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A platform technique for growth factor delivery with novel mode of action

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ABSTRACT

Though growth factors allow tissue regeneration, the trade-off between their effectiveness and adverse effects limits clinical application. The key issues in current growth factor therapy largely derive from initial burst pharmacokinetics, rapid clearance, and proteolytic cleavage resulting in clinical ineffectiveness and diverse complications. While a number of studies have focused on the development of carriers, issues arising from soluble growth factor remain. In this study, we report a prodrug of growth factors constituting a novel mode of action (MoA). To mimic endogenous protein processing in cells, we developed a recombinant BMP-2 polypeptide based on a protein transduction domain (PTD) to transduce the protein into cells followed by furin-mediated protein cleavage and secretion of active growth factor. As proof of concept, a few micrograms scale of PTD-BMP-2 polypeptide sufficed to induce bone regeneration in vivo. As a simple platform, our technique can easily be extended to delivery of BMP-7 and DKK-1 as therapeutics for TGF-β and canonical Wnt signaling, respectively, to suppress the epithelial–mesenchymal transition (EMT), which constitutes a fundamental biological mechanism of many diseases. This technique largely overcomes the limitations of current soluble growth factors and opens the door to next generation growth factor therapeutics.

1. Introduction

The mode of action (MoA) of the soluble growth factors is simply to interact with membrane receptors on target cells, thereby triggering an intracellular signaling cascade for regeneration. Since their approval by the FDA several decades ago, the recombinant bone morphogenic proteins (rhBMPs) have been widely studied for use in bone regeneration. Though the BMPs were initially identified as osteoinductive factors present in demineralized bone matrix [1,2], it is now evident that the BMP family induces mesenchymal-to-epithelial reversion, a major therapeutic goal in treating cancer progression and degenerative disorders [3]. For example, BMP-7 has been identified as an endogenous antagonist of TGF-beta mediated EMT in the kidney and other organs [4–6]. Administration of recombinant BMP-7 in mice with renal fibrosis resulted in reversal of EMT and repopulation of healthy tubular epithelial cells with functional recovery though the current technique for BMP-7 delivery to large visceral organs is very limited [4,6,7]. The insulin-like growth factors, granulocyte–macrophage colony stimulating factor, basic fibroblast growth factor, and platelet-

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derived growth factor have all demonstrated potential for use in regenerative therapy [8].

Despite extensive research into the safety and efficacy of the growth factors in both animal and clinical trials, many complications remain, including ectopic bone formation, osteolysis, pain, and swelling [9,10]. Considering the long-term regeneration process in vivo, it is difficult to resolve the dilemma between efficacy and adverse effects with respect to direct MoA of soluble factors exhibiting an initial burst release pharmacokinetic profile [11]. Because of the short half-life of rhBMP, mainly due to rapid clearance and enzymatic degradation [11,12], excess dosage of soluble proteins, even mg scale (one million times the concentration needed for physiologic regeneration), is often applied to ensure clinical effectiveness. However, high doses of soluble growth factors inevitably increase the chance of diffusion to nearby tissues or blood circulation, with many adverse effects [9,13]. Although many carriers have been adapted for controlled release to overcome the initial burst and rapid clearance rate, the mutual exclusivity between safety and efficacy remains. The cost-effectiveness and legal issues related to soluble recombinant growth factors currently pose a challenge in many medical indications [14,15].

The cell-permeable, protein transduction domain (PTD) peptides have been found to transduce protein across the plasma membrane into the cell as part of a physiologic process [16]. The PTD is widely studied for delivery of macromolecules such as peptides, catalytic proteins, and oligonucleotides [17], providing a therapeutic advantage without raising the safety issues associated with gene delivery. Following initial ionic contact with the cell membrane, PTD-fusion polypeptide is rapidly internalized by lipid raft-dependent macropinocytosis independent of specific receptors [18]. Interestingly, the denatured polypeptides delivered into cells are refolded and elicit physiological protein function in cells and in vivo [19,20], though the mechanism of intracellular processing of delivered protein is still not fully defined [19–21]. Thus, we adopted the PTD technique for post-translational processing and secretion of growth factors mimicking endogenous protein biogenesis to overcome an intrinsic limitation of soluble factor therapy using an indirect MoA. The transduced TAT-BMP-2 polypeptide was properly processed in a furin-dependent manner and secreted as bioactive BMP-2. The hydrogel composite with the polypeptide was functional to promote bone regeneration as proof of concept in vivo. In addition to resolving the issue of initial burst release, the technique was easily extended to BMP-7 and Wnt antagonist Dickkopf-1 (DKK-1), providing a platform for delivery of therapeutic secreted proteins in regenerative medicine.

2. Materials and methods

2.1. Bacterial expression constructs and protein purification

The bacterial expression cassette for PDT-fusion polypeptide was constructed based on pRSET bacterial expression vector (Invitrogen) having His purification tag and Xpress epitope. The TAT sequences (RKKRRQRRR) for the PTD domain were inserted next to the epitope and the precursor cDNAs of BMP-2, BMP-7, or DKK-1 were cloned into a TAT-expression cassette. The precursor cDNA of BMPs and DKK-1 were obtained by PCR amplification of cDNA of Saos-2 osteosarcoma cells and 293T cells, respectively. The TAT sequence was omitted for negative control of protein transduction. Mutant expression vectors for BMP-2 were constructed by deletion of signal peptide (aa 1–24), prodomain (aa 1–270), or mutation of furin cleavage site of REKR to REKA (aa 279–282). Induction and purification of the recombinant protein were followed as described by Dowdy's group [19,22]. Briefly, following transformation of BL21 and IPTG induction, the inclusion bodies were obtained from insoluble fraction of 1% Triton X-100 buffer. The insoluble fraction was solubilized with 8 M urea solution and the recombinant protein purified with Ni–Ti beads and imidazole elution followed by buffer shock to gain high surface energetic (ΔG) properties.

2.2. Cells, western blot analysis, cell migration assay, and immunofluorescence

The murine C3H10T1/2 and osteoblast precursor cell line MC3TC were obtained from ATCC (CCL-226) and culture following manufacturer's recommendations. MCF-7, A549, and 293 cells were described previously [23]. The recombinant protein was directly applied to regular or serum-free culture medium (Opti-MEM) in the indicated amount. The soluble and insoluble fractions of lysate were obtained from Triton X-100 lysis buffer. To detect transduced recombinant protein in cells, the whole fractions of cell were prepared in SDS-RIPA buffer followed by heating at 95 °C for 5 min. The commercially available primary antibodies were directed against anti-Xpress (Invitrogen), BMP-2 (R&D systems), Tubulin (Labfrontier), α1-antitrypsin (Sigma), Snail (Cell Signaling), β-catenin (R&D systems), and E-cadherin (Invitrogen). Cell migration potential of A549 cells was evaluated by transwell assay as described previously [24]. Mammalian expression vector for α1-antitrypsin Portland (α1PDX) was described previously [25]. For immunofluorescent analysis, the cells were treated with TAT-BMP for 1 h and the transduction of recombinant protein was detected against anti-Xpress monoclonal antibody and anti-mouse IgG-conjugated Alexa-Fluor-488 secondary antibody as described previously [23].

2.3. RT-PCR for cbfa-1/Runx2

The murine C3H10T1/2 cells were treated with TAT-BMP-2 for a 16 h culture period and total RNA was harvested with Trizol. The cDNA was synthesized with random hexamer and routine RT-PCR performed. The primer sequences for mouse cbfa-1 and GAPDH of control were as follows: cbfa-1, forward, 5′-ctcttcccaaagccagagtg; cbfa-1, reverse, 5′-cagcgtcaacaccatcattc; mouse GAPDH, forward, 5′-tgaaggtcggagtcaacggattt; GAPDH, reverse, 5′-catgtgggccatgaggtccaccac.

2.4. ELISA assay BMP, alkaline phosphatase, and mineralization assay

To detect secreted BMP-2, 100 ng of recombinant protein were transduced into the 293 cells for 2 h followed by PBS washing and refreshment of serum-free Opti-MEM medium (300 μl in 6-well plate). After 4 h of incubation, the cell lysates and culture medium were harvested. Fifty μl of lysates or culture medium were subjected to ELISA assay for BMP-2 (R&D systems) according to the manufacturer's protocol. Commercially available recombinant BMP-2 (R&D systems) was used as control for the quantitation. To detect alkaline phosphatase activity in cells, a commercially available kit (Wako) was used following the manufacturer's instructions. C3H10T1/2 cells at the late log phase of growth were treated with 10 ng of TAT-BMP-2 or 10 ng of rhBMP-2 (R&D systems) or negative control, and the cells maintained for 4 days before the alkaline phosphatase assay. For mineralization assay of C3H10T1/2 cells, an osteogenesis assay kit (Millipore) based on Alizarin Red S stain was used. The cells were treated with recombinant proteins or negative control for 2 h and the cells incubated in osteogenic culture medium for 14 days. Treatment with the recombinant protein was repeated every 2 days for mineralization assay.

2.5. Furin cleavage assay

Ten units of human recombinant furin (Sigma) were subjected to protein cleavage of the full-length or mutant TAT-BMP-2 protein followed by immunoblot analysis. For in vitro furin cleavage analysis, soluble fractions of recombinant protein were used, the cleaved protein being detected by immunoblot analysis using anti-BMP-2 antibody.

2.6. Reporter assay of E-cadherin, Wnt/TCF, and TGF-β responsive activity

The Wt and E-box mutated E-cadherin proximal reporter constructs (nt-108 to +125) were used as described previously [26]. The luciferase reporter of Topflash (TCF/LEF binding sites upstream of a luciferase reporter), FOPflash (mutated TCF/LEF binding sites), and 3 TP-lux having Smad-binding site on plasminogen activator inhibitor-1 (PAI-1) promoter were obtained from Addgene. The cells were transiently transfected with 100 ng of reporter constructs and 2 ng pSV-Renilla using Lipofectamine2000 (Invitrogen). After 24 h of transfection, the culture medium was refreshed with or without recombinant protein as indicated. The relative ratio of Renilla to firefly luciferase after a 48 h transfection period was determined by dual-luciferase assay kit (Promega) from triplicate experiments.

2.7. Hydrogel preparation and biocompatibility test on HA hydrogel

Hydrogel for animal experiments was prepared from low molecular weight hyaluronic acid (low HA, MW 340kd) as described previously [27]. Briefly, low HA was reacted with adipic acid dihydrazide using N-(3-diethylpropyl)-N-ethylcarbodiimide (EDC) as a cross-linking agent at room temperature to synthesize HA-ADH. HA polymers of HA-Ac and HA-TCEP were prepared from HA-ADH through a reaction with acrylic acid (Ac) or tris(2-carboxyethyl)phosphine (TCEP), respectively. The obtained polymers of HA-Ac and HA-TCEP were precipitated with 95% EtOH and dialyzed for 3 days to remove unbound chemicals followed by freeze drying. Two separated 5% HA-Ac and HA-TCEP solutions were prepared in PBS. After separately sterilizing the HA-Ac and HA-TCEP solutions with a 0.2 μm syringe filter, TAT-BMP-2 was added to the HA-AC solution and hydrogel (HG) was synthesized by mixing the obtained solutions of HA-TCEP and HA-Ac with TAT-BMP-2 on culture plate. Cell culture on HG was performed by seeding MC3T3 cells on the surface of HG for 8 weeks. Cell adhesion and