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DISSERTATION

Titel der Dissertation

**„Characterization of the molecular Mechanisms
underlying Interaction of Cells with the
Fibrin Sealant Matrix“**

Verfasser

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Summary

Wound healing is a complex process with strictly regulated mechanisms to minimize blood loss and to reconstruct the damaged tissue. In this process several cell types such as endothelial cells, fibroblasts, and keratinocytes interact with the primary thrombus that basically consists of a matrix of cross-linked fibrin fibers serving as scaffold for invading cells. Following the initial hemostasis, interaction with this scaffold and ingrowth of cells into the fibrin matrix from tissue surrounding the wound bed is essential for the subsequent stages of the wound healing process. By this process fibrin matrix is degraded and finally replaced by healthy tissue.

Fibrin sealants are widely used as hemostats in various surgical indications and for sealing of soft tissue. Fibrin sealants have also been shown to promote different aspects of tissue regeneration such as cell migration and re-epithelialization in a similar way as their physiological counterpart, the blood clot. Fibrin sealants mostly consist of plasma derived concentrated fibrinogen and thrombin solutions that are mixed immediately before application.

Due to different purification procedures each product has its characteristic formulation. Differences exist e.g. in the concentration of thrombin, fibrinogen, salts or ECM molecules.

In recent clinical studies fibrin sealants have been used as a matrix to promote wound healing. This raised our interest to examine what are the most important components of a fibrin matrix for optimal interaction of the extracellular matrix with cells. In this thesis, we investigated how the presence of proteins present in fibrin sealant affects cell-matrix interaction.

First we characterized two commercially available fibrin sealants Artiss and Evicel in their compatibility with normal human epithelial keratinocytes by several in vitro cell assays. NHEK grown on Evicel with its higher thrombin concentration showed less cell adhesion and viability, deteriorated cell morphology and a higher percentage of dead cells.

Due to the non-covalent bonding of thrombin to fibrin during fibrin clot formation, we wanted to evaluate the impact of fibrin bound thrombin on cell viability. Initially, we quantified the activity of thrombin in 3 different, commercially available fibrin sealants. This information was used to prepare fibrin clots covering a range of thrombin concentrations

from 4 IU/ml to 820 IU/ml, but which were identical with respect to all other constituents. Although these fibrin clots did not differ in their three-dimensional structure, clots prepared with highly concentrated thrombin (820 IU/ml) failed to support adhesion and spreading of primary human keratinocytes (NHEK). The number of attached cells was also significantly reduced on high thrombin activity clots. We hypothesized that these observations are not only the consequence of decreased proliferation but of apoptotic mechanisms, since the expression of cleaved caspase 3 and 7 was strongly enhanced on fibrin clots with high thrombin activity. This was accompanied by an induction of expression of Trail-R2 which is a receptor known to mediate apoptosis signals. Blocking of thrombin activity by hirudin led to an improvement of cell morphology and to an increase in number of attached cells. In addition, the induction of caspase 3 and 7 was also reduced. Thus, here we report for the first time that fibrin bound thrombin does not only decrease proliferation, it also does induce NHEK apoptosis when present at high concentrations.

As a consequence and to confirm our in vitro data, fibrin sealants were tested in an excisional wound healing model in rats. The objective of this study was to compare the different fibrin sealants and formations regarding their effects on cells and wound healing in vivo.

We found a more rapid wound closure (day3 and day7) and less wound severity (day 7) with sealants containing a lower concentration of thrombin compared to wound healing with high thrombin formulations. Furthermore, less new built functional vessels were counted in Evicel treated wounds after seven days which fits to the result that a lower level of VEGF was expressed after two days in such treated wounds. These in vivo results may be partially explained by the biocompatibility data found in vitro. At last, also fibrin degradation was observed: in animals treated with Artiss, the sealant was lysed after ten days compared to Evicel that needed 15 days.

In addition to that, we analyzed the influence of fibronectin on cell-matrix interaction. Since keratinocytes lack of integrin $\alpha\beta 3$ and therefore are not able to bind directly fibrin, other ECM molecules like fibronectin are important for keratinocyte adhesion. We could show that on fibrin sealants depleted of fibronectin, NHEK had less adherence and poor cell morphology and these observations could be improved by addition fibronectin.

Moreover, the influences of constituents on clot structure and further consequences on cell compatibility were observed. By changing the salt concentrations or other constituents (fucoidans instead of thrombin) the clot structure could be modified significantly.

Nevertheless, cell compatibility remained unchanged upon structure is not so important for cell-matrix interaction as ingredients and constituents of the matrix.

Fibrin sealants are important and well established tools in surgery. The aim of this thesis is to help to further understand the principles and pathways of cell-matrix interaction. Numerous studies have been performed to optimize compositions and characteristics of fibrin clots to develop a product which serves clinical requirements and product security guidelines. The results of this project may lead to create a further improved fibrin matrix which activates and promotes tissue regeneration.

Zusammenfassung

Wundheilung ist ein komplexer Prozess mit streng regulierten Mechanismen zur Minimierung des Blutverlusts und zur Rekonstruktion des beschädigten Gewebes. In diesem Prozess spielen verschiedene Zelltypen eine Rolle, wie Endothelzellen, Fibroblasten oder Keratinozyten. Diese Zellen interagieren mit dem primären Thrombus, eine Matrix aus großteils quervernetzten Fibrinfasern, der als erstes Grundgerüst für die einwandernden Zellen dient. Die Interaktionen der Zellen mit diesem Gerüst sowie die Einwanderung der Zellen aus der Gewebeumgebung in diese Matrix stellen eine essentielle Phase der Wundheilung dar. Während dieses Schrittes wird die Fibrinmatrix abgebaut und durch gesundes, körpereigenes Gewebe ersetzt.

Fibrinkleber sind als Hämostat in unterschiedlichen chirurgischen Indikationen sowie als Kleber für weiches Gewebe (soft tissue) etabliert. Sie haben gezeigt, dass sie verschiedene Aspekte der Geweberegeneration unterstützen wie etwa Zellmigration oder Reepithelialisierung – ähnlich wie der physiologisch gebildete Blutklot. Fibrinkleber bestehen großteils aus konzentriertem Fibrinogen und Thrombin humanen Ursprungs. Diese beiden Komponenten werden kurz vor der Applikation vermischt.

Wegen unterschiedlicher Herstellungsverfahren besitzt jedes Produkt seine eigene charakteristische Formulierung. Unterschiede gibt es z.B. in Konzentrationen von Thrombin, Fibrinogen, Salzen oder Molekülen der extrazellulären Matrix (ECM).

In aktuellen klinischen Studien wurden Fibrinkleber auch als Matrix zur Unterstützung der Wundheilung verwendet. Daher untersuchten wir, welche die wichtigsten Komponenten der Fibrinmatrix für optimale Interaktion mit Zellen sind. In dieser Arbeit wurde untersucht, wie sich in der Fibrinmatrix vorhandene Proteine auf Zell-Matrix-Interaktionen auswirken.

Zuerst wurden zwei kommerziell erhältliche Fibrinkleber (Artiss und Evicel) mittels in vitro Zelltests auf deren Kompatibilität mit humanen epithelialen Keratinozyten (NHEK) untersucht. NHEK, die mit Evicel und dessen höherer Thrombinkonzentration in Kontakt gebracht wurden, zeigten weniger Adhäsion und Viabilität, eine degenerierte Morphologie, sowie einen höheren Prozentsatz an toten Zellen.

Aufgrund der nicht-kovalenten Bindung von Thrombin an Fibrin während der Klotentstehung, wurden die Auswirkungen von fibringebundenem Thrombin auf die Zellviabilität getestet. Anfangs wurde die Aktivität von Thrombin in drei verschiedenen,

kommerziell erhältlichen Fibrinklebern gemessen. Mit dieser Information wurden Fibrinklots mit Thrombinkonzentrationen von 4 IU/ml bis 820 IU/ml hergestellt, die sich aber in allen anderen Zusammensetzungen nicht unterschieden. Diese Klots wiesen keine Unterschiede in der Ultrastruktur auf. Bei jenen mit hoher Thrombinkonzentration (820 IU/ml) zeigten NHEK eine schlechtere Adhäsion und weniger Zellspreitung. Die Anzahl der anhaftenden Zellen an den Hochthrombinklot war signifikant niedriger. Der Grund für diese Beobachtungen war nicht nur weniger Proliferation der Zellen, sondern auch erhöhte Apoptose durch maßgeblich verstärkte Expression von Caspase 3 und 7 in Zellen, die auf den Klots mit 820 IU/ml wuchsen. Dies ging einher mit der Induktion der Expression von Trail-R2, ein wichtiger Rezeptor im Apoptosesignalweg. Wurde Thrombin mittels Hirudin geblockt, verbesserte sich die Zellmorphologie und eine größere Zahl an Keratinozyten adhärierte am Fibrinkleber. Weiters war die Expression von Caspase 3 und 7 reduziert. Hier wurde zum ersten Mal beschrieben, dass hohe Konzentrationen von Thrombin im Fibrinkleber nicht nur die Proliferation von Keratinozyten hemmt, sondern auch, dass dies zu vermehrter Apoptose von NHEK führt.

Darauf aufbauend wurden Fibrinkleber in einem Exzisionswundheilungsmodell in Ratten getestet. Das Ziel der Studie war, verschiedene Fibrinkleber, sowie unterschiedliche Formulierungen, auf deren Effekt auf die Wundheilung von Ratten zu untersuchen.

Wunden, die mit einer geringeren Thrombinkonzentration behandelt wurden, zeigten schnelleren Wundverschluss (an den Tagen 3 und 7) und eine geringere Schwere der Wunden (am Tag 7) verglichen mit Wunden, die mit hohen Thrombinkonzentrationen behandelt wurden. Weiters konnte eine geringere Anzahl an funktionellen Gefäßen in Wunden mit Evicel-Kleber gezählt werden. Diese Beobachtung passt zu den Ergebnissen, dass der Level an VEGF in Artiss-behandelten Wunden am Tag 2 höher war. Diese in vivo Resultate können teilweise mit den Beobachtungen zur Zellkompatibilität in vitro erklärt werden. Darüber hinaus zeigte eine Untersuchung des Fibrinabbaus, dass der Artiss-Klot nach zehn Tagen resorbiert wurde, der Evicel-Klot nach 15 Tagen.

Als weiteren Aspekt wurde die Auswirkung von Fibronectin auf Zell-Matrix-Interaktionen untersucht. Da Keratinozyten keinen Integrinrezeptor $\alpha\beta 3$ exprimieren und dadurch nicht direkt an Fibrin binden können, spielen andere ECM-Moleküle wie Fibronectin eine wichtige Rolle in der Zelladhäsion. NHEK zeigten eine schlechtere Anhaftung an den Klot und eine degenerierte Morphologie am Fibrinkleber mit reduzierter Fibronektinkonzentration. Diese Resultate konnten durch Zugabe von Fibronectin in den Klot verbessert werden.

Darüber hinaus wurde die Auswirkung von anderen Bestandteilen des Fibrinklebers auf die Klotstruktur und Zellkompatibilität untersucht. Durch Veränderung der Salzkonzentration oder die Verwendung von Fucoidanen anstelle von Thrombin konnte die Struktur des Klots signifikant modifiziert werden. Trotzdem blieb die Zellkompatibilität unverändert. Dies legt nahe, dass die Struktur nicht so wichtig für Zell-Matrix-Interaktionen ist wie die Zusammensetzung bestimmter Inhaltsstoffe der Matrix.

Fibrinkleber sind wichtige und gut etablierte Werkzeuge in der Chirurgie. Das Ziel dieser Doktorarbeit ist, die Prinzipien und Signalwege der Zell-Matrix-Interaktionen besser zu verstehen. Eine Anzahl an Studien wurde durchgeführt um die Zusammensetzung und Charakteristik von Fibrinklebern zu optimieren und dadurch ein Produkt zu entwickeln, das chirurgischen Ansprüchen entspricht und Produktsicherheit gewährleistet. Die Ergebnisse dieses Projekts sollen zu einer weiteren Verbesserung der Fibrinmatrix führen- zur Unterstützung der Geweberegeneration.

Table of Content

Acknowledgement.....	2
Summary	3
Zusammenfassung.....	6
Table of Content.....	9
Introduction.....	13
1 Hemostasis	13
2 Fibrin Sealants	15
2.1 Fibrin(ogen)	19
2.1.1 Configuration of Fibrinogen.....	19
2.1.2 Conversion of Fibrinogen to Fibrin	20
2.1.3 Clot structure and biomechanical properties.....	22
2.1.4 Fibrinolysis	23
2.2 Thrombin.....	23
2.2.1 Thrombin receptors	24
2.2.2 Thrombin in fibrin sealant clots.....	25
2.2.3 Thrombin inhibition	25
3 Wound healing	27
3.1 Inflammation.....	28
3.2 Granulation and Re-epithelialization	31
3.3 Tissue remodeling and Maturation.....	33
3.4 Wound healing in rats	34
4 The extracellular matrix (ECM).....	35
4.1 Major constituents of the ECM	35

4.2	Adhesion on ECM-molecules	36
5	Cells	38
5.1	Normal Human Epithelial Keratinocytes - NHEK.....	38
5.2	Epithelial Cells	40
5.3	Normal Human Dermal Fibroblasts - NHDF	41
6	Aim	42
	Material and Methods.....	43
	Results	58
1	Comparison of two commercially available fibrin sealants: Artiss vs. Evicel.....	58
1.1	Blocking of thrombin receptor PAR-1	59
1.2	Molecular analysis of NHEK exposed to fibrin sealants.....	60
2	Different Concentrations of Thrombin.....	62
2.1	Thrombin concentration in fibrin sealant extracts	62
2.2	Proteins (undefined) in fibrin sealant extracts	63
2.3	Identification of proteins extracted from fibrin clots	64
2.4	Determination of fibrin-bound thrombin	65
2.5	Activity of fibrin bound thrombin	65
2.6	The influence of thrombin on clot ultrastructure.....	66
2.7	Number of adherent cells on fibrin clots with different thrombin concentrations ..	67
2.8	Morphology of cells on fibrin clots with different thrombin concentrations.....	68
2.9	Inhibition of thrombin activity	69
2.10	Cell death in context with fibrin bound thrombin	73
2.11	Expression of apoptosis marker Trail-R2 in NHEK.....	77
2.12	Expression of apoptosis marker Fas in NHEK.....	78

2.13	Caspase 3 and caspase 7 activity of NHEK seeded on fibrin clots.....	79
2.14	Proliferation of NHEK in context with thrombin	80
2.15	Blocking of PAR-1 thrombin receptor with SCH797979.....	81
2.16	Influence of thrombin on the MAPK expression pattern.....	82
3	Rat excisional wound healing model – thrombin dependent wound healing.....	85
3.1	Planimetry	85
3.2	Histomorphometry.....	87
3.2.1	Superficial Contraction Index	88
3.2.2	Deep Contraction Index.....	89
3.2.3	Wound Severity Index.....	89
3.2.4	Wound Contraction Index	90
3.2.5	Global Healing Index.....	90
3.3	Neo-Vascularization	91
3.3.1	Smooth muscle actin	91
3.3.2	Von Willebrand factor	92
3.3.3	logSMA/vWF	93
3.3.4	Western Blot analysis	94
3.4	Inflammation.....	95
3.5	Fibrin Degradation	97
3.6	Apoptosis.....	98
3.7	Pathological analysis	98
4	ECM molecules in fibrinogen component – Fibronectin.....	100
4.1	Fibrin clot extraction	100
4.2	Integrin β 1 receptor expression on NHEK.....	101

4.3	Depletion of Fibronectin	102
4.4	Plasminogen	106
5	Clot structure.....	107
5.1	Influence of fibrinogen and thrombin concentration on fibrin structure	107
5.2	Influence of salt concentration on fibrin structure.....	108
5.2.1	Morphology	109
5.2.2	Cell adhesion.....	110
5.3	Fucoidans	111
5.3.1	Cell adhesion.....	112
	Discussion.....	113
	Abbreviations	126
	Curriculum Vitae.....	142

Introduction

1 HEMOSTASIS

The blood coagulation system is divided into two initiating pathways: the extrinsic (tissue factor) pathway and the intrinsic (contact activation) pathway which meet at a final common pathway: factor Xa converts prothrombin to thrombin which further cleaves fibrinogen to form an insoluble fibrin clot.

The model of a cascade is a very simplified one as proteins from both pathways can influence one another. Maybe the coagulation system should be seen as an interactive network with carefully placed positive and negative feedback. (1)

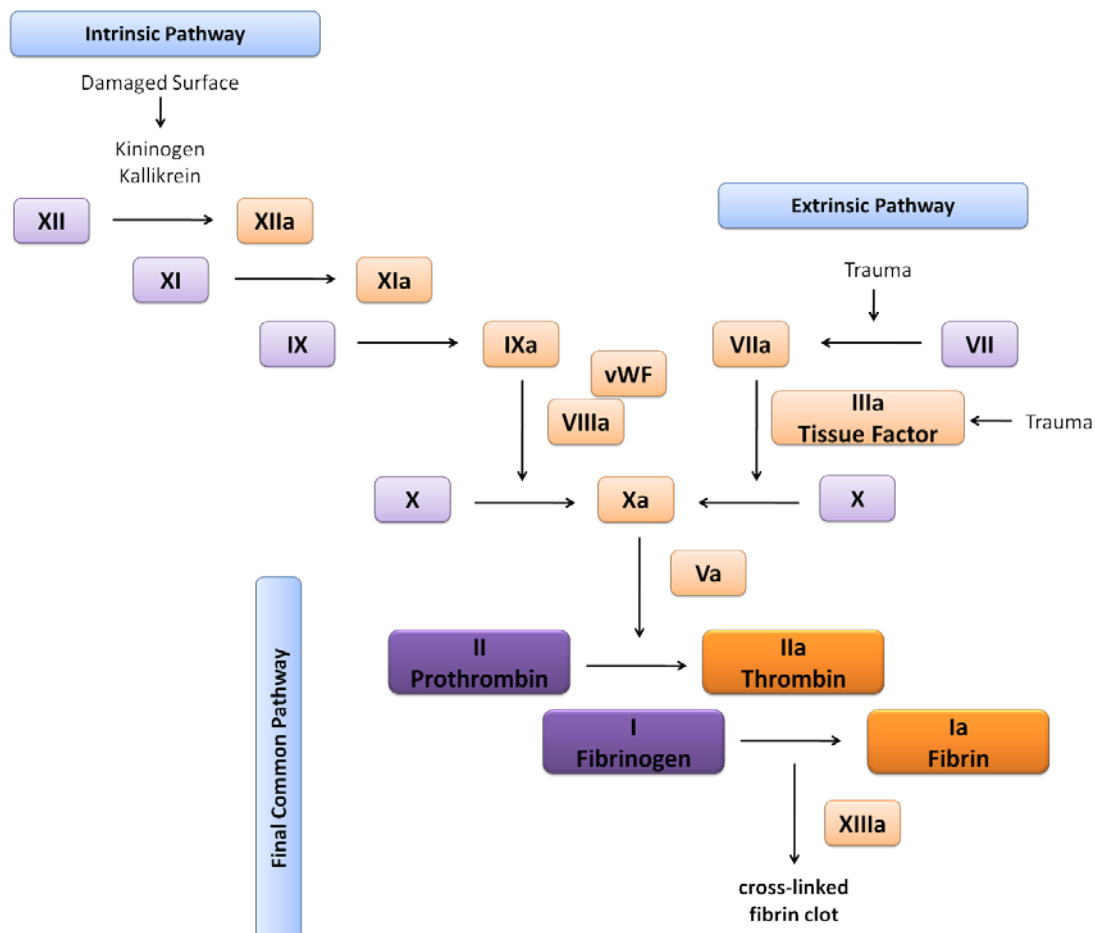


Figure 1 Blood coagulation system (simplified) adapted from: www.frca.co.uk/images/clotting_cascade.gif

The coagulation system works through a group of soluble factors (see table 1) which are synthesized in the liver. They circulate in the plasma in an inactive form (zymogen) and become active after proteolytic cleavage. (2)

Table 1 Coagulation factors (source: Walker and Royston, 2002 (1))

Standard nomenclature	Traditional name	Molecular weight (Da)	Plasma concentration ($\mu\text{g ml}^{-1}$)	Half-life (h)
Factor I	Fibrinogen	340 000	2000 – 4000	90
Factor II	Prothrombin	72 000	120	65
Factor III	Tissue factor	45 000	0	–
Factor IV	Calcium	40	100	–
Factor V	Proaccelerin	330 000	10	15
Factor VII	Proconvertin	48 000	1	5
Factor VIII	Antihæmophilic factor	360 000	0.05	10
Factor IX	Christmas factor	57 500	4	25
Factor X	Stuart-Prower factor	55 000	12	40
Factor XI	Plasma thromboplastin antecedent	160 000	6	45
Factor XII	Hageman factor	85 000	40	50
Factor XIII	Fibrin stabilizing factor	320 000	20	200

In the case of wounding, platelets adhere and aggregate. Shear forces assume that the GPIb-IX-V complex adheres transient to von Willebrand factor (vWF) in exposed subendothelium. This attachment is stabilized by the involvement of integrin $\alpha 2\beta 1$ and GPVI (collagen receptors) and integrin $\alpha \text{IIb}\beta 3$ (Fg, Fn, laminin, vitronectin, TSP, vWF). (3;4)

Activated platelets become procoagulant by exposing anionic phosphatidylserine on their surface. (5) The negatively charged phospholipid remains on the outside surface of the platelet membrane and is not flipped internally by a flipase. (1) Factors VII, IX, X and

prothrombin (vitamin K dependent factors) also possess large amounts of negatively charged glutamic acids at their N-terminal regions. Therefore, calcium ions are important acting as a buffer between the negatively charged areas of activated platelets and these activated coagulation factors.

This surfaces of the platelets are procoagulant and catalytic and therefore accelerate thrombin generation and further fibrin formation. In the fibrin network which surrounds the platelet plug, leukocytes and red cells become trapped. (5) This conglomerate of cells expresses and releases substances that promote tissue repair and influence processes such as angiogenesis, inflammation and the immune response. (5)

2 FIBRIN SEALANTS

Adapted from the principles of the natural occurring final stage of hemostasis (common pathway), fibrin sealants (FS) were developed for surgery, mimicking the coagulation.

Paul Morawitz, a German physiologist, authored in the early 20th century the “chemistry of coagulation” (1905) and laid the headstone for coagulation research. He discovered the important role of thrombin (6): once activated, thrombin cleaves fibrinogen, a fibrin clot is built spontaneously. This rudimentary pathway led in succession to the discovery of the blood coagulation system.

In 1909, Bergel first reported the use of dried plasma as a source of fibrinogen and fibrin fleece as an aid to control hemorrhage in surgery (7), in 1915, Grey used fibrin patches. In 1944, the idea of mixing fibrinogen with thrombin to prepare a fibrin adhesive to anchor skin grafts was described by Tidrick and Warner and Cronkite et al.

Several problems in quality - the inadequate strength and stability of early glues - and the danger of viral infections decreased the interest in this material for the next 30 years. The interest again lit up in the mid-1970s with the development of a commercial process for isolation of large quantities of virally inactivated fibrinogen. (8)

The effective components and main principles of a fibrin sealant system are fibrinogen (Factor I), thrombin (FIIa), calcium (FIV) and fibrin stabilizing factor (FXIII). Thrombin acts as transformer of the precursor fibrinogen to its active form fibrin, calcium as activator of factor XIII and the latter as cross-linker of the fibrin polymer, so that the sealing system could be described as a three-component-sealant: 1. the adhesive part fibrin(ogen), 2. the activator system with thrombin and calcium and 3. the hardener-system (FXIIIa). (9)

Components of fibrin adhesive products include fibrinogen from frozen plasma (cryoprecipitate or Cohn Fraction I) and thrombin (both human and virally inactivated), calcium and a fibrinolytic inhibitor, which is mostly bovine or synthetic aprotinin (8).

When reconstituted, one vial yields a solution of fibrinogen, factor XIII, and often an anti-fibrinolytic agent like aprotinin. The other vial yields a thrombin plus calcium solution. In table 2, representative fibrin sealants available on the fibrin sealant-market with their indication and protein-composition are shown (according to the FDA market authorization).

Table 2 Examples for Fibrin Sealants, their indications and compositions (source: respective package inserts)

Indication (IFU)	Composition
TISSEEL Fibrin Sealant <i>Baxter</i>	
Hemostasis: for use as an adjunct to hemostasis in surgeries involving cardiopulmonary bypass and treatment of splenic injuries due to blunt or penetrating trauma to the abdomen, when control of bleeding by conventional surgical techniques, including suture, ligature, and cautery, is ineffective or impractical. TISSEEL is a satisfactory hemostatic agent in fully heparinized patients undergoing cardiopulmonary bypass. Sealing: TISSEEL has been shown to be an effective sealant as an adjunct in the closure of colostomies. It is not indicated for the treatment of massive and brisk arterial or venous bleeding.	Total protein: 96 – 125 mg/mL Human Fibrinogen: 67 – 106 mg/mL Fibrinolysis Inhibitor aprotinin: 2250 – 3750 KIU/mL Human albumin, tri-sodium citrate, histidine, niacinamide, polysorbate 80 and water for injection (WFI). Human Thrombin: 400 – 625 IU/mL Calcium Chloride: 36 – 44 µmol/mL Human albumin, F XIII, sodium chloride and water for injection (WFI)

ARTISS Fibrin Sealant <i>Baxter</i>	
ARTISS is indicated to adhere autologous skin grafts to surgically prepared wound beds resulting from burns in adult and pediatric populations as well as for facelift. It is not indicated for hemostasis.	Total protein: 96 – 125 mg/mL Human Fibrinogen: 67 – 106 mg/mL Fibrinolysis Inhibitor: 2250 – 3750 KIU/mL Human albumin, tri-sodium citrate, histidine, niacinamide, polysorbate 80 and water for injection (WFI) Human Thrombin: 2.5 – 6.5 IU/mL Calcium Chloride: 36 – 44 µmol/mL Human albumin, F XIII, sodium chloride and water for injection (WFI)
EVICEL Fibrin Sealant <i>OMRIX biopharmaceuticals</i>	
EVICEL Fibrin Sealant (Human) is indicated as an adjunct to hemostasis for use in patients undergoing surgery, when control of bleeding by standard surgical techniques is ineffective or impractical. It is not indicated for the treatment of severe or brisk arterial bleeding.	Human Fibrinogen: 55 - 85 mg/ml Arginine hydrochloride, glycine, sodium chloride, sodium citrate, calcium chloride, water for injection (WFI) Human thrombin: 800 - 1200 IU/ml Calcium chloride, human albumin, mannitol, sodium acetate, water for injection (WFI)

Beriplast® P Combi-Set Fibrin Sealant Set <i>CSL Behring</i>	
Beriplast P can be used locally as supportive treatment in all surgical disciplines, including endoscopic specialities, to achieve – tissue adhesion/sealing, – suture support, – hemostasis, It can further be used for hemostasis in endoscopic treatment of bleeding gastroduodenal ulcers. It is not indicated for arterial and strong venous bleeding.	Fibrinogen (human plasma protein fraction): 90 mg/ml Coagulation factor XIII (human plasma protein fraction): 60 IU Human albumin, L-arginine hydrochloride, L-isoleucine, sodium chloride, sodium citrate dihydrate, sodium L-glutamate monohydrate Bovine lung aprotinin 1000 KIU

The big advantage of current fibrin products is that they are of human origin. As a naturally occurring product they are biocompatible and biodegradable and do not induce an inflammatory response, foreign body reaction, extensive fibrosis or tissue necrosis. (8)

As the body itself degrades the fibrin clot by its fibrinolytic system and the adhesion of the fibrin on the tissue is very important for a sealant, the use of fibrinolysis-inhibitors particularly in tissues with a high fibrinolytic activity (serous cavities, prostate, lung,...) is necessary. The speed of degradation of a fibrin sealant can be reduced by adding an anti-fibrinolytic agent (10) such as aprotinin. Studies have demonstrated that aprotinin, a natural protease inhibitor, has advantages to synthetic anti-fibrinolytic agents e.g. tranexamic acid (tAMCA) (11;12).

Removing plasminogen from the fibrinogen component would be another approach to slow down fibrinolysis. Since the proteolytic activity in wounds is not only related to

plasminogen/plasminogen activator, but also dependent on leukocyte proteases, elastase/cathepsin G inhibitors may also be used to prevent premature clot lysis (13).

In tissues with less or no fibrinolytic activity, such as bradytrophic tissues, such inhibitors negatively affect the wound healing. The explanation therefore lies in the first phase of wound healing: new stroma (granulation tissue) begins to invade the wound space, new capillaries and connective tissue cells are geared to fibrin filaments, dissolve them with their proteolytic (fibrinolytic) activity and replace them. But due to addition of fibrinolysis inhibitors this sprouting of the new tissue into the fibrin clot may happen very slowly and the wound healing is decelerated. (9)

2.1 FIBRIN(OGEN)

2.1.1 CONFIGURATION OF FIBRINOGEN

Fibrinogen is a large, fibrous plasma glycoprotein composed of two sets of three polypeptide chains that are linked by 29 disulfide bonds. The A α -chain consists of 610, the B β -chain 461, and the major γ -chain form (γ A) 411 residues. A minor γ -chain variant termed γ' , arises through alternative processing of the primary mRNA transcript (14). γ' -Chains account for approximately 8% of the total fibrinogen γ -chain population and are mainly found in heterodimeric fibrinogen molecules amounting to approximately 15% of plasma fibrinogen molecules. (15)

Each fibrinogen chain is encoded by a separate gene and these three genes together form the fibrinogen gene cluster that comprises approximately 50 kb and is located on chromosome 4q22-23. Fibrinogen builds a trinodular structure with two identical globular domains at the ends, called D-domains, and one E-domain in the middle, connected by flexible coiled-coil rods. The protein accomplishes a length of 45 nm. (16)

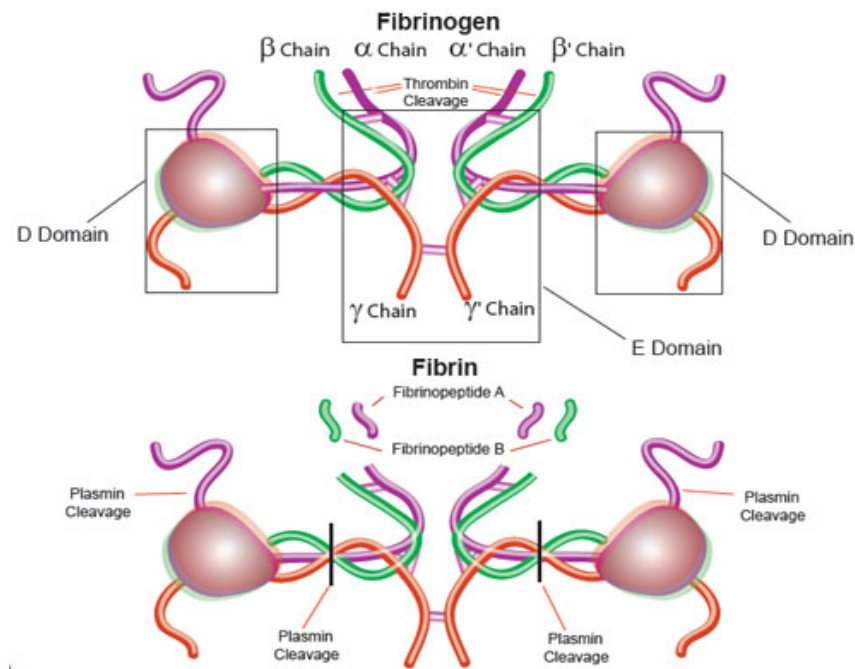


Figure 2 Enzymatic conversion of fibrinogen to fibrin by thrombin results in release of fibrinopeptides A (FPA) and B (FPB) and exposure of EA and EB polymerization sites. (Source: www.sigmaaldrich.com: fibrinogen-cleave.jpg)

The D- as well as the E-domain contains binding sites that play a role in fibrinogen conversion to fibrin, fibrin assembly, cross-linking, and platelet interactions, as well as sites that are available after fibrinogen cleavage (thrombin binding site), and sites that become exposed as a consequence of the polymerization process (tPA-dependent plasminogen activation). (17)

2.1.2 CONVERSION OF FIBRINOGEN TO FIBRIN

In the course of blood coagulation, the protein fibrinogen, which occurs solute in the blood, is converted to an insoluble fibrin polymer: fibrinopeptides A and B (FpA and FpB), which are located at the amino terminal ends of the α and β chains at the E-domain, are cleaved by thrombin. Thrombin is a serine protease generated by activation of prothrombin during the blood coagulation system. Accordingly, the polymerization sites E_A and E_B are laid open, these “knobs” are exposed “holes” (D_a/D_b) at the ends of the molecules (16). The now so-called fibrin monomer then spontaneously assembles with other monomers into linear protofibrils through self-association to make half-staggered oligomers. In succession, the protofibrils aggregate and align in a staggered overlapping end-to-middle domain

arrangement to form double-stranded twisting fibrils which then branch to build a three-dimensional network.

Two types of branch junctions occur in fibrin networks (18): the first occurs when double-stranded fibril converges laterally with another fibril to form a four-stranded fibril, a tetramolecular or bilateral junction (19). The second type of branch junctions, termed trimolecular or equilateral, is formed by convergent interactions among three double-stranded fibrils of equal width. This kind of junction is formed with greater frequency when fibrinopeptide cleavage is relatively slow (18). Under such conditions the networks are more branched and the matrix is tighter (20). Equilateral branching may play an important role in determining the ultimate 3D-structure of the fibrin matrix.

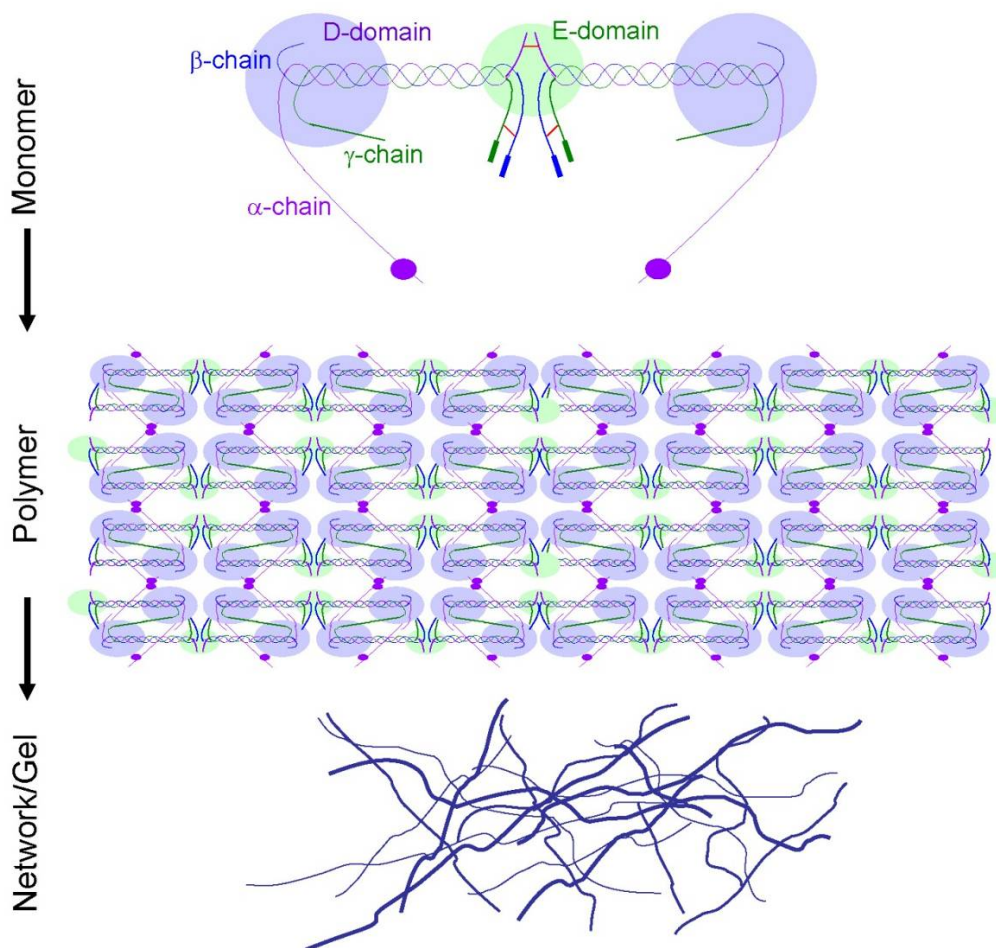


Figure 3 Schematic overview of fibrin polymerization and clotting mechanism. (21)

The coagulation factor FXIII (a plasma transglutaminase) is activated to FXIIIa in the presence of thrombin and calcium ions which lead to an intermolecular cross-linking of fibrin. This is necessary because the “knobs-hole”- assemblies ($E_A:Da$ and $E_B:Db$) are solely constructed by hydrogen bonding. This would be too weak and the fibrin network would be a subject to rapid removal by the fibrinolytic system. This situation is corrected by the introduction of covalent bonds between the assembled fibrin molecules (8): the C-terminal region of each fibrinogen (or fibrin) γ -chain contains one cross-linking site at which factor XIII or XIIIa catalyzes the formation of γ -dimers by introducing reciprocal intermolecular ϵ -(γ -glutamyl)lysine covalent bonds. (22) This stabilizes the fibrin network and creates an insoluble fibrin clot and delays the beginning of the fibrinolytic system. FXIII can increase the elastic modulus fivefold under physiological conditions (23) but it seems to have no influence on fiber thickness. Only an increased density of fibers in the network was seen with the addition of FXIII. (24)

All in all, the resulting insoluble clot does not only stop bleeding but also provides a basis for wound healing by building a provisional matrix for the cell migration and attachment.

2.1.3 CLOT STRUCTURE AND BIOMECHANICAL PROPERTIES

Variables that describe the fibrin structure are e.g. the thickness of the fibers, the number of branch points, the porosity, and the permeability (25). The structure is dependent on chemical factors such as the pH value: for example, at pH 7.0 the fibrin clot occurs as an opaque matrix because of thick fibers that are formed at a faster rate than in a transparent matrix which is formed at pH 7.8 with thinner fibers. Several other conditions may affect the fibrin structure, such as the kinetic of clotting which can be modulated by concentration of thrombin, FXIII and salt content: the fiber structure formed was much finer at the higher salt concentration (NaCl, arginine) with less porosity and many more branch points compared to the thicker, more laterally associated fibers at the lower salt concentration. The rate of polymerization is determined by FXIII concentration and FXIII activation rate, and the rate of lateral polymerization is affected by fibrinopeptide B release and cross-linking sites on α - and γ -chains. Chloride ions control the fiber size by inhibiting the growth of thicker, stiffer and straighter fibers and therefore have an influence on clot porosity (26-28). The concentration of thrombin and thus the release-rate of FpA can also have an important impact on the polymerization process. High concentrations (up to 1 IU/ml) induce the formation of thin fibers, whereas low concentrations (0.001 IU/ml) result in thicker fibers. (29;30) Heparins, in particular low molecular weight (LMW)-heparin, also affect the structure of the fibrin clot, as well as the sensitivity of the clot to plasmin-dependent degradation and this affects invasion by tube-forming EC. (31)

These factors control the physical structure of the fibrin-based biomaterial and have been shown to be important for wound healing. (32)

2.1.4 FIBRINOLYSIS

In a healthy human organism there are hardly any free endopeptidases. The fibrinolytic potential is balanced through a complex activator/inhibitor-system like the blood coagulation system. The fibrin clot is dissolved when the fibrinolytic system is activated and the proenzyme plasminogen is changed into the active protease plasmin. This change is carried out by several activators, present in the tissue. The fission of the fibrin clot also happens by enzymes like factor XIIa, kallikrein, proteases like trypsin or papain, by kinases like strepto- and urokinases. (33)

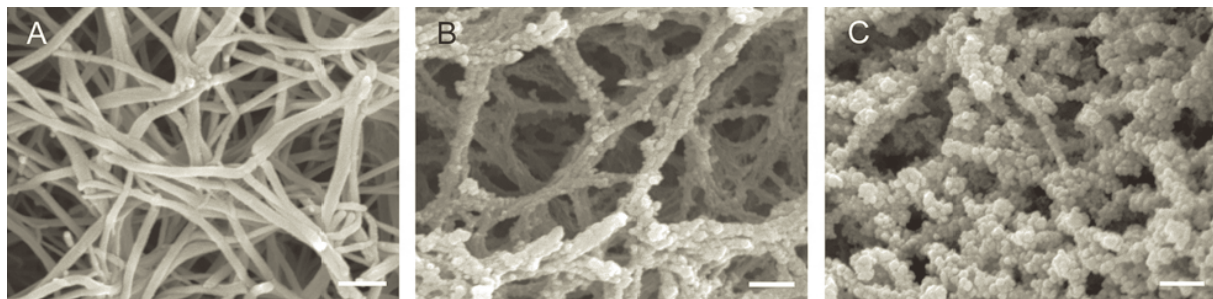


Figure 4 In vitro clot degradation: (A) Intact fibrin network of a clot kept for eight days in PBS with the fibrinolysis inhibitor aprotinin (50 mg/ml fibrinogen, 2 IU thrombin). (B) A clot with the same fibrin formulation as in (A) kept in PBS for eight days without fibrinolysis inhibitor. (C) Advanced fibrinolysis in a clot kept in urokinase. Scale bars: (A–C) 0.5 μ m

In figure 4B, the fibrin network shows signs of fibrinolysis such as a rough and fuzzy surface decorated with fiber residuals. In figure 4C, the fibrous material is entirely granular and the original fiber network is hardly discernible (50 mg/ml fibrinogen, 0.5 IU thrombin).

2.2 THROMBIN

Platelet activation results in a transport of anionic phosphatidylserine to the outer surface of the platelet plasma membrane and in a release of microvesicles. These processes lead to a production of procoagulant surfaces. (34) Furthermore, binding of coagulation factors results in a rapid formation of an activated factor Xa/Va complex that transforms prothrombin into thrombin in presence of calcium ions. The production of thrombin needs

to be explosive at the site of the injury in order to prevent it from being washed away and should occur only where it is directed and required. (35)

Thrombin is a trypsin-like serine protease and interacts with fibrinogen and fibrin through two major types of binding sites:

- The fibrinogen substrate site cleaves fibrinogen by the removal of fibrinopeptides A and B (FpA and FpB) and therefore triggers fibrin polymerization (explained above).
- The thrombin exosite binds at a non-substrate site in fibrin or fibrinogen that is located in the central molecular domain (β -chain). Statistically, there are fewer than one such thrombin binding sites per fibrin molecule suggesting that more than one fibrin molecule is required for this kind of binding. Maybe this binding site becomes expressed only after fibrinogen has been converted to fibrin. (36)

It is noteworthy, that the coagulant potential of fibrin-bound thrombin is retained and that fibrin-bound thrombin cannot be inactivated by heparin-antithrombin III complexes.(37)

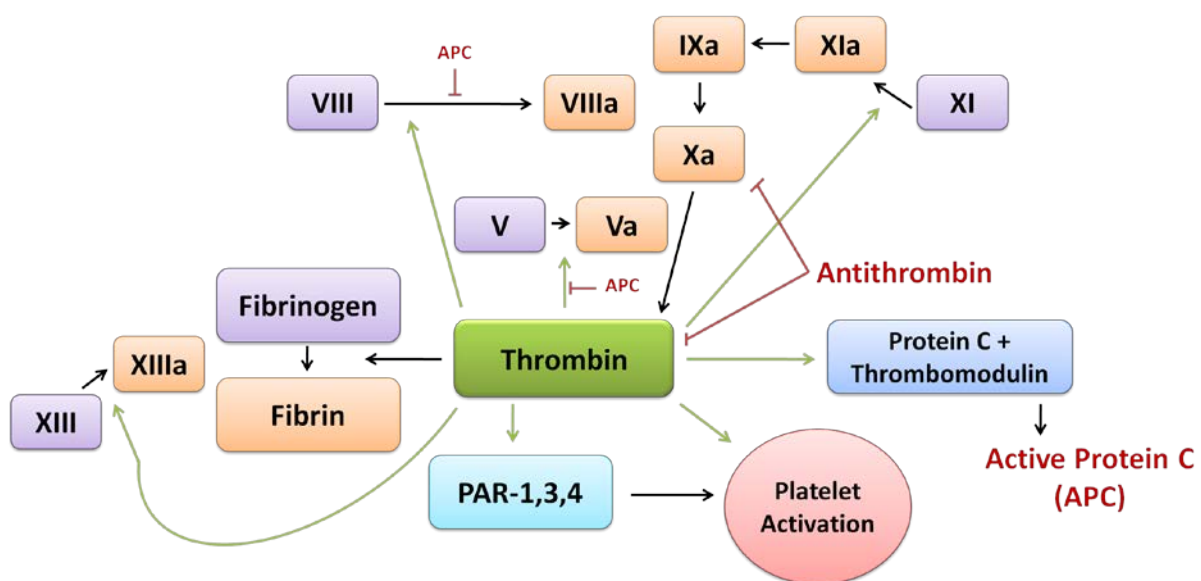


Figure 5 Some of the Effects of Thrombin (pictured within a simplified coagulation system)

2.2.1 THROMBIN RECEPTORS

Protease-activated receptors (PAR) belong to a novel emerging family of seven-transmembrane, G protein-coupled receptors that are activated by proteolysis. Because

proteases are common enzymes in organisms and receptor activation is catalytic, such receptors represent an efficient means to modulate cellular responses. (46)

There are four known members of this receptor family of protease-activated receptors: PAR-1, PAR-3, and PAR-4, which are cleaved and activated by thrombin, and PAR-2, a receptor for trypsin-like enzymes. (47) Based on that, thrombin does not have only pro-coagulant activities, but has also been shown to induce mitogenic responses of different cell types involved in wound healing. For instance, thrombin cleaving of PAR-1 generates an anti-inflammatory and anti-apoptotic response. (48) This response is thought to be mediated through mitogen-activated protein kinase (MAPK) proteins: an increase in phosphorylation of the MAPK proteins p38 and ERK-2 following activated protein C treatment could be observed. (49) Furthermore, a dose-responsive decrease in keratinocyte proliferation could be shown when PAR-1 antibody is applied (to block the receptor). (50)

2.2.2 THROMBIN IN FIBRIN SEALANT CLOTS

Thrombin, also known as coagulation factor IIa, acts as a serine protease that converts soluble fibrinogen into insoluble strands of fibrin. Thrombin is one main component of fibrin sealants and is used in different concentrations: hemostats usually need a higher concentration of thrombin to build a compact clot in few seconds whereas glues used in tissue engineering need to polymerize slowly. Therefore, the thrombin concentration is much lower.

Due to the non-covalent bonding of thrombin to fibrin during fibrin clot formation, thrombin not only plays a role in kinetic of clot building but also influences cells which interact with the sealant hours and days after application. Keratinocytes e.g. get in contact with the clot while re-epithelialization. In vitro studies have shown that high thrombin concentrations (over 2 IU/ml) negatively affect viability of cells like neurons (51), fibroblasts (52) or epithelial cells (53) and even induce apoptosis. The concentration of thrombin required for such effects depends on the cell type.

2.2.3 THROMBIN INHIBITION

Coagulation is intended to be localized to the area where the original platelet plug was formed. There are two methods to achieve this strictly located reaction: on the one hand, reactions are most efficient when restricted to a surface such as platelet phospholipids; on the other hand, by a series of inhibitors. (35)

Thrombin inhibition functions either by having a direct effect on the cleavage site of the molecule or can in some other way inhibit the ability of thrombin to catalyze the conversion of fibrinogen to fibrin. (36)

- **Heparin** increases the affinity of antithrombin to free, unbound thrombin. But when heparin binds to exosite 2 of already fibrin-bound thrombin binding of antithrombin to heparin is impossible; this explains why fibrin-bound thrombin is protected from inhibition by heparin. (38)

- **PPACK** (D-phenylalanyl-L-propyl-L-arginine) is a selective thrombin inhibitor. It inhibits thrombin activity but does not alter its exosite fibrinogen- or fibrin-binding potential. (36;39)

- The **protein C pathway** serves as a natural regulation mechanism against thrombosis. Thrombin is held on the endothelial surface by a co-factor/receptor called thrombomodulin. Thrombomodulin forms with thrombin a 1:1 stoichiometric complex and so promotes protein C activation. Through this TT-complex building the speed of protein C activation is raised thousand fold. Thus, activated protein C (APC) generation is roughly proportional to thrombin concentration. (40) As a consequence, thrombin is converted from a pro-coagulant to an anti-coagulant protein as thrombomodulin-bound thrombin has no pro-coagulant effect.

- The **direct thrombin inhibitors (DTI)** inhibit thrombin by binding to its active site and blocking the interaction with its many, varied substrates. (41) In contrast to e.g. heparin, DTI block both free and fibrin-bound thrombin as well. Bivalent DTI (e.g. lepirudin and bivalirudin) inhibit the active, catalytic center as well as exosite 1 whereas univalent DTI (e.g. argatroban and dabigatran) inhibits only the catalytic center. (38;41)

Another bivalent direct thrombin inhibitor is **hirudin**, a polypeptide originally obtained from the medical leech. It is the most potent natural inhibitor of thrombin and acts by irreversible binding to the active site of unbound and fibrin bound thrombin. Therefore, it inhibits catalytic activity and prevents further thrombin binding to fibrin. (42) Moreover, it inhibits the mitogenic effect of thrombin on fibroblasts and the thrombin activation of platelets. (43;44)

There is no clinically proven antagonist for hirudin and it has been established as an inhibitor of free and fibrin bound thrombin. (45)

3 WOUND HEALING

Tissue injury of any kind implements natural defense and repair mechanisms where a lot of diverse cell types are involved to rebuild the integrity and strength of the tissue.

Several overlapping phases are important for a successful wound healing and repair: hemostasis, inflammation, tissue formation (cell proliferation with angiogenesis), and tissue/matrix remodeling (54;55). A provisional extracellular matrix, the fibrin clot formed at the site of injury, is important to stop bleeding. Furthermore, it is required to support wound healing by providing a structural three-dimensional scaffold for adhesion, proliferation, and migration of invading cells.

Throughout these phases, components of the ECM such as laminin, fibronectin or glycosaminoglycans (GAGs) play an important role in regulating and integrating many key processes of healing (56;57). In the later phases of the healing process the initial ECM of the scar is remodeled (collagen type III is substituted by collagen type I) and the injured tissue is repaired but not regenerated as the architecture of the scar never completely reproduces the pre-wound architecture of the skin tissue.

In healthy individuals the acute wound healing process is guided and maintained through integration of multiple signals (mainly growth factors such as EGF, PDGF, VEGF or members of the TGF-family) released by keratinocytes, fibroblasts, endothelial cells, macrophages, platelets and other cells (31;54). However, there is a number of conditions, which are associated with impaired tissue repair, including old age, steroid treatment and diseases such as diabetes and cancer (55).

Table 3 Phases of wound healing and the causes, results, and effector proteins involved

	Cellular actions	Effector proteins	Results
Phase I	Keratinocyte damage	IL-1	Macrophage and neutrophil recruitment
Inflammation	Extravasation of blood	TNF- α	Fibrin clot formed
	Bacterial load	CSF	

Phase II Granulation and Re- epithelialization	Fibroblasts proliferate, migrate into wound, secrete extracellular matrix (ECM)	PDGF	Fibrin, fibronectin, and hyaluronic acid provide a scaffold for cell migration
	Keratinocytes proliferate, migrate into wound, myofibroblasts cause wound contraction	FGF EGF VEGF KGF TGF IGF GM-CSF MMPs uPA tPA	Angiogenesis Re-epithelialization of wound surface, dissection of eschar, barrier formation
Phase III Tissue remodeling	Fibroblasts produce a collagenous matrix	TGF- β 1 TIMPs MMPs	Formation of scar tissue, apoptosis of fibroblasts, switch from activation to differentiation of keratinocytes

Although each phase has distinct characteristics, these phases are overlapping during normal wound healing (adapted from Singer and Clark and references therein (58;59))

3.1 INFLAMMATION

In the beginning of the phase of inflammation the clotting cascade builds an insoluble clot with fibrin and platelets and other cells to stop blood loss as described above.

Various growth factors (PDGF, IGF-I, TGF- α , EGF) and cytokines are released amongst others by the α -granules in the thrombocytes (60) that attract and activate macrophages to implement phagocytosis at bacteria and debris. It also causes the migration and division of cells into the clot such as fibroblasts. Also the fibrinopeptides FpA and FpB released by thrombin during clot formation are chemo-attractants and activators of leukocytic cells. TGF-

α and tumor necrosis factor (TNF- α) are secreted from vascular endothelium cells, keratinocytes, and fibroblasts inducing the inflammatory stage. (61)

Inflammatory factors such as thromboxanes and prostaglandines are distributed that cause vasoconstriction and call for inflammatory cells and factors in the area. The predominant cells in the wound for the first days are polymorphonuclear neutrophils. (62)

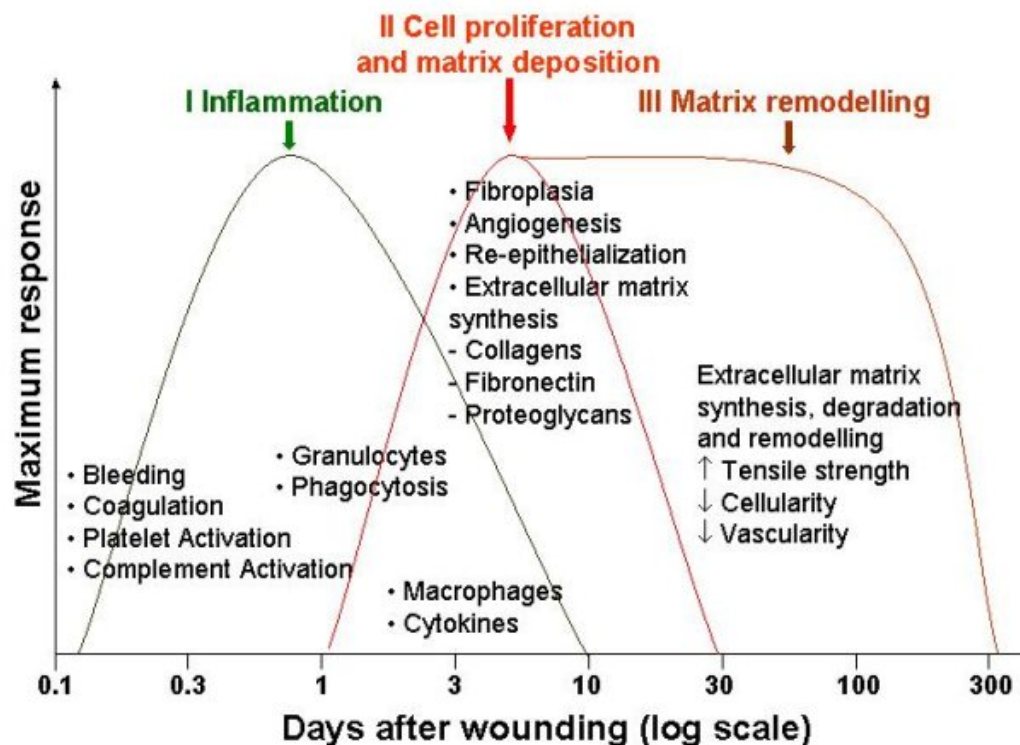


Figure 6 Phases of wound healing (source: World wide wounds: Stuart Enoch)
<http://www.worldwidewounds.com/2004/august/Enoch/images/enochfig1.jpg>

Inflammation plays a role in fighting infection and induces the proliferation phase and therefore is a necessary part of healing. As it wanes after some days, fewer inflammatory factors are produced and the number of neutrophils and macrophages in the wound area is decreasing. This process occurs only in healthy organisms with acute wounds. In chronic wounds like diabetic ulcers, the wound healing never comes beyond the inflammatory phase. (63) This process has to be further elucidated but maybe matrix metalloproteases (64) or other proteases damage components of the extracellular matrix and receptors for growth factors. Without them, a regular wound healing is not possible. (65)

Table 4 Growth factor signals at the wound site (source: <http://emedicine.medscape.com/article/1298196-overview#aw2aab6b4>) "Growth factors represent the intercellular signaling that orchestrates the complex sequence of cell migration, division, differentiation, and protein expression during wound healing. The 8 major families of growth factors are expressed in varying levels by the cells involved with healing." (60)

Growth Factor	Production	Known Effects
1. Epidermal Growth Factor (EGF)	Platelets, macrophages	Stimulates fibroblasts to secrete collagenase to degrade the matrix during the remodeling phase. Stimulates keratinocyte and fibroblast proliferation. May reduce healing time when applied topically.
2. Transforming Growth Factor	Platelets, macrophages, lymphocytes, hepatocytes	TGF-α: Mitogenic and chemotactic for keratinocytes and fibroblasts TGF-β1 and TGF-β2: Promotes angiogenesis, up-regulates collagen production and inhibits degradation, promotes chemoattraction of inflammatory cells. TGF-β3 (antagonist to TGF-β1 and β2): Has been found in high levels in fetal scarless wound healing and has promoted scarless healing in adults experimentally when TGF-β1 and TGF-β2 are suppressed.
3. Vascular Endothelial Growth Factor (VEGF)	Endothelial cells	Promotes angiogenesis during tissue hypoxia.
4. Fibroblast Growth Factor (FGF)	Macrophages, mast cells, T-lymphocytes	Promotes angiogenesis, granulation, and epithelialization via endothelial cell, fibroblast, and keratinocyte migration, respectively.
5. Platelet-Derived Growth Factor (PDGF)	Platelets, macrophages, and endothelial cells	Attracts macrophages and fibroblasts to zone of injury. Promotes collagen and proteoglycan synthesis.

6. Interleukins	Macrophages, keratinocytes, endothelial cells, lymphocytes, fibroblasts, osteoblasts, basophils, mast cells	IL-1: Pro-inflammatory, chemotactic for neutrophils, fibroblasts, and keratinocytes. Activates neutrophils IL-4: Activates fibroblast differentiation. Induces collagen and proteoglycan synthesis. IL-8: Chemotactic for neutrophils and fibroblasts.
7. Colony-Stimulating Factors	Stromal cells, fibroblasts, endothelial cells, lymphocytes	Granulocyte colony stimulating factor (G-CSF): Stimulates granulocyte proliferation. Granulocyte Macrophage Colony Stimulating Factor (GM-CSF): Stimulates granulocyte and macrophage proliferation.
8. Keratinocyte growth factor	Fibroblasts	Stimulates keratinocyte migration, differentiation, and proliferation.

3.2 GRANULATION AND RE-EPITHELIALIZATION

Following the inflammatory stage (about two or three days after injury occurred) vascular angiogenesis with capillary formation occurs in order to ensure that the newly built tissue is provided with nutrients and oxygen. (66) Therefore, cells originated from uninjured blood vessels in the surrounding undergo epithelial-to-mesenchymal transition (EMT), activated through VEGFs. Collagenases, plasmin and plasminogen (produced by the epidermal cells) decompose the extracellular matrix to make way for the new vessels. Then, these cells start to migrate into the matrix and to proliferate by forming new capillary-like tubes (67).

Granulation tissue derives from the visual granular appearance of newly forming tissue and is important to fill up the expansion space after injury with loss of tissue. (68) The intactness of the granulation tissue depends on several factors: the activation of target cells, the presence of biological modifiers like growth factors, and the environment of the ECM. (69) These processes are implemented and regulated by growth factors from the plasma or released by the activated platelets in the wound area or by injured and therefore activated

parenchymal cells. The newly formed clot which serves as first temporary matrix (including fibrin, fibronectin, and hyaluronic acid) also promotes granulation tissue formation.

The fibrin-matrix of a wound also contains other plasma proteins, such as fibronectin and vitronectin which may act as bridging molecules building a link between smooth muscle cells and fibrin by binding to the $\alpha_5\beta_1$ - or $\alpha_v\beta_3$ -receptor on epithelial cells, smooth muscle cells, and other cell types. (70) The appearance of fibronectin and the appropriate integrin receptors that bind fibronectin and fibrin on fibroblasts appears to be the rate-limiting step in the formation of granulation tissue. (71)

When the barrier is disrupted, the keratinocyte becomes activated in a number of different ways to return to hemostasis. If the damage results in a discontinuity of the epidermis, keratinocytes undergo a number of physical and biochemical changes in order to migrate into the wound. The signal for migration is thought to be through the loss of contact inhibition, signaling the cell to switch from a differentiation phenotype (expressing keratins 5 and 14 in the basal layer, or 1 and 10 in the superbasal layers) to a migration phenotype (expressing keratins 6 and 16) (72). Keratinocytes then migrate across the wound bed through the actions of integrin receptors, binding to specific recognition sequences in the granulation tissue (73). Migrating keratinocytes express among others integrin $\alpha_3\beta_1$ and therefore bind to the provisional matrix in the wound bed, rich in fibrin, fibronectin, vitronectin, and laminin-5. Proliferating keratinocytes on the other hand bind via integrin $\alpha_v\beta_5$ and integrins of type β_4 and β_1 . (74)

Once the migration is complete, keratinocytes return to a differentiation scheme. Keratinocyte growth factor (KGF) appears to be the growth factor most closely associated with keratinocyte migration. In addition to migration, hyperproliferation occurs at the wound edge. Migration and hyperproliferation are thought to be the result of cytokine and growth factor stimulation, and involve MAPK signaling (75). This and other data suggest that hyperproliferation and migration of keratinocytes is controlled by multiple overlapping mechanisms, of which endothelial protein C receptor (EPCR) or protease-activated receptor 1 (PAR-1) signaling may have a role. (59)

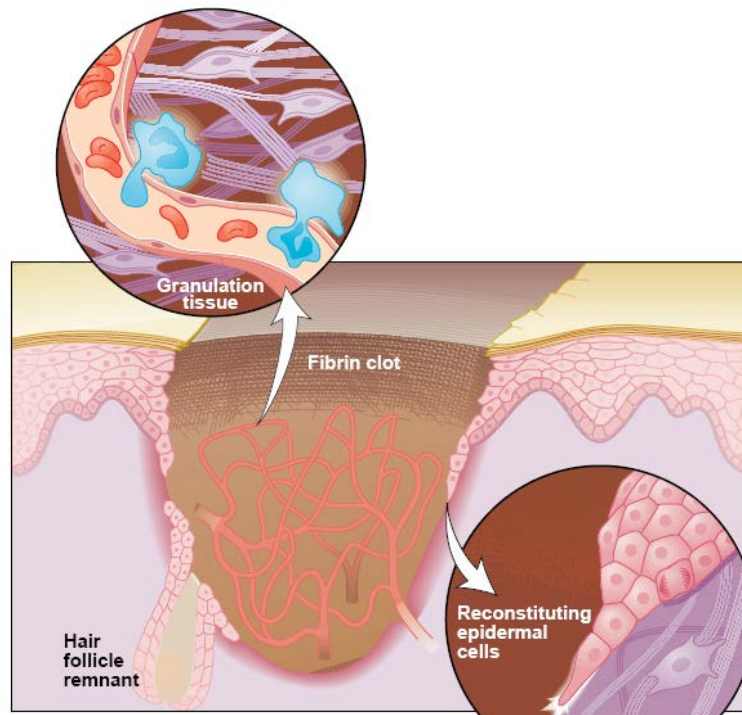


Figure 7 Illustration of the key players in the healing of a skin wound. (Source: Martin, P., et al.: Wound Healing—Aiming for Perfect Skin Regeneration, Science, 1997)(54)

Figure 7 illustrates that the defect is temporarily plugged with a fibrin clot which is infiltrated by inflammatory cells, fibroblasts, and a dense capillary plexus of new granulation tissue. An epidermal covering is reconstituted from the edges of the wound and from the cut remnants of hair follicles. At the migrating keratinocyte leading edge, cells bore a passageway enabling them to crawl beyond the cut basal lamina and over provisional matrix and healthy dermis. Cell division occurs back from the leading edge. Monocytes emigrate from wound capillaries into the granulation tissue, which contracts by means of smooth muscle–like myofibroblasts that tug on one another and the surrounding collagen matrix. (54)

3.3 TISSUE REMODELING AND MATURATION

Fibroblasts and epithelial cells enter the wound site after expressing adequate integrin receptors (63), starting to form a permanent ECM. While the ECM in normal tissue is firmer and more compact, the previous built provisional ECM mainly consists of fibronectin and hyaluronic acid and therefore appears as a hydrated gel-matrix where cells easily can migrate.

The provisional extracellular matrix is gradually replaced with a collagen-containing matrix and the fibroblast-rich granulation tissue is replaced by a relatively acellular scar. (76)

This phase can last over one year. Collagen type I broadens in the matrix and replaces the more present collagen III, the fibers are re-arranged and cross-linked and so the tissue gets stronger. (77)

3.4 WOUND HEALING IN RATS

Rats provide an excellent model for skin wound healing by allowing the standardization of the type, size, shape, and depth of the wound injury, which facilitates comparison of data between studies of healing in all mammalian species. The rat is often selected for skin wound-healing models because of its ready availability, low cost, and small size, which result in a more economical and efficient use of limited laboratory space and housing facilities apart from ethical considerations (78).

One special feature which has to be kept in mind is that rats possess a subcutaneous panniculus carnosus muscle, which humans do not have. This muscle contributes to skin healing by both contraction and collagen formation:

Skin anatomy is important for understanding the wound depth as an aspect of wound healing. Due to their skin's elasticity or redundancy and its lack of a strong adherence to the underlying structures, rats are described as loose-skinned animals. As Mogford and Mustoe (79) explain, the properties of a "loose" skin allow wound contraction to play a significant role in closing rat skin wounds. Consequently, wound contraction, which is usually more rapid than epithelialization, causes a decrease in the overall healing time of rat wounds. Humans have tight skin, and this difference makes comparison with loose skinned animal models difficult, thus transferring this type of animal-derived data to human skin wound healing is problematic (79;80).

But despite these species differences, the availability of animals with well-defined health and genetic backgrounds along with a bounty of literature documenting biological responses and parameters for rats allows the rat to serve as a valuable research tool in the search for faster, stronger, and more anatomically correct wound healing with the ultimate goal of exact skin replacement. (78)

4 THE EXTRACELLULAR MATRIX (ECM)

The ECM is the extracellular part of human tissue, a gel-like matrix which forms the largest component of the dermal skin layer, including the interstitial matrix and the basal membrane. It is produced by the cells which are surrounded by the ECM by exocytose and is composed of water, fibrous proteins, proteoglycans, and glycosaminoglycans. (81) Its appearance is the precondition for wound healing, growth and fibrosis.

4.1 MAJOR CONSTITUENTS OF THE ECM

The major constituents of the ECM are collagens, adhesive glycoproteins such as laminin and fibronectin, and proteoglycans. They not only serve as protein-based scaffold for adhesion and migration but also bind cytokines, growth factors and proteases. (5)

The **collagen** family (16 different types) represents the largest class of fibrous ECM molecules. A normal, healthy skin is composed of 80-85% collagen type I and of 8-11% collagen III. (82) Both types self-assemble to microfibrils (collagen types I – III, V, XI) and get crossed-linked to achieve more stability and strength. Collagen IV molecules form together with laminin and proteoglycans a sheet-like structure called the basal lamina to which epidermal cells attach. This basement membrane separates the epidermis from the dermis and surrounds blood vessels. (77)

As described before, collagenous proteins do not occur always in the same amounts: early granulation tissue contains mainly fibronectin, vitronectin, collagens III, to VI. (83) In the last phase of wound healing, basically collagen I is present. (84)

As collagens give strength, **elastin** molecules give elasticity and stretching ability to the tissue and allow returning in its original stage. This attribute is important especially for blood vessels, lung and skin. Elastin – synthesized by fibroblasts and smooth muscle cells - also builds a cross-linked network with fibrils, which are covered with fibrillin, a large glycoprotein which ensures the integrity of elastic fibers. (81)

Another important glycoprotein is **fibronectin**: it is present in plasma as well as in tissue and its functions are widespread. In the beginning it is secreted in an inactive form and gets activated when binding to integrins. (81) It has different binding sites for collagen, fibrin, and heparin sulfate proteoglycan and links them to different types of ECM molecules. Fibronectin therefore allows interactions between cells and ECM. With its RDG-binding site

(amino acids Arg-Gly-Asp) it binds specific integrin receptors present on the outer membrane of cells. (82)

But also in cell-binding this protein plays a role and allows cells to move through the ECM. Thus, integrins can be linked to collagen, the cytoskeleton of the cell is reorganized and allows cell movement. (81)

For resisting tensile forces the extracellular matrix possesses **laminin**, a large protein which is not arranged in fibers like collagen but forms a network. This molecule assists in cell adhesion and forms bridges to the basement membrane. (81)

Glycosaminoglycans (GAGs) are carbohydrate polymers made up of repeating disaccharide units. Due to its hydrophilic attribute and its high negative charge they attract large amounts of water molecules which are stored in the ECM, keeping it hydrated and enable it to withstand compression forces, forming gels with a high turgor.

A large, special GAG is the hyaluronic acid (HA), a main component of the ECM. It also absorbs a lot of water but also stimulates cytokine production by macrophages and thereby promoting angiogenesis. (77)

Proteoglycans (PGs) also store water in large amounts forming a gel-like substance but also enable ions, hormones and nutrients to move through the ECM. Not only this potential let suggest, that PGs play a role in regulating cell-signaling: heparin sulfate chains of proteoglycans as mentioned above bind to several different growth factors (e.g. FGFs) helping them to bind to their specific cell-surface receptors (77;85). Further, the PG-family is thought to have an influence on selective filtering molecules that pass through the basement membrane beneath the epidermal cells (86).

4.2 ADHESION ON ECM-MOLECULES

Cell adhesion molecules are transmembrane receptors that are important for cell-cell or cell-matrix binding. Most of the CAMs can be assigned to one of four protein families: the integrins, the cadherins, the selectins, and the immunoglobulin superfamily. Other cell adhesion molecules are e.g. the carcinoembryonic antigen, CD22 (binds to sialic acid), CD24, CD44 (receptor for hyaluronic acid), CD146, and CD164.

The adhesion of different cell types to fibrin(ogen) and fibrin matrix is mediated by discrete regions of α and γ chains of the fibrinogen molecule. These regions consisting of short amino acid sequences are recognized by specific cell surface receptors (87-89). Cell adhesion to the fibrin as well as other molecules present in the ECM is predominantly mediated by integrins.

Integrins are structurally and functionally diverse families of cell adhesion molecules, which regulate cell-cell and cell-ECM interactions and in addition mediate signals for cell growth, proliferation, migration, or apoptosis (90). Integrins are heterodimers composed of different, non-covalently associated type I transmembrane α - and β -subunits leading to 25 differentially composed heterodimers with redundant but also specific ligand binding. To date 19 α -subunits and 8 β -subunits were identified in mammals that associate into 25 different integrins. (91)

Integrins connect the extracellular matrix (ECM) with the cytoskeleton (i.e. the microfilaments) inside the cell. The β -subunit is mostly the deciding part which ligands can bind to it. Which matchings of α - and β - chains are found and more or less investigated are listed in figure 8.

Attachment to the ECM allows a cell to resist shear forces and traction without being snatched from the matrix and is a basic requirement to build a multicellular organism. Integrins transmit signals of the surrounding into the cell by mediating the downstream consequences of cell adhesion. This leads to the second major function: signal transduction. Integrins play an important role in cell signaling and can have a relation to cell growth, cell division, cell survival, differentiation and apoptosis. (92;93)

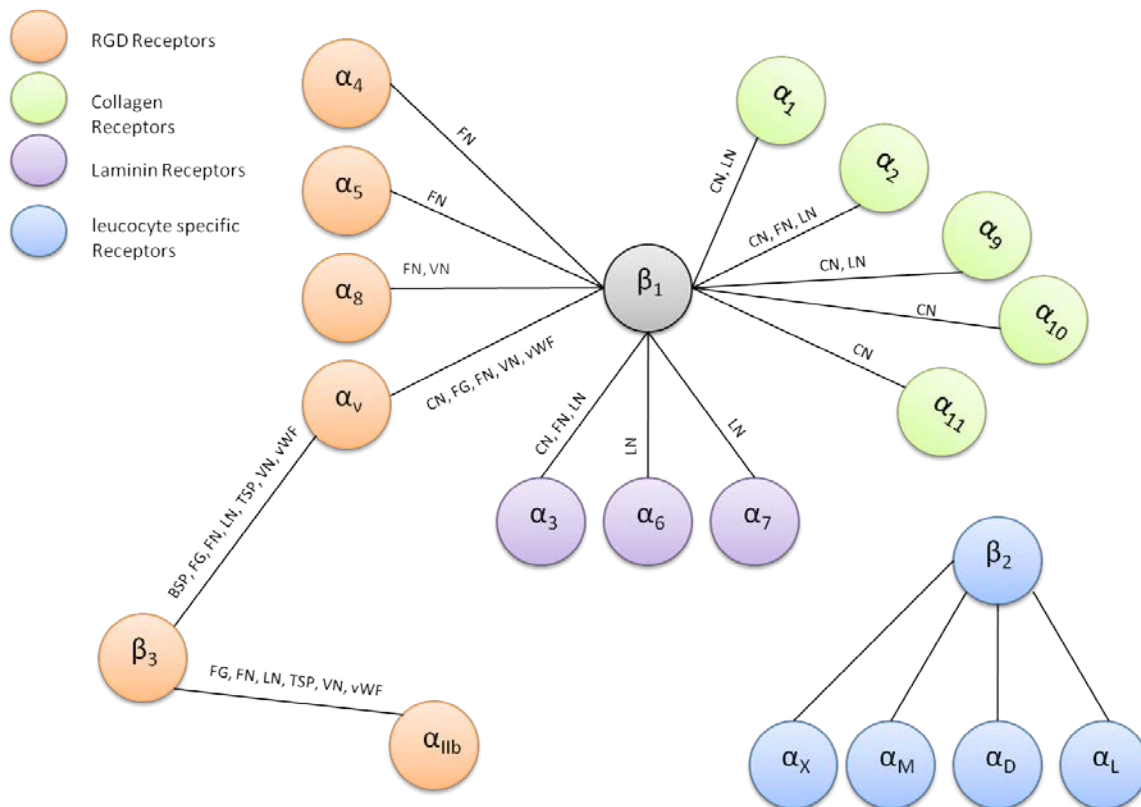


Figure 8 Integrin families and their binding partners (adapted from: <http://www.imgt.org/IMGTEducation/Tutorials/Integrins/image.png>)

Fibroblasts bind directly to fibrinogen through integrin $\alpha\beta_3$ receptors and ICAM-1 (87;94). Fibrin induces attachment, spreading and migration of endothelial cells *via* interaction with integrin $\alpha\beta_3$ and $\alpha\beta_1$ receptors (95;96). Endothelial cells express additional integrin receptors and can interact with other ECM proteins (97). Since keratinocytes lack $\alpha\beta_3$ integrins, adhesion and motility of keratinocytes on fibrin matrix requires a different set of receptors that mediate interaction with other ECM proteins, e.g. fibronectin and vitronectin (98-100).

5 CELLS

5.1 NORMAL HUMAN EPITHELIAL KERATINOCYTES - NHEK

Keratinocytes represent a special form of epithelial cells which contain high amounts of the cytoskeletal protein keratin. They account for 90-95% of epidermal cells in the epidermis.

The epidermis is divided into four or five layers (depending on the type of skin) based on keratinocyte morphology. (101)

Keratinocytes originate from stem cells residing in the basal layer. During their progress through the layers of the epidermis to the skin surface, they differentiate to cells that are enucleated, flattened and keratinized. Keratin gives cells a higher mechanical stability and makes them tough and impermeable. Between the cells in the spinous layer, intercellular adhesion complexes can be detected called desmosomes. In the granular layer keratinocytes synthesize keratohyaline granules, including profilaggrin, loricrin, and cornifin. Finally, they die off and form the stratum corneum, which builds an effective barrier of dead cells.

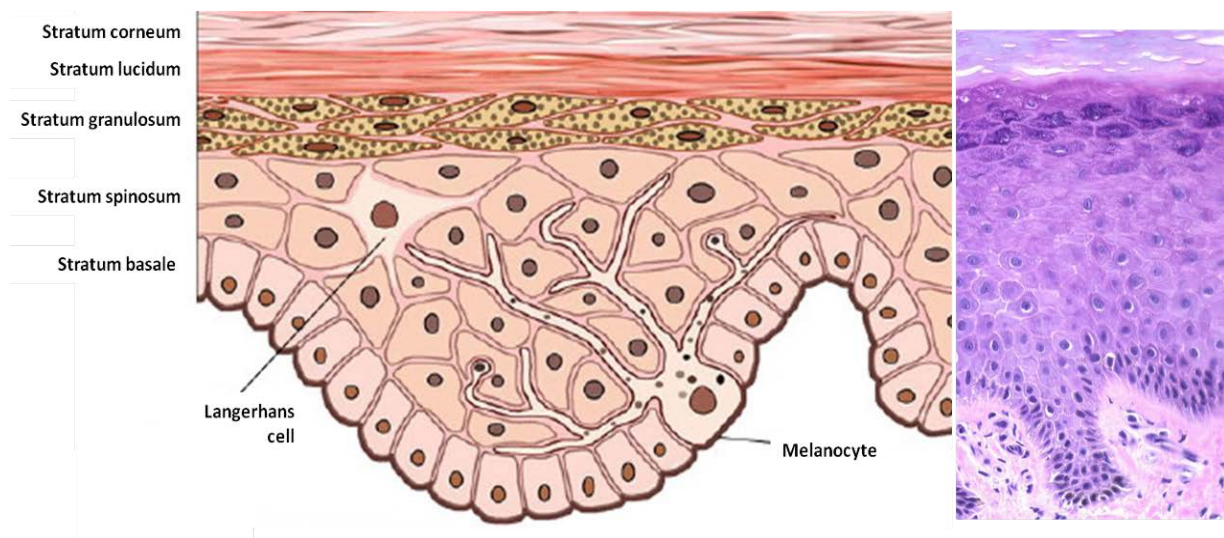


Figure 9 Sublayers of epidermis

(Source: illustration: <http://dermnetnz.org/doctors/principles/images/epidermis.jpg>; histology: http://missinglink.ucsf.edu/Im/DermatologyGlossary/img/Dermatology%20Glossary/Glossary%20Histo%20Images/Epidermis_40x-211.jpg)

Stratum corneum is the outermost layer of the epidermis composed mainly of dead cells. Stratum lucidum is a thin, clear layer of dead skin cells in the epidermis. Stratum granulosum is the highest layer in the epidermis where living cells are found, the stratum lucidum above appears clear due to auto-digestion of cellular organelles. Stratum spinosum is a multi-layered arrangement of cuboidal cells that sits beneath the stratum granulosum. Stratum basale is the layer of keratinocytes that undergo rapid cell division to replenish the regular loss of skin by shedding from the surface.

When the skin is injured a cascade of precisely controlled events is initiated with the aim to restore the protective function of the epithelium. Epidermal keratinocytes play a central role

in the repair of wounded epithelium (102). For this reason the capability of products to support adhesion, growth, and migration of keratinocytes is of vital importance for their performance. (103;104)

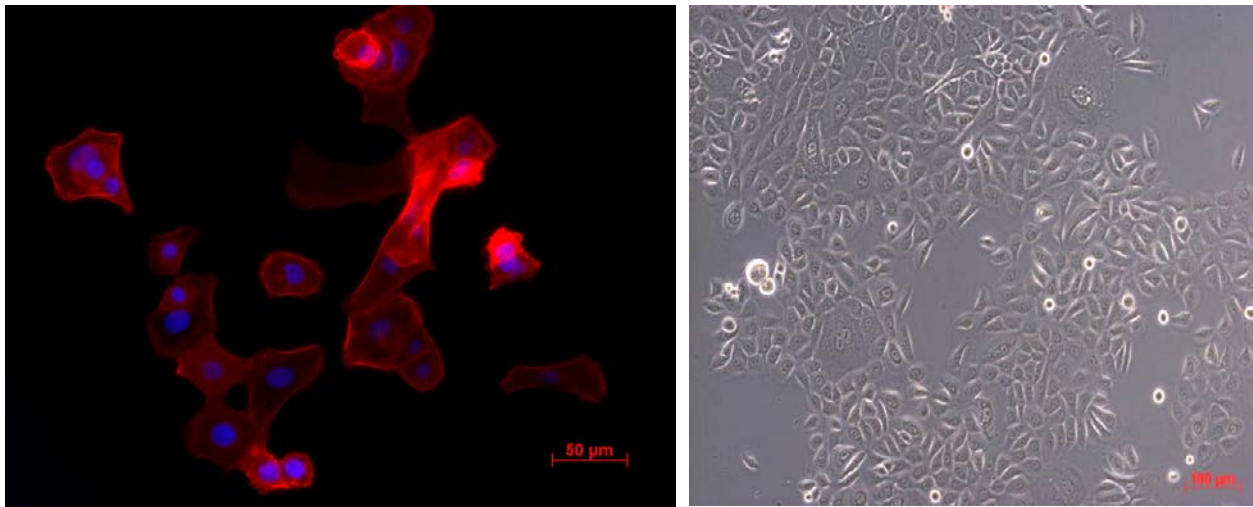


Figure 10 Morphology of NHEK; Phalloidin/DAPI stained (right) and light microscopy (left)

Apart from that, keratinocytes can also act as key immunocytes (105): they have a guarding role through the release of TNF-alpha, an accessory role in the immune response through the production of MHC class II molecules (106) and a target cell role through antigen presentation to cytotoxic T cells.

5.2 EPITHELIAL CELLS

An epithelium is a cell layer that lines the inner and outer surfaces of tissues. Its functions are secretion, absorption, trans-cellular transport, protection, and selective permeability. Histological, epithelia can be divided into three basic types: squamous, cuboidal and columnar. Moreover, the arrangement in layers can be simple (single layer) or stratified. An epithelium often is defined by the expression of the adhesion molecule E-cadherin.

In several cases, an epithelial tissue can change into mesenchymal tissue; a process called epithelial to mesenchymal transition (EMT). This alteration is a highly conserved and fundamental process which governs morphogenesis in multicellular organisms. A phenotypical transition involves the loss of epithelial markers such as components of cell-to-cell contacts (E-cadherin, catenins and cytokeratins), but also needs de novo expression of mesenchymal markers, including vimentin, fibronectin, and N-cadherin. EMT plays a crucial

role during ontogenesis and pathological events like wound healing, but also in late stage tumor progression which leads to invasion and metastasis. (103;104)

HUVEC are isolated – as their name let suggest - from the vein of the umbilical cord. HUVEC express cell adhesion molecules especially after cytokine stimulation (e.g. VEGF) (107) and are commonly used for physiological and pharmacological investigations.

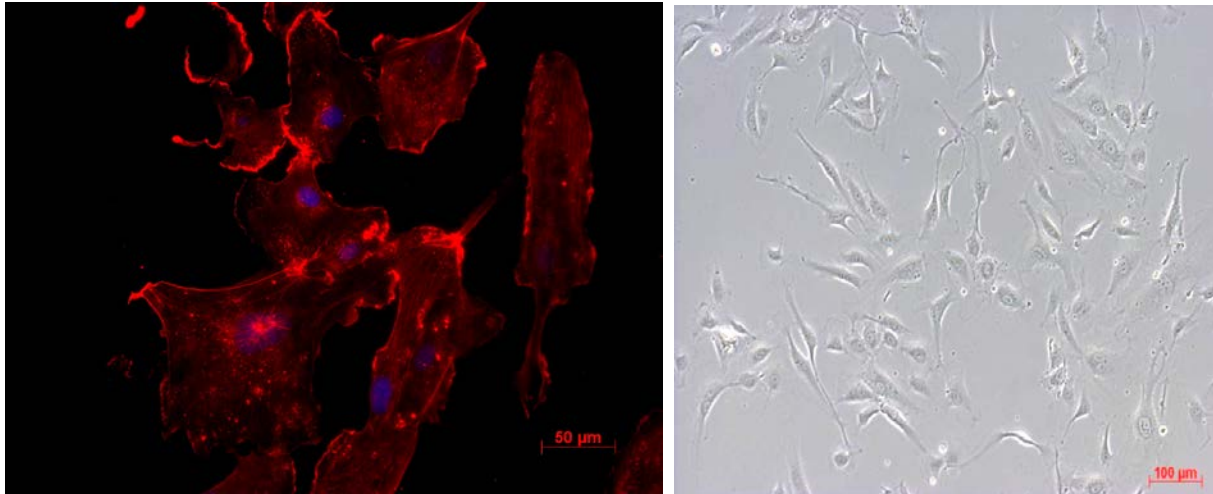


Figure 11 Morphology of HUVEC; Phalloidin/DAPI stained (right) and light microscopy (left)

5.3 NORMAL HUMAN DERMAL FIBROBLASTS - NHDF

Fibroblasts are connective tissue cells that make and secrete collagen proteins and maintain the extracellular matrix. They are morphologically heterogeneous. Their appearance depends on their location and activity. As mentioned above, they express amongst others the intermediate filament vimentin, a feature used as marker for this type of cells. Fibroblasts have a flat, elliptical nucleus, branched cytoplasm and are not restricted by a polarizing attachment to a basal lamina like epithelial cells. (103)

Their big role in wound healing is in the second phase, when fibroblasts and macrophages infiltrate the wound to initiate reconstruction.

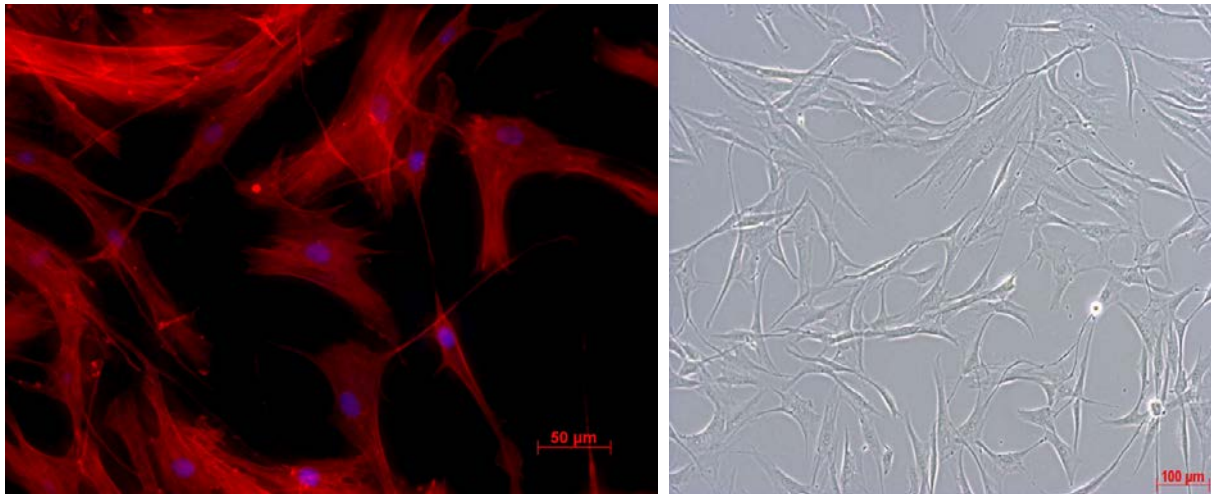


Figure 12 Morphology of NHDF; Phalloidin/DAPI stained (right) and light microscopy (left)

6 Aim

Fibrin sealants are widely used as hemostats in various surgical indications and for sealing of soft tissue. In recent clinical studies fibrin sealants have been used also as a matrix to promote wound healing.

Fibrin sealants are derived from human plasma and therefore they do not only consist of fibrinogen and thrombin, but also of hundreds of constituents present in the plasma. Due to different purification and virus inactivation procedures, variations in salt concentrations and diverse enrichments or depletions, the products not only differ between producers and products but also among lot numbers of the same brand name.

That raised our interest to examine what are the most important components of a fibrin matrix for optimal interaction of the extracellular matrix with cells. In this thesis we examined fibrin sealants regarding their components besides fibrinogen in the final product which is applied – the fibrin clot. We investigated how the presence of proteins present in fibrin sealant affects cell-matrix interaction.

Material and Methods

Fibrin Sealant material

Fibrin sealants Artiss and Tisseel were provided by Baxter AG (Vienna, Austria), Evicel was purchased from Ethicon (Sommerville, NJ, USA).

Cell culture

Cryopreserved normal human endothelial keratinocytes (NHEK), normal human dermal fibroblasts (NHDF) and human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell (Heidelberg, Germany). Cells were thawed and cultivated according to manufacturer's protocol and were grown until 70-80 % confluence before harvesting. Passages 3 to 8 (NHEK) or 3 to 10 (NHDF, HUVEC) were used for experiments.

Fibrin clot preparation

Fibrinogen and thrombin were reconstituted according to the manufacturer's protocol: lyophilized sealer protein component Artiss was equilibrated at room temperature and reconstituted in 3,000 KIE/ml aprotinin solution at 37°C. The room temperature equilibrated lyophilized thrombin component was reconstituted in 40 mM CaCl₂ solution to yield a 4 IU/ml thrombin solution. Thrombin components of 505 IU/ml and 820 IU/ml, but with otherwise identical composition were obtained from a concentrated thrombin stock (1014 IU/ml, product code: VN0301096, Lot Nr.: VNFEK028A, Baxter AG, from production after ultrafiltration 3, endotoxin tested) which was diluted with 25 % HAS, CaCl₂ and NaCl to appropriate concentrations.

Evicel cryo kit was warmed up to 37°C in a waterbath before use.

For Evicel fibrin sealant with 4 IU/ml thrombin, Evicel thrombin component was diluted with the appropriate dilution buffer.

Evicel thrombin dilution buffer:

human serum albumin

mannitol

NaAcetate

CaCl₂.H₂O

NaCl; pH 6.7

Lyophilized FIB3 (Enzyme Research Laboratories; fibrinogen depleted of plasminogen, von Willebrand factor, and fibronectin) was diluted in fibrinogen dilution buffer for Tisseel VH S/D without NaCitrate to ca. 80 mg/ml protein. Clots were made using 4 IU/ml thrombin (= Artiss thrombin component).

For clot formation, equal amounts of fibrinogen and thrombin components were mixed in a tissue culture plate on a vortex mixer. Clots were incubated at 37°C for 1 hour and then washed over night with PBS/aprotinin solution (1500 KIE/ml) at room temperature before seeding of cells. This process was chosen to mimic an in vivo situation where diffusion will take place.

For clot formation in vivo, fibrinogen and thrombin were charged into 1 ml syringes each and put into a “Baxter duplo ject” syringe holder with a mixing piece to get a homogeneously merged fibrin-thrombin-compound. 150 µl fibrin clot were applied to the wound and let polymerize for two minutes.

For fucoidan clot formation, 3.0 mg Fucoidan from *A. nodosum* LMW was lysed in 50 µl water and subsequently mixed with 950 µl Tisseel VH S/D. This fibrinogen/fucoidan mixture was clotted by adding thrombin dilution buffer (no thrombin, only calcium is necessary for building a clot). The mixture was incubated at 37°C for one hour.

For formation of clots with high salt concentration, 200 mM NaCl and 100 mM arginine were added to the thrombin component before mixing with fibrinogen. The resulting clot consists of additional 100 mM NaCl and 50 mM arginine.

Cell quantification by measurement of lactate dehydrogenase

Cells were counted and suspended in cell culture medium. In each experiment, 3 to 6 clots (300 µl) per formulation were seeded with 20,000 cells/clot (in 500 µl medium) in a 24 well plate. Clots overlaid with cell culture medium only served as controls. Clots were incubated with cells at 37°C/5 % CO₂. At selected time points cells were lysed with 9 % TritonX-100. Lysate aliquots (3x 50 µl) from each well were transferred into a 96 well plate and 50 µl LDH/well substrate (CytoTox96 assay substrate mix, Invitrogen, Carlsbad, CA, USA) were added. Plates were then incubated in the dark at room temperature for 30 minutes and absorbance was measured at 490 nm.

Morphology of cells

Clot formation and cell seeding was conducted as described above. Cells were grown on fibrin clots (300 µl) for 24 and 48 hours. After fixation and permeabilization, cells were stained with 10 µg/ml TRITC-phalloidin/PBS and 5 µg/ml 4',6-diamidino-2-phenylinol (DAPI) under light protection. Microscopic analysis was done on an AxioObserver Z1 invert microscope (Zeiss, Jena, Germany).

Fixing solution:

3 % Formaldehyde

0.2 % Glutaraldehyde

in 0.1 M Na-Phosphate buffer; pH 7

Permeabilization solution:

20 mM Hepes

300 mM sucrose

50 mM NaCl

0.5 % Triton X-100

in ddH₂O

Extraction of fibrin sealant clots

By washing with different solutions containing either salts and/or additional denaturing agents (i.e. PBS, 1 M NaCl, 1 M NaCl solution containing 1 % acetonitrile and 0.1 % trifluoroacetic acid, 2 M urea solution) extractable components were removed from fibrin clots. Solutions were changed several times within 24 hours. Subsequently, clots were equilibrated with PBS/aprotinin (1500 KIE/ml) to remove extraction substances. Extractions were observed by SDS-polyacrylamide gelelectrophoresis and Coomassie staining or by Western blot analysis.

FACS Analysis

Artiss fibrin sealant clots were polymerized in 6 well plates and either washed with 1 M NaCl/aprotinin as described above and equilibrated with PBS/aprotinin (washed Artiss clot) or only incubated over night with PBS/aprotinin (untreated Artiss clot). NHEK were seeded 1 million per clot just as per well (cells grown on plate).

After 24 hours, cells were detached with trypsin and fixed in fixing solution (see Morphology of cells). NHEK were washed with PBS/2 % FCS, counted and stained 15 min with integrin β1

antibody (FITC-conjugated; Chemicon). Analysis was done on a BD FACSCalibur flow cytometer.

Purification of fibrinogen from FVIII cryoprecipitate (human plasma)

Approximately 100 g FVIII Cryoprecipitate were thawed and diluted in 10 volumes dilution buffer (DB). This solution was stirred slowly at room temperature for 1 hour.

Dilution Buffer (DB)

NaCitrate

NaCl, pH 7.3

First glycine precipitation: glycine was added to a final concentration of 130 g/L (slow and in small portions). This glycine solution was stirred slowly for 1 hour at room temperature. The solution was centrifuged for 20 min at 8500 rpm at room temperature, the soluble fraction contains fibronectin. The pellet was crushed, weighed, dissolved in 10 volumes of DB and stirred for 30 min. For the second glycine precipitation, glycine was slowly added to a final concentration of 120 g/L and stirred for 1 hour followed by centrifugation for 20 min at 8500 rpm at room temperature. Soluble fraction was discarded.

The resulting pellet was dissolved in 8 volumes of urea buffer and stirred two days at 4°C. This step is required to inactivate FXIII bound to fibrinogen. This sample was dialyzed against 20 volumes of DB at 4°C with three buffer changes.

Urea Buffer:

Urea

NaCl

NaCitrate

Aprotinin

For fibrinogen precipitation ice cold 99 % ethanol was given to the cooled sample in small portions (drop by drop) to a final concentration of 8 % EtOH and stirred at 4°C for 30 minutes. The precipitate was centrifuged for 20 min at 8500 rpm at 4°C. The soluble fraction was discarded and the pellet dissolved in fibrinogen dilution buffer to a final protein concentration of 100 mg/ml.

Fibrinogen Dilution Buffer

Aprotinin

NaCitrate

Niacinamid

Histidin
HSA; pH 7.3

Spiking of FIB3-Clot

As the concentration of fibronectin in the Baxter fibrin sealant is named as 2 – 9 mg/ml (mainly used Artiss lot: ~3 mg/ml), FIB3 was spiked with fibronectin to appropriate concentrations. Lyophilized purified, human fibronectin (Becton, Dickinson and Company, NJ, USA) was reconstituted to a concentration of 5 mg/ml. It was mixed with the fibrin sealant to a final concentration of 2 ml/ml in the resulting clot.

Initiated Clot Lysis

The urokinase stock solution (10,000 IU/ml) was diluted to 1 IU/ml urokinase in 0.9 % NaCl solution. After washing the clots two times for 2 hours with 0.9 % NaCl, 500 µl of lysis buffer were added to each clot and clots were incubated at 37°C shaking. Supernatants were exchanged daily.

Coating of ECM molecules for NHEK adhesion

96-well bacteria plates were treated with different ECM proteins over night at 4 °C. All proteins were used at a concentration of 30 µg/ml, wells were coated with 50 µl (=1.5 µg protein per well).

After washing with PBS, 12,000 cells per well were seeded. After 4 hours and 24 hours LDH assay for cell quantification was performed.

Thrombin activity

The thrombin components of three different commercially available fibrin sealants were tested for thrombin activity by a turbidimetry assay using a Behring Coagulation System (BCS) (Siemens Healthcare Diagnostics, Deerfield, IL, USA).

To measure the activity of thrombin on the surface of the fibrin sealant clot, the Sensolyte[®] 520 Thrombin Activity Assay Kit *Fluorimetric* (AnaSpec, Fremont, CA, USA) was used. Clots of 50 µl were polymerized in a 96 well plate (0.33 cm² surface area per well) and washed

over night with PBS/aprotinin solution (1500 KIE/ml). The assay was performed according to manufacturer's protocol.

Preparation for scanning electron microscopy (SEM)

Fibrin clots (200 µl) were fixed in SEM fixing solution containing 0.1 M sodium cacodylate and 2.5 % glutaraldehyde. Subsequently, clots were washed (0.1 M sodium cacodylate without glutaraldehyde), dehydrated with 2,2-dimethoxypropane and dried with hexamethyldisilazane. Clots were sputter coated with palladium gold in an Emitech (Molfetta, Italy) sputter coater SC7620 and analyzed in a SEM Jeol 6510 (Jeol Ltd, Tokyo, Japan).

Staining of live and dead cells

Cells were grown on top of 300 µl fibrin sealant clots for 24 or 48 hours in 24 well plates. For staining of live and dead cells (Live/Dead® viability/cytotoxicity kit for mammalian cells; Invitrogen, Carlsbad, CA, USA) cells were incubated with calcein AM and ethidium homodimer-1 fluorescent dye solution according to the manufacturer's protocol. Microscopy was done on an AxioObserver Z1 invert microscope (Zeiss, Jena, Germany). Red and green fluorescent signals were quantified with the Plate Reader Synergy MX (Bio-Tek, Winooski, VT, USA) at 485 nm and 528 nm.

Hirudin inhibition of clot bound thrombin

Fibrin clots (100 µl) were prepared with a 820, 505 or 4 IU/ml thrombin component in 96 well plates and washed over night with PBS/aprotinin solution (1500 KIE/ml). Clots were incubated for 1 hour at 37°C either with dH₂O (control), or with 50 µl 100 IU or 200 IU/ml hirudin solution (Sigma-Aldrich, St. Louis, MO, USA) and subsequently washed. Alexa Fluor® 546 conjugated fibrinogen (500 µg) (Molecular Probes, Eugene, OR, USA) was mixed into 1 ml fibrinogen component of the fibrin sealant. A 50 µl aliquot of the mixture was applied to washed fibrin clots in order to examine clot formation induced by fibrin-bound thrombin. After 15 minutes incubation at 37°C, clots were washed to remove unbound fibrinogen and fluorescence was measured (Exc. 520 nm/Em. 580 nm; Plate Reader Synergy MX, Bio-Tek, Winooski, VT, USA).

For cell quantitation on hirudin blocked fibrin clots (300 μ l in 24 well plates), clots were incubated with hirudin one hour prior cell seeding. The hirudin concentrations were deduced from the results of the SensoLyte[®] 520 thrombin activity assay by extrapolating the thrombin values measured in a 96 well plate to the increased surface area of a 24 well plate.

Caspase Activity Assay

Fibrin clots (100 μ l) were prepared in a 96 well plate and washed over night with PBS/aprotinin solution (1500 KIE/ml). Cells (40,000/clot) were seeded onto clots and caspase activity was measured after 4 hours by using an one step fluorometric assay with DEVD-Rhodamine110 substrate according to the manufacturer's protocol (Homogeneous Caspases Assay[®], Roche Applied Sciences, Basel, Switzerland). Cell number was determined as loading control using a LDH assay.

Staining of proliferating cells

NHEK were grown in 24-well plates until 80 % confluence and then incubated with medium containing different concentrations of thrombin taken from the results of the Thrombin Activity Assay Kit described above. After 24 hours incubation, cells were fixed and permeabilized. Immuno-cytochemistry staining against Ki67 was performed, nuclei were counterstained with DAPI.

PAR-1 blocking

For several experiments, thrombin receptor PAR-1 was blocked: e.g. adhesion/viability assay, MAPK Array, morphology of cells grown on clots, pERK western blot analysis. Therefore, NHEK were incubated with 75 nM SCH797979, a selective PAR-1 antagonist, for 30 minutes before seeding. The SCH797979 powder for preparing of the stock (7.5 mM) was reconstituted in DMSO. As control, mock treated cells were incubated with appropriate concentrations of DMSO.

Protein Arrays

Human Apoptosis Antibody Array: The Human Apoptosis Array (R&D Systems) is a rapid and sensitive tool to simultaneously detect the relative expression of 35 apoptosis-related proteins in a single sample and screening of affected proteins.

Fibrin clots Artiss, Evicel and A820 were polymerized in 6 well plates and washed over night with PBS/aprotinin. NHEK were seeded 1 million per clot. After 30 hours, cells were washed with ice cold PBS and lysed with 250 µl lysis buffer containing protease and phosphatase inhibitors. Protein concentration of the lysates was measured by a DC-protein assay. 300 µg protein were applied per membrane. The array was performed according to manufacturer's protocol. Evaluation was done on a ChemiDoc XRS (BioRad) with QuantityOne 4.6.3.

Resultant, Western blots were realized: Trail-R2/DR5, Fas, Caspase 3 and cleaved Caspase 3. Samples were generated as described above for the apoptosis array.

Proteome Profiler Human Phospho-MAPK Array: Screening of levels of phosphorylation of Mitogen-Activated Protein Kinases (MAPKs) and other serine/threonine kinases (R&D Systems).

NHEK were grown in T25 cell culture flasks until 90 % confluence and then were treated with 75 nM SCH797979 or appropriate concentration of DMSO in medium as negative control for 30 minutes and further with 1 IU/ml thrombin or CaCl₂ only (negative control) for 15 minutes. Subsequently, cells were washed with ice cold PBS and lysed with 250 µl lysis buffer containing protease and phosphatase inhibitors. Protein concentration of the lysates was measured by a DC-protein assay. 200 µg protein were applied per membrane. The array was performed according to manufacturer's protocol. Evaluation was done on a ChemiDoc XRS (BioRad) with QuantityOne 4.6.3.

Resultant, Western blots were realized: pAkt and pERK. Samples were generated as described above for the apoptosis array.

Proteome Profiler Human Cell Stress Array Kit: Screening of affected human cell stress related proteins (R&D Systems).

Cells were grown in 6-well plates until 90 % confluence. Then, cells were covered with a fibrin clot (Artiss and Evicel; 1 ml/well) and covered with medium (2 ml/well). As negative control, only the fibrinogen component of the fibrin sealants was applied without adding

thrombin hence without polymerized clot. Fibrin clots were removed after 6 hours. Cells were washed with ice cold PBS and lysed with 250 µl lysis buffer containing protease and phosphatase inhibitors. Protein concentration of the lysates was measured by a DC-protein assay. 300 µg protein were applied per membrane. The array was performed according to manufacturer's protocol. Evaluation was done on a ChemiDoc XRS (BioRad) with QuantityOne 4.6.3.

Proteome Profiler Human Cytokine Array Kit, Panel : Screening of affected human cytokines and chemokines (R&D Systems).

Biopsies of the wound edge (excisional wound healing model) were lysed in lysis buffer containing protease and phosphatase inhibitors. Protein concentration of the lysates was measured by a DC-protein assay. 300 µg protein were applied per membrane. The array was performed according to manufacturer's protocol. Evaluation was done on a ChemiDoc XRS (BioRad) with QuantityOne 4.6.3.

Western Blot

Fibronectin, Thrombin - Clot extractions

For analysis of proteins contained in fibrin clots 50 µL fibrin clots were submerged in 400 µL sample buffer. Complete clot lysis was achieved after incubation for 1 h at 95°C with repeated homogenization. Protein concentration was determined with BCA (Pierce, Rockford, IL, USA) and 200 µg protein were loaded per lane. Proteins were either stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes for Western blotting according to standard procedures. As molecular mass marker the pre-stained See Blue Plus2 standard from Invitrogen (Carlsbad, CA, USA) was used. Fibronectin was purchased from R&D Systems (Minneapolis, MN, USA), and thrombin (Th 622) from Baxter AG (Vienna, Austria). Blots were blocked with 5 % milk powder in TBS 0.1 % Tween. Antibodies against fibronectin were from Santa Cruz (Santa Cruz, CA, USA), anti-thrombin from Cedarlane labs (Burlington, Ontario, Canada). As secondary antibodies rabbit anti-sheep HRP antibody from Pierce (Rockford, IL, USA) and goat anti-mouse IRDye 680 from LiCor (Lincoln, NE, USA) were used. ECL reaction was performed with West Pico Chemoluminescence kit from Pierce (Rockford, IL, USA). Signal detection was monitored with ChemiDoc XRS (BioRad) and with Odyssey imaging system from LiCor (Lincoln, NE, USA).

Trail-R2/DR5, Fas

Cells were grown in 6-well plates until 90 % confluence. Then, cells were covered with a fibrin clot (1ml/well) and covered with medium (2 ml/well). Fibrin clots were removed after 4 hours. Cells were lysed and protein concentration of the lysates was measured by a DC-protein assay (BD Biosciences, San Jose, CA, USA).

Proteins (40 µg/lane) were examined on 10 % Bis-Tris SDS PAGE gels (Invitrogen, Carlsbad, CA, USA). Primary antibodies, Trail-R2/DR5 and Fas (both Cell Signaling Technology, Inc., Danvers, MA, USA) and ERK1/2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), were diluted 1:1000 and 1:500. Secondary antibodies used: IRDye[®] goat-α-rabbit 800 nm, IRDye[®] goat-α-mouse 680 nm, LI-COR, Lincoln, NE, USA; Signal detection was monitored with an Odyssey imaging system from LI-COR (Lincoln, NE, USA). Trail-R2/DR5 and Fas signals were normalized with total ERK1/2 signal.

pAkt, pERK

Cells were grown in 6-well plates until 90 % confluence. Then, cells were treated 30 minutes with 75 nM SCH797979 to block PAR-1. After removing of the PAR-1 antagonist, cells were incubated with medium containing thrombin (pERK: 1 IU/ml for 15 minutes; pERK: 4, 505, and 820 IU/ml for 5 minutes; pAkt; 4, 505, and 820 IU/ml for 4 hours). Cells were lysed and protein concentration of the lysates was measured by a DC-protein assay (BD Biosciences, San Jose, CA, USA).

Proteins (40 µg/lane) were examined on 10 % Bis-Tris SDS PAGE gels (Invitrogen, Carlsbad, CA, USA). Primary antibodies, pERK and pAkt (both Cell Signaling Technology, Inc., Danvers, MA, USA) and ERK1/2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), were diluted 1:1000 and 1:500. Secondary antibodies used: IRDye[®] goat-α-rabbit 800 nm, IRDye[®] goat-α-mouse 680 nm, LI-COR, Lincoln, NE, USA; Signal detection was monitored with an Odyssey imaging system from LI-COR (Lincoln, NE, USA).

Surgical procedure and treatment

The experimental protocol was approved by the Animal Protocol Review Board of Vienna, Austria (Approval Nr. M58/004157/2010/9).

Male Sprague Dawley rats with a weight of 350 – 450 g were purchased by Charles Rivers Laboratories. After initial inhalation narcosis with 2.5 % isoflurane, anesthesia was

maintained by 110 mg/kg ketaminhydrochloride and 12 mg/kg xylazin intraperitoneal. Rats were shaved, depilated and disinfected on the back. Two circular full-thickness excisions with a diameter of 1.3 cm were set paravertebral. One excision was treated the other one on the contra-lateral side remained untreated as intern control.



Rats were divided into 7 groups (n=10). 150 µl fibrin sealant was applied to the wound and let polymerize for two minutes.

Following fibrin sealants were used:

1. Artiss (Baxter): 4 IU/ml thrombin
2. Evicel (Omrix): 820 IU/ml thrombin
3. Artiss fibrinogen mixed with 820 IU/ml thrombin
4. Evicel-fibrinogen mixed with Artiss-thrombin
5. Evicel-fibrinogen mixed with diluted Evicel-thrombin (4 IU/ml)
6. FIB3 (fibrinogen depleted of plasminogen, vWF, and fibronectin)
7. FIB3 spiked with fibronectin

Both wounds were covered by a transparent wound foil (Opsite, Smith and Nephew) and a Fixomull® stretch dressing (BSN Medical, Hamburg, Deutschland) which was changed on day three and when necessary. (After surgery, a viewing window was made – only Opsite on the wound - to recognize possible severe inflammation to set human endpoints, after day three a continuous dressing was applied).

As analgesic treatment, all animals received 1.25 mg/kg Butorphanol s.c. and 0.15 mg/kg Meloxicam 5 mg/ml s.c. after operation and 0.15 mg/kg Meloxicam 5 mg/ml s.c. for three days post-operative.

Animals were housed in pairs, maintained at 22–24°C with a 12-hours light/dark cycle and allowed food and water ad libitum.

After seven days, rats were sacrificed by an intra-cordial injection of 150 mg/kg Nembutal. For histological analysis, tissue was fixed in 3 % phosphate-buffered formalin for 48 hours,

dehydrated and embedded in paraffin. Tissue from the wound edge was fixed in N_2 for protein analysis.

Planimetry

To measure wound closure, excisional wounds were traced on a transparent acrylic sheet after surgery and on day 3 and 7 post-OP. Sheets were digitalized and initial and residual open wound areas were further analyzed with a ChemiDoc XRS (BioRad) with QuantityOne 4.6.3.

Histological analysis

Excisional wounds were pinned on a cork plate to ensure a plain preparation and fixed in 3% neutrally buffered formalin for 24-48 hours. Then, samples were cut with a microtome blade exactly in the middle of the wound (paramedian). After embedding in paraffin, 3 μm tissue sections were cut, de-paraffinized and stained.

Histomorphometry

After hematoxylin and eosin (HE) staining, slides were scanned with an Olympus BX51 microscope, photographed with an Olympus XC10 camera and measured with the program dotSlide2.3.

Parameters were used and calculated as described in Lemo et al. "Cutaneous re-epithelialization and wound contraction after skin biopsies in rabbits: a mathematical model for healing and remodeling index" (108).

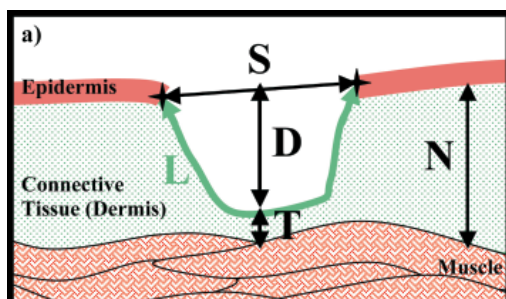


Figure 13 Parameters for Planimetry. (Picture taken from Lemo et al., 2010)

S: distance between the borders of the wound (last keratinocyte)

L: length of re-epithelialization zone

D: depth of the wound from S to deepest point of the wound

T: thickness of the connective tissue (residual dermis or new dermis)

N: thickness of the natural dermis $N=D+T$

Superficial Contraction Index $SCI = (L-S)/L$

Deep Contraction Index $DCI = (N-D)/N$

Wound Severity Index $WSI = (N-T)/N$

Wound Contraction Index $WCI: SCI+DCI$

Global Healing Index $GHI: SCI+DCI-WSI$

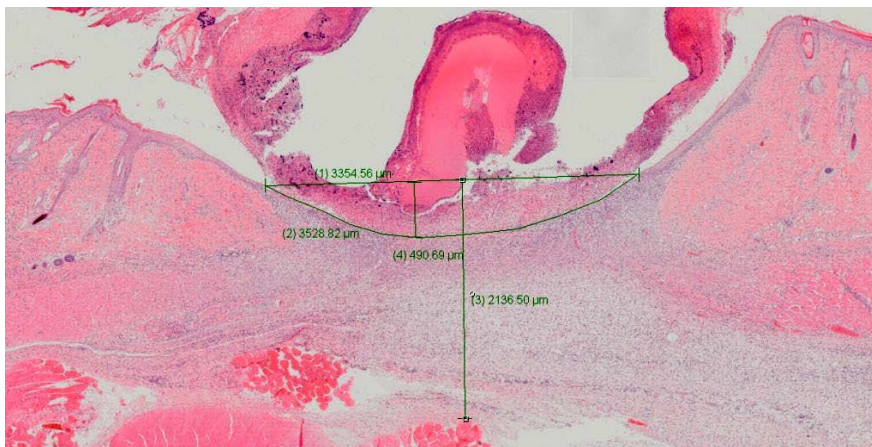


Figure 14 HE-staining of Slide # B100 (wound treated with FIB3 clot). (1)S, (2)L, (3)N, (4)D

Immuno-histochemistry

Endogenous peroxidase was blocked by incubation with H_2O_2 and epitope retrieval was performed by proteinase K treatment or steaming using a conventional vegetable steaming device (Braun). Then, tissue sections were stained with SMA and vWF (vessels) or Ki67 (proliferating cells). After incubation with a HRP labeled polymer, slides were stained with

Vector NovaRed, followed by a nucleus counter staining with Mayer's Hemalaun, dehydration and permanent mounting.

Microscopy was done on an Olympus BX51, photographed with an Olympus XC10 and measured with the program dotSlide2.3 or on a Zeiss Observer.Z1 invert microscope and evaluated with the program AxioVision 4.8.0.

Fibrin Degradation in vivo

For fibrin degradation studies Artiss and Evicel fibrin sealants were mixed with 150 µg/ml human fibrinogen conjugates Alexa fluor 546 (Molecular Probes, Eugene, OR, USA). Fibrin sealant application was done as described above. In one rat, one sample of Artiss and (on the contra-lateral side) Evicel were applied.

Degradation was measured on days 0, 1, 2, 3, 4, 6, 8, 9, 10 and 13 in a Maestro (Cri, part of Caliper Life Sciences, Hopkinton, MA, USA).

Western Blot

IL-1 α , VEGF, Ang-1

Animals were treated as described above in the excisional wound model. Two wounds in one rat were treated either with Artiss or Evicel, in another rat, both wounds remained untreated. On day 2, biopsies were taken from the wound edge and from healthy tissue of the back 4 cm caudal of the Artiss and Evicel treated wounds. The tissue was lysed in 2 % Triton X-100 containing phosphatase and protease inhibitors on 4°C for 4 hours and was periodically homogenized in this time. Protein concentration of the lysates was measured by a DC-protein assay (BD Biosciences, San Jose, CA, USA).

Proteins (150 µg/lane) were examined on 10 % Bis-Tris SDS PAGE gels (Invitrogen, Carlsbad, CA, USA). Primary antibodies, VEGF (1:200), IL-1 α (1:200), ERK1/2 (1:500) (all Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Angiopoietin-1 (Rockland Immunochemicals Inc., Gilbertsville, PA, USA; 1:500), and Actin (Cell Signaling Technology, Inc., Danvers, MA, USA; 1:1,000) were diluted and membranes were incubated at 4°C over night. Secondary antibodies used: IRDye[®] goat- α -rabbit 800 nm, IRDye[®] goat- α -mouse 680 nm, LI-COR, Lincoln, NE, USA; Signal detection was monitored with an Odyssey imaging system from LI-COR (Lincoln, NE, USA). VEGF, IL-1 α and Ang-1 signals were normalized with total ERK1/2 signal or actin, respectively.

In-situ-hybridization (TUNEL)

One of the terminal and unmistakable steps in apoptosis is endonucleolytic degradation of cellular DNA. Detection of degraded DNA fragments by Terminal Transferase nick-end-labeling (TUNEL) is a commonly-used method for quantization of apoptosis.

The DNA strand breaks are detected by enzymatically labeling the free 3'-OH termini with modified nucleotides. These new DNA ends that are generated upon DNA fragmentation are typically localized in morphologically identifiable nuclei and apoptotic bodies. In contrast, normal or proliferative nuclei, which have relatively insignificant numbers of DNA 3'-OH ends, usually do not stain in TUNEL assay.

The ApopTag® Peroxidase in Situ Apoptosis Detection Kit (Millipore, S7100) labels apoptotic cells in research samples by modifying DNA fragments utilizing terminal deoxynucleotidyl transferase (TdT) for detection of apoptotic cells by specific staining.

The in-situ-hybridization was performed according to the manufacturer's protocol, as positive control a mouse embryo was stained.

Pathological analysis

For qualitative analysis, HE stained tissue sections were observed by the head of the pathology of the Landesklinikum Mostviertel Amstetten, Austria.

Following parameters were included: grade of inflammation, neo-vascularization, scar tissue formation (new built collagen), re-epithelialization (wound closure) and possible pathological abnormalities (e.g. seroma, fibrosis).

Indications were given from stage 0 (no inflammation/neo-vascularization,...) to stage 3 (distinct inflammation/neo-vascularization,...).

Statistics

Data are presented as mean $\pm$ SEM of at least three independent experiments (in vitro) and statistical analyses were performed with software MiniTab 15 Statistical comparisons for all experimental settings were based on two sample t-test, ANOVA or General Linear Model using Tukey's test with $p < 0.05$ considered as significant.

Results

1 COMPARISON OF TWO COMMERCIALY AVAILABLE FIBRIN SEALANTS: ARTISS VS. EVICEL

To evaluate the cell compatibility of two different, commercially available fibrin sealants Artiss and Evicel, primary human epithelial keratinocytes (NHEK) were used. They were seeded on these fibrin sealant (FS) clots. After 4 hours, 24 hours and 48 hours cell adhesion/proliferation, morphology and viability assays were performed.

Table 5 Summary of in vitro experiments Evicel/Artiss: A) Cell adhesion: relative % of attached NHEK on Artiss and Evicel fibrin clots +/- treated with hirudin relative to Artiss clots which were set to 100%. Cell number was evaluated after 4, 24 and 48 hours incubation by LDH assay. B) Cell death: live/dead staining of NHEK on Artiss and Evicel fibrin clots after 24 hours given in % of dead cells. Caspase activity of cells cultivated on Artiss and Evicel fibrin clots after 4 hours given in % activity relative to Artiss that was set to 100 %. Data are given in mean percentage.

		Artiss (%)	Evicel (%)	Evicel + Hirudin (%)
A) Cell Adhesion n=3-5	4 hours	100 ± 0	44.6 ± 9.5	78.5 ± 14.0
	24 hours	100 ± 0	34.66 ± 16.1	40.9 ± 4.5
	48 hours	100 ± 0	33.52 ± 5.8	53.0 ± 1.8
B) Cell Death	Live/Dead Staining n=3	2.9 ± 1.2	17.5 ± 8.3	
	Caspase Activity n=2	100 ± 0	292.0 ± 79.9	

To determine cell adhesion on fibrin sealant clots the cell number was measured 4 hours after seeding using an LDH assay. Proliferation of cells was detected at 24 and 48 hours time points. On Evicel clots, significant lower cell numbers were measured at all three time points (up to -66.48 % after 48 hours; $p < 0.000$ for 4, 24 and 48 h) compared with Artiss indicating a higher adhesion and proliferation rate of NHEK on Artiss clots (see table 5, first row).

To block fibrin bound thrombin, clots were incubated with hirudin before cell seeding. Cell numbers on Artiss clots varied between 10.7 % less cells (4 hours time point; $p = 0.033$) and 5.2 % more cells (24 hours; $p = 0.161$; data not shown). In contrast, on hirudin-blocked clots of Evicel fibrin sealant a recovery of cell adhesion and growth could be observed compared to untreated Evicel clots: after 4 hours 33.9 % more cell adhesion was measured than on Evicel clots with intact thrombin activity ($p = 0.001$) and therefore 78.5 % compared to Artiss. After 24 hours, no significant better proliferation was measured ($p = 0.309$) but after 48 hours

19.5 % ($p < 0.000$) more cells could be counted on blocked Evicel clots, compared to untreated Evicel clots.

In order to examine whether the decrease of cells on Evicel fibrin clots is caused by cell death, live/dead staining of NHEK on clot was performed. After 48 hours, 2.9 % dead cells were found on Artiss clots compared to 17.5 % on Evicel clots. To assess whether these dead cells are caused by apoptotic events, caspase 3/7 activity was examined 4 hours after cell seeding. The caspase 3/7 activity level was nearly three-fold higher (292 %) in cells grown on Evicel clots.

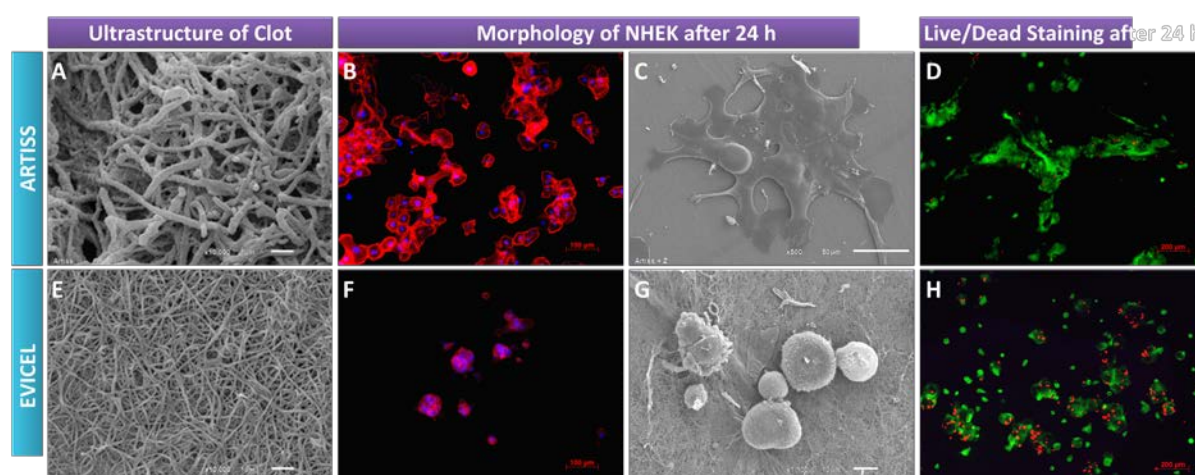


Figure 15 SEM analysis of Artiss and Evicel fibrin clots (A, E). Morphology of NHEK after 24 hours, red stain indicates cytoskeleton, blue stain represents cell nuclei (B, F). Cells on clot after 24 hours in SEM analysis (C, G). Live/dead staining of NHEK on Artiss and Evicel fibrin clots after 24 hours cultivation: green stain (Calcein AM) indicates living cells and red stain (Ethidium Homodimer 1) indicates dead cells (D, H). Size bars: 1 μm (A, E), 100 μm (B, F), 50 μm (C, G), 200 μm (D, H).

Figure 15 shows the different ultrastructures of an Artiss (A) and an Evicel (E) fibrin sealant clot. To examine cell morphology immuno-fluorescence staining was used. A phalloidin/DAPI staining showed deteriorated morphology and minimal spreading of NHEK when grown 24 hours on Evicel clots (figure 15 B/F). Live/dead staining of cells show more dead cells (red stained) in Evicel clots (H).

1.1 BLOCKING OF THROMBIN RECEPTOR PAR-1

To block the thrombin receptor PAR-1, cells were incubated before seeding with SCH797979, a specific PAR-1 antagonist.

Using an LDH assay, cell numbers were evaluated after 4, 24 and 48 hours on Artiss and Evicel fibrin sealant clots with and without SCH79797 treatment. Significantly more cells with blocked PAR-1 attached after 4 and 48 hours on Evicel clots compared to untreated cells. On Artiss a higher number of PAR-1 blocked cells attached after 24 hours while there are no differences at the other time points.

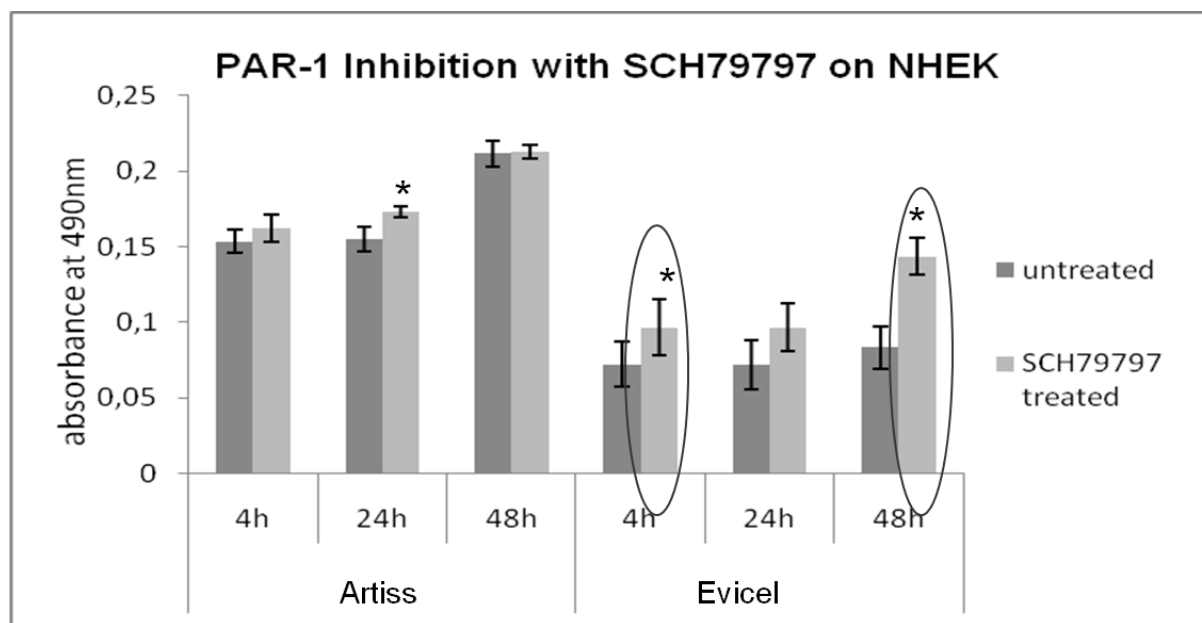


Figure 16 Viability of NHEK on fibrin clots with and without PAR-1 receptor inhibition by SCH79797. NHEK were treated 30 min prior seeding on Artiss and Evicel fibrin sealant clots with PAR-1 inhibitor SCH79797 (75nM). Total cell number adhering to fibrin clots was determined by measuring the amount of lactate dehydrogenase of lysed cells after 4 hours, 24 hours and 48 hours. n=9

1.2 MOLECULAR ANALYSIS OF NHEK EXPOSED TO FIBRIN SEALANTS

To investigate the influence of thrombin on cellular stress level, NHEK were cultivated in 6-well plates until 90% confluence and then covered with Artiss and Evicel fibrin sealant for 6 hours. This experimental setting has been chosen in order to have attached cells which can be exposed to clots for a short time period. As control, NHEK were covered with Artiss and Evicel fibrinogen components without thrombin. Cell lysates were analyzed using a cell stress array.

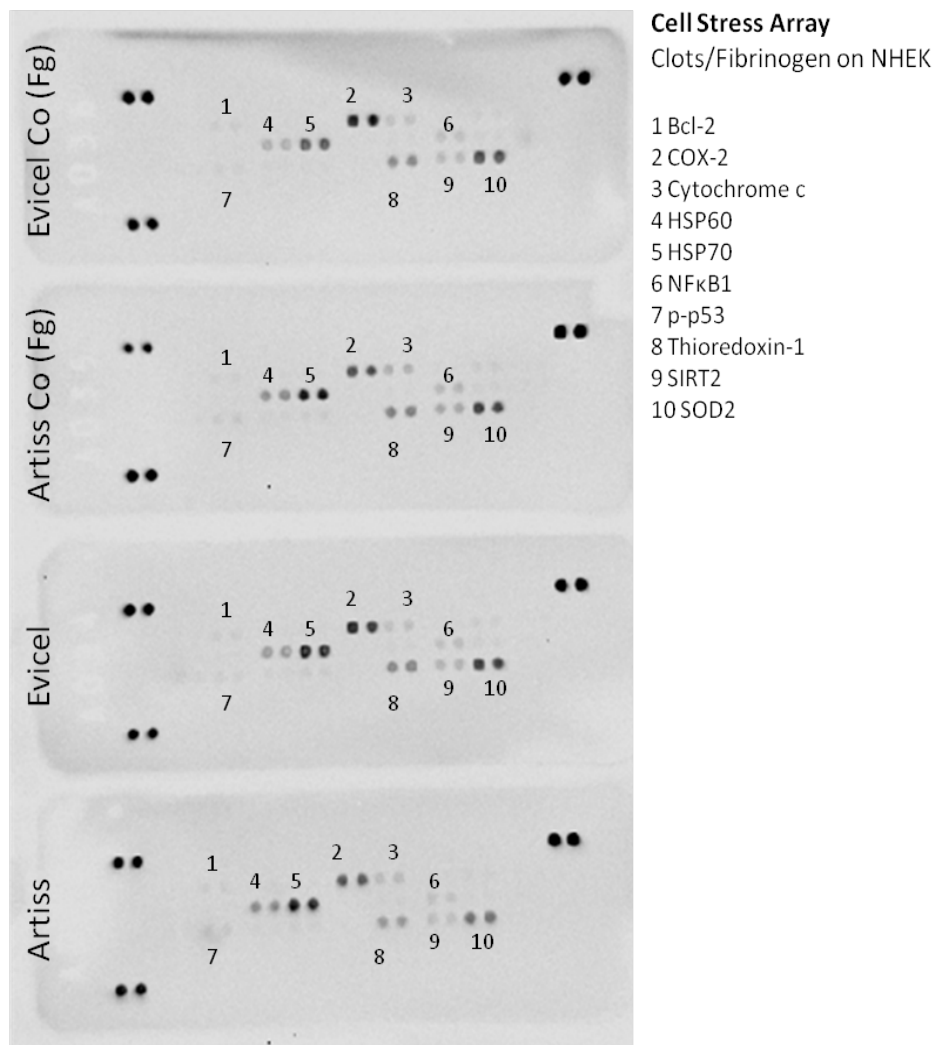


Figure 17 Stress array: NHEK grown in 6 well plates and overlaid with clot or fibrinogen component only (Artiss and Evicel) for 6 hours; LB6 + PI; DC-Kit; 200µg protein/sample. (n=1)

A series of proteins were detectable, for example COX-2, Cytochrome C or Hsp70. Expression patterns between fibrin sealants were varying, however, no differences induced only by thrombin treatment could be observed.

Besides their thrombin component, fibrin sealant products mostly differ in their fibrinogen component. For example the contents of ECM molecules such as fibronectin, laminin or vitronectin or growth factors play an important role in cell compatibility. Again, two common fibrin sealant products, fibrin sealants Artiss and Evicel were compared in vitro regarding cell adhesion, cell viability and apoptosis. Furthermore, these sealants were tested in an excisional wound healing model in rats.

In the next sections we focused on these components and their impact on cell compatibility of different fibrin sealant products.

2 DIFFERENT CONCENTRATIONS OF THROMBIN

2.1 THROMBIN CONCENTRATION IN FIBRIN SEALANT EXTRACTS

To investigate which amount of thrombin can be extracted out of the clot, Western blot analysis was performed.

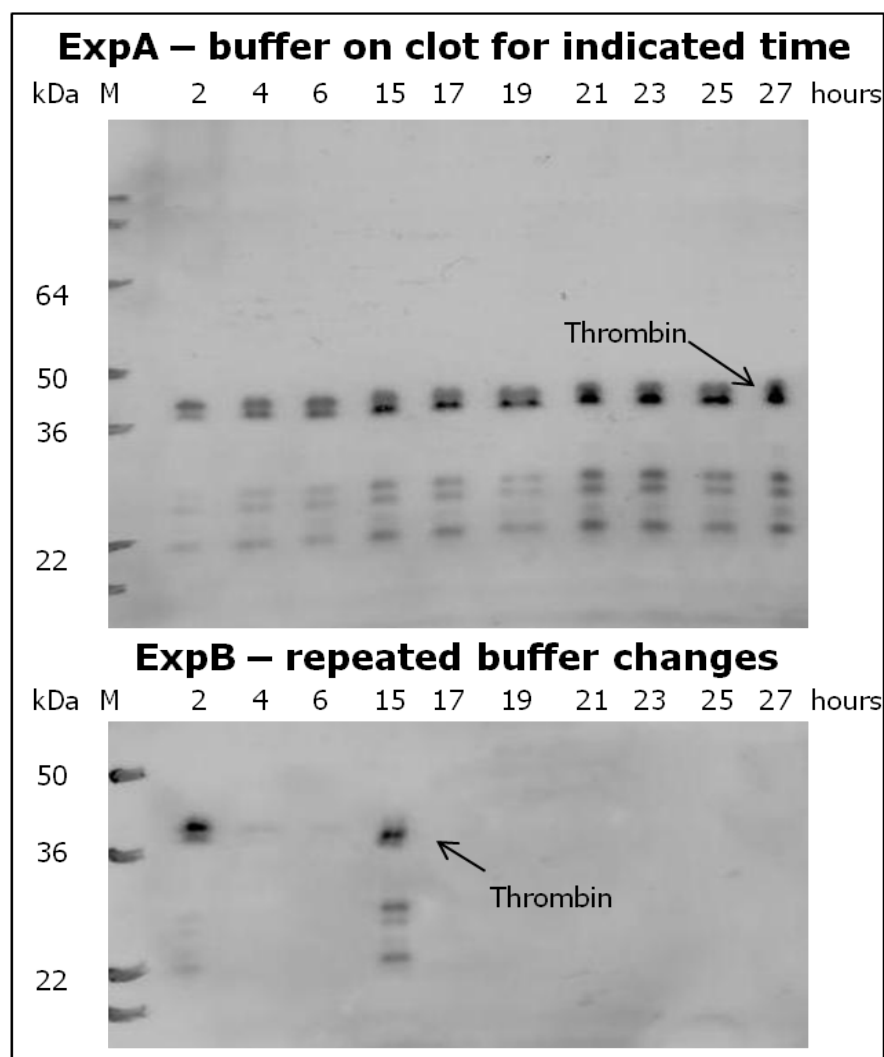


Figure 18 Western Blot: supernatants of ExpA and ExpB (15 μ l); extraction buffer: LMA; M: SeeBlue prestained marker (Invitrogen). Antibodies used: goat anti thrombin, rabbit anti goat HRP; n=2

In experiment design A (ExpA), clots were incubated with extraction buffers for several times and therefore, thrombin concentration in supernatants increased. In experiment design B (ExpB), extraction buffers were changed periodically. The thrombin concentration in the

supernatants decreases, within the first two hours, a burst release occurs. Also at the time point of 15 hours, a high thrombin concentration is visible because this extraction buffer was incubated over night for 9 hours. After this time point, no thrombin could be detected. (n=2)

2.2 PROTEINS (UNDEFINED) IN FIBRIN SEALANT EXTRACTS

Fibrin sealant components not only consist of fibrinogen and thrombin but also of several ECM molecules, growth factors, salts and undefined and in their concentration unknown proteins. The majority of these proteins are washed out in the first hours after polymerization of the fibrin sealant clot because of their weak bonding to the fiber network.

To evaluate these extractable proteins Tisseel clots were treated with different extraction/washing solutions: 1M NaCl, 2M Urea, PBS and LMA (1M NaCl with 1 % acetonitrile and 0.1 % trifluorid acetic acid). Fibrin clot extracts were loaded on an SDS PAGE and Coomassie stained. Results show that this used method is feasible to extract constituents out of the fibrin clot. (n=4)

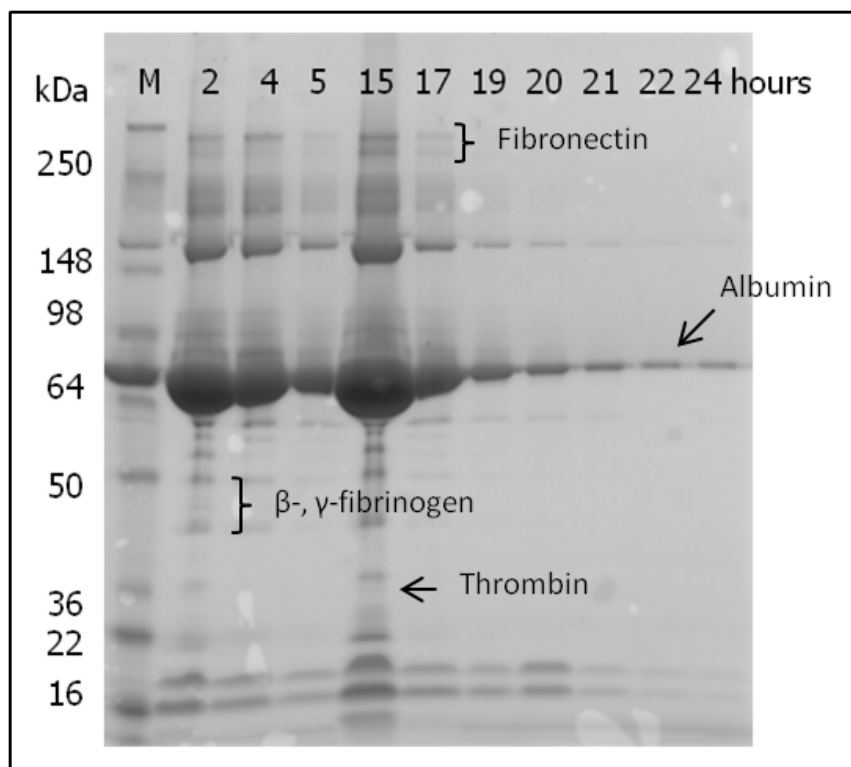


Figure 19 Electrophoretic analysis of Tisseel clot extracts with LMA, Coomassie stained. Buffers changed on distinct time points.

2.3 IDENTIFICATION OF PROTEINS EXTRACTED FROM FIBRIN CLOTS

To elucidate which proteins have been extracted and thus could contribute to cell compatibility sodium chloride solution supernatants from clots of the fibrin sealants Artiss (FS A) and Evicel (FS B) were subjected to SDS PAGE analysis and Coomassie staining and Western blotting.

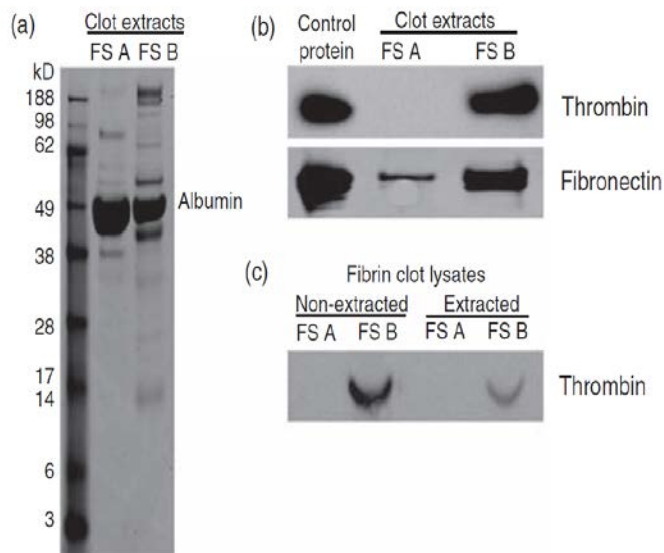


Figure 20 Electrophoretic analysis of fibrin clot extracts and fibrin clots before and after extraction. Supernatants of fibrin clots extracted by washing with 1M NaCl solutions were collected. Fibrin sealant clots (either non-treated or extracted with 1M NaCl solution) were completely lysed in sample buffer. Aliquots of supernatants and lysed fibrin clots were subjected to electrophoresis. Separated proteins were stained with Coomassie Brilliant Blue (a) or analyzed by western blotting (b and c). FS A: Artiss; FS B: Evicel. As positive controls purified thrombin and fibronectin were used (1 µg per lane). n=2

Human serum albumin was abundantly present in the extracts of both fibrin sealants (Figure 19 a). Beside albumin, the protein band patterns of the two fibrin sealants displayed significant differences. This underlines the fact that due to the specific manufacturing and formulation process of Artiss and Evicel the composition is quite different. By Western blotting fibronectin and thrombin were identified as extractable constituents (Figure 19 b). Western blotting of Evicel (FS B) clot lysates before and after extraction demonstrated that substantial but not complete extraction of thrombin was achieved, that is residual amounts were still present in fibrin clots after extraction (Figure 19 c). The amount of thrombin in lysates of Artiss (FS A) clots was below the detection limit.

2.4 DETERMINATION OF FIBRIN-BOUND THROMBIN

Three commercially available fibrin sealants which greatly differ in their thrombin concentration of their thrombin components were selected: Artiss® (Baxter AG; 4 IU/ml), Tisseel® (Baxter AG; 500 IU/ml) and Evicel® (Ethicon; 1000 IU/ml). As the specification ranges of the thrombin component are wide, different methods were used to determine the thrombin concentration.

After washing and extraction of clots and analysis of thrombin in the supernatants, the residual thrombin concentration in the clot was evaluated. Therefore, Artiss, Tisseel and Evicel fibrin sealant clots were washed with PBS over night. Lysates of the clots were observed in a Western blot analysis. The amount of thrombin in Artiss clot was not detectable ($< 0.1 \mu\text{g}$), Tisseel and Evicel lysates showed a clear band ($> 1 \mu\text{g}$). As a positive control purified thrombin (depleted especially of albumin) was used. ($n=7$)

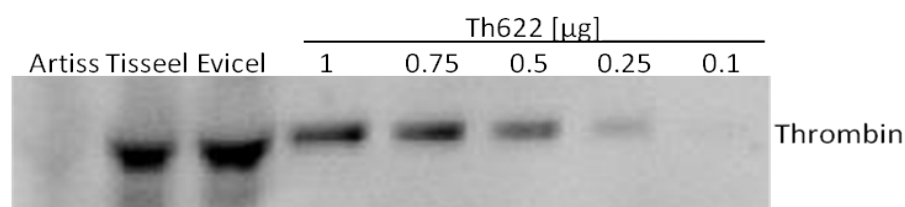


Figure 21 Western blot analysis of lysates of PBS washed fibrin sealant clots. As positive control, Th622 (purified thrombin) was used as a standard curve.

2.5 ACTIVITY OF FIBRIN BOUND THROMBIN

A coagulation assay was used to measure the actual thrombin concentration of each sealant: 4 IU/ml of thrombin was measured in the Artiss thrombin component; 505 IU/ml in the thrombin component of Tisseel, and 820 IU/ml in the thrombin component of Evicel (Table 6).

Since thrombin remains active after clotting, the surface activity of fibrin bound thrombin was evaluated using a Sensolyte test which predominantly measures activity on the clot surface by enzymatic cleavage of a substrate. A clear relation between the thrombin concentration in the thrombin components and the resulting thrombin activity on the clot surface was observed. Interestingly, the activity of fibrin bound thrombin of human blood clots and fibrin clots prepared with a 4 IU/ml thrombin solution was found to be comparable and lies under 1 IU/ml (Table 6).

Table 6 Thrombin activities of commercial fibrin sealants and on fibrin clot surfaces. The activity of the thrombin components of 3 different, commercially available fibrin sealants (Artiss®, Tisseel®, Evicel®) is given in column 3, the activity of fibrin surface is displayed in the last column. Human blood clots served as physiological reference. n=3-5

Materials		Determined thrombin concentration of the thrombin component	Specification as indicated by manufacturer	Thrombin activity on clot surface: (50µl clot volume, surface: 0.33 cm²)
Blood clot				0.079 +/- 0.057 IU
Commercial fibrin sealants	Artiss® (Baxter AG, Vienna)	4 IU/ml	2.5 - 6.5 IU/ml	0.033 +/- 0.003 IU
	Tisseel® (Baxter AG, Vienna)	505 IU/ml	400 - 625 IU/ml	2.24 +/- 0.53 IU
	Evicel® (Ethicon Sommerville, New Jersey)	820 IU/ml	800 -1200 IU/ml	2.91 +/- 0.37 IU
Prepared test items	FS 4 IU "low thrombin"			0.033 +/- 0.003 IU (identical to Artiss®)
	FS 505 IU "medium thrombin"			2.24 +/- 0.53 IU (identical to Tisseel®)
	FS 820 IU "high thrombin"			2.85 +/- 0.22 IU

2.6 THE INFLUENCE OF THROMBIN ON CLOT ULTRASTRUCTURE

The actual thrombin concentrations determined in the thrombin components of Artiss, Tisseel, and Evicel were used as reference to generate fibrin sealants of these thrombin concentrations but with otherwise identical composition of all other ingredients. For this purpose we used the fibrinogen component of Artiss and a high concentrated thrombin solution that was diluted to the required concentrations (505 IU and 820 IU/ml). Analysis by SEM of the three-dimensional structures of fibrin clots prepared with 4 IU/ml and 820 IU/ml thrombin solutions showed that the density as well as the thickness of fibrin fibers was not altered (Figure 22). The SEM pictures indicate that the clot structure was not influenced by thrombin concentrations of 4 IU/ml and 820 IU/ml.

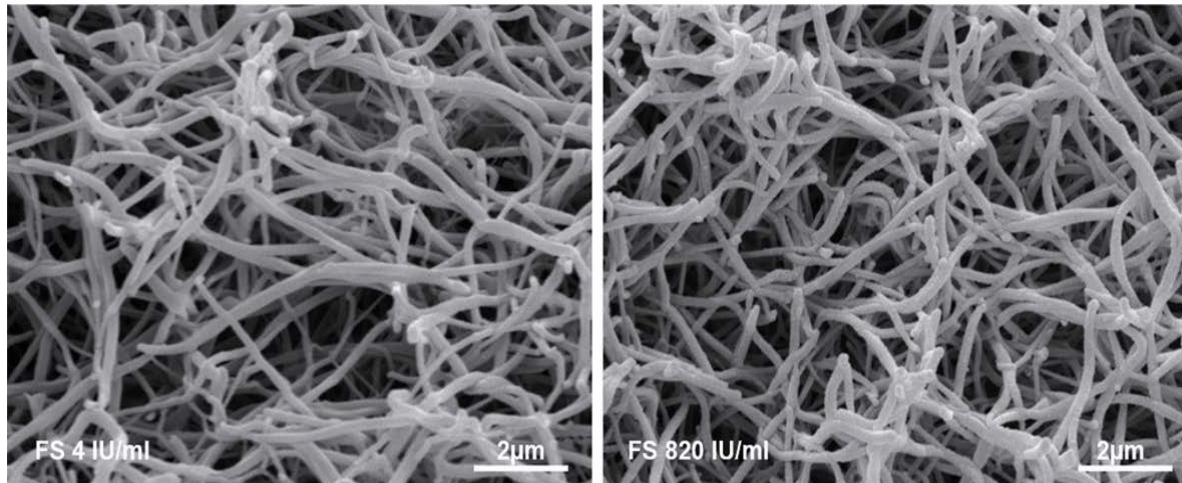


Figure 22 Structures (SEM) of fibrin clots prepared with low (4 IU/ml) and high (820 IU/ml) thrombin concentrations. Size bars indicate 2 μ m.

2.7 NUMBER OF ADHERENT CELLS ON FIBRIN CLOTS WITH DIFFERENT THROMBIN CONCENTRATIONS

To evaluate the interaction of NHEK with fibrin clots prepared with low (4 IU/ml), medium (505 IU/ml), and high thrombin concentrations (820 IU/ml), the number of adherent NHEK cells at different time points was assessed.

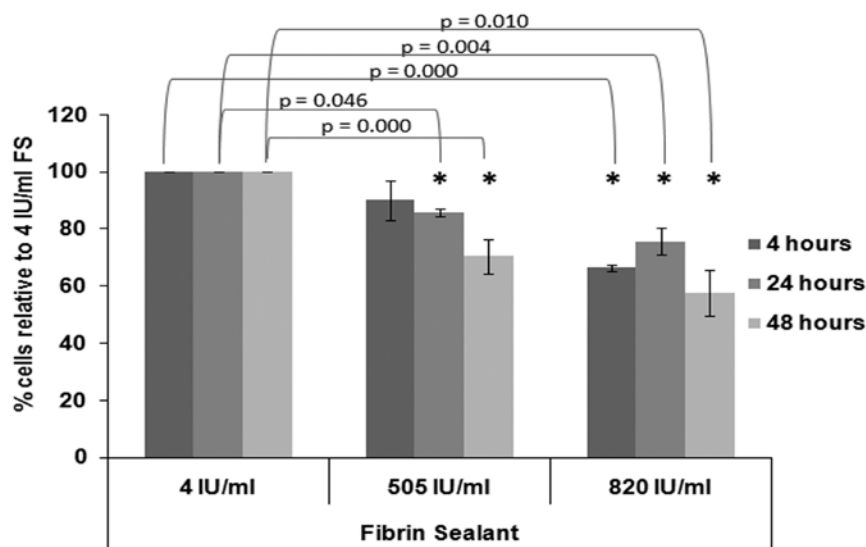


Figure 23 Number of adherent NHEK on fibrin clots prepared with different thrombin concentrations. Cell number was evaluated after 4, 24 and 48 hours incubation by measuring the amount of lactate dehydrogenase. p-values are indicated at the end of the according arrow. *indicate significantly different cell numbers compared to 4 IU/ml thrombin clots; $n=3$

After 24 and 48 hours, significantly fewer cells adhered to fibrin clots prepared with high (24 hours: 75.7 %; 48 hours: 57.7 %) and medium thrombin concentrations (24 hours: 85.6 %; 48 hours: 70.4 %) when compared to fibrin clots prepared with low concentrated thrombin solutions (were set to 100 %) (Figure 23). The cell number on fibrin clots containing high amounts of thrombin was already diminished significantly after 4 hours (66.4 %) adhesion compared to low thrombin concentration.

2.8 MORPHOLOGY OF CELLS ON FIBRIN CLOTS WITH DIFFERENT THROMBIN CONCENTRATIONS

Striking differences in cell morphology were observed between fibrin clots made with low, medium, and high concentrated thrombin solutions. In the presence of low amounts of thrombin spreading of NHEK was seen after 24 hours. With increasing thrombin concentration cell morphology became more and more deteriorated (Figure 24). Fibrin clots prepared with high concentrated thrombin solutions failed to support cell spreading and NHEK displayed a rounded morphology (Figure 24, C and F).

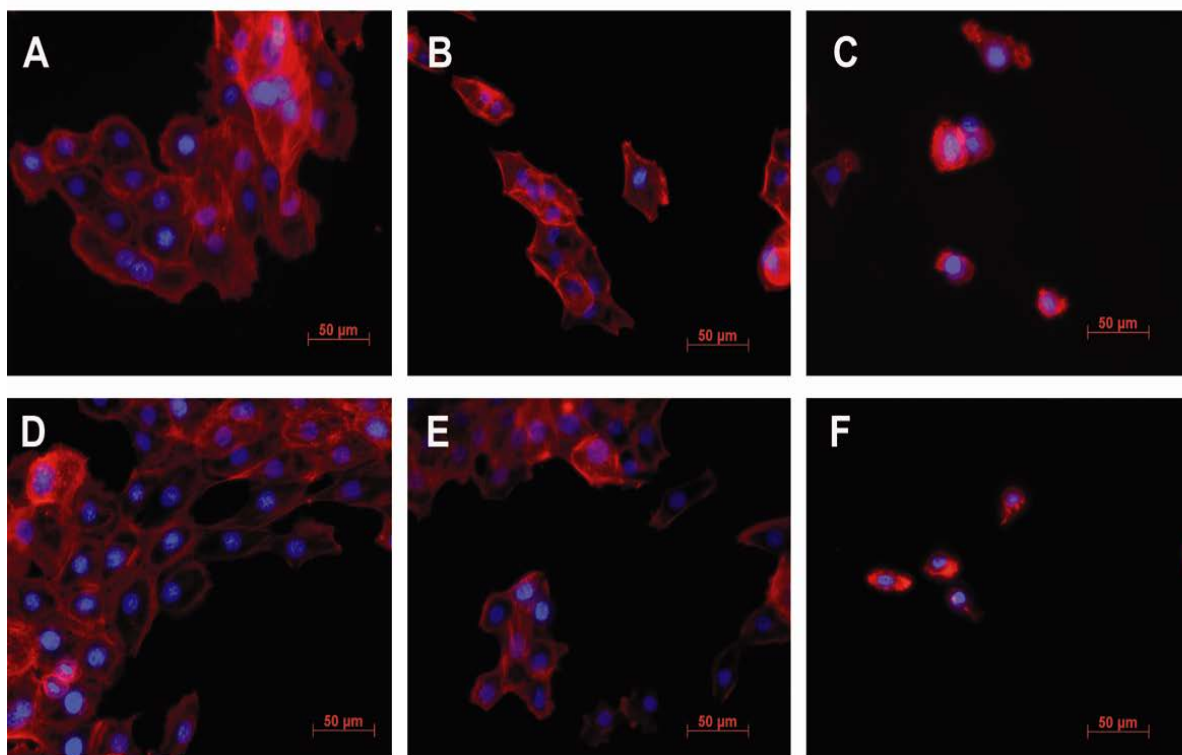


Figure 24 Morphology of NHEK on fibrin clots prepared with low, medium, and high concentrated thrombin solutions. Cells were seeded on top of fibrin clots with different thrombin contents: 4 IU/ml (A and D); 505 IU/ml (B and E); 820 IU/ml (C and F) and were incubated for 24 hours (A-C) or 48 hours (D-F). Cells were stained with TRITC-phalloidin to visualize actin cytoskeleton (red stain) and DAPI to visualize cell nuclei (blue stain).

2.9 INHIBITION OF THROMBIN ACTIVITY

Hirudin has been established as an inhibitor of free and fibrin bound thrombin. We employed hirudin to test the hypothesis that high activity of thrombin in fibrin clots has a negative impact on cell compatibility.

The blocking efficacy of hirudin was verified by an experiment where the capability of fibrin bound thrombin to facilitate fibrin clot growth was monitored. As depicted in Figure 25, hirudin inhibits the assembly of fluorescence-labeled fibrinogen onto a preformed fibrin clot. Compared to unblocked high thrombin clots which were set to 100 % absorbance, assembly of ~73 % and 40 % absorbance labeled fibrinogen was measured for the medium and for the low thrombin clots, respectively. By blocking the thrombin activity with hirudin, the assembly of labeled fibrinogen for the medium and high fibrin clot was decreased significantly to the level of low thrombin clots. The fibrinogen assembly of low thrombin clots remained unchanged upon hirudin blocking.

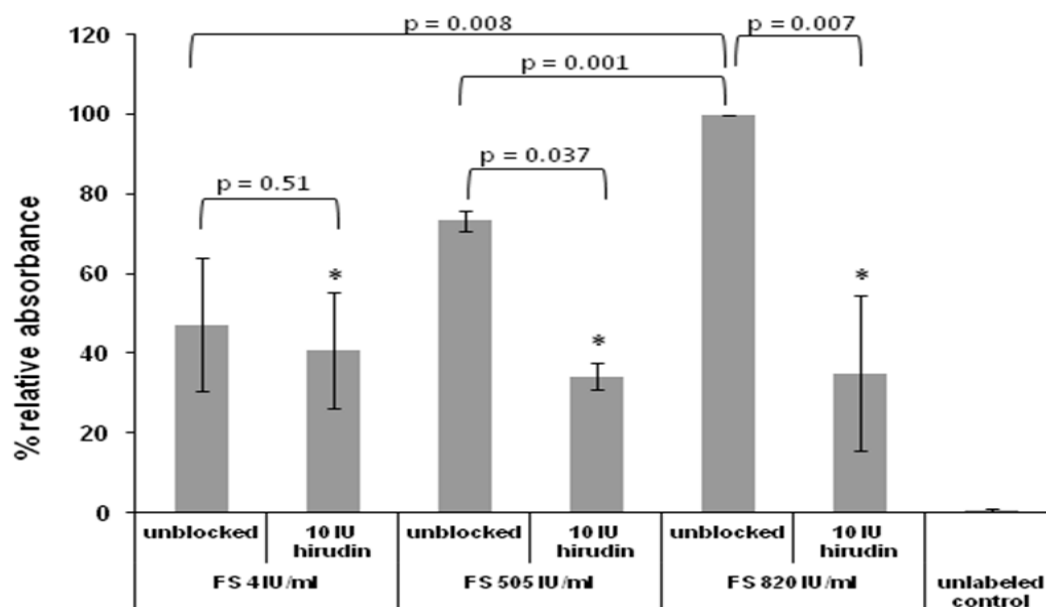


Figure 25 Hirudin blocking of fibrin bound thrombin activity of low, medium, and high thrombin clots. Hirudin blocked fibrin clots and unblocked control clots were incubated with Alexa 568 labeled fibrinogen and fluorescence was measured at 520-580 nm. The absorbance of unblocked high thrombin clots was set to 100%. Relative absorbance is shown for all clots. Unlabeled clots were used as control. * $p < 0.05$ Results of 4 independent experiments are shown ($n = 3$ clots/experiment).

To evaluate the impact of thrombin inhibition on cell compatibility, fibrin clots prepared with low, medium, and high concentrated thrombin solutions were treated with hirudin prior to

NHEK seeding and cell number was evaluated. Cell number was not significantly altered on low thrombin fibrin sealants upon hirudin treatment, whereas, on medium and high concentrated thrombin clots hirudin blocking caused a significantly increased cell number compared to untreated fibrin sealant clots after 24 (505 IU/ml: $p=0.015$; 820 IU/ml: $p=0.01$) and 48 hours (505 IU/ml: $p=0.03$; 820 IU/ml: $p=0.029$) incubation. A representative experiment out of three is shown in Figure 26.

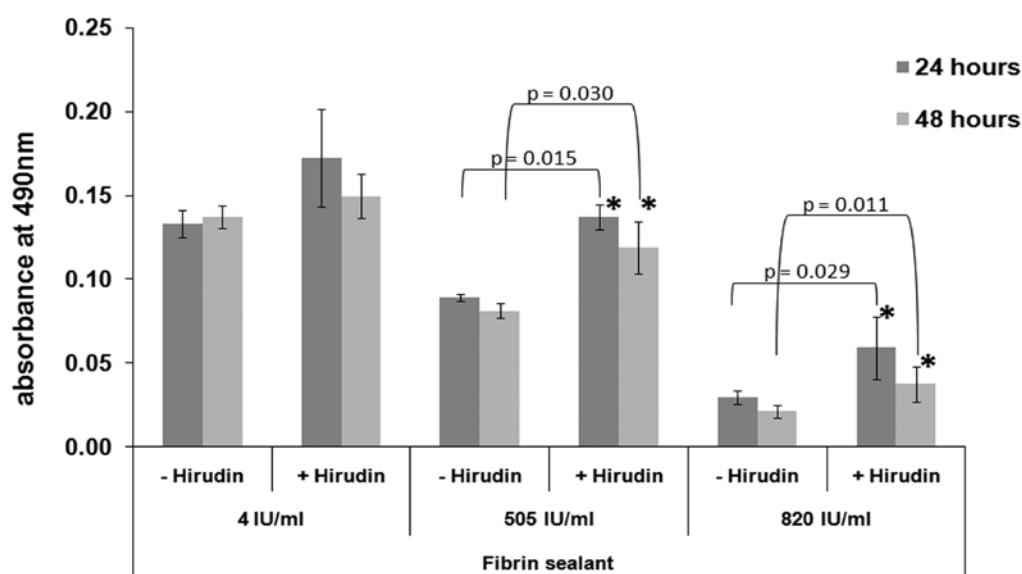


Figure 26 Number of adherent NHEK on fibrin clots with different thrombin concentrations +/- hirudin. Total cell number was determined by measuring the amount of lactate dehydrogenase of lysed cells after 24 and 48 hours incubation. *indicate significantly different cell numbers compared to unblocked clots. Detailed p-values are indicated in the graph. Any out of 3 independent experiments was performed with 3-9 individual clots per group.

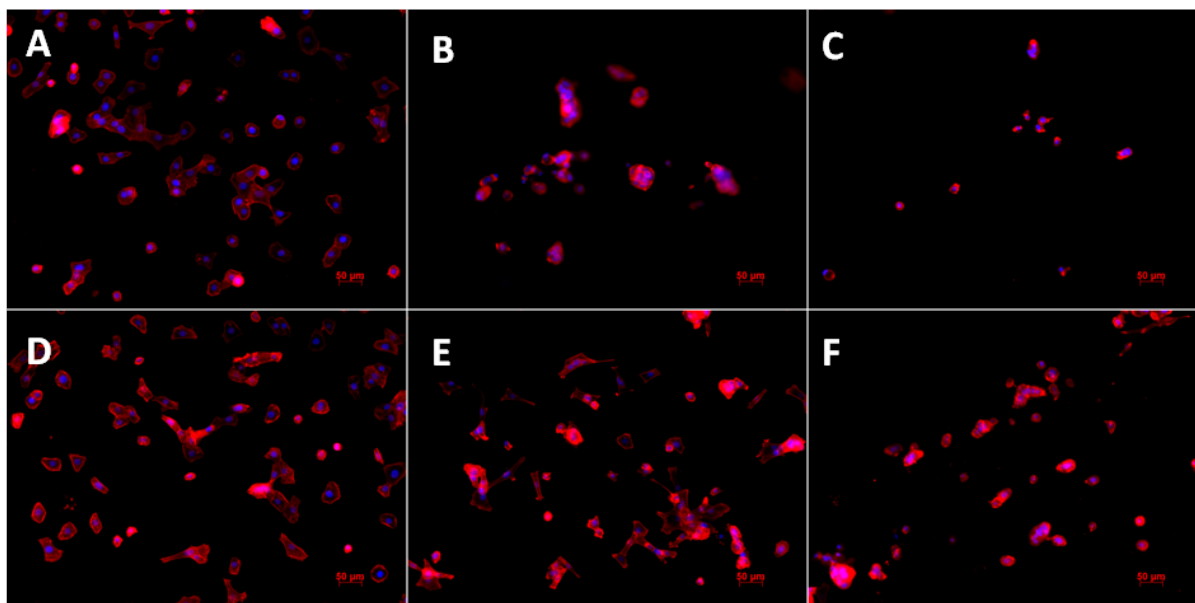


Figure 27 Morphology of NHEK on fibrin clots prepared with low, medium, and high concentrated thrombin solutions after 24 hours. Cells were seeded on top of fibrin clots with different thrombin contents: 4 IU/ml (A and D); 505 IU/ml (B and E); 820 IU/ml (C and F). D-F: Fibrin sealants treated with hirudin before cell seeding (as described above in LDH-experiments). Cells were stained with TRITC-phalloidin to visualize actin cytoskeleton (red stain) and DAPI to visualize cell nuclei (blue stain). n=5

Thrombin inhibition not only affects adhesion and viability but also morphology of cells. Fibrin clots made with low, medium and high concentrated thrombin solutions were incubated with hirudin before cell seeding (Figure 27 D-F). On fibrin sealant clots with 4 IU thrombin we observed no impact on cell spreading upon hirudin blocking. On medium and high thrombin clots NHEK showed a less deteriorated morphology upon thrombin inhibition with hirudin (Figure 27 E and F).

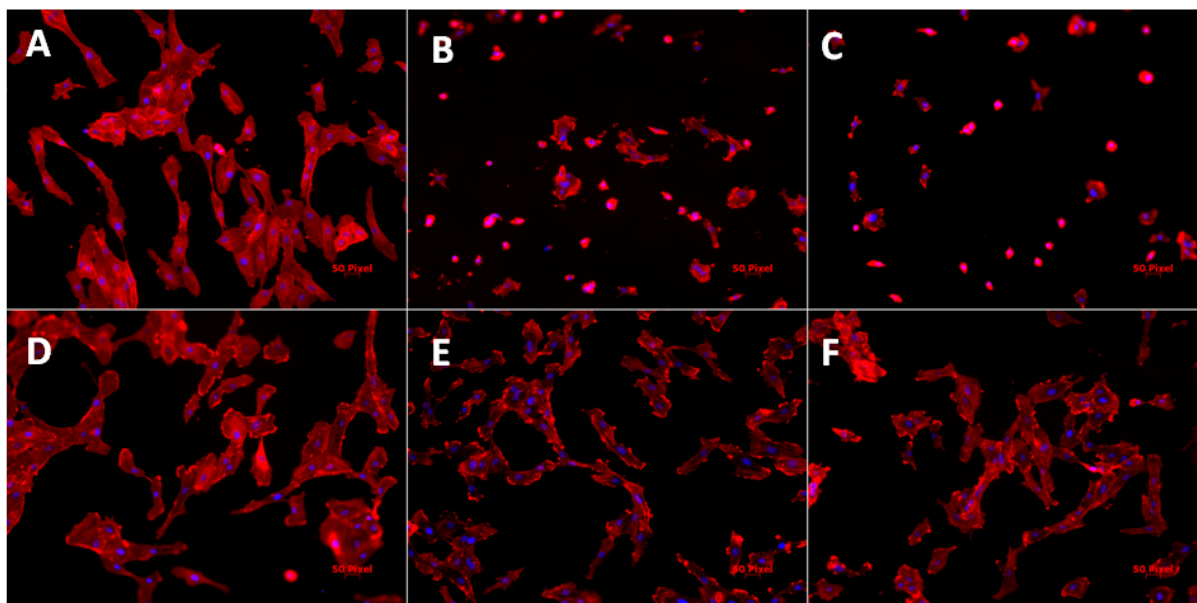


Figure 28 Morphology of HUVEC on fibrin clots prepared with low, medium, and high concentrated thrombin solutions after 24 hours. Cells were seeded on top of fibrin clots with different thrombin contents: 4 IU/ml (A and D); 505 IU/ml (B and E); 820 IU/ml (C and F). D-F: Fibrin sealants treated with hirudin before cell seeding (as described above in LDH-experiments). Cells were stained with TRITC-phalloidin to visualize actin cytoskeleton (red stain) and DAPI to visualize cell nuclei (blue stain). n=2

This effect was also observed with HUVEC after 24 hours even more explicit (Figure 28).

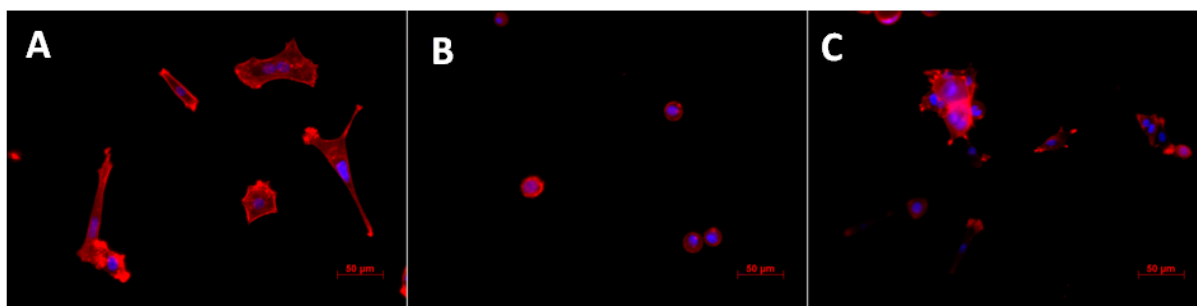


Figure 29 Morphology of NHEK after 24 hours on fibrin sealant clots: Artiss (A), Evicel (B), Evicel treated with hirudin (C) before cell seeding. n=2

Also when Evicel fibrin sealant clots were treated with hirudin, morphology of NHEK was improved.

2.10 CELL DEATH IN CONTEXT WITH FIBRIN BOUND THROMBIN

In order to examine whether the decrease of cells on high thrombin fibrin clots is caused by cell death, live/dead staining of NHEK was performed.

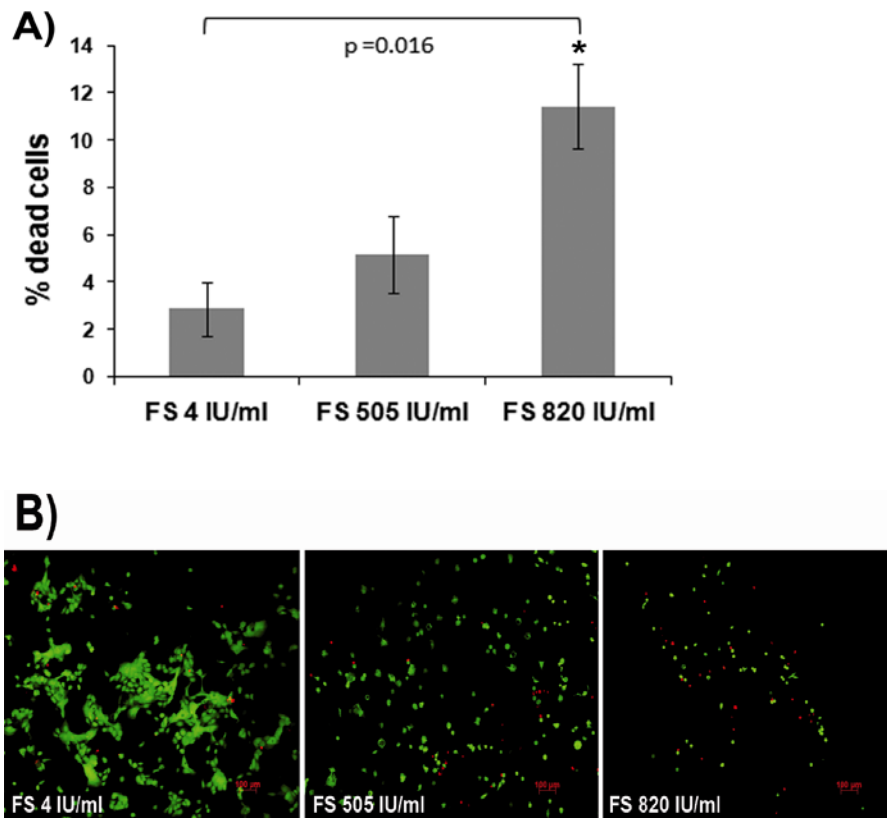


Figure 30 A: Percentage of dead cells on fibrin clots. Red and green signals were evaluated by the plate reader at 530 nm and 620 nm and are given as percentage of dead cells relative to living cells. The graph shows mean results of 3 independent experiments. *indicates the significant difference compared to 4 IU/ml thrombin clots. B: Live/ dead staining of cells on fibrin clots. Cells were cultured for 24 hours on fibrin clots prepared with different thrombin solutions: 4 IU/ml, 505 IU/ml and 820 IU/ml. Green stain (Calcein AM) indicates living cells and red stain (Ethidium Homodimer 1) indicates dead cells.

As shown in Figure 30 A, high thrombin concentrations in fibrin clots led to a significantly increased percentage (up to 11.4 %) of dead cells within 24 hours when compared to low (less than 2.9 % dead cells) and medium (5.2 % dead cells) thrombin clots. Representative immuno-fluorescence live/dead staining is displayed below the graph (Figure 30 B).

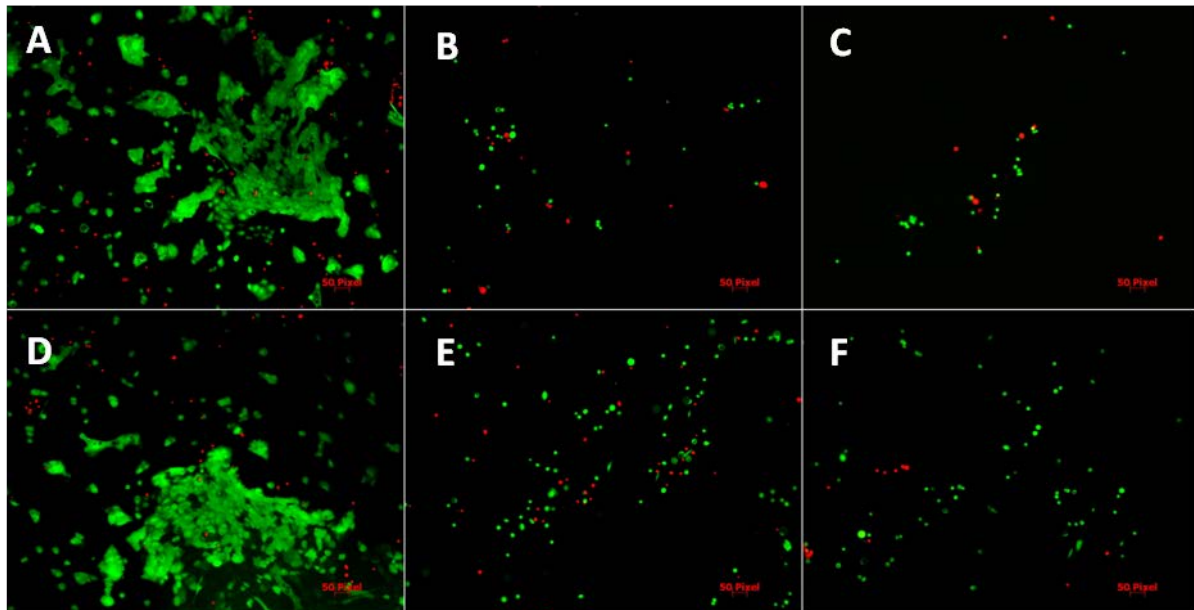


Figure 31 Live/dead stain of NHEK grown on fibrin sealants with different thrombin concentrations for 24 hours. A-C: FS 4 IU/ml, 505 IU/ml, 820 IU/ml. D-F: Fibrin sealants treated with hirudin before cell seeding (as described above in LDH-experiments). Green stain (Calcein AM) indicates living cells and red stain (Ethidium Homodimer 1) indicates dead cells. (n=5)

In order to examine the impact of thrombin inhibition on the number of Ethidium Homodimer 1 stained (=dead) cells we blocked thrombin with hirudin before cell seeding and performed live-dead staining after 24 hours. There were no striking differences in number of dead cells between clots with fibrin-bound thrombin and untreated clots.

To screen other markers for apoptosis and to elucidate their molecular pathways, cell lysates of NHEK grown on fibrin sealant clots were analyzed using an apoptosis array (n=4). Cells were grown on Artiss, Evicel, and 820 IU for 30 hours, as control cells grown in cell culture flasks were used.

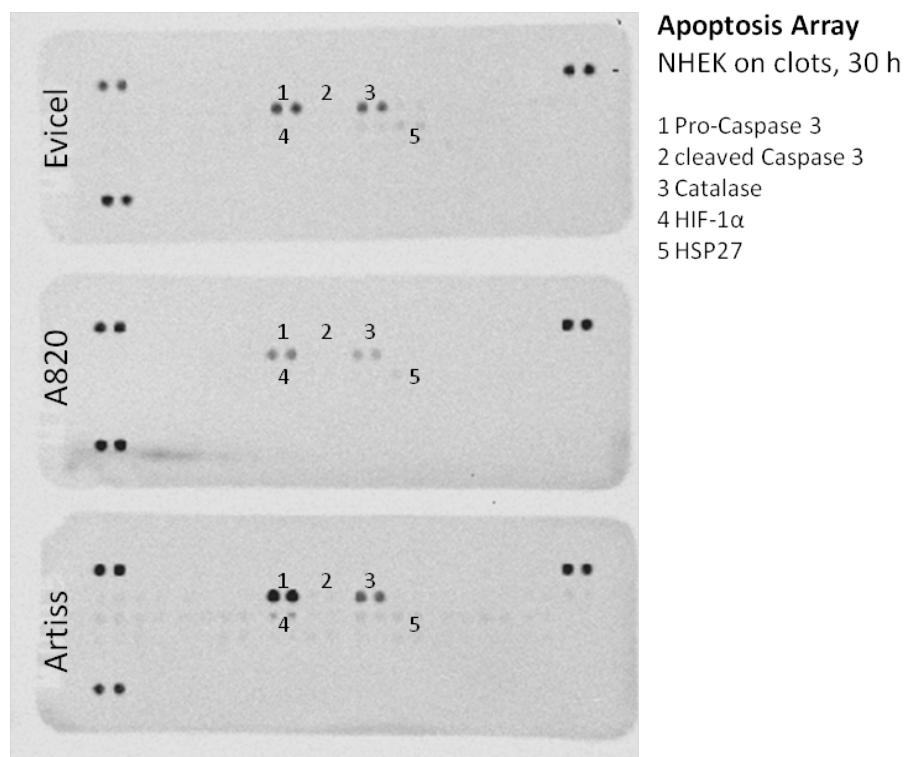


Figure 32 NHEK grown on clots (Artiss, A820 and Evice) for 30 hours; cell lysates were analyzed via apoptosis arrays (300 μ g)

In the array in figure 32 especially pro-caspase 3 has an increased expression level, as well as catalase in cells exposed to Artiss. Other important and remarkable proteins are described in the picture but did not show any differences.

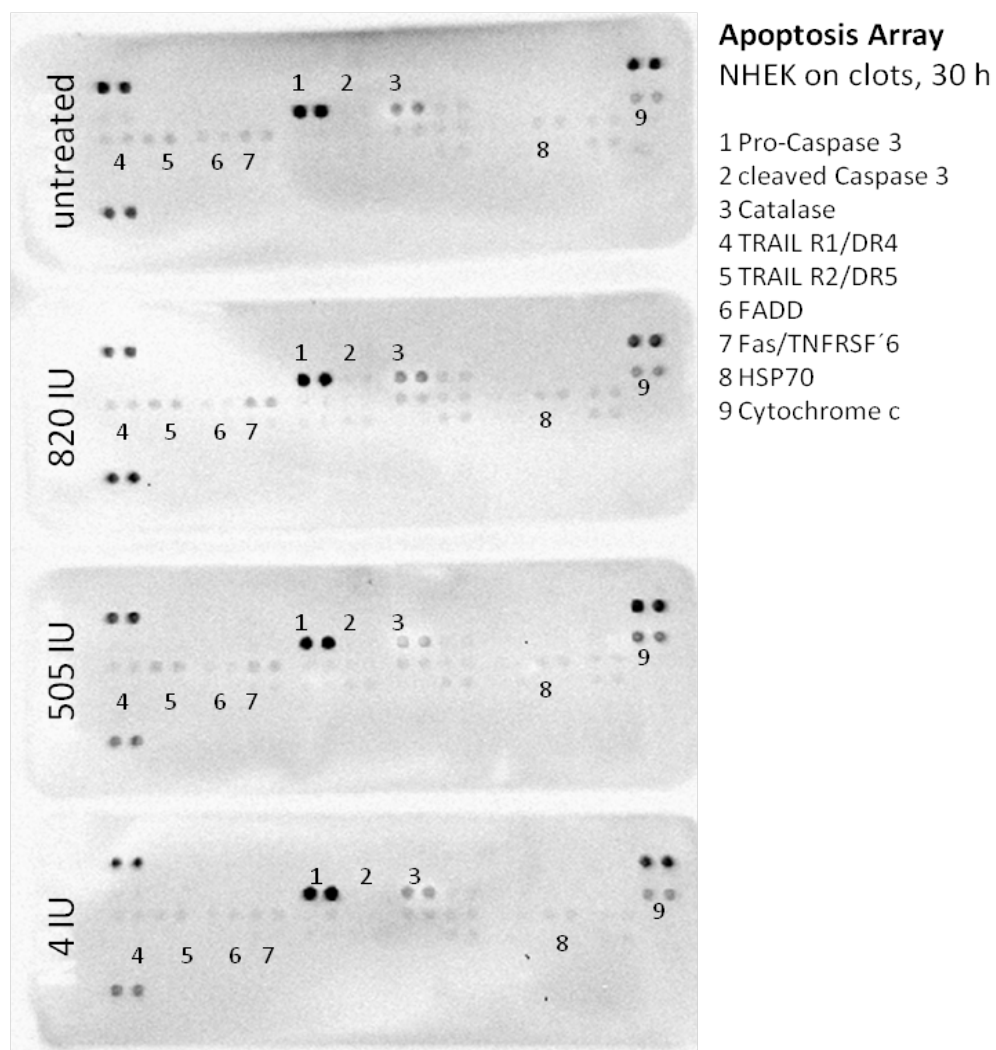


Figure 33 NHEK grown on clots (Artiss 4IU, 505 IU, 820 IU and on plate) for 30 hours; cell lysates were analyzed via apoptosis arrays (300 µg)

In this experiment small differences in catalase, TRAIL receptors and Fas could be observed due to exposure to different fibrin sealant clots. Consequently, Western blot analysis and a caspase 3/7 assay were performed as described below. Other array proteins are listed in the figure which were not regulated differently. For Western blot analyses, cells were grown in 6-well plates and covered with the appropriate clot ("upside down" as done for the array).

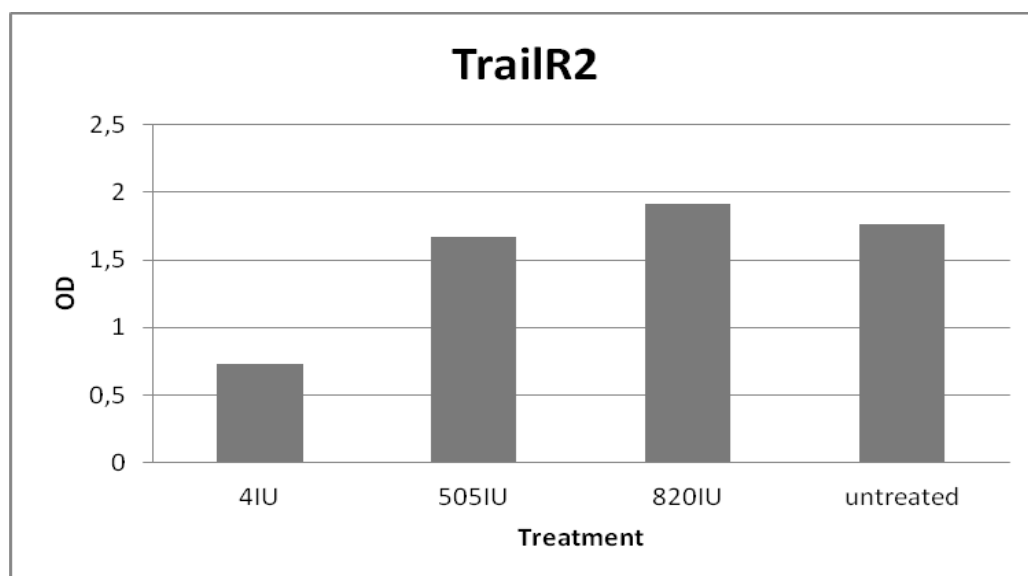


Figure 34 Digital processing of the apoptosis array concerning the apoptosis marker Trail-R2.

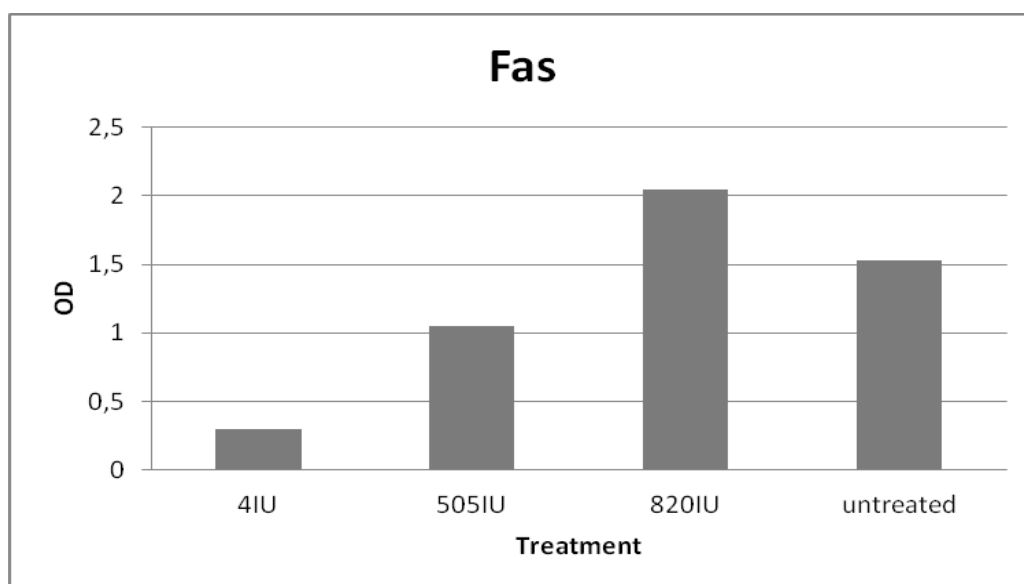
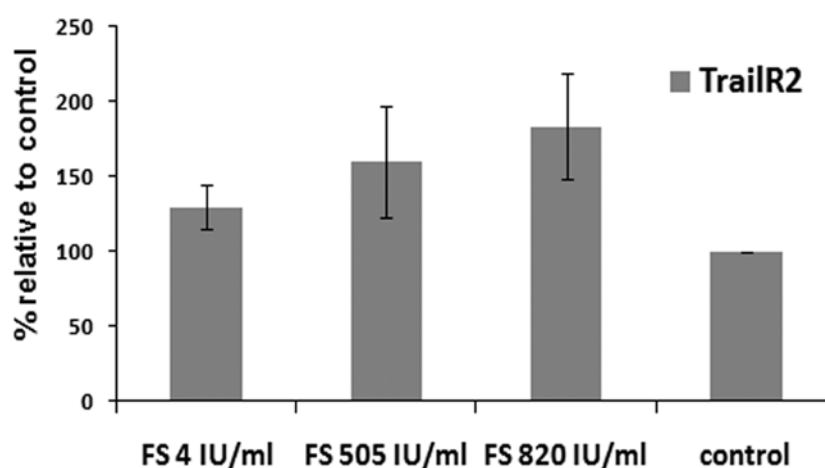


Figure 35 Digital processing of the apoptosis array concerning the apoptosis marker Fas.

2.11 EXPRESSION OF APOPTOSIS MARKER TRAIL-R2 IN NHEK

The expression of Trail-R2 (also known as DR5), a member of membrane bound death receptors was increased on NHEK incubated with fibrin sealant clots prepared with medium (159.6%) and high (183.2%) thrombin concentrations compared to untreated control cells. NHEK incubated with low thrombin fibrin sealant clots demonstrated only 129.4 % Trail-R2 expression relative to untreated cells (Figure 36).

A)



B)

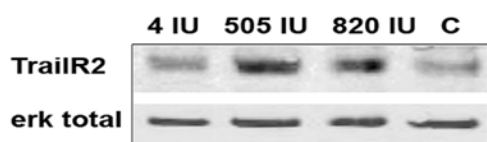


Figure 36 Trail-R2 Western blot of NHEK lysates. Fibrin sealant clots with 4 IU/ml, 505 IU/ml or 820 IU/ml thrombin were used to induce NHEK cells for 4 hours. Cells not exposed to FS clots served as control. A) Summary of 4 independent Western blot experiments. Trail-R2 signal was normalized with total ERK1/2 signal. Results are presented as percentage of signal intensity relative to control. * $p < 0.05$; B) Representative Western blot out of 4 experiments is shown. Total ERK1/2 signal was used as loading control.

2.12 EXPRESSION OF APOPTOSIS MARKER FAS IN NHEK

The Fas ligand belongs to the TNF family and induces trimerization of Fas in the target cell membrane. Fas has been shown to be an important mediator of apoptotic cell death, as well as being involved in inflammation. (109)

As seen in the apoptosis array, Fas expression is upregulated when cells get in contact with higher thrombin concentration. When treated with fibrin sealant clots with 820 IU, an over two fold higher expression could be measured compared to cells treated with Artiss clots.

This observation could not be confirmed in Western blot analysis. In three independent experiments, only a trend could be seen towards up- or downregulation of Fas in cells.

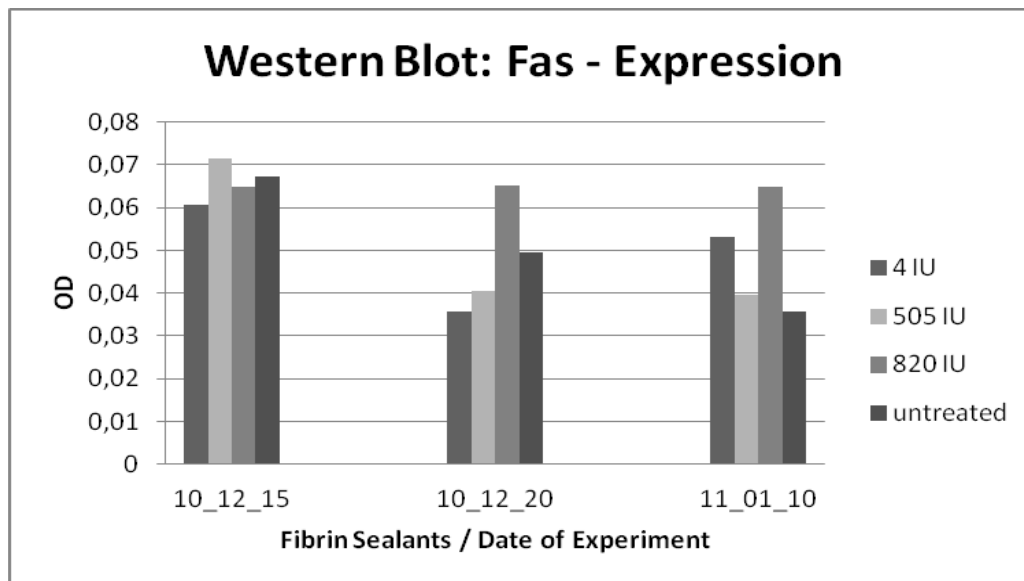


Figure 37 Fas Western blot analysis of cell lysates. Fibrin sealant clots with 4 IU/ml, 505 IU/ml or 820 IU/ml thrombin were used to induce NHEK cells for 4 hours. Cells not exposed to FS clots served as control. n=3

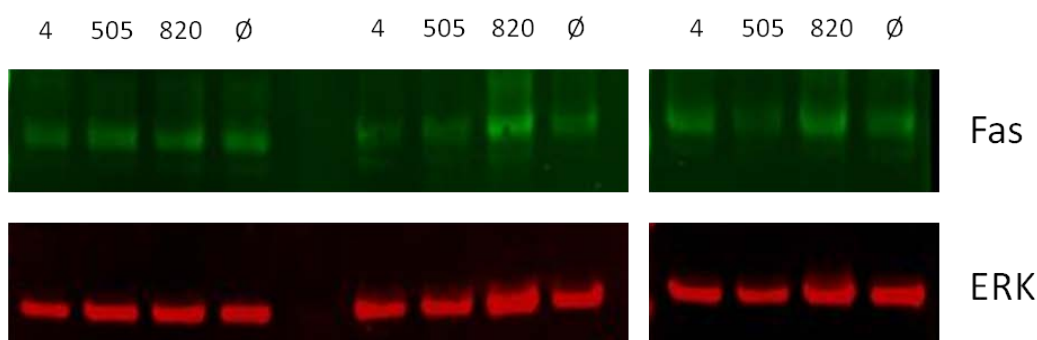


Figure 38 Fas Western blot of NHEK lysates. Fibrin sealant clots with 4 IU/ml, 505 IU/ml or 820 IU/ml thrombin were used to induce NHEK cells for 4 hours. Cells not exposed to FS clots served as control. n=3

2.13 CASPASE 3 AND CASPASE 7 ACTIVITY OF NHEK SEEDED ON FIBRIN CLOTS

To evaluate whether fibrin clots prepared with medium or high concentrations of thrombin trigger apoptotic events in NHEK a caspase activity assay was performed. This test measures the activity of caspases 3 and 7, both important indicators for apoptosis. After 4 hours, cells cultured on fibrin clots with high thrombin concentration showed a significantly higher caspase activity (2.1 relative RFU = 2-fold higher as FS 4 IU/ml, $p=0.02$) as compared to cells seeded on clots prepared with low and medium concentrations of thrombin (Figure 39). The lowest caspase activity was detected on fibrin clots containing the lowest amount of thrombin (was set to 1). Blocking by hirudin reduced the caspase activity of cells cultured on

high thrombin fibrin clots significantly ($p=0.001$). The difference between cells on low and medium thrombin clots regarding their caspase activity was not significant.

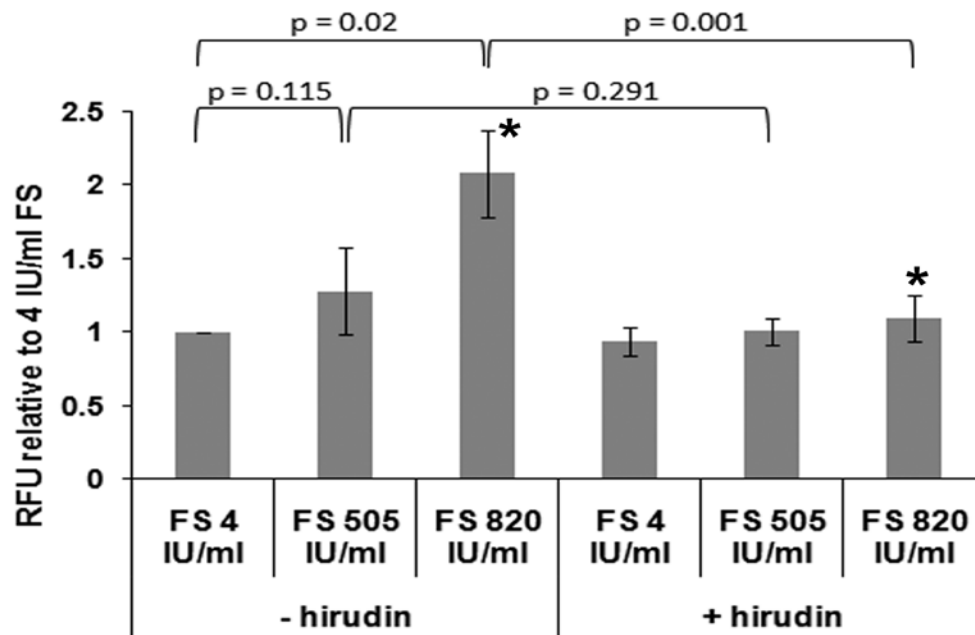


Figure 39 Caspase 3 and 7 activity in NHEK cultured on fibrin clots. Caspase activity was measured after 4 hours by using a one step fluorometric assay. *indicate significantly different caspase activities ($n=6$).

2.14 PROLIFERATION OF NHEK IN CONTEXT WITH THROMBIN

To evaluate the influence of thrombin concentration on cell proliferation, NHEK were incubated with medium containing thrombin (equivalent to Artiss, Tisseel and Evicel thrombin concentrations). No significant differences in the proliferation rate were observed.

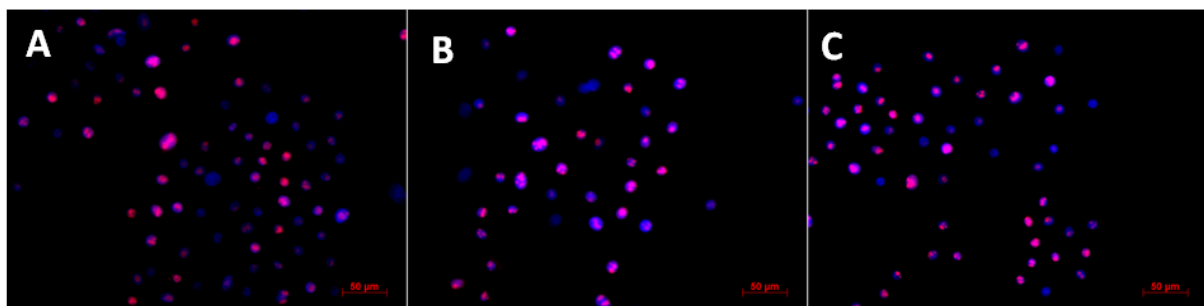


Figure 40 Ki67 immuno-cytochemistry staining of NHEK on cell culture plate incubated with concentrations of thrombin (A: Artiss, B: Tisseel, C: Evicel) over night. ($n=4$)

2.15 BLOCKING OF PAR-1 THROMBIN RECEPTOR WITH SCH797979

The hypothesis behind these experiments was that cell behavior changes when the thrombin receptor PAR-1 is blocked by SCH797979, a selective PAR-1 antagonist. Therefore, cells were incubated with 75 nM SCH797979 for 30 min at 37°C prior seeding. To check morphology, cells were seeded on top of fibrin clots (4 IU, 505 IU and 820 IU thrombin) and after 24 hours they were stained with phalloidin and DAPI. As demonstrated many times before cell morphology was deteriorated on clots with higher thrombin concentration (figure 41 A-C). Upon pre-treatment with SCH797979, cells showed more spreading but did not reach full recovery.

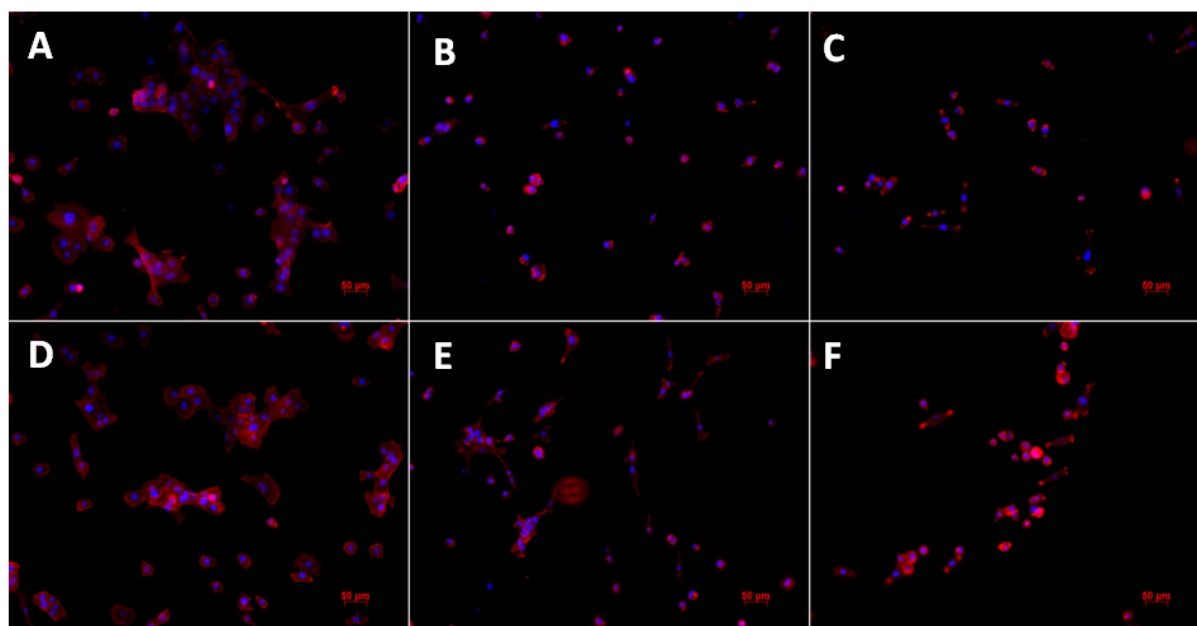


Figure 41 Morphology of NHEK on fibrin sealant clots after 24 hours. A-C: FS 4 IU/ml, 505 IU/ml, 820 IU/ml. D-F: Cells treated with SCH797979 before cell seeding. (n=2)

The same experiment was performed with a live/dead staining.

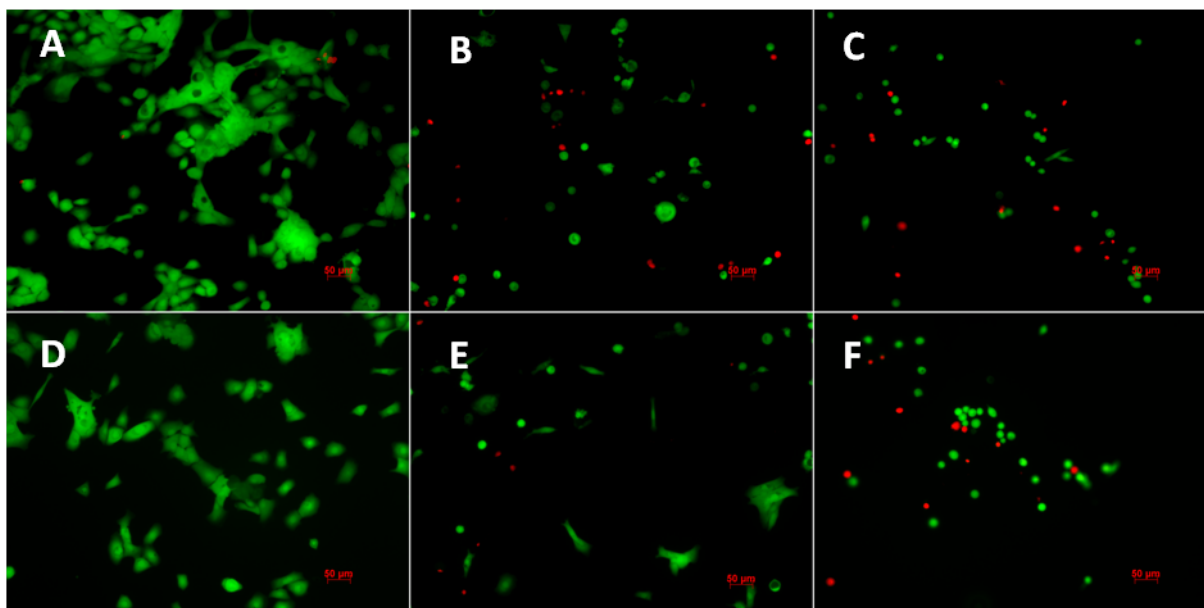


Figure 42 Live/Dead staining of NHEK on fibrin sealant clots after 24 hours. A-C: FS 4 IU/ml, 505 IU/ml, 820 IU/ml. D-F: Cells treated with SCH797979 before cell seeding. (n=2)

In the live/dead staining, no significant differences could be seen between cells treated with or without SCH797979 and thrombin.

2.16 INFLUENCE OF THROMBIN ON THE MAPK EXPRESSION PATTERN

To further observe the pattern of mitogen-activated protein kinases and to elucidate molecular pathways, cell lysates of NHEK treated with SCH797979 and thrombin were analyzed on a MAPK array (n=3). Cells were grown in tissue culture flasks, PAR-1 was blocked and cells were treated with 1 IU thrombin per ml medium for 15 minutes.

In the array in figure 43 especially ERK, RSK and Akt changed expression levels. Other important and remarkable proteins are described in the picture but did not show any significant differences. As a consequence, Western blot analysis was performed for ERK and Akt.

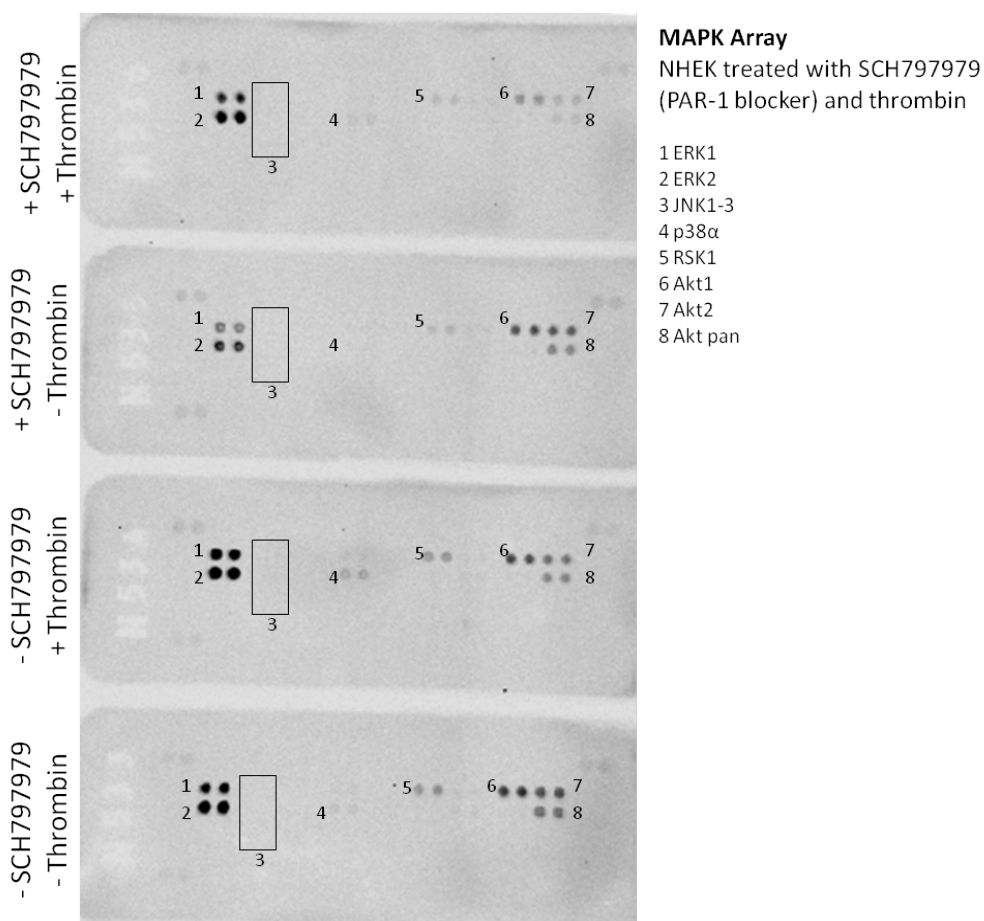


Figure 43 NHEK treated with/without SCH797979 (30 min) and thrombin (1 IU/ml; 15 min); LB6 + PI; DC-Kit; 200 µg protein

For Western blot analysis of pERK, cells were treated with SCH797979 and/or thrombin for 15 minutes. Expression of pERK was activated when cells were incubated with thrombin but no impact of SCH797979 could be detected.

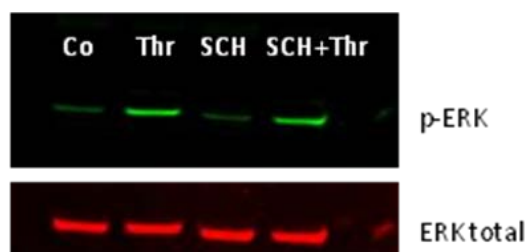


Figure 44 pERK Western blot analysis of cells pre-treated with/without SCH797979 (30 min) and thrombin (1 IU/ml; 15 min).

For further pERK Western blot analyses cells were incubated for 5 minutes with different thrombin concentrations: 4, 505 and 820 IU. As control, cells were only treated with CaCl_2 .

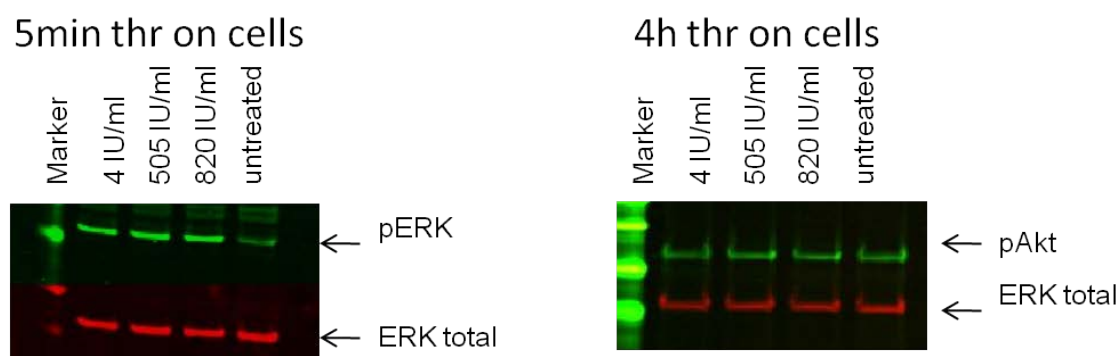


Figure 45 pERK and pAkt Western blot analysis of cells treated with different thrombin concentrations for different times. (pERK: n=3; pAkt: n=4)

The expression level of pERK was induced in cells treated with thrombin but there was no difference between distinct thrombin concentrations.

For analysis of the pAkt expression level, thrombin was given to cells for 4 hours. No difference was seen in the Akt phosphorylation between the samples.

3 RAT EXCISIONAL WOUND HEALING MODEL – THROMBIN DEPENDENT WOUND HEALING

As a continuation of the *in vitro* experiments described above the *in vivo* wound healing performance of fibrin sealants was observed in an excisional wound model in rats. As parameters changes in wound area were monitored by planimetry, histomorphometric analyses gave information about wound severity, wound closure and wound healing in general and progresses in vascularization of wound area was observed immuno-histochemically and by Western blot analysis. Moreover, inflammation was measured on day two, apoptosis of cells was detected by in-situ hybridization and pathological abnormalities were characterized. In addition, fibrin degradation was monitored.

3.1 PLANIMETRY

To analyze the impact of different fibrin sealants on wound healing, Artiss (n=12) and Evicel (n=10) fibrin sealants were applied to excisional wounds (13 mm in diameter) on the back of rats. Furthermore, as control if thrombin is the key player for observed effects, groups treated with Artiss fibrinogen with a thrombin concentration as found in Evicel (named A820; thrombin was diluted from a thrombin concentrate provided by Baxter; n=10) as well as Evicel fibrinogen with a thrombin diluted to the concentration of Artiss (named E4; Evicel thrombin component was diluted with an appropriate dilution buffer; n=8) were tested. Also an Evicel fibrinogen component with Artiss thrombin component (E4Baxter; n=10) was included to the test group.

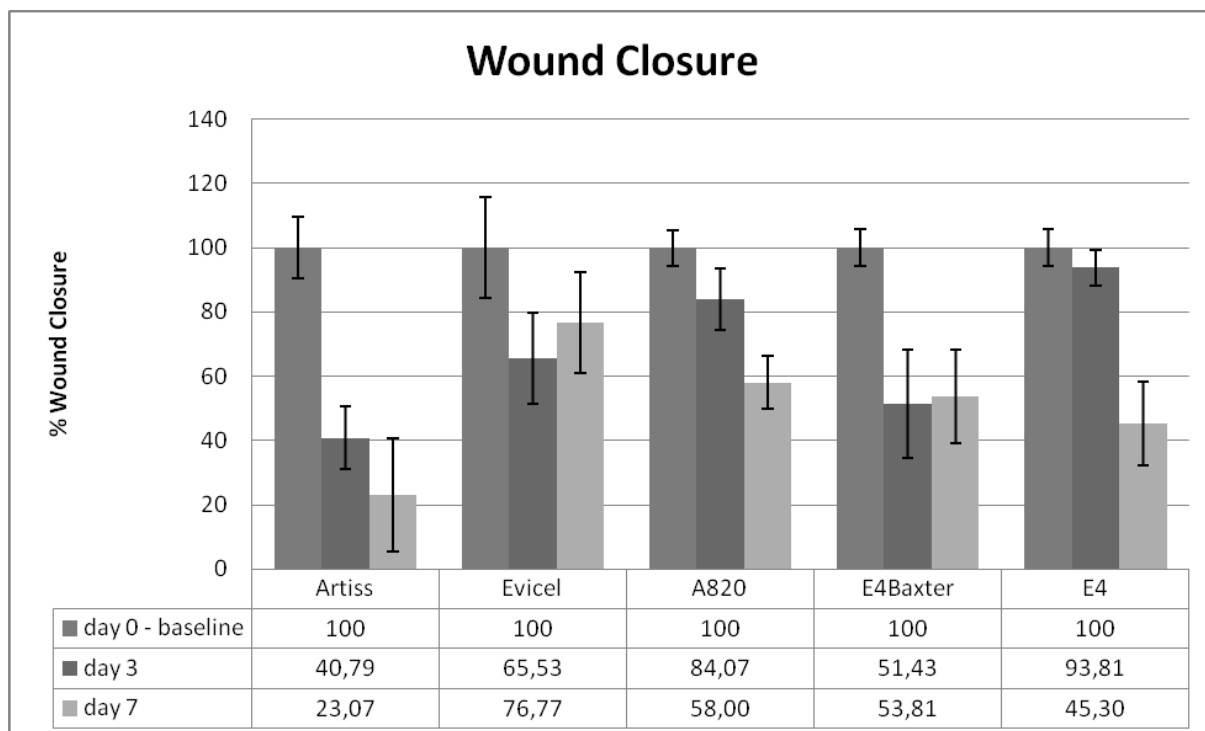


Figure 46 Wound closure: planimetry of wounds treated with fibrin sealants Artiss, Evicel, A820, E4Baxter and E4. Day 0 was set as 100 %, standard error is shown.

To estimate wound closure, wound area was measured via Planimetry. A decrease of wound area of 77 % was observed in the Artiss treated group after 7 days (significant wound closure between day 0 and day 3). Rats treated with Evicel had a decrease of wound area of 24 % after 7 days (not significant). The wound of the A820 and E4Baxter groups shrunk to about 55 % compared to day 0, whereupon the A820 group had a significant wound closure between day 3 and day 7 and the wounds in the E4Baxter group with a lower thrombin concentration significantly reduced their area between day 0 and day 3, therefore had a “faster” wound closure. Rats treated with E4 showed nearly no wound closure in the first 3 days but after 7 days of healing, the wound area amounted to about 45 % compared to the baseline.

In addition to the observations concerning the influence of thrombin in wound healing, also an important ECM molecule was subject of the experiment. Since it is known that keratinocytes – an important cell type in wound healing and most important cells for wound closure – lack the integrin receptor $\alpha v \beta 3$ for fibronectin, a fibrinogen component depleted of fibronectin, plasminogen and vWF was used: FIB3. In one group, FIB3 was mixed with 4 IU

thrombin (Baxter) to form a clot, in another group fibronectin was spiked to FIB3 to a concentration that is comparable to Artiss fibrin sealant (FIB3+Fn).

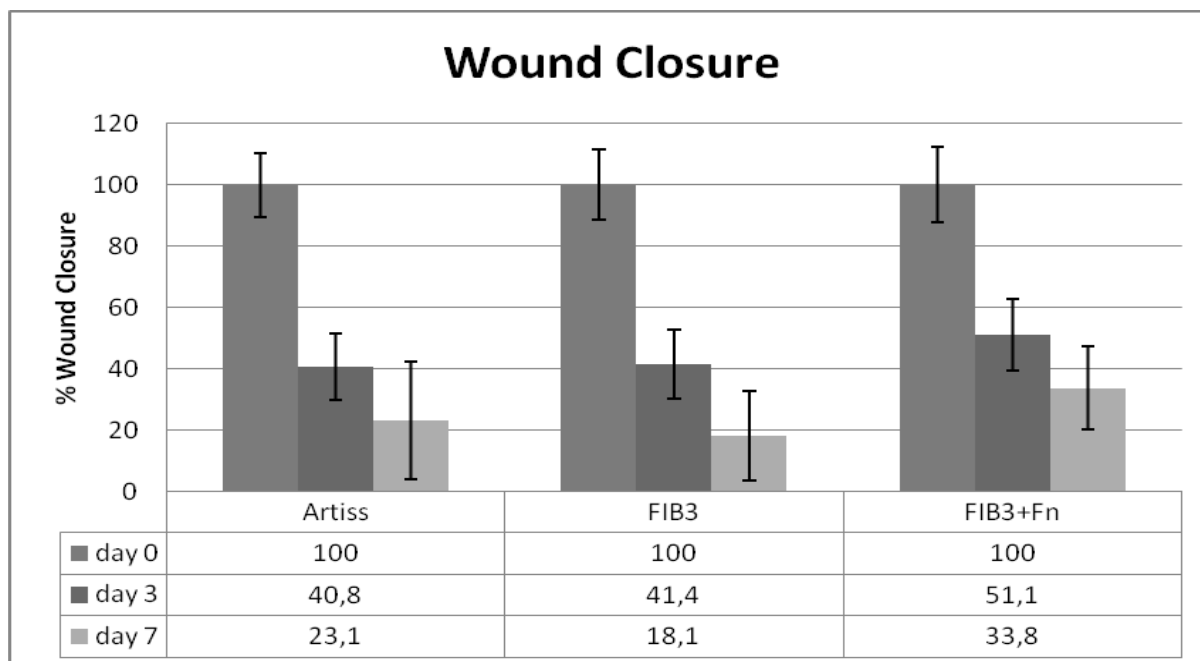


Figure 47 Wound closure: planimetry of wounds treated with fibrin sealants Artiss, FIB3 and FIB3+Fn. Day 0 was set as 100%, standard error is shown.

No differences between the groups could be observed. Wound area decreased significantly between day 0 and day 3 in all groups but not between day 3 and day 7 although a positive trend towards wound closure exists. The FIB3+Fn group showed a slightly decreased wound closure compared to FIB3 and Artiss. (Figure 47)

3.2 HISTOMORPHOMETRY

Advances in wound healing (wound contraction, wound severity, granulation and global healing in general) were measured after day 7 by histomorphometric analysis according to Lemo et al. (108)

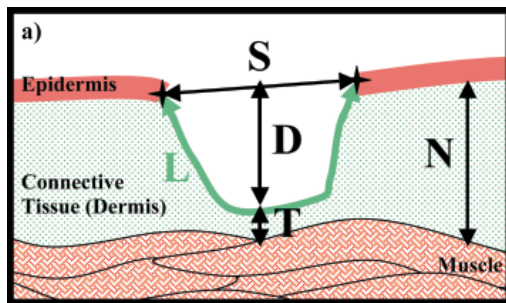


Figure 48 Picture taken from Lemo et al., 2010

S: distance between the borders of the wound (last keratinocyte)

L: length of re-epithelialization zone

D: depth of the wound from S to deepest point of the wound

T: thickness of the connective tissue (residual dermis or new dermis)

N: thickness of the natural dermis $N=D+T$

3.2.1 SUPERFICIAL CONTRACTION INDEX

The Superficial Contraction Index SCI is influenced by the distance between the borders of the wound (last keratinocyte; S) and the length of re-epithelialization zone (L).

$$SCI = (L-S)/L$$

No significant differences could be observed. Artiss and E4Baxter treated wounds had a lower index and therefore more superficial contraction of the wound.

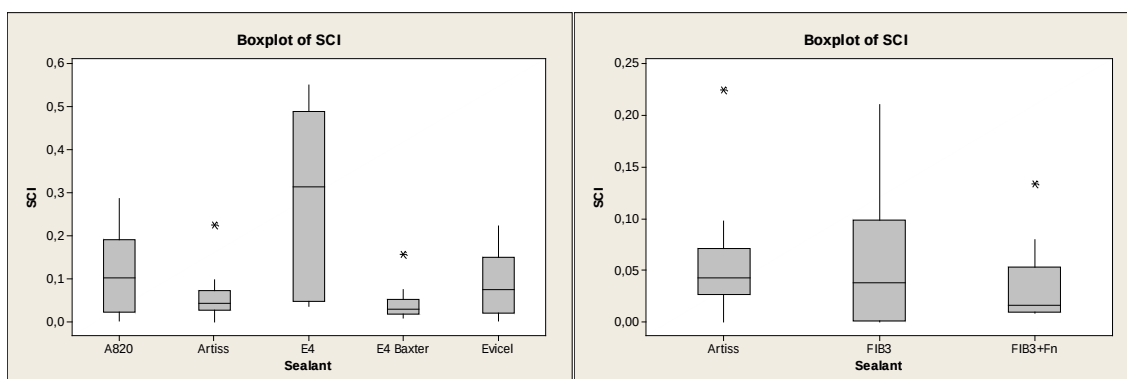


Figure 49 Histomorphometric analysis: Superficial contraction Index on day 7 (n=10).

3.2.2 DEEP CONTRACTION INDEX

The Deep Contraction Index DCI is influenced by the depth of the wound from S to the deepest point of the wound (D) and the thickness of the connective tissue (residual dermis or new dermis; T). It is normalized to the thickness of the natural dermis ($N=D+T$).

$$DCI = (N-D)/N$$

No significant differences could be observed. By trend, Artiss and E4Baxter treated wounds had a higher index and therefore showed more deep contraction.

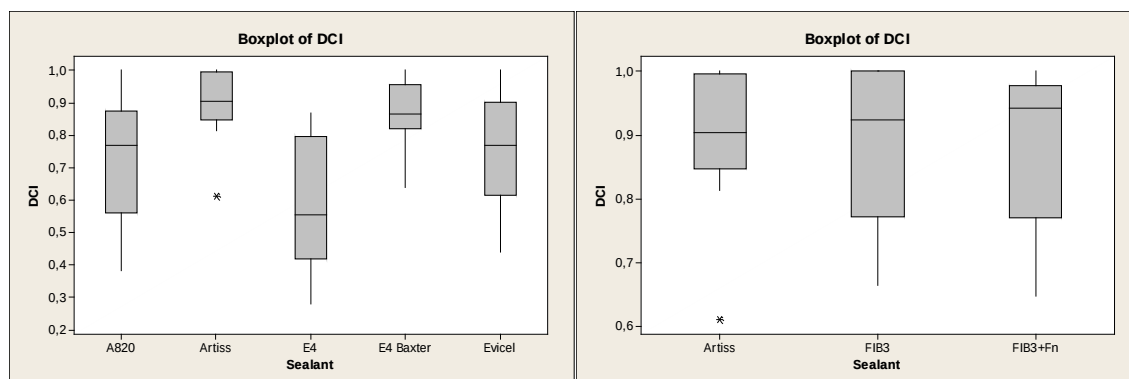


Figure 50 Histomorphometric analysis: Deep contraction Index on day 7 (n=10).

3.2.3 WOUND SEVERITY INDEX

The Wound Severity Index WSI is influenced by the thickness of the connective tissue (residual dermis or new dermis; T) and the thickness of the natural dermis (N).

$$WSI = (N-T)/N$$

No significant differences could be observed. Groups treated with Evicel, E4 and A820 showed more severe (deeper) wounds compared to the other groups. Artiss and E4Baxter had a lower index and therefore indicate a better wound healing.

No differences between Artiss, FIB3 and FIB3+Fn regarding their WSI could be observed.

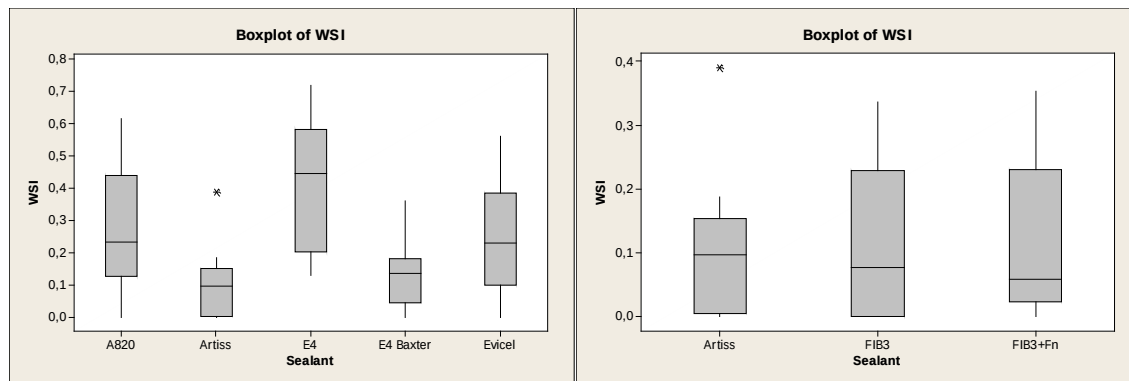


Figure 51 Histomorphometric analysis: Wound Severity Index on day 7 (n=10).

3.2.4 WOUND CONTRACTION INDEX

The Wound Contraction Index WCI is a combination between the Superficial Contraction Index and the Deep Contraction Index and therefore influenced by the distance between the borders of the wound (last keratinocyte), the length of re-epithelialization zone, the depth of the wound, and the thickness of the natural dermis.

$$WCI = SCI + DCI$$

No significant differences between the groups could be measured.

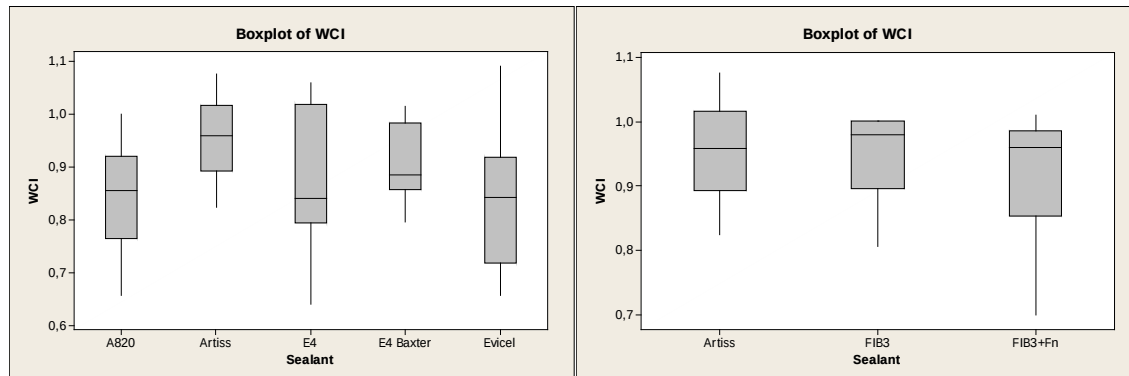


Figure 52 Histomorphometric analysis: Wound Contraction Index on day 7 (n=10).

3.2.5 GLOBAL HEALING INDEX

The Global Healing Index is the difference between WCI and WSI and therefore considers on the one hand contraction - and therefore "closure" of wounds - and its severity. A high GHI indicates a better wound healing.

$$GHI = SCI + DCI - WSI$$

No significant differences could be detected. Evicel, E4 and A820 treated groups demonstrated a lower GHI and therefore a less advanced wound healing. No differences between Artiss, FIB3 and FIB3+Fn could be observed.

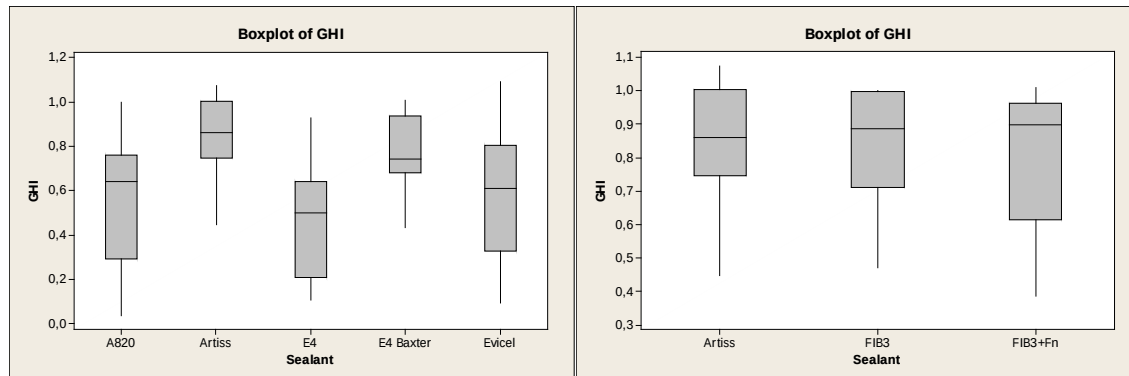


Figure 53 Histomorphometric analysis: Global Healing Index on day 7 (n=10).

3.3 NEO-VASCULARIZATION

Vascularization is an important step towards regeneration of wounds. Therefore, new vessels migrate into the new built tissue to ensure nutrition and can be used as an indicator for wound healing and tissue regeneration.

3.3.1 SMOOTH MUSCLE ACTIN

Smooth muscle actin (SMA) is a marker for functional vessels. All groups treated with fibrin sealants showed a higher number of SMA-positive vessels in the wound area compared to the untreated control group.

The fibrin sealant group with no fibronectin tends to have the lowest number of functional vessels but not significantly lower than the other groups.

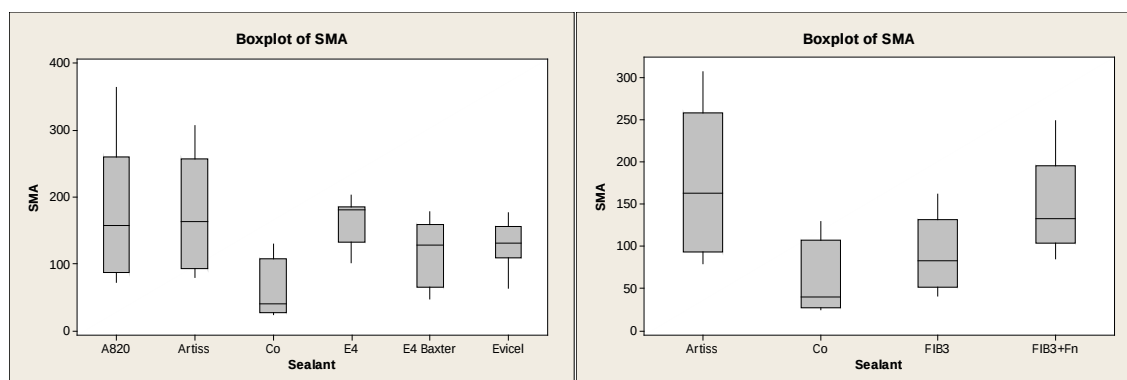


Figure 54 Immuno-histochemical analysis: smooth muscle actin staining (Vascularization) on day 7 (n=10).

Data are illustrated in a logarithmic display because no normal distribution was given (lognormal distribution; pre-condition for ANOVA).

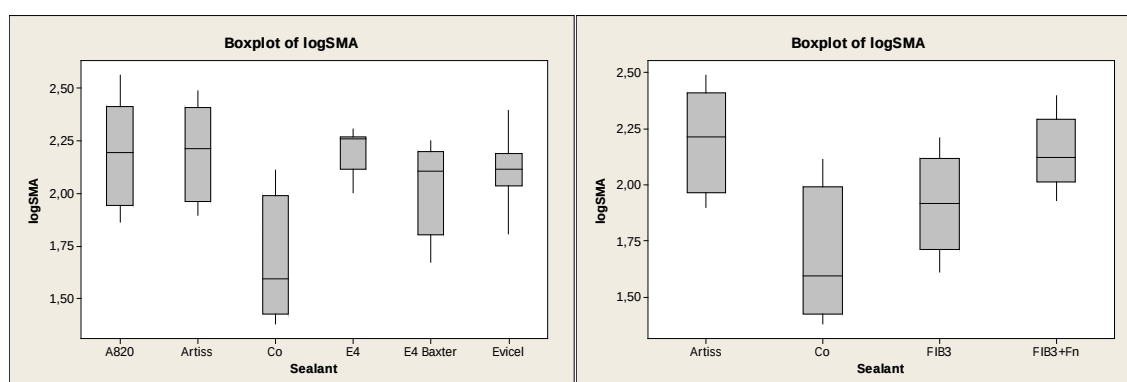


Figure 55 Immuno-histochemical analysis: smooth muscle actin staining (Vascularization) on day 7. Data are given in a logarithmic scale. (n=10).

3.3.2 VON WILLEBRAND FACTOR

The von Willebrand factor (vWF) antibody is a general marker for all kind of vessels.

No significant differences in expression of vWF between the groups could be seen. By trend, E4 treated animals formed a higher number of vessels compared to the others.

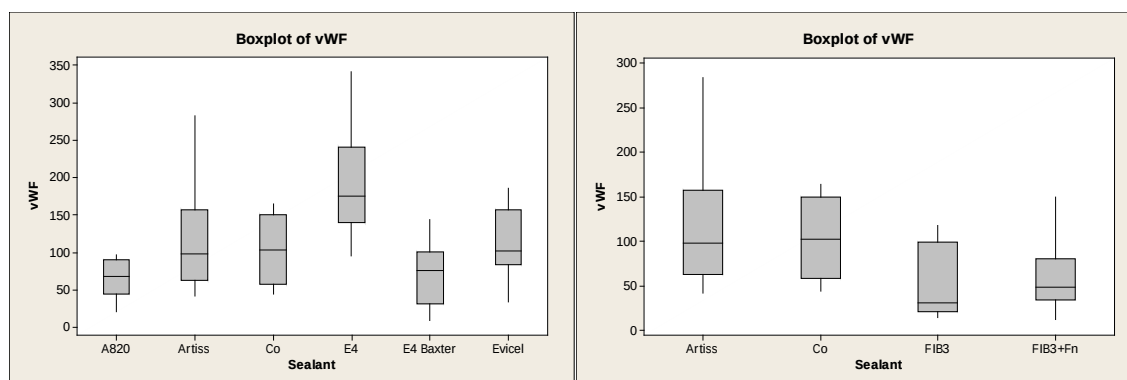


Figure 56 Immuno-histochemical analysis: von Willebrand factor staining (Vascularization) on day 7 (n=10).

3.3.3 LogSMA/vWF

By trend, wounds treated with A820 and Artiss (same fibrinogen component) seemed to have a better neo-vascularization compared to Evicel and E4 treated animals.

Furthermore, FIB3 spiked with fibronectin led to building of more vessels than only FIB3.

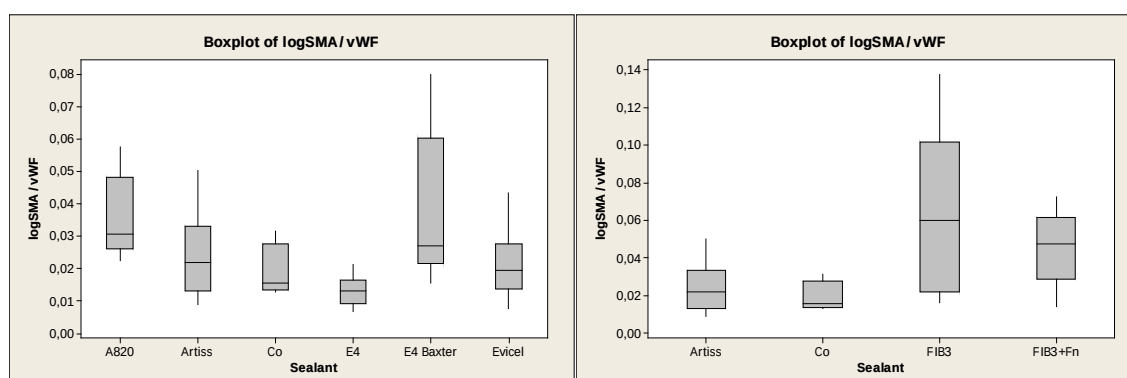


Figure 57 Immuno-histochemical analysis: ratio smooth muscle actin/vWF stained cells (Vascularization) on day 7 (n=10).

To sum up, Artiss treated groups showed a significantly better wound healing after seven days compared to Evicel, A820 and E4Baxter, but not to Evicel with 4 IU/ml thrombin. Furthermore, Evicel did not have any significantly decreased wound area after seven days wound healing.

Concerning wound severity, no significant results could be observed. But a trend is visible that groups treated with Artiss and E4Baxter showed more contraction and a lower wound severity index, indicating a better wound healing than the other groups.

No significant differences in wound healing could be observed between groups with distinct fibronectin concentrations.

Immuno-histochemical analyses for vascularization provided the results that any group treated with a fibrin sealant built more vessels in the wound area than untreated wounds. Between fibrin sealants differing in thrombin concentration there were no significant differences and no trends visible. Fibronectin depleted fibrin sealants by trend initiated the building of a lower number of SMA-positive vessels compared to Artiss and FIB3+Fn. However, this effect was not observed in vWF-positive stained sections.

3.3.4 WESTERN BLOT ANALYSIS

For protein analysis samples of Artiss and Evicel fibrin sealant treated wounds (n=1) as well as untreated wounds (n=1) and healthy tissue was taken by biopsies on day 2. VEGF and angiopoietin are growth factors which are secreted by cells to (among other functions) stimulate the building of vessels and are therefore markers for angiogenesis. Biopsies were processed three times (lysis and Western blot analysis).

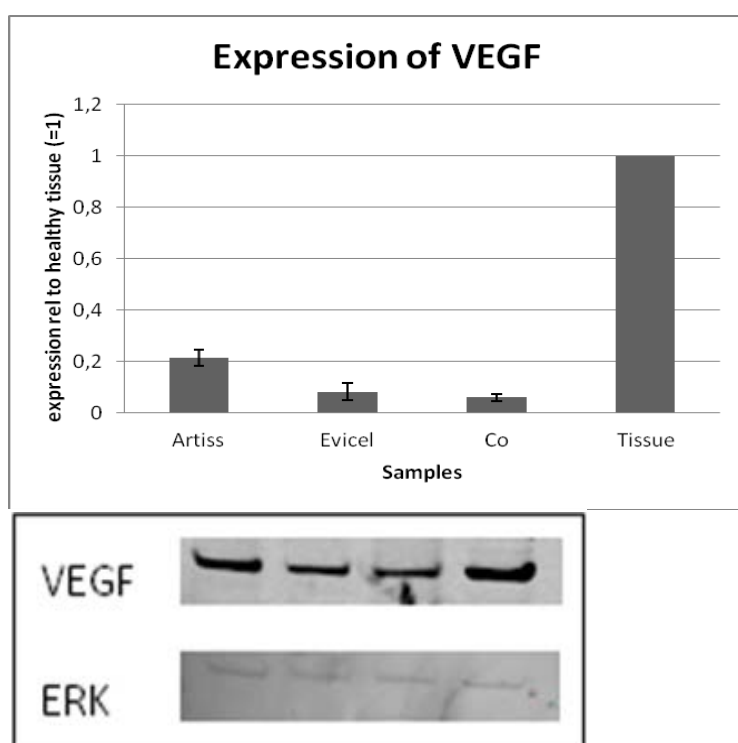


Figure 58 Three Western blot analyses of VEGF expression in biopsies taken of excisional wounds treated with Artiss or Evicel fibrin sealant on day 2. As controls, mock treated wounds and healthy tissue was observed. The graph shows the mean of three blots, one representative Western blot is shown below. (n=3)

Compared to healthy tissue, all samples showed a lower level of VEGF (n=3) and angiopoietin-1 (n=2) expression in wounds. Furthermore, in wounds treated with Artiss, the level of VEGF was significant higher than in Evicel or untreated wounds. These results indicate that Artiss leads to more vessel building compared to the other groups but far less than in healthy tissue. Expression of angiopoietin-1 was not different within the groups.

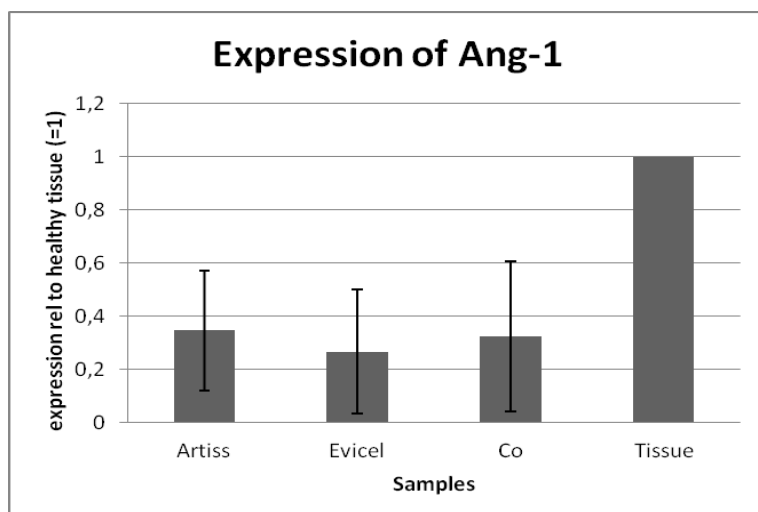


Figure 59 Two Western blot analyses of Ang-1 expression in biopsies taken of excisional wounds treated with Artiss or Evicel fibrin sealant on day 2. As controls, mock treated wounds and healthy tissue was observed. The graph shows the mean of two blots, one representative Western blot is shown below. (n=2)

3.4 INFLAMMATION

To detect the level of inflammation in the wounds, biopsies were taken from the wound edge on day 7, lysed and analyzed by a human cytokine array.

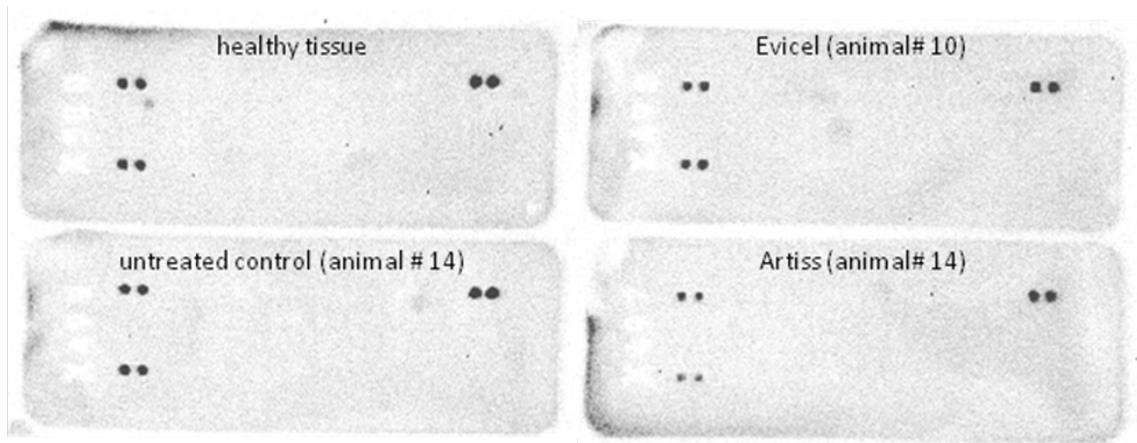


Figure 60 Human cytokine array (R&D Systems) of biopsies taken of excisional wounds treated with Artiss or Evicel fibrin sealant on day 7. As controls, mock treated wounds and healthy tissue was observed. (n=1)

No cytokines and chemokines could be detected (detection limit 83 pg/ml).

For protein analysis samples of Artiss and Evicel fibrin sealant treated wounds (n=1) as well as untreated wounds (n=1) and healthy tissue was taken by biopsies on day 2.

Interleukin-1 is a cytokine that is important in early inflammation and other immune responses. (110) The biopsies were processed four times (lysis and Western blot analysis).

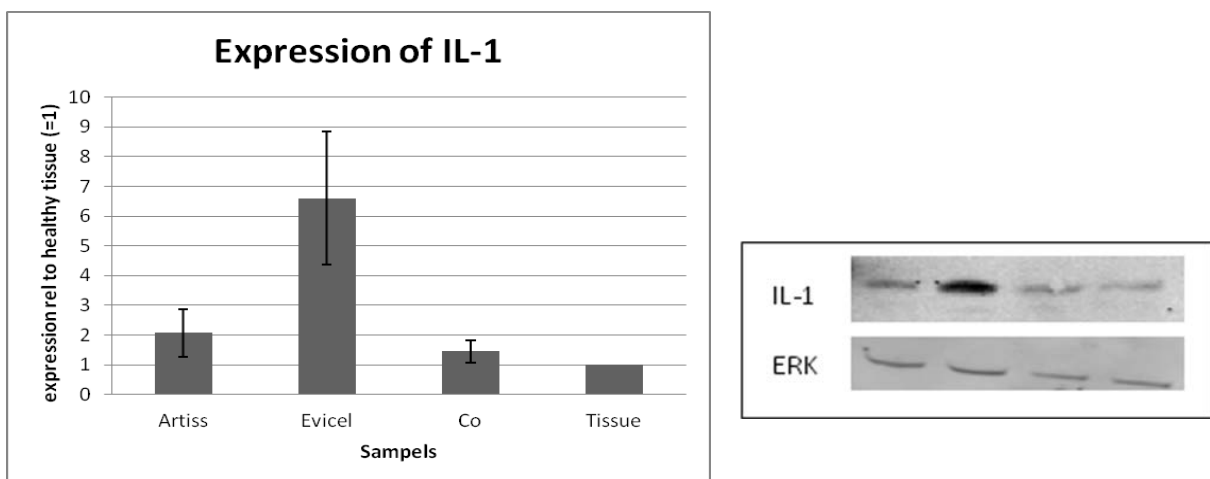


Figure 61 Four Western blot analyses of IL-1 expression in biopsies taken of excisional wounds treated with Artiss or Evicel fibrin sealant on day 2. As controls, mock treated wounds and healthy tissue was observed. The graph shows the mean of four blots, one representative Western blot is shown below. (n=4)

Wounds treated with Evicel fibrin sealant showed a 6.5-fold higher IL-1 α expression (n=2) on day 2 than healthy tissue. Artiss and mock treated wounds had a two-fold higher expression compared to healthy tissue. (n=4)

3.5 FIBRIN DEGRADATION

Fibrin degradation of the fibrin sealants Artiss and Evicel were measured by using Alexa flour 546 labeled fibrinogen (both sealants in one rat; n=3). The decrease of fluorescence in time is directly associated with fibrin degradation.

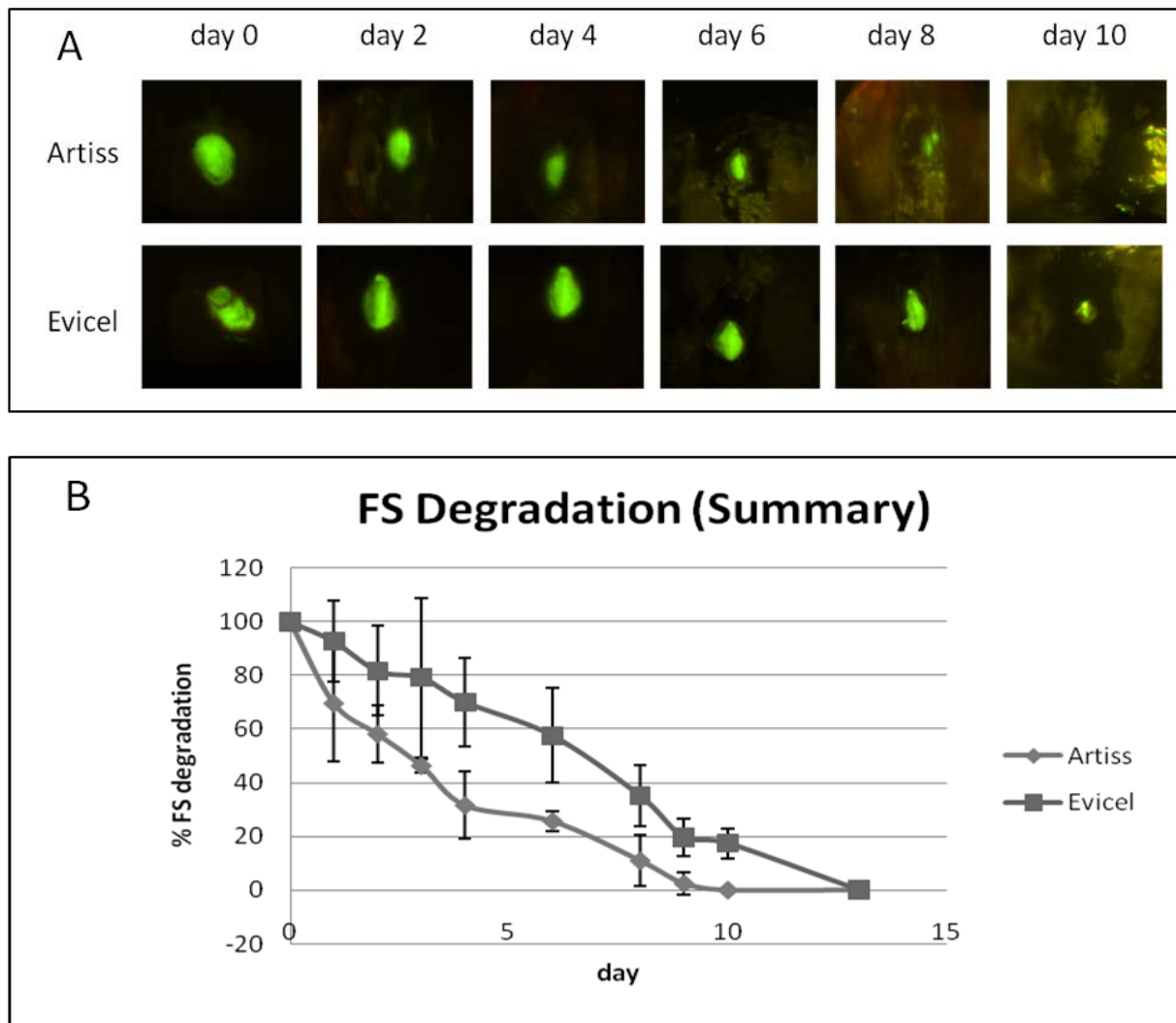


Figure 62 Fibrin clot degradation of Artiss and Evicel: fluorescence in Maestro of representative clots (A) and given in percent relative to day 0 (B). (n=3)

After four days of wound healing, significant differences could be observed: Artiss showed a faster fibrinolysis compared to Evicel which is reduced in plasminogen. (n=3)

3.6 APOPTOSIS

As described before, our *in vitro* studies showed that high thrombin concentrations induced apoptosis in NHEK. To analyze this effect *in vivo*, in-situ hybridization of paraffin embedded cross sections was performed (TUNEL staining).

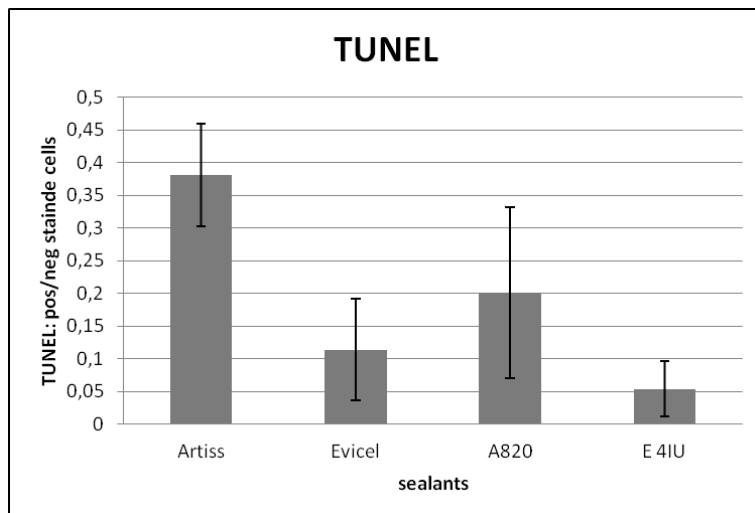


Figure 63 in situ hybridization of apoptosis (TUNEL staining) on day 7.

In contrast to our expectations, Evicel and E4IU showed less dead cells per living cells compared to groups treated with Artiss and A820 fibrin sealants. *In vitro* data showed an induced apoptosis of keratinocytes when getting in contact with high thrombin concentrations.

3.7 PATHOLOGICAL ANALYSIS

To observe any obvious pathological irregularities, H/E stained cross sections were evaluated by a pathologist. Not degraded fibrin sealant remnants and re-epithelialization were evaluated, for inflammatory effects polymorphonuclear leukocytes as well as foreign-body giant cell were rated, the building of vessels was compared and built collagen (scar tissue) and seroma were observed.

Table 7 Pathological Analysis: on the basis of H/E stainings rated according to a scale from 0 (none) to 3 (maximum, severe) by a pathologist. FS: fibrin sealant remnants, Epith Regen: epithelial regeneration, PMN: polymorphonuclear leukocytes, FkRz: foreign-body giant cell, Angio: angiogenesis, Scar/Col: built collagen, Seroma.

Sealant	Rating	FS	Epith Regen	PMN	FkRz	Angio	Scar/Col	Seroma
Artiss	0							
	1							
	2							
	3							
Evicel	0							
	1							
	2							
	3							
A820	0							
	1							
	2							
	3							
E4Baxter	0							
	1							
	2							
	3							
FIB3	0							
	1							
	2							
	3							
FIB3+Fn	0							
	1							
	2							
	3							
untreated	1							
	2							
	3							
	4							

No clear patterns are visible and no qualitative statement could be made upon these results.

4 ECM MOLECULES IN FIBRINOGEN COMPONENT – FIBRONECTIN

4.1 FIBRIN CLOT EXTRACTION

As already mentioned above, fibrin sealant components not only consist of fibrinogen and thrombin but also of several ECM molecules, growth factors, salts and undefined and in their concentration unknown proteins. Some of these proteins are immediately washed out in wash steps because of their weak bonding to the fiber network. One of them is fibronectin as demonstrated by the extraction experiment shown in figure 19.

To evaluate those proteins, Tisseel clots were treated with different extraction solutions: 1M NaCl, 2M Urea, PBS and LMA (1 M NaCl with acetonitrile and trifluoroacetic acid). Fibrin clot extracts were loaded on an SDS PAGE and stained with Coomassie. As depicted in figure 19, results show that this method is feasible to extract constituents from the fibrin clot. (n=4)

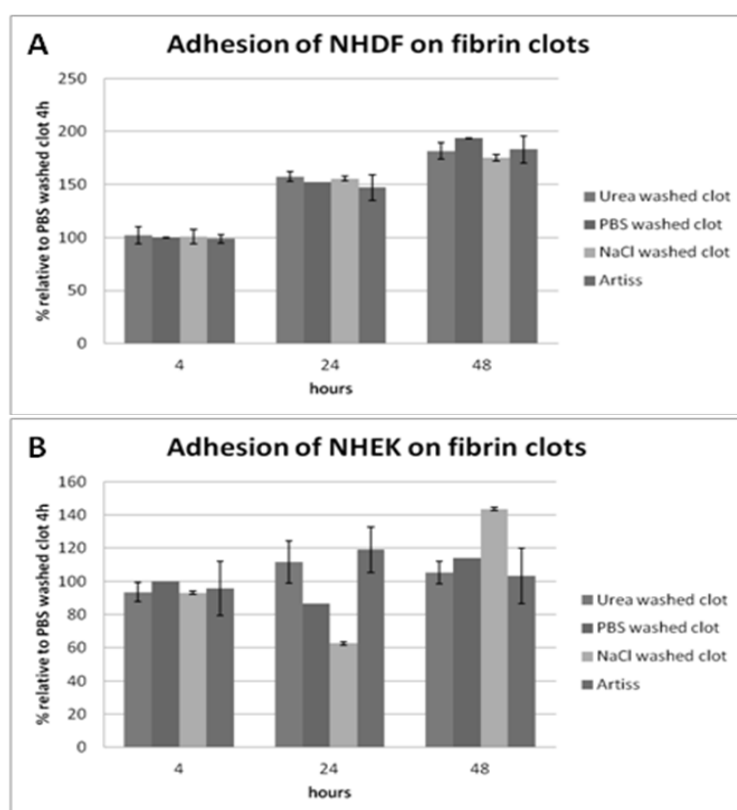


Figure 64 Adhesion Assay: NHDF (A) and NHEK (B) were seeded on fibrin clots extracted for 24 hours with different solutions (2 M Urea, PBS, 1 M NaCl), Artiss = untreated clot. The number of attached cells was quantified after 4, 24 and 48 h by LDH-assay. Data are presented relative to values obtained with PBS washed fibrin clot (=100%). (n=4, in triplicates, mean values and standard deviation are shown)

Extraction (e.g. with 1 M NaCl or LMA) of fibrin clots lead to a reduction of non-covalently bound components within 24 hours. Cell morphology, adhesion and viability of NHDF were not affected by this treatment, which indicated that non-extractable, covalently bound proteins are necessary for interaction with cells (such as fibronectin which is cross-linked to fibrinogen via FXIIIa). NHEK show different adhesion properties when the fibrin clot was extracted with NaCl. Other treated clots did not show significant differences.

4.2 INTEGRIN $\beta 1$ RECEPTOR EXPRESSION ON NHEK

To analyze the expression pattern of integrin $\beta 1$ of cells upon attachment on fibrin sealants, NHEK were cultivated for 24 hours on top of fibrin clots or on culture plate as control. Furthermore, NHEK were seeded on NaCl extracted Artiss clots to determine if removed constituents could have an influence on integrin expression.

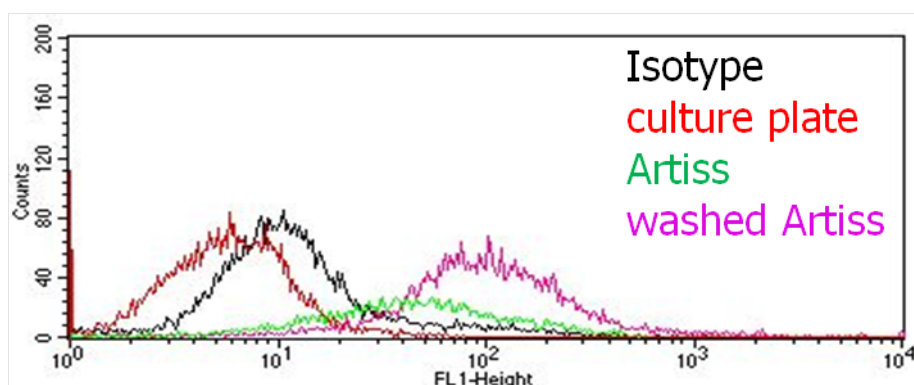


Figure 65 FACS analysis of NHEK cultured 24 hours on cell culture plate (red), Artiss clot (green) or washed Artiss clot (24 h with 1 M NaCl; pink). Expression of integrin $\beta 1$ receptor on NHEK is increased due to contact of cells with fibrin. (n=2)

Expression of integrin $\beta 1$ receptors on keratinocytes was increased upon contact with fibrin clots, which is reflected in a higher percentage of positive cells as well as a higher receptor density per cell. Expression was even more increased when fibrin clots were washed with 1 M NaCl solution. Integrin $\beta 1$ as a proliferation marker indicates that keratinocytes are kept in a proliferating state.

4.3 DEPLETION OF FIBRONECTIN

The ECM molecule fibronectin can be washed out of clots with 1 M NaCl as shown in a coomassie staining in figure 67. But NaCl extraction alone only removes unbound and loosely bound proteins. This treatment does not remove noteworthy amounts of proteins.

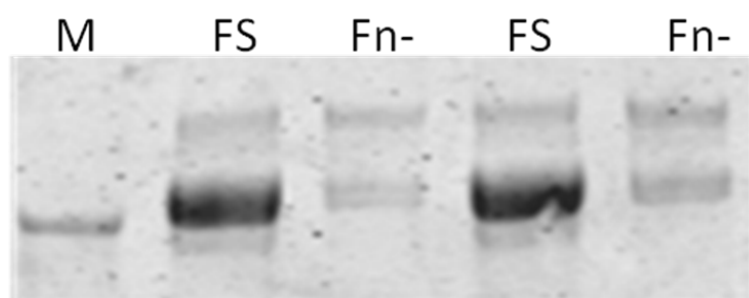


Figure 66 Coomassie staining of sealer protein of Artiss fibrin sealant (FS) and fibrinogen purification (Fn-).

Another approach to evidence the influence of fibronectin on the interaction of cells with the fibrin sealant matrix was the depletion of fibronectin by glycine precipitations of FVIII cryoprecipitate, a precursor product of fibrin sealants.



Figure 67 The residual amounts of fibronectin in the purified fibrinogen of two different experiments were quantified by semi-quantitative Western blot using the Odyssey instrument (LI-COR Biosciences). For each sample, 15 µg of protein were loaded. Fn- 1 and Fn- 2: fibrinogen purification of two experiments, SP: sealer protein of Artiss fibrin sealant, M: marker, Fn-standard: standard curve of fibronectin (5.0-0.6 µg). Fibronectin content of SP was set for 100 % (Artiss: ~3 mg/80 mg fibrinogen), estimated fibronectin content for FIB3: ~0.3 mg/80 mg fibrinogen. Used antibodies: mouse anti fibronectin (St. Cruz, 1:1000); goat anti mouse for Odyssey (LiCOR, 1:10,000). Predicted bands: ~ 230 – 250 kDa.

In Western blot analysis it could be demonstrated that the efficacy of this treatment was at about 30-40 % decrease of fibronectin concentration in the sealer protein. However we did not observe significant differences regarding adhesion and viability assays compared to

untreated Artiss fibrin sealant. This indicates that even small amounts of fibronectin were sufficient. Therefore, FIB3 – pure plasma derived fibrinogen depleted of fibronectin (among others) – was used for cell experiments.

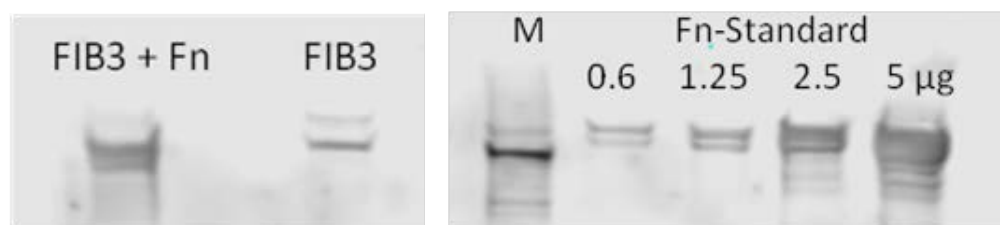


Figure 68 The amount of fibronectin in FIB3 was quantified by semi-quantitative Western blot using the Odyssey instrument (LI-COR Biosciences). 15 µg protein was loaded. FIB3+Fn: FIB3 spiked with fibronectin to a final concentration of 2 mg/ml, FIB3: fibrinogen depleted of fibronectin, plasminogen and vWF (Enzyme Research Laboratories, IN, USA), M: marker, Fn-standard: standard curve of fibronectin (5.0-0.6 µg). Used antibodies: mouse anti fibronectin (St. Cruz, 1:1000); goat anti mouse for Odyssey (LiCOR, 1:10000). Predicted bands: ~ 230 – 250 kDa.

Foremost, residual amount of fibronectin in FIB3 was analyzed by Western blot analysis as shown in figure 68. Compared with the fibronectin standard, about 0.6 µg protein was measured whereas in the spiked FIB3 a content of about 2.5 µg could be observed. It is possible to spike fibronectin into FIB3 to a desired concentration (FIB3 + Fn).

96-well plates not treated for tissue culture are not favorable for cell adhesion. Such plates were coated with fibrinogen, fibronectin and a mixture of both proteins over night at 4°C. Then, keratinocytes and endothelial cells were seeded and adhesion was measured after 2 and 24 hours.

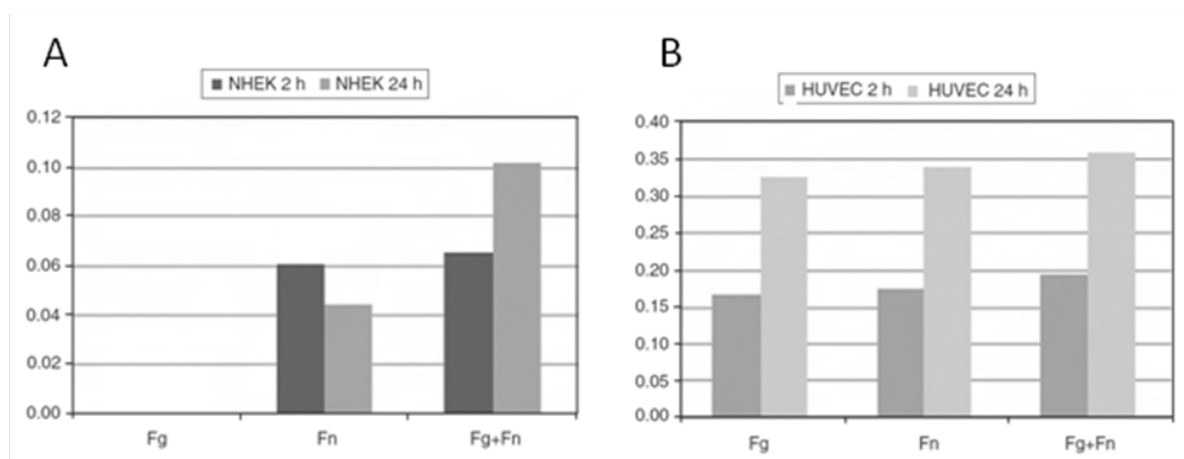


Figure 69 Cell number of human keratinocytes (A) and endothelial cells (B) grown for 2 hours and 24 hours on 96-well plates coated with fibrinogen (Fg), fibronectin (Fn), and a combination of both matrix proteins (Fg + Fn). (NHEK n=4; HUVEC n=3)

HUVEC did not show any significant differences regarding attachment between coatings. But NHEK cells did not adhere on fibrinogen due to the lack of $\alpha\beta 3$ integrins, the most important integrin receptor for fibrinogen. On fibronectin coating, the number of NHEK cells decreased in time whereas on fibrinogen coating with fibronectin, more cells adhered after 24 hours.

On fibronectin-, plasminogen-, and vWF-depleted fibrin clots (FIB3) a reduced attachment and deteriorated morphology of keratinocytes was found. This can be reversed by spiking the fibrinogen with fibronectin demonstrating that fibronectin in fibrin sealants plays a crucial role in interaction with keratinocytes.

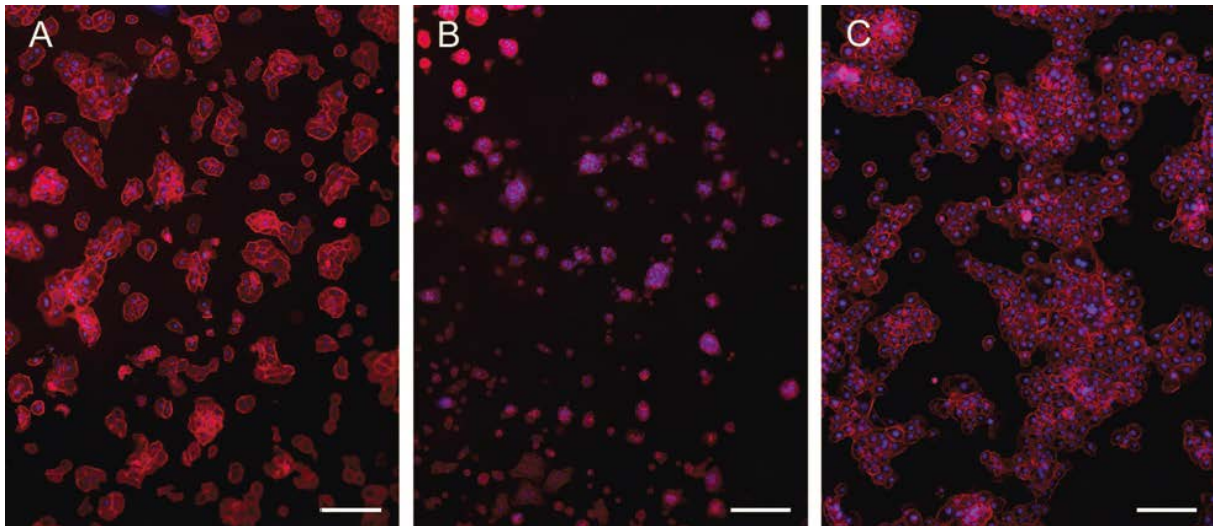


Figure 70 Normal human epithelial keratinocytes seeded on top of Artiss, FIB3 and FIB3+Fn fibrin clots and cultivated for 24 hours. Cellular actin cytoskeleton was labeled with phalloidin (red) and the nuclei with DAPI (blue). (A) On clots made from a commercial product (Artiss) keratinocytes showed a normal polygonal morphotype with a well-developed cytoskeleton. (B) Keratinocytes grown on clots deprived of fibronectin (FIB3) show a rounded shape with little amount of cytoskeleton. (C) Spiking of FIB3 clots with fibronectin to a final concentration of 2 mg/ml prevents the cell morphology alteration. Scale bars: 200 μ m. (n=4)

Observed effects on morphology could be confirmed by adhesion and viability data. More keratinocytes were counted by LDH assay after 24 and 48 hours on Artiss fibrin sealant clot (100 % after 24 hours) and FIB3 spiked with fibronectin (about 95 %). On FIB3 only, significant fewer cells attached (between 70 and 80 %).

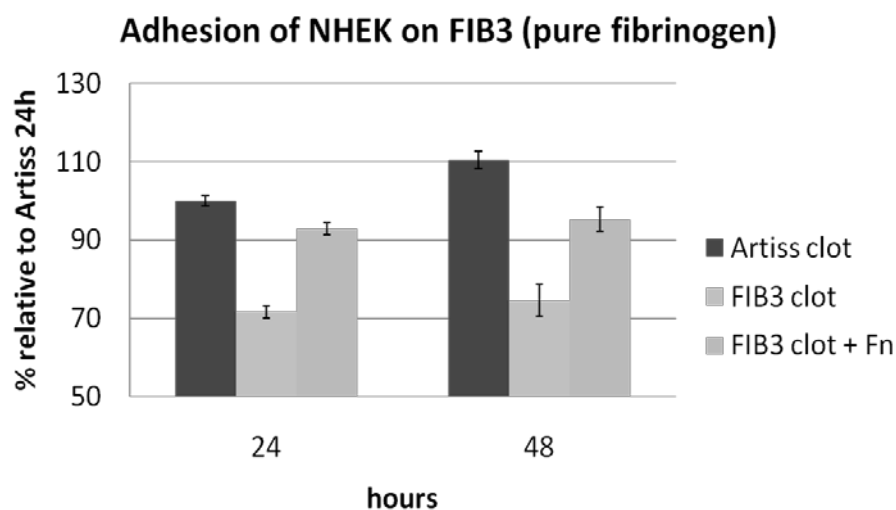


Figure 71 Keratinocytes show differences in adhesion and viability between Artiss and FIB3 clots

Adhesion Assay: Number of attached NHEK was quantified after 24 and 48 hours by LDH-assay. Artiss clot (fibronectin content ~3 mg/ml); FIB3 clot = fibrin depleted of fibronectin, plasminogen, and vWF; FIB3 clot + Fn = FIB3 spiked with fibronectin (final concentration: 2 mg/ml). Data are presented relative to values obtained with Artiss clots 24 hours (=100 %). (n=2, in triplicates, mean values and standard deviation are shown)

4.4 PLASMINOGEN

FIB3 fibrinogen is not only depleted of fibronectin but also of plasminogen. Also the commercially available fibrin sealant Evicel has a reduced plasminogen concentration. To answer the questions whether plasminogen is important for cell adhesion and viability and if it contributes to the differences between Artiss and Evicel, plasminogen was added to Evicel fibrin sealant to a final concentration of 110 µg/ml (to adjust the plasminogen concentration to Artiss). On these clots, NHEK and HUVEC cells were seeded and LDH assay was performed.

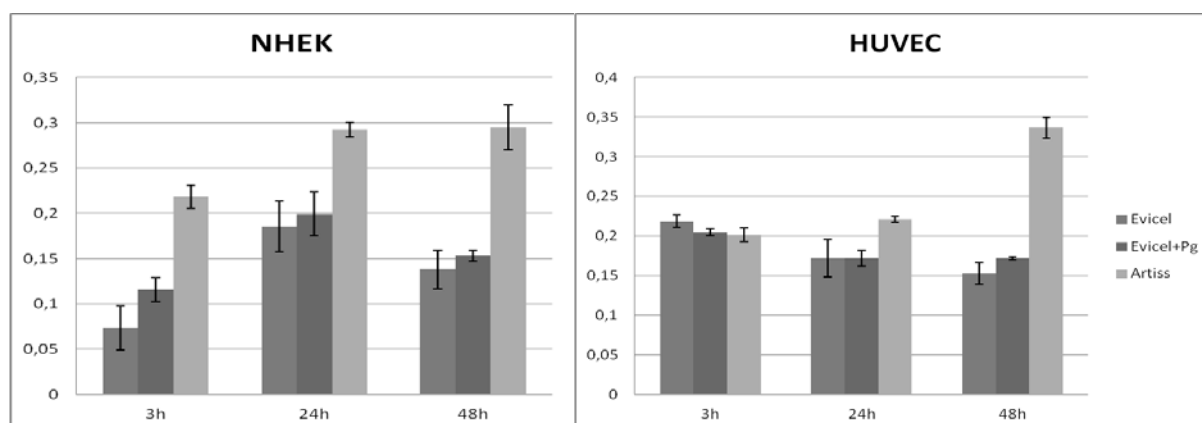


Figure 72 Keratinocytes and endothelial cells were seeded on fibrin sealant clots: Artiss, Evicel and Evicel spiked with 110 µg/ml plasminogen (Evicel+Pg). Number of attached cells was quantified after 3, 24, and 48 hours by LDH assay. n=2

No significant differences between Evicel and Evicel + Plasminogen could be observed indicating that plasminogen has no direct influence on cell adhesion and proliferation

5 CLOT STRUCTURE

During coagulation, fibrinogen is converted to an insoluble fibrin polymer: fibrinopeptides A and B (FpA and FpB) are cleaved by thrombin.

As described in the introduction, the polymerization sites E_A and E_B are laid open, these “knobs” are exposed “holes” (Da/Db) at the ends of the molecules. The now so-called fibrin monomer then spontaneously assembles with other monomers into linear protofibrils through self-association to make half-staggered oligomers. In succession, the protofibrils aggregate and align in a staggered overlapping end-to-middle domain arrangement to form double-stranded twisting fibrils which then branch to build a three-dimensional network.

This event is influenced e.g. by pH, ionic strength, temperature and maybe thrombin concentration.

Different clot structures therefore offer different conditions for adherent cells and result in different wound healing efficiency. (111)

5.1 INFLUENCE OF FIBRINOGEN AND THROMBIN CONCENTRATION ON FIBRIN STRUCTURE

Generally, fibrinogen determines the density and stiffness of the clot in a direct correlation implying that more fibrinogen per volume results in a denser clot with decreased porosity and permeation (112;113).

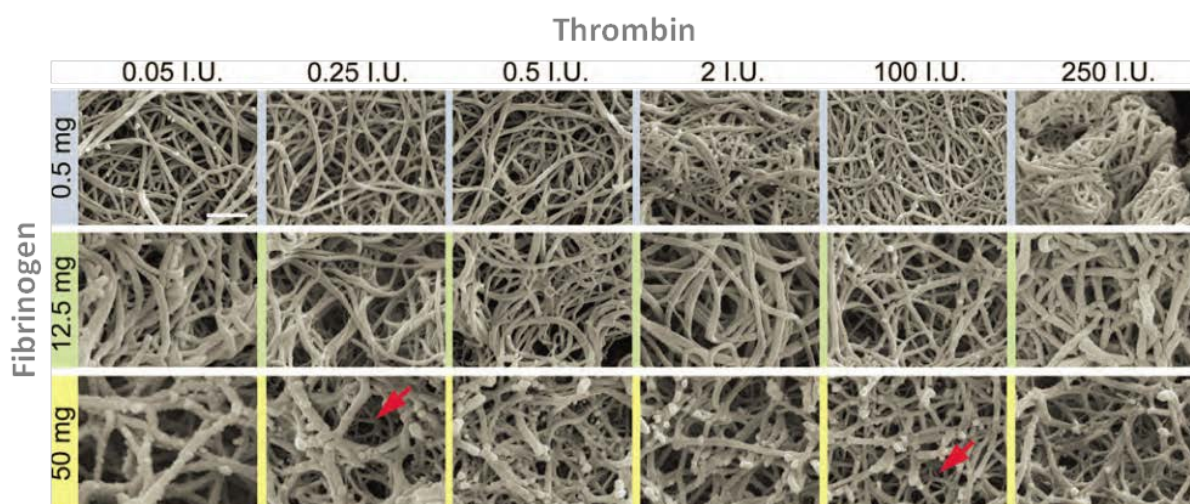


Figure 73 Concentration series of fibrin clots: Thrombin concentrations are horizontally arranged: 0.05 – 0.25 – 0.5 – 2 – 100 – 250 IU/ml and the fibrinogen concentrations vertically 0.5 – 12.5 – 50 mg/ml. Scale bar: 1 μ m.

Figure 73 demonstrates the SEM images of the fiber networks and reveals higher fiber thickness with higher fibrinogen concentrations. With 50 mg/ml a second finer fiber network is visible (arrows).

Histological, this effect becomes visible by a more intense staining of clots with high fibrinogen concentration. Apart from the density, fibrinogen concentration determines the homogeneity of the clot. The enormous variability of structural properties becomes obvious even in a range of 0.5 to 50 mg/ml fibrinogen and varying thrombin concentrations of 0.05 to 250 IU/ml (final clot concentration). Very low fibrinogen concentrations (0.5 mg/ml) cause jelly and translucent gels incapable to keep the shape. They stain very faintly in histology, except of some regions of higher density. Higher fibrinogen percentage (12.5 mg/ml and more) leads to stiff clots whereat the stain affinity is gradually increasing with the fibrinogen content.

Increasing thrombin concentration does not alter fiber structure in a regular manner. Only the highest thrombin concentration in combination with low fibrinogen concentrations results in very dense clots with thick fibers.

5.2 INFLUENCE OF SALT CONCENTRATION ON FIBRIN STRUCTURE

The addition of NaCl (100 mM) and arginine (50 mM) alters the ultra structure of the fibrin clot to a tight fibrin network. Therefore, these clots become clear and fine while untreated Artiss clots have a turbid and smooth occurrence (figure 74).

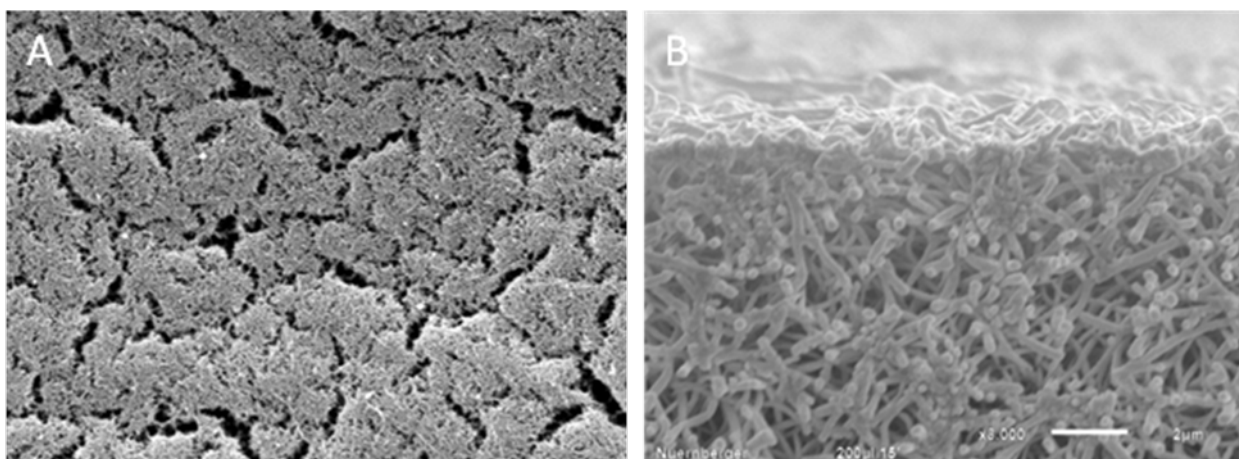


Figure 74 Scanning electron microscopy (SEM) of a salt clot (A) and of Artiss (B). Same magnification.



Figure 75 Photograph of fibrin clots in a 24 well plate (left: salt clot, right: Artiss)

In Figure 76, salts are added in increasing amounts to show possible intermediate steps between turbid and coarse clots.

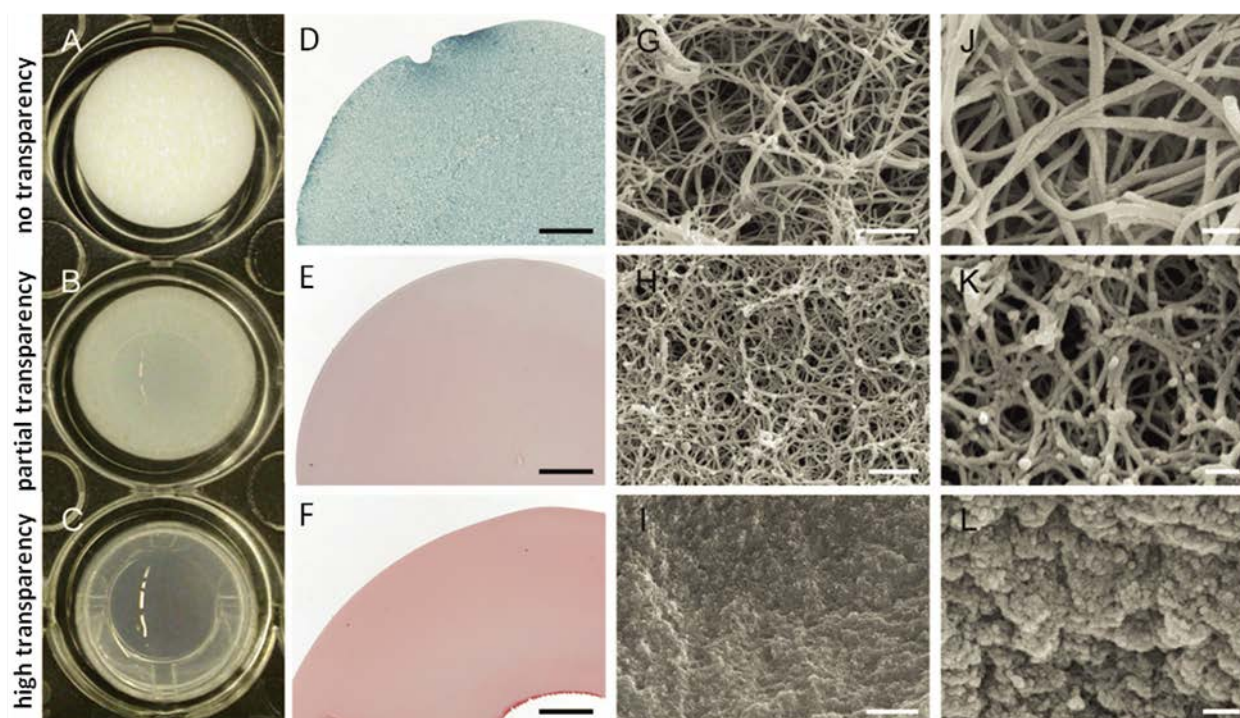


Figure 76 Fibrin clots polymerized (A, D, G, J) without, (B, E, H, K) in the presence of 150 nM NaCl and 75 nM arginine and (C, F, I, L) 200 nM NaCl and 100 nM arginine. Scale bars: (D–F) 1 mm; (G–I) 1 μ m; and (J–L) 0.5 μ m.

The clots show (fig. 76 A–C) a continuous transparency in macroscopic view, (D–F) a color shift from blue to red with MSB-staining and (G–L) decreasing fiber size until total homogeneity in SEM. The clots contain 50 mg/ml fibrinogen and 2 IU/ml thrombin.

5.2.1 MORPHOLOGY

The additional surplus salt in the salt clots can be removed by intense washing thus far that cells can grow as on physiological clots. Morphology of HUVEC after 24 hours was not affected and showed no deteriorated cytoskeleton on these kinds of clots.

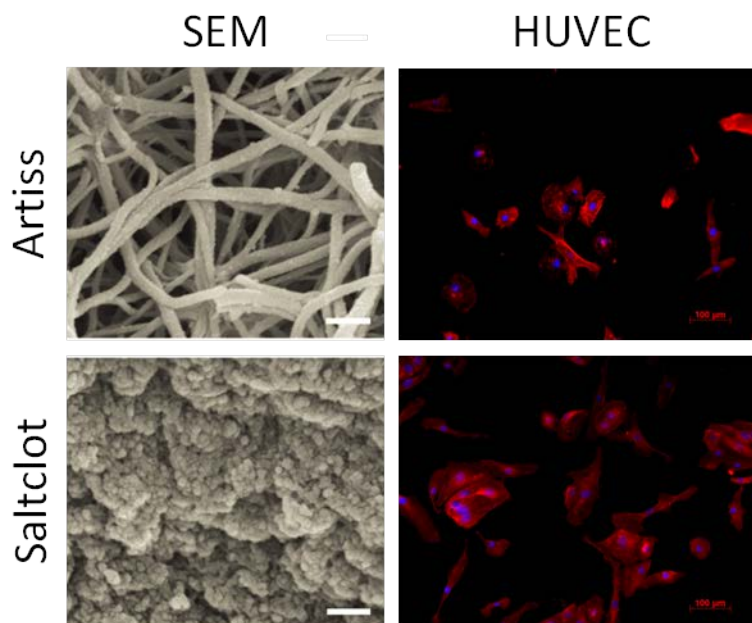
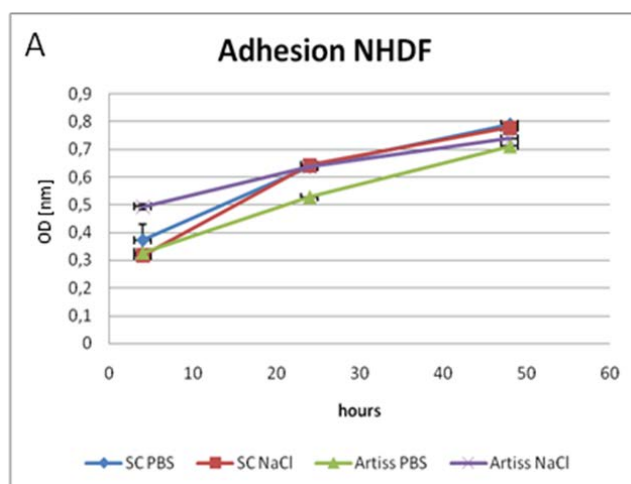


Figure 77 Fibrin clots polymerized without and in the presence of 200 nM NaCl and 100 nM arginine. Scale bars (SEM): 0.5 μm , (Phal/DAPI) 100 μm

Clots with higher salt concentration show a decreased fiber size compared with Artiss clots structure until total homogeneity in SEM (left). The morphology of HUVEC does not differ significantly after 24 hours (right)

5.2.2 CELL ADHESION

Adhesion (4 hours, 24 hours) and viability (48 hours) of NHDF, HUVEC, and NHEK on Artiss and salt clots were compared using an LDH assay.



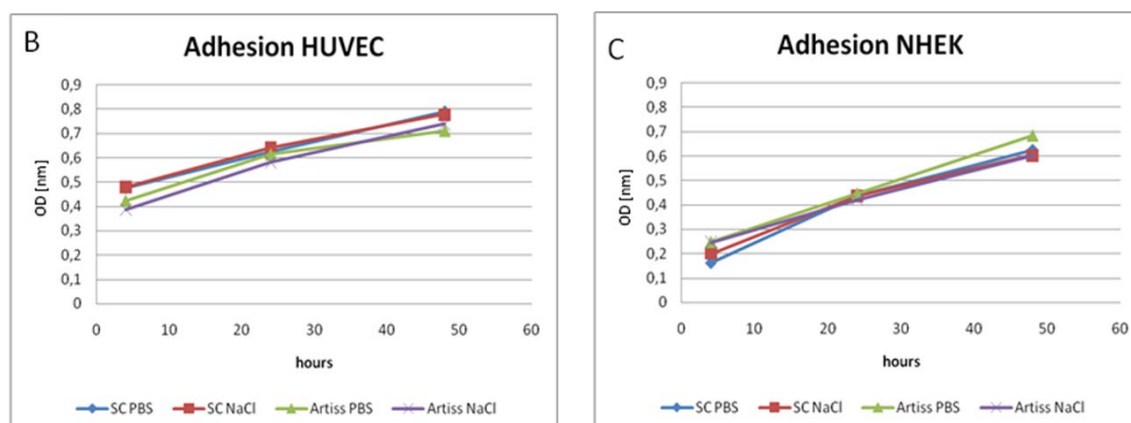


Figure 78 LDH Assay – Adhesion of NHDF (A), HUVEC (B) and NHEK (C). Cell number on fibrin clot after 4, 24, and 48 hours on salt clot (SC) and ARTISS either washed with PBS or extracted with NaCl. n=2

There were no significant differences between cell adhesion between washed salt clot (SC) and Artiss fibrin sealant clots both washed with PBS and extracted with 1 M NaCl followed by PBS washing steps.

5.3 FUCOIDANS

When fibrinogen gets in contact with thrombin fibrinopeptides are cleaved and the so resulting fibrin builds a network as described and shown above. But fibrinogen also polymerizes when getting in contact with other substances. Here, fucoidan from *A. nodosum* LMW was used to generate a clot. This fibrinogen/fucoidan mixture was clotted by adding calcium buffer (no thrombin, only calcium is necessary for building a clot).

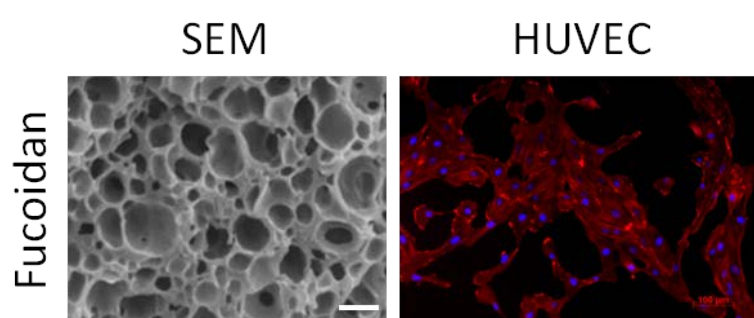


Figure 79 Ultrastructure of fibrinogen/fucoidan (*A. nodosum* LMW)/Ca²⁺ - biomaterial clot (left) and morphology of HUVEC (right) grown on it. Cell morphology was evaluated after staining with TRITC-Phalloidin (red) to visualize actin and DAPI to visualize cell nuclei (blue stain). Scale bars (SEM): 0.5 μ m, (Phal/DAPI) 100 μ m (n=2)

Under the scanning electron microscope an absolutely different structure was observed compared to a clot built with thrombin. On this structure, endothelial cells were seeded. After 24 hours, morphology was monitored by phalloidin/DAPI staining. We could not detect obvious differences compared to HUVEC cultivated on Artiss fibrin sealant clots.

5.3.1 CELL ADHESION

Adhesion (4 h, 24 h) of HUVEC and NHEK on Artiss and fibrinogen/fucoidan (A. nodosum LMW)/Ca²⁺ - biomaterial were compared using cell counting by an LDH assay.

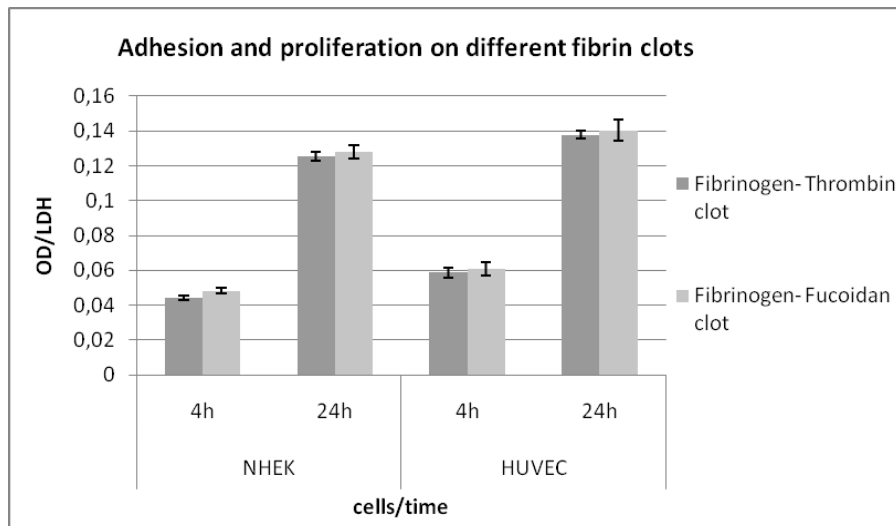


Figure 80 Viability of HUVEC on fibrin clots (Artiss) and fibrinogen/fucoidan (A. nodosum LMW)/Ca²⁺ - biomaterial: Clots were washed with PBS/aprotinin over night and 20,000 cells were seeded/clot. The relative cell number adhering to the clots were determined by measuring the amount of LDH of cell lysates after 4 and 24 hours (n=9).

There were no significant differences between Artiss and the fucoidan clots regarding cell adhesion and proliferation.

Discussion

Comparison of two fibrin sealants: Artiss vs. Evicel

The aim of this thesis was to assess the compatibility of fibrin sealants with different constituents and distinct concentrations of components with cells involved in skin tissue repair: keratinocytes, fibroblasts and endothelial cells (116). For tissue regeneration, the compatibility of fibrin sealants with relevant cell types will be of crucial importance for their application and performance *in vivo*.

In naturally occurring fibrin clots the fibrin fibers form a dense network spinning around erythrocytes, individual blood cells and remaining platelets. (117) The same fibrous substructure occurring in the coagulum *in vivo* can be found in fibrin materials derived from blood plasma such as commercially available fibrin sealants. There are several products available in the market and often used in surgery for years which differ in their compositions. Every fibrin sealant product is approved for its own indications, e.g. Tisseel and Evicel in the US for hemostasis in surgeries (see IFUs) or Artiss to adhere autologous skin grafts especially in burn wounds or facelift. But apart from its basic functionality of sealing or hemostasis, fibrin gels have further advantages: Due to its main components fibrinogen and thrombin and additional natural ingredient such as fibronectin, vitronectin and growth factors it provides a bio-functional environment with beneficial effects on the cell activities and tissue regeneration (118). It has already been used for various tissue engineering approaches including skin, cardiovascular, nerve, cartilage and ocular regeneration (119). Moreover, fibrin sealants are also used as matrix for cell or drug delivery (120).

Initially, we compared the fibrin sealants Artiss and Evicel concerning compatibility with keratinocytes. NHEK on Evicel showed less adhesion and a deteriorated morphology. These results conform to other published data (121). In this work we used hirudin, a direct thrombin inhibitor, to block thrombin which may get in contact with the cells. Because concentrations of thrombin differ very strong between these two fibrin sealants our hypothesis was that this can be a key player for observed results. These effects could be partly reversed with the use of hirudin and significantly more cells were detected on Evicel clots after 4 and 48 hours. Because of the blocking efficiency of hirudin of about 80 % (described by Weitz et al. (37) and affirmed by own experiments) matrix-bound thrombin partly remains active and therefore interacts with cells. Therefore, no full recovery could be expected.

Further, we could show that also apoptosis in keratinocytes was triggered when getting in contact with high thrombin concentrations. A live/dead stain showed more dead cells after 48 hours in Evicel compared with cells on Artiss and activity of caspase 3 and 7 – key proteins in apoptosis pathway (122) – was nearly threefold higher in cells grown on Evicel compared to Artiss fibrin sealant. Thrombin mainly acts as signaling molecule by direct binding to PAR-1 or PAR-3 expressed on the surface of keratinocytes (123). Therefore, PAR-1 was blocked by SCH797979 and also in this case, cell adhesion after four hours and viability of NHEK after 48 hours on Evicel were significantly better compared to cells without SCH797979 treatment. This also indicates – as the experiments with hirudin – that thrombin has an important influence on cell compatibility with the fibrin sealant clot.

To analyze if thrombin is also responsible for cell stress, keratinocytes were incubated with Artiss and Evicel clots for six hours and cell lysates were screened for stress markers with a protein array. Some relevant markers were upregulated upon incubation with Artiss and Evicel but there were no differences between thrombin samples and controls without thrombin indicating that cells were stressed because of mechanical exposure and not because of thrombin.

Our results impressively show clear differences in cell behavior and cell compatibility with low and high thrombin concentrations. But as fibrin sealant products do not only differ in thrombin concentration but also in fibrinogen composition with its various constituents effects cannot be lead back only to thrombin because of the interaction and interplay of hundreds of factors. Therefore, we used clots of the same fibrinogen component and used a high thrombin stock which was diluted to appropriate concentrations, A4, A505 and A820.

Different Concentrations of Thrombin

Based on the data observed with Artiss and Evicel we laid our main focus on the influence of the thrombin component of fibrin sealants.

Therefore, the aim of these studies was to evaluate whether thrombin activity present on the surface of Evicel fibrin sealant is responsible for the impaired cell attachment, proliferation and viability of NHEK, a pivotal cell type for regeneration of the skin (116;124) compared to Artiss clots. Since commercially available fibrin sealants often differ strongly in their composition, knowledge about the factors that influence cell behavior will help to select the appropriate products for tissue regeneration applications (125-127).

As a certain amount of thrombin remains unbound to fibrin upon clotting, this can be washed out easily by a washing step. As a consequence, the exactly amount of fibrin bound

thrombin exposed to cells is unknown. To measure the remaining thrombin concentration in the clot, the matrix bound thrombin concentration, clots of three different fibrin sealants (Artiss, Tisseel and Evicel) were investigated by two different methods: first, clots were lysed and the remaining thrombin was measured by Western blot analysis. This method delivered information about the thrombin concentration of the whole clot. But the more relevant question was how much thrombin affects the cells growing on top of the fibrin sealant clot. Therefore, matrix bound thrombin on the surface of the clot was measured by a thrombin activity assay (thrombin activity assay Sensolyte).

To ensure that thrombin is the main effector for observed results, fibrin sealant clots were prepared from a common fibrinogen component and combined with thrombin components formulated from a single starting material. This strategy ensured that thrombin concentration was the only variable. The selected thrombin concentrations represent the range offered in three commercially available fibrin sealants: Artiss, Tisseel and Evicel.

In addition to its other functions (128-131), thrombin has the capability to influence the three-dimensional clot structure in a certain concentration range (132-134). Moreover, it has been shown that alteration of fibrin structure has implications for wound healing (135;136). Our SEM data revealed no structural differences between fibrin sealant clots prepared with thrombin components ranging from 4 IU/ml to 820 IU/ml. Previous studies described differences in clot structure manifesting only at very low thrombin concentrations (0.001-1 IU/ml) (30;137). In addition to potential structural differences between high and low thrombin clots there is a tremendous variation of structure within one clot group too. Even within one clot areas could be detected which differ from each other. Some clots, especially clots with high thrombin concentration and therefore rapid polymerization time, were mixed inhomogeneously according to mechanical conditions. But apart from this structural variation, cell compatibility remained constant within the same clot group over all experiments indicating that structural changes do not have any significant effect on cell compatibility. Therefore, it is unlikely that the observed effects were caused by structural differences rather than thrombin content.

Our results demonstrate that the surface activity of thrombin on fibrin sealant clots correlates with the amount of thrombin used for clot formation. This is illustrated by an experiment showing the assembly of fluorescence labeled fibrinogen on preformed fibrin clots, and its inhibition by hirudin. All clots treated with 10 IU hirudin showed the same low surface thrombin activity as untreated low thrombin clots. Low thrombin clots remained unchanged upon blocking as the inhibitory effect on the thrombin activity is smaller than the assay variation. The residual fluorescence of 30-40% measured on low thrombin and hirudin blocked clots seems to be due to unspecific assembly of labeled fibrinogen.

We hypothesized that cell signaling events triggered by fibrin bound thrombin are responsible for the observed differences in cell compatibility. Depending on the thrombin concentration, the interaction of NHEK with fibrin sealant clots was either supported or inhibited. When seeded on top of clots prepared with 820 IU/ml thrombin NHEK showed decreased attachment, decreased proliferation, deteriorated morphology, and an increased number of dead cells. These results are inconsistent with previous studies showing that cellular responses to thrombin are increased proliferation, collagen synthesis, migration, and induction of angiogenesis (138;139). Interestingly, in contrast to median thrombin clots, the cell number on high thrombin clots seems to increase significantly between 4 hours and 24 hours - this might be due to delayed attachment caused by the high thrombin concentrations (820 IU/ml) and not due to proliferation as thrombin was described to modulate cell adhesion to fibrin clots dose dependently (121;140;141). Furthermore, similar results were published with NHEK on Evicel fibrin clots (121).

However, thrombin has diverse effects dependent on the concentration and cell type (142). The threshold concentration of thrombin which results in negative effects is often dependent on cell type (129;130;143).

The conflicting effects of thrombin may be related to the receptors that are involved in signal transduction. Thrombin acts as signaling molecule by direct binding to PAR-1 or PAR-3 which are expressed on the surface of many cell types, including keratinocytes (123). Thrombin bound to a fibrin clot is able to activate PAR-1 and to induce cell proliferation (144). This response is thought to be mediated through mitogen-activated protein kinase (MAPK) proteins: an increase in phosphorylation of the MAPK proteins p38 and ERK-2 following activated protein C treatment could be observed. (49) Furthermore, a dose-responsive decrease in keratinocyte proliferation could be shown when PAR-1 antibody is applied (to block the receptor). (50) On the other hand, PAR-1 activation has also been shown to increase epithelial cell apoptosis and intestinal permeability in a caspase 3 dependent manner. (145) Signals induced by high doses of thrombin (above 100 pM) seem to be transduced mainly by PAR-1, and are accompanied by pro-inflammatory events. (146-148)

The given data clearly indicate that the amount of fibrin bound thrombin on fibrin sealant clots prepared with a thrombin concentration of 820 IU/ml negatively influences the fate of NHEK. The observed increased number of dead cells up to 11 % gave rise to the speculation that high amounts of fibrin bound thrombin induce apoptosis. Whereas about 3 % dead cells present on low thrombin clots are in accordance with an average viability of NHEK cells in cell culture (data not shown). Further supporting this hypothesis, the activity of apoptosis markers caspase 3 and 7 was increased on high thrombin clots. Both molecules are situated

at pivotal junctions in apoptotic signaling pathways. To confirm this result and gain more insight into thrombin triggered signaling, apoptosis array analysis was performed (data not shown). One of the regulated proteins identified by this approach was Trail-R2 (TNF-related apoptosis-inducing ligand receptor 2). Trail-R2 expression was elevated in NHEK cultured on clots prepared with 505 or 820 IU/ml thrombin as compared to clots prepared with 4 IU/ml. Activated Trail-R2 signals through caspase 8 to mediate apoptosis (149;150). Simoncini et al. (151) have shown that TRAIL, the ligand of Trail-R2 is a target of thrombin and its expression is highly increased upon thrombin stimulation in endothelial cells thus confirming a direct linkage between thrombin and Trail-R2 signaling. Taken together, these data are consistent with the observed induction of caspase 3 activity and support the finding that high amounts of fibrin bound thrombin induces apoptosis in NHEK.

To further verify that the observed effects are thrombin dependent hirudin was used to block thrombin induced signaling. Hirudin is a physiologic irreversible inhibitor of soluble and fibrin bound thrombin. Blocking of fibrin bound thrombin by hirudin reduced caspase 3 and 7 activity levels of cells cultured on clots prepared with 820 IU/ml thrombin nearly to the level seen on clots prepared with 4 IU/ml thrombin, supporting the hypothesis that thrombin is responsible for the upregulation of these apoptosis indicators. Caspase activity was also tested at other time points. A similar activity pattern at 7 hours, but no up regulation was detected at 30 hours (data not shown). Furthermore, cell number was increased on fibrin sealant clots incubated with hirudin and assessment of the morphology showed an improved morphology of NHEK upon hirudin blocking of high thrombin fibrin clots (data not shown). We assume that the ameliorated cell compatibility is a direct consequence of blocking thrombin dependent detrimental signaling pathways, since hirudin itself was described to have no impact on cell morphology (152). However, capability to support cell proliferation was only partly restored by hirudin blocking. This could be due to the fact that hirudin inhibits fibrin bound thrombin only with an efficacy of 70-80 % (37).

This study evaluated whether thrombin activity present on the surface of fibrin sealant clots influences the compatibility of sealants with NHEK, a pivotal cell type for regeneration of the skin (116;124). NHEK seeded on fibrin clots made with 820 IU/ml thrombin showed decreased attachment, decreased proliferation, deteriorated morphology and an increased number of dead cells. On the other hand, clots prepared with low thrombin concentrations (4 IU/ml) support NHEK attachment and cell spreading. All experiments performed with medium thrombin clots (505 IU/ml) confirmed a strong thrombin dose dependency as the values for attachment, proliferation and caspase activity were found to lay between the values of low and high thrombin clots.

Rat excisional wound healing model – thrombin dependent wound healing

Additional to their haemostatic function, fibrin clots in wounds serve as temporal scaffolds for cells involved in wound healing. After the initial activity of neutrophils, macrophages and lymphocytes, fibroblasts, keratinocytes and endothelial cells prepare and conduct tissue reformation (153;154).

As mentioned above, fibrin sealants are often used as matrix for cell or drug delivery (120), especially in research. The next step after generation of our in vitro results was to evaluate our data in an in vivo wound healing model. We chose the rat excisional wound healing model because rats provide an excellent model for skin wound healing by allowing the standardization of the type, size, shape, and depth of the wound injury. (78) The disadvantage of this animal model is that rats possess the subcutaneous panniculus carnosus muscle which contracts the wound to achieve better wound healing.

We compared the wound healing efficacy of Artiss and Evicel as we did primarily in vitro as described above. Like in our other in vitro experiments, Artiss with a thrombin concentration like Evicel (820 IU/ml), named A820 and – vice versa – Evicel with a low thrombin concentration (4 IU/ml) were applied. On the one hand, Evicel fibrinogen component was mixed with the Artiss thrombin (named E4Baxter) and on the other hand, the Evicel thrombin was diluted to a concentration of 4 IU/ml with an appropriate buffer (named E4). For this reason we could ensure, that salt and protein concentration (except thrombin) were the same as in the original Evicel product. At last, fibrinogen variants FIB3 and FIB3 spiked with fibronectin were applied with 4 IU/ml thrombin.

To measure wound closure, wound area was sized on day 0 (baseline), day 3 and day 7. Wound area of Evicel on day 3 seemed to be by trend smaller than on day 7 that could be due to initial stronger contraction of wounds in rats. However, it was statistically not significant.

Surprisingly E4 showed nearly no wound closure after three days. One explanation could be that the clot is very stiff like an original Evicel clot, the panniculus carnosus is too weak to minimize the wound physically and there is less wound closure in the beginning due to delayed degradation of the clot. Evicel had in the beginning a faster wound closure than E4 because a high thrombin concentration cleaves PAR-1 with high catalytic efficiency, followed by pro-inflammatory and pro-apoptotic effects (155) contributing to a faster clot degradation. According to this, E4 with its low thrombin concentration lead to less inflammation reflected by the much lower expression of IL-1 α compared to Evicel on day 2. Both sealant groups are reduced in plasminogen and as a consequence fibrinolysis was decelerated and other groups showed a faster wound closure.

For histomorphometric analyses, cross sections of the wounds were H/E stained and following data of the wounds were measured: the distance between the epithelial borders of the wound (last keratinocyte at the wound edge), the length of re-epithelialization zone, the deepest point of the wound, the thickness of the connective tissue (new dermis/granulation tissue), and the thickness of the natural dermis. With these benchmark data, several indices were calculated to characterize the wound. Contraction was differentiated in superficial (SCI) and deep contraction (DCI) which makes sense because of the mentioned wound closure via panniculus carnosus. Results were not significant but by trend, Artiss and E4Baxter showed more contraction compared with the other groups. This correlates with the reduction of wound area. The planimetric analyses also showed less wound closure at Evicel and E4. This could also be a result of higher clot stiffness that spreads the wound open.

The indices of severity and wound healing did not differ significantly. Only the trend was visible that Artiss and E4Baxter had a better wound healing compared to the other groups.

Also different fibronectin concentrations in fibrin sealants did not show any clear trend. This was in contrast to our expectations as our in vitro data indicated that fibrinogen with fibronectin performed better because of better cell adhesion and more proliferation of keratinocytes.

Differences between E4Baxter and E4 in the in vivo model were not caused by thrombin concentration or ECM molecules (the fibrinogen components are the same) but by the albumin concentration of the thrombin buffer (0.6 % in E4 compared to 5 % in E4Baxter). Albumin – as some other plasma proteins - is known to have an impact on the kinetics of fibrin fiber growth and therefore on the fibrin clot structure. (156)

An important step in wound healing is vascularization of the newly formed tissue to ensure its supply of nutrition and oxygen. Therefore, endothelial cells invade into the scar tissue and form vessels. To analyze this vessel building cross sections of the wound were stained immuno-histochemically for vWF to image vessels in general and for SMA to make visible more differentiated, functional vessels.

vWF was used for vessel staining although this staining allows only limited predication as it is too unspecific. Also other cells can be vWF-positive: vWF is present in plasma, in the subendothelial matrix of the vessel wall, in the Weibel Pallade bodies of endothelial cells and in the alpha-granule in megakaryocytes and platelets derived from them (157). This could be the reason, that there were any significant differences observed in vWF-staining between the groups. CD31 would be the adequate staining therefore but no antibody for CD31 in

rodents is available. Concerning the SMA-staining, there were no differences neither between the fibrin sealant groups but by trend, all treated groups had more vessel formation compared with the mock treated group without fibrin sealant. Whereas groups with different thrombin concentration perform equally, there was a trend visible for fibronectin: groups treated with FIB3+Fn built more functional vessels compared to FIB3 alone. This fits to the published statements that fibronectin is important for migration of endothelial cell and fibroblasts as it also leads to chemotaxis. (158)

In phase two of wound healing, proteins like VEGF and angiopoietin are secreted by cells to trigger angiogenesis. (159) To further examine vessel formation, these markers for vascularization were analyzed by Western blot analysis. In wounds treated with Artiss fibrin sealant a significant higher amount of VEGF could be detected compared to Evicel or mock treated wounds. Concerning Ang-1 no differences were found. But every wound had a significant lower expression of these two markers compared to healthy tissue.

As inflammation is the first phase of wound healing (159) biopsies were taken on day 7 and analyzed on a protein array to screen inflammation markers. No marker protein could be detected. Therefore in another experiment biopsies of only Artiss and Evicel treated rats were taken on day two because the main effectors of inflammation are expressed between day one and four after injury (160). One of the most important effectors in this phase is the interleukin family, especially interleukin-1 (159). Western blot analyses showed a significant higher expression of IL-1 in Evicel treated wounds compared to Artiss or mock treated wounds or to healthy tissue. As already discussed above, a high thrombin concentration in fibrin sealants results in lower cell compatibility and induces apoptosis in keratinocytes. Moreover, comparing Artiss and Evicel, pathological observations found a higher number of polymorphonuclear leukocytes and foreign-body giant cells, cells typical for inflammation. This could be a reason for higher inflammation marker IL-1 (or vice versa).

Foremost, a fibrin sealant clot stops bleeding and closes the wound. Further, it serves as provisional matrix for cells. But simultaneously, the clot has to be degraded to make place for the granulation tissue. In vivo, fibrinogen clots are degraded in the course of tissue regeneration mainly driven via the plasminogen pathway. It is converted into its enzymatically active form, plasmin, by one of two types of plasminogen activators, the tissue-type or urokinase-type plasminogen activator; tPA and u-PA. Apart from plasminogen, fibrinolysis can also be realized by metalloproteinases (161;162). Both mechanisms are partially mediated by cells, which either synthesize the components or serve as reaction

surface. In addition to that cells remove fibrin by phagocytosis and enzymatic digestion. However, fibrin also degrades in vitro without the presence of any cells. The reason is that both, the precursor of the enzyme (plasminogen) and one of its activators (t-PA) bind fibrin, and are co-isolated with the fibrinogen component and part of the fibrin clot.

To analyze fibrinolysis in vivo, fluorescence stained fibrin sealant Artiss and Evicel were applied on excisional wounds in rats. Degradation was measured over time and after four days Artiss clots were significantly smaller than Evicel clots and completely degraded after about ten days whereas Evicel clots could be detected until day 15. As mentioned above, tPA is the main player in fibrinolysis in vivo. In Evicel fibrin sealants, the plasminogen amount is reduced to enhance fibrin degradation which prolongs the shelf life of the product. As a consequence also natural fibrinolysis is prolonged. Here rises the question if faster – more physiological? – degradation is desirable to afford faster building of granulation building and scar tissue.

ECM molecules in fibrinogen component – Fibronectin as example

Adequate adhesion is the first critical event in cell-matrix interactions in the natural fibrin clots of wounds as well as fibrin sealants and gels and depends on both counterparts: the cells and the matrix. Adhesion involves several types of receptors on the cell membrane (cell adhesion molecules, CAMs) and different ligands on the fibrin matrix. The main ligand is the fibrin(ogen) molecule, which provides specific binding sites on the fibrin(ogen) polypeptide chains α , β and γ . Most of the fibrin(ogen) binding cell receptors belong to the class of integrins. One or several integrin types are in turn represented on most cell types. The most important fibrinogen receptor on fibroblasts and endothelial cells is the $\alpha v \beta 3$ integrin (163;164).

Receptors of other classes binding to fibrinogen include ICAM-1 on fibroblast membranes or VE-cadherin on endothelial cells. Therefore, also other ECM molecules play a very important role in cell adhesion and moreover in cell viability on clots. Because most fibrin sealants are plasma derived also ECM molecules are present in different concentrations due to different production processes (as indicated in the IFUs).

In order to decrease the amount of soluble and non-covalently bound proteins, such as human serum albumin, fibrin clots were washed with several extraction buffers. 1 M sodium chloride solution containing acetonitrile and trifluoroacetic acid reduced the amount of fibrin bound thrombin in Tisseel. Interestingly, fibronectin was also present in the supernatants indicating that crosslinking of fibronectin to fibrin was not complete.

However, clot extractions had no strong influence on adhesion and viability of NHDF but significant differences could be observed with NHEK. After 24 hours a lower number of keratinocytes was detected on these clots. Thus, we conclude that removed ECM molecules do play a role in matrix-keratinocyte interaction. Maybe the lower albumin concentration influences the more “fragile” and pretentious NHEK compared to the robust fibroblasts. But also less fibronectin could be the reason for these results because NHEK are not able to bind directly to fibrinogen via integrin $\alpha v \beta 3$ (165). Thus, interaction of keratinocytes with fibrin depends on additional extracellular matrix proteins which are physiological components of the fibrin clot. One very important protein which is crucial for adhesion and growth of cells on an extracellular matrix is fibronectin (166).

To demonstrate the impact of fibronectin, we tried to minimize the content in fibrin sealants by precipitations with glycine. But only 30% of fibronectin could be depleted. This was too ineffective because this depletion could not be translated into any significant differences in cell behavior compared to untreated clots with normal fibronectin concentration. Therefore, FIB3 was used: it is a human fibrinogen depleted of plasminogen, von Willebrand factor and fibronectin (Enzyme Research Laboratories). Western blot analysis showed that even in FIB3 fibronectin was not completely absent but its concentration was reduced to a small percentage (~7 %) compared to the normal fibrin sealant Artiss. However, NHEK showed less attachment and a deteriorated morphology in FIB3 clots but the detected residual amount of fibronectin could be the reason, why NHEK were still able to attach to fibrin. When FIB3 clots were spiked with fibronectin, spreading of keratinocytes was significantly improved after 24 hours due to the presence of fibronectin.

Another explanation for attachment of keratinocytes to fibrin clots probably is that both the sealant and the FIB3 are plasma products with unknown or not enough identified and characterized components that also could be responsible for adhesion (vitronectin, elastin, collagen, hyaluronic acid, ...).

To demonstrate again the necessity of fibronectin for NHEK attachment without interference of other extra-cellular matrix factors, 96-well bacteria plates were coated with pure proteins: fibrinogen, fibronectin and a mixture of both. Here we did not see an attachment of NHEK on fibrinogen but on fibronectin. Whereas, fibronectin seems to be important for early adhesion (2 hours), fibrinogen with fibronectin seems to allow long term adhesion and proliferation (24 hours). Here it is noteworthy to point out that this approach is a 2D model and lacks of possibly complex interactions with fibrin fibrils.

In all experiments we have performed, HUVEC did not show any significant differences between clots with or without fibronectin because of the available integrin receptor $\alpha v \beta 3$ for fibrin.

FIB3 is fibrinogen depleted not only of fibronectin but also of plasminogen. The fibrin sealant Evicel has a reduced amount of plasminogen in order to elongate its shelf life (about 2-3 $\mu\text{g/ml}$) as plasminogen is the precursor of the serine protease plasmin which is involved in fibrinolysis. Plasminogen k.o. mice displayed severe wound healing defects, probably due to an inhibition of matrix degradation and cell migration. (167) No significant differences in cell adhesion could be shown when Evicel was spiked with plasminogen to reach a concentration of another fibrin sealant. Plasminogen may not be responsible for poorer cell compatibility of Evicel.

Clot structure

Despite the generally fibrous nature of polymerized fibrin, the structure can have a wide range of possible morphological properties such as fiber size, density and homogeneity. The morphology of the fiber network causes the mechanical properties of the clots such as stiffness, tensile strength, clotting time and even the color ("coarseness"). Both, morphological and mechanical parameters have an impact on the behavior of cells in fibrin (168) as already described in this work earlier on.

Those parameters affecting the clot morphology and mechanical properties of a clot are: the fibrin formulation (quantity and ratio between fibrinogen and thrombin and additional ingredients as salts or albumin), the nature of fibrinogen, the pH and the temperature (169-171).

Generally, fibrinogen determines the density and stiffness of the clot in a direct correlation implying that more fibrinogen per volume results in a denser clot with decreased porosity and permeation (172;173). Influence on the fibrin fine structure may further result from different natural sub-variants of the $A\alpha$ $B\beta$ or γ double chains of fibrinogen where either one pair or one single chain is partially altered. The biological reasons for the variations are genetic polymorphisms, alternative splicing, posttranslational modifications or proteolytic degradation (169).

But fibrin clots are also influenced by a number of additional substances, which may be present at the time of polymerization: for instance high concentration of salts which lead to partial or entire transparency of the clots in contrast to the "opaque" appearance of untreated Artiss clots. This clearing effect has primarily been reported by Ferry and Morrison (174). By means of spectrometry they measured the turbidity and suggested, that with increasing salt concentration and transparency the clots became increasingly homogenous (termed "fine" versus "coarse" or "turbid"). Previous studies reported that the effect is restricted to divalent cations (175). More recently, though, the mechanism causing the

structural difference was described as specific effect of Cl^- which opposes the lateral aggregation of protofibrils (176). The structural changes cause further alterations of the mechanical properties.

In figure 73, there are very small fibrin fibers embedded between the large fibers, concluding that high fibrinogen concentrations do not only provoke the increase of fiber size but rather the increase of the range of fiber sizes. Combination of two classes of fiber size in one clot supports one of two dynamic models of polymerization and fiber network formation (114;115). This model says that an initial fiber network is constructed in a first period. In a second period those fibers are enlarged by polymerization and, in addition, new fibers are formed in between until clotting is complete. Therefore, lateral and axial polymerization, proceed simultaneously until clotting is complete. According to this model, large fibers in figure 59 correspond to the initial polymerized ones which increase in size during the whole clotting process. The small fibers represent the “delayed” network forming after the gel point. In contrast, in the second model, network construction is completed in an initial period and afterwards fibers become only enlarged by axial polymerization. (114;115)

The structure of the matrix always is a big discussion between scientists regarding its importance for matrix-cell-interactions. We tested endothelial cells and keratinocytes on three very different structures: Artiss with its typically woven fibers, Artiss with higher concentrations of NaCl and arginine with a very dense fiber network and at least fibrinogen which was polymerized with fucoidans. This treatment resulted in a porous structure. No significant differences could be observed neither in cell adhesion and proliferation nor in cell morphology. Apparently, structure is not a key factor as long as the surface generally is adequate for cells. Other factors such as thrombin, ECM molecules or growth factors have been demonstrated to have much more impact on cell compatibility of fibrin sealant clots.

The aim of this thesis was to further understand the principles and pathways of cell-matrix interactions. A wide range of experiments have been performed to characterize fibrin sealants. Due to a very complex composition of these plasma derived products it is difficult to point out the most important proteins and constituents which are needed for cell compatibility.

For the first time we could show that high concentrations of thrombin can induce apoptosis in normal human endothelial keratinocytes – an important factor especially for cell delivery applications. And as shown in our in vivo results thrombin has to be kept in mind when hemostats with typical high thrombin concentrations are used for wound healing assistance.

Fibronectin is an important ECM molecule for cell-matrix interaction especially with keratinocytes. This could be shown in our experiments where we depleted fibronectin concentration in fibrin sealants. But also other ECM molecules like laminin or elastin, or other proteins like hyaluronic acid or collagen play an important role in cell compatibility and wound healing and need to be further examined. However, structure of fibrin clots does not seem to play such an important role, anyway compared to substances of content.

The results of this project may lead to create a further improved fibrin matrix which activates and promotes tissue regeneration but a lot of research still needs to be done.

Abbreviations

Ang-1	angiopoietin-1
APC	active protein C
CAM	cell adhesion molecule
Co	control
DAPI	4',6-diamidino-2-phenylindole
DB	dilution buffer
DMSO	dimethyl sulfoxide
DTI	direct thrombin inhibitor
ECM	extra-cellular matrix
EGF	epithelial growth factor
ERK-2	extracellular regulated kinase-2
FDA	(US) Food and Drug Administration
Fg	fibrinogen
Fn	fibronectin
FpA (B)	fibrinopeptid A (B)
FS	fibrin sealant
FXIII	Factor XIII
HUVEC	human umbilical vein endothelial cells
IFU	instructions for use
IGF	insulin-like growth factor
IL-1	interleukin-1
IU	international units

LB	lysis buffer
LDH	lactate-dehydrogenase
LMW/HMW	low/high molecular weight
MAPK	mitogen-activated protein kinase
MMP	matrix metalloprotease
NHDF	normal human dermal fibroblasts
NHEK	normal human endothelial keratinocytes
OD	optical density
PAR (-1, -2, -3)	protease-activated receptor (-1, -2, -3)
PDGF	platelet derived growth factor
RFU	relative fluorescence units
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SMA	smooth muscle actin
TGF	tissue growth factor
TNF	tissue necrosis factor
tPA	tissue plasminogen activator
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	transferase nick-end-labeling
uPA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
WFI	water for injection

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Curriculum Vitae

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Work experience

Dates	01.09.2008 – 31.01.2012
Occupation or position held	PhD-Thesis
Main activities and responsibilities	Planning and realization of <i>in vitro</i> and <i>in vivo</i> experiments in tissue engineering and regeneration under supervision of a scientific co-operator. Cell culture (cultivation of primary human skin cells, various cell based assays for viability, proliferation, fibrin migration,...) Molecular biological techniques (Western Blots, Proteome Arrays, IHC, ICC, immuno-fluorescence staining and microscopy, FACS analysis, ELISAs) Statistical analysis (Seminar: 60 basic lectures) <i>in vivo</i> experiments (planning, implementation and evaluation of a rat wound healing model) Experimental planning and creation of standard protocols within GLP environment authoring internal reviews and publications, presentation of data on international conferences
Name and address of employer	Baxter Innovations GmbH, Industriestraße 67, A-1221 Vienna in cooperation with the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Donaueschingenstraße 13, A-1200 Vienna
Type of business or sector	Baxter: BioSurgery (Dr. H. Gulle), Group Cell Biology (Dr. M. Bittner, Dr. W. Pasteiner) LBI: Tissue Regeneration, Wound Healing and Imaging (Prof. Dr. H. Redl, Dr. S. Wolbank)
Dates	02.01.2007 – 31.03.2008
Occupation or position held	Diploma Thesis "Characterization of the potential Tumour Suppressor Gene N33 (TUSC3) in Ovarian Cancer"
Main activities and	Cell culture (cultivation of human cancer cell lines, transfection, varied cell-based

responsibilities	assays)
Name and address of employer	Molecular biological techniques (Western blot, Immunohistochemistry, qRT-PCR, plasmid preparation, RNA-, DNA-, and protein purification, FACS) Univ. Clinic of Internal Medicine I, Währinger Gürtel 18-20, A-1090 Vienna
Type of business or sector	Department of Oncology (Prof. Dr. M. Krainer)
Dates	11.07.2006 – 11.08.2006
Occupation or position held	Individual Laboratory
Main activities and responsibilities	serological methods such as isolating thrombocytes from whole blood, HPA typing, analysis of function of thrombocytes with Diamed Impact, Dade Behring and flowcytometry implementation of molecular biological techniques such as ELISA, PCR practical and theoretical training of another trainee
Name and address of employer	Univ. Clinic of Blood Serology and transfusion medicine, Währinger Gürtel 18-20, A-1090 Vienna
Type of business or sector	Clinical Department of Blood Serology (Prof. Dr. S. Panzer)

Education

Dates	since September 2008
Title of qualification awarded	“Dr. rer. nat.” (PhD)
Principal subjects	Study of Biology, branch of study: Anthropology
Name and type of organisation providing education	University of Vienna, Dr.-Karl-Lueger-Ring 1, A-1010 Vienna, Austria
Level in international classification	ISCED-Level 6
Dates	September 2001 – June 2008
Title of qualification awarded	“Mag. rer. nat.” (MSc)
Principal subjects	Study of Biology, branch of study: Anthropology
Name and type of organisation providing education	University of Vienna, Dr.-Karl-Lueger-Ring 1, A-1010 Vienna, Austria
Level in international classification	ISCED-Level 5
Dates	September 1992 – June 2000
Title of qualification awarded	“Reifeprüfung” (general qualification for university entrance, school-leaving examination)
Principal subjects	humanistic grammar school
Name and type of organisation providing education	Gymnasium Sachsenbrunn, private Grammar School of the Archdiocese Vienna, A-2880 Kirchberg/Wechsel
Level in international classification	ISCED-Level 3A

Mother tongue	German
Other languages	English Level: Advanced Business English, C1 (according to CEFR) French Level: elementary; A2 (according to CEFR) Latin Level of school leaving examination
Social skills and competences	Stress resistant, team oriented, self-reliant, motivated to learn and achieve, willing to bear responsibility
Organisational skills and competences	Scientific Writing, Presentation Techniques, fast perception of tasks, Planning and realization of <i>in vitro</i> and <i>in vivo</i> experiments within GLP environment
Technical skills and competences	Cell culture, sterile working, cell based assays, molecular biological techniques, histology, statistical analysis, animal handling (rats)
Computer skills and competences	Microsoft Office 2007, SPSS, MiniTab, MindManager, Reference Manager, Adobe Photoshop and Illustrator, MSPProject
Driving licence	classes A, B
Additional information	Military service fulfilled (04.09.2000 to 04.03.2001)
Publications	Gugerell A, Schossleitner K, Wolbank S, Nuernberger S, Redl H, Gulle H, Goppelt A, Bittner M, Pasteiner W. High thrombin concentrations in fibrin sealants induce apoptosis in human keratinocytes. J Biomed Mater Res Part A 2012 (accepted). Nuernberger S, Meinel A, Gugerell A, Redl H. The Components of Fibrin Gels and their Influence on Morphology and Function. In von Byern J, Grunwald I (editor). Biological Adhesive Systems. From Nature to Technical and Medical Application. Springer Vienna 2010 November; 1st edition. Nuernberger S, Gugerell A, Wolbank S, Bittner M, Pasteiner W, Redl H. General Characterization of Cell Culture on and in Fibrin. In von Byern J, Grunwald I (editor). Biological Adhesive Systems. From Nature to Technical and Medical Application. Springer Vienna 2010 November; 1st edition. Macasev D, DiOrio JP, Gugerell A, Goppelt A, Gulle H, Bittner M. Cell Compatibility of Fibrin Sealants: In Vitro Study with Cells Involved in Soft Tissue Repair. J Biomater Appl. 2011 Aug;26(2):129-49. Pils D, Wittinger M, Petz M, Gugerell A, Gregor W, Alfanz A, et al. BAMBI is overexpressed in ovarian cancer and co-translocates with Smads into the nucleus upon TGF-beta treatment. Gynecol Oncol 2010 May;117(2):189-97.

- Conferences
- TERMIS-EU 2011 (Granada, Spain): Oral presentation: „Thrombin activity in fibrin sealants –a critical factor for cell compatibility with human keratinocytes”
4. Wiener Biomaterialsymposium (Vienna, Austria): Poster: “„Compatibility of fibrin sealants with primary keratinocytes is influenced by fibrin bound thrombin” (first author and presenter)
- TERMIS-EU 2010 (Galway, Ireland): Poster: „Compatibility of fibrin sealants with primary keratinocytes is influenced by fibrin bound thrombin” (first author and presenter)
- ESB 2009 (Lausanne, Switzerland): Poster: “The Effect of Alterations of Fibrin Composition on Cell Adhesion and Viability” (first author and presenter)
- Poster: “Release of VEGF from a fibrin bio-matrix and its influence on ischemia reperfusion outcome” (Co-author)