Recombinant human TAT-OP1 to enhance NGF neurogenic potential: preliminary studies on PC12 cells

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Osteogenic protein 1 (OP1), also known as bone morphogenetic protein-7 (BMP7), is a multifunctional cytokine with demonstrated neurogenic potential. As the recombinant OP1 (rhOP1) was shown to provide axonal guidance cues and to prevent the reduction of dendritic growth in the injury-induced cortical cultures, it was suggested that an in vivo efficient rhOP1 delivery could enhance neurite growth and functional reconnectivity in the damaged brain. In the present work, we engineered a chimeric molecule in which rhBMP7 was fused to a protein transduction domain derived from HIV-1 TAT protein to deliver the denatured recombinant BMP7 into cells and obtain its chaperone-mediated folding, circumventing the expensive and not much efficient in vitro refolding procedures. When tested on rat PC12 cells, a widely used in vitro neurogenic differentiation model, the resulting fusion protein (rhTAT-OP1) demonstrated to enter fastly into the cells, lose HIV-TAT sequence and interact with membrane receptors activating BMP pathway by SMAD 1/5/8 phosphorylation. In comparison with nerve growth factor (NGF) and BMP7, it proved itself effective to induce the formation of more organized H and M neurofilaments. Moreover, if used in combination with NGF, it stimulated a significant (P < 0.05) and more precocious dendritic outgrowth with respect to NGF alone. These results indicate that rhTAT-OP1 fused with TAT transduction domain shows neurogenic activity and may be a promising enhancer factor in NGF-based therapies.

Keywords: BMP7/dendritic outgrowth/neuronal differentiation/NGF-based therapies/TAT-fusion protein

Introduction

Bone morphogenetic proteins (BMPs) are TGF-β superfamily members involved in nervous system development and regeneration in response to injury (Mehler et al., 1997). Among them, BMP7, also known as osteogenic protein 1 (OP1), was initially considered a trophic factor mainly for non-neuronal tissues, because of the demonstrated potential to increase the proteoglycan synthesis, cartilage formation and bone differentiation (Chang et al., 1994). As already discovered that BMP expression is not limited to the skeletal system, BMP-7 is known to be produced in perinatal neuronal tissues, including hippocampus, cerebral cortex and cerebellum (Liu et al., 2001). Its exogenous application to rat mesencephalic cell cultures showed to increase both the number of tyrosine hydroxylase-positive cells and dopamine uptake (Jordan et al., 1997). The specific receptors for BMP signalling were also demonstrated in neuronal tissue (Söderström et al., 1996) and showed to be upregulated after transient cerebral ischemia (Charytoniuk et al., 2000) and brain contusion (Lewen et al., 1997). Besides neuroprotective effects (Lin et al., 1999), BMP7 induces neuro repair in stroke animals by the recovery of sensorimotor function in the impaired limbs (Kawamata et al., 1998; Schallert et al., 2000). By parenteral administration, it can pass through the blood–brain barrier (BBB) to get ischemic side (Chang et al., 2003). In humans, different growth factors, neurotrophic inducers, cytokines and drugs have been explored as potential therapies (Bani-Yaghoub et al., 2008). However, only a limited number of them may actually have the potential to effectively offset the brain injury or stroke-related problems. BMP7 is considered today for brain repair and is currently used in patients to treat non-neurological diseases, based on its neuroprotective role demonstrated in stroke animal models (Bani-Yaghoub et al., 2008). It is known that BMP7 is produced as a larger precursor (Ozkaynak et al., 1990; Grishina et al., 2005) and then processed to give rise to the biological active carboxy-terminal domain (110–140 amino acids), containing a highly conserved 7-cysteine region (Celeste et al., 1990). Based on in vivo and in vitro studies, BMP7 is secreted as a disulfide linked mature homodimer, or as a heteromeric complex consisting of two propeptides non-covalently associated with a mature disulfide-linked homodimer (Kingsley, 1994; Gregory et al., 2005). To exert its biological activity, BMP7 activates the signal cascade by interaction with specific membrane receptors (Activin RIJA, Activin RIIB, BMPRII, Activin RIA, BMPRIA and BMPRIB) (Sengle et al., 2008). To date, BMP7 and several other BMPs have been produced by recombinant DNA technology for clinical applications (Wang et al., 1990; Sampath et al., 1992; Vaccaro et al., 2003). It is known that heterologous proteins produced by this technique are often accumulated in E. coli as insoluble inclusion bodies, and therefore solubilization and renaturation systems are required to obtain the fully active proteins with native conformation. Protein refolding protocols have been developed on a case-by-case basis and demonstrated to be very expensive (Ho et al., 2001). To overcome these problems, in this study, we have produced the recombinant human OP1 fused with TAT transduction domain, a well established protein delivery system to efficiently introduce into cells and in vivo refold up protein cargoes (Vives et al., 1997; Futaki et al., 2001; Ho et al., 2001; Park et al., 2002;
Muthumani et al., 2009). HIV-1 TAT sequence is a protein transduction domain (PTD) commonly used as carrier for the efficient delivery of bioactive factors difficult to permeate living cells (Muthumani et al., 2009). The short peptide YGRKKRRQRRR is rich in basic amino acids, and allows the fast intracellular transduction and subcellular localization (Futaki et al., 2001; Park et al., 2002) of proteins, ranging in size from 15 to 120 kDa (Vives et al., 1997). The transduction of full-length TAT-fusion proteins across the cell membrane results in an inactivation or denaturation of the delivered product (Bonifaci et al., 1995), and the native protein conformation is restored intracellularly by chaperones (Ellis and Hartl, 1999), a family of proteins located in every cell compartments.

The present work describes the cell trafficking and the neurogenic activity characterization of purified recombinant human OP1 fused with TAT transduction domain. In particular, we demonstrated on PC12 cultures that rhTAT-OP1 enters fastly into the cells, loses TAT sequence and activates the BMP signalling pathway stimulating the production of neurofilaments. The synergy of OP1 with the nerve growth factor (NGF) to promote the precocious neurite outgrowth is restored intracellularly by chaperones (Bonifaci et al., 1995), a family of proteins located in every cell compartments.

Materials and methods

Expression and purification of rhTAT-OP1

The recombinant TAT-OP1 (162 AA) was prepared using the mature part of OP-1 protein (residues 300–431 of human protein precursor) and the TAT-sequence (YGRKKRRQRRR), as protein transduction domain (PTD). The N-terminal end includes a polyhistidine sequence, necessary for the protein purification, and a sequence of six AA corresponding to a peptidase site (SQNRSK) (Fig. 1A). The OP1 coding sequence was obtained from pSV-OP1 plasmid, amplified using polymerase chain reaction (PCR) method and then subcloned into the prokaryotic expression vector pTAT-α-synA30P. The bacterial BL21 lysate was cleared by centrifugation and the supernatants were purified by ion metal affinity chromatography, gel filtration chromatography and reverse phase high-performance liquid chromatography. Purified rhTAT-OP1 was kept at −80°C until use (Fig. 1B).

Cell cultures

PC12 cells, derived from the rat pheochromocytoma, were purchased from the European Animal Cell Culture Collection (EACC, Porton Down, Wilts, UK) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% horse serum (HS), 5% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) (all products from Sigma-Aldrich, St. Louis, MO) at 37°C in a humidified atmosphere with 5% CO2. To be used in this work as in vitro neuronal differentiation model, PC12 cells were first tested for the expression of BMP receptors.

Activin/BMP receptors analysis by RT-PCR

The expression of BMP receptors as mRNA was examined on cells cultured on 25-cm2 culture flasks (Falcon BD Biosciences, San Jose, CA) containing RPMI-1640 added with 10% HS and 5% FCS. Total cellular RNA was extracted using TRIzol (Invitrogen Life Technology, Carlsbad CA), quantified by measuring absorbance at 260 nm and stored at −80°C until use. RNA (1 μg) was amplified by RT-PCR assay, run in a Thermal cycler PTC-100 (MJ Research Inc., Waltham, MA) using the one-step RT-PCR kit (Qiagen, Venlo, NL), according to the manufacturer’s instructions. Primer pairs for BMP receptors and ribosomal protein-L19 were designed as reported by Takeda et al. (2003) (Table I). RT-PCR products were electrophoresed through a 1.5% agarose gel (Invitrogen) and visualized with a UV-transilluminator Gel Doc 2000 Gel Documentation System (Bio-Rad Laboratories Ltd, Hercules, CA).

Treatments

After 24 h from seeding, cultures were treated with rhTAT-OP1 (2 and 200 nM) in combination with or without NGF (1.8 nM) (Sigma-Aldrich). In parallel, positive controls were prepared treating cells with 1.8 nM rhNGF or 2 nM rhBMP7 (Sigma-Aldrich) (Table II). Untreated samples were used as negative controls. After treatment, the cultures were maintained in DMEM added with 0.5% FBS and then submitted to MTS proliferation assay, cell trafficking study and differentiation assays.

MFS proliferation assay

The effects of rhTAT-OP1 on cell proliferation were determined by the colorimetric MTS assay 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (CellTiter 96 Aqueous Assay, Promega, Madison, Wisconsin, USA). PC12 cells (50 × 104 cell/cm2) were seeded on each well of a 96-well tissue culture-treated plates (Falcon BD Biosciences). After 24 h from seeding, cells were cultured for 24, 72 h and 7 days with medium containing rhTAT-OP1 (2 and 200 nM) in combination with or without 1.8 nM rhNGF and 2 nM rhBMP7. Two hours before the end of the incubation period, medium was replaced with a fresh one containing 20% MTS dye and cultures were then incubated for 2 h at 37°C. The medium absorbance of each sample was measured at 490 nm wavelength with ELISA UltraMicroplate Reader (Bio-tek Instruments, Winooski, VT). In parallel, untreated samples were used as negative controls. Results,

![Fig. 1. (A) Aminoacidic sequence of recombinant human TAT-OP1 fusion protein including a polyhistidine-tag (HHHHHHH), TAT sequence (YGRKKRRQRRR) and the sequence (300–431 AA) of human mature OP1. A peptidase cleavage site (SQNRSK) was included. (B) Vydac-C4 chromatography of highly purified product.](image-url)
Table I. Primer sequences for RT-PCR analysis (from Takeda et al., 2003)

<table>
<thead>
<tr>
<th>Target</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>L19</td>
<td>5'-CTGAGGTTAGGAAATGTGGAAG-3'</td>
<td>5'-AATCCTCCTTCTTGTTCC-3'</td>
</tr>
<tr>
<td>BMPR-II</td>
<td>5'-AGGATGATCTTCCTGTCGTC-3'</td>
<td>5'-CTGATACAAAGTATAAGGC-3'</td>
</tr>
<tr>
<td>ActR-II</td>
<td>5'-AGGAAATCGGAGCTGTCGTC-3'</td>
<td>5'-CCGTTAACCCCAAAATGCAC-3'</td>
</tr>
<tr>
<td>ALK2</td>
<td>5'-AGTGCTGCTCAGGGGACTGTTG-3'</td>
<td>5'-GGTCCAAATATCTCCATGTGC-3'</td>
</tr>
<tr>
<td>ALK3</td>
<td>5'-ACATGATTATGTGGAGCC-3'</td>
<td>5'-TGTAACACACAGGAGCTGGAG-3'</td>
</tr>
<tr>
<td>ALK6</td>
<td>5'-ACTCCATCTGCTCAAG-3'</td>
<td>5'-GGTGAAGAACACTTTCACAG-3'</td>
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</tbody>
</table>

Table II. Cytokine cocktails used to induce PC12 cells for 24 h

<table>
<thead>
<tr>
<th>Cytokine cocktail</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>1.8 nM rhNGF</td>
<td></td>
</tr>
<tr>
<td>2 nM rhBMP7</td>
<td></td>
</tr>
<tr>
<td>2 nM rhTAT-OP1</td>
<td></td>
</tr>
<tr>
<td>200 nM rhTAT-OP1</td>
<td></td>
</tr>
<tr>
<td>1.8 nM rhNGF + 2 nM rhBMP7</td>
<td></td>
</tr>
<tr>
<td>1.8 nM rhNGF + 2 nM rhTAT-OP1</td>
<td></td>
</tr>
<tr>
<td>1.8 nM rhNGF + 200 nM rhTAT-OP1</td>
<td></td>
</tr>
</tbody>
</table>

derived from three separate experiments, were expressed as number of cells extrapolated from a standard curve. The linearity of absorbance of MTS over a range of 2.5 × 10^{-3}–50 × 10^{4} cells was established by determining the linear coefficient ($R^2 = 0.9885$). Then, for each experiment, cell number was determined. Their statistical comparison was performed by the analysis of variance, followed by Student’s t-test.

Cell trafficking study

It was performed after 5 min treatment using the following techniques.

Western blot analysis. At different time points (0, 2, 6, 24 and 48 h), cells were washed three times with ice cold phosphate buffer saline (PBS), scraped off in cell lysis buffer (1% v/v Triton X-100, 0.5% p/v sodium deoxycholate, 0.15 M NaCl, 10 mM Tris–HCl pH 7.6, 1% protease inhibitor cocktail) (Sigma-Aldrich) and then lysed by passing through a 29-gauge needle. Lysates were cleared by centrifugation at 14,000 g for 15 min at 4°C, and protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific Inc, Rockford, USA). Equal amounts of protein (50 μg) were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) and probed in SNAP i.d. system (Millipore) for 10 min with a mixture of polyclonal mouse antineurofilament H (200 kDa)/M (160 kDa) (Sigma Aldrich), or rabbit anti-P-SMAD 1/5/8 (Cell Signaling Technology, Inc., Danvers, MA) monoclonal antibodies and polyclonal mouse antineurofilament H (200 kDa)/M (160 kDa) (Sigma Aldrich), or rabbit anti-P-SMAD 1/5/8 (Cell Signaling Technology, Inc., Danvers, MA) both diluted 1:100 in 1% BSA/PBS. All steps were performed at room temperature (RT) with humidified conditions. After washings in cold PBS, samples were incubated for 30 min with goat antimouse Alexa Fluor 488 or Alexa Fluor 594-secondary antibody (Invitrogen) at RT to detect, respectively, mouse anti-HisTag and anti-hBMP7 antibodies. Nuclear staining was performed using Vecta-Shield mounting medium with DAPI (Vector Labs, Burlingame, CA). The fluorescent signals were detected using a Leica TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with Leica application suite advanced fluorescence (LAS AF) software (Leica Microsystems).

Detection of phosphorylated SMAD and neurofilaments.

In order to detect the presence of phosphorylated SMAD 1/5/8 (at 0, 2, 6, 24 and 48 h) and neurofilaments (at 24, 72 h and 7 days), samples were prepared to perform western blotting analysis and immunofluorescence staining.

Immunoassay analysis and confocal microscopy. The samples fixed and permeabilized as previously described, were incubated for 1 h with antineurofilament H (200 kDa)/M (160 kDa) (Sigma Aldrich), at RT using humidified conditions. After washing with cold PBS, specific-binding sites were saturated with 2% BSA solution for 10 min. The samples were then incubated for 40 min at RT with goat antimouse Alexa Fluor 488-secondary antibody (Invitrogen). Nuclear staining was performed as previously described.

Immunofluorescence staining and confocal microscopic analysis. After 2, 6 and 24 h, cultures were fixed at −20°C in methanol for 10 min, permeabilized with 1% Triton X-100/BSA in PBS for 10 min, and finally incubated for 1 h with mouse anti-HisTag (Sigma-Aldrich) or mouse anti-hBMP7 (Santa Cruz Biotechnology, Inc., CA) monoclonal antibodies both diluted 1:100 in 1% BSA/PBS. All steps were performed at room temperature (RT) with humidified conditions. After washings in cold PBS, samples were incubated for 30 min with goat antimouse Alexa Fluor 488 or Alexa Fluor 594-secondary antibody (Invitrogen) at RT to detect, respectively, mouse anti-HisTag and anti-hBMP7 antibodies. Nuclear staining was performed using Vecta-Shield mounting medium with DAPI (Vector Labs, Burlingame, CA). The fluorescent signals were detected using a Leica TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with Leica application suite advanced fluorescence (LAS AF) software (Leica Microsystems).

Differentiation assays

Neurite outgrowth assays. At different time points (24 h and 7 days), cultures were fixed with 4% p-formaldehyde at 4°C overnight and then submitted to toluidine blue staining by incubation for 10 s at RT with 0.5% toluidine blue (Sigma-Aldrich) solution prepared in 20% ethanol. By using
a microscope TL/IL (Leica), one hundred total cells were observed in five random fields and the ones bearing processes longer than cell diameter were considered as positive. All results were expressed as mean ± SD of three separate experiments. Their statistical comparison was performed by analysis of variance, followed by Student's *t*-test.

**Results**

**Activin/BMP receptors analysis**

The expression of activin/BMP system was examined by RT-PCR analysis using total RNA. As shown in Fig. 2, cells expressed BMPR II, ActR-II, ALK2 and ALK3 mRNAs, but not ALK-6 mRNA.

**MTS proliferation assay**

At different time points (24, 72 h and 7 days), the effect of rhTAT-OP1 (2 and 200 nM) on PC12 proliferation was evaluated and compared with that obtained using rhNGF (1.8 nM), rhBMP7 (2 nM) or different combinations of them. As shown in Fig. 3, a decreased proliferation (*P < 0.05) was observed in the treated samples in comparison with control (72 h and 7 days). In particular, at 7 days, although no significant difference was detected among samples treated with 2 nM rhTAT-OP1 and 200 nM rhTAT-OP1, 2 nM rhTAT-OP1 used with 1.8 nM rhNGF induced a stronger inhibition effect on proliferation (*P < 0.05) in comparison with rhNGF.

**Cell trafficking**

In order to make the rhTAT-OP1 signal more detectable, PC12 cells were treated with the highest concentration of rhTAT-OP1 (200 nM).

The *in vivo* transduction of rhTAT-OP1 (200 nM) was evaluated on PC12 cultures at different time points (0, 2, 6, 24 and 48 h) by using a monoclonal antibody directed versus His-Tag sequence present at the N terminal protein end. As shown in Fig. 4A, western blot assay detected the presence of TAT-fusion protein at 0–6 h as a band with molecular size of 18.5 kDa similarly to the positive control rhBMP7. No signal of His-Tag was observed at 24 and 48 h.

To localize the signal coming from both N-terminal and C-terminal ends of rhTAT-OP1 protein during the *in vivo* transduction assay we performed the immunofluorescence staining on PC12 cultures at different time points (2, 6 and 24 h) by using a mouse anti-HisTag and a mouse anti-hBMP7 monoclonal antibodies, respectively, revealed with mouse anti-Alexa Fluor 488 or Alexa Fluor 594-secondary antibody. As shown in Fig. 4B, the presence of His-Tag and OP1 was detected at the membrane and cytoplasm levels within 6 h after the end of treatment with 200 nM rhTAT-OP1. A different localization was observed at 24 h: the signal of His-Tag was detected inside the nucleus while the presence of OP1 was revealed in the membrane.

**Differentiation assays**

**SMAD1/5/8 phosphorylation.** As BMP signalling involves SMAD1/5/8, we evaluated the phosphorylation state of SMAD proteins after the treatment with 2 and 200 nM rhTAT-OP1. Western blotting assay confirmed the presence of phosphorylated SMAD 1/5/8 for all the samples within 24 h from the end of treatment. A decreased signal was observed in rhTAT-OP1 and rhBMP7-treated samples at 48 h (Fig. 5).

**Neurofilaments production.** No difference was detected among samples and controls at 24 h (data not shown). After 72 h and 7 days from the end of treatment (Fig. 6A and B), western blotting assay demonstrated a low expression of neurofilaments H (size band 200 KDa) and M (size band 160 KDa) in samples treated with 2 nM rhBMP7, 2 nM rhTAT-OP1 and 200 nM rhTAT-OP1 in comparison with rhNGF-treated samples. The combination of rhBMP7 and rhTAT-OP1 with rhNGF showed to guarantee a comparable production of neurofilaments with the one obtained by rhNGF treatment. TAT sequence demonstrated no effect on neurofilaments production.

According to the data obtained from western blotting, at 24 h the presence of neurofilaments was well observed in samples treated with rhNGF but a low signal was detected in PC12 cells treated with rhTAT-OP1, or rhBMP7 used in combination with rhNGF (Fig. 7). The immunoreactivity showed to increase from 24 h to 7 days in all samples: in particular, in samples treated with 200 nM rhTAT-OP1, neurofilaments appeared to be well organized and distributed within cytoplasm and neurites.

**Dendritic outgrowth measurement.** The neuritogenic effect of rhTAT-OP1 was studied and compared with that induced by rhNGF and rhBMP7. Table III shows that rhTAT-OP1
(2 and 200 nM) and rhBMP7 (2 nM) were ineffective to induce the neurite outgrowth in the absence of rhNGF, and independently from the stimulation time. In contrast to rhNGF-treated cultures that presented a strong production of neurites only at 7 days, the samples cultured in the presence of rhTAT-OP1 or rhBMP7 both combined with rhNGF showed a precocious sprouting of neurites. Moreover, this effect demonstrated to be time stimulation dependent: a significant increased number \((P < 0.05)\) of cells expressing neurites was detected at 24 h using 2 nM rhBMP7, 2 and 200 nM rhTAT-OP1. The neuritogenic effect of 2 and 200 nM rhTAT-OP1 measured at 7 days was comparable to the ones induced by rhNGF used alone or in combination with rhBMP7.

**Discussion**

Brain injury and stroke are the leading causes of death and disability worldwide (Green and Shuaib, 2006). The treatments available for brain injury patients are very limited and include stabilization, monitoring, surgery and rehabilitation, depending on the case. There is emerging evidence that limited degrees of neurogenesis, neurite growth and functional recovery occur after brain injury or stroke, but unfortunately they are not sufficient to regenerate the damaged brain and stave off the associated sensory, motor and cognitive problems. The potential of OP1 as regulator of in vivo neuroprotection and neuroregeneration (Chiang et al., 2005; Chou et al., 2006) after brain injury has encouraged the idea that such protein could be applied in many diseases with CNS involvement. Besides to regulate critical aspects of development of the nervous system (Mehler et al., 1997; Ebendal et al., 1998; Lein et al., 2002) including specification of cell fate (Furuta et al., 1997), regulation of neural cell survival, determination of neuronal shape and transmitter phenotype (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996), OP1 was found to facilitate the recovery from ischemic injury induced by ligation of the middle cerebral artery (Kawamata et al., 1998; Lin et al., 1999) and to stimulate mature neurons from adult sympathetic ganglia (Lein et al., 1999). There is accumulating evidence that OP1 is abundantly expressed in both meninges and the choroid plexus (Hogan, 1996; Furuta et al., 1997; Wada et al., 2002). Since the telencephalic meninges are derived from cephalic neural crest and they contribute to the formation of choroid plexus...
Detection of neurofilaments (H, M) by confocal microscopy on PC12 cells after treatment with 1.8 nM NGF (A), 2 nM hBMP7 (B), 200 nM rhTAT-OP1 (C) and their combination of 1.8 nM NGF + 2 nM hBMP7 (A + B), 1.8 nM NGF + 200 nM rhTAT OP1 (A + C) (magnification: ×630).
Table III. Dendritic growth measurement by optical microscopy

<table>
<thead>
<tr>
<th>Sample</th>
<th>24 h</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1.8 nM NGF</td>
<td>None</td>
<td>45.97 ± 8.55</td>
</tr>
<tr>
<td>2 nM BMP7</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2 nM rhTAT-OP1</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>200 nM rhTAT-OP1</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1.8 nM NGF + 2 nM BMP7</td>
<td>9.43* ± 2.29**</td>
<td>53.98* ± 12.37**</td>
</tr>
<tr>
<td>1.8 nM NGF + 2 nM rhTAT-OP1</td>
<td>7.43* ± 1.95**</td>
<td>32.17* ± 2.53</td>
</tr>
<tr>
<td>1.8 nM NGF + 200 nM rhTATOP1</td>
<td>11.8* ± 3.04**</td>
<td>42.21* ± 17.25</td>
</tr>
</tbody>
</table>

We considered positive the cells expressing branches whose length was longer than nucleus diameter. Data are reported as % mean of positive cells ± SD detected by 100 cells within five random fields (*P < 0.05 versus untreated sample, **P < 0.05 versus NGF).

Bone morphogenetic proteins regulate many critical aspects of neural development (Mehler et al., 1997; Ebendal et al., 1998). The PC12 phaeochromocytoma cell line is a well-established model for examining the effects of NGF and other factors inducing neuronal differentiation and neurite outgrowth (Greene and Tischler, 1976). NGF is reported to drive the differentiation of PC12 cells, and the NGF-driven neurite outgrowth is sustained by Erk pathway activation (Brightman and Fell, 2000). We presently show rhTAT-OP1 activates BMP signalling by phosphorylation of SMAD1/5/8 within 24 h from treatment.

The induction of neuronal differentiation involves three interrelated cellular processes that are cell cycle arrest, neurofilament production and neurite outgrowth (Liebermann et al., 1995). NGF decreases the growth rate of PC12 cells BMP7 into cells and obtain its chaperone-mediated folding, circumventing the expensive and not much efficient in vitro refolding procedures.

Table III shows the dendritic growth measurement by optical microscopy. The data are reported as % mean of positive cells ± SD detected by 100 cells within five random fields (*P < 0.05 versus untreated sample, **P < 0.05 versus NGF).
(Greene, 1978) and arrests cell cycle in G1 phase (Ignatius et al., 1985). After long-term treatment with NGF, PC12 cells terminally differentiate, resembling sympathetic neurons with a cessation of division, increased substratum adherence, neurite extension and catecholamine synthesis (Greene and Tischler, 1976). rhTAT-OP1 (2 and 200 nM) added to PC12 cells decreased the proliferation from 72 h to 7 days as NGF (1.8 nM), rhBMP (2 nM) or different combinations of all. After 7 days from induction, 2 nM rhTAT-OP1 in combination with rhNGF induced a stronger inhibition effect of proliferation \( (P < 0.05) \) than NGF, suggesting a combined effect between rhTAT-OP1 and NGF to inhibit PC12 growth. When added as a single factor to PC12 cells, rhTAT-OP1 influenced neurofilament expression and neuritogenesis. Likely rhBMP7, rhTAT-OP1 showed a detachable production of neurofilaments H (200 KDa) and M (160 KDa) after 1 week from induction while, when combined with NGF, a precocious expression was induced. The expression of neurofilaments seemed to be not affected by TAT sequence and resulted to be independent on rhTAT-OP1 concentration. As previously reported (Bengtsson et al., 1998; Farkas et al., 1999), BMPs potentiate the response of PC12 cells to NGF, raising the question of convergent signalling pathways.

One of the most prominent activities of BMPs is stimulation of dendritic growth. BMP7-induced dendritic growth requires Smad1 activation (Guo et al., 2001) and this effect has been observed with many types of central neurons (Granholm et al., 1999; LeRoux et al., 1999; Withers et al., 2000).

In contrast to the data reporting the efficacy of rhBMP7 to stimulate the sympathetic neurons either the in vitro and in vivo dendritic outgrowth (Lein et al., 1995) we demonstrated that rhTAT-OP1 and rhBMP7 did not elicit by themselves dendrite outgrowth on PC12 cells. Unlike, NGF is implicated in the regulation of the initial stages of dendritic growth in combination (Bruckenstein and Higgins, 1988) with or without (De Koninck et al., 1993) other bioactive molecules. Up to now, little is known about the molecules and their synergies involved in the precocious phases of dendrite sprouting. In this work, rhTAT-OP1 (200 nM), likely rhBMP7, when used in combination with NGF, showed a precocious neurite outgrowth at 24 h with respect to NGF alone. In contrast, the neuritogenic effect measured at 7 days was comparable to the one induced by rhNGF used as a single factor. One convergence point in the NGF and the BMP pathways suggested by the work of Kretzschmar et al. (1997) is the four putative sites for phosphorylation by Erk in the linker region of Smad1. These authors showed that increased Erk activity as a result of growth factor stimulation of tyrosine kinase receptors yielded increased linker region phosphorylation that inhibited nuclear translocation of Smad1 and reduced its ability to activate reporter genes. Later, similar sites were identified by these authors in the linker region of Smad2 and were shown to suppress Smad activity by Erk-mediated phosphorylation (Kretzschmar et al., 1999).

**Conclusion**

Our findings show that rhTAT-OP1 fastly translocates through the plasma membrane, loses N-terminal end and refolds within the cytoplasm acquiring the biological activity. Furthermore, it is also demonstrated to activate BMP signalling by SMAD 1/5/8 phosphorylation and to stimulate the expression of neurofilaments H and M in PC12 cells. Although TAT-HIV1 by itself did not induce the dendrite outgrowth, it was effective to reduce to differentiate PC12 cells by the synergy with NGF.

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**References**


Recombinant human TAT-OP1 enhances NGF neurogenic potential