Latency can be conferred to a variety of cytokines by fusion with latency associated peptide from TGF-β

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Abstract

Objectives: Targeting cytokines to sites of disease has clear advantages because it increases their therapeutic index. We designed fusion proteins of the latent associated peptide (LAP) derived from TGF-β with various cytokines via a matrix metalloproteinase (MMP) cleavage site. This design confers latency, increased half-life and targeting to sites of inflammation. The aim of this study is to determine whether this approach can be applied to cytokines of different molecular structures and sizes.

Methods: Mature cytokines cloned downstream of LAP and a MMP cleavage site were expressed in 293T cells and assessed for latency and biological activity by Western blotting and bioassay.

Results: We demonstrate here that fusion proteins of TGF-β, erythropoietin, IL-1ra, IL-10, IL-4, BMP-7, IGF1 and IL-17 were rendered latent by fusion to LAP, requiring cleavage to become active in respective bioassays. As further proof-of-principle, we also show that delivery of engineered TGF-β can inhibit experimental autoimmune encephalomyelitis and that this approach can be used to efficiently deliver cytokines to the brain and spinal cord in mice with this disease.

Conclusions: The latent cytokine approach can be successfully applied to a range of molecules, including cytokines of different molecular structure and mass, growth factors and a cytokine antagonist.

Key words: Bioassay, cytokines, Latency associated peptide, protein design, targeted delivery

1. Introduction

Most cytokines are produced in an active form, have a very short half-life, have to be administered frequently at high doses, and the widespread expression of their receptors in many tissues contributes to their untargeted pleiotropic effects. However, contrary to most cytokines, transforming growth factor (TGF)-β is secreted as a latent, inactive cytokine [1].

TGF-β is produced as a long polypeptide with a precursor protein (LAP or latency associated peptide) at the amino terminal end, which is cleaved intracellularly from TGF-β by furin. Both LAP and TGF-β independently dimerise and LAP provides a 'shell-like' structure around TGF-β which is non-covalently maintained within this shell [2]. Cys 33 of LAP forms a disulphide bond with the latency TGF-β-binding protein (LTBP) enabling TGF-β deposition in the extracellular matrix [3,4].

To overcome the limitations of using most cytokines as therapeutics, we have developed a new method of delivery of cytokines to sites of disease by using the LAP of TGF-β. We have made fusion proteins between LAP and several therapeutic agents. We replaced the furin cleavage site in LAP with a collagenous matrix metalloproteinase (MMP) cleavage site and replaced TGF-β with IFN-β [5] or small anti-inflammatory peptides [6]. Metalloproteinases are tightly regulated enzymes involved in development, cell migration and are highly upregulated by pro-inflammatory cytokines and during tissue remodelling [7].

We have also mutated Cys 33 to Ser to avoid interactions of our fusion proteins with LTBP, a modification that did not affect secretion levels of LAP fusion proteins [6].

Previously, we showed that LAP-IFNβ was latent (inactive), but became active when released by cleavage with recombinant MMP-1 or MMP-3. Release of IFN-β biological activity could be detected by incubating LAP-IFN-β with synovial fluid from rheumatoid arthritis (RA) and osteoarthritis (OA) patients or by cerebrospinal fluid (CSF) from meningitis and multiple sclerosis (MS) patients but not by the serum, plasma or CSF from

non–inflammatory brain diseases [5]. We tested this delivery approach initially in a collagen-induced arthritis (CIA) model in DBA/1 mice and showed that plasmid intramuscular injection of LAP-IFN-β was more effective at ameliorating established arthritis than non-latent isoforms containing IFN-β at the amino terminal end of LAP (outside the shell) or as an open shell where the LAP was mutated at the double cysteine (to serine) residues 223 and 225 necessary to maintain the 'shell' as a closed structure. Latent IFN-β has an extended half-life compared to the free IFN-β (37 times longer) or pegylated-IFN-β (7 times longer) [5].

We have also expressed, as LAP fusions, the anti-inflammatory peptides vasoactive intestinal peptide (VIP) and α melanocortin-stimulating hormone (MSH). Both were therapeutic in CIA in DBA/1 mice when delivered by intramuscular DNA delivery after onset of disease compared with the empty LAP construct alone [6]. In addition, LAP-γ_3MSH recombinant protein was demonstrated to be more effective in inhibiting urate crystal-induced peritonitis at a 30 fold lower molar concentration than the free γ_3MSH peptide. LAP-VIP had a thousand fold longer half-life than free VIP [6].

Recently, we have also demonstrated that the original specificity of the cleavage site can be changed to that of an aggrecanase cleavage site that is more specific to arthritic disease [8]. Aggrecanase 1 and 2 (disintegrin-metalloproteinases with thrombospondin motifs; ADAMTS-4 and ADAMTS-5) are regulated by proinflammatory cytokines and are overexpressed in RA and OA. These enzymes cleave aggrecan which is responsible for resisting compressive forces in the joints, followed by essentially irreversible collagen degradation and cartilage destruction [7].

Using a fully mouse recombinant LAP-agg-IFN-β fusion protein we observed slower and more prolonged release with an aggrecanase cleavage site compared to MMP cleavage with synovial fluid of OA patients and improved therapeutic efficacy in CIA. A recombinant

protein without a cleavage site was not therapeutic demonstrating the importance of cleavage for efficacy. We also showed by bioluminescence imaging that the delivery is specific to inflamed tissue without effect in other tissues and this was also confirmed by quantitative RT-PCR of IFN-regulated genes and serum IP-10 concentrations (a serum biomarker of IFN activity). As expected, the half-life of these fusion proteins is reduced by a quarter in arthritic animals compared to naïve mice [8].

The principle of designing a latent cytokine and harnessing the pathological process i.e. the presence of matrix metalloproteinases for drug delivery is an important targeting mechanism, and its applications are broad including autoimmunity, cancer, atherosclerosis and tissue regeneration.

In this manuscript we show that the latent cytokine approach can be applied to a range of other molecules, as latency could be obtained by engineering other cytokines, growth factors and a cytokine antagonist (IL-1ra) that have various molecular configurations. Importantly, this delivery approach, by virtue of its targeting and latency properties, could contribute to the reassessment of therapeutic applications of cytokines that have failed clinical trials due to their unwanted pleiotropism and toxicities.

2. Materials and Methods

2.1 Construction and expression of latent cytokines with human LAP: The mature (without signal peptide) version of all cytokines and growth factors were cloned by PCR using Pfu-DNA polymerase (Promega, Southampton, UK) in frame between the Nco1 and Xba1 sites of the expression vector TGF-β GS-MMP-GS linker [5] in the plasmid vector pcDNA-3 (Invitrogen, Paisley, UK). Table 1 shows the primers used for each gene. All restriction enzymes were from New England BioLabs (Hitchin, UK). DNA sequences of all constructs were verified by sequencing at the Genome Centre, William Harvey Research

Institute. Plasmid DNA was purified from *E. coli* DH5α using Qiagen maxiprep kits (Crawley, UK) and was transiently transfected into 293T cells using the calcium phosphate co-precipitation method as previously described [9]. For biological assays, 293T cells were incubated in 10% heat inactivated FBS in DMEM (Invitrogen) (with 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine) and the supernatant collected 48 hours post-transfection. For Western blotting, the secreted proteins were collected in serum-free DMEM.

2.2 Construction, expression and purification of mouse LAP-IFN-β fusion recombinant proteins: These proteins were expressed and purified as previously reported [8].

2.3 Western blotting: Denaturing SDS-PAGE gels were run at 12% acrylamide with protein molecular weight markers from BioRad (Hemel Hampstead, UK). After electro-transfer onto a Hybond-P:PVDF membrane (GE Healthcare, Little Chalfont, UK). Blocking was carried out using 5 % dried skimmed milk (Marvel) in 0.1 % Tween-20 in PBS (PBST) for 1 hr. Primary antibody (1:200 to 1:1000) was applied for 1 hour at room temperature in the same blocking solution. Following 3 x 30 mins washes in 50 mls PBST, secondary antibody conjugated to HRP (1:1000) (GE Healthcare) was added for 30 mins. After a further 3 x 15 mins washes, the blots were developed using the enhanced chemiluminescence (ECL) reagents (GE Healthcare) and exposed to autoradiography using Hyperfilm (GE Healthcare). Films were developed using an AGFA Curix 60 developer (Gevaert, Germany). Primary antibodies used were as follows: anti-mIFN-β rat clone 7F-D3 (1:200) (Santa Cruz, Heidelberg, Germany); anti-mEPO rat IgG2a, clone 148438, at 0.2 µg/ml (R&D Systems); anti-mIL-4 rat clone BVD4-1D11 at a dilution of 1:100 (Becton Dickinson, Oxford, UK); anti-mIL-10 clone 500-P60 at a dilution of 1:100 (Peprotech, London, UK); anti-human TGF- β mouse clone TB21 at a dilution of 1:500 (Abcam, Cambridge, UK); anti-human LAP goat polyclonal antibody at a dilution 1:1000 from (R&D Systems); anti-human BMP-7 rabbit polyclonal antibody at 1:2500 (Abcam); Anti-IGF1 1:1000 (R&D Systems). Secondary

antibodies used were: rabbit anti-mouse F (ab)2-HRP at 0.5 μg/ml (Zymed, San Francisco, USA); mouse anti-goat-HRP at 1:1000 (Santa Cruz); goat anti-rabbit-HRP at 1:1000 (DAKO, Cambridge UK).

2.4 Assessment of latency: Supernatants from 293T transfected cells were incubated overnight at 37° C with or without recombinant MMP1 (at 30μM) produced in *E. coli* [10] kindly provided by Prof. H. Nagase, Kennedy Institute of Rheumatology, Imperial College, London).

2.4.1 IL-4 and IL-10 biological activity assays

The activity of mouse IL-4 and IL-10 in the 293T cell supernatants was determined by measuring the proliferation of CT6 lymphocytes [11] or the D-36 mast cells [12] respectively, using the CellTiter-Glo^{TM} Luminescent Cell Viability Assay (Promega, Southampton, UK). CT6 cells (starved of IL-2 for 48 hrs) or D-36 cells (starved of IL-4 and IL-3 for 16 hrs) were plated in 96-well plates at a density of 10^4 cells/well in 50 μl. After the addition of 50 μl of supernatant from transfected 293T cells or standards, the plates were incubated at 37° C at 5 % CO_2 for 72 hrs. For the measurement of cell viability, the plates were equilibrated at room temperature for 30 mins and an equal volume (100 μl) of the CellTiter-Glo^{TM} reagent was added. Following induction of cell lysis by repeated pipetting, the luminescent signal was allowed to stabilise by incubation at room temperature for 10 mins. The cells were transferred to opaque-walled plates and the luminescent signal was recorded using a MLX Microtiter® Plate Luminometer (Dynex Technologies Inc., Chantilly, VA, USA).

2.4.2 IL-1ra-mediated inhibition of IL-1-induced NFκB signalling assay

The HeLa 57A cell line that expresses the NF-κB driven firefly luciferase reporter [13] (kindly provided by Prof. R. Hay, University of Dundee, Scotland) was used for this assay. HeLa 57A cells ($1x10^4$)/96 well plate in triplicate were incubated with increasing dilutions of

supernatants from LAP-IL-1ra transfected 293T cells (treated or not with MMP1) for 2 hours and then stimulated for 4 hours with recombinant human IL-1β (10 ng/ml) (PeproTech, London, UK). Cells were lysed with reporter lysis buffer (Promega, Southampton, UK) and luminescence recorded using a Microtiter plate luminometer (Dynex Technologies).

2.4.3 TGF-β assay: Mink lung fibroblast cells expressing the firefly luciferase reporter gene driven by the plasminogen activator-1 promoter as described by Abe et al. [14] (kindly provided by D. Rifkin, New York University, USA) were cultured in DMEM medium supplemented with 10% heat inactivated FBS, 1% penicillin/streptomycin, 1% L-Glutamine and 250μg/ml Geneticin (1mg/ml, G-418 sulphate, Invitrogen, Paisley, UK). 1.6 x 10^4 cells were plated per well of a 96 well plate and allowed to attach for 3 hours. Duplicates of either recombinant human TGF-β (Peprotech) (ranging from 0 pg/ml to 350 pg/ml), or cell culture supernatants from LAP-TGF-β transfected 293T cells (100 μl/well) treated or not with MMP were diluted in medium containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich, Dorset, UK). The cell culture supernatant was then collected and treated with 1 volume 1N HCl to 4 volumes supernatant, incubated for 10 minutes at room temperature (to release TGF-β from the LAP) followed by neutralization (1 volume 1.2N NaOH/0.5M HEPES to 5 volumes supernatant).

The mink cells were treated for 14-16 hours, washed once with 1x PBS before the addition of 50μl of 1x Passive Lysis Buffer (Promega) and subsequent incubation for 15 minutes at room temperature on a platform rocker. The cells were then placed at -80°C for 30 minutes prior to thawing at room temperature for 10 minutes. The freeze/thaw procedure was repeated twice more, followed by titration before transferring 25μl of the cell lysate to assembly strips of white microtiter plates (Thermo Electron Corporation, Vantaa, Finland). The luminescence was read using the MLX Microtiter® Plate Luminometer following the addition of 50μl of the luciferase assay reagent (Promega).

2.4.4 IL-17 assay: The activity of LAP-IL-17 in 293T cell supernatants was determined by measuring the secretion of IL-6 from human foetal fibroblasts (HFF2) cells. HFF2 cells were plated in 96-well plates at a density of 10^4 cells/well in 50 μl. After the addition of 50 μl of supernatant from transfected 293T cells or standards, the plates were incubated at 37° C at 5 % CO_2 for 72 hrs. IL-6 concentration was measured in the HFF2 supernatants by IL-6 duoset ELISA (R&D Systems, Abingdon, UK).

2.4.5 EPO assay: The human megakaryoblastic cell line UT7 [15] was grown in RPMI and 10% FBS in the presence of GM-CSF (50 ng/ml). Prior to assaying EPO-dependent cell proliferation, cells were put in fresh medium without GM-CSF for 16-24 hrs, then plated in 96 well plates at a density of 2000 cells /well. 293T cell supernatants from LAP-EPO transfected cells, with or without MMP treatment, were also added to separate wells in doubling dilutions. After 72 hrs, cell proliferation was assessed using the CellTiter-Glo assay as described above. A standard curve for EPO was obtained by the addition of doubling dilutions of recombinant EPO (R&D Systems, Abingdon, UK) from 100 ng/ml.

2.5 ELISAs

2.5.1 EPO sandwich ELISA: 100μL of anti-mouse EPO rat IgG2a mAb at 5μg/ml (Clone 148438, R&D Systems) was used as capture antibody and 100μl of goat anti-mouse EPO (R&D Systems) for detection with an anti-goat HRP conjugated antibody used at 1μg/ml (Santa Cruz). Alternatively, EPO sandwich ELISA was performed using an ELISA kit (Quantikine mouse/rat immunoassay, R&D Systems). ELISA was carried out on serial dilutions of the supernatant from transiently transfected 293T cells in order to calculate EPO concentration in the linear range of dilutions.

2.5.2 IL-4 ELISA: 96-well microtitre ELISA plates (Nunc, Uxbridge, UK) were coated with 50 μl/well of rat monoclonal anti-mouse IL-4 Ab (BVD4-1D11) at 2 μg/ml in 0.5 M carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4 ^{0}C. After washing once

with PBS, the plates were blocked with 200 µl/well of 2 % casein (VWR, Poole, UK) in PBS for 1 hr. Following two washes in 0.05 % Tween-20 (VWR) in PBST, serial dilutions of standard mIL-4 (Peprotech), starting from 2 ng/ml, and concentrated supernatants from CHO cells were added and incubated in the plate for 3 hrs. After a further 5 washes in PBST, 50 µl/well of biotinylated rat anti-mouse IL-4 monoclonal Ab (BVD6-24G2), at a concentration of 0.5 µg/ml, was added and incubated for 1 hr. The plates were washed 5 times in PBST and 100 µl/well of streptavidin-HRP complex (GE Healthcare) (1:500 dilution) was added for 30 mins. Following 5 washes in PBST, the signal was detected using the TMB microwell substrate system (Kirkguard and Perry, Maryland, USA). The reaction was stopped by the addition of 4.5 M H_2SO_4 and absorbance measurements were performed at 450 nm using a Tecan plate reader as above.

2.5.3 IL-1ra ELISA: 96 well ELISA plates (Nunc) were coated with a mouse monoclonal anti-human IL-1ra antibody (MAB280; R&D Systems) at a concentration of 4 µg/ml diluted in PBS overnight at 4^o C. Plates were then washed with PBS and blocked with a 2% Marvel (Premier International Foods Ltd, Spalding, Lincs, UK) solution in PBS for an hour at room temperature. Plates were washed with PBS containing 0.05% Tween 20 (PBS/Tween) prior to incubation of standards (human IL-1ra; R&D Systems) 1 µg/ml to 1 pg/ml diluted in complete media and samples (culture medium) for 3 hours at room temperature. Plates were washed extensively with PBS/Tween before incubation with biotinylated goat anti-human IL-1ra (BAF280; R&D Systems) at a concentration of 200 ng/ml for 1 hour at room temperature. Plates were again washed with PBS/Tween and incubated with Streptavidin-HRP complex (GE Healthcare). Plates were again washed and the signal was detected using the TMB microwell substrate system and the reaction stopped by addition of 4M sulphuric acid (100 µl) and absorbance measurements were performed at 450 nm using a Tecan ELISA plate reader as above. The detection limit of this ELISA is 10 - 100 pg/ml.

2.6 Culture of chick embryo limb bud cells: fertilised eggs were incubated at 38°C for 3.5 days after which limb buds were microdissected under a microscope. Trypsin dissociated cells were electroporated with 2 μg of DNA expression vectors/$2x10^5$ cells. Alcian blue staining of 4 day stage 20/21 chick micromass cultures was performed as previously reported [16].

2.7 Mice: All animals used in this study were kept according to Institutional and approved Home Office guidelines. DBA/1 and Biozzi AB/H mice were purchased from Harlan Biologicals. Mx2 Luc BAC transgenic mice have a C57/BL6 genetic background and express firefly luciferase under the control of the IFN-responsive Mx2 promoter [17].

2.7.1 EAE in Biozzi AB/H mice: mice were immunised by subcutaneous injection of 1mg of spinal cord homogenate in complete Freund's adjuvant at days 0 and 7 [18]. Animals were injected i.p. on day 10 with a syngeneic fibroblast cell line ($1x10^6$ cells/mouse) which was transduced with lentiviral vectors expressing either LAP-MMP (LAP-MMP.tsF) or LAP-MMP-huTGF-β (LAP-MMP-huTGF-β.tsF) at 40 ng/ml/10^6 cells per 24 hrs. Mice were scored every day for a further 20 days [18].

2.7.2 EAE in Mx2 luc mice: EAE was induced using an EAE induction kit (Hooke Laboratories, Lawrence, MA, USA) according to the manufacturer's instructions. Animals with a clinical score of 3 were injected with 10 μg of recombinant LAP-agg-IFN-β, LAP-mmp-IFN-β or free IFN-β and sacrificed 24 h later. Tissues were dissected from treated mice, snap frozen and stored at -70°C. Serum levels of IP-10 were assessed as previously described [8].

2.8 Luciferase assay: Luciferase activity in various mouse tissues was determined using the Bright-Glo luciferase assay system (Promega, Southampton, UK). Frozen tissues were added to Glo-lysis buffer (Promega) and homogenized in CK28 Precellys homogenisation tubes (Stretton Scientific, Derbyshire, UK) using a Precellys homogenizer (Stretton Scientific).

Samples were then centrifuged (13,000 rpm for 5 min at 4°C) and the lysate collected. Protein concentrations of tissue lysates were determined using the BCA protein assay (Fisher Scientific) and luciferase activity was determined in 25µl aliquot of the sample containing 5µg of total protein, which was mixed with 25 µl of Bright-Glo luciferase assay substrate (Promega), and light emission measured using a MLX Microtiter® Plate Luminometer (Dynex Technologies Inc., Chantilly, VA, USA). Values for luciferase activity were expressed as relative light units per microgram of protein.

2.9 Quantitation of LAP fusion proteins by competitive ELISA: LAP fusion proteins were quantified by competition ELISA as described previously [19] using baculo virus expressed recombinant human LAP as competitor (Sigma –Aldrich).

2.10 Statistical analysis

Descriptive statistics and significant differences between groups were calculated using Mann-Whitney U-tests or one-way ANOVA in Graphpad.

3. Results

3.1 Fusion of LAP-MMP to TGFβ, IL-4, IL-10, IL-17A, BMP-7, EPO, IGF1 and IL-1ra produces latent molecules that upon cleavage with MMP become active

To investigate the applicability of conferring latency to cytokines and growth factors with different structures (dimers such as IL-10, TGF-β, IL-17A, BMP-7 or monomers such as IL-4, EPO, and IL-1ra) and different molecular weights we fused their mature sequence to our original LAP-MMP sequence [5] (fig. 1). Transient expression after 293T cell transfection showed that all proteins were expressed efficiently and were cleaved by recombinant MMP-1 (fig. 2A-H).

To assess their latency, supernatants were incubated in the presence or absence of MMP1 and then added at different dilutions to respective bioassays. Dose response curves were obtained for all proteins after cleavage. LAP-IL-10 increased proliferation of the D36 mast cells (fig. 3A), LAP-IL-4 increased proliferation of the CT6 T cell line (fig. 3B), LAP-IL-1ra inhibited IL-1-β–NFkB-driven luciferase expression in 57A HeLa cells (fig. 3C), LAP-TGF-β also required acid treatment for release from LAP, as expected, and induced luciferase activity from the PA-1 promoter in mink fibroblast cells (fig. 3D), LAP-IGF-1 induced oligodendrocyte CG4 cell proliferation (fig. 3E), LAP-EPO induced proliferation of the megakaryoblastic cell line UT-7 (fig. 3F), LAP-IL17A induced IL-6 secretion from human HFF2 fibroblasts (fig. 3G), LAP-BMP-7 induced luciferase activity from the ld1 promoter in C2C12 cells (not shown) and promoted chondrogenic differentiation (fig. 3H)..

In some of these constructs a $(GGGGS)_3$ linker was used instead of an MMP cleavable site as control. As shown with LAP-EPO this linker is not cleaved by MMP, nor is it active in the bioassay (fig. 2D and 3F).

Metalloproteinases are also active during tissue remodelling and differentiation. Electroporation of plasmids expressing LAP-TGF-β and LAP-BMP-7 into chicken limb bud mesenchymal micromass cultures showed that they facilitate chondrogenesis whilst expression of LAP-MMP alone or empty plasmid cannot (fig. 3H).

3.2 LAP fusion proteins are effective in treatment of EAE.

We have previously shown that plasmid DNA expressing native TGF-β delivered intracranially complexed in liposomes is therapeutic in an animal model of MS [18]. Here we assessed whether intraperitoneal injection of syngeneic fibroblasts engineered with a lentiviral vector to express the same amounts of LAP-TGF-β or LAP-MMP, 10 days post disease induction affected disease progression. Figure 4 shows that LAP-TGF-β but not

LAP-MMP expressing fibroblasts can significantly inhibit the development of the acute phase of the disease.

To investigate whether LAP can be used to deliver a therapeutic entity to the brain and spinal cord, we used the reporter mice that express firefly luciferase under the regulation of the IFN-inducible MX2 promoter [17]. Measurement of luciferase activity 24 hr after injection of latent IFN-β shows that IFN-β is efficiently released from LAP into the brains of mice with EAE (fig. 5A). Interestingly, this release is even more efficient with the aggrecanase cleavage site than with either LAP-MMP-IFN-β or free IFN-β in the spinal cords of EAE mice. Figure 5B shows that the IFN-β biomarker IP-10 is poorly induced in animals treated with LAP-IFN-β constructs in comparison to free IFN-β.

4. Discussion

Here we have described the versatility of our latent cytokine technology. We demonstrate that cytokines whose molecular structure differ substantially, for example EPO, IL-4 and IL-1ra are monomers; IL-10 and IL-17 are dimers; and TGF-β and BMP share similar dimeric folding and cysteine knot structure; could all become latent when cloned after the LAP and the MMP cleavage site. All cytokines could be detected within LAP by their respective antibodies in ELISA and western blotting.

The crystal structure of LAP with TGF-β at its centre has recently been elucidated and the strong interactions of TGF-β with LAP demonstrated [2]. Crucially, TGF-β within the LAP is inaccessible to its receptors (ibid).

Interestingly, BMP-7 is a member of the TGF-β family of growth factors that have a LAP that does not provide latency [20]. This may be due in part to the fact that the LAP of BMP-7 lacks the disulfide bonds provided by cysteines in positions 223 and 225 that dimerise LAP from TGF-β. The hydrophobic residues (leucine and isoleucine) in the α1 helix in the LAP of

TGF-β interacts strongly with tryptophan 279 and tryptophan 281 of TGF-β [2] stabilising the latent structure whilst the opposite face interacts with LTBP [21]. In BMP7, this outward face enables interaction with fibrillin.

We do not envisage that the 'shell' structure provided by LAP is fully closed, particularly with larger cytokines such as IFN-β and neither do we expect that LAP will strongly interact with any other cytokine except for its native TGF-β and some of its family members.

Importantly, secretion of latent cytokines or their latency does not require the presence of LTBP. We have previously demonstrated that overexpression of LAP-IFN-β in CHO cells after methotrexate gene amplification results in only a small proportion of the fusion molecules still interact with the endogenous single gene product of LTBP and the majority of the secreted molecules are LTBP-free and are still latent [5]. Secondly, we have mutated cysteine residue 33 to serine in LAP, preventing its interaction with LTBP, and the constructs tested are well secreted and latent [6,8, 22].

The extent of latency varies in different *in vitro* assays. Our collagenous MMP cleavable site is cleaved by many MMPs including MMP-1, -3, -7 and -13. Most cell lines in culture produce low levels of MMPs which may or may not be active. Addition of our recombinant active MMP-1 may also lead to activation of inactive metalloproteinases in certain culture supernatants. We have detected some gelatinases in many supernatants of cells by zymograms (unpublished). Incubation of some cells with non-specific MMP inhibitors lowers this background activity (fig. 3F).

The modularity of the latent cytokine design was recently exemplified by exchanging the MMP cleavage site for the aggrecanase cleavage site [8]. Importantly, this site is cleaved both by recombinant ADAMTS-4 and -5 and by synovial fluid from OA patients. Release with enzymes from pathological sites is important, showing its clinical applicability, because

most recombinant metalloenzymes are truncated and their substrate specificity may be slightly different than the full enzyme [10,23].

Finally we have also shown that LAP fusion proteins can be used to treat a mouse model of multiple sclerosis and efficiently deliver therapeutics to the brain and spinal cord without systemic side effects. Whether this entry through the blood brain barrier is facilitated by the presence of the RGD sequence in LAP and/or other mechanism(s) requires further investigation.

5. Conclusions

The data presented here clearly demonstrate the applicability of the latent cytokine technology to a number of therapeutic entities including cytokines of different structures and masses, a cytokine antagonist and growth factors. This broad applicability opens up the possibility that these biologically potent molecules may be harnessed for therapeutic use despite their pleiotropic effects and, in some cases, toxicities. This is an important development as there are many pathological conditions where there is an unmet clinical need for new and more targeted therapies, which may now be possible using this approach.

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Table 1. Deoxyoligonucleotide primers used for cloning

Cytokine	**Forward primer**	**Reverse primer**
mEPO	5’ gcgc/g gcc gca CTC TGT GCT CCC CCA CGC	ggt/ctaga cTCA CCT GTC CCC TCT CCT

huIL-1ra	5’gcgc/g gcc gca CGA CCC TCT GGG AGA AAA TCC	5’cct/ctaga g CTA CTC GTC CTC CTG GAA GTA GAA
huTGF-β	5’gcgc/g gcc gca GCC CTG GAC ACC AAC TAT TGC	5’ ggt/ctaga gTCA GCT GCA CTT GCA GGA GCG
mIL-4	5’gcgc/g gcc gca CAT ATC CAC GGA TGC GAC AAA	5’cct/ctaga TTA CGA GTA ATC CAT TTG CAT GAT
mIL-10	5’gcgc/g gcc gca AGC AGG GGC CAG TAC AGC CGG	5’cct/ctaga TCA GCT TTT CAT TTT GAT CAT CAT
huIL-17A	5’ gcgc/g gcc gca GGA ATC ACA ATC CCA CGA AAT	5’ ggt/ctaga TTA GGC CAC ATG GTG GAC AAT CGG
huBMP-7	5’gcgc/g gcc gca TCC ACG GGG AGC AAA CAG CGC	5’cct/ctaga CTA GTG GCA GCC ACA GGC CCG GAC
mIGF1	5’gcgc/g gcc gca GGA CCA GAG ACC CTT TGC GGG	5’cct/ctaga CTA CAT TCT GTA GGT CTT GTT TCC

Primers used for cloning mature cytokines into LAP-MMP. The Not1 and Xba1 sites used for cloning are shown in lower case (with external 2 nucleotides to allow enzymatic cleavage) and stop codons are underlined.

Figure Legends

Figure 1. Structure of latent cytokines. The latency-associated peptide (LAP) from TGF-β forms a shell around the therapeutic cytokine, rendering it biologically inactive. A cleavage site specific for matrix metalloproteinase (MMP) is engineered in between the LAP and the cytokine, enabling the release of the cytokine at the sites of disease. Reprinted from [24] with permission from the publisher.

Figure 2. LAP fusion proteins are expressed and secreted by 293T cells and can be cleaved with MMP1 to release the free cytokine/growth factor. Western blotting of (a) recombinant LAP-mmp-BMP-7 detected witn anti-BMP-7; (b) recombinant LAP-mmp-IL-10 deteced with anti-IL-10; (c) recombinant LAP-mmp-IL-4 deteced with anti-IL-4; (d) recombinant LAP-mmp-IL-EPO deteced with anti-IL-EPO; (f) recombinant LAP-mmp-IL-17 detected with anti-IL-17; (e,g and h) recombinant LAP-mmp-IL-1ra, LAP-mmp-TGF-β and LAP-mmp-IGF1 respectively detected with anti-LAP antibody. All blots indicate via the + or – sign the presence or absence of MMP1.

Figure 3. LAP fusion proteins are latent until activated by cleavage with MMP. Varying dilutions of supernatant from 293T cells transiently transfected with (a) LAP-IL-10 (with or without MMP treatment) were added to D36 cells (mast cell line). *$P \leq 0.05$ **or** (b) LAP-IL-4 (with or without MMP treatment) were added to CT6 cells. The effects of LAP-IL-4 on cell proliferation was measured (n=3) 72 h later (c) HeLa cells permanantly transfected with reporter gene NF-κB-luciferase were treated with IL-1β in the presence of varying concentrations of 293T supernatants containing LAP-IL-1ra (SEM values too small to be plotted). (d) Effect of LAP-TGF-β on mink lung fibroblast cells expressing PA-1 promoter driven luciferase. ***P = 0.0001. (e) Supernatants from transiently transfected 293T cells expressing LAP-IGF1 were added to CG4 cells. ** p=0.001 by paired t-test (f) Proliferation

of the megakaryoblastic cell line UT-7 was measured after incubation with supernatants from 293T cells transiently transfected with LAP-EPO. Inhibition of MMP by TAPI-2 prevents background EPO release (left panel) (g) Human foetal fibroblasts cells were stimulated for 24 hours with 293T cell-supernatants transiently transfected to express human full-length IL-17A or LAP-IL-17A treated or not with MMP1. * p = 0.0002, ** p =0.000001. (h) LAP-TGF-β and LAP BMP-7 promote chondrogenesis *in vitro* in chick embryo limb bud cells. Alcian blue staining of 4 day stage 20/21 chick micromass cultures electroporated with plasmids encoding LAP-MMP (panel A), LAP-BMP-7 (panel B) or LAP-TGF-β (panel C); (panel D) Quantification of the alcian blue staining including that of a control pcDNA3 expression vector. (p=0.005 and 0.001 respectively).

Figure 4. LAP-TGF-β expressed from syngeneic fibroblasts ameliorates EAE. Biozzi mice were immunised by subcutaneous injection of spinal cord homogenate in complete Freund's adjuvant at days 0 and 7. Animals were injected i.p. on day 10 (arrow) with a syngeneic fibroblast cell line ($1x10^6$ cells/mouse) which was transduced with lentiviral vectors expressing either LAP-MMP (LAP-MMP.tsF) or LAP-MMP-huTGF-β (LAP-MMP-huTGF-β.tsF). The results represent the mean clinical disease score ± SEM of all animals within the group (n=8). (**P ≤ 0.005 compared to LAP-MMP.tsF injected mice.

Figure 5. LAP-IFN-β is delivered to the brains and spinal cords of mice with EAE with few systemic effects. (a) Luciferase activity was determined in homogenised organs from mice (*n*=3) with EAE (clinical score of 3) 24 h after injection with saline, or 2.5 $x10^5$U of free IFN-β, LAP-mmp-IFN-β or LAP-agg-IFN-β. (*p≤ 0.005) (b) Systemic effects were also analysed by measurement of serum IP-10 levels taken 24 hours after injection of recombinant proteins (*p≤0.005).