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Fibrin concentration affects ACL fibroblast proliferation and collagen synthesis

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Abstract

Fibrin is a frequently used biomaterial in surgery and tissue engineering. While it has been shown that fibrin supports cellular proliferation and biosynthesis, there is a scarcity of studies focusing on the effects of fibrin concentration. The objective of this study is to assess the effect of fibrin concentrations around the physiological concentration of 3 mg/ml on the behaviors of ligament fibroblasts. Fibroblasts were obtained from the anterior cruciate ligaments of four pigs and seeded throughout fibrin gels of either 1, 3, or 6 mg/ml fibrin. The gels were collected at 2, 6, and 10 days for measurement of DNA and collagen content. We found that both DNA and collagen content increased significantly over time in gels made with all concentrations of fibrin. However, the increases were significantly lower in gels made with the higher concentrations of fibrin (3 and 6 mg/ml). Microscopic assessment of FITC-labeled gels showed a decrease in pore size at high fibrin concentrations, which might be a reason for the observed effect on bioactivity. To enhance cell behavior and thus clinical results fibrin applications should build on physiologic or sub-physiologic concentrations, and those with higher concentrations, such as currently available sealants, should be used cautiously.

Keywords

Fibrin; Fibroblast; Tissue Engineering; Ligament; ACL

1. Introduction

Tears of the anterior cruciate ligament are among the most important disease entities in orthopaedics because of both their increasing incidence in today's highly active population and the associated increase in the risk of osteoarthritic degeneration. Recently, evidence has been presented that the latter remains unabatedly high, even after the current gold-standard treatment, ACL reconstruction[1]. These findings have precipitated a strong interest in regenerative medicine, more specifically tissue engineering, for the management of ACL

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tears[2]. One of the most important aspects in such an approach is the choice of an appropriate biomaterial that supports cellular behavior and thus tissue regeneration[2,3].

Fibrin is a naturally occurring, biocompatible, biodegradable polymer that plays a crucial role in wound healing by entrapping platelets in a scaffold filling the wound site. Thus, fibrin provides a scaffold for cellular migration and, by incorporating the platelets, also facilitates platelet-driven growth factor release in the wound site. Wounds that do not form a fibrin clot such as ruptured ACL or articular cartilage therefore lack the capacity for intrinsic healing, but may heal if a substitute clot or an equivalent is substituted[4-8]. Beyond being used as an adhesive[9,10], fibrin has been investigated as a delivery method for growth factors and cells[11-13]. More complex cell-fibrin compounds are used for skin tissue engineering, injectable bone, or as a biomaterial for autologous chondrocyte implantation[8,14-17]. These facts make fibrin a logical choice for ACL tissue engineering.

While none of these applications depend primarily on biomechanical strength, which generally is low for fibrin clots, all of them do depend on cellular proliferation and, more importantly, on cellular biosynthesis and tissue remodeling. If fibrin had beneficial effects on cellular behavior, a dose-response relationship should be seen. Although there are relatively few studies investigating this dose-response, their results are quite consistent and show reduced cellular activity with increasing levels of fibrin concentration[18,19]. However, these studies focused on fibrin concentrations ranging from 5 mg/ml to 50 mg/ml, which is considerably higher than the physiological concentration of approximately 3 mg/ml[20-22].

It is the objective of this study to assess the effects of three different concentrations of fibrin, centered around the physiological value of 3 mg/ml, on proliferation and collagen biosynthesis of ACL fibroblasts and clot structure. Our hypothesis was that the highest cellular proliferation and collagen production would be seen at the lowest value of fibrin concentration.

2. Materials and Methods

This study was designed as an in vitro assessment of the effects of fibrin concentration on clot structure, and ACL fibroblast proliferation and biosynthetic activity. To study the effect of fibrin concentration on the clot structure descriptively, unseeded fibrin clots were labeled by spiking fibrinogen solutions with 12.5 μ g Alexa Fluor 488 conjugated fibrinogen (Molecular Probes, Eugene, OR) per 1 mg/mL of fibrinogen. Clotting was initiated by adding 50 U/ml thrombin (Jones Plasma, MO, USA) at a 1:0.02 ratio of fibrinogen:thrombin. 50 μ l of labeled solution were placed on microscope slides to gel, cover-slipped, and instantly visualized using a Nikon Eclipse TE2000-U.

Porcine ACL fibroblasts were obtained from explant cultures of biopsies from adolescent Yucatan pigs (n=4). Cells were expanded at 37°C in 95% rH and 5% CO₂ using a standard medium containing DMEM, 5% FBS, 100 IU/ml Penicillin, 100 mg/ml Streptomycin, 0.25 μ g/ml Amphotericin B, and 250 μ M ascorbic acid. Cells from second and third passage were used for all experiments.

Briefly, fibroblasts were trypsinized and resuspended at a concentration of 250,000 cells per ml in HBSS-based fibrinogen solutions of 1, 3, and 6mg/ml (Sigma-Aldrich, MO, USA). The physiological concentration of fibrin has been defined as 3 mg/ml with a variation from 2.3 to 4.9 mg/ml[22]. We chose a higher and a lower concentration, lying well without the range of the physiological concentration but still within biologically reasonable values, to frame this physiologic value. Fibrin gels were formed by adding 50 U/ml thrombin in 40 mM CaCl₂ (Jones Pharma, MO, USA) at a 1:0.02 ratio of fibrinogen:thrombin to 1 ml of

fibrin-cell suspension in a 48-well plate. Suspensions were allowed to gel for 1h in at 37°C before standard medium was added. Medium was changed every other day and triplicates of fibrin gels were obtained at 2, 6, and 10d.

The triplicate gels were lyophilized, weighed, and digested following a standard papain protocol. DNA content of the digested samples was determined with the Quant-iT PicoGreen assay (Molecular Probes, Eugene, OR, USA) and collagen content was measured using the Sircol collagen assay (Biocolor, Carrickfergus, UK). Additionally, cell culture medium was collected throughout the experiment and analyzed for content of collagen using the aforementioned Sircol collagen assay. All analyses were controlled for contents of empty gels.

Total RNA was extracted using the RNeasy kit (Qiagen) as specified by the manufacturer. RNA content was quantified spectrometrically at OD 260/280 nm. One microgram of RNA was reverse-transcribed into cDNA using RETROscript Kit (Ambion, TX). Amplification was performed in ABI PRISM 7900 Sequence Detection System (Applied Biosystems, CA) using SYBRGreen PCR Master Mix Kit (Applied Biosystems, CA). Targeted genes were type I and type III procollagen (COL1A1 and COL3A1). The transcript level of target genes normalized to GAPDH was calculated using the $2^{-\Delta Ct}$ formula.

Results are presented as mean $\pm$ SD with 95% confidence intervals. Groups were compared using oneway ANOVA with Bonferroni adjustment for subgroup comparison. The effect on cellular behavior of different fibrin concentration was assessed by multivariate regression modeling, controlling for the potential confounding by donor, age, gender, and time in culture. A p-value of less than 0.05 was assumed significant. All calculations were done using intercooled STATA 10 (Stata Corp LP, College Station, TX, USA).

3.Results

Microscopic assessment was done to assess the effect of fibrin concentration on fibrin clot structure. While both 1 and 3 mg/ml showed a relatively homogenous degree of porosity, the small increase to 6 mg/ml resulted in obvious decrease in pore size. Furthermore, the 1 and 3 mg/ml clots demonstrated a more homogenous structure, whereas the 6 mg/mL clots had a rather featureless, amorphous appearance. (Figure 1)

Measurement of the DNA content per dry weight of the gel showed statistically significant associations with time ($p=0.014$) and fibrin concentration ($p=0.002$). The subgroup analysis showed that most of the difference existed between 1 mg and 3 mg ($p=0.004$) with no significant difference in the DNA content of gels between 3 mg and 6 mg ($p=1.0$), although this is likely due in part to the rather large standard errors for measurements at 10 days. Data for DNA content were furthermore regressed on concentration and time. The regression analysis, adjusting for potential confounders, showed a increase in DNA of 1.3 $\mu\text{g}/\text{mg}$ dry weight per day in culture (95%CI 0.4 to 2.1) and a 1.82 $\mu\text{g}/\text{mg}$ dry weight decrease (95%CI 3.2 to 0.5) in DNA content with each mg increase in fibrin concentration. (Table 1)

The analysis of variance for collagen content of lyophilized constructs showed significant associations with both time ($p<0.001$) and fibrin concentration ($p<0.001$). Again, the subgroup analysis showed that this significance was based on the difference between the 1 mg and 3 mg group ($p=0.004$), while there was no significance difference between the 3 mg and the 6 mg groups ($p=1.0$). The regression model predicted an increase of 292 μg (95%CI 162 to 421) collagen per mg dry weight per day and a decrease in 352 μg (95%CI 145 to 557) collagen per mg dry weight per mg increase in fibrin concentration. (Table 2) These patterns remained when collagen was adjusted for DNA content with significant associations with time ($p=0.039$) and concentration ($p=0.003$), showing a 0.7% (95%CI 0.01% to 1.5%)

increase in the collagen/DNA ratio per day and a 8% decrease (95%CI 13% to 2.7%) per additional mg of fibrin concentration. The content of soluble collagen in the culture medium varied significantly with time ($p=0.043$) and concentration ($p=0.003$). More specifically, there was an increase in collagen content until 6 days of culture, and later a decrease until day 10. (Table 3)

All samples showed a consistently expression of type I and type III collagen mRNA. Neither type I nor type III collagen mRNA contents were associated with fibrin concentration after adjustment for time in culture ($p=0.318$, $p=0.379$; Tables 4 and 5) Regression of mRNA content on fibrin concentration showed that concentration was not a significant predictor for either type I ($p=0.933$) or type III collagen ($p=0.985$) mRNA expression.

4. Discussion

Tissue engineering holds much promise for regenerative approaches to ACL treatment[2]. Fibrin is a logical candidate as a biomaterial for such ACL tissue engineering. The objective of this study was to assess the effects of fibrin concentration on fibroblast proliferation and biosynthetic activity, effects that will support or hinder the outcome of any potential fibrin-based procedure. We chose to study an adolescent model to account for the age-dependence of ACL fibroblast behavior[23,24], and the fact that adolescent individuals, a group characterized by a sharply increasing incidence of ACL injuries and a lack of treatment options, might profit most from a tissue-engineered approach to ACL treatment[25,26]. Our findings show that while all groups showed increasing DNA and collagen content over time, a lower than physiological concentration of fibrin showed the highest DNA and collagen production

Our study has some potential weaknesses. One is that our gels have a physiological concentration of fibrin, but that does not necessarily mean they have a physiological structure. As a matter of fact, previous in-vitro research in fibrin clot structure has shown structurally homogenous, porous clots at lower concentrations and rapidly polymerizing, non-homogeneously dense clots with very small pores at higher concentrations[19]. Thus our findings are perfectly valid for fibrin-based biomaterials, but not necessarily for biologic wound healing. Finally, we chose a rather narrow range of fibrin, and a wider range would have shown differences in effects more clearly. However, we wanted to build our model on a biological important parameters, thus we chose the physiological concentration of 3 mg/mL[21,22] and a biologically plausible range around this value[27-29]. Finally, we chose to use a porcine model, rather than a human model. Beyond the obvious problem of availability of human tissue, most of the basic science in ACL tissue engineering is based on porcine models[2,5,6,26,30], thus results that pertain to such models and allow for interpretation in their context seem more important immediately, despite the fact that eventually a human application is aimed at. From what is known, extraction of the results of porcine models and application in human disease is possible and valid given the considerable commonalities in cell behavior, tissue composition, and anatomy[2,31].

Our results demonstrated steady increases in DNA and collagen over time in all of the gels. The increase rate in DNA (25% per day) matches commonly accepted doubling times of approximately 3 days for mesenchymal cells. We furthermore interpreted the fact that collagen content in the culture medium decreased after peaking at 6 days while collagen content in the cell-fibrin constructs rose unabatedly as an indicator of incorporation of collagen into newly formed extra-cellular matrix during tissue remodeling. The assumption of high tissue remodeling is supported by the considerably fast rate of degradation seen in most fibrin gels.

In summary, our data suggest that all groups showed ongoing cellular growth and proliferation as well as tissue remodeling, suggesting the fibroblasts in the gels were viable and active. In this model we observed attenuated DNA synthesis and collagen production with increasing fibrin concentration. A very likely reason for such an effect is an increase in biomaterial density beyond the limits of permeability and nutrient transfer. This explanation, rather than a direct suppressive effect of fibrin on cells, is supported by the non-linear nature of this effect as was shown by the larger differences between 1 mg and 3 mg compared to 3 mg and 6 mg and earlier published evidence for increased porosity in gels from diluted fibrin[18]. Our own microscopic analysis, in accordance with data put forward by Cox et al. and Ho et al., showed fairly similar structural characteristics for 1 and 3 mg/mL and a much denser, less porous appearance at 6 mg/ml.

Our interpretation of the effects of fibrin concentration is supported by both statistically significant results and substantive absolute differences. Finally, it is very interesting that there are no significant differences in type I and type III collagen mRNA contents, suggesting that the cellular genetic programming to produce collagen is unaffected, but that biosynthesis may be inhibited on the post-translational level. This finding, too, supports our interpretation of the effects of tissue density, which might inhibit anything from nutrient transfer to extracellular protein assembly, rather than direct cellular effects. From our regression model, we could show that fibrin concentration is not a significant predictor of collagen mRNA production, thus our results may be valid even beyond our chosen range of concentrations. Finally, we interpret the non-significant reduction in mRNA levels over time as a negative feed-back mechanism, which is visible due to the short duration of our observation, and, again, may suggest accumulation of collagen precursor product intracellularly because of inhibit post-translational processing.

Findings from earlier studies corroborate our results. Ho et al studied the combined effects of fibrin concentration and thrombin concentration on fibroblast behavior, yet at much higher than physiological concentrations of fibrin[32]. This group reported decreased cell proliferation with increases in fibrin concentration as well. Additionally, it could be demonstrated that this effect could be countered by increases in thrombin concentration, which might result in more organized clot formation. Cox and colleagues presented similar findings in 2006 for mesenchymal stem cells, but could also show that thrombin effects are most meaningful at low fibrin concentrations. Murray et al., finally, demonstrated that high concentrations of thrombin inhibit cellular migration and reduce biomaterial strength. These findings were included in our study design to avoid interaction with the effects of fibrin concentration.

5. Conclusion

In conclusion our findings suggest that lower concentrations of fibrin produce the highest rates of ACL fibroblast proliferation and collagen synthesis, thus commending low concentrations for fibrin-based biomaterials in ACL tissue engineering. As a corollary, the concentration of fibrin in commercially available fibrin glue might have a detrimental effect on fibroblast behavior according to these results and such adhesives should therefore be used with care.

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Figure 1. shows representative areas of FITC-labeled clots for the three different concentrations. Note the decrease in porosity from low (1 mg/ml and 3 mg/ml) to high (6 mg/ml) concentrations. (100× magnification, size bar represents 100 μ m).

Table 1

shows the DNA content in the cell-fibrin constructs by time and fibrin concentration. Details of statistical significance are given in the text.

DNA content in $\mu\text{g}/\text{mg}$ dry weight (mean $\pm$ sd)				
		days of culture		
		2	6	10
	1	15.58 $\pm$ 3.1	28.6 $\pm$ 22.5	27.03 $\pm$ 16.3
fibrin concentration (mg/mL)	3	7.98 $\pm$ 2.1	11.75 $\pm$ 2.2	15.41 $\pm$ 5.7
	6	8.94 $\pm$ 2.1	12.85 $\pm$ 4.8	20.13 $\pm$ 15.6

across time - $p=0.014$

across concentration - $p=0.004$

Table 2

shows the collagen content in the cell-fibrin constructs by time and fibrin concentration.

Collagen content in mg/ml dry weight (mean $\pm$ sd)				
		days of culture		
		2	6	10
	1	1.76 $\pm$ 0.6	4.27 $\pm$ 1.2	4.67 $\pm$ 2.7
fibrin concentration (mg/mL)	3	0.56 $\pm$ 0.2	1.29 $\pm$ 0.3	2.47 $\pm$ 2.9
	6	0.53 $\pm$ 0.3	1.78 $\pm$ 9.8	2.52 $\pm$ 2.7

across time - $p < 0.001$

across concentration - $p < 0.001$

shows the concentration of collagen in cell culture medium over time. Collagen concentration was not directly compared across groups because this analysis would be confounded by differences in cell permeation of constructs due to differences in construct density at different fibrin concentrations.

Table 3

Collagen content in cell culture medium (mg/mL, mean ± sd)		days of culture				
		2	4	6	8	10
fibrin concentration (mg/mL)	1	69.18 ± 3.3	83.6 ± 10.1	124.72 ± 23.9	120.53 ± 18.3	89.53 ± 16.1
	3	129.8 ± 36.9	430.8 ± 238.4	254.75 ± 73.8	126.25 ± 38.3	94.68 ± 14.3
	6	93.0 ± 17.2	102.7 ± 16.0	171.18 ± 66.6	155.48 ± 12.4	130.05 ± 40.9

Table 4

shows the results from PCR of type I collagen mRNA.

Type I collagen mRNA content (mean $\pm$ sd of delta Ct)				
		days of culture		
		2	6	10
	1	3.39 $\pm$ 5.1	1.13 $\pm$ 0.85	0.89 $\pm$ 1.32
fibrin concentration (mg/mL)	3	0.51 $\pm$ 0.47	0.08 $\pm$ 0.03	0.2 $\pm$ 0.1
	6	3.24 $\pm$ 5.81	0.91 $\pm$ 1.0	0.67 $\pm$ 0.42

across time - $p=0.2313$

across concentration - $p=0.3378$

Table 5

shows the results from PCR of type III collagen mRNA.

Typ III collagen mRNA content (mean $\pm$ sd of kDelta Ct)				
		days of culture		
		2	6	10
	1	0.82 $\pm$ 0.35	0.93 $\pm$ 0.97	0.32 $\pm$ 0.21
fibrin concentration (mg/mL)	3	5.86 $\pm$ 9.29	0.37 $\pm$ 0.05	0.82 $\pm$ 0.9
	6	1.21 $\pm$ 0.94	0.72 $\pm$ 0.14	0.27 $\pm$ 0.17

across time - $p=0.3154$

across concentration - $p=0.4386$