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Short communication

Salmon fibrin supports an increased number of sprouts and decreased degradation while maintaining sprout length relative to human fibrin in an *in vitro* angiogenesis model

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Abstract—Salmon-derived fibrin has been proposed as a preferred alternative to human or bovine fibrin because of its reduced potential for disease transmission. Here we evaluate salmon fibrin as an alternative ECM support for therapeutic angiogenesis applications, such as vascularizing engineered tissues. Human umbilical vein endothelial cells (HUVEC) seeded on gelatin beads and suspended in either salmon or human fibrin sprouted and formed capillary-like structures. Sprout length was generally increased with the addition of bFGF and VEGF and further increased with the addition of phorbol myristate acetate (PMA). The number of sprouts per bead was increased 61–188% in salmon fibrin relative to human fibrin ($\alpha < 0.0005$) in cultures receiving growth factors and PMA, while average sprout lengths were similar for HUVEC within human or salmon fibrin. Additionally, under these conditions in the absence of a protease inhibitor, HUVEC appeared to degrade human, but not salmon, fibrin. These results support the idea that salmon fibrin may be an attractive alternative ECM able to support microvascular network formation.

Key words: Fibrin; angiogenesis; microvascular network; salmon fibrin; extracellular matrix support.

INTRODUCTION

As lack of vasculature is one of the primary limitations in the development of engineered tissues [1], as well as isolation devices and biosensors, there is great interest in investigating materials that induce or support microvascular network formation *in vitro* or angiogenesis *in vivo* [2]. Extracellular matrix (ECM) gels are attractive for this purpose because they provide a native-like environment, including appropriate biological signals, for endothelial cells. In particular, fibrin is an

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attractive support because of its presence in angiogenic provisional wound matrices [3], its ability to support capillary morphogenesis *in vitro* [4–6] and angiogenesis *in vivo* [7, 8], and its use in various tissue engineering applications [9–13]. It has been proposed that fibrin gels derived from cold-water fish plasma have reduced risk of disease transmission relative to those derived from non-autologous human or bovine sources due to the large evolutionary distance and much lower body temperature of fish [14]. Additionally, salmon fibrin gels show low immunogenicity in animal studies [14], although immunocompatibility has not been fully assessed in humans. In this report, we employed an *in vitro* angiogenesis model to evaluate the ability of salmon fibrin, which may be an attractive alternative ECM to support capillary morphogenesis.

MATERIALS AND METHODS

Human umbilical vein endothelial cells (HUVEC, Cascade Biologics, Portland, OR, USA) were cultured in collagen-coated flasks in EBM-2 media (Cambrex Bio Science, Baltimore, MD, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Cytodex3 beads (Amersham, Piscataway, NJ, USA) were hydrated in buffer and autoclaved as directed by the manufacturer and stored at 4°C in media until use. Cells in suspension were seeded onto beads at approximately 50 cells/bead and allowed to grow to confluence for 3 days with gentle agitation.

Salmon (*Salmo salar*) fibrinogen (Sea Run Holdings, Freeport, ME, USA) was obtained from salmon blood anticoagulated with EDTA and fractionated with ammonium sulfate. Though fibrinogen was not depleted of either fibronectin or plasminogen, plasmin activity was inhibited by addition of 30 mM epsilon amino caproic acid. Lyophilized salmon fibrinogen samples were reconstituted in water (5 mg/ml) and further diluted in culture media. Human fibrin (originally 58 mg/ml in 20 mM sodium citrate, Enzyme Research Laboratories, South Bend, IN, USA), plasminogen depleted by the manufacturer using affinity chromatography, was diluted to 5 mg/ml in water and further in culture media. Cell-seeded beads were suspended (125 beads/ml) in either 2 mg/ml human fibrinogen or 2 mg/ml salmon fibrinogen, with or without 200 U/ml aprotinin (American Diagnostica, Greenwich, CT, USA). Fibrin polymerization was initiated by rapidly pipet mixing 200 μl of the fibrinogen/bead solution into 48-well plates containing 10 μl human thrombin (originally 200 U/ml, Sigma-Aldrich, St. Louis, MO, USA). After 30 minutes gels were covered with media, media supplemented with basic fibroblast growth factor (bFGF, R&D Systems, Indianapolis, IN, USA) and vascular endothelial growth factor (VEGF, R&D), or media supplemented with bFGF, VEGF and phorbol myristate acetate (PMA, Sigma-Aldrich). Media was supplimented with 200 U/ml aprotinin as indicated. bFGF, VEGF and PMA were all at 50 ng/ml. Gels were cultured in a humidified 37°C incubator for 4 days and fixed with 4% paraformaldehyde. Twenty beads per group were imaged using a digital

camera (PVC100C, Pixera, Los Gatos, CA, USA) mounted on a Precision Inverted microscope (PIM-III, World Precision Instruments, Sarasota, FL, USA) and number and length of sprouts were quantified using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). Sprout length was measured from the edge of the cell layer, not the bead, to the tip of the sprout. Distance of the cell layer from the bead was measured at 8 points, 45° apart around the circular bead and averaged for each bead. Statistical significance was assessed by performing ANOVA followed by the Tukey–Kramer honestly significant difference test for multiple comparisons using JMP software (SAS, Cary, NC, USA).

RESULTS

After polymerization, both human and salmon fibrin gels were relatively transparent in appearance. After 4 days of culture, HUVEC seeded on gelatin-coated dextran beads and embedded in either salmon or human fibrin had formed capillary-like sprouts (Fig. 1) as previously reported by others using bovine or human fibrin [4].

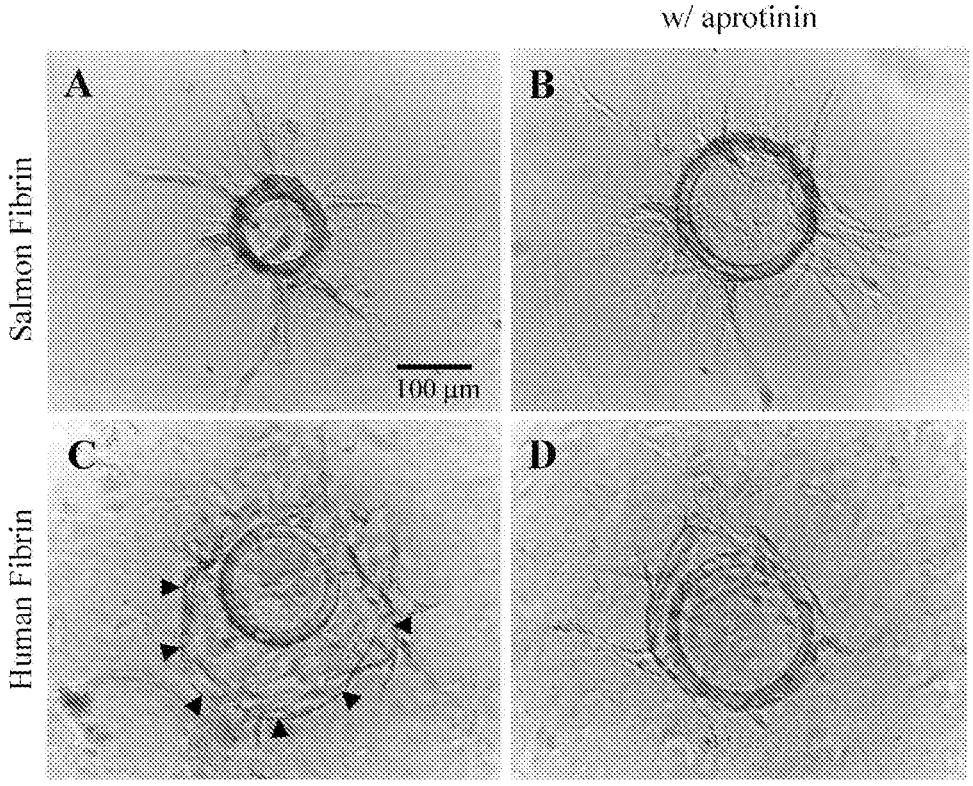

Figure 1. Photomicrographs of HUVEC seeded on dextran beads and suspended in salmon (A and B) or human (C and D) fibrin after 4 days of culture in media supplemented with bFGF, VEGF and PMA (all 50 ng/ml). Some cultures were further supplemented with aprotinin (B and D).

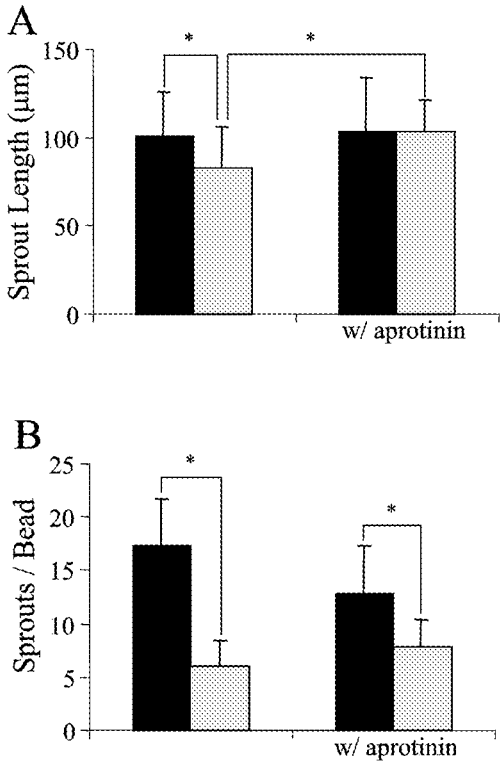

Figure 2. Average sprout length (A) and number of sprouts per bead (B) for HUVEC on dextran beads in salmon (black bars) or human (grey bars) fibrin. Asterisks represents $\alpha < 0.05$, see Tables 1 and 2 for additional statistical comparisons.

Average sprout lengths were similar (Fig. 2A and Table 1) in both salmon and fish fibrin cultured in media supplemented with bFGF, VEGF and PMA, which is commonly used in *in vitro* angiogenesis assays [5, 15–17]. There was a slight, but statistically significant, decrease in sprout length by HUVEC in human fibrin without aprotinin (Fig. 2A and Table 1). The number of sprouts per bead in both salmon fibrin groups was much higher ($\alpha < 0.0005$, Fig. 2B) than in both human fibrin groups cultured with bFGF, VEGF and PMA. Sprout length was similar between all groups receiving media only and was increased by the addition of bFGF and VEGF, and further increased by the addition of bFGF, VEGF and PMA (Table 1). The number of sprouts per bead were similar between groups cultured in media or with bFGF and VEGF (Table 2). Generally, the addition of aprotinin had little to no effect on sprouting behavior in different conditions (Figs 1 and 2, Tables 1 and 2). However, in human fibrin cultures with bFGF, VEGF and PMA, and without aprotinin, the fibrin around the bead appeared to have been degraded, resulting in a cell layer detached from the bead (Fig. 1C) and fewer distinct sprouts. It is interesting to note that sprouts in the human fibrin without aprotinin reached similar distances from beads if the observation that cell layers were 32 ± 20 μm

Table 1.
Average sprout length ($n = 20$ beads per group)

Group	GF + PMA	GF	Media
Salmon fibrin + aprotinin	103 ± 31^{a}	89 ± 32	42 ± 11^{c}
Salmon fibrin	101 ± 25^{a}	87 ± 25	50 ± 27^{c}
Human fibrin + aprotinin	103 ± 18^{a}	73 ± 16^{b}	53 ± 10^{c}
Human fibrin	83 ± 23	80 ± 17	55 ± 11^{c}

[a] Significantly different from human fibrin in same treatment, $\alpha < 0.05$.
[b] Significantly different from GF and PMA in same group, $\alpha < 0.0005$.
[c] Significantly different from GF and GF + PMA in same group, $\alpha < 0.005$.

Table 2.
Sprouts per bead ($n = 20$ beads per group)

Group	GF + PMA	GF	Media
Salmon fibrin + aprotinin	$13 \pm 5^{a,c}$	7 ± 2^{b}	8 ± 3
Salmon fibrin	$17 \pm 4^{a,c}$	8 ± 4^{b}	9 ± 3
Human fibrin + aprotinin	8 ± 2	8 ± 3^{b}	9 ± 3
Human fibrin	6 ± 2^{c}	12 ± 4	10 ± 3

[a] Significantly different from human fibrin and human fibrin + aprotinin in same treatment, $\alpha < 0.0005$.
[b] Significantly different from human fibrin in same treatment, $\alpha < 0.05$.
[c] Significantly different from GF and Media in same group, $\alpha < 0.0005$.

from the edge of the bead is taken into account. Significant degradation of the fibrin surrounding the bead was not observed in other culture conditions with human fibrin or in any conditions with salmon fibrin.

DISCUSSION

Sprout formation by human endothelial cells in fibrin or other ECMs is typically investigated in the presence of angiogenic growth factors, such as bFGF and VEGF, as well as phorbol esters, such as PMA, which are reported to be necessary for significant elongation [15–17]. Because these are the most frequently studied conditions and those which produced maximal sprouting, we focused on this treatment group. Salmon fibrin supported a larger degree of sprouting in that the number of sprouts in salmon fibrin cultures was significantly increased relative to human fibrin in cultures with bFGF, VEGF and PMA, with or without aprotinin, while sprout length was similar. The decrease in sprout length in human fibrin without aprotinin may be related to the degradation of the matrix around the beads that caused a detached cell layer around the bead. Protease inhibitors, such as aprotinin, are typically included in *in vitro* angiogenesis assays to prevent degradation and extreme contraction of bovine or human fibrin [4, 5]. Inclusion of aprotinin in cultures should reduce ambiguity in differences between salmon and human fibrin potentially due to residual plasminogen in the different fibrin preparations. Further, the lack of significant differences in cultures with media

only, in contrast to those with growth factors and PMA supplemented, suggests that observed differences are not due to differences in angiogenic factors that may co-purify with the different fibrins.

In summary, the results from an *in vitro* angiogenesis model indicate that salmon fibrin gels support capillary sprouts similarly to human fibrin, with an increase in sprout number, similar average length, and apparent resistance to degradation. These data, coupled with its reduced potential for disease transmission risk and immunocompatability [14], suggest that salmon fibrin warrants further study into its ability to induce vascularization, particularly for tissue engineering applications.

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