Engineering of targeted protein therapeutics: recombinant N-TIMP-3 with a targeting domain for cartilage

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Statement of Purpose:
Targeted delivery of therapeutic molecules improves their bioavailability in the target tissue while reducing adverse effects by controlling the biodistribution. Targeting functionality has mainly been added to drug delivery systems, but not to the actual therapeutic molecule itself. In the present study, we use a short peptide, which specifically adheres to the cartilage matrix to make a fusion protein with the N-terminal active domain of tissue inhibitor of metalloproteinase-3 (TIMP-3). TIMP-3 is a matricellular protein which inhibits several matrix metalloproteinases (e.g. MMP-1, -2, -9) in addition to aggrecanase 1 and 2, i.e. ADAMTS4 and ADAMTS5. By adding targeting functionality to N-TIMP-3, the bioavailability in articular cartilage is increased, because fast clearance out of the joint is prevented. In addition, the cartilage matrix now serves as a reservoir of immobilized N-TIMP-3 which inhibits proteases where cartilage degradation takes place and may therefore prevent disease progression in osteoarthritis over a prolonged time period.

Methods: *Phage display*: A peptide-on-phage display library (fUSE5/6-mer) with a complexity of $6.4x10^7$ has been screened against bovine cartilage grafts to find a novel targeting peptide for the cartilage matrix. Negative screenings were done against the articular cartilage surface and synovial fluid. The sequence of the displayed peptide was obtained by DNA sequencing after five cycles of affinity purification. Binding assays have been carried out in vitro against bovine cartilage in physiological conditions (37°C, with and without synovial fluid) to probe specificity and competetive binding with 10^8 TU/ml of each phage clone. Titers were determined as transducing units per ml (TU/ml) and binding in % of control.
Cloning: To make the fusion protein, the N-terminal domain of mouse TIMP-3 was cloned. RNA was isolated from primary immature murine chondrocytes and amplified by RT-PCR. In order to attach the targeting peptide to the C-terminal end of the protein, the reverse primer was designed to add the DNA sequence of the targeting peptide with a glycine spacer (GGG) to the PCR amplified fragment. The fragment was then ligated into pGEX-4T-1 (Amersham Biosciences) for expression in E.coli. The construct was confirmed by DNA sequencing.
Protein production and purification: E.coli BL21 were transformed with pGEX-TIMP3 and the best clone selected by anti-GST ELISA. Because expression in the soluble form was not possible, the fusion protein was purified from inclusion bodies, which required refolding over 5 days with buffer shifts every 24 hours. The refolded protein was then purified by FPLC with a GSTPrep FF 16/10 20ml column (Amersham Biosciences), the GST tag cleaved off by incubation with thrombin and purified again by FPLC to remove the GST tag and thrombin with a HiTrap Benzamidine FF column (Amersham Biosciences).

Zymography: The biological activity of the purified protein was determined by MMP-2 zymography. MMP-2 and tr-TIMP-3 at various concentrations were incubated for 1 hour at 37°C before SDS-PAGE and the gel developed overnight.

Results/Discussion: DNA sequencing of phage clones revealed three putative peptide sequences after 5 cycles. All of these sequences were shown to be specific to cartilage versus synovial membrane by two orders of magnitude. A competetive binding assay between the three phage clones and the original library retained only two phage clones, whereas clone C1-3 had a 4-fold higher titer. In addition, the free peptide of C1-3 at a concentration of 10μM resulted in a decrease of phage binding by two orders of magnitude. In contrast, the scrambled sequence of C1-3 did not decrease phage binding. The sequence of C1-3 was therefore used as the affinity motif for articular cartilage binding.
Wild-type TIMP-3 consists of an inhibitory N-terminal domain and a matrix binding C-terminal domain, which is replaced here by the short peptide C1-3, thereby making the N-terminal domain adhesive to articular cartilage. Because TIMP-3 is a relatively insoluble matricellular protein, optimisation of expression in the soluble form could not be achieved. The fusion protein was therefore expressed and purified from inclusion bodies. Refolding was carried out by dialysing against decreasing concentrations of urea with buffer shifts every 24 hours for 5 days. The overall yield was around 1.5mg/l of bacterial culture.
MMP-2 zymography revealed 100% biological activity of the refolded and purified protein, as evidenced by a complete inhibition of MMP-2 at equimolar and above equimolar concentrations.

Conclusions: We have successfully identified a short peptide which exhhibits specific binding to articular cartilage. A fusion protein of N-TIMP-3 and the targeting peptide has been produced and biological activity confirmed by zymography. Current work is probing the targeting of the fusion protein in the cartilage matrix in vitro and in vivo as well as its retention in the joint in vivo. Furthermore, a therapeutic study will investigate the therapeutic effect of targeted N-TIMP-3 vs. wild-type N-TIMP-3 in an osteoarthritis model in mice.