Recombinant fibrinogen synthesis: transient transfection CHO cells in suspension

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Statement of Purpose: Fibrinogen (Fgn), a soluble plasma protein found in all vertebrates, is a covalent dimer composed of monomers with three polypeptide chains called Aα-, Bβ- and γ-chains. Following vascular injury the serine protease thrombin converts fibrinogen into fibrin monomers which then self-assemble into fibrin polymer. Fibrin serves the primary role of hemostasis and as a provisional structural scaffold for invading cells and an immuno-modulator of innate immune cells. It circulates in the plasma at a concentration of approximately 3 mg/ml. Currently, fibrin is used in a multitude of biomedical applications including as a tissue scaffold for tissue engineering constructs. Our ultimate goal is to generate fibrinogen variants with therapeutic value, including variants that tether, at the genetic level, growth factors to the fibrinogen polypeptide backbone. For these reasons we are developing a system capable of producing therapeutically relevant quantities of soluble, bioactive fibrinogen. In this work we are focused on optimizing the capacity of CHO cells, grown in suspension, to take up DNA, to analysing different media formulations to determine ideal conditions for fibrinogen production, and to the establishment of reproducible, cost-effective purification methods for fibrinogen.

Methods: *Cells:* Anchorage and serum-dependent CHO cell lines stably-transfected with fibrinogen Aα, Bβ, and γ chain cDNA (CHO_{fib}) as well as CHO cells stably-transfected with only fibrinogen Bβ and γ chain cDNAs ($CHO_{\beta\gamma}$) were previously established. Cells were placed in rocker incubators to prevent attachment to establish anchorage-independent lines. Subsequently, cell lines were weaned off of serum and into ProCHO4 media (Cambrex Biosciences).

Transfection optimization: Plasmid DNA expression vectors containing Aα-chain and GFP (green fluorescent protein) were amplified in *E.Coli* and extracted using a Plasmid Maxi Kit (QIAGEN GmbH, Germany, cat#12163). Cells transfected with GFP vectors were analysed by FACs (CyAn™ ADP from Dakocytomation, Denmark) and MFI as well as histograms are reported (Fig. 1).

Media screening: Cell number, viability, and aggregate number was counted by a Cedex HiRex cell analysis system (Innovatis AG, Germany) and subsequently fibrinogen expression was determined by standard ELISA techniques using goat anti-human fibrinogen (MP Biomedicals, Ohio, USA, cat#55036) as the capture antibody and HRP-conjugated goat anti-human fibrinogen (MP Biomedicals, Ohio, USA, cat#55239) as the detection antibody.

Purification optimization: Ammonium sulfate precipitation, anionic exchange chromatography and peptide affinity chromotagraphy were used to purify fibrinogen. We used Gly-Pro-Arg-Pro as the peptide and this was synthesized on a solid resin (Fractogel) by standard FMOC synthesis.

Results / Discussion:

$CHO_{\beta\gamma}$ cells are transfected with different PEI: DNA ratio (from 1 to 14) to find the optimal ratio. Two days after transfection, cells are analysed by FACS to see the population of transfected cells (fig. 1). At same time, viable cells are counted using Haemocytometer (fig. 2). The transfection efficiency increases with ratio, but cell viability decrease dramatically with ratio. Due to further experiments with transfected cells, the ratio 2 is chosen for $CHO_{\beta\gamma}$ cells transfection.

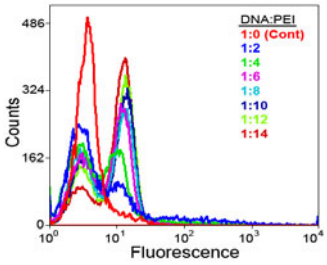

Fig 1: FAcs Analysis

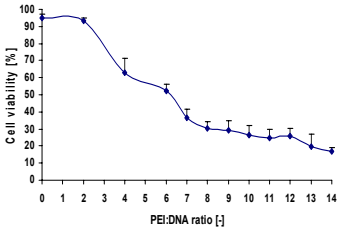

Fig 2: Cell viability vs. ratio

Transfected $CHO_{\beta\gamma}$ cells are used to screen 23 commercial lyavailable medium for 10 days. FACS results indicate that about 41% of cells are successfully transfected. ELISA protein analysis shows that transfected CHO cells are capable of producing significant quantity of fibrinogen in several medium, such as CD CHO, ProCHO5 and ProMedia Select k#10. The cell growth curve (fig 3) indicates that cells pass one lag phase of 1 or 2 days for adaptation to the new medium. Then they can grow more than 4 days in the same medium without exchange and can achieve viability higher than 70% (fig 4). $ProCHO_5$ is shown to be one of best growth and production medium. The only problem is higher cell aggregation rate in this medium.

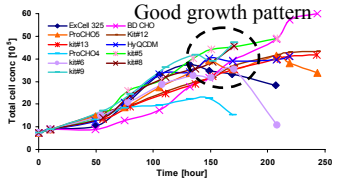

Fig 3: Cell growth curve

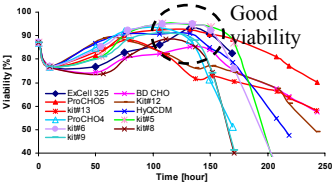

Fig 4:Cell viability curve

Conclusions: Fibrinogen Aα chain can be transfected to $CHO_{\beta\gamma}$ cells to produce significant quantity of secreted, detectable fibrinogen. Cell concentration can attain 6 million cells per ml 10 days after transfection and cell viability is still higher than 50%. It provides a means for the future production of recombinant chimeric fibrinogen and direct incorporation, at the genetic level, of protein therapeutics into fibrin matrices. The optimal production and growth medium for $CHO_{\beta\gamma}$ cells still requires additional development and these data will be presented.

References: Doolittle RF. Blood Review 2003: 17, 33-41
Lord ST. Biochemistry 1996:35, 2342-2348