Development of 3rd generation fibrin delivery systems: recombinant fibrinogen growth factor fusion proteins

Lirong Yang[1], Peter Frey[2], Thomas H. Barker[3], Jeffrey A. Hubbell[1]

[1]Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne, Switzerland; [2]Department of Pediatric Surgery, Centre Hospitalier Universitaire Vaudois Lausanne, Switzerland; [3]Department of Biomedical Engineering, Georgia Tech and Emory University, USA

Statement of Purpose: Fibrinogen (Fgn), a soluble plasma protein found in all vertebrates, is a covalent dimer composed of pairs of three polypeptide chains called Aα-, Bβ- and γ-chains. Fgn is converted to an insoluble fibrin network following activation by thrombin and crosslinking by factor XIIIa following vascular injury and serves the primary role in haemostasis.
Our interest in developing fibrin-based biomaterial technology is based on fibrin's central role in tissue binding and in the initiation of tissue repair and defence. The binding of Fgn/fibrin to hemostasis proteins and platelets as well as to different cells, growth factors, and extracellular matrix proteins is indispensable during the wound repair process. Technologies have been developed with some success for incorporation of growth factors into fibrin, ranging from simple admixing to covalent incorporation using the coagulation transglutaminase factor XIIIa. We are developing a novel platform technology where the growth factor is incorporated into Fgn's Aα-chain at the genetic level. We have chosen this region since evidence suggests that the C-terminus of Fgn's Aα-chain is not required for bioassembly or polymerization. The release profile and functional significance of this novel fibrin-delivery system will be compared to current fibrin delivery schemes.

Methods: Truncated mutant fibrinogen Aα-chain DNA was created by PCR and modified by incorporation of an EcoRI restriction site at 5' terminal and a NotI restriction site at 3' terminal, in order to insert the mutant DNA into the expression vector pMYKpuro. All plasmid sequences were verified by sequencing analysis. The plasmid was used to transfect Chinese Hamster Ovary cells expressing fibrinogen Bβ- and γ-chains ($CHO_{\beta\gamma}$ cells). Expressing of truncated fibrinogen was analyzed by ELISA for assembly capacity and by clotting assay for activity.

To obtain the fusion protein mutant fibrinogen hIGF1, human Insulin-like Growth Factor 1(hIGF1) cDNA was subsequently generated by PCR with both termini containing XbaI restriction sites for incorporation at the 3' terminus of the truncated Aα fibrinogen chain cDNA in the pMYKpuro vector. The modified plasmid DNA was transfected into $CHO_{\beta\gamma}$ cells, followed by puromycin selection. ELISA was used to select the optimal clone for fibrinogen Aα-hIGF1 fusion protein expression. The best clone was verified by FAC for monoclonal existence. The cell line was used to produce recombinant chimeric fibrinogen hIGF1 fusion protein, which was then purified by GPRP affinity chromatography. Polymerization (for the fibrinogen component) and cell proliferation (for the hIGF1 component) were used to verify the functionality of purified fusion protein.

For comparison to our current fibrin delivery system using factor XIII coupling, hIGF1 cDNA was generated by PCR for incorporation into the expression vector pGEX4T1 where the transglutaminase substrate sequence from α2PI (Tg) for crosslinking into fibrin was added at the N-terminus. The cDNA was transformed into BL21 cells after sequence verification. The transformed BL21 cells were selected by ampicillin and produced GST-hIGF1 fusion protein after inoculation in 2xYT medium. GST affinity chromatography followed by thrombin cleavage of the hIGF1 component was used for protein purification. Again, proliferation was used to determine the bioactivity of purified hIGF1.

Results / Discussion: ELISA with the fibrinogen antibody shows fibrinogen expression of all truncated fibrinogen species (Aα189, 194, and 199). This confirms that that C-terminus of fibrinogen Aα-chain is not required for bioassembly and can be cut as short as only 5 amino-acids C-terminal from the second disulfide cluster (FGA5aa). FGA5aa provides at least 45kDa of free space for the fusion protein.

hIGF1 was chosen for its simple active form (single chain) and low molecular weight (18kDa). The new stable cell line $CHO_{fgn5aahIGF1}$, which was confirmed by ELISA with fibrinogen and hIGF1 antibody and by FAC, was created after FGA5aa-hIGF1 plasmid DNA was successfully transfected into $CHO_{\beta\gamma}$ cells. The recombinant chimeric fibrinogen hIGF1 fusion protein displays bioactivity according to clotting assay. We are currently testing the bioactivity of the hIGF1 component.

Tg-hIGF1, produced by BL21 and purified by GST affinity chromatography, showed bioactivity according to the MTT assay.

Conclusions: Fibrinogen Aα chain can be truncated to amino acid 189 while maintaining the capacity to be assembled into a secreted, detectable fibrinogen. It is feasible to produce recombinant chimeric fibrinogen, which directly incorporates, at the genetic level, growth factors of interest into fibrin matrices. We believe this new generation of fibrin delivery system represents a significant advance over current delivery systems. This novel approach allows near-complete incorporation of protein species into fibrin polymers resulting in high levels of control over the delivered protein factor.

References: Yang Z et al. Biochemistry 2000: 97, 3907-3912; Sakiyama-Elbert SE and Hubbell JA. J Cont Release 2000: 69, 149-158.