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TIMP-1/MMP-9 imbalance in an EBV-immortalized B lymphocyte cellular model: evidence for TIMP-1 multifunctional properties

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Abstract

Tissue inhibitors of metalloproteinases (TIMPs) were initially described as agents controlling metalloproteinase activity. The purpose of this study was to investigate the expression and the roles of TIMP-1 secreted by Epstein–Barr-virus (EBV)-immortalized B lymphocytes. TIMP-1 was isolated from conditioned medium of interleukin (IL)-1β stimulated EBV-B lymphocytes; purified TIMP-1 was identified by mass spectrometry and immunochemistry. TIMP-1-free MMP-9 was quantified after purification by zymography and enzyme-linked immunosorbent assay. EBV-B lymphocyte-secreted TIMP-1 inhibited MMP-9 gelatinolytic activity resulting in decreased B-cell transmigration as measured in vitro. The release of huge amounts of TIMP-1 in proportion to MMP-9 from B lymphocytes after EBV transformation was shown to be correlated with secretion of IL-10 and dependent on culture time. In contrast, there was little TIMP-1 and almost no IL-10 released from native B cells, suggesting a possible IL-10 mediated autocrine regulation mechanism of TIMP-1 synthesis. The MMP-9/TIMP-1 imbalance observed in the culture medium of EBV-B lymphocytes (TIMP-1 > MMP-9) and of native B cells (MMP-9 > TIMP-1) is suggestive of a new function for TIMP-1. We propose that TIMP-1 acts as a survival factor controlling B-cell growth and apoptosis through an autocrine regulation process involving IL-10 secreted by EBV-B lymphocytes.

Keywords: B lymphocyte; MMP-9; TIMP-1; Growth factor; Apoptosis

Abbreviations: APMA, *p*-aminophenyl mercuric acetate; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; Con A, concanavalin A; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; EBV, Epstein–Barr virus; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; NBT, nitroblue tetrazolium; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TIMP, tissue inhibitor of matrix metalloproteinase; TLCK, *N*α-*p*-tosyl-L-lysine chloromethyl ketone; MMP-9, gelatinase B, 92 kDa gelatinase, EC 3.4.24.35

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1. Introduction

Zinc metalloproteinases are chiefly responsible for morphogenesis, tissue repair, tumorigenesis and cell death [1–3]. A matrix metalloproteinase called MMP-9, which cleaves type IV collagen (the main component of basement membrane), is assumed to be the protease required for cellular leukodiapedesis during the inflammation process [4].

The tissue inhibitors of metalloproteinases, the TIMPs, are involved in the specific inhibition of metalloproteinase (MMP) activity. This inhibition is likely to be the final point of control limiting matrix proteolysis. Four members of the TIMP family, TIMP-1 to TIMP-4, have been cloned and characterized [5–9]. TIMP-1 is a 28.5 kDa soluble glycoprotein which binds either to active MMPs in a 1:1 molar ratio to form a tight noncovalent complex or to proMMP-9 through an exceptionally strong C-terminal domain interaction [5]. TIMPs have been shown to be produced by many cell types in culture and in vivo through different physiological and pathological processes. Extracellular matrix resorption or fibrosis results from an imbalance of MMPs and TIMPs activity [10].

In human blood cells, TIMP-1 and MMP-9 have been detected in platelets [11], monocytes [12], neutrophils [13], T lymphocytes [14] and recently in B lymphocytes [15,16]. Usually TIMP-1 acts as an MMP inhibitor: it may suppress migration, invasion and metastasis of cancer cells [3–5]. More recently, new properties have been reported: TIMP-1 seems to be a new cell growth factor in serum and to stimulate the cells independently of its inhibitory activity [17,18]. In fact TIMP-1 was initially described as an erythroid potentiating agent [17]. Its properties as a regulatory growth factor were illustrated in the fibrotic process and in scleroderma [19]. Moreover, it is overexpressed in high grade non-Hodgkin's lymphoma and is associated with a poor clinical outcome. Its production by stroma cells may protect tumoral lymphoid cells from apoptosis [20,21] and induce their differentiation [22]. Both TIMP-1 and TIMP-2 were also shown to regulate cell proliferation but apoptosis regulation is tissue-specific.

This study focuses on TIMP-1 and TIMP-1/MMP-9 imbalance in both EBV immortalized and native B cells. The results indicate that TIMP-1/MMP-9 ratio are altered upon EBV transformation and are depending on the extracellular environment. Also, TIMP-1 from immortalized B lymphocytes displays both MMP-9 inhibitory activity and growth factor properties. An interleukin (IL)-10-mediated, autocrine regulation mechanism is hypothesized.

2. Material and methods

2.1. Reagents

Reagents used in this work were obtained from the following sources: RPMI-1640, penicillin, kanamycin, streptomycin, Grace medium (Gibco BRL, Life Technologies SARL, Cergy Pontoise, France); gelatin–agarose, heparin–agarose, *p*-aminophenylmercuric acetate (APMA), phorbol 12-myristate 13-acetate (PMA), dithiothreitol (DTT), iodoacetamide, cyano-4-hydroxy-*trans*-cinnamic acid (4 HCCA), lipopolysaccharide (LPS), leupeptin (Sigma Chemical Co., St Louis, MO, USA); dimethylsulfoxide (DMSO) (Merck, Nogent sur Marne, France); Sephacryl S-200, protein-A Sepharose CL-4B, Ficoll–Hypaque (Pharmacia, Uppsala, Sweden); hTIMP-1 ELISA, recombinant (r)TIMP-1 (Amersham, Buckinghamshire, UK); Bradford reagent (Biorad, Munich, Germany); Centricon 10 (Amicon, Beverly, MA, USA); sequenced grade modified trypsin (Promega, Charbonnières, France); Growth Factor Reduced Matrigel Matrix (Becton Dickinson, Bedford, MA, USA); Dynabead and Detachabead (Dynal, Compiègne, France); innotest human IL-10 ELISA (Innogenetics Valbiotech, Paris, France); Transwell inserts (Corning Costar Corp., Cambridge, MA, USA); Annexin V-FITC and pepstatin (Boehringer Mannheim, Meylan, France); Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 substrate and Mca-Pro-Leu-OH (Novabiochem, Laüfelfingen, Switzerland); gentamycin (Panpharma Laboratories, La Fougère, France); amphotericin B (Bristol-Myers Squibb, Paris La Défense, France); pBlueBacHis C transfer vector, *Autographa californica* nuclear polyhedrosis virus (baculovirus), Insectin insect cell-specific liposomes, *Spodoptera frugiperda* (Sf9) insect cells, ProBond nickel-chelating resin (Invitrogen, Leek, The Netherlands).

Mass spectrometry was performed with HPLC grade water, methanol and acetonitrile. rTIMP-1,

which was used to prepare the antiserum, was a generous gift from Synergen (Boulder, CO, USA).

2.2. B-Lymphocyte culture

Lymphocytes from heparinized sterile venous blood were isolated by Ficoll–Hypaque density gradient centrifugation. For immortalization, the cells were infected with the B95-8 strain of Epstein–Barr virus (EBV) as previously described [23]. The EBV-B lymphocyte cell line was maintained at 37°C in 5% CO_2 atmosphere in RPMI-1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 50 μg/ml kanamycin and 50 μg/ml streptomycin. The medium was changed twice weekly. Before analysis of MMP and TIMP expression and after extensive washing, the EBV-B lymphocytes were grown in serum-free medium containing 0.2% bovine serum albumin (BSA) (w/v) and maintained in the same CO_2 atmosphere for 18 or 72 h. Cell viability was monitored by trypan blue exclusion; it was in the range of 90%. All the experiments were normalized by using an equal number of living cells.

Native B lymphocytes were purified from buffy coats or fresh blood after the Ficoll–Hypaque density gradient centrifugation step by magnetic beads coated with mouse antihuman CD-19 antibody and antimouse Fab antiserum as described previously [24]. Both cell types were identified as B cells by cross-reactivity with anti CD-19 and CD-20 antibodies. Apoptosis was measured by Annexin V staining as following: 10^6 cells were washed in PBS and suspended in 100 μl annexin V-FITC solution prepared from stock reagent by dilution (1:50) in 10 mM Hepes/NaOH, 140 mM NaCl, 5 mM $CaCl_2$ at pH 7.4. The medium was supplemented with 20 μl of 50 μg/ml propidium iodide solution. After 15 min incubation, and washing with the labeling buffer, the cells were analyzed immediately on a FACS Calibur (Becton Dickinson).

2.3. Native TIMP-1 purification procedure

Serum-free conditioned medium from 1 ng/ml rIL-1β stimulated EBV-B lymphocytes was the starting material. Firstly, MMP-9 present in the medium was removed by adsorption onto an affinity gelatin–agarose matrix and eluted further for purification [25]. Approximately 400 ml of serum-free conditioned medium (10^9 equivalent EBV-B lymphocytes) was dialyzed against 0.05 M Tris–HCl (pH 7.6) buffer containing 0.005 M $CaCl_2$, 0.02% (w/v) NaN_3, 0.5 M NaCl, 0.05% (v/v) Brij 35 and applied to a gelatin–agarose column equilibrated in the same buffer, and on which MMP-9 bound. The flow-through was collected and contained TIMP-1 in a free form. The TIMP-1 enriched fractions were pooled and dialyzed against 0.025 M Tris–HCl (pH 7.5) buffer containing 0.05% (w/v) Brij 35. After addition onto a heparin–agarose column equilibrated in the latter buffer and after extensive washing, the bound TIMP-1 was eluted with 0.4 M NaCl. Further purification was performed on an anti-TIMP-1 affinity chromatography equilibrated in the heparin–agarose elution buffer. Bound proteins were eluted with 0.025 M Tris–HCl (pH 10.7) buffer containing 2 M NaCl, 0.05% (w/v) Brij 35. The TIMP-1 enriched eluates were pooled and filtrated through Sephacryl S-200 at a flow rate of 8 ml/h in the latter buffer. The fractions of all chromatographic steps were assayed for TIMP-1 by SDS–PAGE, immunoblotting, and ELISA.

2.4. Purification of B-lymphocyte MMP-9

After extensive washing with the equilibration buffer containing 1 M NaCl, MMP-9, bound to gelatin–agarose, was eluted with dimethyl sulfoxide (DMSO) (0.2%, 0.8%, 10%) added as a stepwise gradient in the latter buffer. Column eluates were monitored for MMP-9 activity by zymography and for TIMP-1 by ELISA. Fractions with gelatinolytic activity and without TIMP-1 were pooled, concentrated by ultrafiltration through Centricon 10 and applied to a Superose 12 column prepared in 0.05 M Tris–HCl (pH 8) buffer containing 0.3 M NaCl and 0.05% (v/v) Brij 35 [25]. Fractions were collected by FPLC at a flow rate of 0.25 ml/min at a pressure of 1 MPa. Eluates showing gelatinolytic activity were pooled and frozen until further use.

2.5. Expression of TIMP-1 in a baculovirus-insect cell system

2.5.1. Insect cells

Recombinant virus was grown and generated in *Spodoptera frugiperda* (Sf9) cell monolayers or sus-

pension cultures in supplemented Grace's medium containing 10% fetal calf serum, 10 μg/ml gentamycin and 2.5 μg/ml amphotericin B.

2.5.2. Construction of recombinant transfer vector

A 0.6 kb cDNA clone for human TIMP-1 was removed from the pGEM3 vector (generous gift from Professor L. Matrisian) and ligated into the baculovirus transfer vector pBlueBacHis C between the *Bam*HI and *Hin*dIII sites.

2.5.3. Recombinant virus production

Recombinant viruses were obtained by co-transfecting monolayer log phase Sf9 cells with 3 pg plasmid DNA and 1 μg linearized *Autographa californica* nuclear polyhedrosis virus DNA diluted in Grace's medium without supplements, using a cationic liposome solution. The recombinant virus was purified and amplified as described [26].

2.6. Purification of recombinant TIMP-1

Expression of recombinant protein was performed on 200–500 ml spinner cultures. TIMP-1 was purified from Sf9 cells 3 days after infection with the recombinant virus (multiplicity of infection close to 5). Extraction of TIMP-1 from intracellular aggregates was performed as recently described [27]. Briefly, 10^7 infected cells were diluted in 10 ml of 100 mM Tris–HCl (pH 7.5) containing 20 mM EDTA, 2 mg/ml lysozyme and 10 μM TLCK, 100 μM PMSF, 1.8 μM leupeptin, 1.5 μM pepstatin as protease inhibitors; the mixture was incubated first for 15 min at 20°C and after addition of 500 mM NaCl and 1% (w/v) Triton X-100 for a further 10 min at 4°C. Extracts were spun at $10\,000 \times g$ (10 min, 4°C) and pellets were resuspended in 50 mM Tris–HCl buffer (pH 7.5) containing 6 M urea and the latter protease inhibitor cocktail. After sonication, homogenate was centrifuged at $10\,000 \times g$ for 10 min at 4°C. Solubilization of the resulting pellet (intracellular aggregates) was achieved in a buffer containing 50 mM Tris–HCl (pH 8.5), 8 M urea, 1 mM EDTA and 200 mM β-mercaptoethanol. The mixture was stirred for 2 h at room temperature and insoluble material was removed by centrifugation at $12\,000 \times g$ and at 4°C for 25 min. Soluble fraction (crude extract) was dialyzed against 10 mM Tris–HCl (pH 7.8) containing 100 mM K_2HPO_4, 4 M urea, 0.05% (w/v) Brij 35 and loaded onto 2 ml of a ProBond nickel affinity matrix equilibrated in the same buffer, with continuous recycling for 4 h at 4°C under gentle mixing. After extensive washing, the recombinant protein was eluted from the matrix with 300 mM imidazole added to the buffer adjusted to pH 4.

2.7. Anti-TIMP-1 affinity column preparation

Twenty ml of rTIMP-1 (generous gift from Synergen) immunized rabbit polyclonal antiserum was applied to 6 ml of protein-A Sepharose CL-4B prepared in 0.1 M KH_2PO_4, 0.1 M K_2HPO_4 (pH 7) buffer. After extensive washing in the same buffer, immunoglobulins were eluted with 1 M CH_3COOH solution. Eleven ml of eluted fractions containing purified immunoglobulins (0.7 mg/ml of proteins) was buffered at pH 8 with 1 M Tris and dialyzed extensively against 0.2 M $NaHCO_3$ (pH 8.5) solution containing 0.5 M NaCl. Then the pooled fractions were coincubated overnight at 4°C with gentle mixing with 1 ml of Sepharose Cyanogen bromide 4B gel equilibrated in the latter buffer. After filtration of the suspension on Büchner, free active sites were blocked with 0.2 M glycine buffer (pH 8) and the gel was suspended in PBS at 4°C until further use (coupling yield was 84%).

2.8. Gelatin zymography

Zymographic analysis was carried out in 10% (w/v) SDS–polyacrylamide gels containing gelatin (0.5 mg/ml), as described [28,29]. Proteins collected from conditioned medium or chromatography eluates were concentrated using Centricon 10. They were applied to the gel in a sample buffer containing 2.3% (w/v) SDS but lacking β-mercaptoethanol, and were not boiled prior to loading. After electrophoresis the gels were washed twice for 15 min in 0.05 M Tris–HCl (pH 7.6) containing 0.005 M $CaCl_2$, 1 μM $ZnCl_2$ and 2.5% (w/v) Triton X-100, in order to remove SDS, followed by 5-min washes in buffer devoid of Triton X-100. After 3.5 h of incubation at 37°C with 1 mM APMA in the same buffer containing 1% (w/v) Triton X-100, the gels were stained with Coomassie Brilliant blue R-250 and destained as de-

scribed [23]. Zones of enzymatic activity corresponding to gelatinolysis were shown by negative staining and quantitated by scanning densitometry (CD 60, Desaga, Sarstedt Gruppe). MMP-9 activity was calculated from a standard curve made as follows: band intensity was expressed as arbitrary units from scanning densitometry and correlated to different concentrations of a reference latent MMP-9 purified from human neutrophils after secretion. Specific activity was expressed per μg of protein in each fraction [25].

2.9. Fluorometric TIMP-1 assay method

The activity of TIMP-1 was assayed by the assessment of inhibition of 3 mM APMA activated MMP-9 (0.5 pmol) purified from human neutrophils [25] using the Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 peptide (1.5 μM) as substrate [30]. The reaction was carried out at pH 7.5 in 0.1 M Tris–HCl buffer containing 0.1 M NaCl, 0.01 M $CaCl_2$, 0.05% (w/v) Brij 35 (625 μl final volume). Hydrolysis of the internally quenched substrate at 37°C resulted in the progressive decrease of the release of the intensely fluorescent methoxycoumarin peptide, Mca-Pro-Leu-OH (emission wavelength: 393 nm; excitation wavelength: 328 nm). The results which are expressed in nmol of degradated substrate/min per ml or mg protein were represented as a percentage of total proteolysis (without TIMP-1). Controls with 10 mM EDTA represent 0% proteolysis.

2.10. Migration assay

Cell migration was quantified using Transwell inserts as described [15]: 10^6 EBV-B lymphocytes in 0.5 ml of serum-free culture medium containing 0.2% (w/v) BSA were added to the 12-mm diameter inserts of Transwell chambers over 12-μm pore polycarbonate filters with a continuous even coating of 100 μl Growth Factor Reduced Matrigel matrix, which separated the cells from 1.5 ml of the same medium in the lower compartment. In this experiment, EBV-B lymphocytes suspended in the 0.2% BSA serum-free medium were cultured in one of two ways: (1) 18 h in the presence or absence of 10 ng/ml LPS or 1 μg/ml Con A in a 5% CO_2 environment at 37°C, or (2) beginning with 18 h in the same conditions as in the first procedure, then added to the upper compartment of the insert for an additional 24 h culture. In some experiments, TIMP-1 purified from EBV-B cell culture medium or recombinant TIMP-1 was added to both compartments at the indicated concentration. Cells in the lower compartment were detached by shaking and collected for counting.

2.11. SDS–PAGE and immunoblotting

Proteins were separated by SDS–PAGE [31] in a 5–15% (w/v) acrylamide gel gradient with a 5% (w/v) stacking gel, and were stained with either Coomassie Brilliant blue R-250 or silver nitrate reagent. In some experiments, the proteins were electrotransferred to nitrocellulose according to Towbin et al. [32]: after blotting, membranes were incubated with specific antisera raised against neutrophil-purified MMP-9 (1:200 dilution) [25] or rTIMP-1 (1:20 dilution) in 0.05 M Tris–HCl, 0.2 M NaCl, 0.05% (w/v) Tween-20, pH 7.5. The Ag/Ab complex was identified by using goat antirabbit IgG alkaline phosphatase conjugate (1:1000 dilution in the same buffer) and stained with the NBT-BCIP reagent according to the manufacturer's instructions.

2.12. Protein identification by mass spectrometry

The silver-stained TIMP band was excised from the gel and digested with trypsin according to the published procedure [33]. A mixture (v/v) of the complete extract peptide with 0.4 ml of cyano-4-hydroxy-*trans*-cinnamic acid in saturation in water containing 0.1% (v/v) trifluoroacetic acid (TFA) and 40% (v/v) acetonitrile was prepared for matrix-assisted laser desorption ionization (MALDI). The excess salts were removed with water containing 0.1% trifluoroacetic acid. MALDI mass spectrometry was performed on a Bruker BIFLEX mass spectrometer (Bruker-Franzen Analytic, Bremen, Germany) and the mass spectra were calibrated using the ion signal from trypsin autodigestion peptides (MH^+ 842.50 and MH^+ 2211.09). Monoisotopic peptide masses were assigned and used in data base searches.

2.13. Biochemical assays

Proteins were estimated by using the Bradford method [34]. TIMP-1 and human (h) IL-10 were as-

sayed by ELISA (Amersham, UK and Innogenetics, Belgium, respectively).

2.14. Statistical methods

Data are presented either as representative traces of at least three experiments or as the mean ± standard error of the mean (S.E.M.) of the number of determinations indicated (*n*).

3. Results

3.1. Isolation of both TIMP-1 and MMP-9 from EBV-B lymphocytes

3.1.1. Approach of MMP-9/TIMP-1 ratio

The tissue inhibitor of metalloproteinase TIMP-1 was purified from conditioned medium of IL-1β stimulated EBV-B lymphocyte medium by a series of four chromatographic steps (Fig. 1). The purification procedure yielded a peptide band of 28 kDa which was identified as TIMP-1 by immunoblotting (Fig. 1c, inset). The EBV immortalization of B lymphocytes and immunotyping argue against any possible contamination by T lymphocytes, neutrophils or monocytes.

After trypsin digestion, MALDI mass spectrometry of the 28-kDa protein band showed the presence

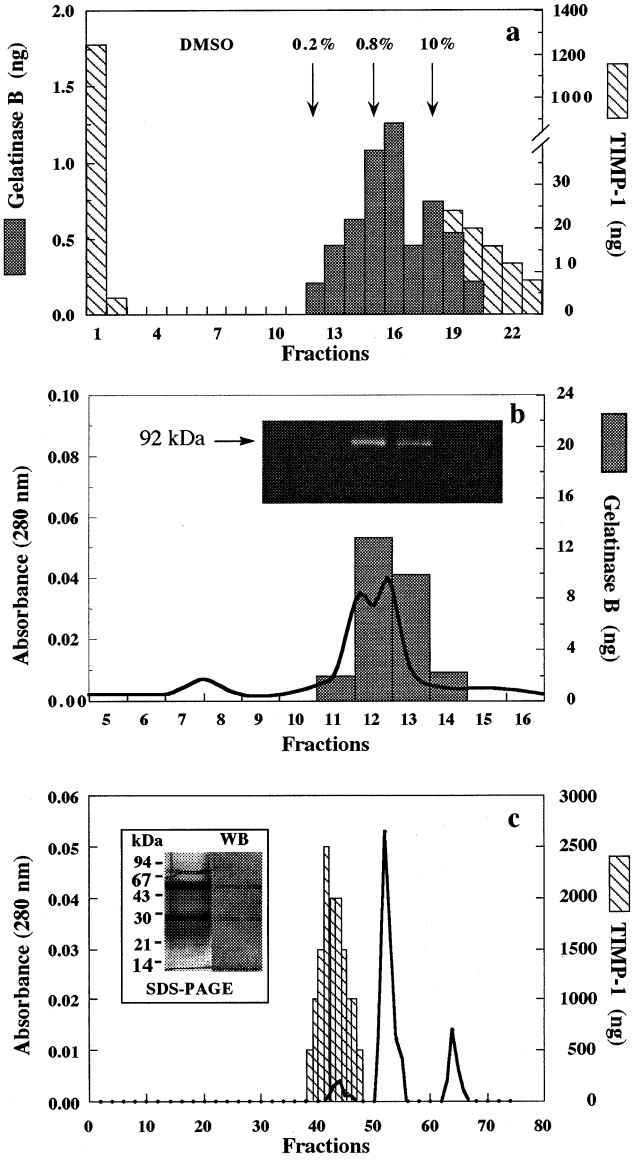


Fig. 1. Purification of TIMP-1 and MMP-9, from serum-free conditioned medium of cultured EBV-B lymphocytes. (a) MMP-9 and TIMP-1 isolation: 400 ml of serum-free conditioned medium of 1 ng/ml rIL-1β-stimulated 10^9 EBV-B lymphocytes cultured at 37°C for 72 h was added to a 2 ml gelatin–agarose matrix as described in Section 2. TIMP-1 filtrated through while MMP-9 adsorbed to the matrix. After washing the column with equilibrium buffer containing 1 M NaCl, an elution of gelatinase was performed by 0.2%, 0.8%, and 10% DMSO added to the NaCl buffer. Each fraction of 2 ml was concentrated onto Centricon 10, submitted to zymography and assayed for TIMP-1 by ELISA. Free TIMP-1 (95% of total TIMP-1) was recovered in the flow through (eluates 1 and 2). First free TIMP-1 proMMP-9 (dark bars) and then TIMP-1 associated proMMP-9 (gray bars) were collected from the gelatin–agarose matrix. (b) MMP-9 purification: FPLC Superose 12 gel filtration of free TIMP-1 proMMP-9. The elution profile was followed by absorbance at 280 nm and zymography (inset). The results are representative of ten experiments. (c) TIMP-1 purification: Sephacryl S-200 chromatography of free TIMP-1. Fractions 1 and 2 (95% of total TIMP-1) collected from the gelatin–agarose matrix were used in three successive chromatographic steps: heparin–agarose, anti TIMP-1 affinity chromatography and Sephacryl S-200 gel filtration. TIMP-1 was quantitated in the eluates by ELISA. TIMP-1 enriched fractions (gray bars) were pooled and subjected to SDS–PAGE and Western blotting. The results are representative of three experiments. Inset: 12.5% SDS–PAGE. One hundred and fifty ng TIMP-1 were loaded on the gel and silver stained. Western blotting was performed by using polyclonal antibodies raised against human rTIMP-1 and NBT/BCIP for staining. Molecular mass standards are expressed in kDa.

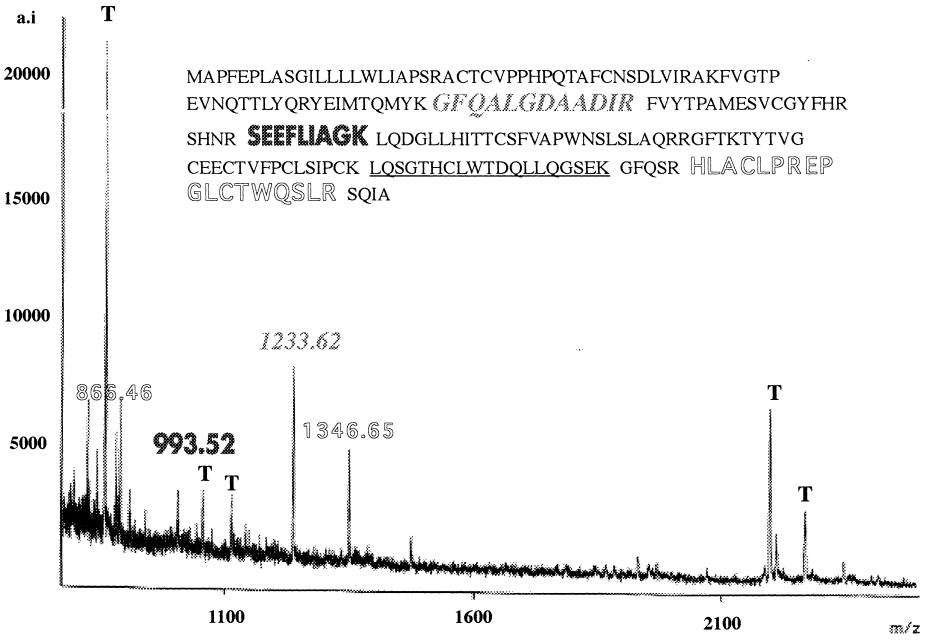


Fig. 2. Mass spectra of TIMP-1. The silver stained 28 kDa band (Fig. 1c, inset) was digested in gel with trypsin according to the published procedure [27]. The mass spectra of TIMP-1 were obtained on a Bruker BIFLEX mass spectrometer (Bruher-Franzen Analytic, Bremen, Germany). Five peaks corresponding to five peptides and to approximately 25% of the published sequence of the human TIMP-1 protein were identified (the signal corresponding to a peptide of 2781 Da is not illustrated in the figure). The other peaks (T) were shown to correspond to trypsin fragments.

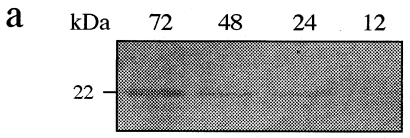
of at least five peptides of 866.46, 993.52, 1233.62, 1346.65 and 2781 Da (Fig. 2), corresponding to sequence residues identified after the MS-Fit search. The analyzed sequences of the residues were, respectively, 186–192 (HLACLPR), 103–111 (SEEFLIAGK), 71–82 (GFQALGDAADIR), 193–203 (EPGLCTWQSLR) and 162–180 (LQSGTHCLWTDQLLQGSEK) (signal not shown), and corresponded to different peptides of the N-terminal and C-terminal domains of TIMP-1, covering 25% of the protein.

Quantification with a competitive ELISA developed for TIMP-1 showed a substantial excess of TIMP-1 versus MMP-9: the total quantity of TIMP-1 purified from 10^9 cells was 12.5 μg from 60 μg in the medium with a recovery of 20% (Table 1). Only a 3–4% proportion of TIMP-1 was associated with MMP-9 and bound to the gelatin–agarose matrix. Free TIMP-1 proMMP-9 and TIMP-1-complexed proMMP-9 were simultaneously eluted from gelatin–agarose by a DMSO stepwise gradient (Fig.

Table 1
TIMP-1 purification table

Fractions	Proteins (mg)	TIMP-1 (μg)	Specific activity (μg/mg)	Yield (%)	Purification factor
CM	18.50	60.0	3.24	100	1
Gelatin–agarose	18.00	58.2	3.33	97	1
Heparin–agarose	0.65	30.0	46.15	50	14
TIMP-1 affinity	0.05	13.5	270.00	22	83
Sephacryl S-200	0.02	12.5	625.00	20	193

IL-1β (1 ng/ml)-stimulated EBV-B lymphocytes (10^9 cells) were cultivated for 72 h under 5% CO_2 atmosphere in 0.2% (w/v) BSA serum-free medium as described in Section 2. Four hundred ml conditioned medium (CM) was filtered through gelatin–agarose matrix. The flow-through containing free TIMP-1 was used for purification in three further purification steps. TIMP-1 was quantitated by ELISA. Results are representative of three experiments.



b

	1									**50**
Human TIMP-1	CTCVPPHPQT	AFCNSDLVIR	AFKVGTPEVN	QTTLY	. . . . .	. .	QRYEIKMT			
Recom TIMP-1				GTPEVN	QTTLY	. . . . .	. .	QRYEIKMT		
	51									**100**
Human TIMP-1	KMYKGFQALG	DAADIRFVYT	PAMESVCGYF	HRSHNRSEEF	LIAGKLQDGL					
Recom TIMP-1	KMYKGFQALG	DAADIRFVYT	PAMESVCGYF	HRSHNRSEEF	LIAGKLQDGL					
	101									**150**
Human TIMP-1	LHITTCSFVA	PWNSLSAQR	RGFTQTYTVG	CEECTVFPCL	SIPCKLQSGT					
Recom TIMP-1	LHITTCSFVA	PWNSLSAQR	RGFTQTYTVG	CEECTVFPCL	SIPCKLQSGT					
	151									**200**
Human TIMP-1	HCLWTDQLLQ	GSEKGFQSRH	LACLPREPGL	CTWQSLRSQI	A					
Recom TIMP-1	HCLWTDQLLQ	GSEKGFQSRH	LACLPREPGL	CTWQSLRSQI	A					

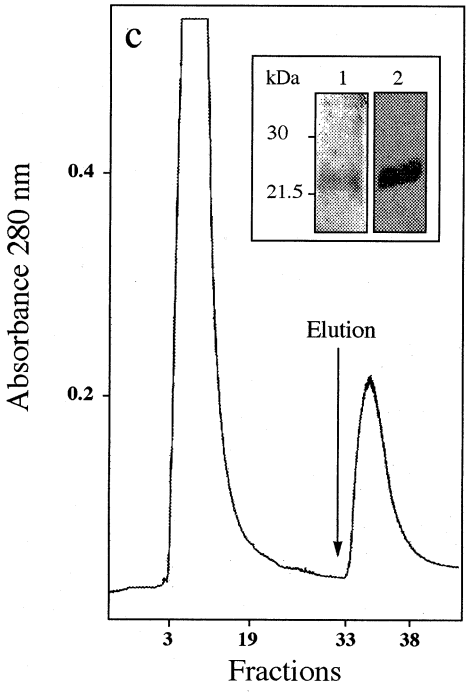


Fig. 3. Expression of recombinant TIMP-1 and purification. (a) Production of rTIMP-1 by infected Sf9 cells. A sample of Grace's culture medium was withdrawn after 12, 24, 48 and 72 h of Sf9 infection with recombinant virus. Thirty μg of culture medium proteins were submitted to 12.5% SDS–PAGE and then transferred onto a 0.45 μm nitrocellulose membrane. rTIMP-1 was characterized using first a rabbit polyclonal anti-TIMP-1 antibody and then a goat antirabbit antibody conjugated with alkaline phosphatase. Staining was performed with NBT-BCIP reagent. (b) Comparison between native and recombinant human TIMP-1 sequences. (c) Elution profile of the Probond affinity column. Two ml of dialyzed soluble extract from inclusion bodies was added onto a 2 ml Probond nickel-chelating resin and eluted after intensive washing by 300 mM imidazole complementing the equilibration buffer (100 mM K_2HPO_4, 10 mM Tris–HCl (pH 7.8), 4 M urea, 0.05% (w/v) Brij 35). The elution profile was followed by absorbance at 280 nm. Inset: 12.5% SDS–PAGE (lane 1) and Western blotting (lane 2) of the Probond affinity column pool eluate. One μg of protein was loaded on the gel and silver stained: the Western blot experiment was performed as described in a.

1a). The resolution of the TIMP-1 complex was performed at 10% DMSO. The eluates which showed gelatinolytic activity, but did not contain TIMP-1, were pooled and used for MMP-9 purification by FPLC filtration onto a Superose 12 column (Fig. 1b). About 10 ng of purified proMMP-9 was isolated from 400 ml of serum-free conditioned medium of 10^9 EBV-B lymphocytes (Table 2). This final material ran as a major 90 kDa peptide band on reducing SDS–PAGE gel and was correlated to the 92 kDa protein with gelatinolytic activity detected by zymography (Fig. 1b, inset). Identification of MMP-9 was performed by immunoblotting with a specific polyclonal antibody raised against neutrophil MMP-9 [24] (not shown).

3.1.2. *Recombinant TIMP-1 overexpression in the baculovirus Sf9 expression system*

The baculovirus expression system has been previously used to overexpress human [35] and murine [36] TIMP-1. Sf9 cells were collected 3 days postinfection with recombinant virus. No rTIMP-1 was secreted in the conditioned medium. rTIMP-1 was found to accumulate within the cells as intracellular aggregates (Fig. 3a). Insoluble rTIMP-1 was extracted from the aggregates and suspended in 50 mM Tris–HCl buffer (pH 8.5) containing 8 M urea, 1 mM EDTA and 200 mM β-mercaptoethanol. Soluble extract was applied to an immobilized nickel-chelating resin [37] on which TIMP-1 had bound. The apparent molecular mass of the rTIMP-1 differed significantly from that of native TIMP-1 (22 and 28 kDa, respectively) (Fig. 3c, inset), as the first 24 amino acid residues of rTIMP-1 N terminal sequence were not in the clone (rΔTIMP-1) (Fig. 3b).

3.1.3. *TIMP-1 isolated from EBV-B lymphocytes displays MMP-9 inhibitory function*

As expected, TIMP-1 purified from conditioned medium of EBV-B lymphocytes inhibited active MMP-9 in a dose-dependent manner (Fig. 4a): the remaining MMP-9 activity was determined by proteolytic degradation of the synthetic peptide Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 as described [30]. 30 ng TIMP-1 (1 pmol) completely abolished MMP-9 activity as did 10 mM EDTA, while half of the activity was inhibited only by 10 pg TIMP-1. The rΔTIMP-1 had no effect on MMP-9 activity and was used as control; indeed the N-terminal portion missing from rΔTIMP-1 was reported to be directly involved in the inhibition of active MMPs [38,39]. The latter inhibitory effect of TIMP-1 on MMP-9 was corroborated in a Matrigel transmigration experiment. Briefly, unstimulated 10^6 EBV-B lymphocytes were layered onto a polycarbonate micropore filter coated with Matrigel consisting of basement membrane matrix constituents. As shown on Fig. 4b, 15% of EBV-B lymphocytes migrated spontaneously through the Matrigel. Twenty pg/ml rTIMP-1 (Amersham) or 6 pg/ml TIMP-1 purified from EBV-B lymphocyte-conditioned medium were shown to inhibit migration by 76% and 95%, respectively. Moreover, 4 pg/ml rTIMP-1 inhibited the spontaneous migration of 10^6 EBV-B lymphocytes by 50% (Fig. 4b, inset).

3.1.4. *Dependence of TIMP-1/MMP-9 imbalance on EBV immortalization and on IL-10 secretion*

In order to investigate the balance between TIMP-1 and MMP-9 secretion and the dependence on EBV transformation of B lymphocytes, native and EBV-

Table 2
MMP-9 purification table

Fractions	Proteins (mg)	MMP-9 (ng)	Specific activity (ng/mg)	Yield (%)	Purification factor
CM	8.400	17.3	2	100	1
Gelatin–agarose	0.045	29.9	665	173	325
FPLC Superose 12	0.010	9.6	994	56	471

MMP-9 from 400 ml conditioned medium (CM) of 1 ng/ml IL-1β-stimulated 10^9 EBV-B lymphocytes cultivated in 0.2% (w/v) BSA serum-free medium for 72 h under 5% CO_2 atmosphere was adsorbed onto gelatin–agarose matrix and eluted in a stepwise DMSO gradient as described in Section 2. Eluted free TIMP-1 MMP-9 was purified further by FPLC Superose 12 chromatography. MMP-9 was quantitated by zymography. Gelatinolytic activity was visualized by negative staining with Coomassie Brilliant blue R-250, and quantitated by scanning densitometry, including neutrophil-purified MMP-9 as the reference enzyme. Results are representative of ten experiments.

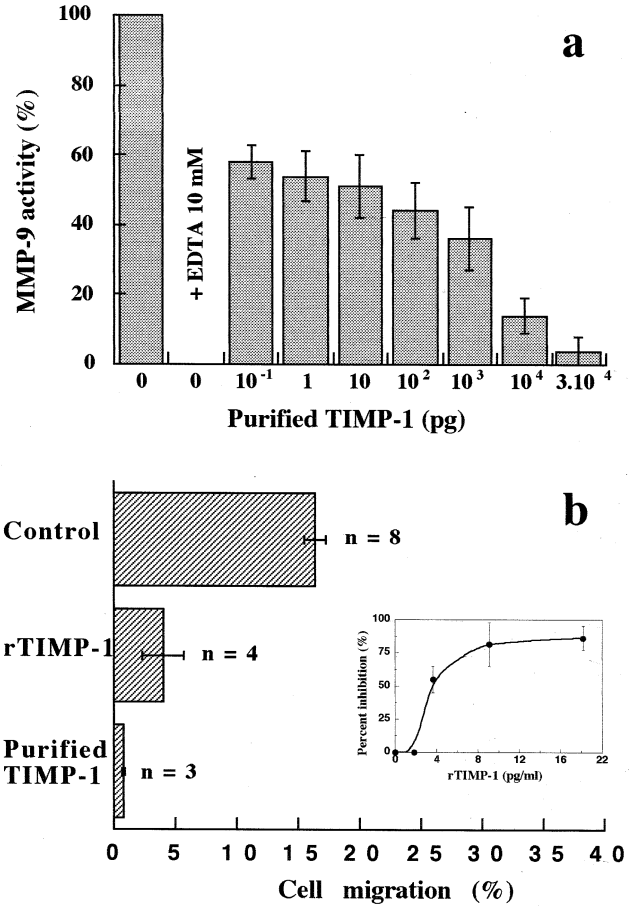


Fig. 4. (a) Inhibition of MMP-9 activity by increasing amounts of purified EBV-B lymphocyte TIMP-1. Increasing amounts of TIMP-1 were incubated at 37°C for 30 min with 0.5 pmol of stimulated MMP-9 purified from neutrophils. The peptide (Mca-Pro-Leu-Gly-Leu-Dpa -Ala-Arg-NH_2) was then added to the mixture at a final concentration of 1.5 μM. The fluorescence of methoxycoumarin peptide resulting from substrate hydrolysis was measured by fluorimetry (emission wavelength: 393 nm; excitation wavelength: 328 nm). Without TIMP-1 the fluorescence was maximum (100%); with 10 mM EDTA the fluorescence was completely eliminated. The results are representative of three experiments. (b) Transwell migration. 10^6 cells nonstimulated EBV-B lymphocytes in 0.5 ml of 0.2% (w/v) BSA serum-free culture medium were added to the upper chamber of a Transwell over a continuous coating of Growth Factor Reduced Matrigel, and incubated for 24 h at 37°C in a 5% CO_2 atmosphere (control). In some experiments, 6 pg/ml of EBV-B lymphocytes purified TIMP-1; in others 20 pg/ml of recombinant TIMP-1 (Amersham, UK) was added to the two chambers. After incubation, the B cells in the lower compartment were collected and counted by trypan blue exclusion. The results are representative of three experiments, each in quadruplicate. Inset: dose–response effect of rTIMP-1 on B-cell migration. 10^6 nonstimulated EBV-B lymphocytes in 0.5 ml of 0.2% (w/v) BSA serum-free culture medium were layered onto the upper Transwell chamber and incubated at 37°C in a 5% CO_2 atmosphere for 24 h. Increasing concentrations of rTIMP-1 up to 18 pg/ml were added to the two chambers. At the end of incubation, the B cells which migrated in the lower chamber were detached and counted. The results are representative of two experiments, each in triplicate.
←

immortalized B cells were cultivated in serum-free medium containing 0.2% BSA. At defined time periods, medium samples were withdrawn and used for MMP-9, TIMP-1 and IL-10 measurements. MMP-9 was isolated on a gelatin–agarose matrix and analyzed for gelatinolytic activity by zymography. TIMP-1 and IL-10 were measured by ELISA. Under the conditions used for short-term cultures, native B lymphocytes prepared from buffy coats secreted a high level of MMP-9 which increased with culture time (Fig. 5a). On the contrary, a slight secretion of MMP-9 was mediated by unstimulated but EBV-transformed B lymphocytes. We had shown previously that this secretion is inducible and can be enhanced by PMA and IL-1β [15]. Significant rates of TIMP-1 ($\approx$30 ng/10^7 equivalent cells) were measured in conditioned medium of EBV-B lymphocytes conversely to that of native B cells after 20 h of culture. TIMP-1 concentration increased with culture time from 20 to 70 h (Fig. 5a).

Two EBV-B lymphocyte culture conditions were used to investigate the modalities of TIMP-1 secretion. The basal concentration of TIMP-1 secreted by suspended EBV-B lymphocytes increased with LPS and Con A stimulation (Table 3). However, contact of unstimulated EBV-B lymphocytes with basement membrane constituents further increased TIMP-1 secretion. This increase was up to 10 times that observed in suspended EBV-B lymphocytes; moreover TIMP-1 concentration increased upon stimulation of the B cells by LPS or Con A (Table 3).

Secretion of IL-10 was correlated with that of TIMP-1: there was no IL-10 secreted by native B lymphocytes, even though it was synthesized and secreted by EBV-B lymphocytes (Fig. 5b). During the experiment, the number of EBV-B lymphocytes doubled in 24 h while the number of native B cells decreased by 50% in 3 days (not shown).

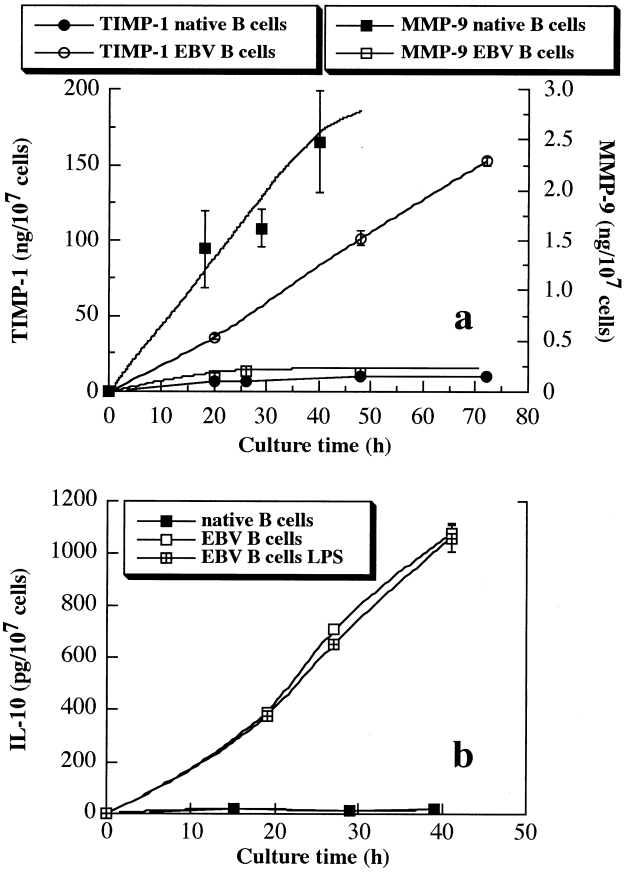


Fig. 5. Secretion of TIMP-1 and MMP-9 (a) and IL-10 (b) by native and EBV-B lymphocytes. (a) Native or EBV-B lymphocytes were cultivated in 0.2% (w/v) BSA serum-free medium at 37°C in a 5% CO_2 atmosphere as described in Section 2. At different incubation times, secreted TIMP-1 was assayed by ELISA in conditioned medium and MMP-9 was quantitated after zymography by using neutrophil purified MMP-9 as standard. The results are representative of at least three experiments. (b) IL-10 was measured by ELISA in conditioned medium of native nonstimulated B lymphocytes (control) or LPS (10 ng/ml) stimulated EBV-B lymphocytes at different incubation times. The results are representative of three experiments.

3.1.5. TIMP-1 can be considered as a survival factor

B-cell apoptosis was studied by flow cytometry (Fig. 6). Native or EBV-B lymphocytes showed a viability close to 100% (Fig. 6A,C). The addition of 0.5 nM PMA to the culture medium of EBV-B lymphocytes did not affect viability (Fig. 6A,B) whereas 21% of native B cells cultivated in the same medium showed an apoptotic phenotype (Fig. 6C,D). Moreover, conditioned medium from EBV-immortalized B cells is TIMP-1 positive whereas native B-cell medium is not. These results suggest that B-cell apoptosis is related to EBV immortalization and that TIMP-1 may be involved in the process.

4. Discussion

4.1. Growth factor properties of TIMP-1

There is growing evidence of multiple properties for TIMP-1. These include MMP inhibition, growth factor activity and antiapoptotic functioning. The present paper highlights an overproduction of TIMP-1 versus that of MMP-9 by Epstein–Barr-virus-immortalized compared to primary B lymphocytes. In this cellular model, secretion of TIMP-1 depends on soluble mediators ([15], this work), the matrix environment and incubation times. The TIMP-1/MMP-9 ratio imbalance measured either in conditioned medium of EBV-transformed B lymphocyte or after purification, asks question about the environment dependence on expression and points

Table 3
Influence of the Transwell coating Matrigel on TIMP-1 production and secretion by EBV-B lymphocytes

Conditioned medium of:	TIMP-1 concentration ($ng/10^7$ cells)	
Native B lymphocytes	6.5 ± 0.6	$n = 3$
EBV-B lymphocytes		
Control	12 ± 9	$n = 8$
LPS-stimulated	207 ± 10	$n = 7$
Con A-stimulated	320 ± 8	$n = 7$
EBV-B lymphocytes in Transwell		
Control	128 ± 9	$n = 9$
LPS-prestimulated	403 ± 49	$n = 8$
Con A-prestimulated	599 ± 37	$n = 9$

10^6 native or EBV-B lymphocytes stimulated by 10 ng/ml LPS or 1 μg/ml Con A or nonstimulated (control), were cultivated for 18 h in 0.2% (w/v) BSA serum-free culture medium as described in Section 2. At the end of incubation, TIMP-1 was measured in conditioned medium by ELISA. In some experiments after 18 h incubation, the 10^6 B cells suspended in 0.5 ml conditioned medium were added to the upper chamber of a Transwell over a continuous coating of Growth Factor Reduced Matrigel matrix and incubated for another 24 h. At the end of incubation, TIMP-1 was quantitated by ELISA in the conditioned medium recovered from the upper chamber. *n*, number of experiments.

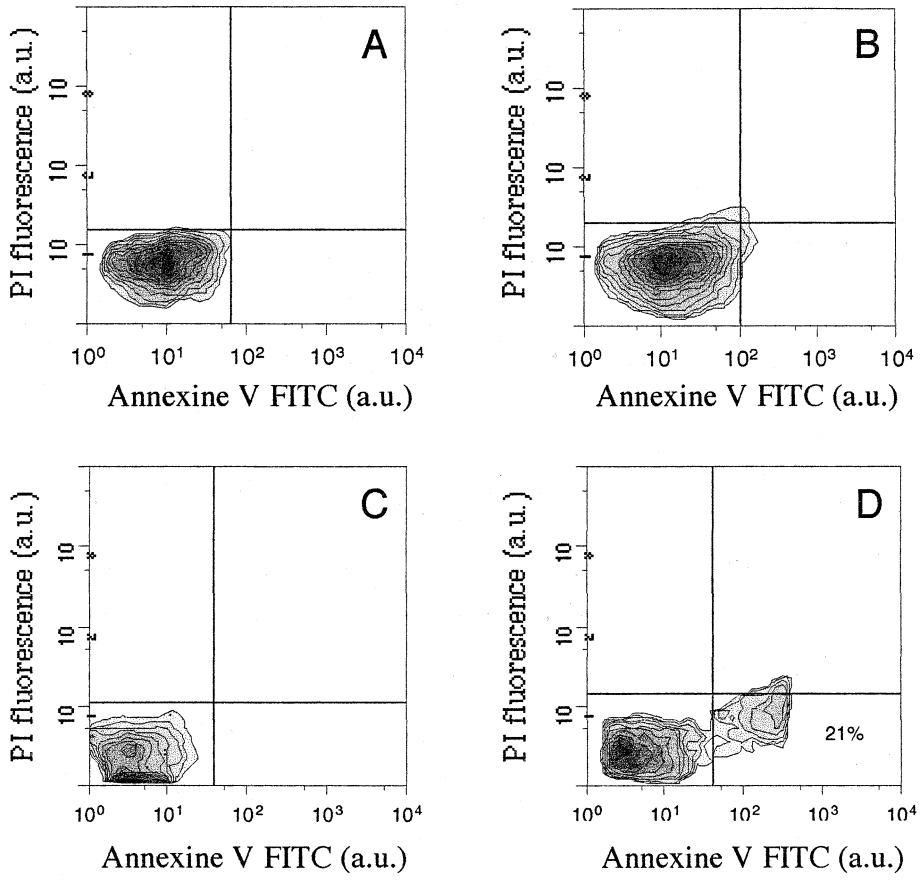


Fig. 6. Apoptosis flow cytometric analysis. 10^6 native (C,D) or EBV immortalized (A,B) B lymphocytes were cultivated for 18 h in a 0.2% (w/v) BSA serum-free culture medium (A,C) supplemented with 0.5 nM PMA (B,D). The cells were then washed and incubated for 15 min in 100 μl of the reaction buffer (10 mM Hepes–NaOH, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4) containing Annexin V FITC and 1 μg/ml propidium iodide. After washing, the cells were analyzed immediately on a FACS Calibur (Becton Dickinson). Apoptosis was measured by Annexin V FITC fluorescence and necrosis by propidium iodide fluorescence, both expressed in arbitrary units (a.u.).

to a novel function of the tissue inhibitor. The high ratio of TIMP-1 versus MMP-9 demonstrated in this report for EBV-B lymphocytes compared to native B cells has also been described in other cell types such as osteoblasts or intestinal smooth muscle [40], suggesting that these cells need TIMP-1 to proliferate. In fact the N-terminal homologous sequence of both granulocyte-macrophage colony-stimulating factor and TIMP-1 may contribute to TIMP-1 mediated growth activity in many cells types including smooth muscle cells, endothelial cells, articular chondrocytes, fibroblasts and human or bovine lymphoblasts [20]. Moreover similar examples of proteins such as TIMP-1, with both inhibitory and growth factor activities have been described in hepatoma cells [41]. Recently TIMP-1 was reported as growth factor in the Burkitt's lymphoma (BL) cells cultured in serum-free conditions where the EBV gene expression controls not only the phenotype but also increases resistance of these cells to apoptosis [42]. TIMP-1 was expressed by the centroblast like BL cell lines but not by the follicular lymphoma cell lines [20]. These findings support a function of TIMP-1 as a survival factor suppressing apoptosis independently of its ability to inhibit MMP-9 activity. These data were corroborated in experiments showing that infection of EBV negative BL cell lines which do not express TIMP-1 readily undergo apoptosis [20]. The results focus on the specificity of TIMP-1 function, demonstrating that TIMP-1 is sufficient to induce the activated phenotype observed in $TIMP\text{-}1^+$, EBV^+ BL cell lines.

4.2. Regulation mechanisms of TIMP-1 expression

In breast epithelial cells, upregulation of TIMP-1 is correlated with the overexpression of bcl-2 resulting in the inhibition of apoptosis [19]. Although in these cells TIMP-1 has no effect on the bcl-2 expression level, it may act in a common signaling pathway of the apoptotic cascade through the focal adhesion kinase activation. The molecular mechanisms by which bcl-2 induces TIMP-1 expression remains to be defined.

The high concentration of TIMP-1 measured in conditioned medium of immortalized B lymphocytes was correlated to the presence of IL-10. The cytokine was recently proposed as an autocrine growth factor for EBV-B lymphocytes [43]. It is known as a pleiotropic factor that enhances proliferation of activated human B lymphocytes and induces them to secrete high amounts of immunoglobulin [44]. Secretion of IL-10 has previously been demonstrated in lymphoblastoid and Burkitt's lymphoma cell lines [44]. Neither IL-10 nor TIMP-1 were detected in culture conditioned medium of primary B cells. EBV-transformed B lymphocytes produce two forms of IL-10, human and viral. Viral IL-10 is expressed during lytic EBV infection and may play a role in the establisment of EBV-related tumors whereas human IL-10 may sustain proliferation of B lymphocytes harboring a latent EBV infection [43]. The release of both TIMP-1 and IL-10 from EBV-B lymphocytes suggest a possible IL-10 involvement in an autocrine regulation process as proposed in monocytes [45] where IL-10 decreased the production of 92 kDa gelatinase while enhancing TIMP-1 expression. Moreover a pivotal role for IL-10 in the development of lymphoproliferative disorders has also been proposed [43].

In the present study we have correlated secretion of TIMP-1 to that of hIL-10 in B lymphocytes transformed through EBV while both TIMP-1 and IL-10 were absent in native B cells. The role of TIMP-1 as antiapoptotic factor previously hypothesized [19] and highlighted in this work needs further investigations especially in order to characterize a specific interaction site of TIMP-1 at the membrane level. Preliminary results performed in human MCF-7 breast carcinoma cells [46] demonstrated the translocation of TIMP-1 to the nucleus suggesting a new function for TIMP-1 as transcription factor involved in the control of growth and apoptosis. These latter aspects are presently under study.

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