Changes in Actin and Actin-binding Proteins during the Differentiation of HL-60 Leukemia Cells

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ABSTRACT

Actin and actin-binding proteins form a peripheral network on the cytosolic side of the plasma membrane. These cytoskeleton proteins are involved in functions that require cellular movement and may also have a role in modulating signal transduction during cellular proliferation and differentiation. To measure changes in F-actin and actin-binding proteins during HL-60 differentiation, cells were induced to mature along the granulocytic pathway by exposure to 1 μM retinoic acid (RA) for 5 days and were analyzed for F-actin and actin-binding proteins by flow cytometry. The amounts of F-actin and spectrin in untreated HL-60 cells and in those undergoing differentiation by treatment with the retinoid did not differ. N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-phallacidin was used to measure F-actin content and a monoclonal antibody followed by fluoresceine isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody was used to measure the content of spectrin; cells were analyzed by flow cytometry. In contrast, cells exposed to RA contained larger amounts of α-actinin, vinculin, talin, lipocortin I, and lipocortin II, as determined with their respective antibodies followed by flow cytometric analysis as described above. An RA-supersensitive clone of HL-60, designated HL-60/S4, exhibited lower constitutive levels of α-actinin, vinculin, and talin but a higher constitutive level of lipocortin II than parental cells. Treatment of HL-60/S4 with RA led to increases in vinculin, talin, lipocortin I, and lipocortin II. An RA-resistant clone, designated HL-60/R3, constitutively expressed larger amounts of α-actinin, vinculin, talin, and lipocortin II than parental HL-60 cells. Treatment of HL-60/R3 with RA resulted in decreases in the amounts of these actin-binding proteins. Changes in actin-binding proteins that occur during the differentiation of HL-60 cells suggest that these proteins may be of importance to the expression of the mature phenotype.

INTRODUCTION

The functions of the cytoskeleton include the maintenance of morphology, cellular motility, and phagocytosis; the formation of the mitotic spindle during cell division; and the transport or localization of cytoplasmic organelles (1). To accomplish these functions, the cytoskeleton forms a structural network of tubules and fibrils, composed of three major elements (microtubules, microfilaments, and intermediate filaments), which connect the plasma membrane to cytoplasmic organelles and the nucleus (2). Modest or virtually no increases in the total cellular content of actin in both murine (3, 4) and human (5–8) myeloid leukemia cells have been reported as a consequence of induced differentiation. However, more marked increases in gelsolin and other actin-binding proteins have been described in differentiated HL-60 leukemia cells (8). Lipocortin I and lipocortin II, which are associated with the cortical cytoskeleton underlying the plasma membrane and bind to actin in a calcium-dependent manner (9–11), have also been shown to increase in HL-60 and U-937 myeloid leukemia cells induced to enter a differentiation pathway (12, 13).

The cytoskeleton plays an important role in the process of cell growth and differentiation (14). In addition, F-actin and actin-binding proteins are involved in the functions of neutrophils (15). Thus, studying changes in F-actin and actin-binding proteins during myelocytic differentiation is important to our understanding of the transition to a more mature phenotype. In this report, we describe changes in F-actin and actin-binding proteins of HL-60 leukemia cells induced to differentiate along the granulocytic pathway by RA. The contents of cytoskeletal components were measured by a newly developed method of fluorescence staining followed by flow cytometric analysis (16). An RA-supersensitive clone called HL-60/S4 and a resistant clone designated HL-60/R3 (16) were used as tools to link changes in cytoskeletal components to the differentiation process rather than to other effects of RA.

MATERIALS AND METHODS

Cell Culture and Treatments. HL-60 human promyelocytic leukemia cells were provided by Dr. Robert C. Gallo of the National Cancer Institute, Bethesda, MD. An RA-supersensitive clone called HL-60/S4 and an RA-resistant clone designated HL-60/R3 were obtained by mutagenization and selection as described previously (16). Cells were routinely passaged in RPMI 1640 medium with 10% fetal bovine serum. Stock cell cultures were passaged twice weekly and maintained in a humidified 37°C atmosphere containing 5% CO2 in air. Cell stocks were routinely screened for Mycoplasma by the gene probe method (Gen-Probe, Inc., San Diego, CA). In all experiments, 1 × 106 exponentially growing cells were seeded per ml of fresh growth medium. Cells were incubated with different concentrations of all-trans-RA (Sigma Chemical Co., St. Louis, MO) for 1–5 days. A stock solution of 1 mM RA was prepared in 100% ethanol and stored at −20°C.

Flow Cytometric Measurement of Cytoskeleton Content. The contents of F-actin and actin-binding proteins were measured by flow cytometry after fluorescent staining as described previously (16). One to 1.5 × 106 cells/ml were fixed in buffer (0.1 M 1,4-piperazinediethanesulfonic acid, pH 6.9–1 mM MgSO4–2 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid–2 mM glycerol) containing 3.7% formaldehyde for 30 min. Fixed cells were incubated in PBS and then in 0.1 M glycine for 5 min each. Cells were then permeabilized by treatment with 10 μg/ml of L-α-lysophosphatidylcholine (Sigma) in PBS for 30 min, washed with PBS twice, and resuspended in PBS containing 0.5% bovine serum albumin. Cells were stained with NBD-phallacidin (Molecular Probes, Inc., Eugene, OR), with mouse monoclonal antibodies against spectrin, α-actinin, or vinculin (Sigma), with antibodies against lipocortins I and II (Oncogene Science, Inc., Manhasset, NY), or with a rabbit polyclonal antibody against talin (supplied by Dr. Keith Burridge, University of North Carolina) for 30 min followed by incubation with FITC-conjugated goat anti-mouse or anti-rabbit immunoglobulin antibody (Cappel, Malvern, PA) for 30 min. Stained cells were analyzed using a Becton Dickinson flow cytometer. The relative fluorescence intensity of cells stained with NBD-phallacidin was measured and compared to unstained cells, and cells stained with anti-cytoskeleton

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antibodies were compared to those stained with secondary antibody alone. The relative fluorescence intensity of RA-treated cells was compared to that of untreated control cells and expressed as a percentage of the control fluorescence.

RESULTS

HL-60 leukemia cells, an RA-supersensitive clone (HL-60/S4), and an RA-resistant clone (HL-60/R3) were exposed to RA, an inducer of granulocytic differentiation. Cell growth and the expression of a mature granulocytic phenotype, as measured by the ability to generate superoxide and the expression of the myelocyte surface marker Mo 1, were determined as a function of time. In HL-60 cells, NBT and Mo 1 positivity were observed by 3 days after exposure to RA and continued to increase through 5 days, achieving 75–80% differentiation-positive cells. No inhibition of growth of HL-60 cells was observed after 3 days of exposure to RA; however, a significant decrease in cellular replication occurred by 4 days of treatment with the retinoid. HL-60/S4 cells exposed to 1 µM RA exhibited 24% more inhibition of growth than HL-60 parental cells treated with the same concentration of retinoid. In contrast, no inhibition of growth was produced by the retinoid in RA-resistant HL-60/R3 cells. NBT positivity was concentration dependent in both parental and HL-60/S4 supersensitive cells. A higher percentage (31, 34, and 18% more at 0.01, 0.1, and 1 µM RA, respectively) of NBT-positive cells was produced in HL-60/S4 cells than in HL-60 parental cells at all of the concentrations of retinoid tested. In contrast, RA-resistant HL-60/R3 cells treated with 1 µM RA for 4 days exhibited only 9% NBT positivity. That the supersensitivity of HL-60/S4 and the resistance of HL-60/R3 cells are specific for RA was shown by the finding that exposure of each of these clones to 1.3% dimethyl sulfoxide, another inducer of the granulocytic differentiation of HL-60 cells, for 5 days resulted in essentially the same percentage of HL-60, HL-60/S4, and HL-60/R3 cells expressing NBT positivity.

The contents of cytoskeletal components were ascertained by flow cytometry after exposure of HL-60 cells to 1 µM RA, fixation with formaldehyde, permeabilization with lysophosphatidylcholine, and staining with NBD-phallacidin or anti-cytoskeleton antibodies. HL-60 cells were fixed with formaldehyde, permeabilized with lysophosphatidylcholine, and stained for fluorescent measurements using NBD-phallacidin (a) or anti-spectrin (b), anti-α-actinin (c), anti-vinculin (d), anti-talin (e), anti-lipocortin I (f), anti-lipocortin II (g) antibodies, followed by staining with FITC-secondary antibody and analysis by flow cytometry. Solid line, unstained cells (a) or cells stained with FITC-secondary antibody alone (b–g); dashed line, cells stained with NBD-phallacidin (a) or specific anti-cytoskeleton antibodies (b–g).

The relative fluorescence intensities of untreated control HL-60 cells stained with NBD-phallacidin, compared to unstained cells, and of cells stained with anti-spectrin, α-actinin, vinculin, talin, lipocortin I, and lipocortin II, compared to those stained with FITC-secondary antibody alone, were 12.3, 5.3, 5.2, 7.3, 22.1, 8.7, and 20.5, respectively.

The contents of F-actin and actin-binding proteins were compared to those of untreated HL-60 cells after 4 days in culture (values equated to 100). Cells used for seeding all experiments were cultured for 4 days; for this reason, 4-day cells were chosen as the baseline with which others were compared. HL-60 cells had relatively high levels of F-actin and actin-binding proteins 1 day after being seeded into fresh culture medium, and the contents of these proteins gradually decreased throughout 5 days. No differences were observed in the contents of F-actin and spectrin between RA-treated cells and untreated control cells (Fig. 2, a and b). In contrast, the contents of α-actinin, vinculin, talin, lipocortin I, and lipocortin II of cells treated with retinoid were higher than those of untreated control cells 4 and 5 days after exposure to RA (Fig. 2, c–g). The contents of F-actin, spectrin, and lipocortin I of parental HL-60 cells and the RA-supersensitive clone, HL-60/S4, were essentially the same (Fig. 3, a, b, and f). However, HL-60/S4 cells exhibited lower constitutive levels of α-actinin, vinculin, and talin (Fig. 3, c and d) but a higher constitutive level of lipocortin II (Fig. 3g) than parental cells. In a manner analogous to parental cells, exposure of HL-60/S4 cells to RA resulted in increases in the amounts of α-actinin, vinculin, talin, lipocortin I, and lipocortin II in differentiated cells (Fig. 3, c–g). The RA-resistant clone, HL-60/R3, contained the same amounts of F-actin and spectrin (Fig. 3, a and b) but larger constitutive amounts of α-actinin, vinculin, talin, lipocortin I, and lipocortin II than parental HL-60 cells (Fig. 3, c–g). In contrast to that occurring in RA-responding HL-60 and HL-60/S4 cells, ex-
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Fig. 2. Effects of retinoic acid on the contents of F-actin and actin-binding proteins in maturing HL-60 leukemia cells. HL-60 cells were treated with 1 μM RA, and, at the indicated times, the contents of F-actin and actin-binding proteins were measured by fluorescent staining using NBD-phallacidin (a), anti-spectrin (b), anti-α-actinin (c), anti-vinculin (d), anti-talin (e), anti-lipocortin I (f), or anti-lipocortin II (g) antibodies and analysis by flow cytometry. The contents of F-actin and actin-binding proteins were expressed as a percentage of that of untreated control cells at day 4. Points, means (bar, ±SE) of at least three separate experiments.

posure of HL-60/R3 cells to RA resulted in a lowering of the contents of α-actinin, vinculin, lipocortin I, and lipocortin II.

**DISCUSSION**

An increase in the contents of total cellular actin and F-actin has been reported in differentiated myeloid leukemia cells compared to their nondifferentiated counterparts (3–7). In contrast, no difference in F-actin content was found in the present studies between RA-treated and untreated HL-60 cells; this finding corresponds to that reported by Kwiatkowski (8) who found little increase in the actin content of HL-60, U-937, and K562 cells after induction of differentiation by 12-O-tetradecanoylphorbol-13-acetate. The difference between these findings may be the result of differences in the methodology used or in the cell line used. The methodology used in our studies was designed to measure cellular structural proteins involved in the cytoskeleton network and proteins associated with this network in situ and was previously used by us to measure microtubules and microtubule-associated proteins in differentiated cells (16). It is important to note that in the present study we have measured the content of F-actin and not the content of total cellular actin.

The functions of mature neutrophils, such as in phagocytosis (15, 17), in the release of proteinases after chemotactic stimulation (18), and in the expression of the oxidase involved in the respiratory burst (19) have all been shown to be associated with F-actin and the actin-binding protein network. We have shown that the kinetics of the expression of the contents of α-actinin, vinculin, talin, lipocortin I, and lipocortin II during the maturation of HL-60 leukemia cells induced by RA coincide with the attainment of markers of the granulocytic phenotype. These findings imply that the changes in the content of actin-binding proteins are the result of the attainment of a mature state and support the contention that actin-binding proteins play an important role in the functions of neutrophils.

The constitutive amounts of actin-binding proteins of the RA-supersensitive clone, HL-60/S4, and the RA-resistant clone, HL-60/R3, were different from those of parental HL-60 cells. RA-supersensitive cells had constitutively lower amounts of α-actinin, vinculin, and talin, whereas RA-resistant cells had constitutively higher amounts of α-actinin, vinculin, talin, lipocortin I, and lipocortin II than parental cells. Exposure of HL-60/R3 cells to RA resulted in decreases in the contents of α-actinin, vinculin, lipocortin I, and lipocortin II. These find-

Fig. 3. Comparative effects of retinoic acid on the contents of F-actin and actin-binding proteins of HL-60, HL-60/S4 supersensitive, and HL-60/R3 resistant clones. Cells were treated with 1 μM RA for 4 days, and the contents of F-actin and actin-binding proteins were measured by fluorescent staining using NBD-phallacidin (a), anti-spectrin (b), anti-α-actinin (c), anti-vinculin (d), anti-talin (e), anti-lipocortin I (f), or anti-lipocortin II (g) antibodies and analysis by flow cytometry. The contents of F-actin and actin-binding proteins were expressed as a percentage of that of untreated control HL-60 cells. Columns, means (bar, ±SE) of at least three separate experiments.
ings were in contrast to the increases in the contents of α-actinin, vinculin, talin, lipocortin I, and lipocortin II of differentiated HL-60 parental and HL-60/S4 RA-supersensitive cells. The results suggest that the constitutive amounts of various actin-binding proteins are important factors in the supersensitivity and resistance to RA of the HL-60/S4 and HL-60/R3 clones, respectively. Further study of the changes in the cytoskeletal network during leukemia cell differentiation may well lead to a better understanding of the role of these structures in the maturation process.

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