FEBS Letters 351 (1994) 215–218

FEBS 14473

Bromelain protease F9 reduces the CD44 mediated adhesion of human peripheral blood lymphocytes to human umbilical vein endothelial cells

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Received 8 July 1994

Abstract The thiol protease bromelain has been shown to remove T-cell CD44 molecules from lymphocytes and to affect T-cell activation. We investigated the effect of a highly purified bromelain protease F9 (F9) on the adhesion of peripheral blood lymphocytes (PBL) to human umbilical vein endothelial cells (HUVEC). Preincubation of the lymphocytes with F9 reduced the adherence to about 20% of unstimulated and to about 30% of phorboldibutyrate ($P(Bu)_2$) stimulated lymphocytes. Using flow cytometry, both crude bromelain and protease F9 reduced the expression of CD44, but not of LFA-1, on PBL. F9 was about 10 times more active than crude bromelain; at 2.5 μg/ml of F9 about 97% inhibition of CD44 expression was found. A mAb against CD44 was tested and found to block the F9-induced decrease in PBL-binding to HUVEC. The results indicate that F9 selectively decreases the CD44 mediated binding of PBL to HUVEC.

Key words: Bromelain F9; CD44; Adhesion; Peripheral blood lymphocyte; Human umbilical vein endothelial cell

1. Introduction

The interaction of lymphocytes with either specialized endothelium in lymphoid organs or activated endothelial cells represents the first critical step in lymphocyte migration into lymphoid tissues or into inflammatory sites. Adhesion of lymphocytes to endothelial cells involves multiple receptor ligand interactions, e.g. LFA-1 to ICAM-1 and ICAM-2, CD44 and VLA-4 to VCAM-1 [1]. Recent studies have demonstrated that CD44 is the primary receptor for hyaluronate [2]. In man, the CD44 molecule (the putative homing receptor) has been of interest because of its multiple proinflammatory functions and regulation of the function of other adhesion molecules [3]. The involvement of CD44 in lymphocytes binding to activated endothelial cells was proven by the use of CD44 specific monoclonal antibodies, which block lymphocyte binding [4]. On the other hand CD44 binding to cultured endothelial cells was blocked by the treatment of these cells with hyaluronidase [2]. However, other studies have shown that the role of CD44 in lymphocyte binding may be indirect, suggesting that CD44 binds to additional ligands [5]. CD44 molecules may be also involved in T-lymphocyte activation. On T-cells, ligand binding to the CD44 molecule promotes T-cell activation, interleukin-2 release and T-cell adhesion to monocytes [6].

Treatment of T-lymphocytes with the thiol protease bromelain has been shown to remove surface CD44 as well as other surface molecules, whereas leaving CD2, CD3 and CD28 molecules intact. This effect is associated with enhanced CD2 mediated T-cell activation and T-cell binding to monocytes [7]. However, since bromelain is a mixture of different basic and acidic proteases as well as undefined different components (T. Harrach et al., submitted for publication), the relationship of these findings to well characterized bromelain derived proteases remains to be determined.

In order to test the role of CD44 molecules on lymphocyte adherence, we investigated the effect of a highly purified basic bromelain protease F9, with defined proteolytic activity, on human peripheral blood lymphocytes (PBL) with respect to their ability to adhere to human umbilical vein endothelial cells (HUVEC). In addition, the protease effects on CD44 and LAF-1 expression on lymphocytes were analyzed using flow cytometry. Our results indicate that F9 selectively decreases the CD44 mediated binding of PBL to HUVEC.

2. Materials and methods

2.1. Preparation of lymphocytes

Lymphocytes (PBL) were isolated by a single-step method. About 20–40 ml of freshly isolated blood were obtained from healthy volunteers, anticoagulated with preservative free heparin (10 U/ml final concentration, from Sigma) and layered onto Polymorphoprep (Nycomed Pharma). After centrifugation (400 × *g*, 30 min) at 37°C the lymphocytes were harvested from the first leukocyte band and washed two times with warm PBS. Non-adherent cells (PBL) were obtained after adherence of peripheral blood mononuclear cells (PBMC) to plastic culture dishes for 60 min at 37°C, yielding > 97% viability by Trypan blue exclusion.

2.2. Endothelial cell cultures

Umbilical cords were obtained from recent deliveries. Endothelial cells (EC) were isolated from individual human umbilical cord veins by collagenase (from Sigma) digestion, using a modified method by Jaffe et al. [8]. EC were suspended in culture medium that contained RPMI 1640 medium, 20% fetal calf serum (FCS), 200 U/ml penicillin and streptomycin (Boehringer Mannheim, Germany), 5 U/ml heparin and 50 μg/ml endothelial cell growth supplement (ECGS, Boehringer Mannheim, Germany). After an overnight incubation at 37°C, 5% CO_2, the EC cultures were washed and cultured to confluency in the medium described above. EC of the first to second passage were used in these studies.

2.3. BCECF-AM labeling of lymphocytes

BCECF-AM (acetoxymethyl-ester of 2',7'-bicarboxyethyl-5,6-carboxy-fluorescein from Molecular Probes, USA) was dissolved in DMSO and kept at −20°C in the dark. The stock solution was diluted with RPMI 1640 to give a 2.3 mM solution and used for labelling at 1:600 dilution. Leukocyte cell suspensions were washed in RPMI 1640 and resuspended in RPMI 1640 to a concentration of 10^6 cells/ml. Cells were labeled with BCECF-AM for 30 min, at 37°C, 5% CO_2 and ≥ 100% relative humidity. After labeling leukocytes were washed twice with RPMI 1640 and resuspended in RPMI 1640 + 10% FCS.

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SSDI 0014-5793(94)00860-4

2.4. Bromelain protease F9 treatment of PBL

Bromelain F9 was prepared from crude stem bromelain using a two step cation-excange chromatography; its proteolytic activity was determined using Pyr-Phe-Leu-pNA as substrate (T. Harrach et al., submitted for publication). Unless stated otherwise batches of F9 with proteolytic activity of 1.91 U/mg were used.

Peripheral blood lymphocytes (PBL) at a concentration of 5×10^6 cells/ml were preincubated in the abcence or presence of crude bromelain or F9 at different concentrations for 60 min, 37°C, 5% CO_2 and washed two times with warm PBS. Inactivation of F9 was performed with iodoacetamide as described (T. Harrach et al., submitted for publication). The cells were resuspended in RPMI 1640 and labeled with BCECF-AM, washed and resuspended in RPMI 1640 + 10% FCS.

2.5. Adhesion assay

EC at confluence were removed from culture flasks with 0.05% trypsin and 0.023% EDTA solution (Boehringer Mannheim, Germany), centrifuged and resuspended in culture medium at a concentration of 7.5×10^4 cells/ml. Samples (0.2 ml) were cultured overnight or longer in gelatine coated 96-well microtiter plates until confluence was reached. Preincubated PBL were added in a final volume of 100 µl (1×10^5 cells/well) to the confluent EC-monolayer in 96 microtiter plates. At this stage the cells were stimulated with $P(Bu)_2$ (100 ng/ml final concentration). The labeled and stimulated cells were allowed to settle and adhere for 60 min at 37°C and 5% CO_2 in an incubator. Non-adherent cells were removed by washing with warm medium and the remaining cells were lysed with a lysis buffer (0.01 M Tris, 0.15 M NaCl, 1.5 mM MgCl, 0.2% Triton X-100, pH 7.3) for 15 min at room temperature. Fluorescence was quantified in a fluorescence spectrophotometer (Millipore, USA). Percent adherence was calculated as follows (FI = fluorescence intensity):

$$\% \text{ adherence} = \frac{\text{FI adherent} - \text{FI (background)}}{\text{FI initial cell number} - \text{FI (background)}} \times 100$$

Under the experimental conditions used, about 1×10^5 labeled PBL gave a fluorescence intensity of about FI = 3,000–5,000.

2.6. Flow cytometry

Isolated PBL were incubated with fluorescein-conjugated monoclonal antibody (Leu-44 antibody from Becton Dickinson) for direct immunofluorescence, or with unconjugated murine mAB (anti-CD11a, clone 25.3.1, from Dianova, anti-CD44, clone J 173, from Dianova) and a FITC-conjugated rabbit anti-mouse IgG as secondary antibody for indirect immunofluorescence. The different probes were analysed by flow cytometry using a FACS scan (Becton Dickinson, Heidelberg, Germany).

2.7. CD44-neutralisation assay

About 7.5×10^5 PBL in 100 µl RPMI 1640 were incubated with 5 µg of anti-CD44 antibody (Leu-44 antibody, Becton Dickinson) or with a CD44 unrelated IgG_1 antibody for 30 min on ice. F9 was added at a final concentration of 50 µg/ml and incubated for 1 h at 37°C, 5% CO_2. F9 treated cells were labeled with BCECF-AM as described above. Following washing to remove the fluorescent dye, the adhesion assay was performed as described above.

3. Results

About 16% of unstimulated PBL adhered to the endothelial cells, stimulation with $P(Bu)_2$ (100 ng/ml) significantly increased the adherence to HUVEC to about 34%.

3.1. Effects of F9 on PBL adhesion to HUVEC

Bromelain protease F9, a highly purified fraction from the crude bromelain protease mixture, was tested for its effects on PBL binding to the endothelial cells. Preincubation of PBL with different concentrations of F9 reduced its adhesion to HUVEC in a dose-dependent manner (Fig. 1). F9 (25 µg/ml) reduced the adherence of unstimulated PBL to about 23%. Higher concentrations up to 75 µg/ml of F9 showed only a small increase of

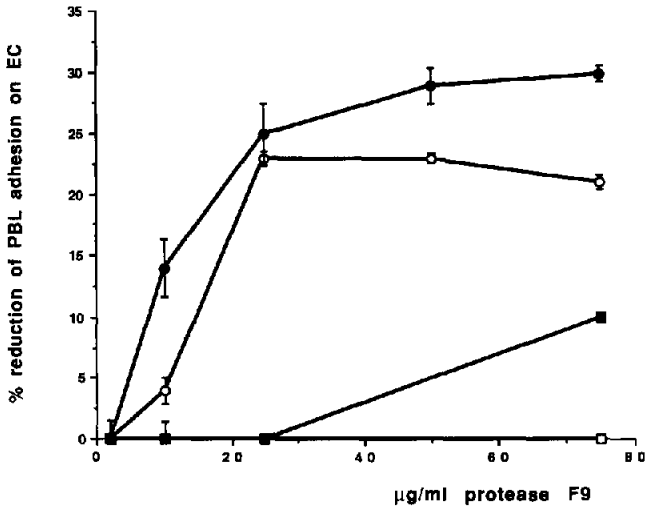

Fig. 1. Dose–response curve of bromelain protease F9 induced reduction of the adhesion of unstimulated (○) or $P(Bu)_2$ (100 ng/ml) stimulated (●) PBL. Protease F9 was inactivated (□) unstimulated, (■) stimulated PBL with iodoacetamide as described. Results are expressed as the mean percentage of protease F9 induced decrease of PBL adhesion to HUVEC as compared with PBL without protease treatment (100%). Values represent the mean ± S.E.M. of triplicates.

reduction, however. $P(Bu)_2$ stimulated PBL were slightly more sensitive to the F9 induced reduction of adherence of the lymphocytes to endothelial cells than unstimulated PBL. At 75 µg/ml of F9, a reduction of 30% PBL-binding was measured. However, iodoacetamide inactivated F9 was not effective in reducing PBL-binding. At the highest protease F9 concentration (75 µg/ml) $P(Bu)_2$ stimulated PBL showed only a slight reduction in adherence to about 10%, possibly due to a persisting proteolytic activity at this high protease concentration.

3.2. Flow cytometry of CD44 and CD11a expression on PBL after protease treatment

Since the adhesion molecules CD44 and LFA-1 (CD11a) are involved in the binding of PBL to HUVEC [9], we analysed the effect of F9 on CD44 and CD11a expression by flow cytometry. Receptor expression was monitored by the respective receptor specific antibody and a FITC labeled rabbit anti-mouse IgG as secondary antibody. In the absence of F9 about 37% CD44 positive and 63% CD11a positive PBL were measured, respectively (Fig. 2). F9 at a concentration of 50 µg/ml reduced the CD44 expression to about 3%. In contrast CD11a expression remained essentialy unchanged and was reduced only slightly to a value of 55%.

Dose–response studies of F9 induced reduction of CD44 expression revealed, that F9 was active at 2.5 µg/ml at least. From this concentration up to 25 µg/ml, no CD44 expression was detected (Fig. 3). For comparison, the crude bromelain extract was significantly less active at 2.5 µg/ml: only 4% reduction of CD44 expression was monitored.

3.3. Monoclonal antibody CD44 effects on PBL adhesion to HUVEC

Next we tested whether an antibody against CD44 was effective in blocking F9 mediated reduction of CD44 expression (Fig. 4). First, CD44 antibody was tested for blocking the

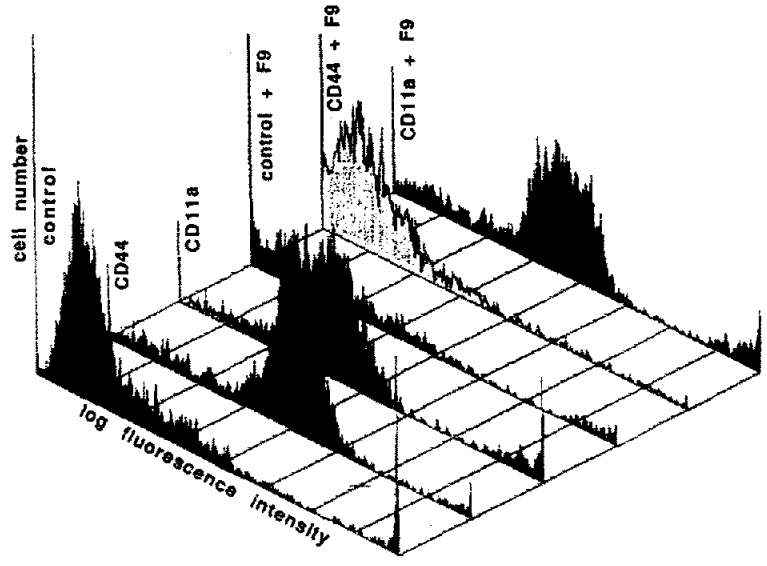

Fig. 2. Effects of bromelain protease F9 on CD44 and CD11a (LFA-1) expression on PBL. Untreated or protease F9 (10 μg/ml) treated PBL were labeled by indirect immunofluorescence with anti-CD44 antibody (clone J173, from Dianova, Germany) or anti-CD11a antibody (clone 25.3.1, from Dianova, Germany). The expression of the adhesion molecules was monitored using a rabbit anti-mouse-IgG-FITC as secondary antibody. Samples were subsequently analyzed by flow cytometry. Values represent the mean ± S.E.M. of two replicates. Similar results were obtained by two other experiments.

PBL-adhesion to endothelial cells in comparison with a CD44 unrelated IgG_1 antibody. Anti-CD44 antibodies reduced the PBL-binding to about 24%, the IgG_1 control antibodies to about 10%. Interestingly, in the same experiment F9, at the optimal concentration of 50 μg/ml, reduced the binding of the PBL to the same value of 24%, as measured by the anti-CD44 antibody.

Incubation of the PBL with anti-CD44 antibodies blocked the proteolytic activity of F9, however not to the extent as by anti-CD44 antibody alone. In contrast, non-specific IgG_1 antibody had no blocking activity towards F9. Under these conditions, about 40% of reduced adherence of PBL to the endothelial cells was estimated, which is somewhat higher than the effect by F9 alone.

4. Discussion

The results of the present paper demonstrate, that the bromelain protease F9 reduces the binding of unstimulated and $P(Bu)_2$ stimulated PBL to HUVEC. The involvement of CD44 molecules in the binding process was derived from the following lines of evidence: first, using flow cytometry bromelain protease F9 reduced the CD44 expression on PBL, without changing LFA-1 expression. Second, a mAb CD44 blocked the adhesion of PBL to the HUVEC to a similar extent as the bromelain protease F9.

CD44 surface molecules are mainly involved in the binding of PBL to mucosal and synovial lymph nodes and but much less to peripheral lymph node cells [10]. CD44 has also been shown to play a role in binding of activated T-cells to IL-1 stimulated endothelial cells [4]. However, measuring the binding of PBL to HUVEC we demonstrated, that only 16% of unstimulated lymphocytes bound to the endothelial cells. $P(Bu)_2$ stimulation of the PBL resulted in an increase to 34% binding. The leukocyte function associated Ag-1 (LFA-1) was reported to play an important role in adhesion (60–90% of total adhesion) to endothelial cells and to be involved in the adhesion of unstimulated and $P(Bu)_2$ stimulated T-cells to unstimulated endothelium [11]. Testing the effects of F9 on PBL-binding to HUVEC, only about 20% of unstimulated and 30% of $P(Bu)_2$ stimulated lymphocyte adhesion were found to be protease sensitive. Therefore, this measured difference of about 10% in reduction of PBL-binding, was somewhat less than the observed $P(Bu)_2$ induced PBL adhesion (about 15%), suggesting that some expressed surface molecules were protease insensitive.

Using flow cytometry, F9 reduced the expression of CD44

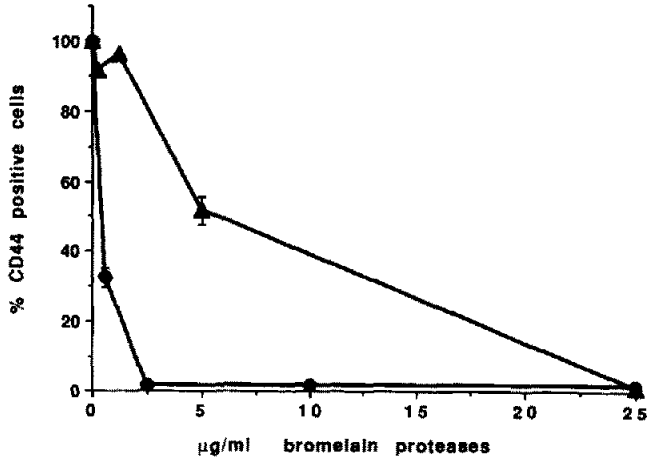

Fig. 3. Flow cytometric analysis of the effects of crude bromelain (▲) or protease F9 (●) on CD44 expression. PBL were incubated with different protease concentrations for 60 min and labeled with Leu-44 antibody as described. Values represent the mean ± S.E.M. of two replicates.

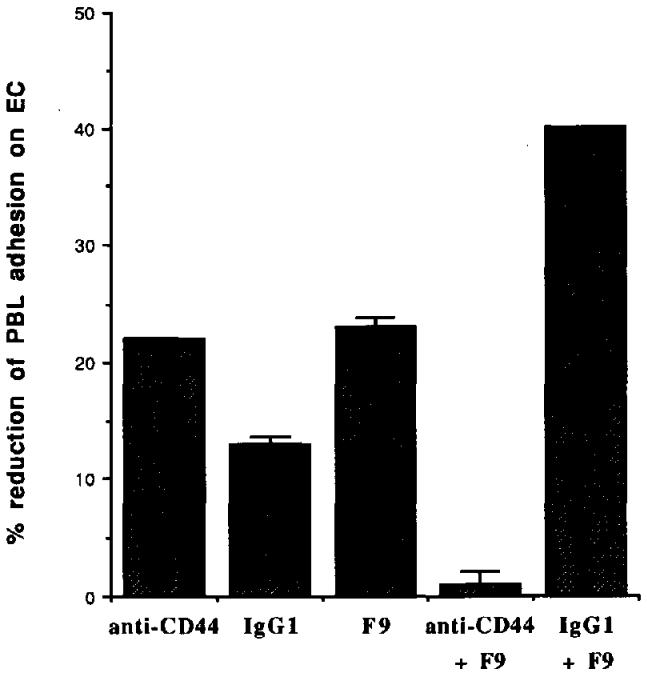

Fig. 4. Effect of anti-CD44 antibody on the bromelain protease F9 induced reduction of PBL adhesion to HUVEC. Anti-Leu-44 and unspecific mAb were applied at 5 μg/ml in the absence or presence of protease F9 at 50 μg/ml. For details see section 2. Data are expressed as the mean percentage of reduction of PBL adhesion compared with PBL adhesion in the absence of any addition. Values represent the mean ± S.E.M. of 4 independent experiments.

molecules on PBL to about 97% leaving LFA-1 expression essentialy unaltered, suggesting an involvement of CD44 molecules in PBL-binding to HUVEC.

Further evidence for the involvement of CD44 came from studies using a neutralising CD44 antibody (Leu-44 antibody). Saturable monoclonal anti-CD44 antibody concentration inhibited the adhesion of unstimulated PBL to the same extent at optimal F9 concentrations (50 μg/ml). In addition, CD44 antibody was able to block the effect of protease F9, comparable to PBL-binding of control cells treated with medium alone. Therefore, we suggest that F9 affected the binding of the CD44 antibody to the lymphocytes, possible by a protease induced structural modification of the antibody.

The mechanism of proteolytic action of optimal concentration (50 μg/ml) of F9 on CD44 remains unclear. However, investigating the effects of high concentrations of crude bromelain (1 mg/ml) on different T-cell functions, cleavage of the CD44 molecule close to the cell membrane lipid bilayer was demonstrated using Western-blot experiments with a polyclonal CD44 antibody [7]. As pointed out, crude bromelain at high concentrations markedly enhanced T-cell activation and induced T-cell monocyte adhesion in response to CD2 mitogenic antibody [7]. It was suggested that CD44 molecules on T-cells have a physiological anti-adhesive effect during macrophage-T-cell interaction, as shown by enhanced binding of T-cells in the presence of mAb against CD44 [12]. However, CD44 on lymphocytes seem to have different adhesion and functional properties against different target cell types.

Comparing the proteolytic activity of F9 in contrast to crude bromelain, purified bromelain F9 was about 15 times more active against a synthetic peptide substrate (T. Harrach et al., submitted for publication). On the other hand, crude bromelain was significantly less active (about 10 times) in reducing CD44 expression, compared to the purified F9. Therefore, the activity of the protease inversely correlated with the number of CD44 molecule left and CD44 function in the process of PBL-binding to HUVEC. Whether other F9 sensitive surface molecules on PBL, besides CD44, were also involved in this process, remains to be demonstrated.

Finally, in view of these results and the proposed antimetastatic potential of bromelain [13], further studies on bromelain effects on CD44 and CD44-splice variant expression on metastatic tumor cells are warranted.

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