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Conformational changes of exogenous deposited fibronectin on decellularized aortic conduits after implantation

Khon Huynh*

School of Biomedical Engineering, International University, Vietnam National University, Ho Chi Minh city

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Abstract:

Fibronectin is an abundant and ubiquitous extracellular matrix component that regulates a wide range of cell functions by directly interacting with cell surface integrin receptors. In a prior study, we observed that fibronectin can accelerate the autologous cellular repopulation of implanted decellularized grafts, enhancing their adhesion capacity and biocompatibility, through fibronectin fibrillogenesis. The supportive effect of deposited fibronectin was shown to be dissimilar in different regions of implanted decellularized grafts. This led us to question whether there is any difference in conformation of fibronectin deposited on the luminal and adventitial surfaces of decellularized aortic conduits after implantation. In this study, we doubly labelled fibronectin with a fluorescent resonance energy transfer (FRET) pair and use this to analyse differences in conformational changes of adsorbed fibronectin at different sites of the decellularized aortic conduits.

Keywords: **conformational changes, fibronectin, shear stress.**

Classification number: **3.6**

Introduction

Plasma fibronectin (FN) is produced by hepatocytes and released into circulation as a soluble compact dimer at concentrations of 200-600 µg/ml. Circulating plasma FN is a glycoprotein dimer and multidomain protein with high molecular weight (440-500 kDa) that is folded into a globular compact structure. Its compact structure leads to the inactivation of FN since major functional binding sites are buried [1]. However, when needed, plasma FN can interact with cell and platelet receptors by which it is unfolded, assembled into fibrillar matrix with exposed functional binding sites and integrated into the extracellular matrix. Alternatively, an FN fibrillar matrix can also be formed under flow conditions [2-4]. Most of the functions of FN in nature have been reported to be related to fibrillar form. However, the mechanism of FN fibril formation and its biological activities are not yet clearly understood.

The hallmark of understanding biological functions of FN is that these functions are extremely complex and appear to be switched back and forth in specific cases. For instance, FN carries domains that can interact with collagen and fibrin at N- terminus and adhesive sites with RGD sequence at position FNIII10 that is capable of interacting with platelet integrins. Hence, this molecule has been shown to contribute to the process of haemostasis. However, results from previous studies on the effect of FN on adhesion and aggregation of platelets demonstrated that the role of FN would change (either by enhanced or inhibited platelet activities) corresponding to different conditions of interaction with platelets [1]. Results from in vivo studies conducted on mice also agreed with this finding. These contradictory findings have given rise to many hypotheses including one that suggests that the functional switching of plasma FN during cellular processes is related to its conformational changes and fibrillogenesis [1, 5].

FN coatings have been shown to accelerate the autologous cellular repopulation of implanted decellularized aortic conduits by enhancing their adhesion capacity and biocompatibility [6, 7]. However, the supportive effect of deposited FN was shown to be dissimilar in different regions of implanted decellularized grafts. To elucidate the biological mechanism that explains this functional dissimilarity, we labelled FN with two fluorophores for Förster (or fluorescence) resonance energy transfer (FRET) to monitor its conformational changes and study how different structures of FN are distributed along the decellularized conduit grafts.

*Email: hckhon@hcmiu.edu.vn

Methods

FN purification and labelling

Plasma FN was purified using a modified protocol of a previously described procedure and was doubly labelled with two types of fluorescence dyes, Alexa Fluor AF488 (488/514 nm) and Alexa Fluor AF546 (517/570 nm), as donor and acceptor pair, respectively, for the FRET experiments [1]. Briefly, FN was incubated in 4-M GdnHCl solution to expose the four cryptic free cysteine residues, which were specifically labelled by adding AF546 at a molar ratio of 30:1 (dye/FN molecule). The reaction was kept for 1 h at room temperature with gentle rotation in the dark. After that, dialysis was performed against phosphate-buffered saline (PBS) at pH 7.3 overnight to remove unbound dyes. The AF546-conjugated FN (FN546) protein was collected, and the concentration was measured by reading the absorption at 280 nm. Next, amine labelling was performed by adding 0.1 m sodium bicarbonate at pH 8.7 followed by the addition of an 80-fold molar excess of AF488 to FN546 solution. The reaction was incubated for 1 h in the dark at room temperature with gentle rotation. Free dyes were again removed by dialysis against PBS pH 7.3 overnight. Concentrations of conjugated FN and the ratio of FN: dyes were determined by reading absorbance at 280, 496, and 556 nm and calculated according to the user manual.

To confirm the sensitivity of FRET in indicating FN conformation changes, FRET signals from labelled FN in solution with increasing concentration of GdnHCl (0-4 M) were measured. FRET signals were determined as the ratio of donor fluorescence intensity divided by acceptor fluorescence intensity (I_A/I_D). FRET signals of soluble FN in solution without GdnHCl were set at 100%.

FN coating of aortic conduit grafts

Decellularized aortic conduit (DAC) grafts were obtained from the Department of Cardiovascular Surgery and Research Group for Experimental Surgery, Heinrich Heine University, Germany [6]. Grafts were incubated with 50 µg/ml mixture of doubly labelled FN: unlabelled FN (1:10 ratio) for 24 h at 37ºC. FN-coated grafts were implanted into systemic circulation of recipient rats as previously described [6]. Samples of DACs were fixed with 3.7% formaldehyde and stored in PBS buffer containing 0.1% NaN_3 at 4ºC.

FRET analysis

At 1, 3, and 6 h after implantation, implanted conduits were thoroughly excised and collected from region A and B corresponding to ascending and descending aortic conduits. Tissue samples were further fixed with 3.7% formaldehyde, cryo-embedded, and sectioned for FRET analysis. For each sample, 15 random ROI from three representative sections 200 µm apart from one another were observed and captured by fluorescence microscopy DM2000 (Leica, Wetzlar, Germany). FRET signals were calculated as the ratio of fluorescence signals of AF488 and AF546 (AF546/AF488) in ROI regions. In parallel experiments, FN-coated conduits were incubated with PBS pH 7.3 buffer or blood plasma for 1, 3, and 6 h followed by FRET analysis without implantation.

Results

FRET changes with FN conformation

In principle, in solution without GdnHCl, FN exposes a compact structure. As GdnHCl in solution increases from 0 to 1, 2, 3, and 4 M, FN molecules become extended either by being partially and completely unfolded (Fig. 1). Accordingly, the average intramolecular distance between donors and acceptors gradually increases leading to the reduction of FRET signals. FRET signals of FN in its compact conformation (0 m GdnHCl) were set at 100% and decreased to 61% as the FN molecule extended in the 1 m GdnHCl solution. Further unfolding of FN at concentrations of 2, 3, and 4 m GdnHCl reduced the FRET signals by 48, 46, and 40, respectively.

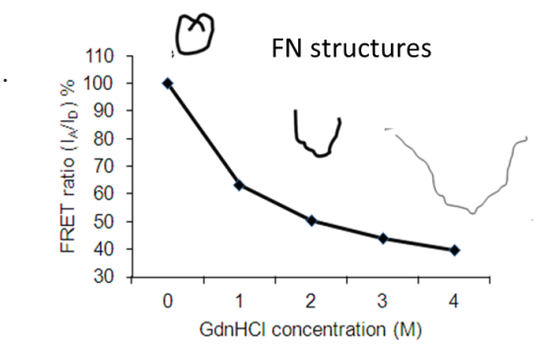

Fig. 1. Conformational changes of FN monitored by FRET.

FN conformation changes in GdnHCl solutions correlated with FRET signals (ratio of I_A/I_D). As GdnHCl concentration in solution increases from 0 to 4 M, causing FN to be partially unfolded or unfolded, respectively, the FRET signal decreases.

FRET changes upon implantation

Labelled FN was mixed with unlabelled FN at a ratio of 1:10 to prevent energy transfer between adjacent molecules that can influence FRET measurement. To observe the changes in conformation of deposited FN on decellularized aortic conduits (DAC), 50 µg/ml mixture of

labelled and unlabelled FN was used to coat the adventitial and luminal surfaces of DAC. By using fluorescence microscopy to excite donor fluorophores and record both donor and receptor fluorescence emission, we were able to monitor conformational changes of FN on DAC. For better visual observation, microscopic images were set so that regions of high FRET level appeared violet, and regions of low FRET level appeared grey indicating a more compact or more extended state of FN, respectively.

To confirm that the decrease in FRET signals was due to cell invasion after implantation, DAC after FN coating was incubated in PBS buffer pH 7.3 and blood plasma collected from experimental mice (Fig. 2A-B). FRET was monitored within 6 h of incubation. Results showed that while FRET signals of deposited FN did not reduce at the adventitial surface, the FRET signals at the luminal side decreased slightly from 100% to 92±2.6% after 6 h of incubation in PBS (Fig. 2A(b-d)). In blood plasma, at the adventitial side, FRET signals of deposited FN decreased from 100% to 86.2±2.6%. The decrease in FRET signals at the luminal side was less pronounced (from 100% to 94.6±7.9%) (Fig. 2A(e-g)).

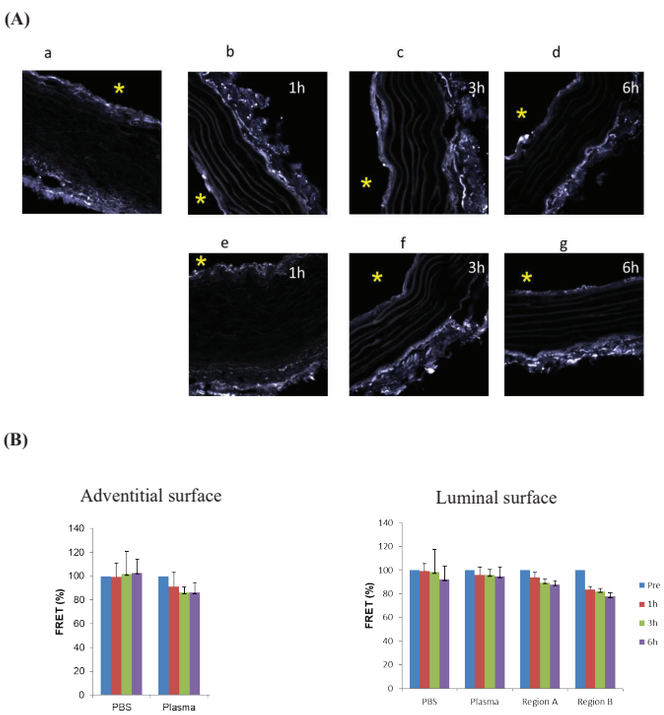

Fig. 2. FRET signal changes of FN incubated in PBS and mouse blood plasma. (A) Representative images of pre-incubation doubly labelled FN (a) and incubated in PBS (b, c, d) and blood plasma (e, f, g) after 1, 3 and 6 h. Areas with high and low FRET signal appear in violet and grey, respectively. Vessel luminal side is indicated by asterisk. **(B)** FRET analysis of FN incubated in PBS and blood plasma at the adventitial (left) and luminal side (right).

To study the conformational changes of deposited FN on DAC after implantation, FRET signals of the adventitial and luminal side at ascending aorta (region A2) and descending aorta (region B1) of DAC were recorded. In contrast to control experiments in which DACs were incubated with PBS buffer and blood plasma (Fig. 2), implanted DACs showed a significant FRET decrease even after 1 h of incubation. The decrement in FRET was pronounced during the first hour of incubation and after that slowed down with only few percent decrement at 3 and 6 h. Among the analysed regions, FRET signals of deposited FN on adventitial side of region A2 showed the most reduction (from 100% to 67.4±4.5%, 63.5±2.6%, and 63.8±3.2% at 1, 3 and 6 h after implantation, respectively). However, in the same region but at the luminal side, FRET decrease was the least going from 100% to only 93.9±4.2%. Changes in FRET signals of the adventitial and luminal sides of region B were similar to each other with about 15% FRET decrement after 1 h followed by 3-5% decrement at every 3 h thereafter (Fig. 3).

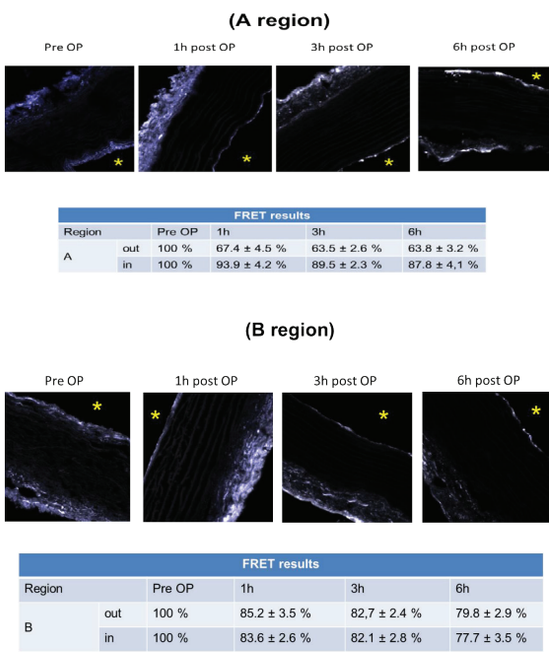

Fig. 3. Changes in FRET signal of adventitial and luminal sides at ascending aorta (region A) and descending aorta (region B).

Discussion

Our previous data has shown that FN surface coating of DAC accelerates the autologous *in vivo* recellularization after implantation when compared to the control group without FN coating [6]. The supportive effect of deposited FN was shown to be dissimilar in different regions

of DAC. FN's biological activity is dependent on its conformation and fibrillogenesis [2, 4, 8]. This led us to a question whether there is any difference in conformation of FN deposited on the luminal and adventitial surfaces of DAC after implantation.

FRET measurement was successfully used to monitor conformational changes of FN [1, 9, 10]. The fact that FRET signals of deposited FN did not change during the 6-h time period when DACs were incubated with PBS buffer confirms that coating FN on the surface of DAC did not influence its conformation. However, when blood plasma was used instead of PBS buffer for the incubation, FRET signals of FN on DACs slightly but continuously decreased to 86% and 94.6% during the 6-h observation time at the adventitial and luminal sides, respectively, indicating conformational changes of deposited FN upon contacting with components of the plasma.

In vitro study of FN incorporation into extracellular matrix of NIH 3T3 fibroblasts using FRET measurement showed that within 1-2 h of incubation, cell-bound FN showed initial unfolding and small fibrils formation from the cell edge [11]. Our observation that FRET signals were dramatically decreased after implantation confirms the activity of living cells and the physiological condition on conformation of FN. Our previous study had shown that, during the recellularization process, the cells dominantly invaded from the adventitial side in region A2 but not region B1 of the DAC [6]. This data agrees with the presented FRET data in which we observed that FRET signals of the same region showed a strongest reduction. The effect on FRET decrease can be seen even after 1 h of implantation. The changes of FN conformation can be caused by biomechanical forces generated by invaded cells or the high activity of MMP around the media-repopulating cells. Recent studies have shown the effect of different shear stresses on FN conformation [2-4]. Our data also demonstrates the effect of shear stress on conformation of deposited FN on DACs by the different changes in FRET signals between the adventitial and luminal sides of region A and region B.

Conclusions

FRET measurement was successfully used to monitor conformational changes of deposited FN on DACs. The differences in FRET signal reduction between the adventitial and luminal sides of region A and region B demonstrated the effect of shear stress on the deposited FN's conformation. Taken together, our data suggests a morphological-dependent biological activity of FN.

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COMPETING INTERESTS

The author declares that there is no conflict of interest regarding the publication of this article.

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