NOTE

Enhanced Expression of CD69 and CD25 Antigen on Human Peripheral Blood Mononuclear Cells by Prolactin

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Abstract. Several clinical reports have suggested that prolactin (PRL) plays an important role in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE). We have investigated the influence of PRL on immune system, by evaluating the effects of PRL on the expression of CD69 and CD25 on human peripheral blood mononuclear cells (PBMCs). Human PBMCs obtained from healthy female volunteers were incubated with phytohemagglutinin (PHA) in the presence or absence of various concentrations of PRL. The expression of CD69 and CD25 was monitored using immunofluorescence staining and flow cytometry. PRL significantly enhanced the expression of CD69 and CD25 on activated PBMCs compared with that in the absence of PRL (p<0.05, paired t-test). Increasing doses of PRL enhanced the expression of CD69 up to 2 μg/ml and CD25 up to 1 μg/ml. The enhanced expression of CD69 was observed on CD8+ T lymphocytes but not on CD4+ T lymphocytes. Our data suggest that PRL can significantly enhance the expression of CD69 and CD25 molecule on human PBMCs when induced by PHA. However, PRL would have to be at optimal concentration in order to enhance their expression.

Key words: Prolactin (PRL), CD69, CD25, Phytohemagglutinin (PHA)

PROLACTIN (PRL) is a pituitary hormone which is known to induce the synthesis of milk proteins in women during breastfeeding [1]. Of particular interest, however, is the role of PRL in immune regulation. Evidence suggests that the immune system is an important target of pituitary PRL, and that PRL may play a physiological role in the regulation of cell-mediated and humoral immune response [2]. It has been shown that PRL has an important role in regulating T lymphocyte-mediated immune functions [3, 4]. Receptors for PRL on human lymphocytes have been identified on T and B lymphocytes [5–7].

On the other hand, T lymphocytes express de novo several cell surface glycoproteins, which are also called activation inducer molecules, in a characteristic order during activation. CD69, one of the earliest of the cell surface activation markers, is a phosphorylated 28–32-kilodalton disulphide linked homodimer. Once expressed, CD69 is thought to take part in the ongoing activation process. CD25 (the α chain of the IL-2 receptor), a phosphorylated 55–60-kilodalton cell surface glycoprotein, is also an activation marker induced during lymphoid cell activation. The synthesis of CD25, along with the IL-2 itself, is induced by the activation of T lymphocytes on the initial encounter with specific antigen.

In this report, we have examined the effects of PRL on the expression of CD69 and CD25 antigen on human peripheral blood mononuclear cells (PBMCs). We have found that exogenously-added PRL could significantly enhance the expression of CD69 and CD25 antigen on human PBMCs.

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Materials and Methods

PBMCs

Human PBMCs were separated from fresh blood samples obtained from 16 healthy female volunteers aged 20 to 62 years old using the standard Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) method. The cells were resuspended in RPMI 1640 medium supplemented with L-glutamine 3 mg/l (Gibco BRL, Paisley, Scotland), and 10% fetal bovine serum (FBS) (Gibco BRL).

Cell cultures

The cells were adjusted to a final concentration of $1 \times 10^6$ cells/ml and then incubated at 37°C for 24 hr (CD69) or 72 hr (CD25) in a 48-well flat-bottomed plate (Asahi Techno Glass Corp., Tokyo, Japan), in the absence or the presence of various concentrations of recombinant human PRL (Genzyme, Cambridge, MA). Phytohemagglutinin (PHA) (Difco, Detroit, MI) was used to stimulate PBMCs to mimic polyclonal activation and was added at a final concentration of 1/10000 and 1/5000 v/v which was found to be optimal to evaluate the effect of PRL on CD25 and CD69 expression, respectively. Negative controls were simultaneously prepared and treated identically in every way to the test samples.

In order to eliminate the influence of bovine estrogen ($E_2$) or some other steroid hormones present in the media, stripped FBS was prepared. FBS was stripped of estrogen and other steroid hormones, using dextran-coated charcoal as previously described [8]. After this treatment, the FBS had no detectable levels of $E_2$ or cortisol estimated by radioimmunoassay in contrast to the concentrations in conventional FBS ($E_2$: 20.2 pg/ml and cortisol: 1.2 µg/dl). The above-mentioned cultures were also performed identically using stripped FBS.

Fluorescence-activated cell sorter (FACS) analysis

Cells were centrifuged, washed, resuspended in phosphate buffered saline (PBS), and stained at 4°C for 30 min with phycoerythrin (PE)-labeled anti-CD69 (Immunotech, Marseille, France) or fluorescein isothiocyanate (FITC)-labeled anti-CD25 (Immunotech). After washings, in order to investigate the difference in CD69 expression in lymphocyte subsets, the cells stained with PE-labeled anti-CD69 were resuspended in PBS, and then restained at 4°C for 30 min with FITC-labeled anti-CD3, CD4, CD8 MAb (Immunotech). After additional washing, the cells were resuspended in PBS supplemented with 0.5% paraformaldehyde, and then analyzed using Epics XL (Beckman Coulter, Miami, FL) flow cytometer and Epics XL software (Beckman Coulter). Using $5 \times 10^5$ cells, the light scatter characteristics of lymphocytes were gated to minimize interference from other cell types. Antigen density was evaluated by assessing the mean fluorescence intensity (MFI) of antigen expression and the percentages of the positive cells. FITC- and PE-conjugated mouse IgG with irrelevant specificities was used to differentiate between positive and negative cells. The fluorescence background of the control IgG was subtracted. The control Abs were always stained <0.5% of the cells in assessment of the percentages of antigen positive cells.

Statistical analysis

The data are presented as the mean ± SEM of each experiment. Statistical analyses were performed as follows: the effect of PRL on CD69 or CD25 expression on PHA-activated PBMCs and the effect of PHA on CD25 expression on resting PBMCs were evaluated by paired t-test, and the effect of PHA on CD69 expression on resting PBMCs was evaluated by Welch’s t-test. P value <0.05 was considered as significant.

Results

We investigated the effect of PHA on CD69 expression on PBMCs. CD69 was not detected on resting PBMCs (MFI = 0.04 ± 0.02) and the expression of CD69 was strongly induced by PHA stimulation (PHA 1/10000: MFI = 0.85 ± 0.07, PHA 1/5000: MFI = 1.22 ± 0.10) ($p<0.01$, Welch’s t-test) in the 24 hr cell culture (Fig. 1a). Increasing doses of PHA progressively enhanced the expression of CD69 on PBMCs. Elevation of CD69 expression on PBMCs was noted after 12 hr, with the highest CD69 expression after 24 hr cell culture as shown in Fig. 1b. There were no differences observed by age in the effect of PHA inducing CD69 expression (data not shown).

PHA alone did not induce the CD69 expression on human PBMCs (data not shown). With PHA (1/5000
v/v), the expression of CD69 was significantly enhanced in the presence of 2 μg/ml PRL (MFI = 1.20 ± 0.12) compared with that in the absence of PRL (MFI = 0.98 ± 0.11) (p<0.05, paired t-test) (Fig. 2a). The results presented were taken from 24 hr cell culture, which demonstrated the most significant elevation of CD69. Fig. 3a shows the effects of PRL in concentrations ranging from 1 μg/ml to 4 μg/ml on the CD69 expression on human PBMCs. Increasing doses of PRL progressively enhanced the expression of CD69 on PHA-activated PBMCs. The maximal effect was observed at 2 μg/ml followed by a fall at the higher concentration (4 μg/ml). Elevation of CD69 expression on PHA-activated PBMCs was already noted after 6 hr exposure to PRL (2 μg/ml) and was still noted after 36 hr of culture. The highest CD69 expression was recognized after 24 hr culture as shown in Fig. 4. In additional experiment using stripped FBS,
the expression of CD69 on PBMCs was also enhanced at 2 μg/ml of PRL (data not shown), suggesting that no other constituents in FBS contributed to the results. Since the expression of CD69 as a lymphocyte activation marker is induced within only two hours of stimulation, the effects of concurrently yielded cytokines could also be eliminated. There were no observed differences by age of the subjects in the effect of PRL inducing CD69 expression on PHA-activated PBMCs (data not shown). In other experiments using two-color immunofluorescence staining in order to investigate the difference in lymphocyte subsets, increased expression of CD69 was observed on CD8+ T lymphocytes but not on CD4+ T lymphocytes both in the MFI and the percentage of positive cells, although it failed to reach statistical significance (Table 1, Fig. 5).

PRL alone did not induce the CD25 expression on human PBMCs (data not shown). With PHA (1/10000 v/v), the expression of CD25 was significantly enhanced in the presence of 0.2 μg/ml PRL (MFI = 2.96 ± 0.35) compared with that in the absence of PRL (MFI = 2.50 ± 0.49) in the 72 hr cell culture.
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(p<0.05, paired t-test) (Fig. 2b). Fig. 3b shows the effects of PRL ranging from 0.2 μg/ml to 2 μg/ml on the CD25 expression on human PBMCs. Increasing doses of PRL progressively enhanced the expression of CD25 on PHA-activated PBMCs. The maximal effect was observed at 1 μg/ml followed by a fall at 2 μg/ml.

Discussion

Evidence has accumulated to suggest that the immune system is an important target of pituitary PRL. Russell et al. [7] have identified PRL receptors on human PBMCs and have indicated that they were specific for PRL. It has already been reported that PRL could induce IL-2 receptor (CD25) expression in murine lymphocytes [9, 10], but the effect of PRL on the expression of CD25 and other activation markers in human lymphocytes has rarely been evaluated. In this paper, we have shown that PRL could significantly enhance the expression of CD69 and CD25 antigen on human PBMCs.

The expression of CD69, which was not detected on resting PBMCs, was strongly induced by PHA stimulation, and the highest expression occurred after 24 hr cell culture. Cebrian et al. [11] have previously demonstrated that the peak of CD69 expression in human peripheral blood T lymphocytes was reached at 24 hr by phorbol ester (PMA). In our experiment, the kinetics of expression of CD69 showed a similar pattern using PHA as a mitogenic stimulus. PMA is a compound that strongly activates protein kinase C (PKC) and stimulates many types of cells, whereas PHA mainly stimulates T lymphocytes. Therefore, our data using PHA brought out a more T lymphocyte-specific response.

A number of observations have suggested an immunostimulating effect of PRL. The proliferative response of mouse spleen cells exposed to Con-A was significantly potentiated by PRL [12]. Ornithine decarboxylase (ODC) in human lymphocytes [7] and the formation of IL-2 cell surface receptors on lymphocytes [9, 10, 13] were induced by PRL. In our experiment, PRL at 1–2 μg/ml significantly enhanced the expression of CD69 and PRL at 0.2–1 μg/ml significantly enhanced the expression of CD25 induced by PHA stimulation, respectively, but PRL alone did not induce the expression of CD69 and CD25. PHA may be a useful mitogen as a co-stimulatory factor when PRL exerts its immunostimulating effect. It has been shown that the stimulation of murine lymphoid cells with Con-A promotes the enhancement of the density of PRL receptors [14]. PHA might also have similar effect on human PBMCs. PRL concentrations were determined after preliminary experiments. The difference of PRL concentrations between the two activation markers might be due to the difference of incubation times for the induction of their expressions. Since it takes a longer time for the induction of CD25 expres-
sion than for that of CD69, autocrine factors such as cytokines might be produced by stimulated lymphocytes. Even though PRL concentrations which induced CD69 and CD25 were supraphysiological, high concentrations of PRL might be induced in the microenvironment of the inflammatory sites in autoimmune diseases. In patients with rheumatoid arthritis (RA), synovium infiltrating T cells which play an important role in the pathogenesis, might synthesize PRL in situ [15]. Moreover, PRL is produced by PBMCs as well as the anterior pituitary gland [7–9].

On the other hand, several reports have suggested that PRL in high concentrations is immunosuppressive. High levels of PRL inhibited the activity of ODC in human lymphocytes [7], and inhibited PHA-induced mitogenesis of chicken lymphocytes [16]. In our experiment, PRL enhanced the CD69 expression up to 2 μg/ml and also enhanced the CD25 expression up to 1 μg/ml, but it reduced their expressions above these concentrations. PRL would have to be at optimal concentration in order to exert its immunostimulating effect. Experiments which evaluated the immunological effect of PRL were performed under various conditions using lectins [7, 8, 13, 17], hence the optimal concentration of PRL could not be established definitively. Further study is needed to clarify the best condition in which PRL exerts its immunostimulating effect.

Several clinical reports have also suggested an important role for PRL in the pathogenesis of autoimmune disease, with hyperprolactinemia being implicated in the pathogenesis of systemic lupus erythematosus (SLE) [18–24]. A correlation between increased PRL levels and disease activity as measured by joint swelling was observed in RA [25]. On the other hand, the relationship between CD69 and various autoimmune diseases has been demonstrated. The CD69 to CD3 ratio of PBMCs was correlated with SLE disease activity index score in SLE [26]. The percentage of T lymphocytes bearing CD69 was significantly increased in synovial fluid from patients with RA [27]. Although Athreya et al. [8] have shown that physiological concentrations of PRL did not have any consistent effects on the T lymphocyte subsets in vitro, our experiment the enhancement of CD69 expression by PRL was significantly recognized on CD8+ cells. Of particular interest, Crispin et al. [28] have reported the expression of CD69 was upregulated in freshly isolated CD4+ and CD8+ T-cell subsets from SLE patients. Their finding that the difference of CD69 expression between SLE and normal subject was statistically significant only for the CD8+ subset, is consistent with our data. PRL is thus one of the prime candidates in the immunostimulating state of SLE. Our data confirm that PRL in optimal concentration exerts an immunostimulating effect, and suggest that CD69 and CD25 might be useful markers to evaluate the immunostimulating effect of PRL in autoimmune diseases.

References


