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Comparative

Fibrin matrix supports endothelial cell adhesion and migration in culture

SI Almelkar[1*], AM Patwardhan[2], SA Divate[3], NB Agrawal[1], RR Bhonde[4], AP Chaukar[1]

Abstract

Introduction

Fibrin glue (FG) mainly contains fibrin which is formed as a result of physiological blood coagulation. Fibrin plays an important role in arresting bleeding. The sealant fibrin glue is organized by using two components such as fibrinogen and thrombin. The transformation of fibrinogen into fibrin gel involves various enzymatic cascade cleavage and polymerization reactions. Enzymatic cascade reactions are thrombin dependent and involves cleavage of fibrinopeptides A and B from fibrinogen to form fibrin monomers. In the current study we investigated the role of fibrin glue which plays a part in the support of adhesion and expansion of vascular endothelial cells.

Materials and methods

Human saphenous vein endothelial cells (HSVECs) and sheep external jugular vein endothelial cells (SEJVECs) were cultured onto the fibrin glue coated tissue culture plate surfaces. HSVECs & SEJVECs cultured separately onto the fibrin glue coated surface resemble the wound healing type of morphology that is scar (healing) like formation.

Results

The results demonstrate that endothelial cells also play an important role in migration, wound healing and support scar development.

Conclusion

It was concluded that fibrin glue (FG) provides a suitable biological matrix surface for the adhesion and migration of endothelial cells which are also involved in scar like morphology development (wound healing) in the culture.

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Introduction

Fibrin glue (FG) is a biological tissue adhesive which initiates the final stages of coagulation when a solution of fibrinogen is activated by thrombin[1]. Abundant uses of fibrin glue (FG) as a sealant, hemostatic agent and adhesive have been reported in various research and surgical disciplines1. Fibrinogen associated proteins can be isolated from blood plasma using cryoprecipitation and centrifugation. The concentrated fibrinogen can be mixed with thrombin in calcium carbonate solution to form a stable adhesive gel.

Fibrin is distinguished from fibrinogen chiefly due to its insolubility under physiological conditions. Due to cross linking insoluble fibrin formed in the presence of factor XIIIa cannot be dissolved in urea and other similar solvents.

With respect to toxicity fibrin is a safe matrix. Fibrin breakdown products are well established as being toxicologically innocuous. The hemostatic properties of fibrin helps in promoting wound healing. Rapid and fast healing was reported after using fibrin powder on wounds. The coagulation pathway generates numerous vasoactive mediators and chemotatic factors which recruit cells to the wounded site. Fibrin deposition is consistent in wound healing and progresses to new blood vessel ingrowths[2]. Fibrin gel induces angiogenesis in the absence of platelets and this process was enhanced when mitogens were added in the gel. Generation of granulation tissue requires the coordinated activated and migration of vascular endothelial cells, macrophages and fibroblast into fibrin gel with resulting new blood vessel formation[3]. Application of fibrin glue provides a 3 dimensional biological matrix for wound healing which has a strong sealant property and provides support to the infiltrating vascular endothelial cells.

We have prepared the fibrin glue with plasma components of fibrinogen and thrombin. Human plasma containing fibrinogen was cryo-preserved and fresh frozen plasma (FFP) was used for thrombin preparation. The HSVECs and SEJVECs adhesion was high on fibrin coated surfaces compared to uncoated. HSVECs and SEJVECs when plated and expanded onto fibrin glue (FG) coated surface showed migration as well as spreading. This established the wound healing (scar like formation) type of major mechanisms in expanding cells, this concludes that endothelial cells are involved in healing wounds, migration and plays a significant role for scar like development. The aim of this study was to investigate role of fibrin matrix in supporting the endothelial cell adhesion and migration in culture (development of scar like morphology).

Materials and Methods

Isolation and expansion of HSVECs and SEJVECs

Institutional ethical committee CARE (Humans and Animals) permission was taken to procure the sample from patient and animal samples from the abattoir. In brief, the human saphenous vein (HSV) and sheep external jugular

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vein (SEJV) were separately cannulated and flushed with sterile PBS to remove blood and blood clots. One end of the vein (HSV & SEJV) was clamped using artery forceps and an enzyme cocktail of 0.15% collagenase type IV (Sigma Aldrich, St. Louis, MO) and dispase II (Roche, Nutley, NJ) was injected in the vein (HSV & SEJV) for detachment of ECs from the luminal wall. The entire vein was incubated for 20 min at 37°C. At the end of the incubation period, the vein was flushed using M199 medium (Invitrogen, Carlsbad, CA), and the digest was centrifuged at 1,500 rpm for 10 min. Cell pellet was resuspended in the endothelial cell growth medium (ECGM) (PromoCell GmbH, Germany) containing 20% foetal bovine serum (FBS) (Invitrogen) and seeded onto culture flasks. After 12 h of incubation, ECs were fed with complete ECGM containing 20% FBS as described by Baudin et al.[4]

Characterization of ECs by confocal microscopy

The cells from early passages were used for the characterization studies. For immunocytochemistry, study cells were grown in monolayers on coverslips and were fixed using 4% paraformaldehyde as described by Wagner et al.[6] The cells were then permeabilized using 50% methanol for 5 min followed by treatment with 5% bovine serum albumin (BSA) in PBS for 1 h to block nonspecific binding sites. The cells were then exposed to anti-von-Willebrand factor (vWF), a primary non-labelled antibody IgG fraction of antiserum developed in the rabbit (1:200 dilution) (Chemicon, Temecula, CA), for 12 h at 4°C, followed by respective FITC-tagged antirabbit secondary antibody (Invitrogen) for 1 h at 37°C. The coverslips were mounted on a mounting medium containing antifade (Vectashield, Vector Laboratory, Burlingame, CA) and 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). The slides were then viewed using a confocal laser-scanning microscope—LSM 510 Zeiss workstation (Carl Zeiss Meditec AG, Jena, Germany). DAPI was used for nuclei visualization[5].

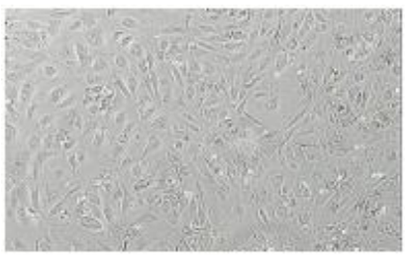

Figure 1: HSVECs expanded in cobblestone morphology on FG non-coated surfaces.

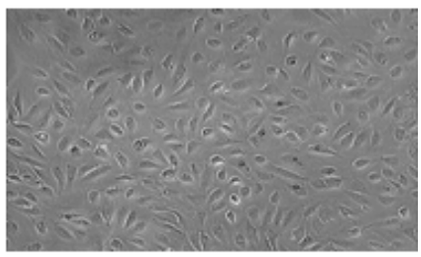

Figure 2: SEJVECs expanded in cobblestone morphology on FG non-coated surfaces.

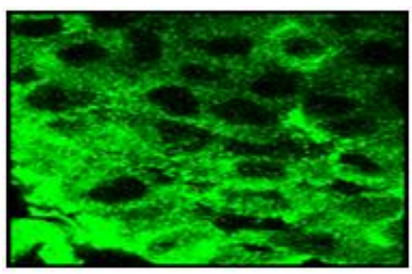

Figure 3: HSVECs showed positive expression of vWF.

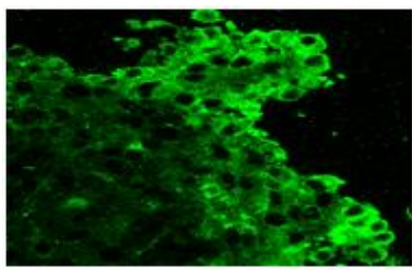

Figure 4: SEJVECs showed positive expression of vWF.

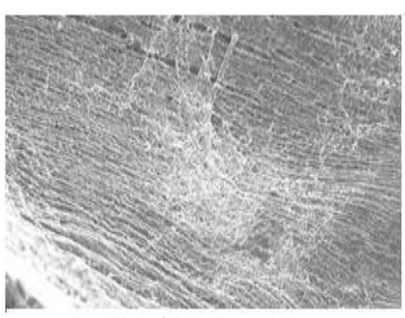

Figure 5: SEM of FG Matrix Meshwork.

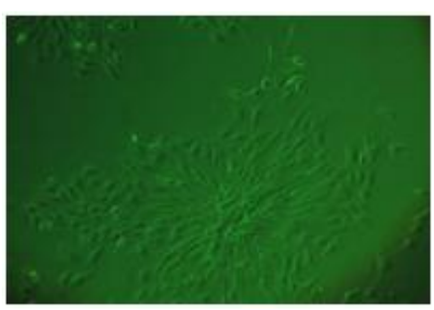

Figure 6: Fibrin Glue (FG) plated HSVECs showing wound healing type of appearance.

Preparation of Fibrinogen

Fresh 10 ml of venous blood was drawn in a heparinised centrifuged tube and further centrifuged at 3000 rpm for 10 mins. Separated plasma was aspirated and stored at -200 Centigrade(C)[6].

Preparation of Thrombin

This was prepared according to the technique described by Saxena et al.[6] Briefly 1ml of Fresh frozen plasma (FFP) was thawed at 2-40C.FFP was further diluted with distilled water (1:10) making the volume of 10ml. 0.1ml of 1 percent (%) acetic acid was added to make pH 5.3.After addition of acetic acid precipitate was developed and it was further kept for exactly 30 mins. Centrifugation was done at 3000 rpm for 5 mins followed by precipitate collection and normal saline (10ml) was added and pH was brought to 7 by addition of sodium carbonate. This was put in water bath (370C) and 0.1 M Calcium Chloride (CaCl2) was added. The clot formed in 45-120 seconds was removed. Thrombin solution formed was watery clear.

Fibrin Glue (FG) preparations

Fibrinogen 50µl and thrombin 50µl was added and mixed well on the tissue culture plate surface[6]. Further the FG mixture was dried under the laminar flow and immediately plate surface was used for cell seeding. Scanning Electron

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Microscopy (SEM) was done for observation of the FG matrix.

Proliferation of HSVECs and SEJVECs on FG coated surfaces and wound healing type of morphology

The split ratio for the subculture was kept at 1:2, and cells were seeded in fresh tissue culture plate coated with and without FG. Sub-culturing of ECs was done at 70% confluence. The HSVECs and SEJVECs were observed under phase contrast microscope to note the wound healing type of mechanism. The proliferation rate was calculated by plating fixed population densities of ECs and their recovery after a number of days. Growth curve was plotted for HSVECs and SEJVECs for with and without coating. Wound healing type of mechanism for expanded HSVECs and SEJVECs were studied for FG coated surface.

Statistical analysis

Mean ⁺ SD, t' test was performed using Microsoft excel statistical software and $P<0.05$ & $P<0.005$ was considered as statistically significant.

Results

Isolation and expansion of HSVECs and SEJVECs

Isolated HSVECs and SEJVECs showed nearly about 20-30 clusters. These clusters were expanded in vitro and reach 70% confluence with cell density of 4.5 x 10^5 cell/ cm2 for HSVECs while SEJVECs showed 5 x 105 cells/cm^2. These freshly isolated cells adhered to the culture well within 4 h of seeding. Proliferated HSVECs (Figure 1) and SEJVECs (Figure 2) showed perfect cobblestone morphology.

Characterization of ECs by confocal microscopy

HSVECs (Figure 3) and SEJVECs (Figure 4) characterized by immunocytochemistry (confocal microscopy) and showed positive phenotypic expression for von-Willebrand factor ($vWF^{+/+}$).

Preparation of Fibrinogen

The separated human plasma stored at -20°C was a rich source of natural fibrinogen.

Preparation of Thrombin

Thrombin solution prepared to a concentration which gave a thrombin time of 20 seconds was stored at -200°C till further use.

Fibrin Glue (FG) preparation

HSVECs and SEJVECs showed excellent attachment on the FG (1:1 mixture) coated surface. Scanning electron microscopy (SEM) confirmed the web like meshwork of Fibrin matrix (clot) formation on the tissue culture surface (Figure 5). 3.6 Expansion of HSVECs and SEJVECs on FG coated surfaces. HSVECs and SEJVECs showed best adhesion on FG coated surface as compared to non-coated FG surface. After 2h of seeding about maximum 80% of the cells were completely spread on the surface and by 4h the remaining attached cells were also organized in the culture area. It is clearly observed that cytoplasm occupied maximum area on the FG coated surface. Growth curve showed that there was not much difference for the cell density of HSVECs and SEJVECs after reaching on 70% confluence (mean + S.D) (Figure 8).

HSVECs and SEJVECs coated on FG surface showed faster proliferation as compared to non-coated surface.

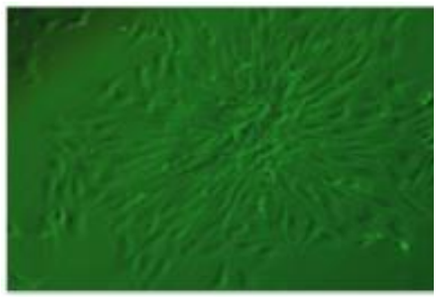

Figure 7: Fibrin Glue (FG) plated SEJVECs showing wound healing type of appearance.

HSVECs ($P<0.005$) and SEJVECs ($P<0.004$) reached confluence on day 6 while non-coated surface expanded HSVECs ($P<0.05$) and SEJVECs ($P<0.05$) reached confluence on day 12. Cell migration, spreading and wound healing type of scar formation was observed on 2th day which further showed expansion of HSVECs (Figure 6) and SEJVECs (Figure 7) in culture.

Discussion

Majority of tissue adhesives are organic compounds especially long chain covalently linked polymer. There two types of tissue adhesives 1. synthetic-n-butyl-2-cyanoacrylate. 2.Biological source-Fibrin glue. The isolated endothelial cells were characterized by immunocytochemistry for the presence of von-Willebrand factor ($vWF^{+/+}$).

Wounds that are only a few cells wide repair themselves through cell spreading. While medium sized wound require both spreading and migration

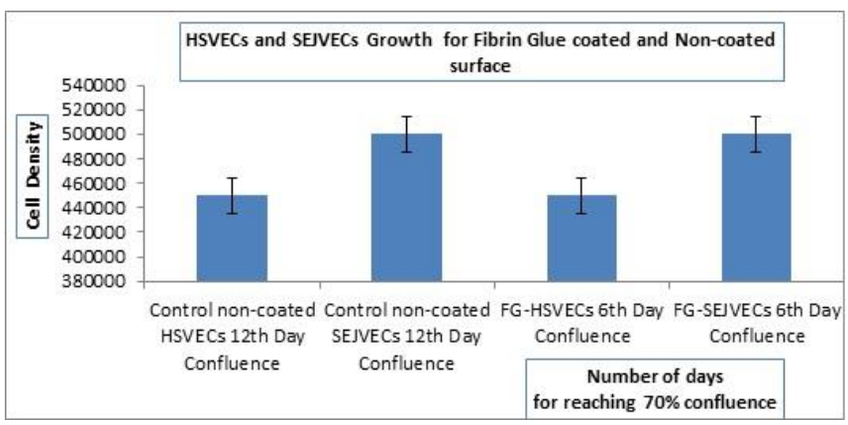

Figure 8: HSVECs and SEJVECs Growth period for Fibrin Glue coated ($P<0.005$) and Non-coated surface ($P< 0.05$).

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of endothelial cells to fit a wide injury and heal (scar formation).

It is well-established that fibrin glue brings about wound healing and angiogenesis by speeding the expansion and migration of cells which are actively involved in wound repair process like endothelial cells. Fibrin glue matrix behaves as a biological scaffold to entrap the released components needed for spreading, cell growth and support on which the endothelial cells migrate. Fibrin glue has numerous advantages for cell adhesion and tissue adhesion. Fibrin glue reduces total cell adhesion time on tissue culture plate surface. No adverse reactions to fibrin glue have been reported when used with endothelial cells. The thrombogenicity of fibrin glue coated surface is also less as compared to other cell adhesives.

In our study endothelial cells were expanded on fibrin glue coated and uncoated surfaces. Thrombin is very expensive and has storage problems so we have obtained fibrinogen from human blood and thrombin from fresh frozen human plasma. The isolated endothelial cells were characterized by immunocytochemistry for the presence of von-Willebrand factor (vWF+/+). The endothelial cells were noted to undergo well characterized architechtural and cytoskeletal changes during migration. The HSEVCs and SEJVECs showed complete attachment, spreading in cytoplasmic area, migration and wound healing (scar formation) type of appearance in the culture.

Endothelial cells are likely to have higher number of adhesive bonds to increase its attachment strength. This emphasizes that endothelial cells also plays an important role in migration, wound healing and supports scar development at in-vivo conditions.

Conclusion

Our study concludes that fibrin glue (FG) meshwork supports adhesion and migration of endothelial cells in development of scar like morphology (wound healing) in the culture.

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Abbreviations

FG = Fibrin glue, vWF=von-Willebrand Factor

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