Incorporation of fibronectin domains into fibrin matrices for stem cell delivery

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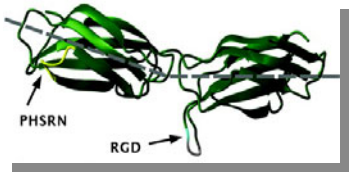
Statement of Purpose: A potential therapy for repairing damaged tissues and organ systems is the delivery of mesenchymal stem cells or precursors cells to the site of injury[1]. Fibrin is ideal for cell delivery since the polymer is derived from the normal hemostatic pathway and is degraded by biofeedback loops linked to tissue repair. However, fibrin alone does not sustain adequate cell attachment required for migration. Fibronectin, a multidomain extracellular matrix protein, allows cell attachment through its central binding domain. The 9th TypeIII repeat within this region contains the so-called synergy sequence PHSRN whereas the 10th TypeIII repeat contains the traditional integrin binding peptide RGD. In its unmodified state, fibronectin, through it's 9th and 10th TypeIII repeats, engage cells in a way that induces proliferation, an effect that often leads to scar tissue formation rather than organized tissues. Recently it has been shown that the biological activity of fibronectin can switch between stimulation of proliferation and differentiation through different integrin binding affinities[2,3]. Thus the integration of 9th and 10th TypeIII repeats domains with different integrin binding affinities in a fibrin matrix would provide instructive information that would direct stem or progenitor cells towards proliferation or differentiation depending on the design requirements. In this study we will determine how five modified 9th and 10th TypeIII repeats incorporated in a fibrin matrix alter the homeostasis of osteoblastic precursors.



9th and 10th TypeIII repeats

Methods: *Construction of FNIII9-10 mutants in pGEX4T-1 vector:* cDNAs encoding wild-type FNIII9-10, FNIII9 and FNIII10 were amplified by PCR from a human FN cDNA fragment, pFH154, with the α2 plasmin inhibitor transglutaminase-sensitive sequence that allows covalent crosslinking of the species into fibrin matrices[4] and inserted into pGEX4T-1 (Amersham Pharmacia Biotech). For the insertion of flexible linkers between the 9th and 10th repeats FNIII9 and FNIII10 were individually and sequentially cloned into the pGEX4T-1 vector using a complementary *Xma*I/*Sma*I site that adds one additional proline and glycine between the two domains (FNIII9-PG-10). Subsequently, a sequence encoding four glycine residues was inserted into the *Sma*I site of FNIII9-PG-10. Finally a point mutation of Leu[1408] to Pro was made in the wild-type FNIII9-10 construct following the Quickchange protocol (Stratagene).

GST-protein purification: E. coli BL21 transformed with pGEX4T-1 constructs were grown O/N in 2xYTA and the protein expression was induced by addition of 1mM IPTG. The purification procedure was performed using ÄKTA*FPLC* (Amersham Pharmacia Biotech) with a GSTPrep FF 16/10 20ml column (Amersham Pharmacia Biotech). Thrombin was used to remove the GST tag and thrombin removed from preparations with a HiTrap Benzamidine FF column.

Fibrin-fibronectin matrix construction: Fibrin polymers were made at a physiologic ratio of fibrinogen:fibronectin (2 mg/ml fibrin : 0.3 mg/ml fibronectin with 0.5U/ml thrombin, 0.3U/ml factor XIII).

Cells: Immature osteoblast like cells MC3T3 cells (RIKEN Cell Bank, Tokyo, Japan) were seeded into the matrices and Alkaline Phosphatase (an indicator of differentiation state) activity was quantified.

Results / Discussion: The activation of integrin $\alpha_v\beta_3$ by the 10th TypeIII repeat (RGD) directs the cells down proliferation pathways whereas the activation of $\alpha_5\beta_1$ by the synergistic 9th domain and the RGD within the 10th domain directs the cells to differentiation. The incorporation of spacer amino acids between the 9th and 10th domains decreases the affinity of $\alpha_5\beta_1$ for the fibronectin by a destabilization of the two domains[5]. Conversely, stabilization via simple point mutations (Leu[1408] to Pro) can increase their affinity[6]. The construction of FNIII 9-10 mutants was verified by sequencing and the expression of recombinants proteins was checked by ELISA. The proteins were purified by FPLC and excellent purity was obtained. The capacity of Fibrin-FNIII9-10 matrix to instruct differentiation and proliferation of the osteoblastic precursor MC3T3 will be checked by microscopy and measurement of ALP activity reported. The matrices that bind mainly $\alpha_v\beta_3$ (FNIII10 > FNIII9-P[G]$_5$-10 > FNIII9-PG-10) should direct the cells towards a way of proliferation. Conversely those which binds mainly $\alpha_5\beta_1$ (FNIII9-10* > FNIII9-10) should promote the differentiation.

Conclusions: This new generation of fibrin matrix would allow increased cellular adhesion while being probably effective to promote the proliferation and/or the differentiation of osteoblastic and myogenic precursors. Moreover, it could be used on mesenchymal stem cells in partnership with other component like growth factors or transcription factors.

References:

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