Expression of 85-kDa Protein of Adriamycin-resistant Tumor Cells during Hematopoietic Differentiation of THP-1 and HEL Cells

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An 85-kDa protein was identified in adriamycin-resistant tumor cells recognized by monoclonal antibody MRK-20. Recently, the monoclonal antibody MRK-20 was found to be reactive to human peripheral mononuclear cells. In order to investigate the molecular function of the 85-kDa protein, we carried out flow cytometric analysis of the expression of the 85-kDa protein during monocytic differentiation of the hematopoietic cells. Human myelomonocytic leukemia THP-1 cells were induced to differentiate into macrophage-like cells by treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). A maximum of 55% of the cells expressed the 85-kDa protein along with the CD-14 antigen, which is a surface marker of monocytes and macrophages. The kinetic analysis revealed that the 85-kDa protein appeared prior to the expression of the CD-14 antigen. The 85-kDa protein was also coexpressed with CD-14 in THP-1 cells that were induced to differentiate by recombinant tumor necrosis factor- α , which is one of the physiological inducers of monocytic differentiation. In human erythroleukemia, HEL cells, the 85-kDa protein was constitutively coexpressed with CD-14. The expression of both the 85-kDa protein and CD-14 was drastically reduced during the megakaryocytic differentiation of the HEL cells with TPA. These results suggest that the 85-kDa protein could be expressed on monocytic cells as well as CD-14 and that the expression of the 85-kDa protein might be regulated at an earlier stage of monocytic differentiation of hematopoietic cells than the expression of CD-14.

Key words: 85-kDa protein — Differentiation-related antigen — Drug resistance

An 85-kDa protein, which is recognized by monoclonal antibodies MRK-20 and MRK-4, was originally found in adriamycin-resistant variants of human myelogenous leukemia K562 cells (K562/ADM) and ovarian cancer cells (2780AD).¹⁾ The expression of the 85-kDa protein in K562/ADM cells decreased when the cells were cultured in the absence of adriamycin, and this diminished expression was restored by the treatment of the cells with adriamycin.¹⁾ The 85-kDa protein was also expressed in other cell lines resistant to anthracyclines, mitoxantrone, etoposide and bleomycins.²⁾ These observations suggest that the 85-kDa protein might be involved in a novel multidrug resistance mechanism of the cells. The molecular function of the 85-kDa protein, however, is unknown.

To study the function of the 85-kDa protein, we have investigated the expression of the 85-kDa protein in various cell lines and normal cells. Besides the adriamycin-resistant cells, the protein is also expressed in human monocytes, granulocytes, endothelial cells and some monocytic cell lines.³⁾ In the course of the studies on the expression of the 85-kDa protein in the monocytic cell lines, we found that the 85-kDa protein was expressed in human myelomonocytic leukemia THP-1 cells

during differentiation. In the research reported here, we found that the THP-1 cells expressed the 85-kDa protein during monocytic differentiation and that the human erythroleukemia HEL cells decreased the expression of the 85-kDa protein during megakaryocytic differentiation. The function of the 85-kDa protein is discussed.

MATERIALS AND METHODS

Chemicals 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was obtained from Sigma Chemical Co., St. Louis, MO, and recombinant human tumor necrosis factor- α (rTNF- α) was from Genzyme Corporation, Boston, MA.

Cell culture and induction of differentiation Human myelomonocytic leukemia THP-1 cells⁴⁾ and human erythroleukemia HEL cells^{5,6)} were obtained from the Japanese Cancer Research Resources Bank. The cells were cultured in RPMI 1640 medium (Nissui, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY) and 100 μ g/ml of kanamycin (Meiji Seika, Tokyo) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

To induce differentiation of THP-1 cells into macrophages, the cells were seeded at an initial density of 2×10^5 cells/ml and incubated with various concentra-

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tions of TPA or with 100 U/ml of rTNF- α . The medium containing TPA was changed every three days. After incubation for the indicated time, the numbers of adherent cells and nonadherent cells were counted, and the percentage of the adherent cells was calculated. In the experiments with HEL cells, the cells were seeded at the same initial density, treated with 3 nM TPA and incubated as above.

Immunoprecipitation Membrane fraction of the cells was prepared as described previously,⁷⁾ and protein was measured by the BCA method.⁸⁾ Ten milligrams of protein of the membrane fraction was solubilized in buffer A

[50 mM Tris-HCl (pH 8.0), 150 mM NaCl] containing 1% Triton X-100 for 1 h on ice, and the sample was centrifuged at 2000g for 30 min at 4°C. The supernatant was incubated with 10 μ g/ml of MRK-20 for 1 h on ice, and then 100 μ l of protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) (20% by volume in PBS) was added. After constant mixing for 1 h at 4°C, the precipitate was washed with buffer A containing 0.1% Triton X-100 four times, and the final precipitate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 4–20% gradient polyacrylamide gel (Daiichi Chemical, Tokyo) according to the procedure

Fig. 1. Morphological change and immunostaining with MRK-20 of TPA-induced THP-1 cells. THP-1 cells were cultured with (A, B) or without (C, D) 3 nM TPA for 3 days and stained with MRK-20 and FITC-labeled anti-mouse IgG (F(ab')₂ fragment). The cells were then observed by light (A, C) and fluorescence (B, D) microscopy, and photomicrographs were taken on the same fields.

of Laemmli.⁹⁾ The gel was subsequently stained with 2D-Silver Stain "DAIICHI" (Daiichi Chemical).

Flow cytometry Approximately 1 million cells were reacted with 10 μ g/ml of the first antibody, such as MRK-20, anti-CD14, anti-CD41 (Cosmo Bio, Tokyo), or nonimmune mouse IgG (Sigma) on ice for 30 min. After washing of the cells twice with PBS, they were reacted with 1/20-diluted FITC-labeled anti-mouse IgG (F(ab')₂ fragment) (Zymed Laboratories, San Francisco, CA) for 30 min on ice. Then the cells were washed twice with PBS. The fluorescence staining of the cells was recorded on an EPICS-IV (Coulter Electronics, Hialeah, FL). Thresholds were arbitrarily set so that the positive cell population for the negative control antibody would be less than 2%. Results were expressed as the percentage of positive cell population.

RESULTS

Expression of 85-kDa protein in THP-1 cells during monocytic differentiation THP-1 cells were induced to differentiate into macrophage-like cells by the treatment of the cells with TPA for 3 days (Fig. 1). In addition to the morphological change of the cells, the reactivity of MRK-20 antibody to the cells was drastically changed during the differentiation. Most of the differentiated adherent THP-1 cells were immunostained with MRK-20, as shown in Fig. 1D. Since the 85-kDa protein was immunoprecipitated with the MRK-20 from the differentiated THP-1 cell membrane, but not from the undifferentiated THP-1 cell membrane (Fig. 2), the 85-kDa protein expressed in the differentiated THP-1 cells was identical to the adriamycin resistance-related 85-kDa protein expressed in K562/ADM cells.

Flow cytometric analysis of THP-1 cells We analyzed the expression of 85-kDa protein in THP-1 cells quantitatively. Figure 3 shows the dose-dependent expression of the 85-kDa protein in THP-1 cells after the treatment

with TPA. When THP-1 cells were treated with 3 nM TPA for 3 days, 52% of the cells became adherent (Fig. 3A), and 44% of the cells expressed 85-kDa protein (Fig. 3B). The adherent cell population was increased to 84% after the treatment with 10 nM TPA, while the 85-kDa positive cell rate remained at 42%. The expression of CD-14, a differentiation marker of monocytes,¹⁰⁾ in the THP-1 cells increased in a dose-dependent manner up to 100 nM TPA (Fig. 3B).

Fig. 3. Expression of 85-kDa protein in THP-1 cells by treatment with TPA. THP-1 cells were seeded at 2×10^5 cells/ml and treated with various concentrations of TPA for 3 days. (A) The number of cells (open triangles) and the percentage of adherent cells (open circles) were measured. The treatment with 300 nM TPA was slightly cytotoxic. (B) The percentage of 85-kDa protein-positive cells (circles) or CD-14-expressing cells (squares) was measured by means of flow cytometry. Results of two separate experiments are shown (experiment I, closed symbols; experiment II, open symbols).

Fig. 2. Immunoprecipitation of 85-kDa protein with MRK-20. Plasma membrane fractions containing 10 mg (lanes 2, 3, 5, 6 and 7) or 1 mg (lane 4) protein of cells were immunoprecipitated with MRK-20 monoclonal antibody, as described in "Materials and Methods." Lanes 1, without membrane fraction; 2, K562; 3, 4, K562/ADM; 5, TPA-treated THP-1; 6, THP-1; 7, HEL.

Fig. 4. Time course of expression of the 85-kDa protein in THP-1 cells by TPA. THP-1 cells (2×10^5 cells/ml) were treated with 3 nM TPA. Medium containing TPA was changed every 3 days. (A) The percentage of adherent cells and (B) the percentage of 85-kDa protein-(circles) or CD-14-(squares) expressing cells were measured as described in "Materials and Methods."

Next, the time-dependent expression of the 85-kDa protein was examined in THP-1 cells after treatment with 3 nM TPA (Fig. 4). The adherent cell population increased linearly in a time-dependent manner, and more than 90% of the cells became adherent after 5 days. The 85-kDa protein-positive cells increased to 44 and 55% after 3 and 5 days, respectively; however, the 85-kDa protein-positive cells gradually decreased during prolonged incubation. In contrast, the CD-14-positive cells increased gradually until day 9, and 15, 33, 56 and 65% of the THP-1 cells expressed CD-14 antigen after 3, 5, 7 and 9 days, respectively.

Fig. 5. Change in the expression of surface antigens on HEL cells during differentiation induced by TPA. HEL cells (2×10^5 cells/ml) were treated with 3 nM TPA and the medium containing TPA was changed every 3 days. At the indicated days, cells were immunostained with MRK-20 (circles), anti-CD-14 (squares) or anti-CD-41 (triangles), and the percentage of positive cells was measured by means of flow cytometry.

The THP-1 cells were also induced to differentiate into macrophage-like cells by the treatment with rTNF- α . After the treatment of the THP-1 cells with 100 U/ml of rTNF- α for 3 days, 10 and 21% of the cells expressed 85-kDa protein and CD-14 protein, respectively.

Diminished expression of 85-kDa protein in HEL cells during megakaryocytic differentiation Most HEL cells constitutively express 85-kDa protein and CD-14 under the culture conditions (Fig. 2, lane 7 and Fig. 5). When the HEL cells were treated with 3 nM TPA, the cells expressing CD-14 and 85-kDa protein decreased (Fig. 5). CD-14 diminished more rapidly than the 85-kDa protein. After 3 days of culture with TPA, only 7% of the cells expressed CD-14, whereas 66% of the cells still expressed 85-kDa protein. The cells expressing 85-kDa protein decreased, and 12 and 5% of the cells were positive after 5 and 7 days, respectively. In contrast to the CD-14 and 85-kDa protein, the expression of CD-41, which is a surface marker of megakaryocytic cells,¹⁰ increased in a time-dependent manner. These results suggest that the expression of the 85-kDa protein in the HEL cells was diminished during the megakaryocytic differentiation of the cells.

DISCUSSION

Since the 85-kDa protein is expressed in cells resistant to anthracyclines, mitoxantrone, etoposide and bleomycins,² it has been speculated that the 85-kDa protein could be involved in a novel multidrug resistance mecha-

nism of the cells. However, no molecular mechanism of the resistance involving the 85-kDa protein has been reported. Furthermore, no molecular functions of the 85-kDa protein have been described. As MRK-20 against the protein was reactive to human peripheral mononuclear cells, we investigated the expression of the 85-kDa protein in various cell lines.

The expression of the 85-kDa protein in the THP-1 cells was dramatically increased during monocytic differentiation of the cells. The estimation of differentiation of the THP-1 cells was based on two criteria, the morphological changes, including adhesive properties of the cells, and the expression of the monocyte/macrophage marker protein CD-14. When the THP-1 cells were treated with TPA, they exhibited the morphological characteristics of macrophages and express the CD-14 antigen. The differentiated THP-1 cells coexpressed the 85-kDa protein. The 85-kDa protein was also coexpressed with CD-14 in the THP-1 cells after differentiation by rTNF- α , which is one of the physiological inducers of the monocytic differentiation.^{11,12} The 85-kDa protein was coexpressed with CD-14 in the undifferentiated HEL cells, and the expression of both the 85-kDa protein and CD-14 was extremely reduced during the megakaryocytic differentiation of the HEL cells. These results suggest that the expression of the 85-kDa protein was a characteristic property of the monocytic cells along with the expression of CD-14.

We also examined the expression of the 85-kDa protein during monocytic differentiation in other cell lines. Promyelocytic leukemia HL-60¹³ and histiocytic lymphoma U-937¹⁴ could be induced with TPA to differentiate into monocyte/macrophages. The HL-60 cells were morphologically differentiated to monocyte/macrophage-like cells by the treatment with TPA, but the differentiated HL-60 cells did not express CD-14 or the 85-kDa protein (data not shown). In the case of the U-937 cells, a significant population of the cells (about 20%) intrinsically expressed both the CD-14 and the 85-kDa protein. The treatment with TPA marginally decreased the expression of the CD-14 and the 85-kDa protein (data not shown). These results indicated that the HL-60 and the U-937 cells we used were differentiated morphologically into monocyte/macrophages with TPA, but they did not express the surface marker CD-14. The expression of the 85-kDa protein was always comparable

to that of CD-14. Therefore the 85-kDa protein could be expressed on the monocytic cells as well as CD-14.

The expression patterns of the 85-kDa protein and the CD-14 antigen were slightly different from each other. The kinetic studies revealed that the 85-kDa protein appeared earlier than CD-14 in the TPA-treated THP-1 cells. The 85-kDa protein-expressing cells decreased during prolonged incubation with TPA, whereas the CD-14-expressing cells increased in a time-dependent manner. It was reported that tissue macrophages did not express the 85-kDa protein, although a large population of peripheral monocytes did express the 85-kDa protein.³⁾ These results suggest that the expression of the 85-kDa protein might be regulated at an earlier stage of monocytic differentiation of the cells than the expression of CD-14.

In addition to peripheral monocytes, the 85-kDa protein was expressed in endothelial cells in various normal tissues.³⁾ This suggests that the protein plays an important role physiologically. We assume that the 85-kDa protein might be involved in the mechanism of intracellular adhesion because the expression of the protein occurs concomitantly with the appearance of adhesive properties in THP-1 cells. The K562/ADM cells, which were found initially to express a large amount of the 85-kDa protein, tend to aggregate loosely by themselves. Furthermore, the treatment of the K562/ADM cells with MRK-20 reduced the adhesion of the K562/ADM cells to human endothelial cells to some extent (unpublished observation). The 85-kDa protein might be one of the cell adhesion proteins that may be involved in the monocytic differentiation process. However, the role or the molecular function of the protein is unclear, especially in relation to drug resistance. The cDNA cloning of the 85-kDa protein is in progress in our laboratory. Analysis of the sequence and a transfection experiment should reveal the molecular function of the 85-kDa protein and the mechanism(s) of drug resistance.

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