Tissue Inhibitor of Metalloproteinases-3 Inhibits Shedding of L-selectin from Leukocytes*

(Received for publication, August 10, 1998, and in revised form, October 14, 1998)

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L-selectin is one of three members of the selectin family and is expressed on leukocytes (for review, see Ref. 1). Through binding to mucin-like carbohydrate ligands on vascular endothelium, L-selectin mediates the tethering and rolling of leukocytes, which precedes their migration into lymphoid organs and sites of inflammation. L-selectin is rapidly down-regulated from the leukocyte surface within minutes by a number of stimuli including chemoattractants (2), phorbol ester (3, 4), and cross-linking of cell surface L-selectin (5), or more slowly by cross-linking of the T cell CD3 complex (6). L-selectin is also down-regulated on leukocytes that have migrated across cultured endothelial cells (7, 8) or into sites of inflammation (9). The latter observation led to the suggestion that L-selectin down-regulation from the leukocyte surface may be required for transendothelial migration, although it is not necessary for migration of neutrophils across human umbilical vein endothelial cells (10).

Rapid down-regulation of L-selectin has recently been shown to be the result of proteolytic cleavage in the membrane proximal extracellular domain (11). The cleavage site has been mapped in human L-selectin to Lys283–Ser284. The activity mediating this proteolytic cleavage (L-selectin sheddase) is inhibited by the hydroxamic acid-based inhibitors of zinc-dependent metalloproteinases Ro 31-9790 (4), KD-IX-73-4 (12), and TAPI (13), but not by inhibitors of other classes of proteases (4). L-selectin sheddase is also not inhibited by tissue inhibitor of metalloproteinases (TIMP)-1 (13), an endogenous inhibitor of matrix metalloproteinases (MMPs).

To characterize L-selectin sheddase further, we have tested the ability of two additional members of the TIMP family, TIMP-2 and TIMP-3 (for review, see Ref. 14), to inhibit L-selectin shedding from leukocytes. These inhibitors are structurally homologous to TIMP-1, which we have already shown does not inhibit shedding of L-selectin (4). However, the expression patterns and inhibitory specificities of the TIMPs differ (15, 16), suggesting that although all three proteins inhibit the MMPs, they have some functional differences that may be useful in the characterization of L-selectin sheddase.

Proteolytic cleavage is a commonly used mechanism for down-regulation of cell surface proteins (17). Like L-selectin, the shedding of a number of these proteins is inhibited by the hydroxamic acid-based inhibitors at concentrations much greater than those required to inhibit the MMPs (18, 19). Recently, a cell surface metalloproteinase has been identified which mediates cleavage of one of these cell surface molecules, the pro-form of tumor necrosis factor (TNF)-α (20, 21). This enzyme belongs to the ADAM (a disintegrin and metalloproteinase) family of zinc-dependent metalloproteinases. To determine the relationship between these enzymes and L-selectin sheddase, we compared the sensitivities of TNF-α shedding and L-selectin shedding from human peripheral blood monocytes to inhibition by TIMPs and synthetic hydroxamic acid-based inhibitors.

MATERIALS AND METHODS

Reagents and Antibodies—Phorbol myristate acetate (PMA; Sigma) was dissolved in dimethyl sulfoxide to a concentration of 1 mm and stored at −20 °C in aliquots. Ro 31-9790 (Roche Products, Herts.) was dissolved in dimethyl sulfoxide to a concentration of 30 mM and stored at −20 °C in aliquots. Lipopolysaccharide (LPS; Sigma) was dissolved in RPMI 1640 to a concentration of 1 mg/ml and stored at −20 °C in aliquots. Recombinant TIMPs were produced as described in (22–24). Antibodies used are described in Table I.

Cells—Mouse lymphocytes were isolated from pooled axillary and cervical and mesenteric lymph nodes of 6–8-week-old BALB/c mice bred in the SPF unit at the NIMR. Tissues were collected into calcium- and magnesium-free phosphate-buffered saline (PBS-CMF) at 4 °C and cell suspensions prepared. Human lymphocytes and monocytes were prepared from buffy coat preparations of peripheral blood obtained from 1 The abbreviations used are: TIMP, tissue inhibitor of metalloproteinases; MMP, matrix metalloproteinase; TNF-α, tumor necrosis factor-α; ADAM, a disintegrin and metalloproteinase; PMA, phorbol myristate acetate; LPS, lipopolysaccharide; PMA, phorbol myristate acetate; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; FCS, fetal calf serum; FACS, fluorescence-activated cell sorter; ELISA, enzyme-linked immunosorbert assay; MT-MMP, membrane-type matrix metalloproteinase; ACE, angiotensins-converting enzyme; TACE, TNF-α-converting enzyme.

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1 The abbreviations used are: TIMP, tissue inhibitor of metalloproteinases; MMP, matrix metalloproteinase; TNF-α, tumor necrosis factor-α; ADAM, a disintegrin and metalloproteinase; PMA, phorbol myristate acetate; LPS, lipopolysaccharide; PMA, phorbol myristate acetate; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; FCS, fetal calf serum; FACS, fluorescence-activated cell sorter; ELISA, enzyme-linked immunosorbert assay; MT-MMP, membrane-type matrix metalloproteinase; ACE, angiotensins-converting enzyme; TACE, TNF-α-converting enzyme.
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Table I

| Antibodies used      | Antibody          | Isotype    | Source                     |
|----------------------|-------------------|------------|----------------------------|
| MEL-14 (control)     | Mouse L-selectin  | Rat Ig2a   | ATCC                       |
| MAC193 (control)     | Mouse L-selectin  | Rat Ig2a   | Dr. G. Butcher, Babraham Institute, Cambridge, U. K. |
| T28.45               | Mouse L-selectin  | Mouse Ig2b | Dr. U. Hammerling, Sloane-Kettering Memorial Cancer Center, New York |
| FMC46 (control)      | Human L-selectin  | Mouse Ig2b | DACO                       |
| 11.52.1.9 (control)  | Human L-selectin  | Mouse Ig2b | Dr. G. Klaus, NIMR, London |
| LAM1.3               | Human L-selectin  | Mouse Ig1  | Prof. T. Tedder, Duke University Medical Center, Durham, NC |
| 101/4                | Human TNF-α       | Mouse Ig1  | Dr. A. Nebshut, Celltech, Berkshire, U. K. |
| OX5 (control)        | Rat CD4           | Mouse IgG2a| ECACC                      |
| UCHM1                | Mouse CD4         | Mouse Ig1  | Serotec                    |
| Anti-mouse IgG1-phycocerythrin | Mouse IgG1       | Polyclonal | Southern Biotechnology     |

Results

The North London Blood Transfusion Service (Colindale, London). Peripheral blood mononuclear cells (PBMC) were isolated by flotation over Ficoll-Paque (Amersham Pharmacia Biotech). E6.1 Jurkat T cells were grown in RPMI 1640 containing 10% fetal calf serum (FCS), 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in 5% CO₂. For experiments, mouse lymphocytes and Jurkat cells were resuspended at 5 × 10⁵/ml in RPMI 1640 supplemented with 5% FCS and 10 mM Hapes. Human PBMC were resuspended at ≥ 2 × 10⁶/ml in RPMI 1640 supplemented with 10% FCS.

The L-selectin shedding assay—Mouse lymphocytes or Jurkat T cells (2.5 × 10⁶ in 50 μl) were incubated with 100 nM PMA or an equivalent volume of dimethyl sulfoxide for 60 min. Cells were pretreated with inhibitor for 20 min and throughout the assay. Inhibitors and PMA were removed by washing in PBS-CMF containing 0.2% bovine serum albumin, and cell surface L-selectin expression was determined by flow cytometry. This assay was also carried out using PBMC (10⁶ in 0.5 ml) within 90 min of isolation from buffy coat.

The TNF-α shedding assay—PBMC (10⁶ in 0.5 ml) were incubated in the presence or absence of inhibitor for 20 min followed by the addition of 1 μM LPS for 3 h. Inhibitors and LPS were removed by washing in PBS-CMF containing 5% FCS, and cell surface TNF-α expression was determined by flow cytometry. The washing and staining procedures for flow cytometric analysis were carried out on ice, using chilled buffer to prevent proteolytic cleavage of cell surface TNF-α after washing out of inhibitor.

Flow Cytometry—L-selectin was measured on mouse lymphocytes and Jurkat T cells by direct immunofluorescence using fluorescein isothiocyanate-conjugated MEL-14 and fluorescein isothiocyanate-conjugated FMC46, respectively. L-selectin and cell surface TNF-α on human PBMC were measured using two-color indirect immunofluorescence. LAM1.3 was used to detect L-selectin and 101/4 to detect TNF-α.

Inhibition of Mouse Lymphocyte L-selectin Shedding by TIMP-3—We have shown previously that PMA-induced shedding of L-selectin from mouse lymphocytes is inhibited by the synthetic metalloproteinase inhibitor Ro 31-9790 but not by the endogenous MMP inhibitor TIMP-1 (4). These studies have been extended by including TIMP-2 and TIMP-3 in the same in vitro L-selectin shedding assay. Lymphocytes isolated from mouse lymph nodes were treated with PMA in the presence or absence of varying concentrations of each of the inhibitors, and L-selectin remaining on the cell surface was detected by flow cytometry. As reported previously, TIMP-1 had no effect on PMA-induced shedding of cell surface L-selectin at concentrations up to 1 μM (Fig. 1, A and B), whereas the hydroxamic acid-based inhibitor Ro 31-9790 completely inhibited PMA-induced shedding at a concentration of 10 μM (Fig. 1B). TIMP-2 was also unable to inhibit L-selectin shedding (Fig. 1, A and B), but TIMP-3 inhibited PMA-induced L-selectin shedding in a dose-dependent manner with complete inhibition at 0.8 μM (20 μg/ml; Fig. 1B). To confirm that PMA was inducing shedding of L-selectin, rather than internalization, soluble L-selectin was quantitated by ELISA in supernatants from untreated, PMA-treated, and PMA + inhibitor-treated mouse lymphocytes. Although there was a background level of soluble
L-selectin present in cell supernatants in the absence of PMA, an increase of approximately 100% was seen in response to phorbol ester (Fig. 1C); this PMA-induced increase was inhibited completely by TIMP-3 and Ro 31-9790 but not by TIMP-1 or TIMP-2. This background level of L-selectin shedding, detectable as soluble L-selectin in the supernatants of untreated mouse lymphocytes, explains the >100% inhibition of PMA-induced shedding by TIMP-3 and Ro 31-9790 (Fig. 1B); these inhibitors prevent both the PMA-induced and the background shedding, allowing cell surface L-selectin to increase to a level greater than that seen on untreated lymphocytes. However, even in the presence of TIMP-3 or Ro 31-9790 some residual soluble L-selectin can be detected in cell supernatants by ELISA.

**TIMP-3 Inhibits L-selectin Shedding from Human Lymphocytes**—The TIMPs used in the experiments described above were recombinant human TIMPs. It was possible that the absence of inhibition of L-selectin shedding by TIMP-1 and TIMP-2 was caused by the inability of the human TIMPs to function in a mouse cell system. Therefore, the ability of the TIMPs to inhibit shedding of L-selectin from human cells was investigated. Jurkat T cells were treated with PMA in the presence and absence of inhibitor, or untreated lymphocytes stained with control monoclonal antibody (open histogram) or MEL-14 (shaded histogram); b–f, lymphocytes stained with MEL-14 after treatment with δ, PMA alone; e, PMA + 1.1 μM TIMP-1; d, PMA + 1.44 μM TIMP-2; e, PMA + 0.83 μM TIMP-3. Vertical line indicates marker used to determine percent positive; number in each box indicates percent cells positive for cell surface L-selectin. Profiles are representative of several independent experiments. Panel B, cells were treated as in panel A, and cell surface L-selectin was detected by flow cytometry. Results are presented as the percent inhibition of shedding ± S.E. Panel C, inhibition by TIMP-3 and Ro 31-9790 of soluble L-selectin appearance in the supernatants of mouse lymphocytes treated as in panel A, detected by ELISA. Background shedding of L-selectin (at approximately 350 ng/10⁶ cells under these conditions) in the absence of PMA can be seen. Results for inhibitor-treated samples are from a representative experiment; the points showing the levels of soluble L-selectin for untreated and PMA-treated are the mean ± S.E. for at least three experiments and indicate the variable levels of soluble L-selectin obtained from different samples. In panels B and C, closed circles represent Ro 31-9790-treated samples, open circles represent TIMP-1-treated samples, closed squares represent TIMP-2-treated samples, and open squares represent TIMP-3-treated samples. In panel C alone, the closed triangle represents untreated samples, and the open triangle represents PMA-treated samples; these values are the mean ± S.E. for six independent experiments.

**FIG. 1. TIMP-3 and Ro 31-9790 inhibit L-selectin shedding from mouse lymphocytes.** Panel A, flow cytometry profiles showing inhibition of L-selectin down-regulation from cells treated with 100 nM PMA in the presence and absence of inhibitor. a, untreated lymphocytes stained with control monoclonal antibody (open histogram) or MEL-14 (shaded histogram); b–f, lymphocytes stained with MEL-14 after treatment with δ, PMA alone; e, PMA + 1.1 μM TIMP-1; d, PMA + 1.44 μM TIMP-2; e, PMA + 0.83 μM TIMP-3. Vertical line indicates marker used to determine percent positive; number in each box indicates percent cells positive for cell surface L-selectin. Profiles are representative of several independent experiments. Panel B, cells were treated as in panel A, and cell surface L-selectin was detected by flow cytometry. Results are presented as the percent inhibition of shedding ± S.E. Panel C, inhibition by TIMP-3 and Ro 31-9790 of soluble L-selectin appearance in the supernatants of mouse lymphocytes treated as in panel A, detected by ELISA. Background shedding of L-selectin (at approximately 350 ng/10⁶ cells under these conditions) in the absence of PMA can be seen. Results for inhibitor-treated samples are from a representative experiment; the points showing the levels of soluble L-selectin for untreated and PMA-treated are the mean ± S.E. for at least three experiments and indicate the variable levels of soluble L-selectin obtained from different samples. In panels B and C, closed circles represent Ro 31-9790-treated samples, open circles represent TIMP-1-treated samples, closed squares represent TIMP-2-treated samples, and open squares represent TIMP-3-treated samples. In panel C alone, the closed triangle represents untreated samples, and the open triangle represents PMA-treated samples; these values are the mean ± S.E. for six independent experiments.
the abilities of TIMP-1, TIMP-2, TIMP-3, and Ro 31-9790 to inhibit shedding of L-selectin and TNF-α from human monocytes in order to investigate the relationship between these enzyme activities on the same cell. Human peripheral blood monocytes express L-selectin, therefore PMA was used to induce shedding, and cell surface L-selectin was measured as described for lymphocytes. Cell surface TNF-α is not normally detectable on monocytes. To measure the effect of metalloproteinase inhibitors on TNF-α shedding, monocytes were stimulated with LPS, to up-regulate TNF-α synthesis, and the level of TNF-α which accumulated at the cell surface, and which was released into the cell supernatant, in the presence and absence of inhibitors was determined. As found for lymphocytes, TIMP-3 and Ro 31-9790 (0.83 and 0.30 μM, respectively) blocked PMA-induced L-selectin shedding from human monocytes (Fig. 3 and Table II). TIMP-3 and Ro 31-9790 also mediated accumulation of TNF-α on the surface of human monocytes (Fig. 3 and Table II), indicating a block in the TNF-α shedding pathway. This accumulation of TNF-α on the cell surface was mirrored by decreased soluble TNF-α in supernatants of cells treated with LPS and TIMP-3 (data not shown). In contrast, TIMP-1 and TIMP-2 had no effect on shedding of L-selectin from monocytes (Table II). TIMP-1 was also without effect on cell surface TNF-α on monocytes, but inclusion of TIMP-2 at

**TABLE II**

Inhibition of L-selectin and TNF-α shedding from human monocytes by TIMP-3 and Ro 31–9790

| Treatment                  | L-selectin (PMA-treated) | TNF-α (LPS-treated) |
|----------------------------|--------------------------|---------------------|
| Negative control           | 0.5                      | 0.5                 |
| Untreated                  | 92.3                     | 5.4                 |
| Stimulated (PMA or LPS)    | 1.2                      | 39.3                |
| Stimulus + Ro 31–9790 (50 μM) | 85.7             | 86.6                |
| Stimulus + TIMP-1 (1.10 μM) | 1.9                      | 38.3                |
| Stimulus + TIMP-2 (1.44 μM) | 3.3                      | 55.7                |
| Stimulus + TIMP-3 (0.83 μM) | 99.1                     | 95.5                |
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**Table III**

IC$_{50}$ values for TIMP-3 and Ro 31–9790 on L-selectin and TNF-α shedding

| Activity                        | TIMP-3  | Ro 31–9790 |
|---------------------------------|---------|------------|
| Mouse lymphocyte L-selectin     | 0.31 ± 0.07 | 4.82 ± 0.75 |
| Jurkat L-selectin shedding      | 0.37 ± 0.15 | 1.16 ± 0.27 |
| Human lymphocyte L-selectin     | 0.39 ± 0.21 | 0.70 ± 0.66 |
| Human monocyte L-selectin shedding | 0.39 ± 0.18 | 4.47 ± 1.27 |
| Human monocyte TNF-α shedding   | 0.11 ± 0.04 | 0.38 ± 0.05 |

1.44 μM resulted in an increase in the percentage of cells positive for TNF-α, from 39.3% to 55.7% (31% inhibition), although this was less than that obtained with TIMP-3 (Table II).

IC$_{50}$ Values—The IC$_{50}$ values obtained for TIMP-3 and Ro 31–9790 on each of the cell types tested are shown in Table III. TIMP-3 gave very similar IC$_{50}$ values for inhibition of L-selectin shedding on all cell types tested (0.30–0.40 μM). However, this inhibitor prevented TNF-α shedding with a lower IC$_{50}$ of 0.1 μM, indicating possible differences between TNF-α and L-selectin shedding activities. IC$_{50}$ values obtained for Ro 31–9790 on L-selectin shedding showed more variation between cell types, but, like TIMP-3 this inhibitor was more potent in inhibiting TNF-α shedding on monocytes than inhibiting L-selectin shedding on any cell type tested. IC$_{50}$ values obtained for both TIMP-3 and Ro 31–9790 by measurement of soluble L-selectin released from mouse lymphocytes were similar to the values obtained by analysis of flow cytometry data (data not shown). The IC$_{50}$ for inhibition by TIMP-3 of soluble TNF-α release from LPS-stimulated human monocytes (0.17 ± 0.02 μM) was also similar to that obtained by measurement of cell surface TNF-α under identical conditions.

DISCUSSION

The enzyme or enzymes mediating L-selectin shedding from leukocytes have yet to be identified. However, using a new category of MMP inhibitors based on hydroxamic acid, L-selectin shedding has been shown to be blocked in a variety of cell types in response to both physiological and nonphysiological stimuli (4, 10, 12, 13), suggesting that L-selectin shedding in these different circumstances is mediated by similar metalloproteinase dependent mechanisms. In this report, we extend our previous work and demonstrate that the endogenous metalloproteinase inhibitor TIMP-3 inhibits L-selectin shedding from mouse and human lymphocytes and human monocytes. We have shown previously that TIMP-1 did not inhibit L-selectin shedding (4), and we demonstrate here that the closely related inhibitor TIMP-2 is also ineffective.

Some MMPs, and collagenase in particular, mediate limited L-selectin proteolysis (4), suggesting that the L-selectin sheddase activity may be an MMP or an MMP-related enzyme. However, the work reported here and a previous study (4) demonstrate that phorbol ester-induced L-selectin shedding is not inhibited by two members of the TIMP family, TIMP-1 and TIMP-2. Between them, these two proteins inhibit the catalytic activity of all known MMPs, and the inability of TIMP-1 and TIMP-2 to prevent L-selectin shedding of L-selectin therefore suggests that L-selectin sheddase is not a known MMP. This is supported by the evidence that L-selectin sheddase is a cell-associated, rather than a soluble, activity (4, 25), as the MMPs are secreted enzymes. Recently, a subgroup of transmembrane MMPs (membrane-type MMPs or MT-MMPs) has been identified (26). Like L-selectin sheddase, MT1-MMP is not inhibited by TIMP-1 (27). However, this cell surface enzyme can be excluded as a potential candidate for the L-selectin sheddase because it is inhibited by both TIMP-2 and TIMP-3 (27), and the data presented here show that L-selectin shedding is not inhibited by TIMP-2.

The hydroxamic acid-based inhibitors were originally designed as inhibitors of MMPs, and Ro 31–9790 inhibits recombinant human fibroblast collagenase (MMP1) with an IC$_{50}$ of 4.9 nM. As described here and elsewhere, hydroxamic acid-based inhibitors prevent phorbol ester-induced shedding of cell surface L-selectin with IC$_{50}$ values in the low micromolar range (4, 12, 13) i.e. they are about 1,000-fold less potent in inhibiting L-selectin shedding in cell-based assays than in inhibiting recombinant MMPs in cell-free assays. When considered together with the inability of TIMP-1 and TIMP-2 to inhibit L-selectin shedding from all of the cell types tested, it is unlikely that the L-selectin-shedding enzyme(s) is an MMP. Proteolytic cleavage of a number of other cell surface molecules, such as angiotensin-converting enzyme (ACE) (28), amyloid precursor protein (29), transforming growth factor-α (18), and the pro-form of TNF-α (20, 21) and this study), is also inhibited by synthetic hydroxamate inhibitors at micromolar concentrations, suggesting that the activities mediating shedding of these molecules and L-selectin shedding may be related.

An enzyme has recently been cloned, called TNF-α converting enzyme (TACE; ADAM17) which correctly cleaves both a pro-TNF-α cleavage site peptide and full-length pro-TNF-α (20, 21). In addition, leukocytes from TACE-deficient mice have a much reduced ability to secrete soluble TNF-α, suggesting an important role for TACE in mediating TNF-α shedding in vivo. TACE is a member of a recently characterized family of zinc-dependent metalloproteinases known as the ADAM family (for review, see Ref. 30). An association between TACE and L-selectin shedding has recently been identified: thymocytes from TACE-deficient mice do not shed L-selectin in response to PMA stimulation (2). In addition, high concentrations of recombinant catalytic domain of TACE have been shown to cleave a peptide representing the cleavage site of L-selectin. This suggests that TACE may be a physiological L-selectin sheddase. A second member of the ADAM family (ADAM10) also cleaves a TNF-α cleavage site peptide but has yet to be shown to cleave full-length pro-TNF-α correctly (31, 32). We therefore investigated the possible relationship between enzymes mediating shedding of L-selectin and TNF-α by comparing the inhibitor profiles of the TIMPs and Ro 31–9790 on L-selectin and TNF-α shedding from human monocytes. We show here that TIMP-3 mediates accumulation of TNF-α on the surface of human monocytes *in vitro*. TIMP-1 has no effect, and TIMP-2 is less effective than TIMP-3. Solomon et al. (33) have shown that although the level of cell surface TNF-α on human peripheral blood monocytes may not quantitatively reflect alterations in TNF-α shedding (because of degradation of unprocessed pro-TNF-α), this measurement can be used as a marker of inhibition of shedding at the time point utilized in our TNF-α-shedding assays (3 h). Measurement of soluble TNF-α in supernatants from monocytes stimulated with LPS in the presence or absence of TIMP-3 gave an IC$_{50}$ very similar to that obtained from cell surface expression. This indicates that both types of measurement can be used in our assay. Therefore differences can be found in the inhibitory profiles of the TIMPs and Ro 31–9790 on shedding of L-selectin and TNF-α from human monocytes and of Ro 31–9790 on shedding of L-selectin from different cell types, suggesting the potential involvement of more than one

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31-970). This raises the possibility that shedding of L-selectin and TNF-α from human monocytes may be mediated by different enzymes, although other explanations exist.

Acknowledgments—We thank Chris Atkins for help with flow cytometry and Frank Johnson for preparing the figures. We also thank Dr. Andrew Nesbitt and Dr. Andrew Docherty of Celltech for gifts of anti-body and useful discussions.

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