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DIFFERENTIATION OF A HISTIOCYTIC LYMPHOMA CELL LINE BY LIPOMODULIN, A PHOSPHOLIPASE INHIBITORY PROTEIN

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SUMMARY: When U 937 cells, a human histiocytic lymphoma cell line, were cultured with purified lipomodulin for 3 days, morphological and functional differentiation was induced as detected by microscopical examination of Giemsa stained smears, expression of mature monocyte antigen, and antibody dependent cellular cytotoxicity tests. Essentially similar differentiation was observed by the treatment with dexamethasone for 6 days and this differentiation by dexamethasone was blocked by monoclonal anti-lipomodulin antibody. Furthermore, the synthesis of immunoprecipitable lipomodulin in these cells was induced by dexamethasone treatment. These results, taken together, suggest that the induction of lipomodulin synthesis might be the primary event in dexamethasone-induced cellular differentiation of U 937 cells.

The anti-inflammatory action of glucocorticoids has been proposed to be associated with induction of phospholipase inhibitory protein(s) in neutrophils (lipomodulin) (1) and macrophages (macrocortin) (2). Induction of such protein factor(s) by glucocorticoids has been also reported in kidney cells and adrenal cells (3,4). These proteins inhibit the release of free arachidonic acid from phospholipids by the action of phospholipase(s). This in turn, reduces the subsequent formation of inflammatory agents, prostaglandins and hydroperoxides of this fatty acid. In addition to their anti-inflammatory effects, glucocorticoids have many other biological actions such as promotion of cellular differentiation (5-9). Although morphological and functional alterations induced by glucocorticoids have been described in various cells, the molecular mechanism remains to be explored. Recently, we have isolated lipomodulin from glucocor-

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ticoid-treated neutrophils and have prepared a monoclonal antibody against this protein (10-13). To study cellular differentiation induced by glucocorticoids at the molecular level, we compared the effects of purified lipomodulin and dexamethasone on the morphology and function of U 937 cells, a human histiocytic lymphoma cell line. We find that purified lipomodulin induces cellular differentiation of U 937 cells faster than glucocorticoids do, and that anti-lipomodulin antibody can block the differentiation of U 937 cells induced either by lipomodulin or by dexamethasone.

MATERIALS AND METHODS

The human histiocytic lymphoma cell line, U 937, was maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin and 10 mM HEPES buffer, pH 7.4 (complete medium) (13). In the experiments described, cells were plated on 1.6 cm-diameter wells (Costar, 3529), with 2 ml of complete medium, and cultured in an atmosphere of humidified 95% air-5% CO_2 gas. Morphological changes were examined under a microscope after staining with Giemsa. Expression of cell surface antigen was detected by indirect rosette formation, using the monoclonal antibody, B 43, 4, 1, which is associated with human monocytes, natural killer (NK) cells and neutrophils (14). Antibody dependent cellular cytotoxicity (ADCC) was measured, using ^{51}Cr labeled chicken red blood cells (CRBC) as described (15).

RESULTS AND DISCUSSION

Like other cells (5-9), U 937 cells underwent a distinct morphological change when they cells were cultured with 10^{-5}M dexamethasone (Dx) for 6 days. In Giemsa-stained smears, the control cells had large kidney-shaped nuclei, moderate amounts of cytoplasm, and coarse granular chromatin (Fig. 1a). The Dx treated cells became larger, their cytoplasm more vacuolated and their membrane more villous (Fig. 1b). The Dx-treated cells were more adhesive and aggregated, and Dx-treatment caused inhibition of cell growth by approximately 20%. These changes in U 937 cells resulting from Dx treatment were almost completely blocked by monoclonal anti-lipomodulin antibody, while control immunnoglobulins had no effects. These results suggest that the morphological changes observed might be a consequence of induction of lipomodulin in the cells by the glucocorticoid. To confirm this interpretation, U 937 cells were cultured with purified lipomodulin (5×10^{-9}M). Lipomodulin had essentially same effects on morphology of the cells as Dx did (Fig. 1c). The inhibition of cell growth by lipomodulin

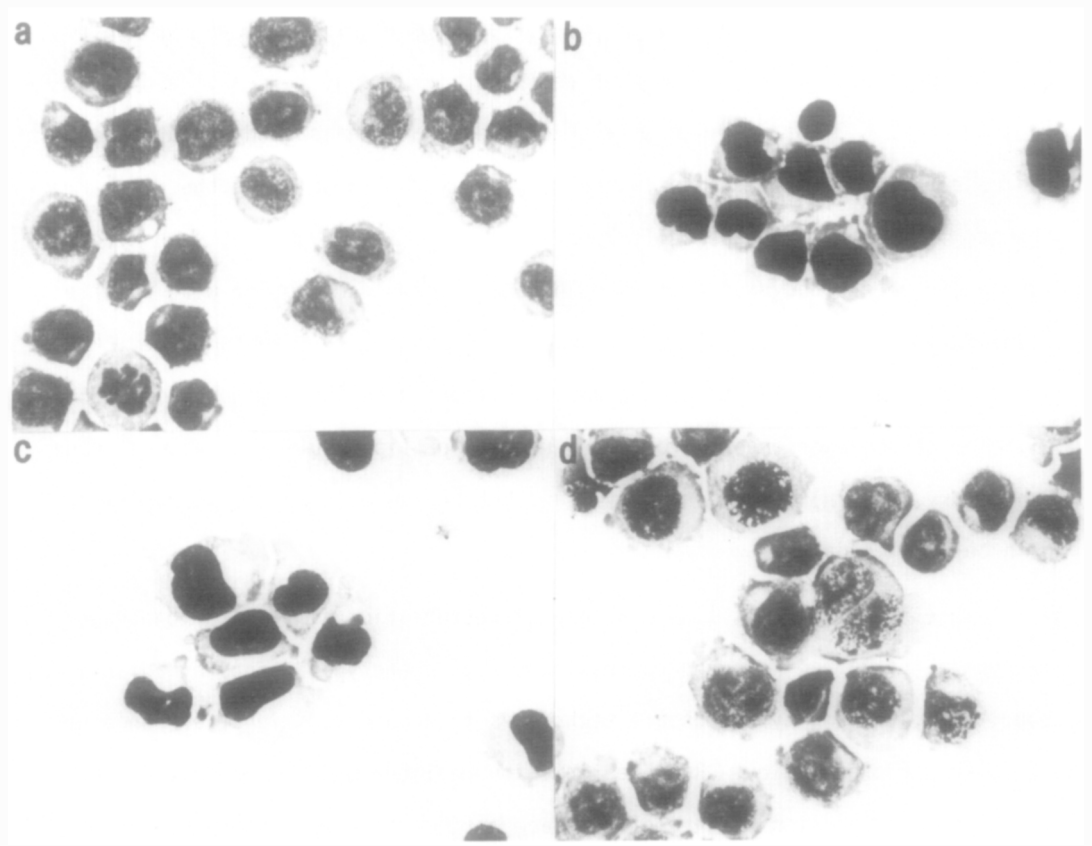

Figure 1. Morphological changes of U 937 cells by Dx, lipomodulin and anti-lipomodulin antibody. U 937 cells (1.5×10^5/ml) were cultured in the absence (a) or presence (b) of 10 μM Dx for 6 days as described in the text. Lipomodulin was purified from rabbit neurotrophils treated with glucocorticoids as described previously (12). The purity of this preparation as measured on SDS-gel electrophoresis was approximately 80%. The cells were treated with 5 nM purified lipomodulin for 3 days (c). Anti-lipomodulin antibody (10) obtained from the ascite fluids of mice bearing hybridoma was diluted with complete medium. Anti-lipomodulin antibody was added to the culture at a final dilution 1:4000 for 6 days (d). Cells were harvested by costar 3010 scraper and resuspended at a concentration of 1×10^6 cells/ml. One-fifth ml of the cell suspension were smeared on slide glasses by the use of a cytospin centrifuge. The smears were stained with Giemsa and were microscopically examined at 499 times magnification.

(87.5%), however, was much greater than that produced by Dx. In addition, the differentiation of the cells by Dx took 6 days to occur, but lipomodulin induced similar changes within 3 days. Anti-lipomodulin antibody blocked the effects of lipomodulin. These results suggest that Dx induces the synthesis of lipomodulin, which in turn, differentiates cells.

The antibody alone reduced the viability of cells from 90% in the control cells to 68% in the antibody treated cells as measured by trypan blue dye exclusion test. Giemsa stained smears revealed that the antibody treated cells had condensed nuclei, vacuoles in their cytoplasm and bubbles in the plasma membranes, indicating that the cells were dying (Fig. 1d). However, such cytotoxicity of the antibody was diminished by the previous absorption with purified lipomodulin (90% viable). Control immunoglobulins had no effects on cell viavilities. The cytotoxicity of the anti-lipomodulin antibody might be due to activation of phospholipase(s) following removal of lipomodulin (a phospholipase inhibitory protein) by the antibody (10), but the details of the mechanism remain to be elucidated.

Since the U 937 cell line has some characteristics similar to monocytes/macrophages (13), we measured expression of the phenotype defined by an antibody, B 43,4,1, employing rosette techniques to obtain additional evidence of cellular differentiation. Rosette formation mediated by the antibody was attributed to expression of the cell surface antigen, but not to expression of Fc receptors, because control immunoglobulins (IgG IIa) from mouse myeloma cells did not cause significant rosette formation. Dx treatment of cells resulted in a moderate increase in antigen expression, while lipomodulin caused a marked increase (Fig. 2a and b). The increase in antigen expression was dependent on the amount of lipomodulin or Dx added to the culture. Anti-lipomodulin antibody blocked the increase in antigen expression caused by Dx as well as by lipomodulin. To confirm the functional differentiation of U 937 cells by lipomodulin, we examined activities of antibody dependent cellular cytotoxicity (ADCC), one of the principal functions of macrophages (Fig. 3). Dx treatment significantly increased ADCC activities after 6 days of culture and anti-lipomodulin antibody blocked this increase. Lipomodulin markedly enhanced ADCC activities after 3 days of culture. Neither differentiated nor control cells were cytotoxic against CRBC in the absence of anti-CRBC antibody. Since ADCC activities depend on Fc receptor expression on the cell surface, the inhibitory effects of anti-lipomodulin antibody might be due to its occupation of the Fc receptors.

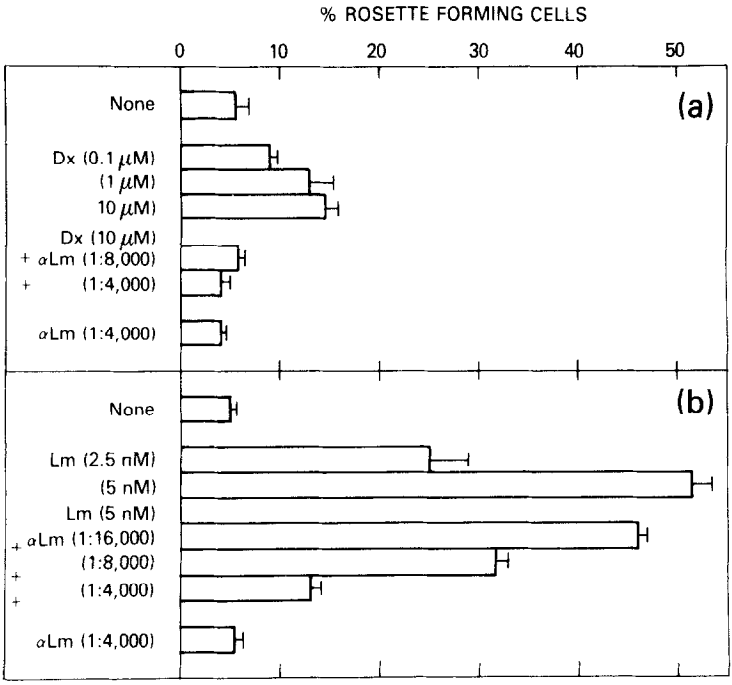

Figure 2. Effects of Dx and lipomodulin on expression of cell surface antigen. The cell surface antigen was assayed by an indirect rosette formation. B 43,4,1 antibody was kindly provided by Dr. Trinchieri at Wistar Institute (14). The culture conditions of U 937 cells were the same as described in the legend to Fig. 1. One hundred μl of cell suspensions (2 to $3x10^6$/ml) were incubated with an equal volume of monoclonal antibody (1:500 dilution) at room temperature for 30 min, washed 3 times with 2 ml of complete medium and resuspended in 100 μl of complete medium. Twenty-five μl of 1% solution (v/v) of ox-red blood cell (OX-RBC) conjugated with affinity purified anti-mouse rabbit IgG (Kirkegaard and Perry, Gaithersburg, MD) were added, centrifuged for 5 min at 500 rpm and kept for 1 hr at 4°C. The pellets were gently suspended and 50 μl of trypan blue were added. Rosette forming cells were counted in a haemocytometer. More than 200 viable cells were counted for each test in duplicate. The results were expressed as mean ± S.D. The cells which bound 3 or more OX-RBC were defined as positive cells. Lm, lipomodulin; alpha-Lm, anti-lipomoduin antibody.

However, $F(ab)'_2$ fragments of anti-lipomodulin antibody also blocked the differentiation of cells by lipomodulin as measured by ADCC activities. In addition, control immunoglobulins from mice myeloma cells (IgG IIa) had no effect on the increase of ADCC activities in the cells treated with Dx or lipomodulin.

The data presented in this communication suggest that the morphological and functional differentiation of U 937 cells by Dx is mediated through lipomodulin induced in the cells. In keeping with this interpretation, purified lipomodulin is effective after a shorter period of incubation than required by Dx, probably because of a lag time between Dx administration and induction of

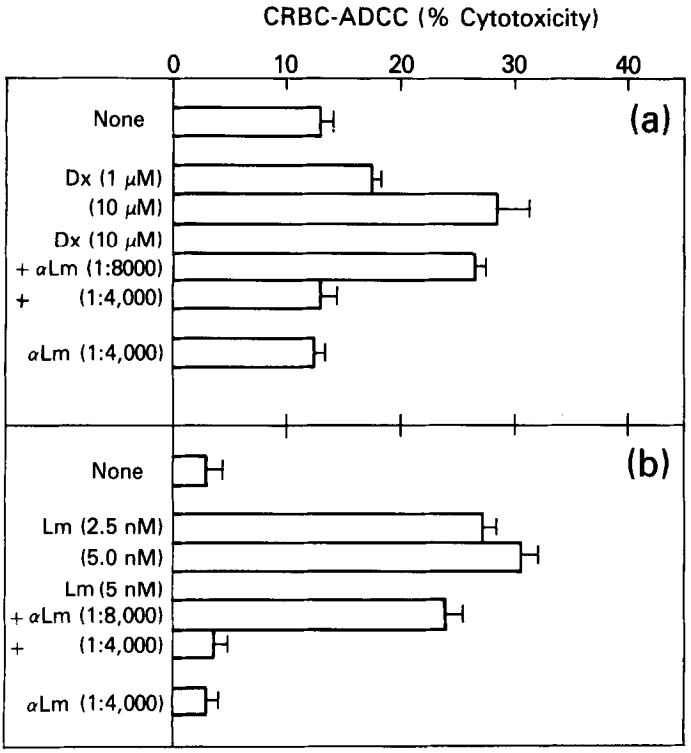

Figure 3. Effects of Dx and lipomodulin on CRBC-ADCC activities. The cells were cultured as described in the text. After harvesting, numbers of viable cells were counted by trypan blue dye exclusion test. Viabilities were always over 90% except when cells were cultured with 1:4000 diluted anti-lipomodulin antibody for 6 days (68%) or when cells were cultured with 10 μM dexamethasone and anti-lipomodulin antibody (1:4000 dilution) (62%). CRBC-ADCC activities were measured as described by Perlmann et al. (15). Briefly, CRBC were radiolabeled with ^{51}Cr (New England Nuclear, Boston, MA). After washing, 1 x 10^4 radiolabeled CRBC (50 μl) and 50 μl of anti-CRBC antiserum (1:5000) (Cappel Lab., Cochranville, PA) were mixed with 100 μl of effector cells to make an E:T ratio at 10:1 in the well of 96-well round bottomed microtiter plates (Linbro Scientific Co., Hamden, CT). The plates were incubated for 4 hr and the supernatant was carefully removed by the Titerteck automatic harvesting system (Flow Laboratoies, Rockville, MD) and counted in a gamma counter. Supernatants from microwells containing target cells alone in 0.2 ml complete medium served as controls for the release of ^{51}Cr. Percent cytotoxicity was calculated as follows: percent cytotoxicity = (cpm in the presence of target cells - cpm in control medium) x 100 / (total cpm of 10^4 target cells - cpm of counter backgrounds). All these experiments were done in triplicates. To keep the number of cells constant under these assay conditions, equal numbers of viable effector cells were added. Abbreviations are as described in the legend to Fig. 2.

the protein. To confirm that Dx causes induction of lipomodulin synthesis in U 937 cells, the cells were cultured for 6 days with [^{35}S]methionine in the presence and absence of Dx. The cells were solubilized with 2% Nonidet P40 containing 1% aprotinin, a protease inhibitor, and then the aliquots were immunoprecipitated with anti-lipomodulin antibody. SDS electrophoresis of the radio-

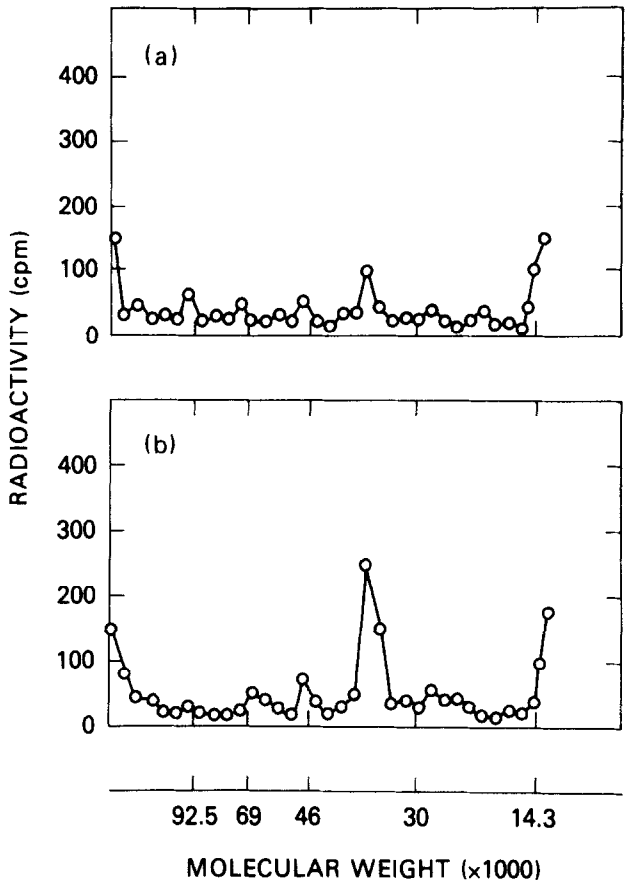

Figure 4. SDS gel electrophoresis of immunoprecipitates from control and Dx-treated cells. U 937 cells (1.5 x 10^6/5ml) were cultured with 1 mCi of ^{35}S-methionine in PPMI 1640 medium supplemented with 10% FCS in the absence (a) and presence (b) of 10 μM dexamethasone for 6 days. The cells were solubilized with 1 ml of 2% nonidet P 40 and centrifuged at 27,000 x g for 60 min. Aprotinin (Sigma) was added to aliquots at a final concentration of 1%. Immunoprecipitation was carried out by 24 hr incubation with 50 μl of anti-lipomodulin antibody, followed by another 24 hr incubation with 20 μl of anti-mouse IgG (light and heavy chains) antibody. SDS-gel electrophoresis was performed using 10% polyacrylamide slab gel as described (11).

active immunoprecipitates showed one major band of radioactive protein which had Mr around 36,000 (Fig. 4). Control immunoglobulins or antibody preabsorbed with purified lipomodulin failed to precipitate this protein. The radioactivity in this peak increased 3 fold in the Dx treated cells, suggesting that the increase in cellular lipomodulin parallels the degree of differentiation caused by Dx. Lipomodulin purified from rabbit neutrophils treated with glucocorticoids has Mr = 40,000 (11). The difference in the molecular weights may be due to use of different species in the two studies.

Differentiation or maturation of cells generally requires arrest of cell growth and mitogenesis. Phospholipase activation is necessary for lymphocyte activation by mitogens (16). Treatment of 3T3 mouse fibroblasts with phospholipase activators such as melittin results in morphological changes associated with the transformed cells (17,18), and in biochemical changes which take place after mitogenic stimulation (19). Transformation or increased mitogenesis of the cells infected with tumor viruses such as polyoma virus and sarcoma virus appears to be due to the induction of a protein with Mr = approximately 60,000, which has tyrosine-phosphorylating activity in plasma membranes (20,21). Activation of such a tyrosine phophorylating kinase by mitogens such as phorbol esters and epidermal growth factor has also been reported (22). Cells infected with viruses or stimulated with mitogens have a higher capacity to generate prostaglandins; this is probably attributable to activation of phospholipase(s) (23). Since lipomodulin regulates phospholipase activities in intact cells by phosphorylation-dephosphorylation (11), it is likely that exogenously added lipomodulin exceeds the abilities of such kinase(s) to block phospholipase activation. Thus, it is possible that lipomodulin, a phospholipase inhibitory protein whose synthesis is induced in membranes by glucocorticoids (1), exerts its activity in arresting cell growth and inducing differentiation of the cells by the inhibition of phospholipase(s), but the precise mechanisms remain to be elucidated.

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