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EXPRESSION OF A CALCIUM BINDING PROTEIN pEL98 (*MTS1*) DURING DIFFERENTIATION OF HUMAN PROMYELOCYTIC LEUKEMIA HL-60 CELLS

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Summary: The expression of pEL98 (also termed *mts*1), an S100-related calcium-binding protein, was induced during macrophagic or granulocytic differentiation of human promyelocytic leukemia HL-60 cells in response to phorbol 12-myristate 13-acetate or dimethylsulfoxide, respectively. However, pEL98 expression remained at the low level during granulocytic differentiation of the cells by the treatment with *all-trans* retinoic acid. The expression of cell motility, but not adhesiveness or phagocytic ability, was found to be coincided with that of pEL98 (*mts*1), suggesting that pEL98 (*mts*1) is involved in regulating cell motility. Immunocytochemical analysis demonstrated that pEL98 (*mts*1) was expressed in human monocytes, macrophages and polymorphonuclear leukocytes.

pEL98, initially identified as a mRNA expressed in immortalized cell line (1), is an S100-related EF-hand type calcium-binding protein. The amino acid sequence of pEL98 was revealed to be identical to that of *mts1*, identified as a mRNA distinguishing metastatic tumor cells from nonmetastatic cells (2), and 18A2, identified as a serum-inducible mRNA in BALB/c3T3 cells (3), and shows 96% homology with that of p9Ka, identified as a mRNA induced during differentiation of rat mammary epithelial stem cells to myoepithelial-like cells (4). Although definite functions of these related proteins still remain uncertain, there is accumulating evidence that these are partly involved in regulating cytoskeletal organization and thereby cell motility. We have recently found that pEL98 (*mts*1) associates with nonmuscle tropomyosins and exists along actin microfilament bundles (5). p9Ka has also been shown to be associated along actin stress fibers in a

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Abbreviations: PMN, polymorphonuclear leukocyte; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethylsulfoxide; RA, *all-trans* retinoic acid; NBT, nitro blue tetrazolium; fMLP, N-formyl-methionyl-leucyl-phenylalanine; DME, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline.

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rat mammary epithelial cell line transfected with the gene for p9Ka (6). In addition, we have demonstrated that there is a close correlation between the expression level of pEL98 (*mts*1) and cell motility in various murine cell lines (7).

We have recently found that mouse bone marrow cells express a very low amount of pEL98 (*mts*1), while it is expressed abundantly in mouse peritoneal macrophages (8). These results suggest a possibility that pEL98 (*mts*1) expression is related to differentiation of myeloid cells. To test this possibility, we examined pEL98 (*mts*1) expression during differentiation of human promyelocytic leukemia HL-60 cells that can be induced to differentiate into macrophage- and granulocyte-like cells in response to PMA (9) or either DMSO (10, 11) or RA (12, 13), respectively, and also in mature myeloid cells. The present study demonstrates that pEL98 (*mts*1) expression is induced in PMA- and DMSO-treated HL-60 cells, whereas it remains at the very low level in RA-treated cells. In addition, we demonstrate that human monocytes, macrophages and PMN express a significant amount of pEL98.

MATERIALS AND METHODS

Materials: PMA was purchased from LC Services Co., Wobburn, and RA and fMLP were from Sigma, St.Louis, MO. Esterase stain kit was provided by Muto Pure Chemicals Co. Ltd., Tokyo, Japan. NBT was obtained from Nacalai Tesque, Inc., Kyoto, Japan. NycoPrep 1068, NycoPrep 1063 and Polymorphprep were purchased from NYCOMED PHARMA AS, Oslo, Norway.

Cell culture: The human promyelocytic leukemia HL-60 cells were grown in RPMI1640 supplemented with 10% fetal bovine serum (13). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Isolation of human mononuclear cells and PMN: Human peripheral blood mononuclear and PMN were isolated as described previously (14), using NycoPrep 1068 and Polymorphprep , respectively. Platelets were removed by using NycoPrep 1063. Human colostral macrophages and PMN were prepared by the method described elsewhere (15). Peripheral blood and colostrum were collected from healthy donors and a normal donor on days 2-4 postpartum, respectively.

Evaluation of cell differentiation: Nonspecific esterase activity was determined by cytochemical assay using an esterase stain kit with α-naphthyl butyrate as a substrate (16). NBT reduction was assayed as reported previously (12). Phagocytic activity was measured by counting the number of cells that phagocytosed polystyrene latex particles (1.02-μm diameter, Sigma) in the presence of 5% fresh human serum (13). The percentage of nonspecific esterase positive, NBT reduction positive cells and phagocytic cells were determined by counting at least 200 cells. Directed cell motility (chemotaxis) and random (spontaneous) motility were assayed as described previously (17, 18), with some modifications. For chemotaxis assay, polycarbonate filters (5-μm pore size, Coaster) were placed in the blind well Boyden chambers. The cells (3×10^5) suspended in DME containing 0.1% bovine serum albumin were placed in the upper compartment of the chamber. fMLP (10^{-7} M) was added to the lower chamber. After incubation for 5 h at 37°C, the cells in the lower chamber which migrated through the pores but not adhered to the filters were counted. The cells adhered to the bottom side of the filters were also counted. For random motility assay, differentiation-inducing agents were added to 10-day-old colonies in semi-solid agar medium and 2 or 5 days later the percentage of dispersed colonies due to migration of colony-forming cells was determined .

Northern blot analysis: Total RNA was extracted with guanidium thiocyanate (19). Ten μg of total RNA were electrophoresed on 1% agarose gel containing formaldehyde and transferred onto nylon filters (19). Blots were hybridized with a ^{32}P-labeled pEL98 cDNA probe (1). Filters were finally washed at 50°C in 30 mM NaCl, 3 mM sodium citrate and 0.1% SDS.

Immunoblot analysis: Cells were lysed in extraction buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM EGTA and 1 mM PMSF) for 10 min on ice. The lysates were centrifuged at 10,000*g* for 10 min and the supernatant (20 μg protein) was used for immunoblot analysis as described previously (5). The pEL98 protein was detected using polyclonal anti-pEL98 antibodies (5) and an enhanced chemiluminescence Western blotting detection kit (Amersham, UK).

Immunocytochemistry: Cells on cover slips prepared by cytocentrifugation were fixed for 30 min with 4% formaldehyde and 5% sucrose in DPBS. After rinsing with DPBS, the cells were permeabilized with 0.5% Triton X-100 in DPBS for 4 min, and rinsed again with DPBS. After blocking nonspecific binding sites with 3% BSA in DPBS containing 0.1% glycine for 1 h, the cells were incubated for 1 h with affinity-purified anti-pEL98 antibodies in DPBS containing 0.1% BSA. The first antibodies were localized with TRITC-labeled goat anti-rabbit IgG (5).

RESULTS

We examined pEL98 expression during differentiation of HL-60 cells. The differentiation of HL-60 cells cultured with PMA (16 nM) into macrophage-like cells was confirmed by the appearance of cells with nonspecific esterase activity. As shown in Fig. 1, the differentiation was induced in a time-dependent manner (Fig. 1A). Approximately 80% of the cells treated with 16 nM PMA for 3 days was nonspecific esterase positive. The expression of pEL98 (*mts*1) at mRNA and protein levels in PMA-treated HL-60 cells was examined by Northern and Western blot analyses, respectively. A nearly maximal induction of the pEL98 (*mts*1) mRNA was already seen 24 h after the treatment with 16 nM PMA (Fig. 2A), while the induction of the protein was detected 24-48 h after the treatment (Fig. 3A). Notably, the expression of pEL98 (*mts*1) at the protein level was in parallel with

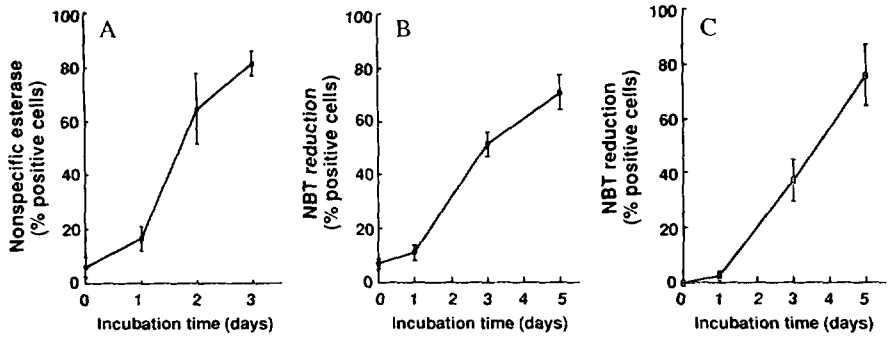

Figure 1. Differentiation of HL-60 cells into macrophagic or granulocytic cells. HL-60 cells were cultured with 16 nM PMA (A), 1.25% DMSO (B) or 10 μM RA (C) for various periods. After each treatment, nonspecific esterase activity or NBT reduction activity was determined as a macrophagic or granulocytic differentiation marker, respectively. Values are mean ± S.D. of triplicate determinations.

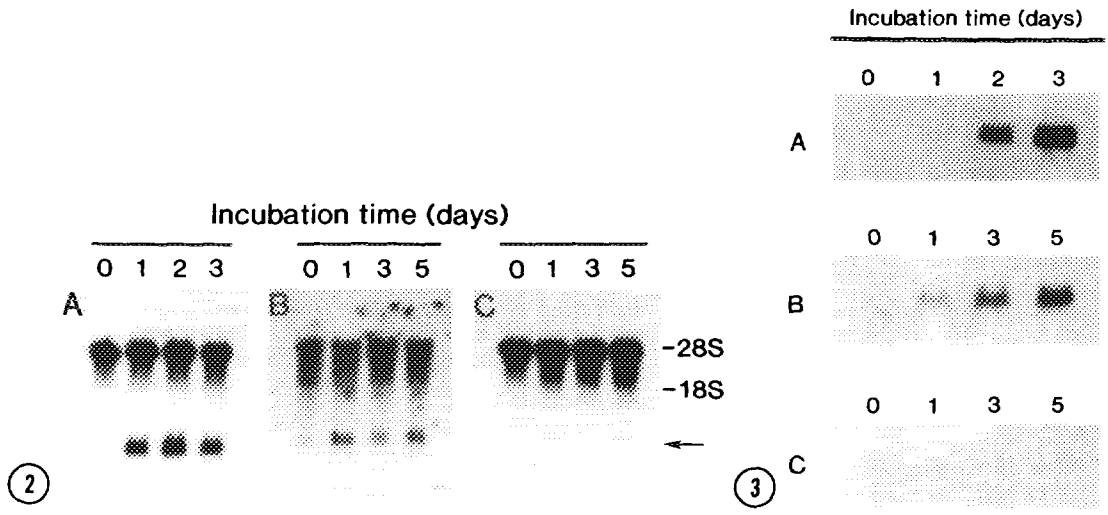

Figure 2. Expression of pEL98 (*mts*1) mRNA during differentiation of HL-60 cells. HL-60 cells were treated with 16 nM PMA (A), 1.25% DMSO (B) or 10 μM RA (C) for the indicated periods. Expression of the pEL98 mRNA was examined by Northern blot analysis. The arrow indicates the position of pEL98 (*mts*1) mRNA (approximately 0.6 kb in size). The upper bands probably represent non-specific hybridization of the probe with 28S ribosomal RNA.

Figure 3. Expression of the pEL98 (*mts*1) protein during differentiation of HL-60 cells. HL-60 cells were treated with 16 nM PMA (A), 1.25% DMSO (B) or 10 μM RA (C) for the indicated periods. Expression of the pEL98 protein was examined by Western blot analysis. Only the region of interest of the gel is shown.

the appearance of cells with nonspecific esterase activity (Fig. 1A), indicating that pEL98 (*mts*1) expression was accompanied by macrophagic differentiation of HL-60 cells.

Next, HL-60 cells were induced to differentiate into granulocytic cells by treatment with either DMSO or RA and the differentiation was assessed by NBT reduction. As shown in Fig. 1B and C, treatment of the cells with either DMSO (1.25%) or RA (10 μM) resulted in the appearance of cells with NBT reduction. Approximately 70% and 80% of the cells treated with 1.25% DMSO or 10 μM RA for 5 days was NBT reduction positive, respectively. A nearly maximal induction of the pEL98 (*mts*1) mRNA was evident as early as 24 h after the treatment of the cells with DMSO (Fig. 2B) and the protein was expressed in a time-dependent manner (Fig. 3B). In a separate experiment, we evaluated the expression level of the pEL98 (*mts*1) protein in both HL-60 cells treated with 1.25% DMSO for 5 days and the cells treated with 16 nM PMA for 2 days and found that the amount of the protein expressed in DMSO-treated cells was approximately 60% of that expressed in PMA-treated cells (data not shown). On the other hand, only a marginal amount of pEL98 was expressed in RA-treated cells (Fig. 2C and Fig. 3C).

In order to find out what cellular function is associated with the expression of pEL98 (*mts*1), we examined various cellular phenotypes of PMA-, DMSO- or RA-

Table 1. Functional phenotypes of HL-60 cells treated with PMA, DMSO and RA

Treatment	Adhesive property (% of cells adhered)	Phagocytic ability (% of phagocytic cells)	Motility	
			Random (% of colonies dispersed)	Directed (No. of cells penetrated, x10⁴)
NONE	0	2.2 ± 0.9	7.8 ± 3.7	0.3 ± 0.1
PMA	89.9 ± 2.6	22.8 ± 3.1**	70.0 ± 4.6**	ND
DMSO	1.0 ± 1.0	29.1 ± 6.7**	41.0 ± 3.3**	7.3 ± 0.7**
RA	0.8 ± 1.2	46.4 ± 3.8**	24.4 ± 4.4*	0.2 ± 0.1

HL-60 cells were treated with 16nM PMA for 2 days, 1.25% DMSO for 5 days or 10 μM RA for 5 days. The assays were performed as described in Materials and Methods. Values are mean ± standard deviations. ND; not determined. * $p < 0.01$, ** $p < 0.001$.

treated HL-60 cells including adhesiveness, phagocytosis and cell motility. As shown in Table 1, surface adhesive property was expressed predominantly in PMA-treated cells, but not in DMSO- or RA-treated cells. Phagocytic ability was expressed in all of them. Therefore, there was no relationship between the expression of these phenotypes and that of pEL98 (*mts*1). Random motility in semi-solid medium was significantly induced in both PMA- and DMSO-treated cells. However, it was weakly induced in RA-treated cells (Table 1). It should be noted that, as described previously (17), the halo formed around each colony as the result of cell migration after the addition of RA was much smaller than that appearing after the addition of PMA or DMSO (data not shown), indicating that random motility was incompletely expressed in RA-treated cells. Directed motility to the formylated peptide (fMLP) was also induced in DMSO-treated cells, but not in RA-treated cells. These results suggest that the expression of cell motility is coincided with that of pEL98 in these cells.

Monocytes and PMN in peripheral blood and tissue macrophages and PMN in colostrum were examined for pEL98 expression in these cells by indirect immunofluorescence study. As shown in Fig. 4, a bright fluorescence was observed in the cytosol of these cells when affinity-purified anti-pEL98 antibody was used. Only background level of fluorescence was evident when preimmune serum was used (data not shown).

DISCUSSION

The present study demonstrated that monocytes, macrophages and PMN were all positive for pEL98 (*mts*1) expression. In addition, we demonstrated that untreated HL-60 cells expressed a very low amount of pEL98 (*mts*1), while its expression was significantly induced during macrophagic or granulocytic differentiation in response to PMA or DMSO, respectively. In contrast, pEL98 expression remained at the low level during granulocytic differentiation of HL-60 cells treated with RA.

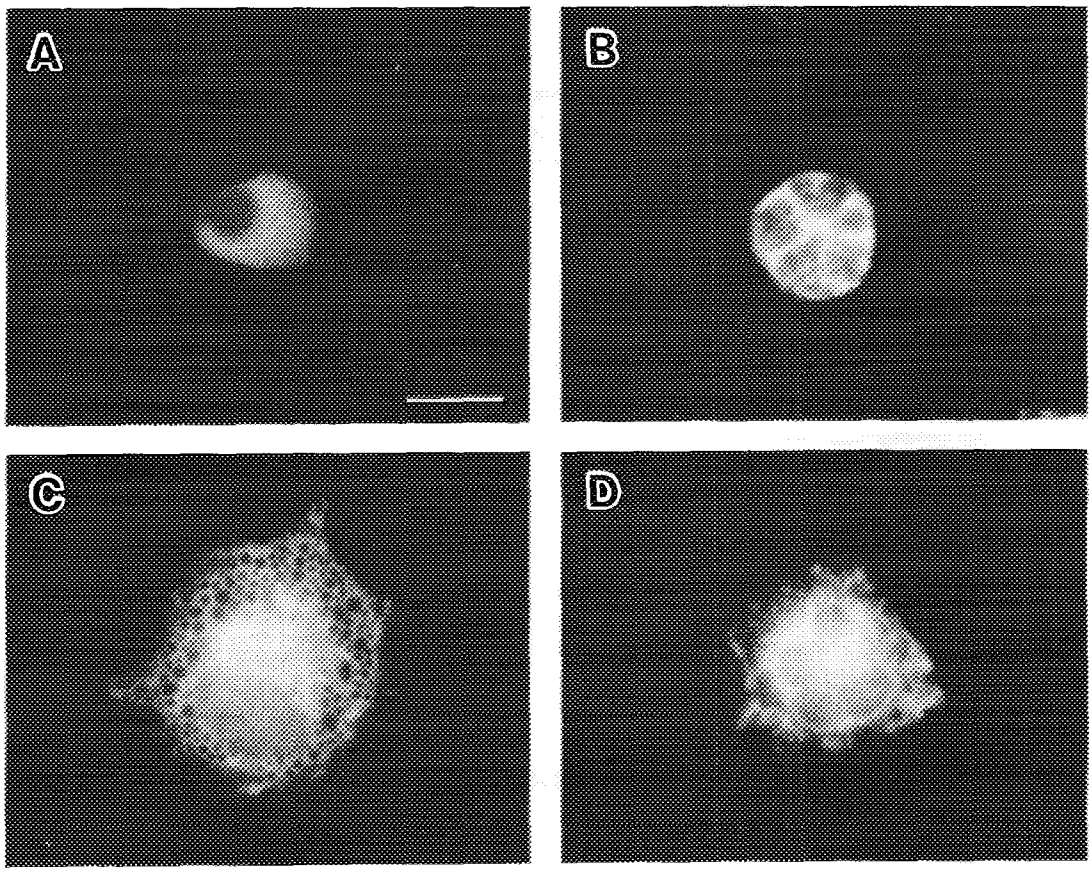

Figure 4. Immunofluorescence staining of human peripheral blood monocytes (A) and PMN (B) and colostral macrophages (C) and PMN (D) with the affinity-purified anti-pEL98 (*mts*1) antibodies. Only background level of fluorescence was observed when preimmune serum was used (data not shown).

At present, we do not know the reason why pEL98 (*mts*1) expression is induced differentially in DMSO- and RA-treated cells, in spite of that a similar degree of granulocytic differentiation (as assessed by the appearance of cells with NBT reduction) was observed in both cells. However, it is possible that the difference reflects a functional difference(s) of these cells. To examine what cellular phenotype is associated with the expression of pEL98, we evaluated various functional phenotypes of PMA-, DMSO- and RA-treated cells including adhesive property, phagocytic ability and cell motility. The results showed that the expression of cell motility, especially directed motility toward fMLF, but not of phagocytic ability or adhesiveness, was coincided well with pEL98 expression. Macrophages, PMA and monocytes are known to be highly motile. In addition, we have recently found that there is a close correlation between pEL98 expression and cell motility in various murine fibroblasts and epithelial tumor cells (7). Thus, these observations

collectively suggest that pEL98 is involved in regulating cell motility in mature myeloid cells as well.

MRP-8 and MRP-14, other S100-related calcium binding proteins, are reported to be primarily expressed in monocytes and granulocytes, while normal tissue macrophages do not synthesize these MRPs (20, 21). The syntheses of these proteins were induced during differentiation of HL-60 cells induced by RA, but not by PMA (22), indicating that the expressions of pEL98 and the MRPs are regulated separately. Unfortunately, the function of the MRPs also remains obscure, but they may be associated with both monocyte and neutrophil activation (20). Although pEL98 is not myeloid cell-specific, it may also be associated with leukocyte activation. Further studies on the function(s) of these S100-related calcium-binding proteins might provide clues as to the mechanisms of activation of monocytes/ macrophages and neutrophils.

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