, ALL, Reh-6, and KM. The G/total-actin ratio in PBL

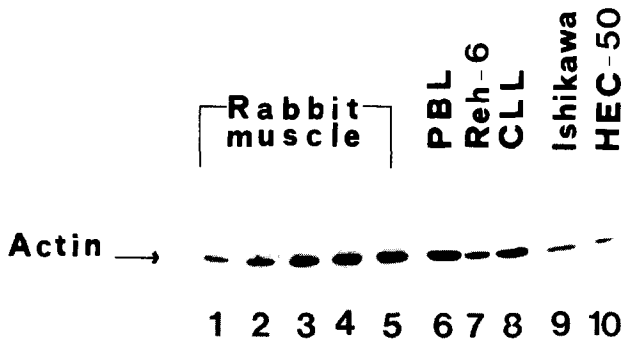

FIG. 2. Quantitative Western blot analysis (representative of a total of 3 experiments) depicting amounts of total cellular actin in normal and malignant human lymphocytes and endometrial cells. Lanes 1–5: Standard curve of isolated pure rabbit muscle actin (0.2–1.0 µg). Lanes 6–8: Amounts of total actin in 10-µg cellular protein aliquots from PBL, Reh-6, and CLL cell extracts, respectively. Lanes 9, 10: Amounts of total actin in 10-µg cellular protein aliquots from Ishikawa and HEC-50 cell extracts, respectively.

was 0.43 ± 0.03 (mean ± SE, N = 10), and the corresponding values in all malignant human lymphocytes were 1.49- to 1.81-fold higher compared to normal PBL.

The monomeric-to-total actin ratio was similarly determined in normal and malignant human endometrial cells. In primary cultures of normal endometrial cells, the G/total-actin ratio was 0.35 ± 0.07 (N = 5). On the other hand, the corresponding values for Ishikawa and HEC-50 cells were significantly higher: 1.37- and 1.74-fold, respectively (Table 2).

Malignant Cells and F-Actin

The above findings suggest reduced amounts of polymerized actin in malignant cells. This was examined in control experiments by direct fluorescence measurements of the F-actin content of normal and malignant cells. Indeed, the rhodamine-phalloidin-labeled F-actin amounts were found to be significantly decreased by 56% in the Reh-6 leukemic lymphocytes compared to normal human PBL (22.2 ± 4.9 vs 50.2 ± 5.4 units/mg total protein, N = 4, $P < 0.01$). Similarly, the poorly differentiated HEC-50 cells express 24% lower F-actin amounts compared to the well differentiated Ishikawa cells (13.9 ± 0.9 vs 18.3 ± 2.4 units/mg total protein, N = 4, $P < 0.01$). These results, showing decreased amounts of polymerized actin in malignant cells, fully support the G/total-actin ratios.

Effect of CB on Human Lymphocyte Viability

The increased actin ratio and the decreased amounts of total- and F-actin measured in malignant cells may result in a higher susceptibility of these cells to anticytoskeletal agents. Thus, we studied cell viability of PBL and leukemic cell cultures (10^6 cells per mL) in the presence of various CB concentrations. As is shown in Fig. 3, viability of PBL cultured for 24 hr in the presence of 10^{-6} M and 5×10^{-6}

TABLE 1. Percentage (%) of monomeric G- and polymerized F-actin content and the G/total-actin ratio in primary cultures of PBL, CLL, and ALL, and in the leukemic cell lines Reh-6 and KM

	Monomeric actin	Polymerized actin	G/total ratio	Mean-fold increase
PBL (N = 10)	43.4 ± 4.1	54.5 ± 5.3	0.43 ± 0.03	
CLL (N = 6)	77.1 ± 5.2	22.9 ± 4.6	0.77 ± 0.05*	1.79
ALL (N = 4)	78.2 ± 3.1	21.8 ± 2.5	0.78 ± 0.04*	1.81
Reh-6 (N = 6)	64.3 ± 2.9	35.7 ± 1.7	0.64 ± 0.02*	1.49
KM (N = 4)	69.3 ± 2.8	30.7 ± 2.4	0.69 ± 0.05*	1.60

Determined by the DNaseI inhibition assay. The results are the mean values ± SE (N = number of patients or number of cell preparations from the leukemic cell lines).

* Denotes statistically significant differences in G/total-actin ratios compared to PBL ($P < 0.01$).

M CB was found to be 87% of control cells (not exposed to CB). On the other hand, in all tested cultures of leukemic cells in the presence of 10^{-6} M and 5×10^{-6} M CB, cell viability was significantly lower (48–69% of control cells). These results suggest a higher susceptibility of malignant lymphocytes to specific agents acting *via* depolymerization of microfilaments.

In a control experiment, we examined the permeability of normal and leukemic cell preparations to CB. For this, PBL and Reh-6 cells in culture were incubated with 8×10^{-8} M [^{3}H]-CB (1 μCi/mL, spec. activity 12.5 Ci/mmol) for 24 hr. At the above conditions, cell viability was shown to be unaffected (data not shown). The incorporation of [^{3}H]-CB in PBL and Reh-6 cells was 3280 ± 134 cpm/10^6 cells and 2495 ± 253 cpm/10^6 cells, respectively (N = 4) or 32194 ± 1802 and 16095 ± 788 cpm/mg total protein, respectively (N = 4). These results indicated clearly that the increased sensitivity of Reh-6 cells to CB action could not be attributed to a higher CB uptake in the leukemic cells.

Dose-Dependent Effect of CB on Human Endometrial Cell Proliferation

Results very similar to those described above regarding the differential susceptibility of malignant cells to CB, were also obtained for human endometrial cells. In these experiments, cell proliferation was assessed by ^{3}H-thymidine incorporation. As shown in Fig. 4, thymidine incorporation was clearly decreased in the poorly differentiated HEC-50 cells treated for 24 hr with 10^{-6} M, 3×10^{-6} M, and 6×10^{-6} M CB, compared to the equally treated well-differentiated Ishikawa cells. These results revealed a higher responsiveness of HEC-50 cells to the cytoskeletal inhibitor. They suggest destabilization of their microfilaments, a notion compatible with the higher G/total-actin ratio and the lower amounts of F-actin measured in the poorly differentiated adenocarcinoma cell line.

Fluorescence Microscopy of Endometrial Cells

The above observations on endometrial cell proliferation were further supported by direct fluorescence microscopy of Ishikawa and HEC-50 cells, studying the responsiveness of their microfilaments to the shortening action of graded CB concentrations, as previously described [5, 13, 27]. The microfilament network of Ishikawa cells incubated for 3 hr with 10^{-6} M of CB appeared unaffected, showing normally distributed cytoplasmic stress fibers with some small spots (Fig. 5a). HEC-50 cells, treated in the same way with CB, showed decreased fluorescence intensity of the stress fibers and a high number of small and larger spots (Fig. 5a'; arrows). Incubation of Ishikawa cells for 3 hr with 3×10^{-6} M of CB induced a weak depolymerization of actin filaments, because small and larger spots became apparent; the microfilament network, however, remained partially intact (Fig. 5b). On the other hand, HEC-50 cells treated with equal concentration of CB displayed an almost complete disappearance of intact microfilaments (Fig. 5b'). Even more prominent were the differences observed when cells were incubated for 3 hr with 6×10^{-6} M of CB. In Ishikawa cells, stress fibers were still visualized (Fig. 5c), but in HEC-50 cells, microfilaments seemed to be totally depolymerized (Fig. 5c'). These results, which indicate a lower resistance of HEC-50 cells to CB treatment, imply microfilament de-

TABLE 2. Percentage (%) of monomeric G- and polymerized F-actin content and the G/total-actin ratio†

	Monomeric actin	Polymerized actin	G/total ratio	Mean-fold increase
Endometrial normal cells (N = 5)	34.6 ± 4.3	65.4 ± 5.3	0.35 ± 0.07	
Ishikawa cells (N = 24)	47.8 ± 1.2	52.2 ± 1.6	0.48 ± 0.03*	1.37
HEC-50 cells (N = 12)	64.0 ± 2.1	36.0 ± 2.5	0.61 ± 0.08*	1.74

Determined by the DNaseI inhibition assay. The results are the mean values ± SE (N = number of patients or number of cell preparations from Ishikawa and HEC-50 cell lines).

* Denotes statistically significant differences in G/total-actin ratios compared to normal human endometrial cells ($P < 0.01$).

† In primary cultures of normal human endometrial cells in a well-differentiated human endometrial adenocarcinoma cell line (Ishikawa cells) and a poorly differentiated human endometrial adenocarcinoma cell line (HEC-50).

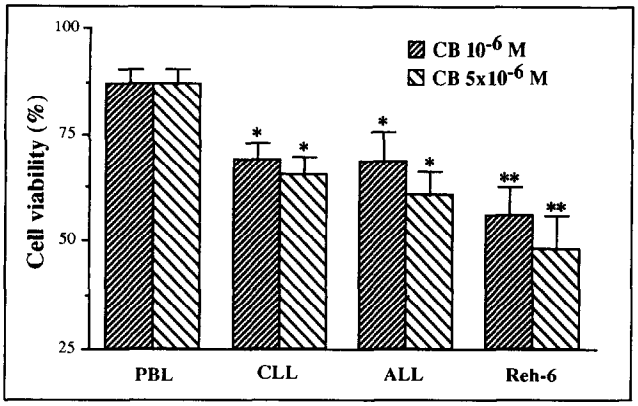

FIG. 3. Cell viability assessed by Trypan Blue dye exclusion from normal PBL (N = 10 patients) and mononuclear cells from CLL (N = 6 patients), ALL (N = 4 patients), and Reh-6 (N = 6 cell preparations), cultured for 24 hr in the presence of 10^{-6} M and 5 x 10^{-6} M CB. Results are normalized as percentage of control (cells not exposed to CB). Percentage of decrease in cell viability is expressed as mean values ± SE. *denotes $P < 0.05$ and **$P < 0.01$ for statistically significant differences between equally treated PBL and leukemic cells.

stabilization in the poorly differentiated HEC-50 cells compared to the well-differentiated Ishikawa cells.

To evaluate the differential susceptibility of Ishikawa and HEC-50 cells to CB treatment, we studied the permeability of these cell preparations to exogenously applied [^{3}H]-CB in control experiments. For this, equal numbers of Ishikawa and HEC-50 cells (10^6) were incubated with 8 × 10^{-8} M [^{3}H]-CB (1 μCi/mL, spec. activity 12.5 Ci/mmol) for 3 and 24 hr. Under the above conditions, cell viability was shown to be unaffected (data not shown). Both cell lines showed very similar CB-uptake rates: 6321 ± 282 (3-hr incubation)

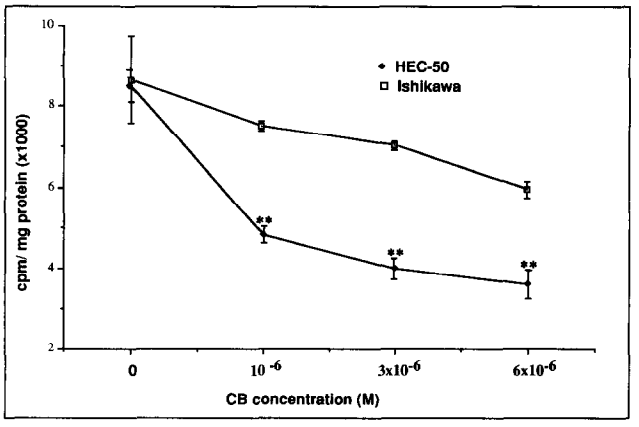

FIG. 4. Dose-dependent effect of CB on cell proliferation of endometrial cells. ^{3}H-thymidine incorporation in human endometrial adenocarcinoma Ishikawa and HEC-50 cells, untreated or treated for 24 hr with 10^{-6} M, 3 x 10^{-6} M and 6 x 10^{-6} M CB. Results are expressed in cpm per mg of total cellular protein as mean values ± SE of triplicates from 6 distinct experiments. **denotes statistically significant differences between equally treated Ishikawa and HEC-50 cells ($P < 0.01$).

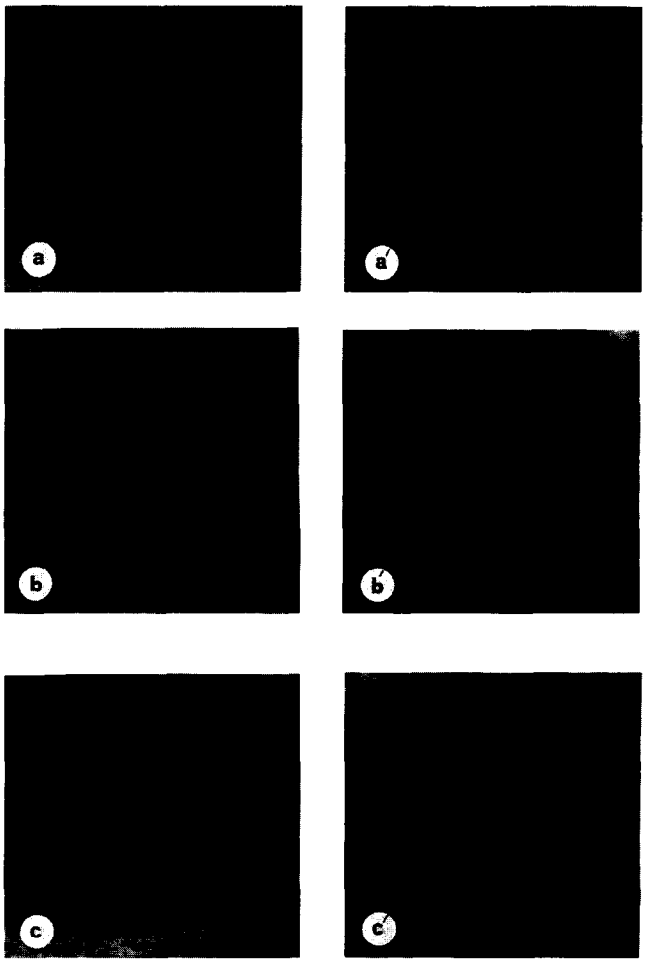

FIG. 5. Rhodamine-phalloidin staining of actin microfilaments in Ishikawa (a,b,c) and HEC-50 cells (a',b',c'). Filament stability was assessed by a 3-hr incubation of cells with graded concentrations of CB. (a,a') 10^{-6} M CB; (b,b') 3 x 10^{-6} M CB; (c,c') 6 x 10^{-6} M CB. (Magnification x 500).

and 14653 ± 579 (24-hr incubation) cpm/mg total protein for Ishikawa cells and 6803 ± 391 (3-hr incubation) and 14919 ± 863 (24-hr incubation) cpm/mg total protein for HEC-50 cells (N = 4). These findings indicate that the observed CB effects were not due to increased permeability and accumulation of CB in the poorly differentiated HEC-50 endometrial cells.

DISCUSSION

In the nonmuscle cell, actin exists in monomeric and polymeric forms maintained in a dynamic equilibrium. Because actin cytoskeleton is an important framework upon which the processes of growth and differentiation are accomplished, quantitative characterization of these monomeric and polymerized forms would appear to be important for the understanding of modified cellular functions, including malignant transformation. Thus, in the present study, we measured modifications of the dynamic equilibrium between the monomeric and polymeric actin forms by quantitative

biochemical, immunoblot, and fluorescence assays, as well as the organization and stability of microfilaments in various normal and malignant human cell types.

The results reported here demonstrate clearly that all malignant cells investigated are characterized by significantly decreased total actin levels. This was deduced by the DNase I inhibition assay (Fig. 1) and confirmed by immunoblot analysis (Fig. 2), indicating that both approaches offer similar quantitative results for actin determinations. Additionally, the state of actin polymerization was different in normal and malignant cells, because neoplastic cells showed a 1.37- to 1.81-fold increase in the G/total-actin ratios (Tables 1, 2). The increased G/total-actin ratios reflect the reduced proportion of the microfilamentous content in malignant compared to healthy cells, a conclusion fully supported by direct fluorescence determinations of the amounts of F-actin in control experiments. It should be mentioned that both biochemical and fluorescence observations disclose the overall cellular actin, and do not address the issue of the eventually occurring compartmental differential changes in actin polymerization.

Our data are in line with recently reported results showing differential G/total-actin ratios in normal and malignant human keratinocytes [28]. Moreover, they are compatible with other findings, which revealed a shift in the actin monomer/polymer equilibrium in rat fibroblasts transformed by the Rous sarcoma virus [29]. In this work, the protein product of the *src* oncogene was shown to induce substantial depolymerization of actin filaments. On the other hand, other studies reported an increase in actin content and actin synthesis during maturation of HL60 human promyelocytic leukemia cells [30]. Similarly, the actin synthesis and content, as well as the F-actin ratio, were also reported to increase during induced differentiation of a mouse myeloid leukemia cell line (M1) [31]. These reports, in relation to our findings, support the notion that increased F-actin and total actin levels may represent characteristics of mature, well-differentiated cells, and malignant cells may be characterized by decreased amounts of total- and F-actin. Although this suggestion seems to be valid for cells derived from different tissues, including lymphocytes, endometrial cells, and keratinocytes, whether it can be generalized to any cell type needs to be corroborated. Subsequently, quantitative biochemical characterization of actin polymerization dynamics might be useful as an indicator for the assessment of malignant transformation and dedifferentiation.

The observed alterations in the distribution of G- and F-actin and the increased monomer-polymer actin ratio in neoplastic cells reflect a decreased proportion of polymerized actin and suggest a higher sensitivity of malignant cells to microfilament inhibitors. Experimental evidence for this was provided by cell viability and cell proliferation measurements in leukemic and endometrial adenocarcinoma cell cultures (Figs. 3, 4), which showed significantly higher responsiveness of the malignant cells to this agent. This hypothesis was further supported by the fluorescence microscopy data (Fig. 5), which showed an increased destabilization of microfilaments in the presence of CB in the poorly differentiated HEC-50 endometrial cells compared to the well-differentiated Ishikawa cells.* In addition, our findings demonstrating that CB uptake is not increased in neoplastic cells are in line with earlier observations [13] and support the notion that the differential CB effects cannot be attributed to a simple modification of cell permeability due to malignant transformation. Rather, they seem to be related to a differential shortening activity of CB, most probably due to the decreased amounts of polymerized actin measured in malignant cells. Consequently, the results suggesting an increased sensitivity of neoplastic cells to this specific anticytoskeletal agent may represent an interesting feature that may be useful for further evaluation of the responsiveness of malignant cells to other drugs acting *via* the actin cytoskeleton.

In summary, our data showing that all tested neoplastic cells are characterized by lower total- and F-actin content and increased G/total-actin ratio may be of important biological interest. They seem to be correlated to the higher sensitivity of neoplastic cells to the anticytoskeletal agent CB. Thus, the ability to study the polymerization state and stability of the actin cytoskeleton quantitatively may provide important clues and a valuable tool for evaluation of novel pharmacological approaches to the manipulation of cancer cells.

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