Expression and modulation of the Lewis x antigen (CD15) on the T cell line Molt-4

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Abstract The T cell lines Molt-4 and H9 exhibited a characteristic distribution of the cell adhesion molecule Lewis x (CD15, lacto-*N*-fucopentanose III) showing an unusually broad peak by flow cytometry ranging from cells without CD15 to cells with high expression. The cytokines IL-1, IL-2, IFN-β, IFN-γ, and TNF-α, known to activate T cells, did not affect CD15 expression. However, phorbol myristate acetate and the thymic peptide extract Thymex-L were able to enhance both the number of CD15-positive cells and the median fluorescence. The effects of both inducers were dose- and time-dependent. An additive or synergistic effect was not seen. These data suggest that phorbol esters and distinct thymic peptides can modulate the expression of the cell surface antigen CD15.

Key words: CD15 expression; Phorbol ester; Thymic peptide; Molt-4 cell

1. Introduction

The selectin family is involved as part of the cell adhesion molecule system in the initial stage of leukocyte extravasation [1–3]. E- and P-selectin are expressed on activated endothelia and are recognized by various leukocyte populations including activated T cells [4,5]. Their counterparts on leukocytes are cell surface carbohydrates [6] including the Lewis x antigen (CD15, lacto-*N*-fucopentanose III). CD15 is found on numerous cellular proteins involved in cellular adhesive events and has a broad cellular distribution also on leukocytes [7] and tumor cells [8–10]. In addition to its well-recognized adhesive role, the engagement of CD15 results in monocyte activation [11] suggesting CD15 as part of a signaling pathway. Only little information is available about the modulation of CD15. In the breast cancer cell line MDA-MB-468 CD15 was downregulated by TNF-α, PMA, IL-1α, IFN-γ, and all-*trans*-retinoic acid, whereas 1,25$(OH)_2$-vitamin D_3 upregulated its expression [12]. In contrast CD15 remained unchanged after treatment of two human myeloid leukemia cell lines (NB4, HL60) with all-*trans*-retinoic acid, bryostatin 1, or PMA [13].

We investigated the T cell leukemia line Molt-4 clone 8 for expression of CD15. Molt-4 cells are widely used as a model for T lymphocytes and share many features like cytokine secretion [14] and receptor expression [15] with normal T cells. The modulation of CD15 was tested using known cytokines. Additionally the phorbol ester PMA was included. PMA has been found to enhance IL-2 secretion [14], increase TNF-α mRNA production [16] and modulate leukocyte surface antigens [17] of Molt-4 cells. Moreover, since the induction of specific cell surface receptor molecules like CD4 and CD8 on T cells normally takes place in the thymus during T cell maturation (reviewed in [18]) and may be mediated by so-called thymic hormones, we also tested the influence of several thymic peptides and extracts on CD15 modulation. In the present study we provide evidence that PMA and a total thymus peptide extract are able to enhance CD15 expression in Molt-4 cells.

2. Materials and methods

2.1. Materials

Thymex-L, TFX, prothymosin α_1 [19], and thymosin β_4 [20] were gifts of Thymoorgan-GmbH (Vienenburg, Germany). Thymex-L is a total water extract from juvenile calf thymus, prepared by homogenization, centrifugation, and sterile filtration. The final lyophilisate contains 0.6 mg protein/mg, a variety of enzymes, and thymic peptides. TFX (Thymomodulin) is prepared by water extraction of calf thymus, centrifugation, 3 times ultrafiltration, and gel filtration. The thymic fraction with molecules < 10 kDa contains main peptide components around 4200 Da. THFγ2 is a chemically defined octapeptide isolated from dialysates of calf thymus homogenates [21] (Pharmacia, Milan, Italy). TP5 was from Ferring Co. (Kiel, Germany). IL-1, IL-2, and TNF-α were from Boehringer Mannheim (Germany). Phorbol myristate acetate (PMA) was from Sigma (Deisenhofen, Germany). IFN-γ was from Rentschler Co. (Laupheim, Germany).

2.2. Cell culture

The cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin/0.1 mg/ml streptomycin. The cells were subcultured twice a week and seeded at an initial density of 2.5×10^5 cells/ml. Cell viability was assessed by trypan blue exclusion and found to be routinely $>95\%$.

2.3. Cell induction

7×10^5 Molt-4 clone 8 cells were suspended in 1 ml RPMI 1640, 5% FCS in a 24-well microtiter plate and treated with the indicated substances for 24 h at 37°C in a humidified incubator. Subsequently the viability was estimated using trypan blue exclusion and the CD15 expression was measured using flow cytometry. All experiments were repeated at least three times. Representative experiments are shown.

2.4. CD15 expression by flow cytometry

2×10^5 cells were stained with a monoclonal anti-CD15 antibody (clone 80H5, Dianova, Hamburg, Germany) for 60 min at room temperature, washed, incubated with FITC-conjugated goat anti-mouse IgG antibody (Sigma) for 45 min at 4°C, and washed again. For each sample a control reaction omitting the CD15 antibody was performed. Flow cytometric analysis was executed on a FACSCalibur (Becton Dickinson) using the CellQuest software. During flow cytometry, dead cells and debris were excluded by appropriate scatter gates. Control values were subtracted from all samples.

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Abbreviations: PMA, phorbol myristate acetate; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor

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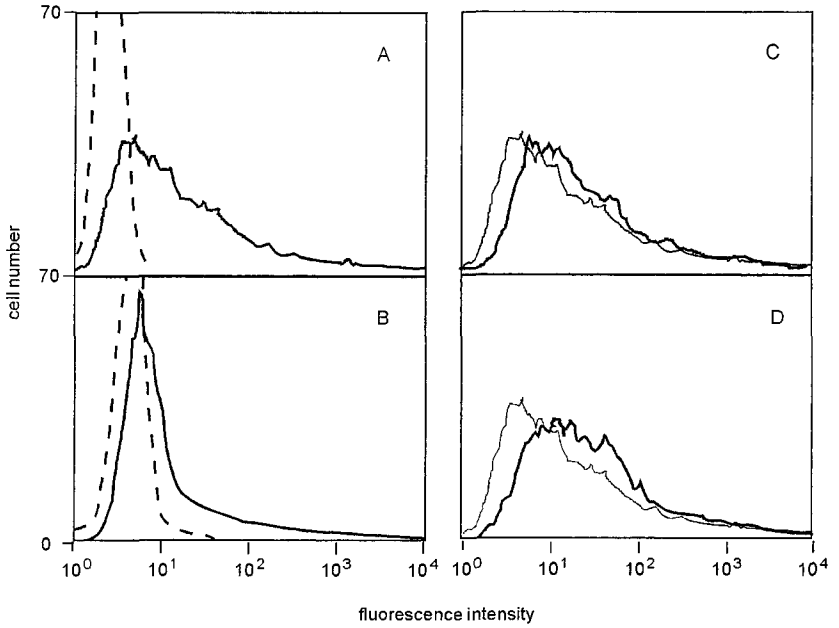

Fig. 1. Flow cytometric analysis of CD15 in Molt-4 and H9 cells. A: Basic expression in Molt-4 cells (solid line); control (dashed line). B: Basic expression in H9 cells (solid line), control (dashed line). C: Expression in Molt-4 cells after treatment with 1 ng/ml PMA (thick line) and 800 μg/ml Thymex-L (line); untreated cells (dashed line). D: Expression in Molt-4 cells after treatment with 800 μg/ml Thymex-L (thick line); untreated cells (dashed line).

3. Results

Molt-4 cells exhibited a characteristic CD15 distribution on flow cytometry: a broad sloping peak containing the majority of cells in the range of weak expression (Fig. 1A). While the level of expression increased, the number of Molt-4 cells continuously decreased. Nevertheless cells with extremely high expression were also found. This was indicated by the discrepancy between the mean (84.1 relative units) and the median (7.0 relative units) fluorescence. For comparison, the expression of another T cell line was investigated. The expression profile of H9 cells shared important features with Molt-4 cells: the broad peak, most cells with weak expression, but cells with a high CD15 level were also present (Fig. 1B). The number of positive cells (about 30%) was less than the number of positive Molt-4 cells (about 60%).

Several T cell inducing cytokines were tested for their ability to change CD15 expression in Molt-4 cells (Fig. 2). None of them was effective. However, PMA was able to enhance both the number of positive cells and the median fluorescence. Moreover, different kinds of peptide preparations from calf thymus were tested at the concentrations given in Fig. 2: (i) oligopeptides (TP5, 5 amino acids; THFγ2, 8 amino acids); (ii) polypeptides (thymosin β_4, 43 amino acids; prothymosin α_1, 109 amino acids); (iii) aqueous extracts (TFX, < 10 kDa; Thymex-L, < 70 kDa).

Only Thymex-L showed an effect comparable to PMA. Fig. 1C,D show that both inducers shifted the expression curves of Molt-4 cells in the same direction. The treatment mainly enhanced the expression of Molt-4 cells with weak constitutive CD15 expression (below 10^2 relative fluorescence units). Whereas PMA treated Molt-4 cells showed a depressed proliferation rate, Thymex-L did not affect cell viability and proliferation. The addition of unspecific proteins such as serum albumin was ineffective (data not shown).

Fig. 3 shows the dose dependence of both activators after 24 h of incubation. Maximal effects of PMA were reached with 1 ng/ml, higher concentrations inhibited the stimulation. The variation of PMA concentration between 0.5 and 5 ng/ml showed less effect. Using Thymex-L up to 5 mg/ml, the effect increased up to 383% of the untreated control. A plateau was

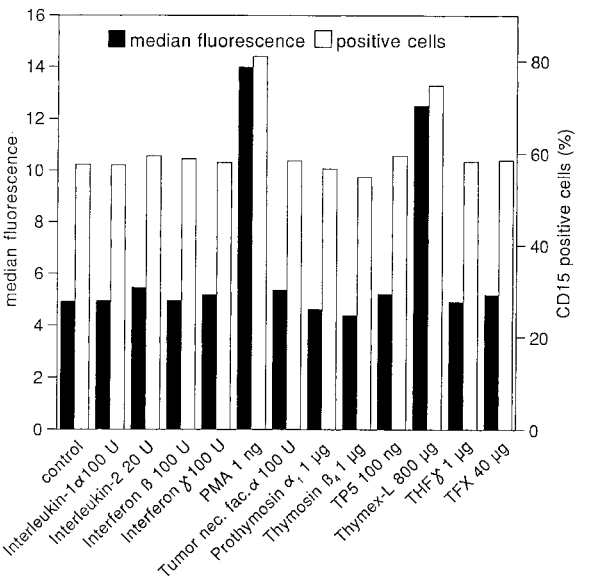

Fig. 2. Modulation of CD15 expression in Molt-4 cells by various cytokines and immunomodulators The cells were treated for 24 h with the indicated substances (all concentrations per ml). Subsequently cell surface CD15 expression was determined by flow cytometry, measuring the binding of an anti-CD15 antibody followed by FITC-conjugated anti-murine IgG antibody. Control values obtained from samples incubated only with the FITC-labeled antibody were subtracted.

not seen. A 48 h incubation with either inducer led to a stronger increase than 24 h of incubation (data not shown).

In addition, the effect of simultaneous treatment of Molt-4 cells with both inducers was investigated: 0.5 or 1.0 ng/ml PMA and 0.5 or 1.0 mg/ml Thymex-L did not augment CD15 expression over the level reached with either of the inducers (Fig. 4). Therefore an additive or synergistic effect was not seen. In one combination a partial antagonism was even observed: 1.0 ng/ml PMA stimulated the median fluorescence by 197% of the control, but in combination with 0.5 mg/ml Thymex-L only 152% was found.

4. Discussion

Here we report the expression and modulation of the adhesion molecule Lewis x (CD15) in Molt-4 cells as a T cell model. This cell surface carbohydrate participates at the first step of leukocyte extravasation and reacts with its counterpart ligand P-selectin. The constitutive CD15 expression is characterized by an unusually broad distribution ranging from cells without CD15 to cells with very high CD15 expression. This was also seen with another T cell line (H9 cells).

The thymus provides a unique environment for the development of T cells. To modulate CD15 expression we investigated cytokines known to be present in the thymus and to influence T cell development. IL-1 is produced by stromal cells of the thymus [22]; its receptor has been isolated from T cells [23]. Also for IL-2, IFN-γ, and TNF-α functions related to T cells have been reported (reviewed in [24]). But none of these cytokines showed a positive or a negative influence on the CD15 number at the Molt-4 cell surface.

A second class of immunoregulatory substances present in the thymus comprises the so-called thymic peptides or hormones. Isolation, characterization and elucidation of their functions are well documented [25,26]. Here we showed that a total thymus water extract has a strong enhancing effect on CD15 expression. Various biological activities of Thymex-L have been demonstrated in vitro and in vivo. Thymex-L has beneficial effects on depressed immunological functions of patients with disseminated cancer [27]. In lung cancer patients, Thymex-L enhanced the lymphoproliferative response in combination with indometacin in vitro [28]. In a clinical trial,

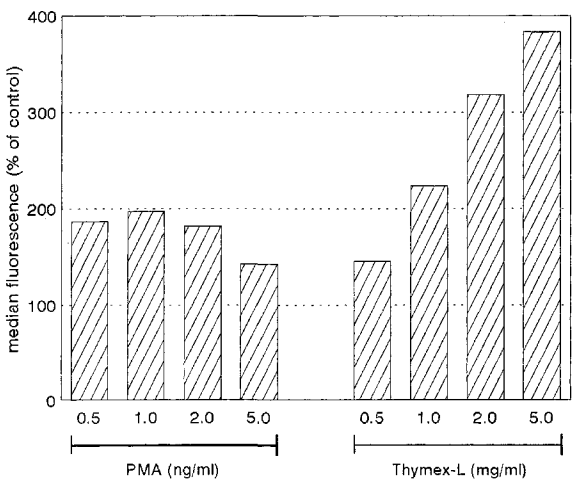

Fig. 3. Dose dependence of CD15 expression in Molt-4 cells Treatment for 24 h and analysis as described in the legend to Fig. 2.

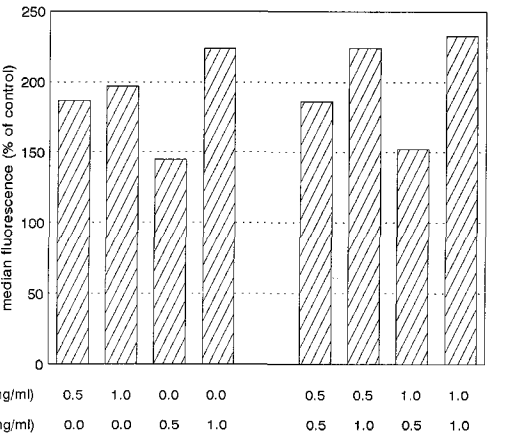

Fig. 4. CD15 expression after 24 h of simultaneous treatment of Molt-4 cells with PMA and Thymex-L. The cells were analyzed as described in the legend to Fig. 2.

Thymex-L showed a protective effect on lymphocytes of lung cancer patients who had been exposed to irradiation [27] and partially normalized depressed monocyte functions in selected melanoma patients [29]. Moreover, Thymex-L potentiated the all-*trans*-retinoic acid-induced differentiation of the human cell line HL60 [30].

The other CD15 activator identified in our investigation was PMA. The biological effects of phorbol esters are known to be mediated through the stimulation of protein kinase C (PKC) [31]. It seems likely PKC may also be involved in CD15 regulation. Interestingly, the PMA effect on CD15 expression is different in various cell lines: in the breast cancer cell line MDA-MB-468 CD15 was downregulated [12], in NB4 and HL60 cells CD15 remained unchanged [13], and here we report an enhanced expression to our knowledge for the first time.

The treatment of Molt-4 cells with PMA and Thymex-L, two completely different agents, resulted in similar effects. The change in flow cytometry was practically not distinguishable. The median fluorescence, a sensitive marker, was increased about 2-fold with PMA (1 ng/ml) and about 3.8-fold using Thymex-L (5 mg/ml). The number of positive cells was only moderately influenced, because Molt-4 cells exhibited a basal CD15 expression of about 60%. Surprisingly, when both inducers were used simultaneously, an additive or synergistic effect was not seen. This suggests that the action of the two substances was mediated by different pathways. It is remarkable that for comparable effects a seemingly 10^6 times higher amount of Thymex-L than of PMA is necessary. This may be caused by the complex composition of the extract containing a variety of molecules not involved in the enhancement of CD15 expression. The purification of an active component may drastically reduce the amount in the assay.

The physiological significance of our results remains to be shown. In general, an enhanced expression of adhesion molecules may be followed by a stronger cell-cell interaction. Granulocytes from patients with chronic myeloid leukemia exhibit reduced binding to P-selectin [32]. In contrast to normal granulocytes, the binding of these cells cannot be blocked with an anti-CD15 antibody suggesting repressed CD15 expression. Other reports confirming this hypothesis are available for the related sialyl Lewis x antigen. In vitro treatment of human gastric adenocarcinoma (NUGC4) cells with di-

methyl sulfoxide resulted in enhanced expression of sialyl Lewis x and in increased adhesion to activated human endothelial cells [33]. An influence of thymic proteins on adhesion is also known. Prothymosin α1 stimulates the binding of peripheral blood lymphocytes to human umbilical vein endothelial cells in vitro [34]. This effect is mediated by an elevated expression of CD16, CD18, and CD56.

It is noteworthy that none of the pure thymic peptides up to 1200 Da was effective in the induction of CD15 expression. This supports the usage of extracts containing high molecular mass, yet uncharacterized molecules. The isolation of such active molecules using Molt-4 cells may lead to the discovery of new biomolecules.

A standardized in vitro bioassay for Thymex-L or its components is not available. None of the known effects described above was suitable for developing such an assay. The effects are mostly seen only on primary cells with the disadvantage of great cell variability. HL60 differentiation is a time consuming process (5 days of incubation) [30]. Our described assay using a well characterized cell line that gives results within 2 days avoids these problems.

Our data demonstrate that a distinct thymic extract can modulate the expression of the cell surface molecule Lewis x. The expression of specific molecules on the cell surface regulates cellular signal recognition, signal transduction, and cell-cell interactions. Therefore these processes may be influenced by thymic preparations. It must be elucidated further whether the therapeutic potential of Thymex-L is related to this property.

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