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ORIGINAL ARTICLE

Gene therapy for long-term restoration of circulating thymulin in thymectomized mice and rats

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Thymulin is a thymic peptide possessing hypophysiotropic activity and antiinflammatory effects in the brain. We constructed a synthetic DNA sequence encoding met-FTS, a biologically active analog of thymulin, and subsequently cloned it into different expression vectors. A sequence optimized for expression of met-FTS in rodents, 5′-ATGCAGGCCAAGTCGCAGGGGGGGTCGAACTAGTAG-3′, was cloned in the mammalian expression vectors pCDNA3.1(+) and phMGFP (which expresses the Monster Green Fluorescent Protein), thus obtaining pcDNA3.1-metFTS and p-metFTS-hMGFP, which express met-FTS and the fluorescent fusion protein metFTS-hMGFP, respectively. The synthetic sequence was also used to construct the adenoviral vector RAd-metFTS, which expresses met-FTS. Transfection of HEK293 and BHK cells with pcDNA3.1-metFTS (experimental groups) or pcDNA3.1 (control), led to high levels of thymulin bioactivity (>600 versus <0.1 pg/ml in experimental and control supernatants, respectively). Transfection of HEK293 and BHK cells with pmetFTS-hMGFP revealed a cytoplasmic and nuclear distribution of the fluorescent fusion protein. A single intramuscular (i.m.) injection (10^7 plaque forming units (PFU)/mouse or 10^8 PFU/rat) of RAd-metFTS in thymectomized animals (nondetectable serum thymulin) restored serum thymulin levels for at least 110 and 130 days post-injection in mice and rats, respectively. We conclude that RAd-metFTS constitutes a suitable biotechnological tool for the implementation of thymulin gene therapy in animal models of chronic brain inflammation.
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Keywords: *thymulin; synthetic gene; adenoviral vector; thymectomy*

Introduction

Thymulin is a thymic hormone involved in several aspects of intra- and extrathymic T-cell differentiation.[1,2] Thymulin, which is exclusively produced by the thymic epithelial cells (TEC), consists of a nonapeptide component (termed thymus serum factor or FTS), coupled in an equimolecular ratio to the ion zinc,[3] which confers biological activity to this molecule.[4] The metallopeptide active form bears a specific molecular conformation that has been evidenced by nuclear magnetic resonance.[5]

There is increasing evidence that, besides its immunomodulatory actions, thymulin possesses hypophysiotropic activity.[6] More recent studies point to an antinflammatory and analgesic activity of thymulin and thymulin analogs in the brain, which makes this peptide a molecule of clinical interest.[7,8]

Unfortunately, efforts to clone the gene for thymulin have been unrewarding so far. This has prevented the assessment of thymulin gene therapy strategies in relevant animal models.[9] This situation led us to undertake the design and construction of a synthetic DNA sequence encoding a biologically active analog of thymulin. We report here, the successful construction of such synthetic DNA sequence and the subsequent construction of an adenoviral vector which, when injected intramuscularly (i.m.) in thymectomized mice and rats, induced long-term expression of high circulating levels of thymulin.

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Results

Design of a DNA sequence expressing met-FTS

An open reading frame (ORF) encoding the peptide component of FTS was designed by choosing for each amino acid, the codon which is most frequently used in rat cells.[10,11] An ATG starting codon and two TAG termination codons were added immediately upstream and downstream of the FTS coding sequence, respectively (Figure 1a). When cloned into appropriate mammalian expression vectors, the peptide expressed by this sequence (here referred to as the met-FTS gene) was met-FTS (Figure 1a). The insertion of two contiguous termination codons at the end of the FTS coding sequence assured efficient termination of the translation process.

Cloning of the met-FTS synthetic gene in pcDNA3.1(+)

In order to clone the met-FTS gene into the multiple cloning site (MCS) of pcDNA3.1(+), a variant (variant #1) of the basic sequence, consisting of the met-FTS gene

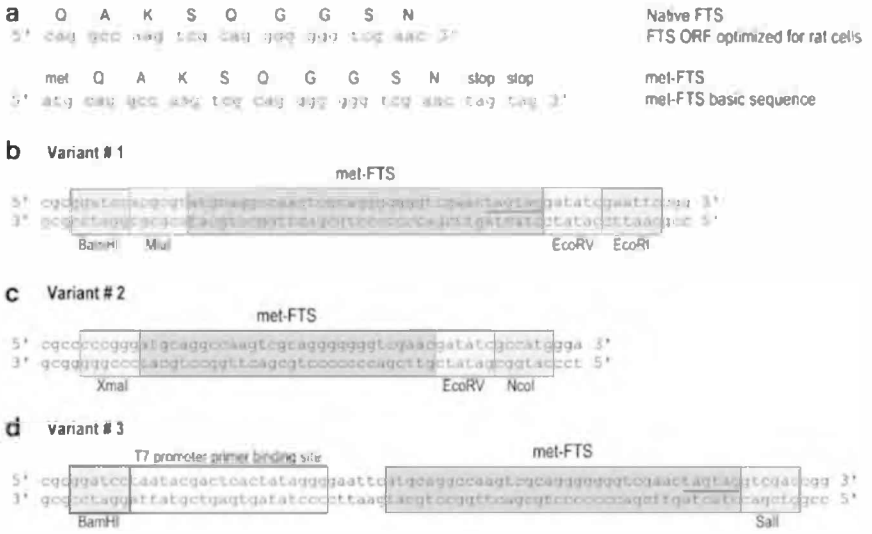

Figure 1 DNA constructs encoding met-FTS. A DNA sequence coding for native FTS was designed for optimal expression in rat cells. By adding an ATG starting codon upstream and two stop codons downstream this sequence, respectively, it was converted into an ORF for the analog met-FTS (**a**). In turn, this met-FTS ORF was used to generate appropriate synthetic constructs to be cloned in the expression vectors pCDNA3.1(+) (**b**), phMGFP (**c**), and pD515 (**d**). For further technical details see the M&M section.

flanked upstream, by a *Bam*HI and *Mlu*I site and downstream, by an *Eco*RV and *Eco*RI site (Figure 1b), was prepared by annealing of the corresponding sense and antisense DNA strands (see Materials and methods (M&M) for technical details). In order to identify the more efficient construction method, the same double-stranded (ds) DNA was prepared by polymerase chain reaction (PCR) using the sense DNA strand as template and appropriate primers (see M&M section). As expected, both methods generated identical ds oligonucleotides with the annealing method being simpler and the PCR variant having a higher yield. In subsequent preparations, only the annealing method was used. The dsDNA obtained by annealing was digested with *Bam*HI and *Eco*RI and ligated into the *Bam*HI and *Eco*RI sites of the MCS of pcDNA3.1(+), which yielded pcDNA3.1-metFTS, a met-FTS expressing vector (Figure 2a).

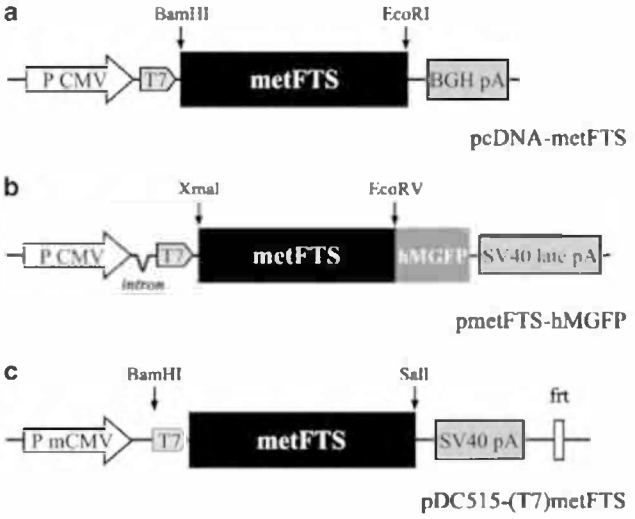

Figure 2 Mammalian viral and nonviral expression vectors harboring the synthetic gene for met-FTS. pcDNA-metFTS was generated by cloning the synthetic met-FTS gene into the *Bam*HI and *Eco*RI sites of the MCS of pcDNA3.1(+) (**a**). pmetFTS-hMGFP, was generated by inserting the met-FTS gene (lacking the two stop codons) into the *Xma*I and *Eco*RV sites of the MCS of phMGFP. This insertion left the met-FTS sequence in frame with the gene for hMGFP, so that a metFTS-hMGFP could be expressed (**b**). pDC515-metFTS was generated by inserting the T7-metFTS sequence into the *Bam*HI and *Sal*I sites of the MCS of the shuttle pDC515 (**c**).

Biological activity and immunoreactivity of transgenic met-FTS

In vitro *tests.* Baby Hamster Kidney (BHK) and Human Embryo Kidney (HEK) 293 cells transfected with pcDNA-metFTS, but not those transfected with empty pcDNA3.1, showed a high level of expression of biologically active and immunoreactive thymulin (Figure 3). Although thymulin concentration was much lower in the cell supernatants than in the corresponding lysates, transgenic thymulin in the supernatants from pcDNA-metFTS-transfected cells was much higher than the thymulin bioactivity found in the supernatants of rat TEC cultures (1.15 ± 0.32 pg/ml, $n = 4$), which are the natural source of thymulin.

Incubation of the lysates from pcDNA3.1-metFTS-transfected HEK293 cells with a polyclonal anti-FTS serum completely quenched their thymulin bioactivity (Table 1).

In vivo *tests.* In order to confirm that the thymulin bioactivity present in the transfected cells was not an *in vitro* artifact, lysates from HEK293 and BHK cells transfected with pcDNA-metFTS, were intraperitoneal (i.p.) injected in thymectomized (Tx) mice, which had nondetectable preinjection serum thymulin activity. Significant levels of biologically active thymulin were detected in the serum of the animals 45 min after lysate

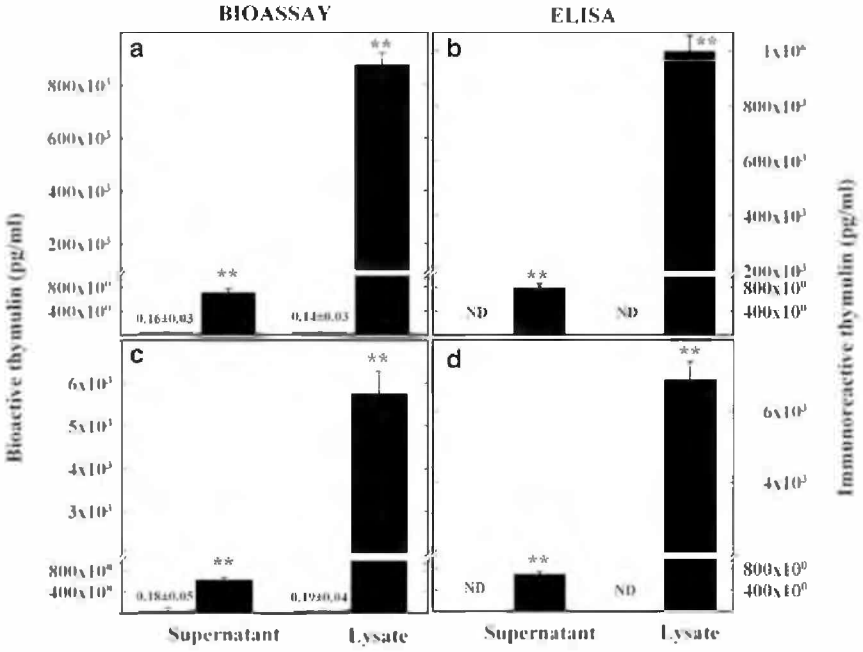

Figure 3 Bioactive and immunoreactive thymulin activity in pcDNA3.1 or pcDNA-met-FTS transfected cells. Thymulin levels were measured by bioassay (**a** and **c**) and ELISA (**b** and **d**) in supernatants and lysates from HEK293 (upper panels) and BHK (lower panels) cells transfected with either pcDNA3.1(+) (open columns) or with pcDNA-metFTS (solid columns). Bioactive thymulin concentrations in the pcDNA transfected cells are indicated over the corresponding columns (the height of the control columns has been multiplied by 10 in order to allow visualization). Error bars represent s.e.m. Double asterisks on columns indicate highly significant differences ($P<0.01$) from the corresponding pcDNA transfected group. *N*-value is 3 in all cases.

Table 1 Immunoneutralization of thymulin activity in lysates from HEK293 cells transfected with pcDNA-metFTS

Antiserum used for incubation	*Anti-FTS*[a]	*Anti-KLH*[b]
Thymulin in incubation mix (fg/ml)	2500[c]	2500[c]
Thymulin in postincubation filtrate (fg/ml)	2±1 (4)	2625±315 (4)

Abbreviations: FTS, thymus serum factor; KLH, Keyhole Limpet Hemocyanin.
Lysates mixed with an equal volume (100 μl) of the appropriate undiluted antiserum were incubated for 90 min at 37°C. The incubation mix was filtered through an Amicon membrane (30 kDa cutoff) and bioactive thymulin measured in the filtrates.
[a]Anti-FTS was raised by immunizing rabbits with synthetic FTS coupled to KLH as carrier.
[b]Anti-KLH, used as an irrelevant antiserum, was raised by immunizing rabbits with KHL alone.
[c]Bioactive thymulin concentration was measured in a lysate from HEK293 cells transfected with pcDNA-metFTS and an appropriate dilution was made so that the final thymulin concentration in the incubation mix was 2500 fg/ml.

injection. In contrast, injection of lysates from HEK293 cells transfected with pcDNA3.1 alone did not induce the appearance of detectable thymulin in Tx animals (Figure 4). Since thymulin is known to restore the sensitivity to azathioprine (Az) in spleen cells from Tx or nude mice, the splenocytes of Tx mice injected with experimental cell lysates were assessed 24 h postinjection. It was found that lysate injection restored the splenocyte sensitivity to Az in the Tx animals. Injection of control lysates did not affect splenocyte sensitivity to Az (Table 2).

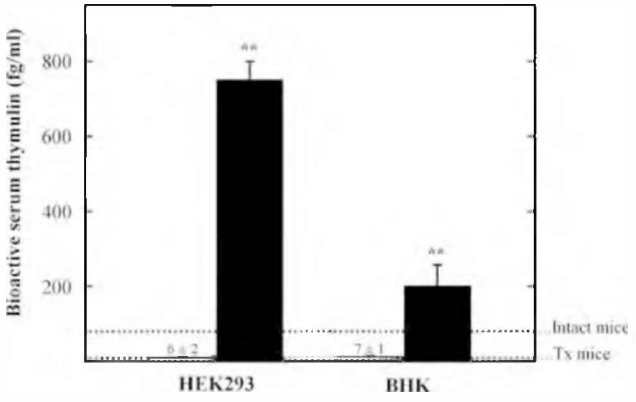

Figure 4 Serum thymulin in thymectomized (Tx) mice injected with lysates from cells transfected with pcDNA3.1(+) or pcDNA-metFTS. Animals received 100-μl i.p. injections of lysates from HEK293 and BHK cells transfected with either pcDNA3.1(+) (open bars) or pcDNA-metFTS (solid bars). The lysates were supplemented with $ZnCl_2$ (final $ZnCl_2$ concentration, 1.86 μM). At 45 min after injection, blood was taken and thymulin measured in sera by bioassay. Dotted lines represent average serum thymulin levels in Tx and intact young (2 months) mice, 6±1 and 80±16 fg/ml, respectively ($N=4$). Error bars represent s.e.m. Double asterisks on bars indicate highly significant differences ($P<0.01$) from the corresponding pcDNA3.1(+) transfected group. *N*-value is 4 in all cases.

Construction of a mammalian expression vector for a metFTS-hMGFP fusion protein

In order to obtain information about the intracellular localization of transgenic met-FTS, a second ds oligonu-

cleotide variant (variant #2) was designed and synthesized. It consisted of the met-FTS gene lacking its STOP codons and flanked upstream by a *Xma*I site and downstream by *Eco*RV and *Nco*I sites (Figure 1c). This dsDNA was digested with *Xma*I and *Eco*RV and cloned into the same sites of the MCS of an expression vector harboring the gene for humanized monster green fluorescent protein (phMGFP). This procedure yielded p(metFTS-hMGFP), a vector expressing a N-fusion of met-FTS to hMGFP (Figure 2b).

Transfection of this vector in HEK293 and BHK cells revealed a high level of fluorescence, distributed in both the nuclear and cytoplasmic compartments (Figure 5).

Table 2 Restoration of splenocyte sensitivity to Az in Tx mice injected with lysates from HEK293 cells transfected with pcDNA-metFTS

Experimental group	*Plasmid used for transfection*	*Az sensitivity (μg/ml)*
Tx mice	None	37.1±7.1 (4)
Tx mice	pcDNA3.1	33.2±8.3 (3)
Tx mice	pcDNA-metFTS	2.3±0.5 (4)
Intact euthymic mice	None	1.0±0.3 (3)

Abbreviations: Az, azathioprine; Tx, thymectomized.
Mice were i.p. injected with 100 μl vehicle or lysate from HEK293 cells transfected with the indicated plasmid. On the following day the animals were killed, splenocytes prepared as indicated for the thymulin bioassay (see Materials and methods section) and incubated with sheep erythrocytes in the presence of varying concentrations of Az. The inhibitory activity of Az on rosette formation was determined and expressed as the minimum concentation of Az sufficient to inhibit rosette formation. This concentration is inversely related to the circulating levels of thymulin in the animals.

Construction and testing of an adenoviral vector expressing met-FTS

In order to construct an adenoviral vector expressing met-FTS, a third ds oligonucleotide variant (variant #3) was designed and synthesized. It consisted of the met-FTS gene flanked upstream by an *Eco*RI site and downstream by a *Sal*I site. For sequencing purposes the phage T7 promoter, preceded by a *Bam*HI site, was included upstream the met-FTS sequence (Figure 1d). The resulting dsDNA construct was digested with *Bam*HI and *Sal*I, and cloned into the same sites of the MCS of the shuttle pDC515, which yielded pDC515-metFTS (Figure 2c). The shuttle plasmid so loaded was co-transfected with the Ad5 genomic plasmid pBHGfrt(del)E1,3FLP, in HEK293 cells, which led to the generation of an adenoviral vector, RAd-metFTS, expressing met-FTS under the control of the mouse cytomegalovirus (mCMV) promoter.

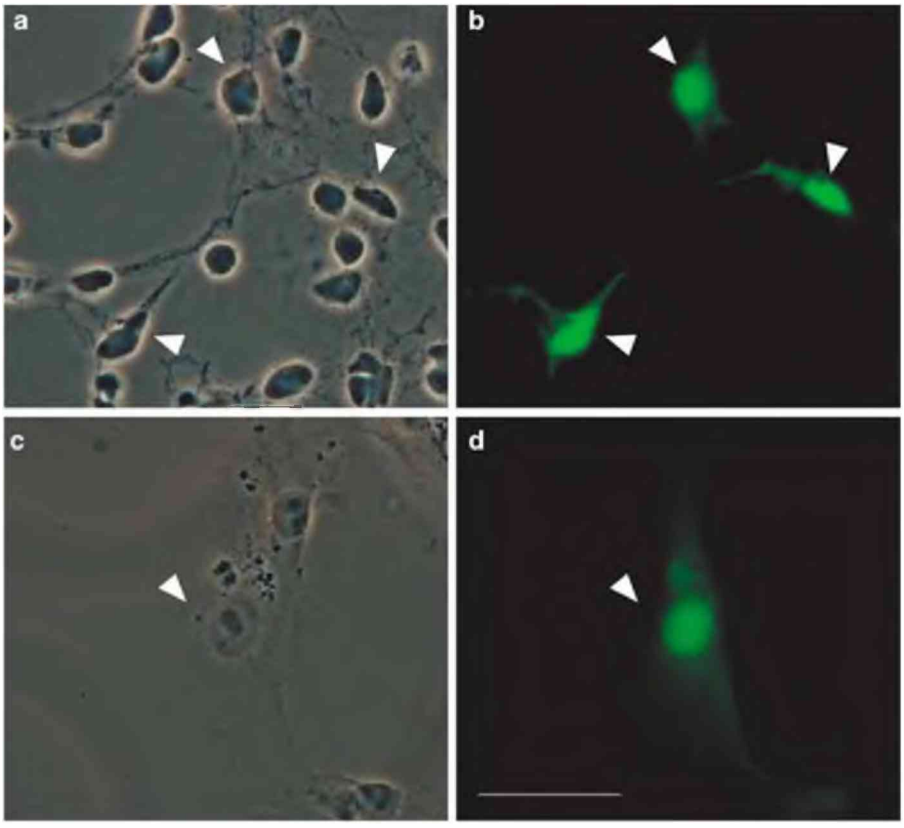

Figure 5 Intracellular localization of metFTS-hMGFP. Cultures of HEK293 (**a** and **b**) and BHK (**c** and **d**) cells grown on coverslips were transfected with pmetFTS-hMGFP using lipofectamine, according to the manufacturer's protocol. Two days later cells were fixed and mounted. (**a** and **c**) Phase constrast micrographs. (**b** and **d**) Fluorescence images of the corresponding phase images. Arrowheads identify cells expressing the transgenic fusion protein. Higher fluorescence intensity in nuclei is probably due to the fact that on the cover slips nuclei are thicker than the surrounding cytoplasm. Scale bar = 50 μm.

Supernatants and lysates from HEK293 and BHK cell cultures incubated for 3 days with 10^8 plaque forming units (PFU)/ml RAd-metFTS, possessed high levels of bioactive thymulin (Table 3). Neither BHK cells nor TEC from rat or human origin showed cytopathic effects after 2 or 4 days incubation with RAd-metFTS (data not shown).

Thymectomized mice and rats (which had nondetectable serum thymulin) respectively, injected i.m. with 10^7 and 10^8 PFU RAd-metFTS showed high levels of serum thymulin for at least 110 and 130 days, respectively (maximum expression time tested in both cases). In both species, i.m. injection of an adenoviral vector expressing β-galactosidase (RAd-βgal) in Tx animals failed to induce detectable serum thymulin levels (Figures 6 and 7). Female rats exposed for 100 days to high circulating levels of transgenic met-FTS showed body weight and patterns of estrous cyclicity comparable to those of their RAd-βgal-injected counterparts (data not shown).

Discussion

The present study demonstrates the feasibility of constructing a synthetic gene for the thymulin analog met-FTS, which retains the bioactivity of the native peptide. This is in line with an early study reporting the construction and expression in bacteria of a synthetic gene encoding a chimeric protein consisting of FTS fused to the C-terminus of *Escherichia coli* β-gal. Subsequent cleavage of the purified fusion protein with BrCN allowed these workers to obtain preparative amounts of the peptide.[12]

Analog studies have demonstrated that the N-terminal Glu of FTS is not critical for the biological activity of the peptide. Thus, des Glu[1] FTS as well as the synthetic dodecapeptide Val-Lys-Arg-(N)-FTS have the same biological activity as that of FTS.[13,14] Consequently, at the time of gene design, met-FTS was expected to be biologically active and therefore was considered to be an appropriate thymulin analog to be expressed by our prospective synthetic gene.

The *in vitro* and *in vivo* experiments reported here, show that our synthetic gene is efficiently expressed in mammalian cells under the control of both the mouse and human CMV promoter. It is worth noting that despite the extremely high intracellular levels of transgenic thymulin activity achieved *in vitro*, none of the cell lines (except HEK293 cells where the vector is replicative) transduced with RAd-metFTS showed signs of cytotoxicity. This is in line with toxicologic studies performed in various species and early clinical studies documenting that administration of pharmacological doses of synthetic thymulin did not have toxic effects even in long-term treatments.[15]

In the mouse thymus, thymulin is immunocytochemically detected in small cytoplasmic vacuoles of reticuloendothelial cells[16,17] and is thought to originate from a high molecular weight precursor targeted to the secretory pathway.[18] The pancellular localization of the transgenic metFTS-hMGFP fusion protein generated in this study suggests that met-FTS is initially targeted to the cytoplasm but diffuses freely into the nuclear compartment.

Table 3 Thymulin levels in HEK293 and BHK cells transduced with RAd-metFTS

Thymulin conc. (pg/ml)	*Cell type (treatment)*		
	HEK293 (RAd-FTS)	*BHK (RAd-FTS)*	*HEK293 (RAd-β gal)*
Supernatants	267±53 (3)	133±27 (3)	0.064±0.023 (3)
Lysates	1066±213 (3)	533±107 (3)	0.072±0.020 (3)

Abbreviations: BHK, baby hamster kidney.
Transduced cells were incubated for two days with the indicated vector at a MOI = 2. Thymulin activity was measured by bioassay. Data are expressed as mean±s.e.m. (number of wells tested).

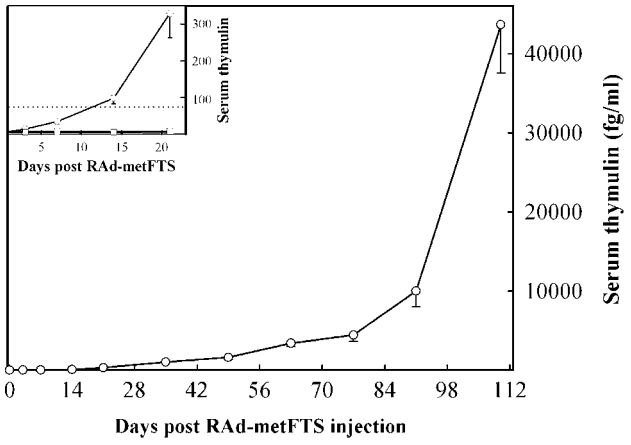

Figure 6 Time–course of serum thymulin activity in thymectomized (Tx) mice injected with RAd-metFTS. Animals received a single i.m. (hindlegs) injection of of 10^7 PFU in 100 μl RAd-metFTS (open circles) and were bled by exanguination at the indicated post-injection times. Inset displays an expanded portion of the main plot (day 0–21), showing serum thymulin levels in intact mice (dotted line) and in Tx mice injected with RAd-βgal (open squares). Data are expressed as mean±s.e.m. (N=4). A single experiment was performed using a total of 60 mice.

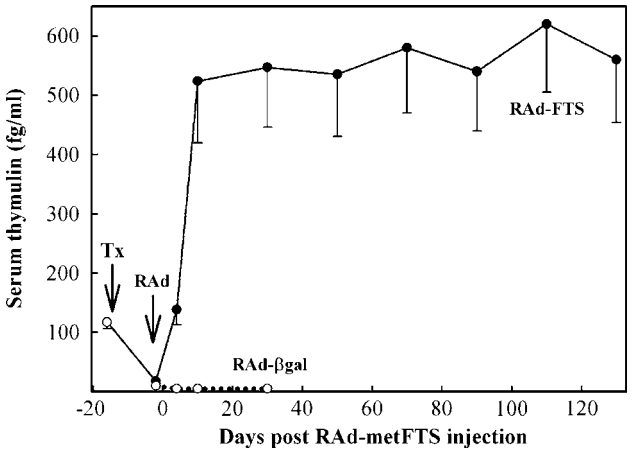

Figure 7 Time–course of serum thymulin activity in thymectomized (Tx) rats injected with RAd-metFTS. Animals were thymectomized at Experimental, day −16 (gray circle) and at Experimental, day 0 received a single i.m. (hindlegs) injection of 10^8 PFU in 200 μl vehicle, of either RAd-metFTS (solid circles) or RAd-βgal (open circles) and were bled from the tail veins at the indicated post-injection times. Data are expressed as mean±s.e.m. (N=6). Similar results were obtained in a second independent experiment (data not shown).

Our *in vivo* experiments with RAd-metFTS indicate that transduced myocytes release met-FTS into the bloodstream and can therefore be used as an ectopic source of this thymulin analog in gene therapy strategies. This is in line with previous studies showing that skeletal muscle is a well-suited tissue for efficient viral–vector-mediated peptide gene transfer.[19,20] The prolonged *in vivo* expression of met-FTS in our Tx mice and rats (which were fully immunocompetent) is in contrast with the usually short-lived transgene expression achieved by adenoviral vectors in immunocompetent animals. We hypothesize that the long-term expression of RAd-metFTS *in vivo* may be due to the known anti-inflammatory activity of thymulin, which could prevent the immune system of the animals from mounting a destructive response against the transduced myocytes. This feature of RAd-metFTS makes it a suitable tool for the implementation of long-term thymulin gene therapy.

There are a number of potential uses for thymulin as a therapeutic agent. Thymulin has been used in the treatment of certain autoimmune pathologies.[21] Interestingly, recent evidence suggests that thymulin may play a role in the brain as an endogenous anti-inflammatory factor.[7,8] Since thymulin traverses the brain–blood barrier and has no toxic effects even in high doses, i.m. injection of RAd-metFTS could generate sustained, pharmacologically effective levels of serum and brain thymulin for the amelioration of pathologies involving chronic brain inflammation.

We conclude that the construction of an adenoviral vector able to induce sustained high circulating levels of a biologically active thymulin analog represents a significant initial step in the exploration of the therapeutic potential of this molecule.

Materials and methods

Animals and in vivo *procedures*

Young (4–9 week) C57BL/6 male and female mice as well as young (8–10 week) Sprague–Dawley female rats were raised in our animal facilities (INIBIOLP). Animals had free access to food and water and were kept at 22°C with a light/dark cycle of 12/12 h. All experiments on animals were performed in conformity with the Guidelines on Handling and Training of Laboratory Animals, published by the Universities Federation for Animal Welfare.[22]

Thymectomy was performed by aspiration with the animals under Avertin (2,2,2 tribromoethanol) anesthesia (0.4 mg/g BW, i.p.). Thymectomized animals were used at least 10 days after surgery in order to ensure absence of circulating thymulin.

Cell cultures

HEK293, BHK cell lines as well as the rat thymic epithelial cell (TEC) line IT-76M1 were used. The TEC IT-76M1 cells were originally produced by Kasahara and Itoh,[23] who described their morphologic and functional characteristics. Cell lines were grown in Eagle's minimum essential medium (MEM), 16.8 mM HEPES buffer (pH 7.0), 2 mM glutamine, 0.1 mM nonessential amino acids, 20 mg/l penicillin/streptomycin, 3.3 mg/l amphotericin B, 2.2 g/l $NaHCO_3$ and 10% (v/v) fetal bovine serum. They were grown at 37°C in a humidified atmosphere of 95% air–5% CO_2. Cells were fed every 3–4 days and split when confluent.

Mammalian expression vectors

The pcDNA3.1(+) eukaryotic expression vector (Invitrogen Corp., Carlsbad, CA, USA) was used to clone and express the first version (Variant #1) of our FTS-encoding DNA construct. This vector uses the human cytomegalovirus (CMV) immediate early promoter to drive the cloned gene, with the bovine growth hormone polyadenylation signal located downstream the polylinker.

The humanized Monster Green™ Fluorescent Protein (phMGFP) mammalian expression vector (Promega Corp., Madison, WI, USA) was used to fuse our thymulin analog to the N-terminus of hMGFP in order to monitor the intracellular targeting of the analog. The hMGFP synthetic gene is driven by the hCMV promoter, with the late SV40 polyadenylation signal located downstream the gene.

Preparation of double-stranded oligonucleotides coding for met-FTS

Each of the three FTS-encoding ds deoxyoligonucleotide variants used in this study (see Results) was prepared employing the corresponding sense and antisense synthetic single-stranded (ss) oligonucleotides which were purchased from Invitrogen as reverse phase chromatography-purified (cartridge) preparations. Each of the dsDNA variants was prepared by mixing 1 nmol of each of the appropriate sense and antisense met-FTS oligonucleotides in 50 μl annealing buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA). The mix was placed in a thermocycler (Hybaid, model PCR Express, UK) and submitted to a two-step heating sequence (95°C for 10 min followed by 89°C for 15 min). The heating process was followed by a programmed stepwise cooling sequence consisting of a decrease in 1°C/min until 4°C. The resulting synthetic dsDNA oligonucleotides were cut with appropriate restriction endonucleases and purified by native 20% polyacrylamide gel electrophoresis (PAGE) in 1 × TBE buffer. Once the DNA constructs were cloned in the indicated expression vectors, they were sequenced in order to confirm that they were in frame and without error. Cloning was performed by standard procedures,[24] using for transformation the *E. coli* K-12 strain JM109 (Promega) which was grown aerobically in Luria–Bertani (LB) medium at 37°C. In some cases ds oligonucleotides coding for FTS were prepared by PCR, using the following primers (Invitrogen): 5′-GGATCCACGCGTATGCAGG-3′ (sense) and 5′-CCGGAATTCGATATCCTACTAGTTCG-3′ (antisense), and standard PCR protocols.[24] Briefly, the appropriate sense FTS ss oligonucleotide was mixed with the primers and submitted to 30 thermal cycles consisting of 30 s at 95°C, 30 s at 53°C and 1 min at 72°C, for each cycle. Finally the mix was left 5 min at 72°C and then cooled at 4°C. The resulting dsDNA oligonucleotides were cut with appropriate restriction endonucleases and purified by PAGE as indicated above.

Adenoviral vector construction

A recombinant adenoviral (RAd) vector harboring the met-FTS synthetic gene was constructed by a variant of the two plasmid method[25] employing the AdMax®

plasmid kit (Microbix, Canada). This kit uses a shuttle plasmid (pDC515) containing a MCS and a FRT recognition site for the yeast FLP recombinase. This cassette is flanked by sequences of the adenovirus type 5 (Ad5) E1 region. The second plasmid of the kit, the genomic plasmid pBHGfrt(del)E1,3 FLP, consists of the entire genome of Ad5, containing deletions in the regions E1 and E3. Upstream the E1 deletion, pBHGfrt(del)E1,3FLP contains an expression cassette for the gene of yeast FLP recombinase and immediately downstream the E1 deletion, a FRT recognition site has been inserted. Once the thymulin synthetic gene was inserted into the shuttle, both plasmids were co-transfected into HEK293 cells. In co-transfected HEK293 cells, FLP recombinase is expressed and efficiently catalyzes the site-directed recombination of the expression cassette of pDC515-metFTS into the left end of pBHGfrt(del)E1,3FLP, thus generating the genome of the desired recombinant adenoviral vector, RAd-metFTS. The newly generated RAd was rescued from HEK293 cell lysates and plaque purified. It was further purified by ultracentrifugation in CsCl gradient. Final virus stocks were titrated by a serial dilution plaque assay.

Recombinant adenoviral vector expressing β-galactosidase

This RAd was kindly provided by Dr Michel Perricaudet, Institut Gustave Roussy, Villejuif, France. In this vector, the E1 genomic region has been replaced by an expression cassette containing the *E. coli lacZ* reporter gene under the control of the Rous sarcoma virus long-terminal repeat (RSV-LTR). The vector was expanded in HEK293 cells and purified and titrated as indicated for RAd-metFTS.

Thymulin bioassay

Biologically active thymulin was measured in cell cultures and serum by a rosette bioassay described in detail elsewhere.[26] This method is based on the ability of thymulin to restore the inhibitory effect of Az on rosette formation in spleen cells from Tx mice. Briefly, sera, cell lysates or supernatants were filtered through membranes (30 kDa cutoff; YMT micropartition system, PMS-I Amicon Corporation, Denver, CO, USA) and the ultrafiltrates were diluted serially and incubated for 90 min at 37°C with spleen cells from C57BL/6 Tx mice in the presence of 10 μg Az/ml, a concentration that inhibits rosette formation in normal but not Tx mice. Rosettes were then allowed to form by centrifugation with sheep red blood cells and counted in a hemacytometer after gentle resuspension. In the presence of thymulin, rosette formation was inhibited by Az. The inhibitory activity of samples was compared with that of a standard curve using synthetic thymulin (a kind gift of Dr Peter Heinklein, Humboldt University, Berlin). Values were expressed as bioactive thymulin concentration in the sample. The sensitivity of the assay is 1×10^{-15} g thymulin/ml.

Thymulin ELISA

Immunoreactive thymulin (i.e., FTS and FTS-Zn) was determined by a previously described ELISA with some modifications.[27] Briefly, in low-binding microtiter plates 60 μl sample or synthetic FTS standard were mixed with 60 μl rabbit anti-FTS (prepared by JMP; final dilution 1/2000) and incubated overnigth at 4°C. The mix was transferred to high-binding microtiter plates coated with 100 ng synthetic FTS per well. After 1 h incubation at 37°C, plates were washed three times with PBS containing 0.1% triton X-100. The amount of anti-FTS bound to the solid phase was determined colorimetricaly using the Universal ABC *elite*® kit (Vector Laboratories Inc., Burlingame, CA, USA), employing the ABTS peroxidase substrate for color development. The sensitivity of the assay is 2×10^{-10} g FTS/ml.

Statistical analysis

Data were expressed as mean ± s.e.m., unless otherwise indicated. Statistical comparisons between experimental groups were performed by ANOVA followed by Duncan's multiple range test when appropriate.

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