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Activation of latent transforming growth factor beta 1 and inhibition of matrix metalloprotease activity by a thrombospondin-like tripeptide linked to elaidic acid

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Abstract

Impaired wound healing and skin aging are characterized by neutral protease-mediated destruction of matrix macromolecules associated with disturbance in tissue repair. We synthesized a fatty acyl-peptide derivative at aims to simultaneously activate latent TGF-β through its peptide domain, KFK, and inhibit MMPs through its lipophilic moiety, elaidic acid. Elaidyl-KFK as well as KFK were shown to activate LAP-TGF-β both in vitro, using a solid phase assay with immobilized LAP-TGF-β, and ex vivo using human dermal fibroblasts cultures. In both assays, as much as up to 10% of LAP-TGF-β added could be recovered as active form. KQK, KQFK as well as their lipopeptide counterparts were inactive. Elaidyl-KFK-mediated LAP-TGF-β activation led to up-regulation of collagen and TIMP-1 production and down regulation of PMA-induced MMP-1 expression in fibroblasts cultures. Those effects could be suppressed by supplementing cell culture medium with blocking TGF-β antibody. Elaidyl-KFK inhibited MMP-2, MMP-9, MMP-3, MMP-1, in vitro with IC_{50} equal to 1.2, 1.0, 0.24 and 8.9 μM, respectively. Its ex vivo inhibitory capacity, as assessed using skin tissue sections, towards the elastin-degrading capacity of MMP-9 was even more pronounced. At a 1 μM concentration, the lipopeptide decreased by up to 80% enzyme activity. Thus, ''Lipospondin,'' i.e. elaidyl-KFK might be considered as a promising model compound to prevent age-associated dermal alterations.

Keywords: Matrix metalloprotease; Tissue inhibitor of metalloprotease; Transforming growth factor beta 1; Fatty acyl-peptide derivative; Thrombospondin-1; Aging

1. Introduction

Tissue homeostasis is regulated by the balance between synthesis and degradation of matrix molecules. Aging-associated diseases are characterized by a disturbance of such equilibrium towards increased catabolism and imperfect repair [1,2]. As an example, alteration of the dermal collagen and elastin fibers networks constitutes the hallmark of human skin aging [3,4]. Several lines of evidence indicate that modifications affecting those extracellular matrix structures originate from a gradual imbalance between biosynthesis and degradation of collagen and elastin [3,5], that progressively lead to skin atrophy and loss of recoil [3,4]. Collagen and elastin degradation is catalyzed mainly by neutral proteinases belonging to the MMP family [6–8]. Among that clan, Collagenases 1 and 3 (MMP-1; MMP-13), Gelatinase A (MMP-2) and Membrane-type 1 Matrix Metalloprotease (MMP-14) display collagenolytic activity and gelatinases A and B (MMP-2 and MMP-9) have been described as true elastases [9–11].

Abbreviations: TGF-β1, transforming growth factor beta 1; MMP, matrix metalloprotease; TIMP, tissue inhibitor of metalloprotease; TSP-1, thrombospondin-1; LAP, latency-associated protein; EA, elaidic acid; BSA, bovine serum albumin; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris buffer saline; PMA, phorbol myristate acetate

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Other MMP members, as stromelysin-1 (MMP-3) can equally participate in the lysis of collagen and elastin fibers, acting either upstream in the proteolytic cascade leading to procollagenases and proelastases activation or degrading collagen or elastin-associated matrix macromolecules [11]. Innate, as well as photodamage aging, is characterized by an increased expression of most of those MMPs from dermal fibroblasts that is not counterbalanced by TIMP-1 and TIMP-2 production [12–14].

A series of investigations demonstrated that retinoids could re-establish a correct homeostasis of dermal tissue, as impaired during aging and exacerbated with chronic non healing wounds [14], by enhancing collagen biosynthesis while inhibiting MMP expression [5]. We turned here our attention to TGF-β1, a factor also exhibiting the capacity to increase collagen, elastin, and TIMP-1 expression while decreasing collagenase-1 production by fibroblasts in culture [15–18]. TGF-β is secreted from cells as a latent form, where the TGF-β receptor interacting sequence is hidden by its non covalent association with a dimer of its N-terminal propeptide, designated as LAP [19]. Revelation of this cryptic site, as occurred extracellularly, is considered as the main regulatory step in the control of TGF-β activity [20]. Under physiological or pathological conditions, generation of mature TGF-β can result from the action of reactive oxygen species, deglycosylation of the latent protein, or through LAP proteolysis by several enzymes as cathepsin(s) or plasmin [21,22]. However, TSP-1 was evoked as the major activator of LAP-TGF-β1 both in vitro or in vivo [23,24]; this activation was attributed to conformational change mediated by the interaction between a KRFK sequence within the properdin-like domains of TSP-1 and an LSKL complementary sequence in LAP [25]. Indeed, peptides with the consensus BFB sequence (B: a basic amino acid) were consistently found to activate LAP-TGF-β in several in vitro and ex vivo experimental conditions [26].

In the present investigation such tripeptide, i.e. KFK was covalently coupled to a long-chain unsaturated fatty acid, i.e. EA, known to potently inhibit MMP activity [27]. The bifunctionality of this compound at activating LAP-TGF-β1 and at inhibiting MMPs is here reported, using in vitro and ex vivo experimental models.

2. Experimental procedures

2.1. Peptides and lipopeptides syntheses

Tri peptides (KFK; KQK) and their elaidyl derivatives were synthesized by Neosystem (Neosystem groupe SNPE). Their purities were up to 95% and their molecular weights as determined by mass spectrometry corresponded to calculated values. They were solubilized in DMSO and solvent concentration never exceeded 0.1% in all experiments.

2.2. Real-time biomolecular interaction analysis of elaidyl-KFK binding to LAP-TGF-β

The interaction between LAP-TGF-β1 and elaidyl-KFK was studied using Biacore X system (Pharmacia Biotech). Sensor chips HBA (Biacore AB) were used for all experiments. A flow rate of 5 μl/min was applied and the instrument was thermostated at 25 °C. The surface of the sensorchip was washed with 40 mM *n*-octoyl-β-D-glucopyranoside in water for 5 min. Elaidyl-KFK (20 μl of a 1 mM solution in 50% (v/v) DMSO/water) was then injected. Spontaneous fatty acid adsorption of the lipopeptide to the sensorchip was monitored and subsequent injections were performed until the sensorgram reading reached a stable level. The formation of multiple lipid layers on the sensor chip surface was eliminated by increasing the flow rate to 100 μl/min for 5 min and by an additional injection of 10 mM NaOH. To assess the extent of coverage of the surface, 10 μl of 0.1 mg/ml BSA in a 0.1 M Tris–HCl, pH 7.8 were injected. The amounts of BSA bound to the sensor chip surface never exceeded 43 resonance units (RU). An uncoated octylglucoside surface bound an average of 1000 RU. LAP-TGF-β (from 20 to 100 nM) binding experiments were performed in the same buffer; after each cycle, the sensor chip was regenerated by injection of 10 mM HCl/Glycine pH 1.1, that allowed to restore the baseline level observed before each injection. Equilibrium dissociation constants were determined as described [28]. Briefly, at steady state:

$$\frac{dR}{dT} = k_{ass} \times C(R_{max} - R_{eq}) - k_{diss} \times R_{eq} = 0 \quad (1)$$

which may be rearranged as follows:

$$R_{eq} = \frac{C \times R_{max}}{(K_D + C)} \quad (2)$$

where $K_D = k_{diss}/k_{ass}$ is the equilibrium constant, R_{eq} is the response value at steady state, R_{max} is the maximal capacity of the sensor chip for binding analyte, and C is the molar concentration of analyte. K_D was determined by non-linear regression analysis by fitting the (R_{eq}, C) pairs to Eq. (2) using BIAevaluation software (BIAcore) and according to a single site model.

2.3. In vitro activation of LAP-TGF-β1

96-well plates were coated with an anti LAP monoclonal antibody (R&D Systems) (800 ng/well) in 25 mM bicarbonate buffer, pH 9.6 for 18 h at 4 °C. After washings, LAP-TGF-β1 (R&D Systems) was added in 20 mM Tris–HCl buffer, pH 7.6 containing 50 mM NaCl and 1% (w/v) BSA, for 2 h at 20 °C. Under our experimental conditions, 5.25 ng of LAP-TGF-β1 were bound per well, representing 1.25 ng (5×10^{-5} nmol) of mature TGF-β, as determined by ELISA. In some experiments 400 ng/well of LAP antibody were coated allowing immobilization of 2.2 ng

of LAP-TGF-β1. Various concentrations of peptides or elaidyl-peptides (from 1 to 75 μM) were then added in PBS buffer pH 7.4, and incubated for various times (1–24 h) at 37 °C; samples were then tested for increased TGF-β1 activity using the TGF-β1 Emax™ immunoassay system (Promega), following the manufacturer's instructions. Increased TGF-β activity was quantified using a TGF-β standard curve. LAP-TGF-β incubated with 1N HCl for 1 h at 4 °C, leading to the release of 660 pg/well, was used as a positive control for LAP-TGF-β activation.

2.4. Activation of LAP-TGF-β1 in fibroblasts cultures

Skin biopsies were obtained from healthy individuals (age range 12–50) during aesthetic or reconstructive surgery. The informed consent of the donors was obtained. Fibroblasts were grown from skin explants in DMEM supplemented with 10% (v/w) FCS, and cultured at 37 °C in a humidified incubator in an air/carbon dioxide atmosphere. Cells were used between the 4th and 8th passages and cultured in 24 wells in DMEM medium supplemented 10% (v/v) FCS. Then, cells were incubated at confluency in 0.2 ml DMEM supplemented with 0.5% (v/v) FCS for protein and collagen synthesis. They were serum-deprived for MMPs and TIMPs determination. Peptides and/or lipopeptides (=20 μM) were added to LAP-TGF-β (10 ng/well)-containing culture medium and TGF-β activity was quantified following 24 h incubation with cells using the TGF-β1 Emax™ immunoassay as above described. Cell viability was assessed by the Trypan blue exclusion test.

2.5. Protein and collagen syntheses'

At subconfluency, LAP-TGF-β1-containing dermal fibroblasts cultures were exposed to increasing concentrations of peptides or lipopeptides (up to 20 μM) for 24–72 h in DMEM supplemented with 0.5% (v/v) FCS, 50 μg/ml β-amino propionitrile, 10 μg/ml ascorbic acid, 23 μg/ml proline and 5 μCi/ml ^{3}H-proline (NEN, Life Science). Culture medium and cell monolayer were collected separately at the end of incubation and aliquots were withdrawn for DNA measurements [29]. *Clostridium histolyticum* collagenase (2 U/ml; Worthington) was used for the quantification of collagen synthesis according to Peterkofsky and Diegelmann [30].

2.6. Determination of MMP-1 and MMP-3 by western blotting

MMP-1 and MMP-3 expressions were determined following treatment of confluent dermal fibroblasts with 0.1 μM PMA for 24 h by western blotting. Conditioned media were supplemented with active TGF-β (2 ng/ml), elaidyl peptide (10 μM) or LAP-TGF-β1 (10 ng/well) and elaidyl-KFK (10 μM). Concentrated aliquots from culture medium were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in 10% (w/v) acrylamide under reducing conditions according to Laemmli [31]; proteins were then transferred to PVDF membranes (Immobilon-P, Millipore). The membranes were blocked with 5% (w/v) non fat dry milk (Biorad) in 50 mM Tris–HCl pH 7.5 containing 150 mM NaCl (TBS), for 2 h at room temperature. The blotted proteins were probed with MMPs primary antibodies (VWR International), diluted 1/5000 in TBS-Tween (0.1% v/v) (TBS-T), containing 1% non fat dry milk (w/v) for 18 h at 4 °C. After extensive washings with TBS-T, immunoreactive proteins were revealed by staining with enhanced chemiluminescent detection reagents (Amersham). Rec MMP-1 and rec MMP-3 were incorporated as controls in all experiments.

2.7. Reverse zymography

Tissue inhibitors of metalloproteases (TIMP-1 and TIMP-2) production by dermal fibroblasts was evaluated by gelatin-reverse zymography as described [32]. Briefly, 12% (w/v) separating SDS acrylamide gels were copolymerized with 2 mg/ml gelatin containing 1% (v/v) SDS and proMMP-2 (10 ng/ml) (VWR). Culture media (10 μl) from fibroblasts treated with active TGF-β or LAP-TGF-β and elaidyl-peptide were loaded and subjected to electrophoresis. Reverse zymography revealed inhibitory activity, which appeared as blue zones against a clear background, demonstrating inhibition of gelatin lysis in the gels. Human recombinant TIMPs (0.1 ng) were used as markers in the gels. TIMP-1 was quantified by an automated image analyzer (Vilbert-Lourmat).

2.8. Inhibition of MMP activity

2.8.1. In vitro assays

The influence of EA and elaidyl-KFK on recombinant MMP-2, MMP-3 and MMP-9 (VWR) activity was determined using the fluorescent quenched substrate Mca-Pro-Leu-Gly-Leu-Dpa-Leu-Ala-Arg (where Mca, (7-methoxycoumarin-4-yl)acetyl and Dpa, (3-(2′,4′-dinitrophenyl)-L-2,3-diaminopropionyl)), (Bachem) [27]. Each enzyme was active site-titrated using a standard preparation of human recombinant TIMP-1 for MMP-1 and TIMP-2 for MMP-2, MMP-9 and MMP-3 [33]. Recombinant TIMP-1 and TIMP-2 were from Calbiochem. Two hundred picomolar of MMP-2 or MMP-9, 400 pM of MMP-3 or 3 nM of MMP-1 were mixed with increasing concentrations of EA or elaidyl-KFK up to 20 or 50 μM in a 50 mM HEPES buffer, pH 7.5 containing 150 mM NaCl and 5 mM $CaCl_2$ and assay was initiated by adding 2 μM of substrate. The rate of substrate hydrolysis was linear up to 30 min at 22 °C and reaction was stopped by adding 10 mM EDTA. The rate of the reaction was measured in triplicate for each EA or EA-KFK concentration examined, using Perkin-Elmer

LS50B spectrofluorometer with excitation and emission wavelengths of 325 and 375 nm, respectively. Less than 5% of the substrate was hydrolyzed during the rate measurements. IC_{50} (μM) were determined by plotting V_i/V_o, where V_i is the rate of substrate hydrolysis in the presence of inhibitor, V_o is the rate in its absence, as a function of EA or EA-KFK concentrations and non linear regression analysis using the Grafit 4 computer program (R. Leatherbarrow, Erithacus, Software).

2.8.2. Ex vivo assays

Assays were performed using tissue sections as described [11]. A set of three foreskins sections (8 μm) was laid on polylysine-coated microscopy slide (Biorad) and overlaid with 10 μl of 50 mM Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$ pH 7.5 containing 50 pM of APMA-activated MMP-2 or MMP-9. Each enzyme was active site-titrated using a standard preparation of TIMP-2 [33].The preparations were then incubated for 4 or 18 h at 37 °C in a moist chamber, rinsed thoroughly with Tris buffer and stained either with catechin/fuscin for elastin fibers revelation or with sirius red for identifying collagen fibers. For inhibition assays, either MMPs or skin tissue sections were preincubated with various concentrations of elaidyl-KFK. The microscopic slides were observed under a Zeiss standard 14 microscope equipped with a CF 126 PHR video camera and computerized morphometric analyses of elastin and collagen fibers were carried out. Our program determined the area fraction (A_A) occupied by the elastin and collagen fibers and represented the surface of fibers as a function of the tissue area analyzed. For elastin fibers, the volume fractions (V_V) was also quantified; V_V is equal to a $A_A \times k$ where k represented the Weibel correction factor, i.e. $k = d/(d + t)$, (d is the diameter of the elastin fibers (μm) and t is the section of thickness (μm)). The average diameter of the elastin fibers was determined semi-automatically using skin tissue sections treated or not with enzymes, in presence or absence of inhibitor and a calibrated slide.

2.9. Statistical analysis

Results were presented as means ± S.E. Experimental results were statistically analyzed using Student's t-test. P values <0.02 were regarded as indicating significant differences.

3. Results

3.1. KFK and elaidyl-derivative activate LAP-TGF-β1, in vitro

EA was chosen as the fatty acid moiety of our bifunctional molecule in keeping with its potent inhibitory capacity towards gelatinases [27]. It also presented the advantage, contrary to its *cis* counterpart, not to influence to any significant extent the basal expression of collagenase-1 or stromelysin-1 by dermal fibroblasts in culture [34]. It was initially attempted to couple EA to RFK, a TSP-1 tripeptide motif, reported to activate, both in vitro and ex vivo, LAP-TGF-$\beta_{1,2}$ [26]. Synthesis of elaidyl-RFK appeared rather difficult, due to problems encountered in the deprotection of the arginine residue. Elaidyl-KFK was instead synthesized since KFK tripeptide was shown to activate the small form of LAP-TGF-β to similar extent as RFK peptide. KQK and elaidyl-KQK were synthesized as control peptide and control lipopeptide respectively as Phe_{414} in TSP-1 was found essential for LAP-TGF activation [25,26].

To evaluate whether elaidyl-KFK could interact with LAP-TGF-β1, similarly as its peptide counterpart [25], surface plasmon resonance studies were initially performed with chip-immobilized fatty acyl-peptide and injected LAP-TGF-β1. Interaction was specific with K_D (M) values equal to $0.94 \pm 0.35 \times 10^{-9}$.

To determine whether elaidyl-KFK binding was associated with LAP-TGF-β1 activation, we used an in vitro "template device" where LAP-TGF-β1 was immobilized on plastic wells coated with LAP monoclonal antibodies. Released active TGF-β from the surface was measured without acid or heat treatment in the quantitative assay. Fatty acyl-peptide derivative mediated active TGF-β release in a time and concentration manner, in such an assay system (Fig. 1). A 2 μM minimal concentration of compound was required for detecting active TGF-β, a reaction that occurred as early as following 15 min incubation. Whatever lipopeptide concentration, amounts of liberated active growth factor leveled of following 4 h incubation, and steady state levels were attained at 10 h.

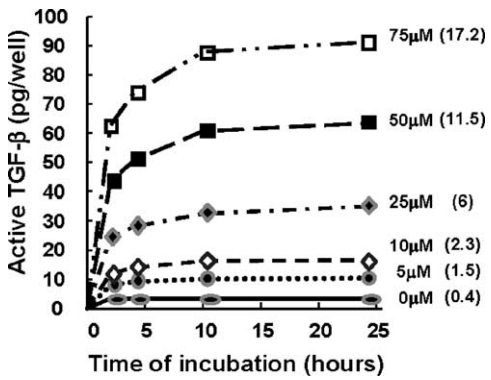

Fig. 1. Kinetics of LAP-TGF-β1 activation by elaidyl-KFK in a solid phase assay. Ninety-six wells-containing plastic plates were coated with anti-LAP antibody (400 ng/well) that allowed LAP-TGF-β1 immobilization (2.2 ng), corresponding to 524 pg of mature TGF-β. Plates were further incubated with increasing concentrations of elaidyl-KFK (0–75 μM) in PBS, pH 7.4, at 37 C°, for various periods. Concentration of active TGF-β1 was determined by ELISA using the TGF-β1 Emax™ immunoassay. Values under brackets represent the percentage of total activable mature TGF-β1; it refers to (active TGF-β1 × 100)/total mature TGF-β1.

Table 1
In vitro capacity of several tripeptides and their elaidyl counterparts to activate LAP-TGF-β1

	Active TGF-β1 (pg)	Percent of total activable mature TGF-β1 (%)
Control	10 ± 0.1	0.8
EA	38 ± 8.4	3
KFK	140 ± 8.3	11.2
KQK	18 ± 0.3	1.4
EA-KFK	150 ± 11	12
EA-KQK	20 ± 0.2	1.6
HCl	660	52.8

Ninety-six wells-containing plastic dishes were coated with anti LAP antibody (800 ng/well) allowing immobilization of 5.25 ng LAP-TGF-β1 that correspond to 1.25 ng mature TGF-β1. Plates were further incubated with 75 μM EA, KFK, KQK or EA-KQK in PBS, pH 7.4, for 24 h at 37 °C. Amount of released active TGF-β1 was determined by the TGF-β1 Emax™ immunoassay as described in Section 2. Level of acid-activable latent TGF-β1 was taken as reference.

The highest concentration of elaidyl-KFK used: 75 μM, i.e. 7.5 nmol could activate under our experimental conditions, 3.6×10^{-3} pmol of LAP-TGF-β representing 17.2% of total activable mature TGF-β and 32.5% of acid-activable latent growth factor. We then evaluated the specificity of this activation process and compared, using such an assay system, the ability of free fatty acid, peptides and lipopeptides to activate LAP-TGF-β. Data from Table 1 indicates that EA, KQK as well as elaidyl-KQK were inactive. On the contrary, KFK and elaidyl-KFK generated 140 and 150 pg of active TGF-β1, respectively. Those quantities corresponded to 11.2 and 12% of total activable mature TGF-β. For the sake of comparison, HCl treatment of 5.25 ng small LAP-TGF-β1 led to the liberation of 660 pg of active TGF-β representing 52.8% of total activable mature TGF-β. the ability of elaidyl-KFK to activate LAP-TGF-β in solution was very low and did not exceed 1% of total activable mature TGF-β.

3.2. KFK and elaidyl derivative activate LAP-TGF-β1 in fibroblasts cultures and stimulate collagen synthesis

Above depicted experiments indicated that elaidyl-KFK via its peptide moiety was able to activate LAP-TGF-β1, in vitro. To investigate whether this lipopeptide could reproduce such an effect, ex vivo, either elaidyl-KFK or KFK were preincubated with LAP-TGFβ1 and complexes were added to human dermal fibroblasts at subconfluency. Lipopeptide was used at 10–20 μM concentration. Cells were maintained for 24 h in serum-deprived DMEM, at which time conditioned medium was analyzed for TGF-β1 activity using TGF-β1 Emax™ immunoassay. Fig. 2A indicates that KFK (10 μM) and elaidyl-KFK (10 μM) but not elaidyl-KQK (10 μM) can induce LAP-TGF-β activation at levels similar to that attained in an in vitro solid phase assay. Again, EA and several scrambled peptides (KQK, KQFK) had no significant influence on LAP-TGF-β1 activation (not shown). Up regulation of collagen production by TGF-β1 in fibroblasts cultures is well documented [15]. We thus verified whether KFK or elaidyl-KFK-mediated LAP-TGF-β activation in fibroblasts cultures could led to increased collagen anabolism. Fig. 2B shows that KFK as well as elaidyl-KFK could significantly enhanced collagen biosynthesis as determined either as soluble secreted or fibroblasts-associated forms.

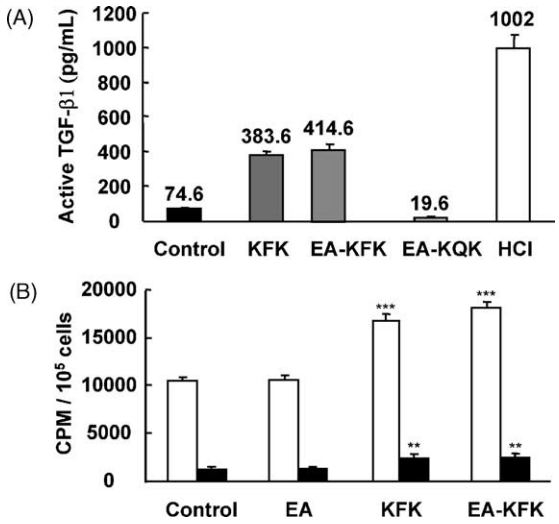

Fig. 2. Influence of EA, KFK and elaidyl-KFK on LAP-TGF-β1 activation (A) and collagen synthesis (B) by human dermal fibroblasts in culture. (A) Conditioned media from human dermal fibroblast cultures were supplemented with LAP-TGF-β1 (10 ng/well) and without or with KFK, elaidyl-KFK, elaidyl-KQK each at 10 μM concentration. Active TGF-β1 was determined in conditioned medium by the TGF-β1 Emax™ immunoassay, following 24 h of culture. Level of acid activable LAP-TGF-β1 was taken as reference. (B) Similarly, collagen synthesis is quantitated in conditioned medium (□) and cell extracts (■) according to Peterkofsky and Diegelmann [30]. Bars represented standard error of the mean; statistical significance is appreciated by the Student–Fisher t test. $^{**}P < 0.02$; $^{***}P < 0.01$.

3.3. Elaidyl-KFK-mediated activation of LAP-TGF-β1 regulates MMPs and TIMPs expression in fibroblast cultures

As reported in the literature, active TGF-β1 had little influence on basal levels of MMP-1 and MMP-3 production by dermal fibroblasts while repressing the PMA-induced level of those enzymes [35,36]; that is documented in Fig. 3 for MMP-1. PMA-induced MMP-1 and MMP-3 expression could be significantly inhibited by supplementing cell culture medium with active TGF-β1 (2 ng/well) or with LAP-TGF-β1 (10 ng/well) in the presence of 10 μM of elaidyl-KFK (Fig. 3B). To verify that elaidyl-KFK influence on MMP production was attributed to LAP-TGF-β1 activation, lipopeptide and LAP-TGF-β1-treated cultures were supplemented with blocking anti-TGF-β1 activity antibody. In such conditions, level of MMP production was similar to that determined in the culture medium of PMA-treated cells. Elaidyl-KFK (10 μM) and anti-TGF-β1 antibody (5 μg/ml) had no influence on the amount of enzyme secreted by PMA-stimulated

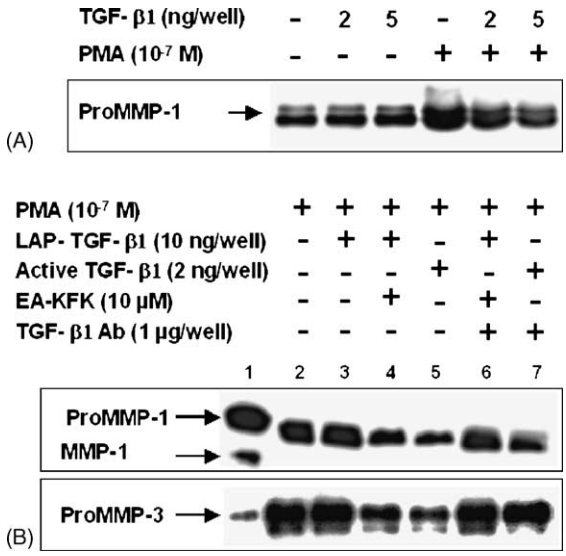

Fig. 3. Elaidyl-KFK-mediated activation of LAP-TGF-β1 down-regulates PMA-induced expression of collagenase-1 (MMP-1) and stromelysin-1 (MMP-3). (A) Influence of active TGF-β1 on the production of basal and PMA-stimulated levels of MMP-1 by human dermal fibroblasts in culture. Untreated or PMA-treated (10^{-7} M) human dermal fibroblasts were incubated in the presence of 2 or 5 ng/well of active TGF-β1 for 24 h. Production of MMP-1 is determined by western-blot from concentrated aliquots of culture medium. (B) PMA (10^{-7} M)-treated human dermal fibroblasts are incubated in the presence of LAP-TGF-β1 (lanes 3, 4 and 6), active TGF-β1 (lane 5), elaidyl-KFK (lanes 4, 6 and 7) or active TGF-β1 blocking antibody (lanes 6 and 7). MMP-1 and MMP-3 production are determined by western-blotting. Lane 1 corresponds to standards of proMMP-1, MMP-1 and proMMP-3. Their respective position is indicated by arrows.

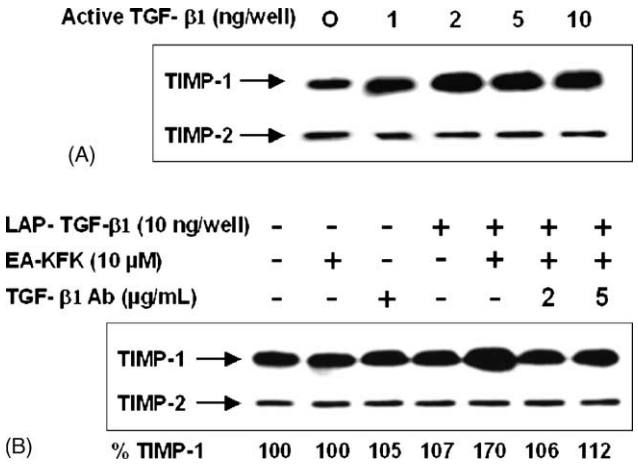

Fig. 4. Activation of LAP-TGF-β1 by elaidyl-KFK up-regulates the basal level of TIMP-1 expression by human dermal fibroblasts in culture. (A) Active TGF-β1 increases the production of TIMP-1, but not TIMP-2, by dermal fibroblasts as assessed by gelatin reverse zymography. (B) Fibroblasts were cultured in the presence of LAP-TGF-β1 (10 ng/well) and in the absence or presence of elaidyl-KFK and active TGF-β1 blocking antibody; TIMPs secretion are visualized by gelatin reverse zymography, following 24 h of culture. TIMP-1 was quantified by an automated image analyzer (Vuilbert-Lourmat) and expressed as percentage relative to control.

fibroblasts (not shown). The enhancing effect of active TGF-β1 on basal expression of TIMP-1 [18], but not TIMP-2 [37], by dermal fibroblasts was confirmed (Fig. 4A). TIMP-1 raises a plateau at a concentration of 2 ng/well TGF-β, while latent growth factor was ineffective (Fig. 4B). Supplementing LAP-TGF-β1 culture medium with 10 μM elaidyl-KFK reproduced active TGF-β1-induced up regulation of TIMP-1 with a 1.7-fold increase, an effect that could be nearly suppressed by TGF-β1 antibodies with an inhibition value of 91% in the presence of 2 μg/ml TGF-β1 antibodies (Fig. 4B). It indicated that fatty acid peptide influence on TIMP-1 production was mediated through its ability to activate LAP-TGF-β1. Interestingly, basal levels of TIMP-1 expression was also reduced in presence of the highest TGF-β1 antibody concentration used, suggesting that TIMP-1 production could be partly controlled by TGF-β1 expression in dermal fibroblasts.

3.4. Potent inhibition of matrix metalloprotases by elaidyl-KFK

In vitro experiments: we studied the contribution of the tripeptide moiety on the MMP inhibitory capacity of the fatty acyl peptide derivative. To that end, the kinetics of inhibition (IC_{50} values) of gelatinases (MMP-2 and MMP-9), collagenase-1 (MMP-1) and stromelysin-1 (MMP-3) by EA and elaidyl-KFK were compared using Mca-Pro-Leu-Gly-Leu(-Dpa)-Leu-Ala-Arg synthetic peptide (Table 2). As previously reported, EA inhibited gelatinases to a greater extent as compared to other MMPs, as here MMP-1 or MMP-3, due to its preferential binding mode to MMP-2 and MMP-9 fibronectin-like domains, not found in other members of this enzyme family [27]. Strikingly, elaidyl-KFK inhibited 3.2-, 4.1- and 77-fold more potently MMP-2, MMP-9 and MMP-3, respectively as compared to EA (Table 2).

3.4.1. Ex vivo experiments

The influence of elaidyl-KFK on the activity of MMP-2 and MMP-9 was then explored ex vivo using skin tissue section as substrate [11]. Although both MMPs were

Table 2
MMP inhibitory capacity of EA and elaidyl-KFK

	IC_{50} (μM)			
	MMP-2	MMP-9	MMP-3	MMP-1
Elaidic-acid	3.8 ± 0.3	4.1 ± 0.7	18.5 ± 4.0	>25
EA-KFK	1.2 ± 0.3	1.0 ± 0.4	0.24 ± 0.17	8.9 ± 1.7

The influence of EA and elaidyl-KFK on recombinant MMP-2, MMP-3 and MMP-9 activity was determined using the fluorescent quenched substrate Mca-Pro-Leu-Gly-Leu(-Dpa)-Leu-Ala-Arg. Two hundred picomolar of MMP-2 or MMP-9, 400 pM of MMP-3 or 3 nM of MMP-1 were mixed with increasing concentrations of EA or elaidyl-KFK up to 20 or 50 μM in a 50 mM HEPES buffer; assay was initiated by adding 2 mM of substrate. The rate of the reaction was measured in triplicate for each EA or EA-KFK concentration examined, using spectrofluorometer with excitation and emission wavelengths of 325 and 375 nm, respectively. IC_{50} were determined by plotting V_i/V_o as a function of EA or EA-KFK concentrations and non linear regression analysis using the Grafit 4 software.

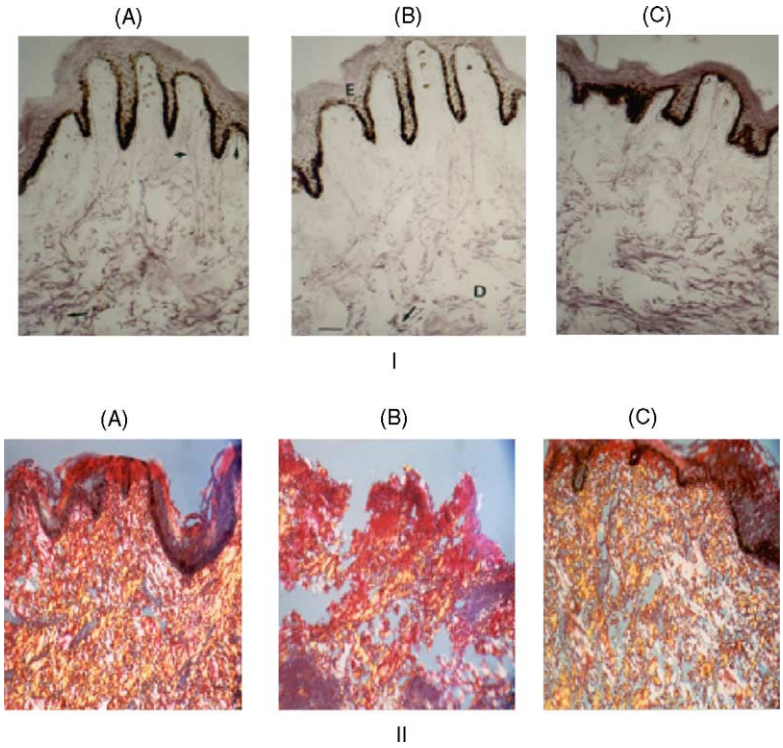

Fig. 5. Ex vivo protection of elaidyl-KFK (10 μM) on MMP-9 induced elastolysis (I) and MMP-2-induced collagenolysis (II). Elastin and collagen fibers in skin tissue sections (8 μm) are revealed by catechin-fuscin and Sirius red stains, respectively following 4 h of incubation at 37 °C with TBS alone or TBS-containing MMPs. I: Elastin fibers: (A) control skin; (B) skin section treated with 50 nM active MMP-9; (C) skin section treated with 50 nM active MMP-9 and 10 μM EA-KFK. II: Collagen fibers: (A) control skin; (B) skin section treated with 50 nM active MMP-2; (C) skin section treated with 50 nM active MMP-2 and 10 μM EA-KFK.

described to display, in vitro, both collagenolytic and elastinolytic activities [9,10], they appeared to have a more restricted pattern of action in our ex vivo assay. Consistent with our previous report [11] MMP-2 was found to degrade preferentially dermal collagen fibers while MMP-9, lysed essentially elastin fibers (Fig. 5). The inhibition of MMP-2 and MMP-9-mediated degradation of collagen fibers and elastin fibers, respectively, by elaidyl-KFK could be demonstrated using this ex vivo system (Fig. 5). Data were analyzed by computerized morphometry and ex vivo inhibition of gelatinases was compared to the in vitro data (Fig. 6). Extent of MMP-2 inhibition similar, whatever substrates (synthetic ●; collagen fibers △); and effector (EA (C); EA-KFK (D)) was similar. On the contrary, a more potent influence of free fatty acid (A) and elaidyl-KFK (B) on the ex vivo activity of MMP-9 towards elastin fibers (□) could be evidenced.

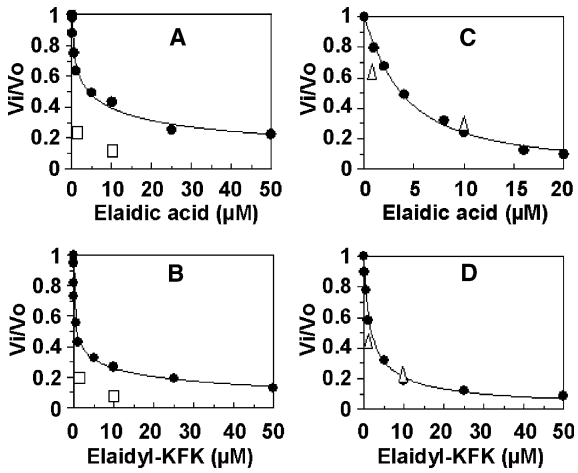

Fig. 6. Comparison between the in vitro and ex vivo MMP inhibitory capacity of EA and elaidyl-KFK. (A and B) MMP-9: in vitro (●) and ex vivo (□) MMP-9 inhibitory capacity was determined using MCA-Pro-Leu-Gly-Leu(-Dpa)-Leu-Ala-Arg (●) and elastin fibers substrates (□), respectively, (C and D): MMP-2: in vitro (●) and ex vivo (△) MMP-2 inhibitory capacities were determined as for MMP-9 using synthetic substrate (●) and collagen fibers substrates (△), respectively. For ex vivo assay MMP inhibitory capacity was defined as: 1 − (volume (elastin fibers) fraction or surface (collagen fibers) area occupied following enzyme action) × 100/(initial volume fraction or surface area).

4. Discussion

The aim of our investigation was to engineer a model compound that might display regenerating tissue activity (RGTA compound) [38]. We focused our interest to matrix metalloprotease clan and TGF-β1 since both families are widely described as pivotal elements in dictating tissue degradation and repair [8,20]. Their respective contribution is particularly evident in dermal homeostasis since aging-associated alteration of fibrillar structures was commonly attributed to increased MMP activity [5,12–14] coupled to imperfect repair possibly due to impaired LAP-TGF-β1 activation [39]. Thus, our basic approach

consisted in designing a molecule exhibiting both properties, i.e. activating LAP-TGF-β1 while inhibiting MMPs.

TSP-1-mediated activation of LAP-TGF-β1 was found to involve a unique RFK sequence located between the first and the second type 1 repeat of TSP-1 [25,26,40,41] which binds to a LSKL sequence within LAP molecule [25,26]. Such interaction was proposed to induce a conformational change in LAP-TGF-β1 molecule revealing an hidden epitope in the active domain of the growth factor [26]. The presence of the central phenylalanine residue is crucial but the first arginine residue could be substituted by a lysine residue, so that KFK tripeptide reproduced the effect of RFK [26]. LAP-TGF-β1 activation experiments as reported by Murphy-Ullrich and colleagues [25,26,42,43] were performed using rat kidney cells colony formation in soft agar assay and reacting entities (LAP-TGF-β1, TSP1 or peptides) in solution. In such conditions, LAP-TGF-β activation was fast, occurring within 2 min of incubation and involved equimolecular concentration of LAP-TGF-β1 and either thrombospondin or KRFK peptide [26]. However, extent of LAP-TGF-β1 activation was low, never exceeding 0.4% of potentially activable LAP-TGF-β1 [26]. Similar level of activation was recently reported using different preparations of rec TSP-1 and rec LAP-TGF-β1 in test tube experiments [42]. We used, here, a different in vitro assay system, where LAP-TGF-β1 was immobilized onto LAP coated dishes and were able to reproduce the main structure-dependence of TSP-1 peptides involved in LAP-TGF-β1 activation. Nevertheless, the main characteristics of activation were quite distinct than previously reported ones since much higher concentration of peptide (KFK) or elaidyl KFK was required for inducing activation. At opposite, level of LAP-TGF-β activation was two orders of magnitude higher as compared to other models. Such difference could be accounted for by antibody-mediated conformational change in LAP subunit rendering LSKL domain more accessible to peptide or lipopeptide. This assay system might somewhat reproduce in vivo conditions where LTBP could maintain LAP-TGF-β in a more susceptible conformation for TSP-1 activation [43]. Alternatively, as already reported [44], LAP-TGF-β1 can bind to various integrins through its RGD sequence present in the amino terminal part of LAP; such interactions favor growth factor activation process by distinct mechanisms including spatially restricted conformational changes [45]. Thus, contrary to experiments performed in soluble phase, immobilization of LAP by antibodies could reproduce such a template mechanism catalyzing the rate of LAP-TGF-β1 activation by elaidyl-KFK.

We reported that the first FN-II module of MMP-2 served as a nucleating site to form oleic acid micromicelles [27], a property that derived the inhibitory capacity of such fatty acid towards this enzyme. Possibly, hydrophobic domains in LAP could similarly act as nucleating sites that might favor kinetics of LAP-TGF-β1 activation obtained and might explained the proportionality between the maximal amounts of TGF-β1 liberated and the amounts of lipopeptide added.

Human skin fibroblasts in culture were used as a living system to test the ex vivo ability of KFK peptide and lipopeptide to activate LAP-TGF-β1. Substantial amount of active TGF-β, representing 16.1 and 17.4% of potentially activatable latent growth factor, was recovered, following 24 h of culture, by supplementing cell culture medium with KFK or elaidyl-KFK, respectively. On the contrary, it was reported that no change in TGF-β associated biological activity could be observed in either mink lung epithelial cells or rat aorta smooth muscle cells cultures in the presence of TSP-1 or TSP-1-derived peptides [42]. It suggested that, in cell culture systems, cofactors might be necessary for effective TSP-1-mediated LAP-TGF-β1 activation. To that respect, we noted that dermal fibroblasts could form, following 12 days of culture in presence of 3 ng/ml of LAP-TGF-β1, an array of LTBP1-containing fibers. The formation of such structures was significantly enhanced when cells are grown in presence of KFK or elaidyl-KFK (unpublished histochemical observations).

Lipopeptide-induced LAP-TGF-β1 activation in dermal fibroblasts cultures, led to known biological activity exerted by this growth factor on this cell type. Elaidyl-KFK action resulted in increased collagen and TIMP-1 biosyntheses while inhibiting the PMA-induced MMP-1 and MMP-3 expression. Those effects were suppressed by a TGF-β blocking antibody demonstrating that lipopeptide action on the fibroblast anabolic–catabolic balance was essentially mediated through LAP-TGF-β activation. Interestingly, blocking of TGF-β1 activity considerably reduced TIMP-1 basal expression suggesting that inhibitor production is controlled, mainly in young and aged dermis through the regulation of LAP-TGF-β1 activators, as also previously reported [39].

EA was chosen as the TSP-1 like peptide partner, in keeping with our earlier data demonstrating the preferential binding mode of long-chain unsaturated fatty acids to Fn(II)-like domains of gelatinases [27]. That has been confirmed here, since such fatty acid could inhibit gelatinases with IC_{50} values 5- to >10-fold lower than those determined with MMP-3 and MMP-1, respectively. In our previous data [27], Dixon and Cornish-Bowden plots were consistent with oleic acid acting as a mixed competitive inhibitor against MMP-2, although, we did not here evaluate the mode of inhibition of elaidyl-KFK against MMPs. Although KFK itself did not exhibit any MMP inhibitory capacity, covalent coupling of this peptide to EA greatly improved the MMP inhibitory capacity of the fatty acid. Part of this enhancing influence could be attributed to a greater solubility of the lipopeptide decreasing its critical micelle concentration as compared to the free fatty acids [27]. Its high inhibitory potential towards MMP-3 ($IC_{50} = 2.4 \times 10^{-7}$ M) was unsuspected but it suggested that lysine binding sites at the vicinity of the enzyme active

site might participate to enzyme–lipopeptide interactions. Elaidyl-KFK, as well as free fatty acids, proved to act as potent ex vivo protecting agent against elastin fibers degradation by MMP-9. That extents previous investigations using distinct lipopeptides and emphasizes the pharmacological potential of such class of compounds as regulators of elastolysis catalyzed by elastase-type proteases [43,46]. Such a property is related to the high affinity of lipids [47] and fatty acids, particularly [43,46], for elastin; thus it might be suspected that within dermis, elastin fibers might represent the main docking site for elaidyl-KFK, providing a reservoir of potentially active sites for generation of mature TGF-β.

The in vitro and ex vivo bifunctionality of a model fatty acid peptide, we named ''Lipospondin,'' to simultaneously inhibit matrix metalloprotease and activate LAP-TGF-β1, was here demonstrated. Experiments at aims to evaluate its beneficial influence in skin aging or as a tissue regenerating agent are currently under progress.

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