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DISSERTATION

„Modification of fibrin to improve applications in regenerative medicine“

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Contents

List of Abbreviations

A		Adenosin
AA		Acrylamide
aa		Amino acid
ACL		Anterior cruciate ligament
as		Antisense
ASC		Adipose-derived stem cell
αSMA	**α-SMA**	alpha smooth muscle actin
Bis		N,N-Methylenbisacrylamide
BMP-2		Bone morphogenic protein-2
BMPR-1a		Bone morphogenic protein receptor-1a
bp		Base pairs
BrdU		5-bromo-2-deoxyuridine
BSA		Bovine serum albumin
BW		Body weight
βactin	**β-actin**	Beta actin
C		Cytosine
CCD		Charged-coupled device
cDNA		Complementary DNA
CFL		Cofilin
Col III		Collagen III
Col IIα1		Collagen II alpha1
Col Iα1		Collagen I alpha1
Col Iα2		Collagen I alpha2
Col XII		Collagen XII
COMP		Cartilage oligomeric matrix protein
CTO		Cell tracker orange
Des		Desmin
DMEM		Dulbecco's Modified Eagle Medium
DMSO		Dimethylsulfoxide
DNA		Deoyribonucleic acid
dNTP		2´-deoxynukleoside-5´-triphosphate
DTT		1,4-Dithiothreitol
E. coli		*Escherichia coli*
ECM		Extracellular matrix
EDC		1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA		Ethylenediaminetetraacetic acid
ELISA		Enzyme-linked immunosorbent assay
EYFP		Enhanced yellow fluorescent protein
fbg		Fibrinogen
FCS		Fetal Calf Serum

FGF		Fibroblast growth factor
FGF-1	**aFGF**	Acidic fibroblast growth factor
FGF-2	**bFGF**	Basic fibroblast growth factor
FGFR		Fibroblast growth factor receptor
FPA		Fibrinopeptide A
FPB		Fibrinopeptide B
FS		Fibrin sealant
fXIII		Factor XIII
G		Guanin
GAPDH		Glyceraldehydes-3-phosphate dehydrogenase
GFP		Green fluorescent protein
GRF		Gelatin-resorcinol-formaldehyde
GST		Glutathione S-transferase
h		Hour
H&E		Hematoxylin/eosin
HAM		Human amnion-derived mesenchymal stem cell
HFP		1,1,1,3,3,3-hexafluoro-2-propanol
His		Histidine
HMW		High molecular weight
IL-1		Interleukin-1
IL-10		Interleukin-10
IPTG		Isopropyl β-D-1-thiogalactopyranoside
IU		International unit
kb		Kilo base pairs
kDa		Kilo Dalton
LB-medium		Luria Broth medium
M		Marker
M		Molar
M.S.B.		Martius/scarlet/blue
mg		Milli gram
ml		Milli liter
mM		Milli molar
Mono		Monoclonal
mRNA		Messenger ribonucleic acid
MSC		Mesenchymal stem cell
MTT		*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*
µg		Micro gram
µl		Micro liter
NaCl		Sodium chloride
ng		Nano gram
NHS		N-hydroxysuccinimide
Ni-NTA		Nickel-nitrilotriacetic acid
nm		Nano meter

OC	Osteocalcin
OD_{600}	Optical density at 600 nm
OP	Osteopontin
OPD	O-Phenylenediaminehydrochlorid
P	Pellet
PAGE	Polyacryl gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pep	peptide
PGLA	Poly-(DL-lactic-co-glycolic acid)
pH	potentia Hydrogenii
pI	Isoelectric point
pmol	Pico mol
POX	Peroxidase
PPACK	D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone
rpm	Rounds per minutes
RT	Room temperature
s	Sense
S	Supernatant
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
sec	Seconds
SEM	Scanning electron microscopy
STD	Standard deviation
T	Thymine
t-AMCA	Tranexamic acid
TBE	Tris boric acid EDTA
TEMED	N,N,N’,N’-Tetramethylethylenediamine
TNF	tumor necrosis factor
TNS2	Tensin2
Tris	2-Amino-2-(hydroxymethyl)-propan- 1,3-diol
Trx	Thioredoxin
TWEEN	Polyoxyethylene Sorbitan Monooleate
U	Unit
V	Voltage
VEGFR	Vascular endothelial growth factor receptor
Vim	Vimentin

Chapter I

General Introduction

Tissue engineering approaches urge us to develop and create engineered matrices or scaffolds biomimicking tissue in vitro. The main objective of that is to stimulate temporarily some aspects present in vivo during normal tissue development. Tissue engineering implies the presence of reparative/regenerative cells, biodegradable scaffolds, and bioreactors to control the cellular environment. Cells and biomaterial scaffolds can be utilized in many ways. Fibrin represents a useful biodegradable matrix, that also binds to regulatory signals such as growth factors.(1-5)

Fibrinogen and fibrin

Fibrinogen (factor I) is the most important factor in the blood coagulation process. It is a soluble ~ 340 kDa serum protein with an isoelectric point of 5.1. Fibrinogen is composed of 3 globular units, two outer D domains, each connected by helical coil to a central E domain (Figure 1.1). They are composed of two sets of three polypeptide chains termed Aa, Bb, g, which are linked within its E domain by five disulfide bonds.(6,7)

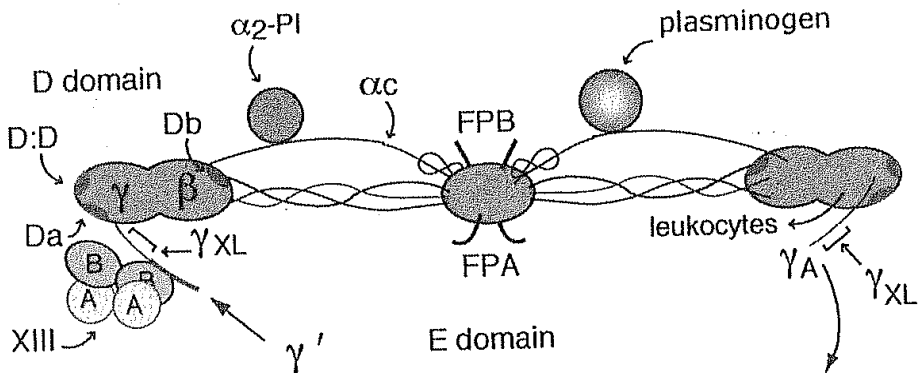

Figure 1.1. Schematic diagram of fibrinogen showing the major structural domains D and E. FPA and FPB are the association sites that participate in fibrin polymerization (adapted from Mosesson, 2000 (8)).

For conversion of fibrinogen to fibrin a 16 amino acid region (fibrinopeptide A, FPA) of each Aα chain and 14 amino acids (fibrinopeptide B, FPB) from the Bβ-chains in the E domain are cleaved by thrombin (factor II).(9,10) The thereby free N-terminal regions of the Aα and Bβ chains bind to the γ chains in the D domains of the fibrinogen and build protofibrils.(11,12) That association results in double-stranded fibrils in which fibrin molecules become aligned in an end-to-middle staggered overlapping arrangement.(13) The protofibrils undergo lateral associations and form branches of a fiber network (Figure 1.2).(8,14) The fibrin network is described in different ways and the structure depends on the enviroment. Thus, fibers formed in vivo are longer and thinner than fibers formed outside the biological organism.(15)

In vitro, the properties and structure can also be modified by various preparation methods, such as electrospinning technique.(16) The consistency of fibrin can be artificially changed by additives such as the fibrin stabilizing factor XIII (fXIII), the fibrinolysis inhibitors aprotinin and tranexamic acid (t-AMCA), or various growth factors.(4,5,17-20)

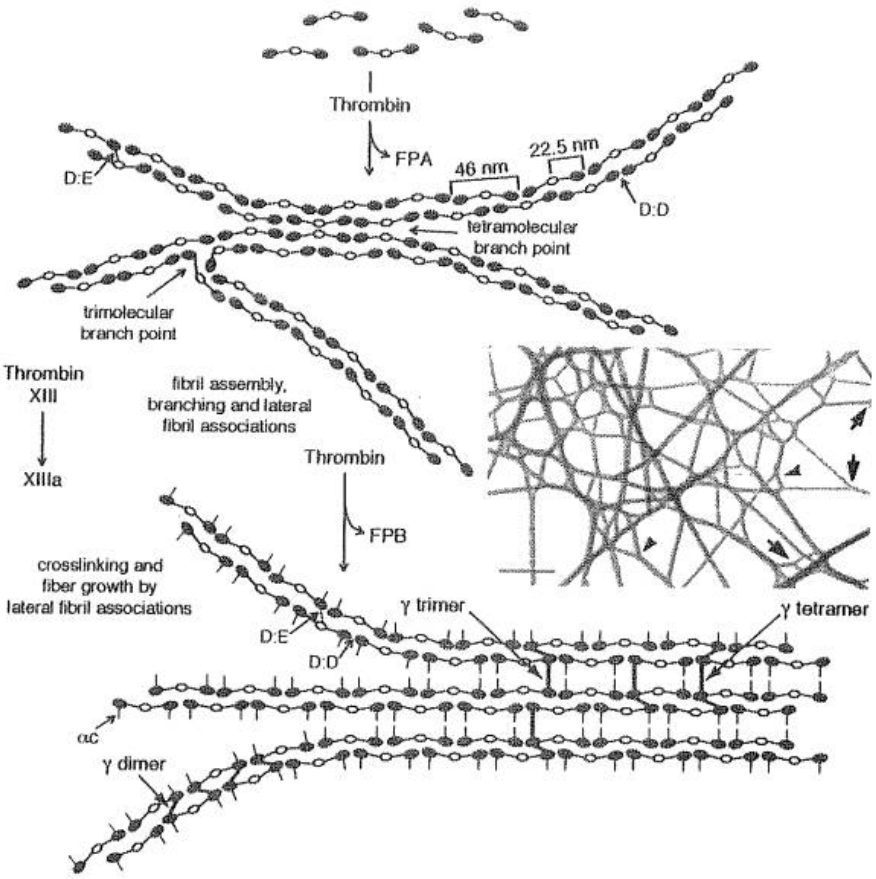

Figure 1.2. Fibrin polymerization and crosslinking. The association results in double-stranded fibrils in which fibrin molecules become aligned in an end-to-middle staggered overlapping arrangement. The protofibrils undergo lateral associations and form branches of a fiber network (modified from Mosesson, 2000 (8)).

Fibrin by itself and in a modified form is commonly used in surgical procedures to achieve rapid hemostasis and tissue sealing.(21) Commercial available fibrin sealant products consist of a highly concentrated fibrinogen complex and a high potency thrombin. After reconstitution and mixing of the components, a fibrin-based clot is rapidly formed at the site of application. These products have been clinically approved for use as hemostatic and tissue sealing agents in surgical procedures.(22,23) The application of fibrin gel is widely-used due to its high biological tolerance and its naturally proteolytic resorption. Therefore fibrin gels are perfectly suited as a matrix or depot for living cells and bioactive agents which have an intrinsic binding capacity to fibrin.(24,25) Due to the hydrated and porous structure of fibrin, however, substances without specific affinity to fibrin will exit the gel by diffusion relatively fast.

These substances have to be linked either directly or by a linking substance to fibrinogen and fibrin. One possible linking anchor can be fibroblast growth factor-2, which is naturally associated to fibrinogen and fibrin by a 15 amino acid domain.(26)

Human basic fibroblast growth factor

Fibroblast growth factor (FGF) constitutes a family of related proteins controlling normal growth and differentiation of mesenchymal, epithelial, and neuroectodermal cell types.(27,28) Two main groups of FGF are known; acidic and basic FGF. One type of FGF was isolated initially from brain tissue and was identified initially by its proliferation-enhancing activity for myoblasts. Due to its acidic pI (pI = 5.5-6) the factor was named aFGF (acidic FGF or FGF-1).(29,30) Another factor, isolated also initially from brain tissue, is bFGF (basic FGF, heparin-binding growth factor, prostatropin or FGF-2) with an isoelectric point of 9.6. It was identified by its proliferation-enhancing activities of murine fibroblasts (3T3 cells).(31,32) This factor is the prototype member of the FGF family and shows a homology of 55% to FGF-1. The FGF-2 gene is expressed in bone marrow, lymph node, pancreas, thymus and probably spleen.(31,33) The observation that FGF-2 mRNA is below the detection limit in adrenal, spleen, heart, kidney, liver, stomach, small intestine, large intestine, testis and ovary support the notion that the high abundance of FGF-2 mRNA in specific tissues is due to the storage of mitogen in the extracellular matrix and not continuous gene expression. (34) FGF-2 is a single copy gene, located on the 4th chromosome 4q26-q27 (Figure 1.3) and encodes by alternative splicing of multiple FGF-2 isoforms, with molecular weights ranging from 18 kDa to 24 kDa.(35)

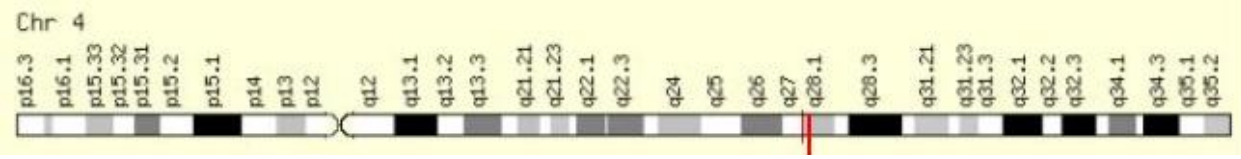

Figure 1.3. Chromosomal location of human FGF-2 on the 4th chromosome (red line) (adapted from http://www.ensembl.org).

Four high molecular weight isoforms (HMW-FGF-2s) and one low 18 kDa protein exist. The low molecular weight isoform, characterized by Florkiewicz and Sommer, lacks the motif of a N-terminal nuclear localization signal and remains localized in the cytosol.(36) It is a single-chain polypeptide composed of 146 amino acids and does not contain disulfide bonds and is not glycosylated.(31) The structure of FGF-2, revealed by X-ray crystallography, shows a similar folding structure as seen with Interleukin-1(IL-1).(37,38)

Because of the many different receptor phenotypes expressed in various cell types, FGF-2 is a multifunctional protein with a wide spectrum of biological activities. FGF-2 stimulates a variety of physiological processes, including cell proliferation, cell differentiation and cell migration.(27) FGF-2 plays an important physiological role in tissue regeneration and wound healing, and is involved also in angiogenesis by controlling the proliferation and migration of vascular endothelial cells.(27,39,40) The expression of plasminogen activator and collagenase activity by these cells is enhanced by FGF-2.(4,41) It is probably one of the factors responsible for the out-growth of new capillary blood vessels in the mesencephalon and telencephalon during embryogenesis, at a time when these structures are essentially free of blood vessels.(42)

More recently, FGF-2 has been further recognized as a hematopoietic cytokine. Receptors for human FGF are expressed on the surface of peripheral B and T cells and a variety of leukemic cell lines. T-lymphocytes express FGF-2 mRNA and produce heparin-binding FGF-like bioactivity, raising the possibility of an autocrine or paracrine role for human FGF in hematopoietic cell function.(43,44) The membrane-associated factor,

FGF-2, can be phosphorylated by a protein kinase also located on the cell surface, which may alter its activity and bioavailability. Heparin protects FGF-2 from inactivation by proteases, acids, and heat. It also improves its capacity to bind to the receptors and hence potentiates the biological activities of FGF-2. This feature may be of physiological importance because mast cells, for example, contain high levels of heparin, which could be released during degranulation. In addition, heparin also increases the biological half life of FGF-2.(45,46)

Study aims

The general purpose of our studies was to modify fibrin for applications in regenerative medicine for skin, connective tissue, bone, and cartilage.

Specific purposes were:

1. to find truncated FGF-2 peptides with a high affinity to fibrin (**chapter 2**)
2. to analyze the biological activity of the truncated FGF-2 peptide compared to FGF-2 (**chapter 3**)
3. to test controlled release of substances bound to fibrin by fibronectin, thrombin, and DNA (**chapter 4**)
4. to test electrospun fibrin for tissue engineered application of skin (**chapter 5**)
5. to stimulate fibrin-cell constructs for tissue engineered applications of tendons and ligaments (**chapter 6**).

Future perspectives

Future steps of our research would be first the testing of FGF-2 peptide as a fibrin anchor for controlled release from fibrin of substances without a binding affinity to fibrin. Secondly, our focus would be on the optimization of the electrospinning process for fibrin. Finally, our interest would be on further stimulation processes of fibrin-cell constructs. It needs to focus on standardized strength and stability testing of the produced constructs and moreover *in vivo* testing.

References

1. Hubbell JA. Tissue and cell engineering. Curr Opin Biotechnol In press.

2. Tisseel Engineering - Fibrin Matrix Technology. 2001. Baxter Hyland Immuno. The Fibrin Network in Tissue Engineering.
   Ref Type: Report

3. Sahni A, Odrljin T, Francis CW. Binding of basic fibroblast growth factor to fibrinogen and fibrin. J Biol Chem In press.

4. Sahni A, Francis CW. Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation. Blood In press.

5. Campbell PG, Durham SK, Hayes JD, Suwanichkul A, Powell DR. Insulin-like growth factor-binding protein-3 binds fibrinogen and fibrin. J Biol Chem In press.

6. Zhang JZ, Redman CM. Identification of B beta chain domains involved in human fibrinogen assembly. J Biol Chem In press.

7. Huang S, Cao Z, Davie EW. The role of amino-terminal disulfide bonds in the structure and assembly of human fibrinogen. Biochem Biophys Res Commun In press.

8. Mosesson MW. Fibrinogen functions and fibrin assembly. Fibrinolysis and Proteolysis In press.

9. Liu CY, Koehn JA, Morgan FJ. Characterization of fibrinogen New York 1. A dysfunctional fibrinogen with a deletion of B beta(9-72) corresponding exactly to exon 2 of the gene. J Biol Chem In press.

10. Pandya BV, Cierniewski CS, Budzynski AZ. Conservation of human fibrinogen conformation after cleavage of the B beta chain NH2 terminus. J Biol Chem In press.

11. Mosesson MW. Fibrinogen and fibrin polymerization and functions. Blood Coagul Fibrinolysis In press.

12. Everse SJ, Spraggon G, Veerapandian L, Riley M, Doolittle RF. Crystal structure of fragment double-D from human fibrin with two different bound ligands. Biochemistry In press.

13. Mosesson MW, Siebenlist KR, Amrani DL, DiOrio JP. Identification of covalently linked trimeric and tetrameric D domains in crosslinked fibrin. Proc Natl Acad Sci U S A In press.

14. Mosesson MW, DiOrio JP, Siebenlist KR, Wall JS, Hainfeld JF. Evidence for a second type of fibril branch point in fibrin polymer networks, the trimolecular junction. Blood In press.

15. Weisel JW, Phillips GN, Jr., Cohen C. The structure of fibrinogen and fibrin: II. Architecture of the fibrin clot. Ann N Y Acad Sci In press.

16. Wnek GE, Carr ME, Simpson DG, Bowlin GL. Electrospinning of Nanofiber Fibrinogen Structures. Nano Lett In press.

17. Board PG, Losowsky MS, Miloszewski KJ. Factor XIII: inherited and acquired deficiency. Blood Rev In press.

18. Furst W, Banerjee A, Redl H. Comparison of structure, strength and cytocompatibility of a fibrin matrix supplemented either with tranexamic acid or aprotinin. J Biomed Mater Res B Appl Biomater In press.

19. Smith JD, Chen A, Ernst LA, Waggoner AS, Campbell PG. Immobilization of aprotinin to fibrinogen as a novel method for controlling degradation of fibrin gels. Bioconjug Chem In press.

20. Cox S, Cole M, Tawil B. Behavior of human dermal fibroblasts in three-dimensional fibrin clots: dependence on fibrinogen and thrombin concentration. Tissue Eng In press.

21. Sierra DH. Fibrin sealant adhesive systems: a review of their chemistry, material properties and clinical applications. J Biomater Appl In press.

22. Schlag G, Redl H, Turnher M, Dinges HP. Importance of fibrin in wound repair. Rev Laryngol Otol Rhinol (Bord) In press.

23. Martin P. Wound healing--aiming for perfect skin regeneration. Science In press.

24. Sahni A, Odrljin T, Francis CW. Binding of basic fibroblast growth factor to fibrinogen and fibrin. J Biol Chem In press.

25. Newton, C., Goodwin, C., Helgerson, S., and Spaethe, R. Human Growth Factors in the Fibrinogen Complex Component of Fibrin Sealant Products. 7th World Biomaterials Congress 2004 . 2004.
Ref Type: Abstract

26. Peng H, Sahni A, Fay P, Bellum S, Prudovsky I, Maciag T, Francis CW. Identification of a binding site on human FGF-2 for fibrinogen. Blood In press.

27. Gospodarowicz D. Biological activities of fibroblast growth factors. Ann N Y Acad Sci In press.

28. Bikfalvi A, Klein S, Pintucci G, Rifkin DB. Biological roles of fibroblast growth factor-2. Endocr Rev In press.

29. Burgess WH, Shaheen AM, Hampton B, Donohue PJ, Winkles JA. Structure-function studies of heparin-binding (acidic fibroblast) growth factor-1 using site-directed mutagenesis. J Cell Biochem In press.

30. Weich HA, Iberg N, Klagsbrun M, Folkman J. Expression of acidic and basic fibroblast growth factors in human and bovine vascular smooth muscle cells. Growth Factors In press.

31. Florkiewicz RZ, Shibata F, Barankiewicz T, Baird A, Gonzalez AM, Florkiewicz E, Shah N. Basic fibroblast growth factor gene expression. Ann N Y Acad Sci In press.

32. Abraham JA, Whang JL, Tumolo A, Mergia A, Fiddes JC. Human basic fibroblast growth factor: nucleotide sequence, genomic organization, and expression in mammalian cells. Cold Spring Harb Symp Quant Biol In press.

33. Bugler B, Amalric F, Prats H. Alternative initiation of translation determines cytoplasmic or nuclear localization of basic fibroblast growth factor. Mol Cell Biol In press.

34. Shimasaki S, Emoto N, Koba A, Mercado M, Shibata F, Cooksey K, Baird A, Ling N. Complementary DNA cloning and sequencing of rat ovarian basic fibroblast growth

factor and tissue distribution study of its mRNA. Biochem Biophys Res Commun In press.

35. Shibata F, Baird A, Florkiewicz RZ. Functional characterization of the human basic fibroblast growth factor gene promoter. Growth Factors In press.

36. Florkiewicz RZ, Sommer A. Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. Proc Natl Acad Sci U S A In press.

37. Zhang JD, Cousens LS, Barr PJ, Sprang SR. Three-dimensional structure of human basic fibroblast growth factor, a structural homolog of interleukin 1 beta. Proc Natl Acad Sci U S A In press.

38. Sahni A, Guo M, Sahni SK, Francis CW. Interleukin-1beta but not IL-1alpha binds to fibrinogen and fibrin and has enhanced activity in the bound form. Blood In press.

39. Fujita M, Ishihara M, Shimizu M, Obara K, Nakamura S, Kanatani Y, Morimoto Y, Takase B, Matsui T, Kikuchi M, Maehara T. Therapeutic angiogenesis induced by controlled release of fibroblast growth factor-2 from injectable chitosan/non-anticoagulant heparin hydrogel in a rat hindlimb ischemia model. Wound Repair Regen In press.

40. Marra KG, Defail AJ, Clavijo-Alvarez JA, Badylak SF, Taieb A, Schipper B, Bennett J, Rubin JP. FGF-2 enhances vascularization for adipose tissue engineering. Plast Reconstr Surg In press.

41. Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. J Investig Dermatol Symp Proc In press.

42. Alarid ET, Cunha GR, Young P, Nicoll CS. Evidence for an organ- and sex-specific role of basic fibroblast growth factor in the development of the fetal mammalian reproductive tract. Endocrinology In press.

43. Araujo DM, Cotman CW. Basic FGF in astroglial, microglial, and neuronal cultures: characterization of binding sites and modulation of release by lymphokines and trophic factors. J Neurosci In press.

44. Benezra M, Vlodavsky I, Yayon A, Bar-Shavit R, Regan J, Chang M, Ben Sasson S. Reversal of basic fibroblast growth factor-mediated autocrine cell transformation by aromatic anionic compounds. Cancer Res In press.

45. Gospodarowicz D, Cheng J. Heparin protects basic and acidic FGF from inactivation. J Cell Physiol In press.

46. Klagsbrun M. The affinity of fibroblast growth factors (FGFs) for heparin; FGF-heparan sulfate interactions in cells and extracellular matrix. Curr Opin Cell Biol In press.

Chapter II

Fibroblast growth factor-2 peptides bind to fibrinogen and fibrin and exert biological activity

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Manuscript submitted

Abstract

Full length human FGF-2 is a single chain polypeptide of 162 amino acids and a high binding affinity to fibrinogen and fibrin The putative fibrin(ogen)-binding site of FGF-2 has been localized to an approximately 40-residue domain near the N-terminus of FGF-2. This domain observation allowed us to begin our analyses by further characterizing and truncating the sequences within this domain required for the FGF-2/fibrinogen interaction. Complementary DNAs of FGF-2 peptides ranging from 37 to 58 amino acids (aa) were created by PCR, ligated into the pGEX-6P-2 vector system and expressed in *E. coli* BL21(DE3) bacteria cells. Peptides smalller than 37 aa were synthesized. Only purified peptides were tested for their binding affinitiy to fibrin(ogen) and their biological activity on mouse myoblast cells (C2C12).

The results of the fibrinogen and fibrin binding assay demonstrated a high affinity of native FGF-2 and peptides longer than 37 aa. The smallest fragment of 20 aa had nearly no binding affinity in various concentrations. Native FGF-2 also shows the highest proliferative activity in C2C12 cells. Similar to the fibrinogen and fibrin binding fragments ranging from 58 aa to 37 aa promoted cell proliferation almost in the same dimension. The smallest fragment pep5 did not stimulate C2C12 cell growth.

The results indicate that the domain for biological activity and the binding domain to fibrinogen are located on the same segment of FGF-2 cDNA. It is necessary to use more than the 15 aa, which demonstrates the putative binding domain.

Introduction

Fibroblast growth factor-2 (FGF-2, basic FGF, heparin-binding growth factor or prostatropin) belongs to a family of heparin-binding growth factors.(1) As a single-chain protein, FGF-2 has 146 amino acids, an isoelectric point of 9.6 and a molecular weight of about 17,400 Dalton. (2,3) The human FGF-2 gene is expressed in bone marrow, lymph node, pancreas, thymus and assumable spleen.(4,5) The observation that FGF-2 mRNA is below the detection limit in adrenal, spleen, heart, kidney, liver, stomach, small intestine, large intestine, testis and ovary support the notion that the high abundance of human FGF-2 mRNA in specific tissues is due to the storage of mitogen in the extracellular matrix and not continuous gene expression.(6) FGF-2 was characterized by Florkiewicz and Sommer(7) and is the prototype member of the FGF family. It does not contain any disulfide bonds and is not glycosylated.(2) The structure of FGF-2, revealed by X-ray crystallography, shows a similar folding structure as seen with Interleukin-1(IL-1).(4,8)

Binding of FGF-2 to one of its receptors (FGFR-1, FGFR-2, FGFR-3, FGFR-4) requires the interaction with heparan sulfate and heparan sulfate proteoglycans (Syndecan) of the extracellular matrix before reaching full functional activity.(9) This is also demonstrated by the ability of heparinase to inhibit receptor binding and biological activity of FGF-2.(10) Because of the many different receptor phenotypes expressed in various cell types, FGF-2 is a multifunctional protein with a wide spectrum of biological activities. FGF-2 stimulates a variety of physiological processes, including cell proliferation, cell differentiation and cell migration.(11) More recently,

FGF-2 has been further recognized as a hematopoietic cytokine and may play an important role in wound healing and angiogenesis.(12) It is released after tissue injury and during inflammatory processes, and also during the proliferation of tumor cells.(13) It is probably one of the factors responsible for the out-growth of new capillary blood vessels in the

mesencephalon and telencephalon during embryogenesis, at a time when these structures are essentially free of blood vessels.(14)

The expression of FGF-2 and related factors seems to be regulated differentially, depending on cell type and developmental age.(15,16) The FGF-2 can be phosphorylated by a protein kinase also located on the cell surface, which may alter its activity and bioavailability. Heparin protects FGF-2 from inactivation by proteases, acids, and heat. It also improves its capacity to bind to the receptors and hence potentates the biological activities of FGF-2. This feature may be of physiological importance because mast cells, for example, contain high levels of heparin, which could be released during degranulation. In addition, heparin also increases the biological half life of FGF-2. (17,18) The expression of plasminogen activator and collagenase activity by primary endothelial cells is enhanced by FGF-2.(12,19)

The high-affinity and saturable binding of FGF-2 to fibrinogen and fibrin indicates an important level of influence at sites of injury between growth factors with critical cell regulatory functions and the fibrin matrix.(20) Fibrin is naturally associated with a number of growth factors that binds to fibrinogen and may promote wound healing.(19,21) Such growth factors may attract and/or stimulate cells involved in tissue repair.(4,12) The application of fibrin gel is widely-used in hemostasis and tissue sealing due to its high biological tolerance and its naturally proteolytic resorption. Therefore, fibrin gels are perfectly suited as a matrix or depot for living cells and bioactive agents acting as a slow release delivery system.(20,22) Due to the hydrated and porous structure of fibrin, however, substances without specific affinity to fibrin will exit the gel by diffusion relatively fast. In comparison, natural fibrin binding proteins, or binding sequences thereof, can be linked to target substances without a natural binding affinity to fibrin, thereby retaining them in the fibrin matrix. FGF-2 holds significant potential as a therapeutic additive to sealant products, because FGF-2 can stimulate wound healing, tissue regeneration, and angiogenesis. Therefore, our aim was to characterize the fibrinogen

binding domain of FGF-2 that has a high affinity to fibrinogen and fibrin. (20) The putative fibrinogen-binding site of FGF-2 has been localized to an approximately 40-residue domain near the N-terminus of FGF-2 (Figure 2.1a).(21) This domain observation allowed us to begin our analyses by further characterizing and truncating the sequences within this domain required for the FGF-2/fibrinogen interaction (Figure 2.1b).

Figure 2.1a. Three-dimensional structure of human FGF-2 (~ 17.4 kDa protein) The red part identifies the putative binding domain (15 bp) of FGF-2 on fibrinogen (plotted using Visual Molecular Dynamics).

hFGF-2	base pairs	amino acids	kiloDalton
full length	486	162	~ 17.4
pep5	60	20	~2.1
pep1	174	58	~ 6.2
pep2	144	48	~ 5.2
pep3	111	37	~ 4.0
pep4	87	29	~3.1

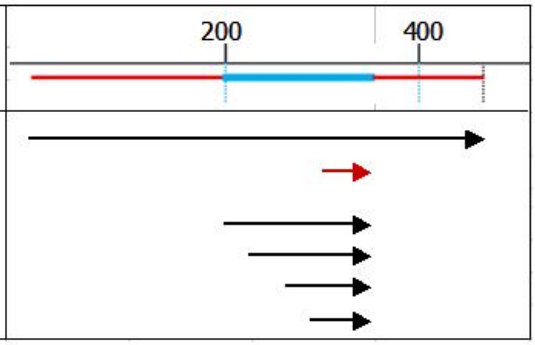

Figure 2.1b. Schema of the truncated FGF-2 fragments pep1, pep2, pep3, pep4, and pep5 in comparison to full length human FGF-2.

Materials and methods

Construction of rhFGF-2 and various truncated hFGF-2 peptides

Using a standard polymerase chain reaction (95°C, 30 sec; 60°C, 30 sec; 72°C, 30 sec; 25 cycles), full length cDNA for human FGF-2 contained in the cloning vector system pCR2.1 (Invitrogen, Germany) was amplified with the following primer pairs in table 2.1. The primers contained *EcoR*I and *Xho*I restriction sites and a 6x HIS-tag on the C-terminus. The PCR products, ranging from 486 bp down to 111 bp were digested with EcoRI and XhoI and then ligated to the same restriction enzyme site in the multicloning region of the pGEX-6P-2 plasmid vector (Pharmacia Biotech, Vienna) using DNA Ligation Kit (Roche Diagnostics, Vienna). The pGEX vector systems contained an N-terminal glutathione S-transferase (GST) site, which results in a fusion protein of GST and the protein of interest. It also included a PreScision protease site to cleave the GST from the fusion protein. After transformation into Escherichia coli Top10 (Invitrogen, Germany), the plasmid DNA of positive clones were isolated and purified (Mini Preparation Kit, Sigma-Aldrich, Vienna) and then sequenced by Boehringer Ingelheim Austria GmbH, Vienna.

Expression and purification

Positive clones plasmids were transformed into BL21(DE3) an Escherichia coli strain used for protein expression (Invitrogen, Germany), which was induced with isopropyl-β-D-thiogalactopyranoside (IPTG). After 5 hours of expression cell lysates were dissolved in phosphate buffered saline (PBS, pH 7.5), 5 mM 1,4-dithio-DL-threitol (DTT), 1% Triton X-100, protease inhibitor cocktail (Roche Diagnostics, Vienna), and 0.5 µg/ml lysozyme. The bacteria lysate was mixed, incubated for 15 minutes on ice, sonicated 6 times for 10 seconds and centrifuged for 15 minutes. The supernatant was then incubated with 10% glutathione Sepharose slurry (Pharmacia Biotech, Vienna) over night at 4°C. Afterwards the column was washed once with the lysis buffer and twice with a cleavage buffer (50 mM Tris-

HCl, pH 7.0; 150 mM NaCl; 0.01% Triton X-100). Then the GST fusion protein was cleaved with Precision protease (Amersham Biosciences, Germany) over night at 4°C and only the recombinant hFGF-2 peptides were eluted using 50 mM Tris-HCl (pH 7.0) and 10 mM reduced glutathione. Purified proteins were dialyzed against PBS using a minidialysis system (Pierce, THP Medical Products, Vienna), and then stored at -80°C.

Electrophoresis and Western blot analysis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue staining, was performed at each step of the recombinant protein production as previously described.(23) Peptides (<7 kDa) were separated by discontinuous tricine SDS-PAGE according to manufacturer's instruction (Bio-Rad Laboratories, Vienna).

For Western blot analysis, samples in the gel after SDS-PAGE were electrophoretically semi-dry transferred to a nitrocellulose membrane (Bio-Rad, Vienna). The membrane was first immersed in a blocking solution (PBS and 2% bovine serum albumin (BSA)) and then incubated with monoclonal anti-His6-peroxidase antibody diluted 1:1.000 in PBS containing 0.2% TWEEN (PBST 0.2). After washing three times with PBST 0.2, the membrane was covered with a chemiluminescent detecting reagent (Roche Diagnostics, Vienna) according to manufacturer's instruction. Immuno-reactive bands were visualized in the Multiimage™ Light Cabinet (Biozym, Vienna).

Synthetic peptides

Due to the small sizes and therefore difficult expression procedure peptides pep3 (37 aa), pep4 (29 aa) and pep5 (20 aa) were synthesized by the company PiChem (Graz). The sequences are shown in Table 2.1.

Primers	sense (s) and antisense (as); ٥' - ٣'
***EcoR*I restriction site**	
hbFGF full lenght sense	GGA ATT CCC ATG GCA GCC GGG AGC ATC
hbFGF pep1 sense	GGA ATT CCC GAA GAG AGA GGA GTT GTG
hbFGF pep2 sense	GGA ATT CCC GTG TGT GCT AAC CGT TAC
***Xho*I restriction site**	
hbFGF full lenght antisense	CTC GAG TCA ATG ATG ATG ATG ATG ATG GCT CTT AGC AGA CAT TGG
hbFGF pep antisense	CTC GAG TCA ATG ATG ATG ATG ATG ATG CCT TGA CCG GTA AGT ATT

Synthetic peptides	
pep 3 (37 aa)	EDGRLLASKC VTDECFFFER LESNNYNTYR SRHHHHH
pep 4 (29 aa)	KCVTDECFFF ERLESNNYNT YRSRHHHHH
pep 5 (20 aa)	FERLESNNYN TYRSRHHHHH

Table 2.1. Primers with *EcoR*I and *Xho*I restriction sites for ligation into pGEX-6P-2 expression vector system. The restriction sites are underlined and the 6x HIS-tag on the C-terminus is marked in red. The sequences of the synthetic peptides #3, #4, and #5 contain a 5x HIS-tag at the C-terminus (red).

Biological activity of recombinant hFGF-2 and peptides

Mouse myoblast (C2C12) cells were purchased from ECACC (Sigma-Aldrich, Vienna). They were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (FCS). At 50-60% confluence cells were trypsinized and transferred to a 24-well plate (1 x 10^4/well). Following a 48-hour-culture period at 37°C, cells were washed with DMEM only and then covered with fresh medium (DMEM + 1% FCS) containing various concentrations of rhFGF-2 or rhFGF-2 peptides. After 48 hours the number of viable cells was determined by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, Vienna) as previously described.(24) MTT is converted to formazan by mitochondrial succinate dehydrogenase. The amount of formazan produced is proportional to the number of viable cells and its absorbance was read at 550 nm.

Fibrin(ogen) binding assay

A polystrene 96-well plate (Nunc® Maxisorp ELISA-plates) was coated with 1 mg/ml fibrinogen (Tisseel, Baxter AG, Vienna) overnight at 4°C, and blocked with blocking solution (PBS and 1% BSA) at room temperature (RT) for 2 h. After washing three times with PBS, purified recombinant proteins and peptides were added in various dilutions to the plate and incubated for 1 hour at RT. After washing procedure, monoclonal anti-His6-peroxidase antibody diluted in PBST 0.1 was added and incubated for 1 hour at RT. Finally, the plate was washed again and samples were incubated with o-phenylenediaminehydrochlorid substrate for color reaction. The absorbance was measured at 492 nm and 620 nm.

For fibrin binding analysis the plate was first coated with 2 mg/ml fibrinogen overnight at 4°C and then incubated with 10 I.U./ml thrombin (Tisseel, Baxter AG, Vienna) for 30 minutes at 37°C. The following procedure was the same as described above.

Results

rhFGF-2 and hFGF-2 peptides

Recombinant human FGF-2 was expressed as a fusion protein using the pGEX-6P-2 system. Western blot technique revealed the detection with monoclonal anit-6His-peroxidase antibody (Figure 2.2a). It showed enough correctly sized protein in the supernatant for purification steps. The result of cleavage and purification of rhFGF-2 was confirmed by SDS-PAGE (Figure 2.2b).

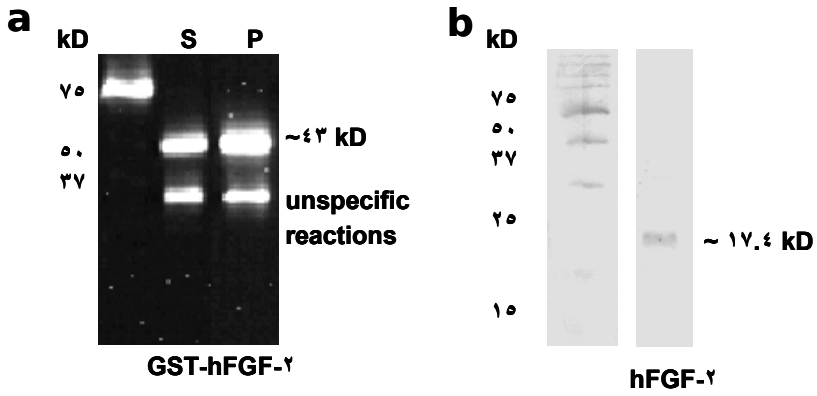

Figure 2.2. Expression and purification of FGF-2. (**a**) The expression of fused GST-FGF-2 (~ 43 kDa) was analysed by Western blot technique developed with a monoclonal anti-6His-peroxidase antibody. S supernatant, P inclusion bodies. (**b**) The cleaved and purified FGF-2 (~ 17.4 kDa) was confirmed by SDS-PAGE stained with Coomassie brilliant blue.

As a result of deletion of human FGF-2 cDNA parts, amino- and carboxyl-terminally truncated rhFGF-2 peptides (pep1 to pep3) were successfully expressed as a GST fused protein (Figure 2.3a). The Western blot analysis represented similar protein amounts in both supernatant and inclusion bodies. It also showed unspecific reactions of the antibody. Purified and cleaved peptides pep1 (~32 kD), pep2 (~ 5.2 kD), and pep3 (~ 29 kD) were analyzed by tricine SDS-PAGE (Figure 2.3b). Fragments smaller than 37 amino acids were sythesized. Discontinuous tricine SDS-PAGE confirmed the different sizes of the peptides pep3, pep4, and pep5 (Figure 2.4).

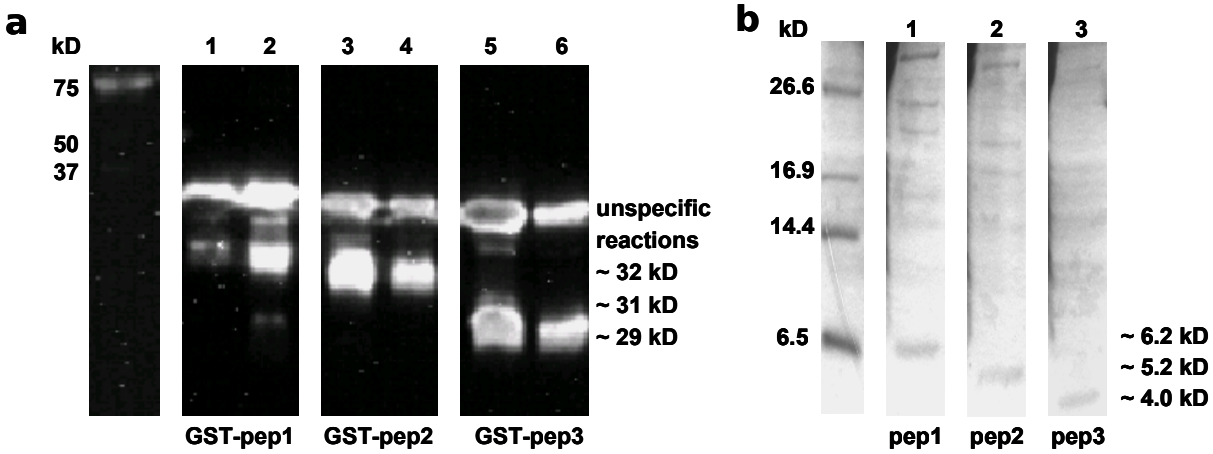

Figure 2.3. The expression and purification of FGF-2 peptides. (**a**) The expression of fused GST-pep1 (~ 32 kDa), GST-pep2 (~ 31 kDa), and GST-pep3 (~ 29 kDa) was analysed by Western blot technique developed with a monoclonal anti-6His-peroxidase antibody. Lane 1, 3, 5 supernatant; lane 2, 4, 6 inclusion bodies. (**b**) The cleaved and purified pep1 (~ 6.2 kDa), pep2 (~ 5.2 kDa), and pep3 (~ 4.0 kDa) were confirmed by discontinuous tricine SDS-PAGE stained with Coomassie brilliant blue.

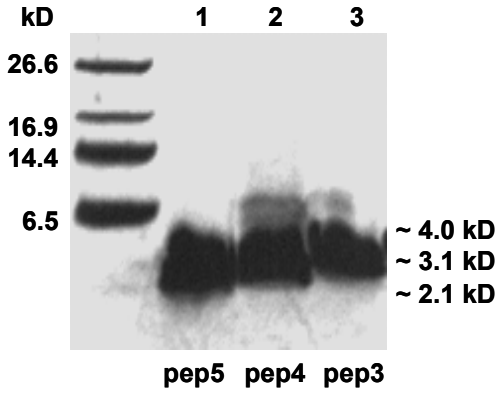

Figure 2.4. Synthetized peptides pep3 (~ 4.0 kDa), pep4 (~ 3.1 kDa), and pep5 (~ 2.1 kDa) were detected by discontinuous tricine SDS-PAGE stained with Coomassie brilliant blue

Binding assay

Only purified proteins and peptides in different concentrations were used for testing the binding affinity to fibrinogen and fibrin (Figure 2.5). The results of the fibrinogen and fibrin binding assay demonstrated a high affinity of native rhFGF-2, pep1, pep2, and pep3 to fibrinogen. Peptide pep4 showed a significantly lower and pep5 has nearly no binding affinity in various concentrations.

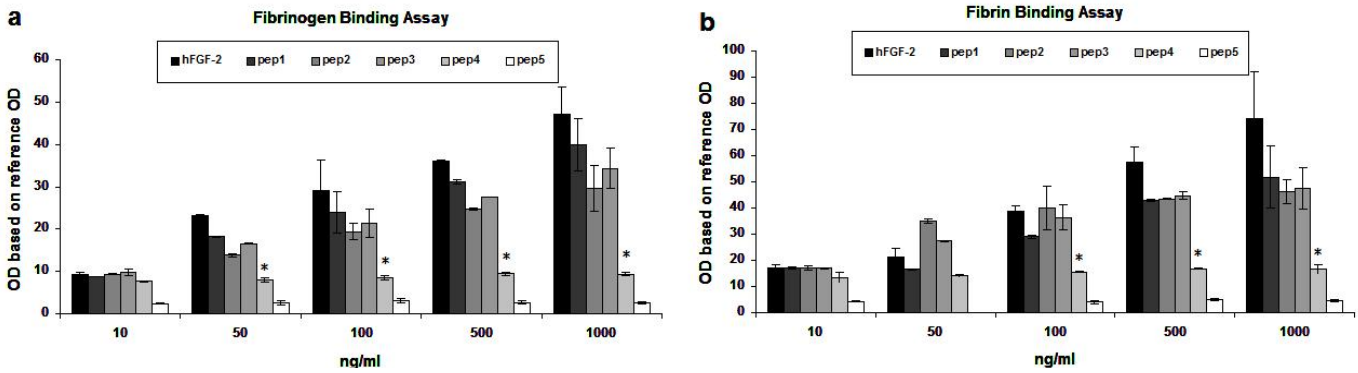

Figure 2.5. Fibrin(ogen) binding assay with different concentrations of FGF-2 and FGF-2 peptides (pep1 – pep5). (**a**) The graph represents the binding affinity of the purified (FGF-2, pep1, and pep2) respectively synthesized (pep3, pep4, and pep5) samples to fibrinogen. (**b**) The graph demonstrates the binding affinity to fibrin. The values are expressed as mean ± SD (*p<0.01).

Biological activity

Recombinant human FGF-2 showed the highest proliferative activity in mouse myoblast cells. Similar to the fibrinogen and fibrin binding assay pep1 to pep3 promoted cell proliferation almost in the same dimension. The smallest fragment pep5 did not stimulate C2C12 cell growth (Figure 2.6).

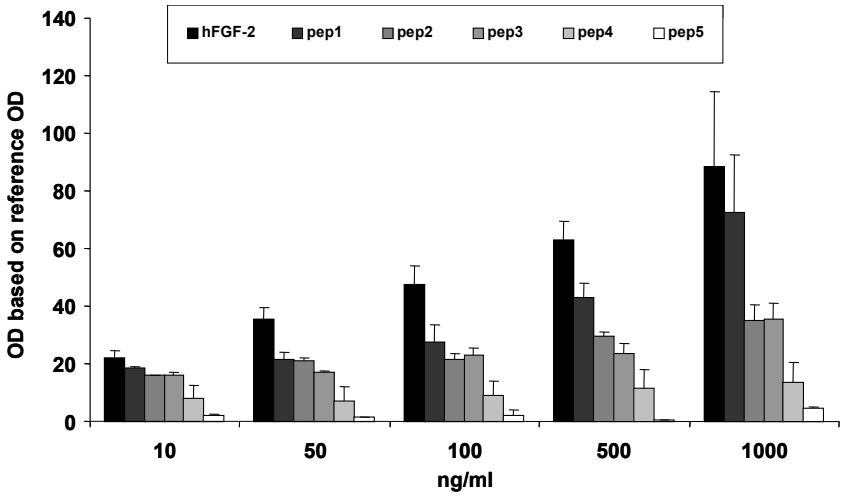

Figure 2.6. MTT assay of mouse myoblast (C2C12) cells incubated with different concentrations of FGF-2 and FGF-2 peptides (pep1 – pep5). The values are expressed as mean ± SD.

Discussion

Full length human FGF-2 is a single chain polypeptide without any disulfide bonds and glycosylation. It is suitable to be expressed in *E. coli*, especially in *E. coli* BL21(DE3) and glutathione S-transferase fusion system. Amplified FGF-2 was ligated into pGEX-6P-2 vector which contains a GST at the N-terminus. The N-terminal fusion with GST led to a higher expression rate of FGF-2 than in the pET-11a (direct expression without a fusion protein) and pEYFP-His (C-terminal fusion with EYFP, that encodes a green-yellow variant of the Aequorea victoria green fluorescent protein (GFP)) vector systems (data not shown). After expression, this system permits convenient site-specific cleavage and simultaneous purification on Glutathione Sepharose™. For FGF-2 digestion and purification it is necessary to work at low temperature (5°C) to minimize degradation of the protein and peptides.

The high expression rate of a N-terminal fused protein and the following simultaneous purification and cleavage of the protein of interest allows the production of the smaller FGF-2 peptides pep1 - pep4. According to Peng *et al.* the putative fibrinogen-binding site of FGF-2 has been localized near the N-terminus of FGF-2.(21) That allowed us to begin our analyses by further characterizing the sequences within this domain required for the FGF-2/ fibrinogen interaction.

The smallest FGF-2 peptide pep5 with 20 aa, which characterizes the binding domain of FGF-2 to fibrinogen and fibrin, has to be synthesized. This domain incorporated in the whole FGF-2 protein sequence represents over 90% of the binding affinity of FGF-2 to fibrinogen and fibrin(21). Only pep5 (20 aa) shows no binding affinity to both fibrinogen and fibrin. In contrast to Peng *et al* our aim was not only to identify the fibrinogen and fibrin binding domain of FGF-2 but also produce FGF-2 truncated peptides including this binding area.(21)

The pGEX-6P-2 vector system with the N-terminal fusion of GST allows a high level expression of fused peptides and a well working cleavage and

purification down to 37 amino acids. Consequently, to eliminate all sorts of unspecific reactions for the binding assays to fibrinogen and fibrin peptides pep3, pep4, and pep5 were synthesized.

The results of the binding assays demonstrate that there is a binding affinity to both fibrinogen and fibrin down to 87 base pairs or 29 amino acids (pep4). Based on these results, we assume that there are more than the 15 amino acids of pep5 necessary for the binding of hFGF-2 to fibrinogen. The extension has to be on the N-terminal site and needs a minimum of 4 amino acids (pep4).

Another point of interest is the biological activity in a mouse myoblast cell line. The results demonstrate a concentration dependent biological activity of FGF-2 and the FGF-2 peptides pep1 to pep4. Full length FGF-2 showed the highest cell growth effect. In contrast pep2 and pep3 decreased these effect down to about 50%. The smallest peptide pep5 possesses an extremely weak or no biological activity in all concentrations.

Consequently, the results indicate that the domain for biological activity and the binding domain to fibrinogen are located on the same segment of FGF-2 cDNA. And it is necessary to use more than the 15 aa, which represents the putative binding domain.

Therefore it might be possible that hFGF-2 fragments act as a linking agent between fibrinogen and target substances in fibrin matrices.(25) And they could stimulate cell proliferation and thereby have beneficial effects on wound healing and angiogenesis.

Acknowledgement

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References

1. Gospodarowicz D, Ferrara N, Schweigerer L, Neufeld G. Structural characterization and biological functions of fibroblast growth factor. Endocr Rev In press.

2. Florkiewicz RZ, Shibata F, Barankiewicz T, Baird A, Gonzalez AM, Florkiewicz E, Shah N. Basic fibroblast growth factor gene expression. Ann N Y Acad Sci In press.

3. Shibata F, Baird A, Florkiewicz RZ. Functional characterization of the human basic fibroblast growth factor gene promoter. Growth Factors In press.

4. Sahni A, Guo M, Sahni SK, Francis CW. Interleukin-1beta but not IL-1alpha binds to fibrinogen and fibrin and has enhanced activity in the bound form. Blood In press.

5. Bugler B, Amalric F, Prats H. Alternative initiation of translation determines cytoplasmic or nuclear localization of basic fibroblast growth factor. Mol Cell Biol In press.

6. Shimasaki S, Emoto N, Koba A, Mercado M, Shibata F, Cooksey K, Baird A, Ling N. Complementary DNA cloning and sequencing of rat ovarian basic fibroblast growth factor and tissue distribution study of its mRNA. Biochem Biophys Res Commun In press.

7. Florkiewicz RZ, Sommer A. Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. Proc Natl Acad Sci U S A In press.

8. Zhang JD, Cousens LS, Barr PJ, Sprang SR. Three-dimensional structure of human basic fibroblast growth factor, a structural homolog of interleukin 1 beta. Proc Natl Acad Sci U S A In press.

9. Partanen J, Vainikka S, Korhonen J, Armstrong E, Alitalo K. Diverse receptors for fibroblast growth factors. Prog Growth Factor Res In press.

10. Ornitz DM, Yayon A, Flanagan JG, Svahn CM, Levi E, Leder P. Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. Mol Cell Biol In press.

11. Gospodarowicz D. Biological activities of fibroblast growth factors. Ann N Y Acad Sci In press.

12. Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. J Investig Dermatol Symp Proc In press.

13. Folkman J. Anti-angiogenesis: new concept for therapy of solid tumors. Ann Surg In press.

14. Alarid ET, Cunha GR, Young P, Nicoll CS. Evidence for an organ- and sex-specific role of basic fibroblast growth factor in the development of the fetal mammalian reproductive tract. Endocrinology In press.

15. Bikfalvi A, Klein S, Pintucci G, Rifkin DB. Biological roles of fibroblast growth factor-2. Endocr Rev In press.

16. Mignatti P, Morimoto T, Rifkin DB. Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex. J Cell Physiol In press.

17. Gospodarowicz D, Cheng J. Heparin protects basic and acidic FGF from inactivation. J Cell Physiol In press.

18. Klagsbrun M. The affinity of fibroblast growth factors (FGFs) for heparin; FGF-heparan sulfate interactions in cells and extracellular matrix. Curr Opin Cell Biol In press.

19. Sahni A, Francis CW. Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation. Blood In press.

20. Sahni A, Odrljin T, Francis CW. Binding of basic fibroblast growth factor to fibrinogen and fibrin. J Biol Chem In press.

21. Peng H, Sahni A, Fay P, Bellum S, Prudovsky I, Maciag T, Francis CW. Identification of a binding site on human FGF-2 for fibrinogen. Blood In press.

22. Newton, C., Goodwin, C., Helgerson, S., and Spaethe, R. Human Growth Factors in the Fibrinogen Complex Component of Fibrin Sealant Products. World Biomaterials Congress, Australia . 2004.
Ref Type: Abstract

23. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature In press.

24. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods In press.

25. Morton TJ, Furst W, van Griensven M, Redl H. Controlled release of substances bound to fibrin-anchors or of DNA. Drug Deliv In press.

Chapter III

FGF-2 peptide enhance adipose-derived stem cell differentiation into tendon-like tissue

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Manuscript submitted

Abstract

Fast growth, proliferation and differentiation of cells, which were isolated from waste material, is one of the aims for tissue engineering applications. Isolated human adipose-derived stem cells (ASCs) were stimulated with 3 ng/ml (low-dose) and 30 ng/ml (high-dose) recombinant fibroblast growth factor 2 (FGF-2) and a 32 amino acid FGF-2 peptide over 28 days. Results showed on one hand the estimated growth and proliferation effect of FGF-2 in both the low-dose and high-dose group. On the other hand FGF-2 peptide stimulation had no effect on cell growth and cell proliferation. In contrast to cell growth and cell proliferation, stimulation with both 3 ng/ml and 30 ng/ml of FGF-2 peptide upregulated collagen Iα2 already within 7 days compared to the addition of FGF-2. Another marker for development into connective tissue and tendon, collagen III, showed a higher mRNA expression level in the low-dose group within 7 days, as well. The effects of the low-dose concentration of FGF-2 peptide on ASCs were reflected in the ratio of collagen Iα2 to collagen III. The high differentiation effect of FGF-peptide also was detected on the mRNA expression of alpha smooth muscle actin (α-SMA) and desmin, which are essential extra-cellular matrix proteins and cytoskeletal elements for tissue engineering applications.

The stimulation with FGF-2 and FGF-peptide led to differentiation patterns in ASCs for essential extracellular matrix proteins and cytoskeletal elements, which are key for tendons and ligaments.

Introduction

Mesenchymal stem cells (MSCs) are mostly isolated from bone marrow. However, other sources exist such as adipose tissue, muscle, connective tissue, skin, and placenta with similar surface expression patterns on the MSCs.(1) Recent studies have confirmed the existence of an abundant and easily accessible source of multipotent stem cells in subcutaneous or articular adipose tissue.(1-5) Human adipose tissue is vascularized and represents a source of autologous multipotent cells, such as pericytes and marrow-derived MSCs.(6,7) Cells isolated from adipose tissue, which were obtained from elective liposuction procedures under local anesthesia, have been termed processed adipose-derived stem cells (ASCs). Many studies analyzed and documented the cellular behaviour of the ASCs. Erickson et al. described the high chondrogenic potential of ASCs in vitro and in vivo. (8) Different culture conditions induced surface expression patterns on ASCs associated with the adipocyte, osteoblast, and myocyte pathways. (1,9-11)

Some groups as Marie and Varkey et al. showed an osteogenic effect of human basic fibroblast growth factor (heparin-binding growth factor, prostatropin, bFGF or FGF-2) on osteoprogenitor and bone marrow cells. (12,13) Dvorak et al. proposed that the endogenous FGF signaling pathway can be implicated in differentiation of human embryonic stem cells.{18} Cell differentiation, proliferation, and migration are often induced by FGF during development in the same cell type. Therefore, the same receptor for FGF-2 can produce various biological effects.

Fibroblast growth factor constitutes a family of related proteins controlling normal growth and differentiation of mesenchymal, epithelial, and neuroectodermal cell types.(14,15) FGF-2 was isolated initially from brain tissue. Its gene is expressed in bone marrow, lymph node, pancreas, thymus and presumably spleen.(16-18) The observation that hFGF-2 mRNA is below the detection limit in adrenal, spleen, heart, kidney, liver, stomach, small intestine, large intestine, testis and ovary support the

notion that the high abundance of hFGF-2 mRNA in specific tissues is due to the storage of mitogen in the extracellular matrix and not continuous gene expression. (19)

Because of the many different receptor phenotypes expressed in various cell types, FGF-2 is a multifunctional protein with a wide spectrum of biological activities. FGF-2 stimulates a variety of physiological processes, including cell proliferation, cell differentiation and cell migration.(14) More recently, FGF-2 has been further recognized as a hematopoietic cytokine. Receptors for human FGF are expressed on the surface of peripheral B and T cells and a variety of leukemic cell lines. T-lymphocytes express FGF-2 mRNA and produce heparin-binding FGF-like bioactivity, raising the possibility of an autocrine or paracrine role for human FGF in hematopoietic cell function.(20,21)

Binding of FGF-2 to one of its receptors (FGFR-1, FGFR-2, FGFR-3, FGFR-4) requires the interaction with heparan sulfate and heparan sulfate proteoglycans (Syndecan) of the extracellular matrix before reaching full functional activity.(22) This is also demonstrated by the ability of heparinase to inhibit receptor binding and biological activity of FGF-2.(23)

Because of the many different receptor phenotypes expressed in various cell types, FGF-2 is a multifunctional protein with a wide spectrum of biological activities. FGF-2 stimulates a variety of physiological processes, including cell proliferation, cell differentiation and cell migration.(14) More recently, FGF-2 has been further recognized as a hematopoietic cytokine. Receptors for human FGF are expressed on the surface of peripheral B and T cells and a variety of leukemic cell lines. T-lymphocytes express FGF-2 mRNA and produce heparin-binding FGF-like bioactivity, raising the possibility of an autocrine or paracrine role for human FGF in hematopoietic cell function.(20,21)

FGF-2 plays an important physiological role in tissue regeneration and wound healing, and is involved also in angiogenesis by controlling the proliferation and migration of vascular endothelial cells. The expression of plasminogen activator and collagenase activity by these cells is enhanced

by FGF-2.(24,25) It is probably one of the factors responsible for the out-growth of new capillary blood vessels in the mesencephalon and telencephalon during embryogenesis, at a time when these structures are essentially free of blood vessels.(26) The high-affinity and saturable binding of FGF-2 to fbrinogen and fibrin indicates an important level of coordination at sites of injury between growth factors with critical cell regulatory functions and the fibrin matrix.(27) In previous studies the binding domain of FGF-2 to fibrinogen was characterized by truncating the protein from the carboxyl- and amino-terminus.(28,29) One of these peptides is 32 amino acids long and showed both binding affinity to fibrinogen and fibrin and biological activity on fibroblast cells.(30)

The effect of FGF-2 on bone marrow stromal cells in two different concentrations was analyzed by Hankemeier et al. The increase in proliferation and the stimulation of the mRNA expression of specific extracellular matrix proteins and cytoskeletal elements by low-dose FGF-2 treatment established an interesting background for our experiments.(31) This study analyzes the effect of FGF-2 and a truncated synthesized FGF-2 peptide in two different concentrations on adipose-derived stem cells. Cell growth, proliferation and differentiation of collagen Iα2, collagen III, vimentin, desmin, and α-smooth muscle protein (α-SMA) mRNA expression are observed.

Materials and methods

ASC isolation and cultivation

Human subcutaneous adipose tissue was collected during liposuction procedures in plastic and reconstructive surgery. The study was approved by the local Ethical Review Board. Adipose-derived stem cell (ASC) isolation was modified form Zuk et al.(1) and followed a procedure as described previously.(32) Finally, cells were cultured in Dulbecco's Modified Eagle's Medium mixed 1:1 with Ham's F-12 (DMEM/Ham's F-12) containing 1% penicillin/streptomycin, 2 mM glutamine and 10% fetal calf serum (FCS; Sigma-Aldrich, Vienna) at 37°C, 5% CO_2 and 95% air humidity to a subconfluent state.

Stimulation of human ASC cultures

In the following experiments, cells up to passage 4 were seeded at a density of 150 cells/well in 96-well plates and 5,0 x 10^3 cells/well in 6-well plates (Nunc, Vienna). Cells were covered with the medium described above supplemented with low-dose (3 ng/ml) recombinant human full-length FGF-2 (154 amino acids, PeproTech, Vienna) or synthetic FGF-2 peptide (37 amino acids, PiChem, Graz), high-dose (30 ng/ml) of both agents, or without any growth factor as the control. The supplemented medium was changed once per week and samples for analysis were collected after 7, 14, 21, and 28 days. Due to the low number of seeded cell, three replicates for the first collection time point on day 7 were prepared for cell density analysis and reverse transcription-polymerase chain reaction (RT-PCR).

Cell count, cell viability, and cell proliferation

Numbers of viable cells were counted on days 7, 14, 21, and 28 by trypan blue exclusion in a Neubauer counting chamber. Analysis of cell viability was performed using on one hand an MTT assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-

Aldrich, Vienna) was added to the cells as previously described.(33) MTT is converted to formazan by mitochondrial succinate dehydrogenase. The amount of formazan produced is proportional to the number of viable cells and its absobance was read at 550 nm. On the other hand the proliferation of the cells was analyzed with a BrdU kit (Roche Diagnostics, Vienna) according to manufacturer's instruction. 5-bromo-2-deoxyuridine (BrdU) is a synthetic nucleoside, which is incorporated into newly produced DNA of replicating cells.(34) Cells were incubated for 24 hours at 37°C with the BrdU labeling solution. During this period the BrdU, which is analogue to pyrimidine was incorporated in place of thymidine into the newly synethized cellular DNA. The labeling medium was removed, then cells were fixed and incubated with the anti-BrdU conjugate. Finally samples were washed, covered with the substrate and measured at 450 nm.

RT-PCR

RNA was extracted from cells according to manufacturer's instruction (GenElute Mammalian Total RNA Kit, Sigma-Aldrich, Vienna) Purified RNA was quantified using a spectrophotometer at 260 and 280 nm, and then aliquoted and stored at -80°C. 2 µg of RNA were first treated with RNase-free DNase (Promega GmbH, Germany) according to the manufacturer's instruction and then transcribed into cDNA using AMV reverse transcription system (Promega GmbH, Germany). Primer sequences for the genes of interest for the RT-PCR were described in table 3.1. PCR conditions were: 5 min at 94°C, and then 39 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. The PCR reactions were separated by gel electrophoresis and the product bands were visualized and photographed under ultraviolet light densiometry. The results were expressed as relative quantification of mRNA levels compared to mRNA levels of the internal reference gene GAPDH and the control group without stimulation.

Gene	Primers: sense (s) and antisense (as); 5' - 3'		accession#
Col I α 2	s	ATG GTC GCA CTG GAC ATC C	NM_000089.3
	as	GGT GGC TGA GTC TCA AGT C	
Col III	s	TGG AGT GTC TGG ACC AAA AG	NM_000090.2
	as	ACC ATC TGA TCC AGG GTT TC	
Vim	s	CTG CCA ACC GGA ACA ATG AC	NM_003380.2
	as	CAC GAA GGT GAC GAG CCA TTT	
α -SMA	s	CGA CCG AAT GCA GAA GGA GA	NM_001613.1
	as	TTT GCG GTG GAC AAT GGA AG	
Des	s	ATG TGG AGA TTG CCA CCT ACC GGA AGC TG	NM_001927.3
	as	GTG TCC TGG GAT GGA AGA AGG CTG GCT T	
GAPDH	s	TTA GCA CCC CTG GCC AAG G	NM_002046.3
	as	CTT ACT CCT TGG AGG CCA TG	

Table 3.1. Human specific oligonucleotide primers used for polymerase chain reactions. Col Iα2, collagen I alpha2; Col IIIα1, collagen III alpha1; Vim, vimentin; α-SMA, alpha smooth muscle actin; Des, desmin; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

Statistical analysis

The means ± SEM were calculated for all variables tested. Statistical analysis of data was performed by unpaired t-test and statistical significance was accepted at $p<0.05$.

Results

Light microscopic analysis showed that cultured cells were healthy in all groups (Figure 1). Cells treated with low-dose FGF-2 (3 ng/ml) reached higher cell densities than cultures in all other groups and possessed a more homogeneous spindle-shaped morphology at day 7, 14, 21, and 28 (Figure 1e-h). Both FGF-2 peptide groups (3 ng/ml and 30 ng/ml) showed similar cell morphology and growth behaviour as cells cultured without stimulation over the whole time period (Figure 3.1a-d, 3.1m-p). Low-dose FGF-2 peptide treated cells arranged to stellar cell clusters on day 28 (Figure 3.1a-d).

Cell count and cell viability, and cell proliferation

Generally, the cell density was significantly higher on day 21 and 28 in the ASC cultures with low-dose (3 ng/ml) FGF2- or FGF-2 peptide compared with cultures of high-dose (30 ng/ml) FGF-2 or FGF-2 peptide on days 7, 14, 21, and 28 (Figure 2a). The cell density of both FGF-2 peptide groups was below or on the level of the control group without FGF-2 or FGF-2 peptide. The cell number increased significantly in the FGF-2 low-dose group on day 21 compared to the FGF-2 peptide and control group ($p<0.01$). The proliferation of both FGF-2 peptide groups were almost on the same level as that of the control group at all time points (Figure 3.2b-c). The FGF-2 groups showed higher proliferation as all other groups. The MTT and BrdU assay detected the highest signal in both FGF-2 groups on day 14.

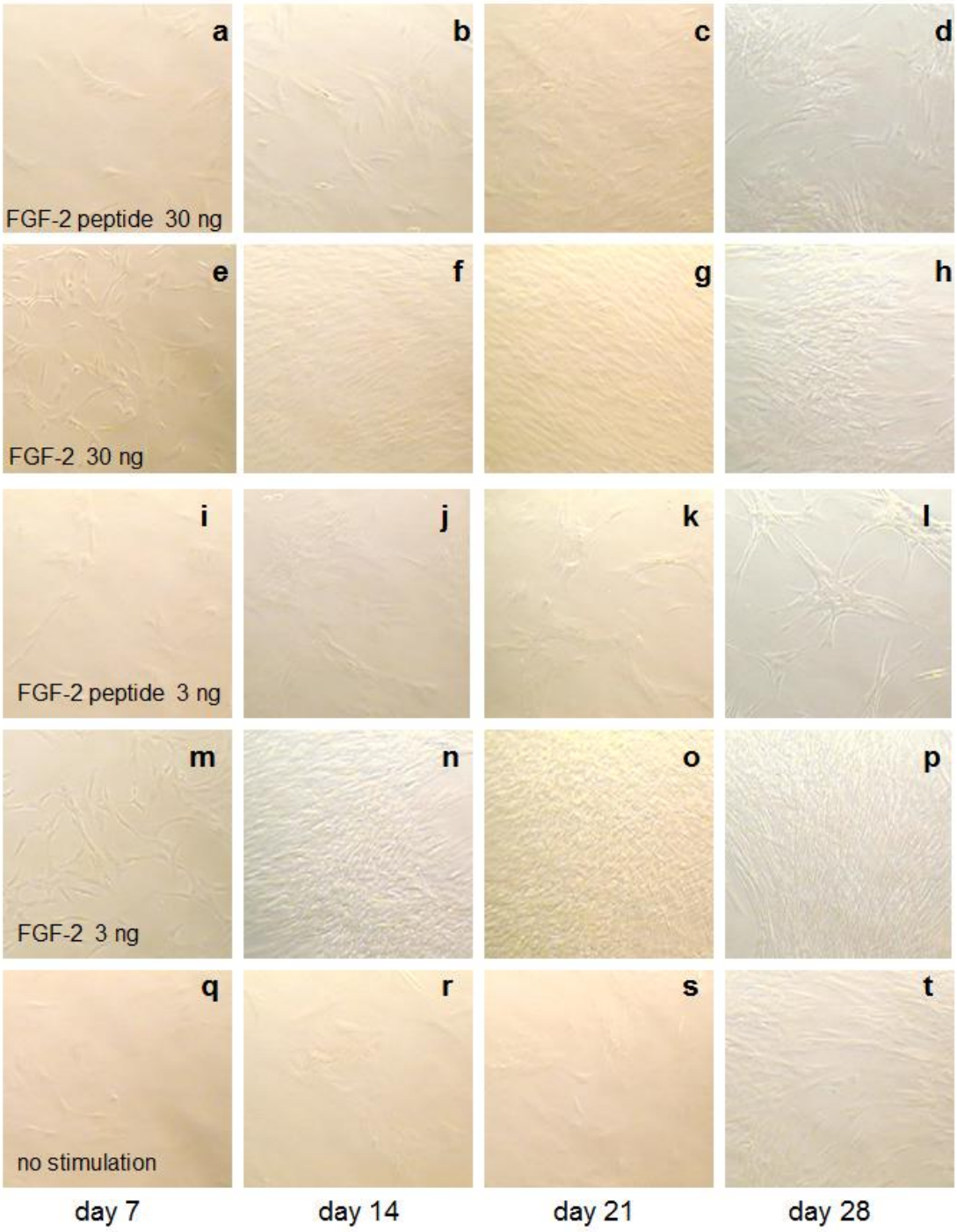


Figure 3.1. Light micrographs from ASCs stimulated with FGF-2 peptide (**a-d** and **m-p**) and FGF-2 (**e-h** and **q-t**) in 2 different concentrations in comparison to the control group (**i-l**) over 28 days (magnification, x100).

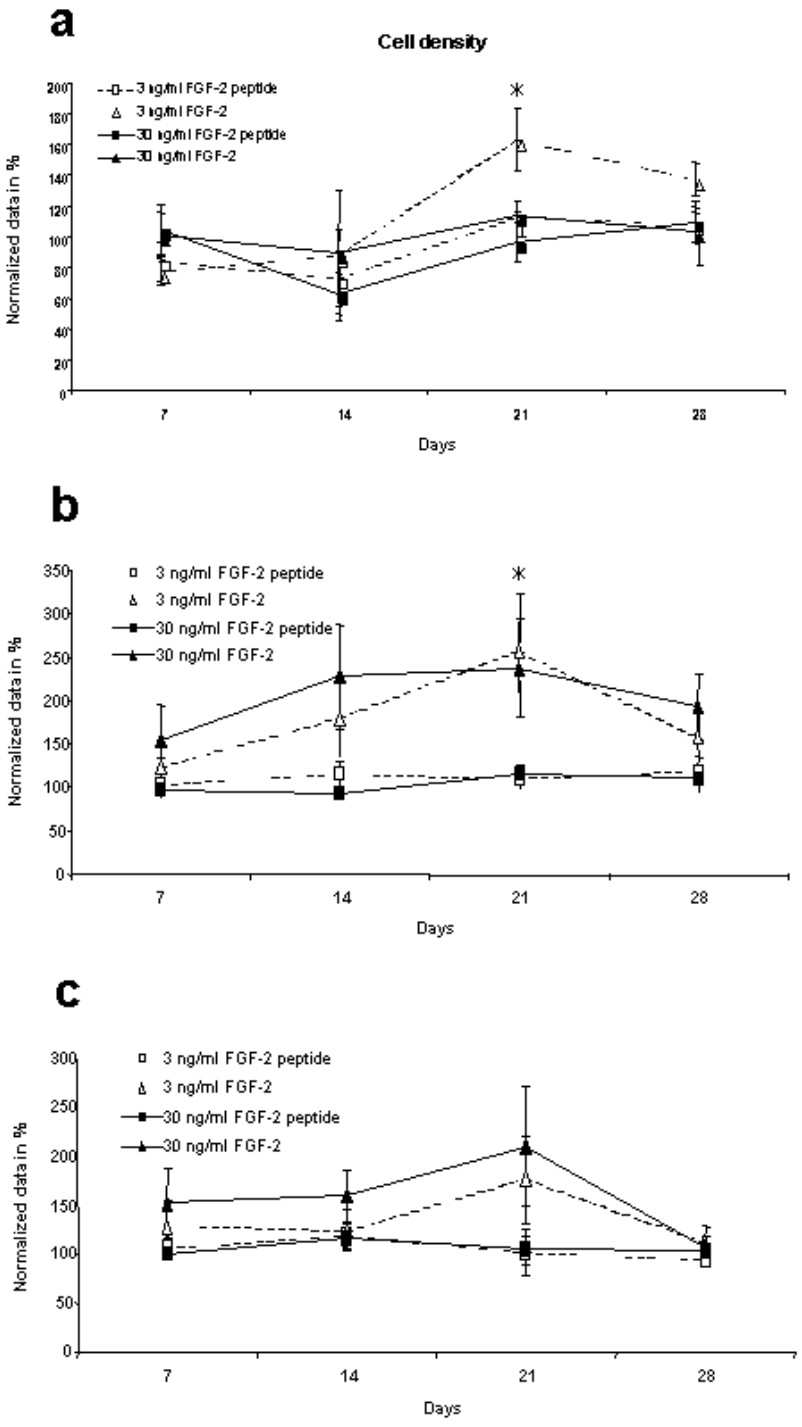
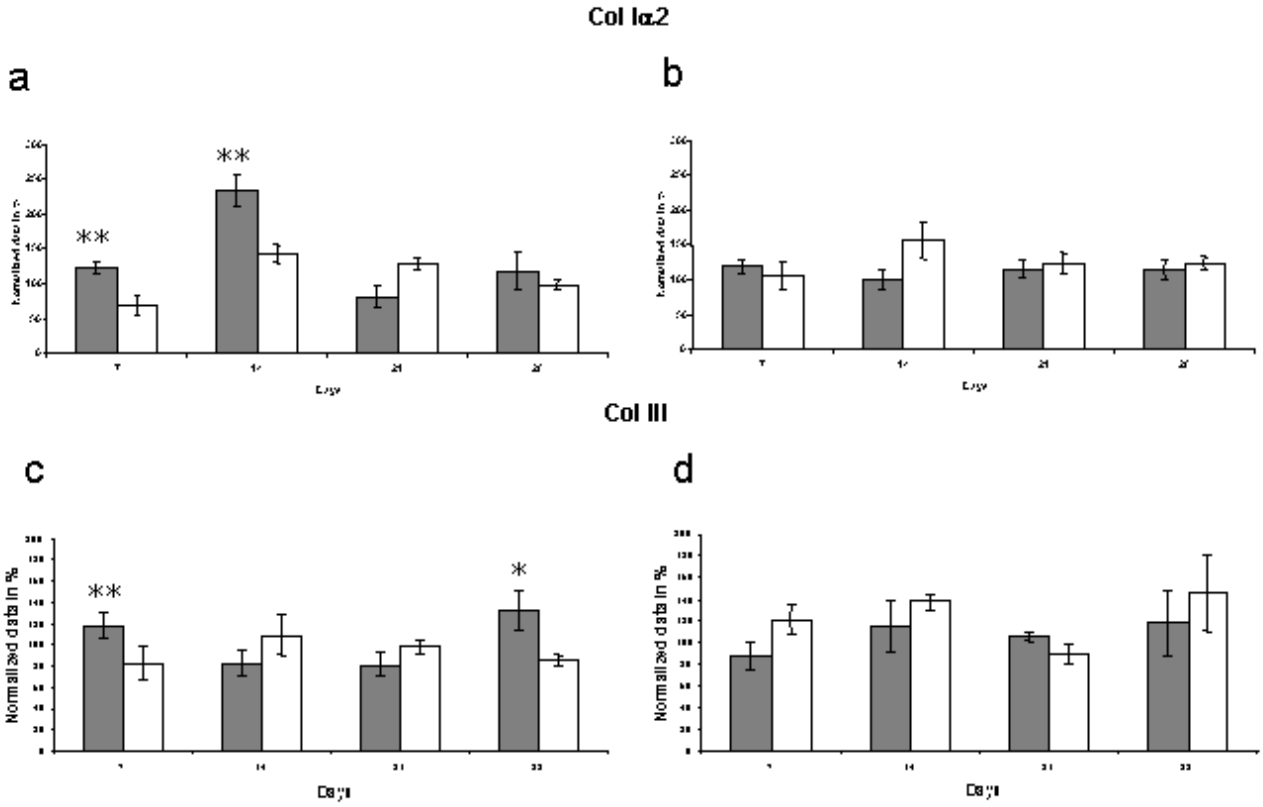


Figure 3.2. Cell count (**a**), cell viability measured by MTT assay (**b**), and cell proliferation (**c**) analyzed by BrdU incorporation of human ASCs on day 7, 14, 21, and 28 after stimulation with low-dose (3 ng/ml) and high-dose (30 ng/ml) FGF-2 peptide and FGF-2 (*$p<0.05$).

Gene expression

As measured by PCR, the growth factors FGF-2 and synthetic FGF-2 peptide utilized in this study displayed differentiation in osteogenetic direction on ASCs. In these experiments, expression of mRNA for a positive marker of osteogenesis Col Iα2 and for a fibrous scleroprotein Col IIIα1 was determined. For Col Iα2 and Col IIIα1 gene expression (Figure 3.3a-d), only the treatment with 3 ng/ml FGF-2 peptide for 7 days led to significantly enhanced gene expression of 35% and 96% respectively as compared with the addition of FGF-2 (**p<0.01). ASCs treated with 30 ng/ml of FGF-2 peptide showed a continuous expression pattern of Col Iα2 over the whole time period of 28 days. The ratio of Col Iα2 to Col IIIα1 was significant higher in both FGF-2 peptide groups (Figure 3.3e-f).



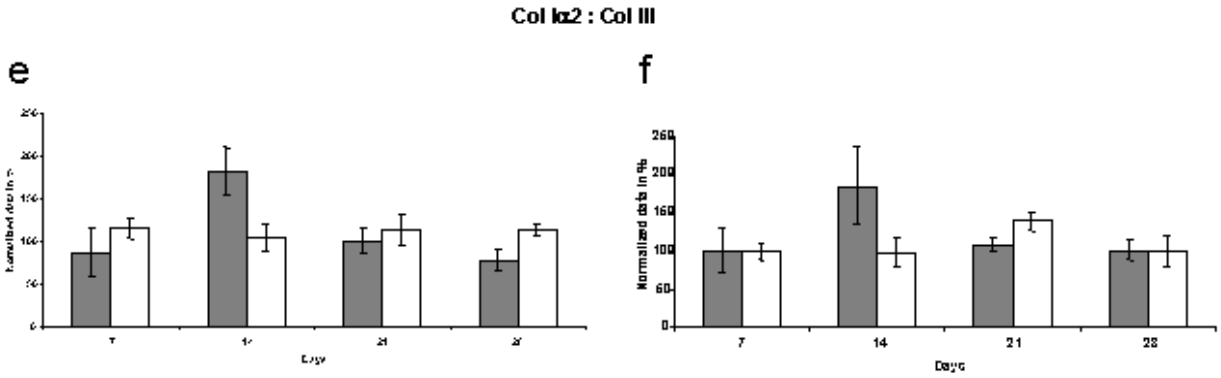
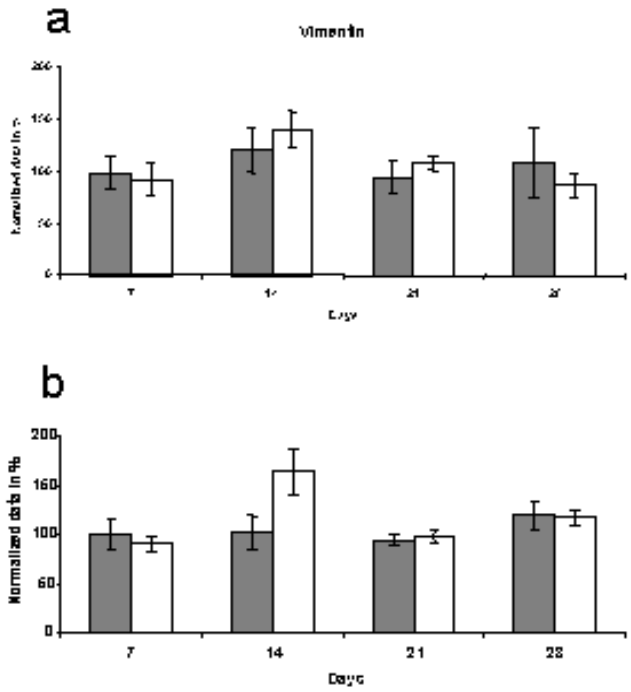


Figure 3.3. Comparison of mRNA expression of collagen Iα2 and collagen IIIα1 in the low-dose (**a**, **c**) and the high-dose group (**b**, **d**) of FGF-2 peptide and FGF-2. The ratio of collagen Iα2 to collagen IIIα1 showed a significant increase in both FGF-2 peptide groups (e, f; *, $p < 0.05$). The results were expressed as relative quantification of mRNA levels compared to mRNA levels of the internal reference gene GAPDH and the control group without stimulation. Open bar: FGF-2 peptide; closed bar: FGF-2 stimulation.

Vimentin, desmin, and α-SMA mRNAs were detected in each group at all time points (Figure 3.4a-f). The 3 ng/ml and 30 ng/ml FGF-2 peptide groups showed a significantly higher desmin expression on day 7 and 28 compared to FGF-2 stimulation (*$p<0.05$). In both FGF-2 peptide groups the expression levels of vimentin, desmin, and α-SMA reached highest levels on day 28. The addition of 3 ng/ml and 30 ng/ml of FGF-2 resulted in the highest mRNA levels already on day 14.



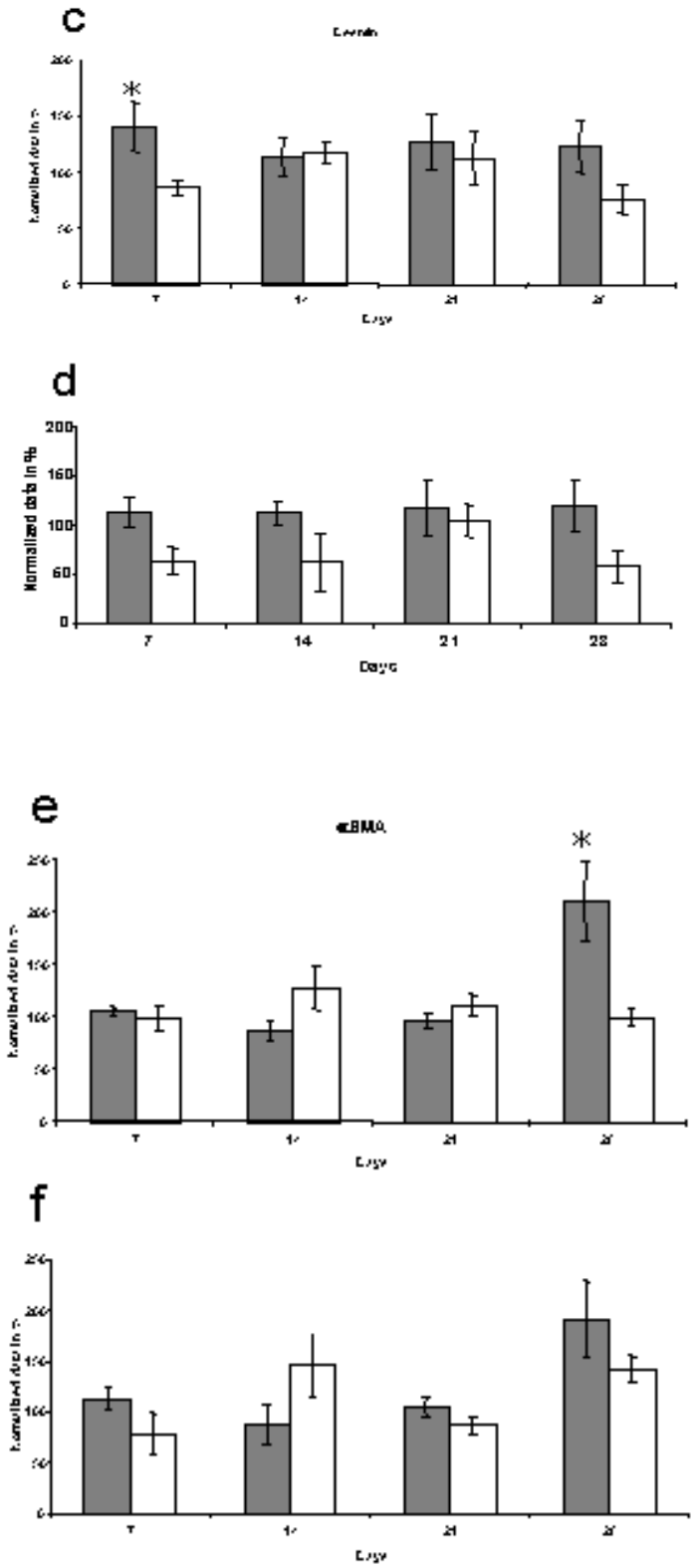


Figure 3.4. Expression patterns of mRNA of vimentin (**a**, **b**), desmin (**c**, **d**), and α-smooth muscle actin (**e**, **f**). The results were expressed as relative quantification of mRNA levels compared to mRNA levels of the internal reference gene GAPDH and the control group without stimulation. Open bar: FGF-2 peptide; closed bar: FGF-2 stimulation.

Discussion

This study demonstrates various effects of recombinant human fibroblast growth factor-2 (FGF-2) and of one synthetic FGF-2 peptide on proliferation and differentiation of isolated human adipose-derived stem cells (ASCs). FGF-2 often induces proliferation and migration in the same cell type during development, angiogenesis, regeneration, and cancer.(35) Koumoto et al. showed an upregulation of osteoblast migration and a decrease of alkaline phosphatase activity and osterix mRNA expression by FGF-2.(36) Dvorak et al. found that FGF-2 can be implicated in the self-renewal process and the differentiation of human embryonic stem cells.(37,38) Some other studies also approved in vitro mineralization, elevation of proliferation, and stimulation of osteogenic parameters of human and rat bone marrow stromal cells.(12,13,39-43) FGF-2 also showed a high potency in cell growth and differentiation of human adipose-derived stem cells.(44,45)

In comparison to previous studies especially of bone marrow stromal cells treated with FGF-2 our results present an interesting effect into the development of connective tissue and tendon with ASCs. Similar to Hankemeier et al. the data of this study showed on one hand the estimated growth effect of FGF-2.(31) The low-dose FGF-2 enhanced cell growth within 21 days and cell proliferation within 14 days. In contrast, the high-dose group led to an increase in cell growth within 7 days. However, it showed a subsequent decrease to the values of the control group without stimulation. Cell proliferation reached its maximum within 14 days and decreased moderately afterwards. Generally, the cell growth was higher in the low-dose FGF-2 group than in the high-dose one.

On the other hand FGF-2 peptide stimulation had no effect on cell growth and cell proliferation in both the 3 ng/ml and 30 ng/ml group compared to the control group. For cell growth the method of cell counting and the MTT assay led to similar tendency in the results.

In contrast to cell growth and cell proliferation, stimulation with both 3 ng/ml and 30 ng/ml of FGF-2 peptide upregulated collagen Iα2 already within 7 days compared to the addition of FGF-2. Another marker for development into connective tissue and tendon, collagen III, showed a higher mRNA expression level in the low-dose group within 7 days, as well. The effects of the low-dose concentration of FGF-2 peptide on ASCs were reflected in the ratio of collagen Iα2 to collagen III. The lower values (as a result of high collagen III expression) in the FGF-2 peptide group suggested differentiation into the development of connective tissue and tendon. The high differentiation effect of FGF-peptide also was detected on the mRNA expression of alpha smooth muscle actin (α-SMA) and desmin, which are essential extra-cellular matrix proteins and cytosceletal elements for tissue engineering applications. Desmin elevated in the low-dose and the high-dose FGF-2 peptide group within 7 days and showed a second maximum after 28 days. In contrast, α-SMA was highly upregulated in both FGF-2 peptide groups only within 28 days. Vimentin showed similar expression levels as the control group during the entire observation period.

Moreover, there was no mRNA expression signal detected in all groups of stimulation and in the control group as well for the adipogenic marker lipoprotein lipase (data not shown). Therefore, we could exclude differentiation of ASCs into adipocytes.

The normal growth and proliferation behaviour of ASCs in both FGF-2 peptide groups during the whole time period led to the conclusion that FGF-2 loses its domain or configuration with the truncation, which is responsible for increasing proliferation. In contrast to that, especially the low-dose FGF-2 peptide group possessed a very early differentiation potential of the ASCs for collagen Iα2, collagen III, and desmin.

For further experiments stimulation with a combination of FGF-2 and FGF-peptide might lead to a high cell number and an early differentiation of isolated ASCs.

Conclusion

Fast growth, proliferation and differentiation of cells, which were isolated from waste material, is one of the aims for tissue engineering applications. The stimulation with FGF-2 and FGF-peptide led to differentiation patterns in essential extracellular matrix proteins and cytoskeletal elements, which are key patterns for tendons and ligaments development.

Acknowledgement

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References

1. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng In press.

2. McIntosh K, Zvonic S, Garrett S, Mitchell JB, Floyd ZE, Hammill L, Kloster A, Di Halvorsen Y, Ting JP, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM. The immunogenicity of human adipose-derived cells: temporal changes in vitro. Stem Cells In press.

3. Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, Di Halvorsen Y, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells In press.

4. Halvorsen YC, Wilkison WO, Gimble JM. Adipose-derived stromal cells--their utility and potential in bone formation. Int J Obes Relat Metab Disord In press.

5. Guilak F, Lott KE, Awad HA, Cao Q, Hicok KC, Fermor B, Gimble JM. Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. J Cell Physiol In press.

6. Schor AM, Allen TD, Canfield AE, Sloan P, Schor SL. Pericytes derived from the retinal microvasculature undergo calcification in vitro. J Cell Sci In press.

7. Doherty MJ, Ashton BA, Walsh S, Beresford JN, Grant ME, Canfield AE. Vascular pericytes express osteogenic potential in vitro and in vivo. J Bone Miner Res In press.

8. Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H, Guilak F. Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. Biochem Biophys Res Commun In press.

9. Saladin R, Fajas L, Dana S, Halvorsen YD, Auwerx J, Briggs M. Differential regulation of peroxisome proliferator activated receptor gamma1 (PPARgamma1) and PPARgamma2 messenger RNA expression in the early stages of adipogenesis. Cell Growth Differ In press.

10. Halvorsen YD, Bond A, Sen A, Franklin DM, Lea-Currie YR, Sujkowski D, Ellis PN, Wilkison WO, Gimble JM. Thiazolidinediones and glucocorticoids synergistically induce differentiation of human adipose tissue stromal cells: biochemical, cellular, and molecular analysis. Metabolism In press.

11. Halvorsen YD, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL, Paschalis EP, Wilkison WO, Gimble JM. Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Eng In press.

12. Marie PJ. Fibroblast growth factor signaling controlling osteoblast differentiation. Gene In press.

13. Varkey M, Kucharski C, Haque T, Sebald W, Uludag H. In vitro osteogenic response of rat bone marrow cells to bFGF and BMP-2 treatments. Clin Orthop Relat Res In press.

14. Gospodarowicz D. Biological activities of fibroblast growth factors. Ann N Y Acad Sci In press.

15. Bikfalvi A, Klein S, Pintucci G, Rifkin DB. Biological roles of fibroblast growth factor-2. Endocr Rev In press.

16. Bugler B, Amalric F, Prats H. Alternative initiation of translation determines cytoplasmic or nuclear localization of basic fibroblast growth factor. Mol Cell Biol In press.

17. Florkiewicz RZ, Shibata F, Barankiewicz T, Baird A, Gonzalez AM, Florkiewicz E, Shah N. Basic fibroblast growth factor gene expression. Ann N Y Acad Sci In press.

18. Abraham JA, Whang JL, Tumolo A, Mergia A, Fiddes JC. Human basic fibroblast growth factor: nucleotide sequence, genomic organization, and expression in mammalian cells. Cold Spring Harb Symp Quant Biol In press.

19. Shimasaki S, Emoto N, Koba A, Mercado M, Shibata F, Cooksey K, Baird A, Ling N. Complementary DNA cloning and sequencing of rat ovarian basic fibroblast growth factor and tissue distribution study of its mRNA. Biochem Biophys Res Commun In press.

20. Araujo DM, Cotman CW. Basic FGF in astroglial, microglial, and neuronal cultures: characterization of binding sites and modulation of release by lymphokines and trophic factors. J Neurosci In press.

21. Benezra M, Vlodavsky I, Yayon A, Bar-Shavit R, Regan J, Chang M, Ben Sasson S. Reversal of basic fibroblast growth factor-mediated autocrine cell transformation by aromatic anionic compounds. Cancer Res In press.

22. Partanen J, Vainikka S, Korhonen J, Armstrong E, Alitalo K. Diverse receptors for fibroblast growth factors. Prog Growth Factor Res In press.

23. Ornitz DM, Yayon A, Flanagan JG, Svahn CM, Levi E, Leder P. Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. Mol Cell Biol In press.

24. Sahni A, Francis CW. Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation. Blood In press.

25. Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. J Investig Dermatol Symp Proc In press.

26. Alarid ET, Cunha GR, Young P, Nicoll CS. Evidence for an organ- and sex-specific role of basic fibroblast growth factor in the development of the fetal mammalian reproductive tract. Endocrinology In press.

27. Sahni A, Odrljin T, Francis CW. Binding of basic fibroblast growth factor to fibrinogen and fibrin. J Biol Chem In press.

28. Peng H, Sahni A, Fay P, Bellum S, Prudovsky I, Maciag T, Francis CW. Identification of a binding site on human FGF-2 for fibrinogen. Blood In press.

29. Sindelar, T. Characterization and identification of fibrin binding domain of hFGF-2. 2004. Diploma Thesis.
Ref Type: Thesis/Dissertation

30. Morton, T. J., Kloesch, B., van Griensven, M., and Redl, H. Production of rhFGF-peptides. 2009.
Ref Type: Unpublished Work

31. Hankemeier S, Keus M, Zeichen J, Jagodzinski M, Barkhausen T, Bosch U, Krettek C, van Griensven M. Modulation of proliferation and differentiation of human bone marrow stromal cells by fibroblast growth factor 2: potential implications for tissue engineering of tendons and ligaments. Tissue Eng In press.

32. Wolbank S, Peterbauer A, Wassermann E, Hennerbichler S, Voglauer R, van Griensven M, Duba HC, Gabriel C, Redl H. Labelling of human adipose-derived stem cells for non-invasive in vivo cell tracking. Cell Tissue Bank In press.

33. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods In press.

34. Porstmann T, Ternynck T, Avrameas S. Quantitation of 5-bromo-2-deoxyuridine incorporation into DNA: an enzyme immunoassay for the assessment of the lymphoid cell proliferative response. J Immunol Methods In press.

35. Boilly B, Vercoutter-Edouart AS, Hondermarck H, Nurcombe V, Le B, X. FGF signals for cell proliferation and migration through different pathways. Cytokine Growth Factor Rev In press.

36. Koumoto, T., Masaki, C., Yamanaka, T., and Akagawa, Y. Effects of basic fibroblast growth factor (FGF-2) on cell migration and differentiation of MC3T3-E1 osteoblast-like cells. 2006.
Ref Type: Unpublished Work

37. Dvorak P, Dvorakova D, Koskova S, Vodinska M, Najvirtova M, Krekac D, Hampl A. Expression and potential role of fibroblast growth factor 2 and its receptors in human embryonic stem cells. Stem Cells In press.

38. Doukas J, Blease K, Craig D, Ma C, Chandler LA, Sosnowski BA, Pierce GF. Delivery of FGF genes to wound repair cells enhances arteriogenesis and myogenesis in skeletal muscle. Mol Ther In press.

39. Hori Y, Inoue S, Hirano Y, Tabata Y. Effect of culture substrates and fibroblast growth factor addition on the proliferation and differentiation of rat bone marrow stromal cells. Tissue Eng In press.

40. Lisignoli G, Zini N, Remiddi G, Piacentini A, Puggioli A, Trimarchi C, Fini M, Maraldi NM, Facchini A. Basic fibroblast growth factor enhances in vitro mineralization of rat bone marrow stromal cells grown on non-woven hyaluronic acid based polymer scaffold. Biomaterials In press.

41. Martin I, Muraglia A, Campanile G, Cancedda R, Quarto R. Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. Endocrinology In press.

42. Frank O, Heim M, Jakob M, Barbero A, Schafer D, Bendik I, Dick W, Heberer M, Martin I. Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro. J Cell Biochem In press.

43. Ozono S, Fujita T, Matsuo M, Todoki K, Ohtomo T, Negishi H, Kawase T. Co-treatment with basic fibroblast growth factor and 17beta-estradiol in the presence of dexamethasone accelerates bone formation by rat bone marrow stromal cell culture. Nihon Hotetsu Shika Gakkai Zasshi In press.

44. Inoue S, Hori Y, Hirano Y, Inamoto T, Tabata Y. Effect of culture substrate and fibroblast growth factor addition on the proliferation and differentiation of human adipo-stromal cells. J Biomater Sci Polym Ed In press.

45. Suga H, Shigeura T, Matsumoto D, Inoue K, Kato H, Aoi N, Murase S, Sato K, Gonda K, Koshima I, Yoshimura K. Rapid expansion of human adipose-derived stromal cells preserving multipotency. Cytotherapy In press.

Chapter IV

Controlled release of substances bound to fibrin-anchors or of DNA

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Abstract

Fibrin sealants have been proposed as depot matrices for substances due to their biocompatibility, advantageous biological properties, and widespread use in wound healing. Our study showed possibilities for a continuous and controlled release of pharmaceutically active substances out of a fibrin matrix. Substances of interest were linked to naturally occuring fibrin-anchors, i) thrombin, ii) fibronectin, and iii) DNA. Fibronectin and thrombin bind fibrin by a specific binding moiety and DNA by charge. Fibrin clots were prepared from Tisseel Fibrin Sealant (Baxter AG, Vienna) by mixing 100 mg/ml fibrinogen, the substance of interest and 4 U/ml of thrombin. Chemical crosslinking of proteins was performed with EDC using standard reaction conditions. Modification of proteins with biotin and PPACK was performed with N-hydroxysuccinimid activated compounds. With fibrin-anchors pharmaceutically active substances, i.e. tumor necrosis factor (TNF), albumin and plasmid-DNA were continously released over 10 days. In conclusion, the naturally occuring proteins fibronectin and thrombin with a fibrin binding moiety or DNA can be used as fibrin-anchors.

Introduction

Natural extracellular matrices (ECMs) of tissues are regarded as depots for growth factors, which affect many physiological processes of surrounding tissues (1-3). Similar to ECMs, biomatrix preparations, such as fibrin-based biomaterials, may act as temporary depots for the sustained release of substances or drugs. Fibrin sealants, a fibrin-based biomaterial, are optimally suited as drug depots, because of their biocompatibility, advantageous biological properties, and established use in haemostasis, tissue sealing and support of wound healing (4-6). Commercially available fibrin sealants contain fibrinogen and thrombin isolated from human plasma (7).

Fibrin sealants (FS) rapidly, consistently and easily form a clot when fibrinogen is mixed with thrombin. Fibrinogen, a soluble plasma protein, is converted into insoluble fibrin monomers, via proteolytic cleavage by thrombin. These monomers aggregate into fibrils to form a three-dimensional biopolymer clot (8). The 3-D clot is, ultimately, degraded via proteolysis by plasmin, and the degradation products are then resorbed by phagocytosis (7). The natural degradation of fibrin sealants is a prerequisite for a controlled release drug depot.

Therefore, if a pharamceutically active substance is linked to a fibrin clot, the substance can be released in a controlled fashion. This also implies that the substance must be bound to the fibrin clot in either a reversible or an irreversible way. A pharmaceutically active substance can be modified or the fibrin matrix can be modified to bind the substance in the matrix.

A fibrin binding moiety, or fibrin-anchor, can be directly linked to a pharmaceutically active substance or indirectly linked to a drug binding moiety. The resulting structure, whether directly or indirectly bound, are termed fibrin conjugates (Figure 4.1). Previous studies evaluated vascular endothelial growth factor, basic fibroblast growth factor and interleukin-1β as naturally occurring fibrin-anchors (3,9,10).

Alternatively, tranexamic acid (t-AMCA, 4-(aminmethyl)cyclohexane carboxylic acid), an antifibrinolytic agent, can modify the density and structure of the fibrin matrix. Tranexamic acid decreases the density and increases the free space within the clot to retain affinity bound substances (11).

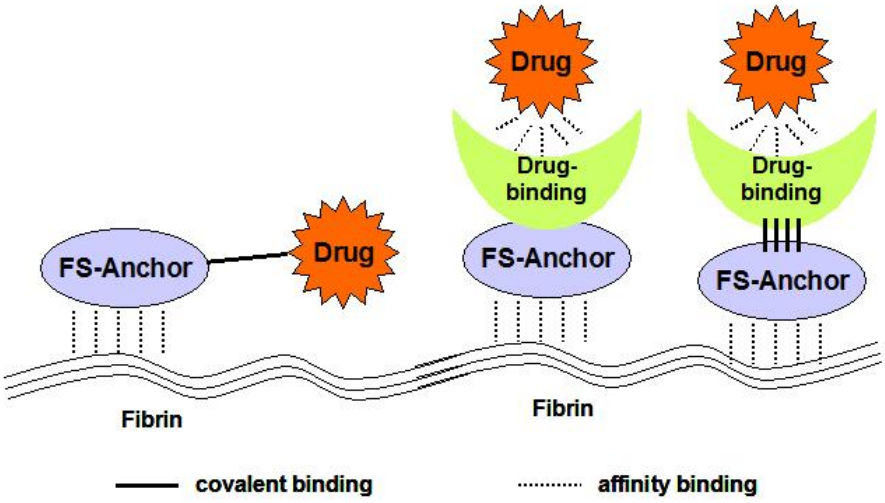

Figure 4.1. Fibrin conjugates consist of a fibrin-anchor directly bound to a pharmaceutically active substance (left) or indirectly bound via a drug binding moiety (right). Moieties are either affinity bound or covalently bound.

The aim of the work is to evaluate thrombin, fibronectin and a specific plasmid-DNA (pGEM-IL10) as naturally occurring fibrin-anchors and to compare diffusion rates of high and low weight molecules from a fibrin clot modified by tranexamic acid. Tranexamic acid is one of the most common fibrinolysis inhibitors and influences the structure and mechanical properties of fibrin (12-14). The release of cytochrome C as an example for a low molecular weight substance and the the release of a high molecular β-galactosidase, both without intrinsic affinity to fibrin was tested.

Secondly, the diffusion was tested of substances linked to fibrin-anchors based upon naturally occuring proteins with a fibrin binding moiety, such as i) thrombin ii) fibronectin, or due to charge iii) DNA. Fibrin clots for

release experiments were prepared from Tisseel Fibrin Sealant and after polymerization incubated in phosphate-buffered saline (PBS).

Thrombin as a natural byproduct in fibrin formation has a tight binding to fibrin with high binding capacity but without crosslinking. Binding of proteins to thrombin by random chemical crosslinking reactions bears the risk that lysin residues within the fibrin binding exo-loop of thrombin become modified and the fibrin/fibrinogen binding activity is lost. To avoid this effect, a modified form of the irreversible thrombin inhibitor PPACK was used to bind proteins to a specific site on thrombin. The modified PPACK is bound easily to a protein of interest and will direct the protein to the active site of thrombin and form a covalent link without affecting the fibrin/fibrinogen binding activity of thrombin.

Fibronectin, the second natural byproduct in fibrin formulation, is a large molecule and binds to fibrin via affinity and FXIII-crosslinking. Linking to fibrin via fibronectin binding was done by covalently 1-etyl-3-3-dimethyl-aminopropylcarbodiimide (EDC) binding of fibronectin to a tumor necrosis factor (TNF) antibody for further affinity binding of TNF (15).

Compared to thrombin and fibronectin DNA binds because of its charge strongly to fibrin and fibrinogen. First, plasmid-DNA encoding for a gene is embedded in the fibrin matrix and its affinity for fibrin and fibrinogen allows a slow but sustained release of DNA over a long period of time. The use of DNA as a binding moiety is a new concept of controlled and consistant release of substances of interest and should not be mixed up with genetransfer.

Materials and methods

Thrombin as a fibrin-anchor

The fibrin conjugate was prepared by dissolving 8.8 µM of biotin-N-hydroxysuccinimide (biotin-X-NHS; Calbiochem, CA, USA) in N,N-dimethylformamide (DMF; Fluka, Germany) and then incubated with 9.54 µM D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK: NHS-PPACK; Pichem, Austria) in 10 mM hydrochloric acid (HCl; Sigma, Vienna, Austria) buffer at room temperature (RT) for 30 minutes. Unbound PPACK was saturated with 0.13 µM valin (Merck, Germany) at RT for 30 minutes (16). Following saturation, 322 µl of PPACK bound biotin and PPACK bound valin solution was added to 66 µg of thrombin dissolved in 400 µl phosphate buffered saline (PBS). After a 30-minute incubation at RT, a size exclusion column (Amicon Y10; Millipore, MA, USA) was used to isolate thrombin bound PPACK from unbound PPACK, biotin, valin and other forms. The resulting fibrin conjugate consists of thrombin as the fibrin anchor, PPACK as the drug binding moiety, and biotin as the pharmaceutically active substance. Fibrin clots were spiked with 64 µl of the PPACK-thrombin-biotin binding conjugate.

The release of biotin was measured using streptavidin-peroxidase (streptavidin-POX) immuno assay. A microtiter plate was incubated with diluted supernatant fluids over night at 4°C to allow proteins to attach to the plate. Then the plates were washed, 2 hours saturated with 1% bovine serum albumin (BSA) at RT, washed again with PBS and incubated for 1 hour with streptavidin-POX conjugate (Sigma-Aldrich, Vienna) diluted 1:5000 in 0.1% BSA. After washing the plates again in PBS, o-phenylenediaminehydrochloride (OPD) solution was added, incubated, and the color reaction was stopped with 3 M sulfuric acid (Sigma, Vienna, Austria). The absorbance at 492 nm and 620 nm for the reference were measured with a microplate reader (Spectra, SLT Labinstruments).

Fibronectin as a fibrin-anchor

The fibronectin binding conjugate was prepared by dissolving 9 mg/ml tumor necrosis factor (TNF) antibody (American Laboratories Inc., Connecticut, USA) in 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Aldrich, USA) solution. After 5 minutes of incubation at RT 4.5 mg/ml fibronection (gift from T. Seelich, Baxter AG) was added. The reaction was incubated for 4 hours at RT and stopped by adding sodium acetate (Sigma, Vienna, Austria) to a final concentration of 100 mM. The EDC-fibronectin-TNF antibody binding conjugate were dialysed against PBS using an Amicon Y100; (Millipore, MA, USA) size exclusion column (17).

The degree of coupling was analyzed via a sandwich-enzyme linked immuno assay (ELISA). Briefly, a microtiter plate was coated with fibrinogen overnight at 4°C and subsequently incubated with EDC-fibronectin-TNF-antibody binding conjugate or an uncoupled mixture of TNF-antibody and fibronectin for 1 hour at RT. After washing the plate with PBS, recombinant human TNF (Knoll AG, Germany) was added and incubated for 1 hour at RT. After an other wash with PBS, the plate was first incubated with a secondary TNF antibody (Jackson Immuno Research Europe Ltd., Great Britain) recognizing another epitope for 1 hour at RT and then 15 minutes with a goat anti rabbit peroxidase conjugate. OPD was used as substrate for peroxidase. The colour reaction was stopped after 2 minutes with 0.5 M sulfuric acid and the absorbance measured at 492 nm and 620 nm for reference with a microplate reader.

Same parts of EDC-fibronectin-TNF antibody binding conjugate and TNF were incubated for 2 hours at RT and then mixed to the fibrinogen component before adding thrombin. The polymerized clots were coated with PBS and incubated over a time period. The TNF concentrations in the supernatant fluids were analyzed by ELISA. Microtiter plates were coated with the supernatants of the clots and incubated for 24 hours at 4°C. After that it followed the same procedure as described above.

DNA as a fibrin-anchor

The full length cDNA for baboon IL-10 was amplified using a standard polymerase chain reaction (PCR) (94°C-30'', 58°C-30'', 72°C-30'', 40 cycles) (18). The forward (5'-CCAGGCCAGGGCACCCAGTCTGA-3') and the reverse (5'-ATAGAGTCGCCACCCTGATGTCTC-3') primer obtained a ~500 base pairs PCR product, which was ligated into pGEM-T Easy vector (Promega, Germany). The product pGEM/IL-10 was used to transform *Escherichia coli* DH5α (Invitrogen, Lofer, Austria). Plasmid DNA from positive bacteria colonies were isolated from 5 ml overnight cultures using Miniprep kit (Qiagen, Germany). DNA concentration was measured at 260 nm and 280 nm as the reference using a photometer (SmartSpec™3000, Bio-Rad Laboratories, Vienna, Austria).

Fibrin clots were spiked with either 2 or 20 µg plasmid-DNA (pGEM-IL10) and incubated in PBS or 25000 U/ml urokinase (Sigma-Aldrich, Vienna).

Released plasmid-DNA was purified with Qiagen DNA extraction kit following the manufacture's instructions (Qiagen, Hilden, Germany). After amplification of the IL-10 gene using polymerase chain reaction (94°C-30'', 50°C-30'', 72°C-30'', 30 cycles) with the primer pair (forward: GATTCTACGTCGACCGGTCAT, reverse: CAGTCGAGGCTGATAGCGAGCT), the PCR products were visualized on a 1% agarose gel. The amount of released DNA was densitometrically assessed in a semiquantitative manner (The Mini Cycler, MJ Research, INC, MA, USA).

Fibrin clots without FS-anchor

Tranexamic acid (6.67 mM and 667 mM, Sigma, Vienna, Austria) was added to the fibrinogen component to decrease density and crosslinking and increase fibril size of the fibrin network. Cytochrom C (20 mg/ml; Sigma, Vienna, Austria) or β-galactosidase (10 mg/ml; Sigma, Vienna, Austria) was added to the fibrinogen component as the low and high molecular weight compound, respectively.

The release of cytochrome C and β-galactosidase was measured photometrically at 409 nm and 420 nm, respectively, using a DU-70 Spectrophotometer (Beckmann Instruments, New Jersey, USA).

Preparation of Fibrin Clots

Tisseel® VH Fibrin Sealant (Baxter AG, Vienna, Austria) was used to prepare 200 µl clots. The fibrinogen component was reconstituted with an aprotinin solution and the thrombin component with a 40 mM calcium chloride solution according to the manufacturer's instruction. The final concentration of fibrinogen was 50 mg/ml and of thrombin 2 IU/ml, respectively.

Polymerized fibrin clots were then transferred into cryo-tubes, covered with 1 ml PBS and incubated at 37°C. The supernatant fluid was collected at 2 hours and every 24 hours thereafter for 12 days and stored at -20°C until analysis. PBS or urokinase were replaced and the clot was continuously incubated at 37°C between collections. On the12th day, remaining clots were lysed with 25 mg/ml trypsin and the substance content in the lysate was analyzed.

Statistic analysis

Experiments were run in triplicates (N = 3) and repeated three to five times (3-5). Results were displayed as mean and standard deviation.

Results

Thrombin as a fibrin-anchor

Thrombin -PPACK coupling showed a higher thrombin binding capacity to fibrin than free active thrombin, (data not shown). Conjugation of biotinylated albumin to thrombin via the NHS-PPACK allows over 75% retention of albumin in a fibrin clot. In contrast, free albumin without binding to thrombin is released mostly within 24 hours from the clot (Figure 4.2a). The release of PPACK-coupled and uncoupled streptavidin without thrombin as a fibrin-anchor shows no difference of retardation in the measured supernatants (Figure 4.2b).

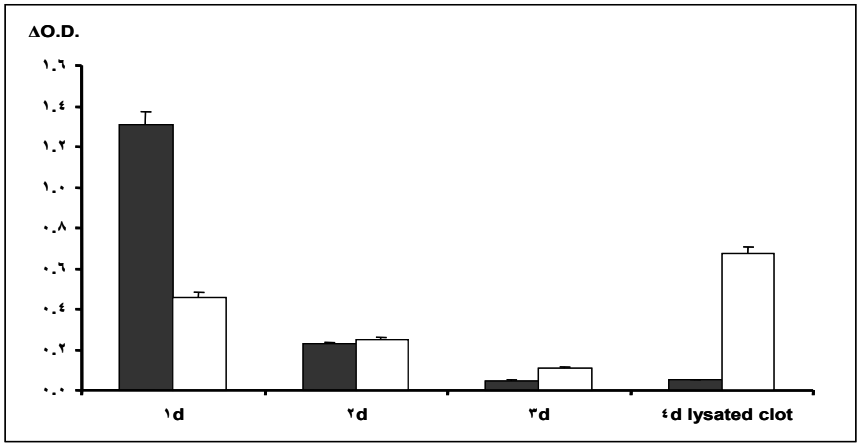

Figure 4.2a. Covalently bound labeled albumin to thrombin via PPACK showed a reduced release rate of albumin than free albumin over 4 days. Nearly 75% of bound albumin was retained in the fibrin clot. Closed bar: free albumin, open bar: bound albumin.

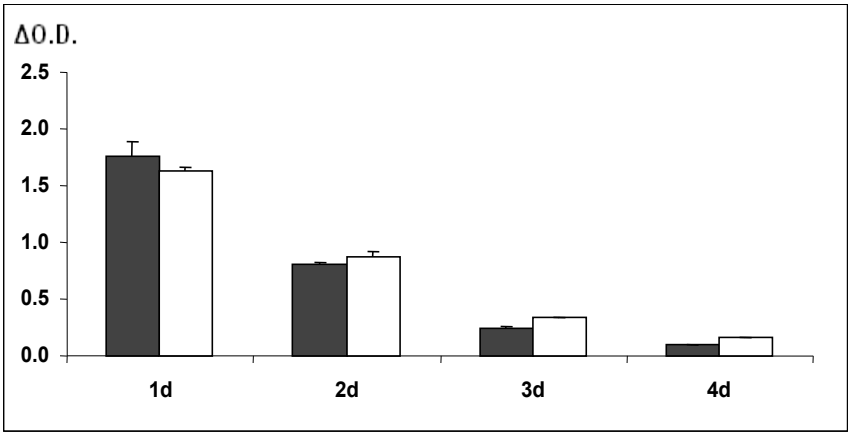

Figure 4.2b. Covalently bound streptavidin-POX to PPACK without thrombin as a fibrin-anchor showed no significant difference to unbound streptavidin-POX over 4 days. Closed bar: uncoupled strepdavidin-POX, open bar: PPACK coupled streptavidin-POX.

Fibronectin as a fibrin-anchor

The results of Figure 4.3 demonstrated the high affinity of biotinylated fibronectin to fibrin in comparison to biotinylated albumin without fibronectin as a fibrin-anchor. Only the controll substance, biotinylated albumin, was released from fibrin within 8 days. Biotinylated fibronectin was continuously released in small quantities over the time-period. For demonstration of a continuous and retarded release of a substance coupled to fibronectin as a fibrin-anchor, TNF was bound to an EDC-fibronectin-TNF antibody binding conjugate. Fibrin with fibronectin bound TNF showed a slow and delayed release (Figure 4.4). Fibrin containing unbound TNF showed a high release of TNF within the first 4 days. After clot lysis on day 10 nearly 30% of TNF were measured in remained fibrin clots.

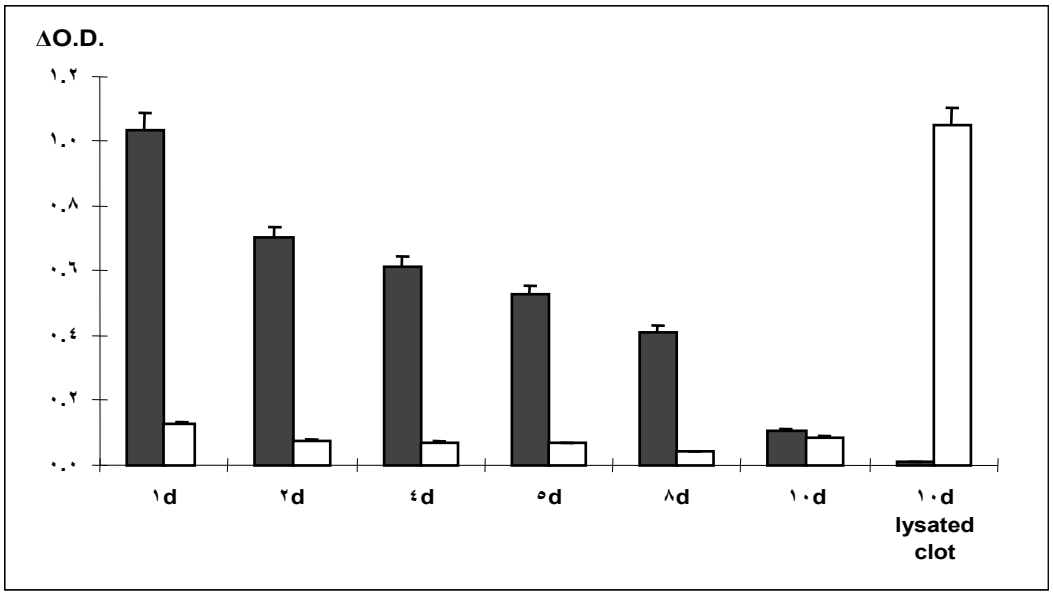

Figure 4.3. Graph illustrates the high affinity of fibronectin to fibrin. Biotinylated albumin without fibronectin as a fibrin-anchor showed a significant higher release than biotinylated fibronectin over 10 days. Closed bar: biotinylated albumin, open bar: biotinylated fibronectin.

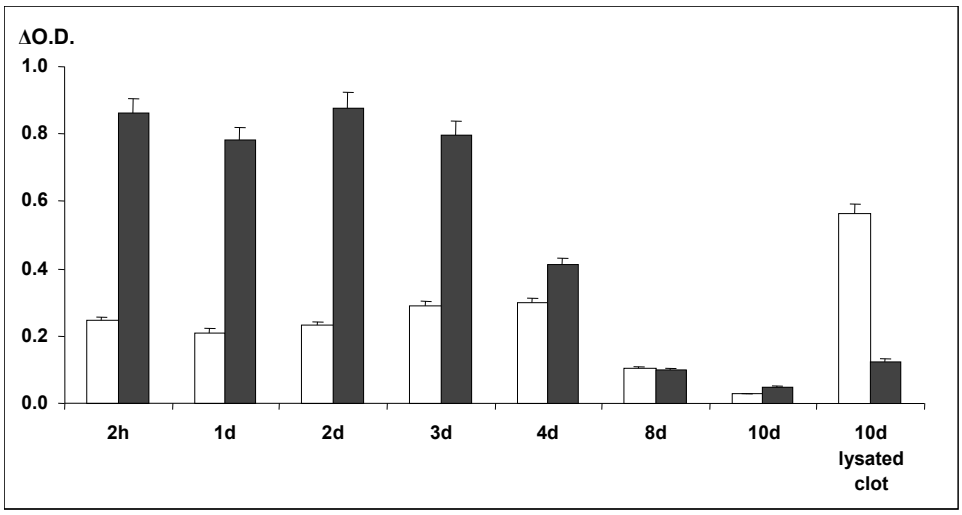

Figure 4.4. Covalently bound anti-TNF antibody to fibronectin via EDC showed a reduced release rate of TNF than the unbound anti-TNF antibody over 10 days. Bound anti-TNF antibody and so TNF was retarded in the fibrin clot. Closed bar: fibronectin + TNF-antibody (uncoupled mixture), open bar: fibronectin-TNF-antibody (EDC-coupled).

DNA as a fibrin-anchor

Fibrin clots spiked 20 µg plasmid DNA and incubated with PBS showed a higher release of plasmid DNA than fibrin containing 2 µg over the first 4 days. In general, the incubation of the fibrin clots with urokinase led to a higher and more continous release of plasmid DNA. The best result was obtained with fibrin spiked with 20 µg (Figure 4.5).

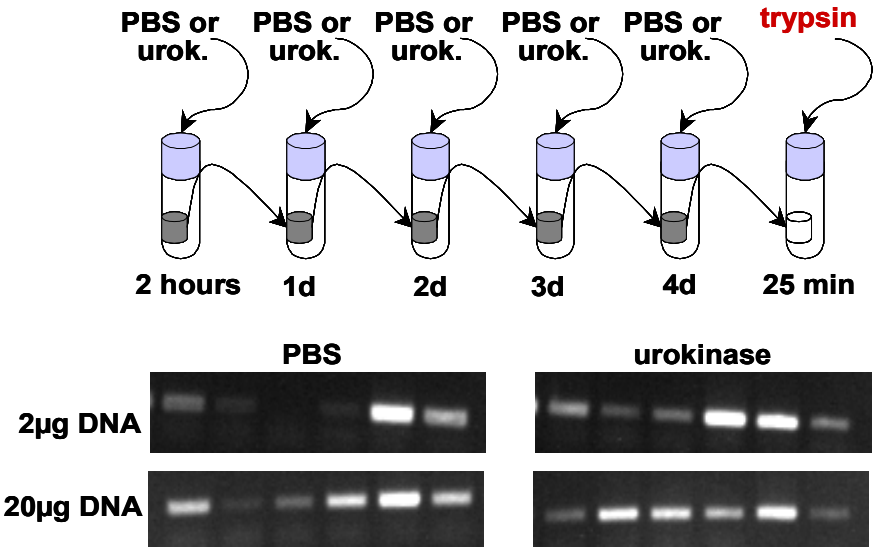

Figure 4.5. Fibrin clots spiked with 2 or 20 µg of plasmid-DNA (pGEM/IL-10) were incubated in PBS of urokinase over 4 days. Release rate of the supernatants was analysed by PCR. Fibrin clots containing 20 µg plasmid-DNA and incubated with urokinase showed the most effective release of pGEM/IL-10.

Fibrin clots without FS-anchor

Diffusion of a low molecular weight substance, cytochrom C, and a high molecular weight substance, β-galactosidase, was independent of molecular weight of the substance and density of the fibrin network. Over 50% of cytochrom C and β-galactasidase added to fine and coarse fibrin clots was found in the supernatants after 24 hours and another 40% was released during the next 2 days (Figure 4.6).

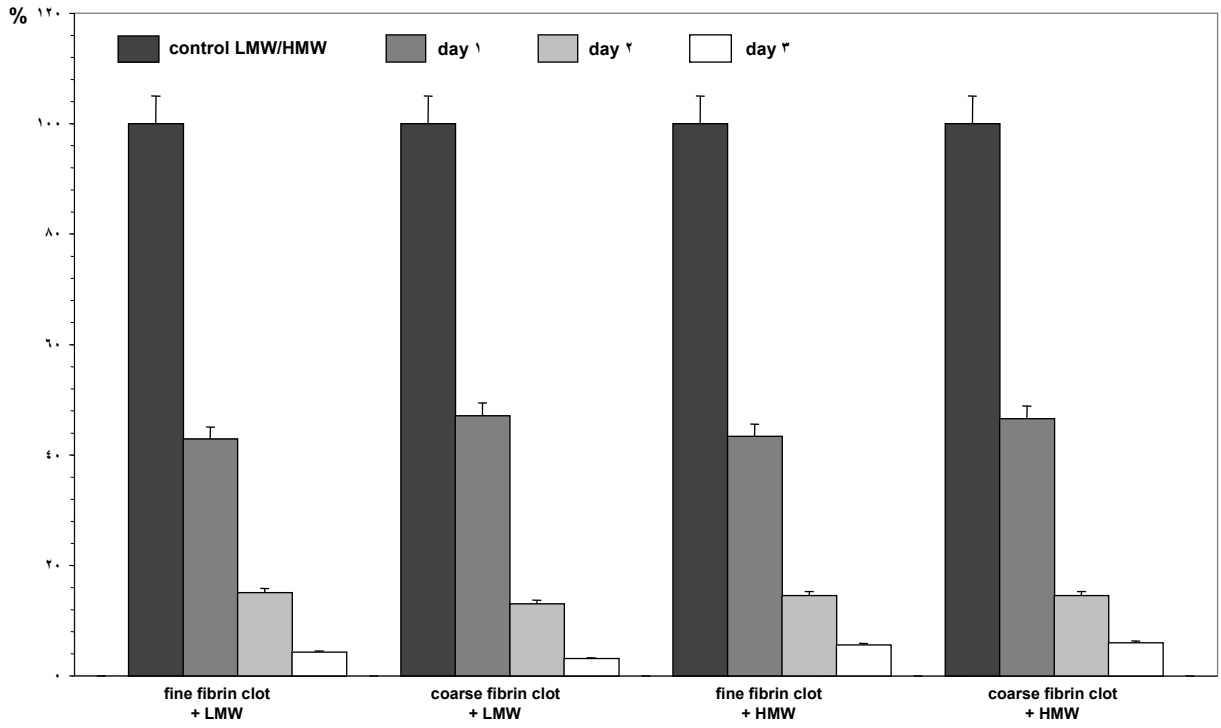

Figure 4.6. Release rate of cytochrom c (LMW) and β-galactosidase (HMW) was independent of the molecular weight of these substances and the density of the fibrin network.

Discussion

This study documents fibrin-based delivery systems for a sustained and controlled release of substances by using fibrin anchors. Because of its advantageous biological properties, fibrin gels have been generally proposed as preferred matrices for regeneration in wound healing (3,19,20). Thus, it is important finding possibilities to bind substances of interest to fibrin. Fibrin-anchors as thrombin, fibronectin and DNA have a high binding capacity to fibrin and binding oportunities to any substances. Thrombin and fibronectin are proteins and have a high natural binding affinity to fibrin.

Modification of substances and linking to fibrin via thrombin binding was done covalently to a specific site on thrombin utilizing a modified form of the irreversible thrombin inhibitor PPACK. Conjugation of a labeled protein, i.e. biotinylated albumin, to thrombin allow retardation of this protein on fibrinogen coated plates (data not shown) as well as in a fibrin clot. In contrast, free albumin, as an example of a labeled substance of interest, showed a high release in the first days.

Fibronectin the second natural byproduct in fibrin formulation is a large molecule and binds to fibrin via affinity and FXIII-crosslinking. Biotin-labeled fibronectin was slowly released from a fibrin clot over 14 days and still most of the labeled fibronectin was found in lysates from the residual clot. In contrast, biotin-labeled albumin was released quickly within 4 days resulting in high concentrations in the clot-supernatant and nothing was left in the lysed clot after 14 days (data not shown). Conjugation of an anti-TNF antibody (using EDC-coupling technique) to fibronectin and incorporation of TNF-loaded conjugates into fibrin clots allowed retardation on TNF in a fibrin clot. Thus, the high initial concentrations of TNF detected in supernatants from clots with free TNF were avoided. Conjugation of aprotinin to fibronectin and incorporation into fibrin clots increases persistance of clots with bound aprotinin compared to clots with free aprotinin. This led to more restistant fibrin gels with a slower

degradation rate. Therefore, to fibrin bound substances will be released in smaller amounts over a longer period of fibrin degradation.

Compared to thrombin and fibronectin DNA binds through charge strong to fibrin and fibrinogen. First plamid-DNA encoding for a gene is embedded in the fibrin matrix and its affinity for fibrin and fibrinogen allows a slow but sustained release of DNA over a long period of time. Fibrin clots treated with urokinase showed a more continuous release of plasmid-DNA than clots covered with PBS. This result showed the strong binding of DNA to fibrin/fibrinogen and that DNA will just be released by degradation of fibrin. Transfection reagents resulting in condensed and neutralized DNA are expected to reduce the affinity of DNA to fibrinogen. For a more consistent release of plasmid-DNA it actually is necessary to use a retrenching acting substance such as urokinase. The results showed then a more consistent and controlled release than by using PBS. It also will be possible to use single- or a double stranded, linear or circular DNA as a fibrin/fibrinogen binding moiety (data not shown). In this case DNA parts bind via affinity or covalent binding substances of interest.

Finally, results of released substances with different molecular weights (β-galactosidase and cytochrom C) and without a binding affinity to fibrin demonstrated an immediate release after clot formation. Also the addition of tranexamic acid for a more structured fibrin network with smaller cavaties did not affect the fast release of β-galactosidase and cytochrom C within three days. Therefore, substances without specific affinity to fibrin were released from the fibrin gel by diffusion. The molecular weight and the difference in fibrin structure by addition of tranexamic acid did not effect the delivery rate of these substances.

In conclusion, the feasibility of using affinity based fibrin binding conjugates for specific drug binding to fibrin gels has been shown for three binding moieties: thrombin, fibonectin and DNA. By use of these fibrin-anchors a slower release of different substances without a natural affinity to fibrin components could be achieved.

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References

1. Vlodavsky I, Bar-Shavit R, Ishai-Michaeli R, Bashkin P, Fuks Z. Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism. Trends Biochem Sci In press.

2. Richardson TP, Peters MC, Ennett AB, Mooney DJ. Polymeric system for dual growth factor delivery. Nat Biotechnol In press.

3. Wong C, Inman E, Spaethe R, Helgerson S. Fibrin-based biomaterials to deliver human growth factors. Thromb Haemost In press.

4. Spotnitz WD. Commercial fibrin sealants in surgical care. Am J Surg In press.

5. Van Hinsbergh VW, Collen A, Koolwijk P. Role of fibrin matrix in angiogenesis. Ann N Y Acad Sci In press.

6. MacPhee MJ, Singh MP, Brady R, Akhyani N, Liau G, Lasa C, Hue C, Best A, Drohan W. Fibrin Sealant: A versatile delivery vehicle for drugs and biologics. In: Sierra DH, Saltz R, editors. Surgical Adhesives and Sealants. Lancaste, Basel: Technomic Publishing CO. INC, 2001:109-20.

7. Martinowitz U, Saltz R. Fibrin sealant. Curr Opin Hematol In press.

8. Mosesson MW, Siebenlist KR, Meh DA. The structure and biological features of fibrinogen and fibrin. Ann N Y Acad Sci In press.

9. Sahni A, Odrljin T, Francis CW. Binding of basic fibroblast growth factor to fibrinogen and fibrin. J Biol Chem In press.

10. Sahni A, Guo M, Sahni SK, Francis CW. Interleukin-1beta but not IL-1alpha binds to fibrinogen and fibrin and has enhanced activity in the bound form. Blood In press.

11. Furst W, Banerjee A, Redl H. Comparison of structure, strength and cytocompatibility of a fibrin matrix supplemented either with tranexamic acid or aprotinin. J Biomed Mater Res B Appl Biomater In press.

12. Liu CY, Nossel HL, Kaplan KL. The binding of thrombin by fibrin. J Biol Chem In press.

13. Mosher DF, Johnson RB. Specificity of fibronectin--fibrin cross-linking. Ann N Y Acad Sci In press.

14. Richardson TP, Murphy WL, Mooney DJ. Polymeric delivery of proteins and plasmid DNA for tissue engineering and gene therapy. Crit Rev Eukaryot Gene Expr In press.

15. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell In press.

16. Lyon ME, Fine JS, Henderson PJ, Lyon AW. D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK): alternative anticoagulant to heparin salts for blood gas and electrolyte specimens. Clin Chem In press.

17. Harlow E, Lane D. Antibodies. New York: Cold Spring Harbor Laboratory Publications, 1988.

18. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning. A Laboratory Manual. Cold Spring Habor: Cold Spring Harbor Laboratory Pres, 1989.

19. Currie LJ, Sharpe JR, Martin R. The use of fibrin glue in skin grafts and tissue-engineered skin replacements: a review. Plast Reconstr Surg In press.

20. Lee H, Chung HJ, Park TG. Perspectives On: Local and Sustained Delivery of Angiogenic Growth Factors. Journal of Bioactive and Compatible Polymers In press.

Chapter V

Electrospun fibrin nanofibers for the use in tissue engineering

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Manuscript submitted

Abstract

Electrospinning has been recognized as an efficient technique for the fabrication of polymer nanofibers. In this study, we describe electrospinning of fibrin nanofibers in an attempt to create biomimicking tissue-like material *in vitro* for use as a tissue scaffold and *in vivo* for angiogenesis.

We have used lyophilized human fibrinogen and thrombin of the product Tisseel® VH (Baxter AG) to demonstrate fibrin electrospinning. The mixture dissolved in an appropriate dilution of 1,1,1,3,3,3-hexafluoro-2-propanol and sodium chloride solution was electrospun under various conditions. The quality of electrospun fibers were analyzed by scanning electron microscopy (SEM). For *in vitro* tests sterile matrices were seeded with human adipose derived stem cells for 14 days. *In vivo* experiments were done using an excision model on the dorsal site of VEGFR-2-luciferase transgenic mice.

Due to the small diameters of the electrospun fibrin nanofiber, they are more attractive for cell attachment. Their similarity in size to native extracellular matrix components and the 3-dimensional structure allows cells to attach to several fibers in a more natural geometry. In addition, seeded cells showed different proliferation patterns on matrices containing growth factors in comparison on nanofibers without additives. Furthermore, VEGFR-2-luciferase transgenic mice treated with electrospun fibrin containing growth factors showed a higher VEGFR-2 promoter activity during 3 weeks of observation. From the results of these studies we hypothesize that it may be possible to construct fibrous scaffolds composed of nanofibers for tissue engineering and wound repair using the process of electrospinning with fibrin.

Introduction

Tissue engineering has pursued a variety of materials and manufacturing processes over the last decades to develop and create engineered matrices or scaffolds biomimicking tissue in vitro. The majority of these attempts have focused on materials such as poly-(lactic acid), poly-(glycolic acid), polycaprolactone, other biocompatible polymers, and collagen for use in matrix construction with limited success. Many of these scaffold materials lack mechanical integrity and often induce an inflammatory response.(1-4) Moreover, typical scaffold fiber diameters approximate 10 μm, which is comparable to the diameter of a cell. Constituents of the natural extracellular matrix (ECM) exhibit fiber diameters that are in the range of 50-150 nm, a cross-sectional diameter far smaller than can be achieved with conventional processing strategies. Electrospinning represents a fabrication technique that makes it possible to produce fibrils of various materials with a cross-sectional diameter that resembles the native profile.(5-7)

Electrostatic spinning, or electrospinning, is a process that utilizes electrostatic forces to create small diameter fibers from the solution of a polymer or proteins.(8,9) The process can generate generous amounts of fibers at the sub-micron level, smaller in diameter than any standard extrusion process.(10) The efficacy of this process, as well as the final fiber product, are affected by a litany of factors, including, but not limited to solution polymer or protein concentration, viscosity of solution, voltage between solution and ground electrode, the distance between the Taylor cone and the ground electrode, and environmental conditions such as humidity and temperature.(11,12)

Electrospun fibers, because of their small diameters, have been of much interest not only in the textile field, but also in that of biomedical research. The small diameter fiber is more attractive to cell attachment, because of its similarity in size to native ECM components, which allow for the cell to attach to several fibers in a more natural geometry, rather than

the singular flattened orientation that an attached cell would experience on a large diameter fiber. Much research had been conducted with synthetic resorbable polymers, such as poly-(DL-lactic-co-glycolic acid (PGLA), and natural polymers(10), such as collagen.(3)

In addition to collagen(3,4,6), fibrinogen(13) and fibrin represent materials for use in the development of an electrospun tissue engineering scaffold.(14-16) These materials have additional potential uses in wound dressings and hemostasis products.(13,17)

The use of fibrin in wound treatment is storied, with reporting of such dating to 1909 by Bergel et al., who used dried plasma to arrest surgical bleeding. Patches of pure fibrin have documented use in the decade to follow. Early uses of fibrin included aid to skin grafting and nerve repair. (18,19) The current fibrin-based products come in two forms, dry and liquid. Both involve simultaneous application of fibrinogen and thrombin, sometimes with the addition of Factor XIII and calcium. The dry product contains fibrinogen and thrombin, in a freeze-dried or frozen state, to prevent their reacting. Stored in air-tight packaging, this product will yield fibrin upon exposure to air humidity, and more appropriately, to a wound. The liquid product is delivered as a combination of fibrinogen and thrombin, forming a layer of fibrin that immediately clots the bleeding. (20,21) Both technologies show great efficacy in their ability to deliver concentrations of these proteins at levels much higher than in blood. Both of these products, however, have their drawbacks. The dry sealant has difficulty in handling because of its activation upon exposure to moisture (even humid air will begin the reaction). The liquid version has a long preparation time because of the necessary dissolution of components, and therefore will probably not be optimal in an emergency setting.(22-24)

However, the rapid attainment of fibrin with better handling could make such a product more viable and practical. With the technology of electrospinning, an electrospun fibrin wound dressing would have the added benefit of small fiber diameter and a big surface which could accelerate wound healing. Wnek *et al.* demonstrated the feasibility of

electrospinning fibrinogen through the determination of an appropriate addition (10X minimal essential medium) to a common electrospinning organic solvent 1,1,1,3,3,3-hexafluoro-2-propanol. Once the appropriate solvent was determined, Wnek *et al.* electrospun different concentrations of bovine fibrinogen and measured the fiber diameters of the final product using SEM microscopy and image processing software.(13) Their results indicated that fiber diameter could be controlled by the adjustment of the concentration of the spinning solution. However, this was only fibrinogen and not the endproduct fibrin.

In addition, an electrospun mat of synthetic or natural polymer or protein could be used as a vehicle for drug delivery, which enhances the application in wound healing therapies. Bioactive agents such as growth factors could be incorporated into the polymer or protein solution to be spun, and should be uniformly distributed as the final nanofiber product. These added substances support cell proliferation, migration and differentiation.(25-27) In comparison to other well-proven useful technologies for drug delivery matrices, none would show the full cell-friendly benefits of electrospun materials.(11,28,29) The use of a protein, such as fibrinogen and fibrin should reduce much concern of immunogenicity and offer a more favourable attachment scaffold for cell infiltration. Several of these growth factors, including fibroblast growth factor 2 (FGF-2) are involved in vascular responses by increasing endothelial cell proliferation, stimulating migration and promoting angiogenesis.(30) FGF-2 also increases secretion of collagenase and urokinase plasminogen activator, and has its influence on human bone marrow stromal cells as a potential implication for tissue engineering.(31)

Another growth factor of interest has a critical role in bone formation and regeneration. The bone morphogenic protein 2 (BMP-2) has been studied using a variety of delivery systems(32-34) and showed strong influence in mesenchymal stem cell osteogenic differentiation.(35)

Therefore, this study aimed at electrospinning fibrin with and without growth factors and secondly the possibility to use electrospun fibrin fibers both *in vitro* and *in vivo*.

Materials and methods

Electrospinning

Lyophilized human fibrinogen and thrombin (Tisseel Baxter AG, Vienna) were suspended in a solution of 9 parts 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) and 1 part of 10X minimal essential medium (MEM) without L-glutamine (Sigma-Aldrich, Vienna).(13) The final solution with concentrations of 75 mg/ml of fibrinogen, 125 IU/ml of thrombin and 12.5 µg/ml fluorescent labeled fibrinogen 488 (Invitrogen GmbH, Lofer) for detection of electrospun fibrin nanofibers was placed in a 2 ml syringe with 20 gauge blunt needle. Either 200 ng/ml recombinant human basic fibroblast growth factor (rhFGF-2; ProSpec-Tany TechnoGene Ltd., Israel) or 200 ng/ml recombinant human bone morphogenic protein 2 (BMP-2; InductOs®, Wyeth Pharmaceuticals, Vienna) was mixed into the fibrin solution. In addition for a better stability of the electrospun structure, 10 mg/ml poly-(DL-lactic-co-glycolic acid (PGLA)) was added to the final mixture. A syringe pump was maintained for a continous flow rate of 0.01 ml/min during the spinning process. A high voltage power supply imparted a voltage of 25 kV at the syringe needle. The electrospun fibers were collected on a rotating aluminium cylinder of 12 cm in length and 3 cm in diameter, which roated at a rotational velocity of approximately 400 rpm. The distance from the needle tip to the cylinder was 12 cm. After 120 min the electrospinning process was stopped. The nanofiber mats were removed from the cylinder and, wrapped in aluminium foil and stored at 4°C until further use.

Scanning electron microscopy of electrospun fibrin

To analyze the morphology of the nano-scaffold, scanning electron microscopy (SEM) was used. The samples of each mat were cut into appropriate size, coated with gold using Edwards S150 sputter coater and then SEM imaging was carried out using JEOL T100 (JEOL Ltd. Tokyo, Japan) microscope. SEM images were obtained at magnifications of 500x,

2000x and 5000x and scanned into digital computer images. Fiber diameter was determined by means of Image J 1.33u (Wayne Rasband National Institute of Health, USA). These values were averaged and standard deviations calculated.

Experimental setup *in vitro*

Cells and cell culture

Isolated human adipose-derived stem cells in passage 2 were cultured in a mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 medium in the same volumes supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, penicillin and streptavidin (PAA Laboratories, Pasching) at 37°C, 5% CO_2 and 95% air humidity up to a subconfluent state of 80%.(36,37)

For the in vitro experiments adherent cells were stained with CellTracker™ Orange (CTO) CMRA (Invitrogen GmbH, Lofer) according to manufacturing instructions. After effective labeling, cells were removed from cell culture plates by incubating with 10X trypsin (PAA Laboratories, Pasching) and centrifugation (5 min, 1500 rpm). 10^6 cells/mL were then seeded on sterile electrospun nanofiber fibrin scaffolds. After 2 hours, seeded cells were covered with 1 ml fresh medium supplemented with 1% FCS.

On day 14, seeded CTO-labeled cells were first stained with 5 µM calcein AM (Invitrogen GmbH, Lofer) for 15 min at 37°C, washed once with phosphate buffered saline without $MgCl_2$ and $CaCl_2$ (PAA Laboratories, Pasching). Subsequently, cells were stained with 300 nM 4´,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 2 min at room temperature. After washing, cells were covered with cell culture medium and imaged for live/dead viability.

Fluorescence microscopy

The fluorescent signal generated by the labeling of the electrospun nanofiber fibrin with fluorescent labeled fibrinogen 488 nm and by the cells with CTO, calcein AM and DAPI was detected by fluorescence

microscopy using a UV-microscope (Zeiss, Axiovert 10/AttoArc HBO100W). Signals were detected on days 1, 5, 7, 14.

RT-PCR

RNA was extracted from cells after 14 days of the experiment using trizol. Phase separation was conducted using chloroform. Tubes were centrifuged and only the aqueous phase containing the RNA was transferred to a fresh tube for further precipitation and purification. RNA precipitation was performed using isopropyl alcohol, and RNA pellets were purified using 70% ethanol (Sigma-Aldrich, Vienna). Purified RNA was quantified using a spectrophotometer, and then aliquoted and stored at -80°C. 2µg of RNA were first treated with Rnase-free Dnase (Promega GmbH, Germany) according to the manufacturer's instruction and then transcribed into cDNA using AMV reverse transcription system (Promega GmbH, Germany). For RT-PCR the following primer sequences (Table 5.1) and conditions were used: 5 min at 94°C, and then 39 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. The PCR reactions were separated by gel electrophoresis and the product bands were visualized and photographed under ultraviolet light densiometry.

Gene	Primers: sense (s) and antisense (as); 5' - 3'		accession#
Col Iα1	s	AGC TGT CTT ATG GCT ATG ATG	NM_000088.3
	as	GCA CCA TCC AAA CCA CTG AA	
Col IIα1	s	GTA ACC CTG GAA CAG ATG GA	NM_001844.4
	as	CAC CTC TCT TGC CTT CTT CA	
OC	s	CCC TCA CAC TCC TCG CCC TAT TGG	NM_199173.3
	as	GCC TCC TGA AAG CCG ATG TGG TC	
OP	s	TCC TAG CCC CAC AGA CCC TTC C	NM_000582.2
	as	TCT CAT CAT TGG CTT TCC GCT TAT	
Vim	s	CTG CCA ACC GGA ACA ATG AC	NM_003380.2
	as	CAC GAA GGT GAC GAG CCA TTT	
αSMA	s	CGA CCG AAT GCA GAA GGA GA	NM_001613.1
	as	TTT GCG GTG GAC AAT GGA AG	
Des	s	ATG TGG AGA TTG CCA CCT ACC GGA AGC TG	NM_001927.3
	as	GTG TCC TGG GAT GGA AGA AGG CTG GCT T	
GAPDH	s	TTA GCA CCC CTG GCC AAG G	NM_002046.3
	as	CTT ACT CCT TGG AGG CCA TG	

Table 5.1. Human specific oligonucleotide primers used for polymerase chain reactions. Col Iα1, collagen I alpha1; Col IIα1, collagen II alpha1; OC, osteocalcin; OP, osteopontin; Vim, vimentin; αSMA, alpha smooth muscle actin; Des, desmin; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

Experimental setup *in vivo*

Excision model in transgenic VEGFR-2 luciferase mice

In vivo models were approved by the local Committee on Animal Experiments, Vienna, Austria, and all experimental procedures were consistent with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH Publication No. 85-23, revised 1996). Transgenic FVB/N-Tg(VEGFR-2-luc)Xen mice (Xenogen Corporation, CA, USA) were used for non-invasive, real-time assessment of the vascular endothelial growth factor receptor 2 (VEGFR-2 / Flk-1 / KDR) induction using an in vivo imaging system (VivoVision® IVIS®, Xenogen, Alameda, CA). In these mice the VEGFR-2 promoter is fused with the firefly luciferase gene, thus resulting in a co-transcription of the VEGFR-2 and firefly luciferase. VEGFR-2 expression was monitored by luciferase co-expression after injection of the substrate luciferine *in vivo*.

After the initial isoflurane (2 Vol% + 300 ml/min air) anesthesia mice were further anesthetized with intraperitoneal (i.p.) 1:10 dilution of

ketamine:xylazine (60 mg/kg BW, 7.5 mg/kg BW, respectively). Mice were injected i.p. with luciferine (150 mg/kg BW) and imaged using a CCD camera IVIS imaging system in order to acquire a background image signal. After background imaging each back was shaved and sites of excision were disinfected. Two round skin excisions (1 cm in diameter) were prepared and either covered with appropriate electrospun fibrin nanofiber mats or 0.2 ml fibringel (Tisseel Duplojet, Baxter AG, Vienna). Finally the shaved part of the back was dressed with Tegaderm™ (3M Austria GmbH, Wr. Neustadt). As a control, excisions were only covered with Tegaderm™.

Follow up

2 hours postoperatively, animals were imaged again. A luciferin injection preceeded each imaging sequence. The bioluminescence signal was quantified using LivingImage software (Xenogen Corporation, Alameda, CA) from the in vivo luciferace activity (indirect sign of VEGFR-2 activity) measured in emitted photons per second. Pre-surgical activity was set to 100% (=baseline) and the subsequent measurements were referenced to this baseline. Bioluminescence images were obtained over a period of 3 weeks (1 day, 2, 5, 7, 10, 13, 15, 17, and 20 days post OP).

Planimetric analysis

Digital images were taken under standardized conditions (light, distance, magnification) and then transferred to a personal computer. Subsequent analysis of wound closure area was performed with a specific planimetric software program (Lucia G®, Version 4.8, Laboratory Imaging Ltd., Czech Republic).

Statistical analysis

The medians and deviations (Q1, Q3) were calculated for all variables tested. Statistical significance was accepted at $*p < 0.05$ and $**p < 0.01$.

Results

The quality of electrospun fibers were analysed using the scanning electron microscopy SEM evaluation. Figure 5.1 show that there is a difference in using the same conditions for spinning only fibrin and a mixture of fibrin and PGLA. There are much more fibers in mixed matrix therefore it has a higher density in the structure. The fibers are more orientated and have a better regular structure and are similar in diameter than in the matrix just made out of fibrin.

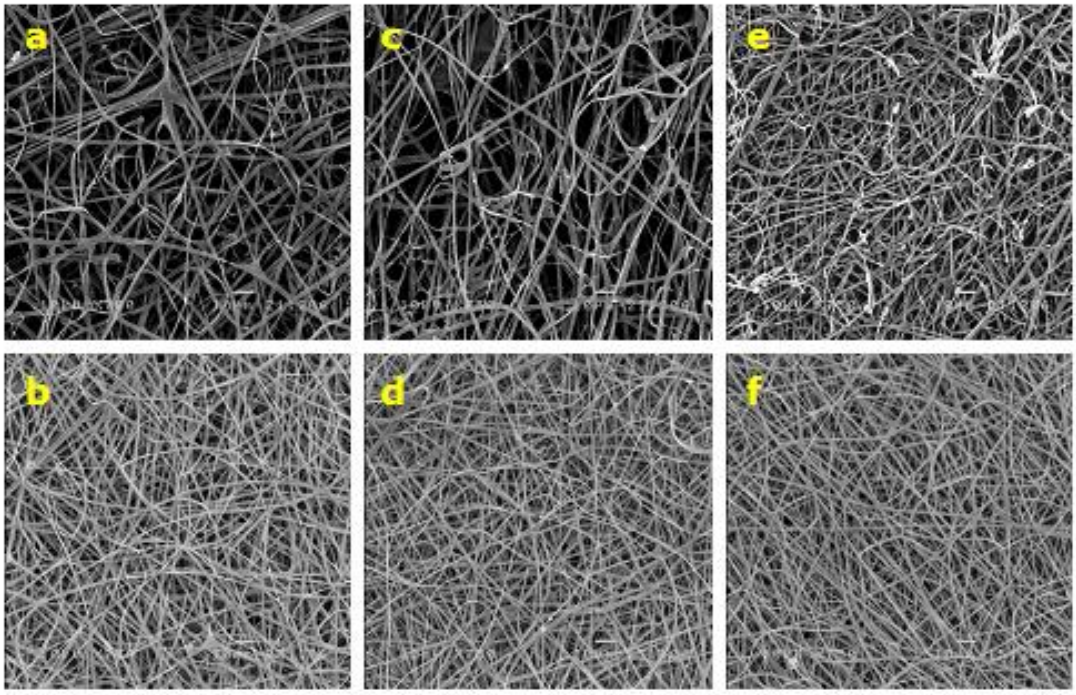

Figure 5.1. SEM micrographs illustrating the fibrous structure of electrospun fibrin (a, c, e) and fibrin+PGLA (b, d, f) scaffolds with additives as rhFGF-2 (c, d) and rhBMP-2 (e, f).

Experimental setup in vitro

Labeled adipose derived stem cells were seeded on sterile electrospun fibrin nanofiber mats. After 48 hours analysed samples showed cells well distributed in and also on the biodegradable matrix (Figure 5.2a). CTO labeled cells could be well detected for 5 days, then the staining faded and became more granular. Therefore, it was necessary to label the cells in

the matrix after 14 days again (calcein AM). Further added proteins as human FGF-2 and human BMP-2 showed different patterns of the cells in orientation after 14 days in culture (Figure 5.2b). Human FGF-2 treated adipose derived stem cells showed an equal orientation and had a thin body. In contrast to that, cells on the scaffolds containing BMP-2 formed circular structures.

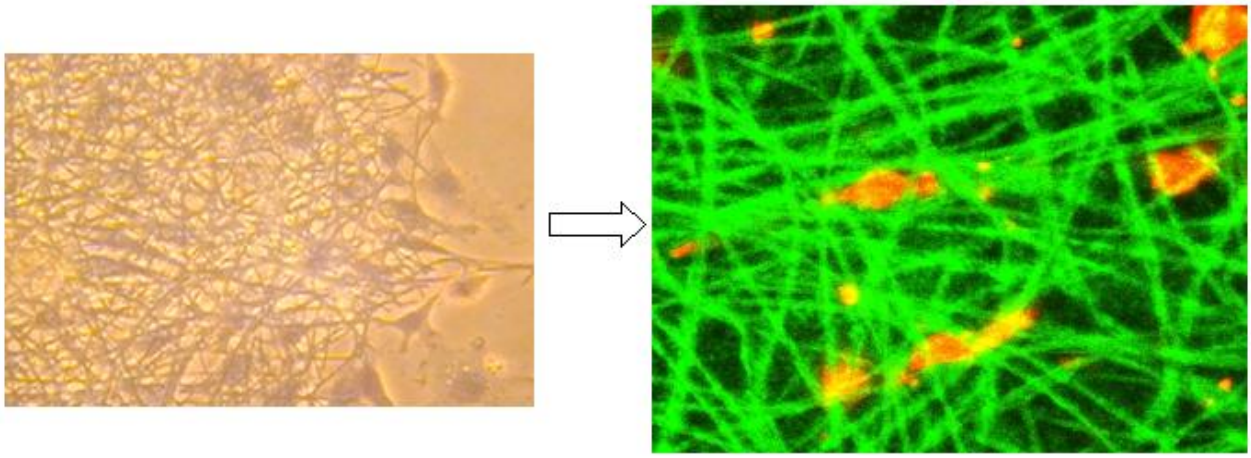

Figure 5.2a. Electrospun fibrin colonized with adipose derived stem cells, cultured in DMEM medium containing 5% FCS. After 48 hours cells were stained with a hematoxyline solution and photographed with a magnification of 200x. The photograph of CTO labeled cells points up the well-distribution of cells specially in the electrospun fibrin nanofiber matrix.

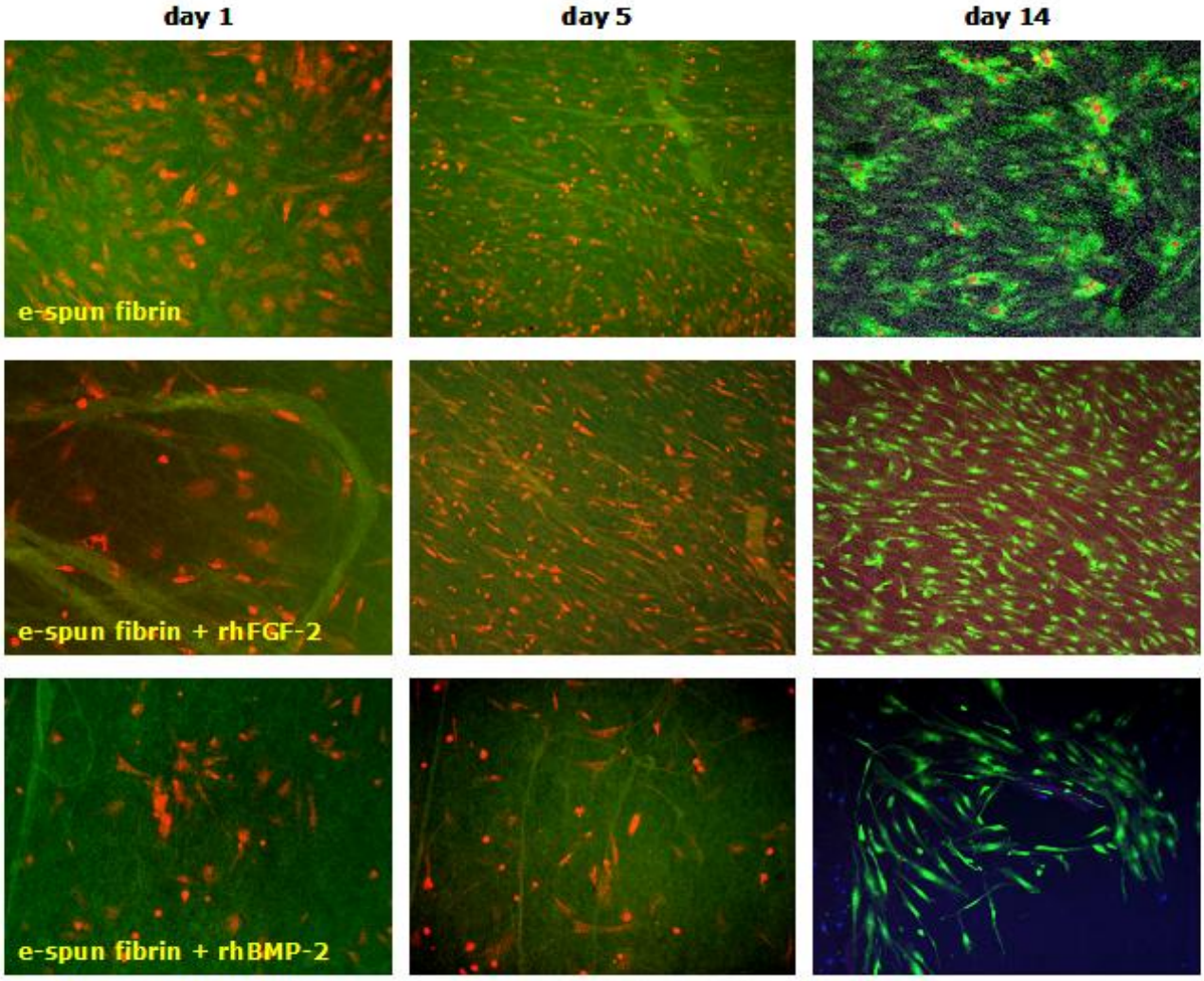

Figure 5.2b. Human adipose derived stem cells were seeded on electrospun fibrin/PGLA with and without rhFGF-2 and rhBMP-2. After 14 days cells showed different cell morphology.

The difference in behavior is also reflected in the gene expression results (Figure 5.3). The messenger RNA (mRNA) levels of vimentin, alpha smooth muscle actin, and desmin were highly expressed only in the control group. High levels of the chrondrogenic markers collagen IIα 1 (**p < 0.01 control vs. BMP-2 group) and osteopontin (*p < 0.05) were also observed only in the cells without any scaffold. The cells showed a significant high expression of collagen Iα 1 (**p < 0.01) and osteocalcin (**p < 0.01) in cells on the electrospun fibrin containing rhBMP-2 in

comparison to all other groups. Cells only showed no collagen Iα 1 and osteocalcin expression.

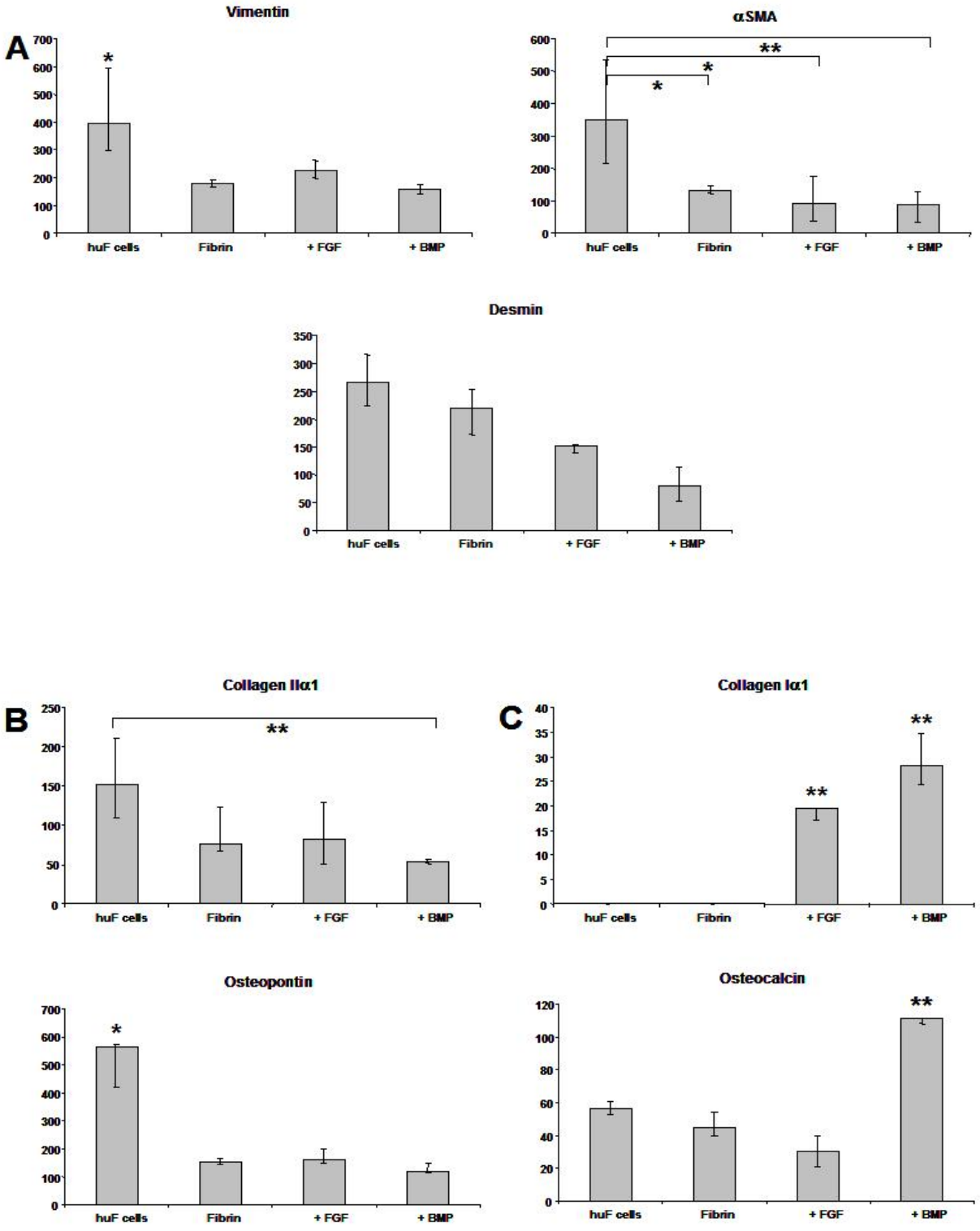

Figure 5.3. Different expression levels of osteogenic and endothelial markers could be observed in adipose derived stem cells on fibrin/PGLA, fibrin/PGLA+FGF-2 and fibrin/PGLA+BMP-2 (n = 6, median ± Q1, Q3).

Experimental setup in vivo

Appropriate cut electrospun fibrin nanofiber mats +/- human FGF-2 were applied on the skin excisions of transgenic FVB/N-Tg(VEGFR-2-luc)Xen mice (Figure 5.4).

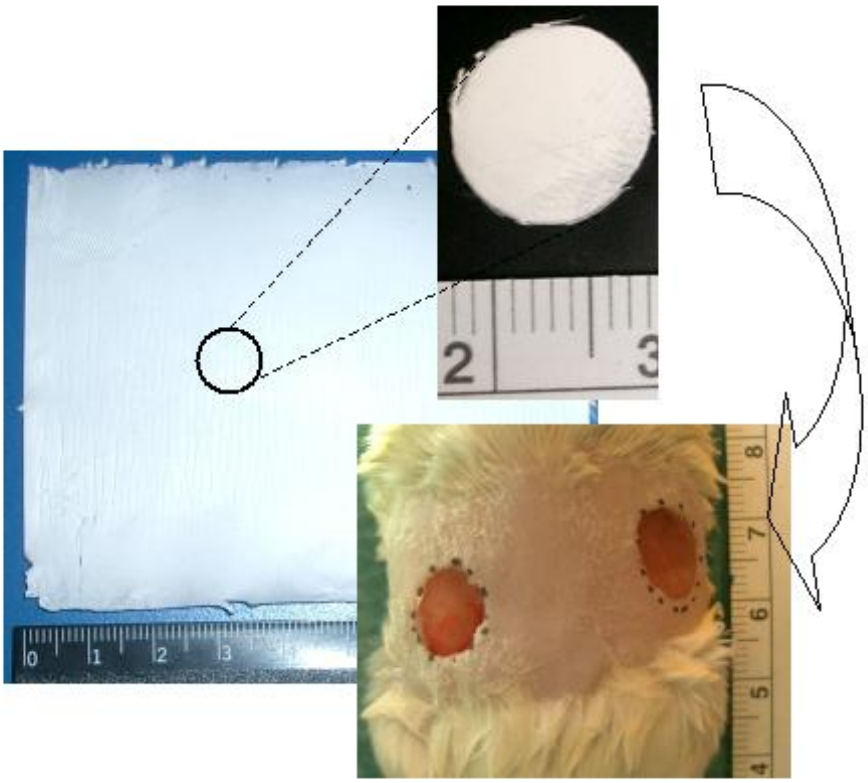

Figure 5.4. Photograph of an electrospun fibrin/PGLA mat produced from 75 mg/ml fibrinogen, 125 IU/ml thrombin and 10 mg/ml PGLA solved in HFP/MEM for use as a wound dressing in our transgenic mouse skin excision wound model.

For the control groups either 0.2 ml fibringel or no therapy were applied. Associated bioluminescence imaging (Figure 5.5a) generally showed higher VEGFR-2 expression levels on day 1 and 10 after injury. There was a high increase in VEGF-R2 expression in the group of electrospun nanofiber mats +FGF-2 on day 12 in comparison to all other groups. Both electrospun fibrin without FGF-2 and fibringel led nearly to the same results and induced a higher VEGFR-2 signal from day 7 to day 20 than the control group without any therapy (Figure 5.5b).

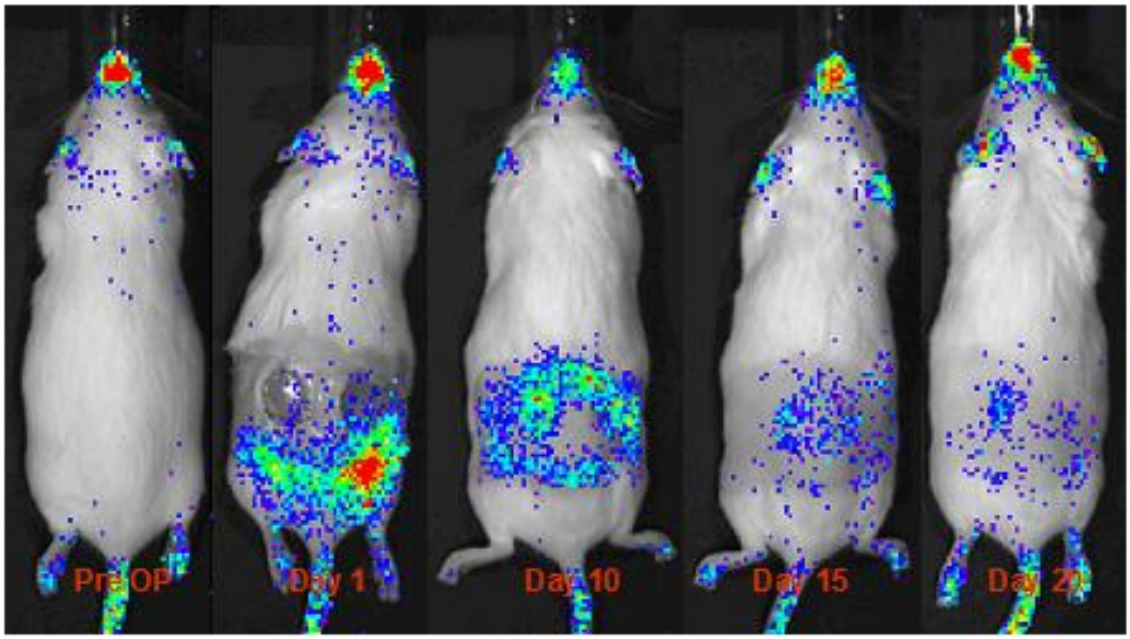

Figure 5.5a. Photographs show mouse dorsal skin excision wounds at days 1, 10, 15 and 20 post injury with corresponding image after analysis and color representing of photon quantification. The photon quantification increases, as color progresses from blue to red.

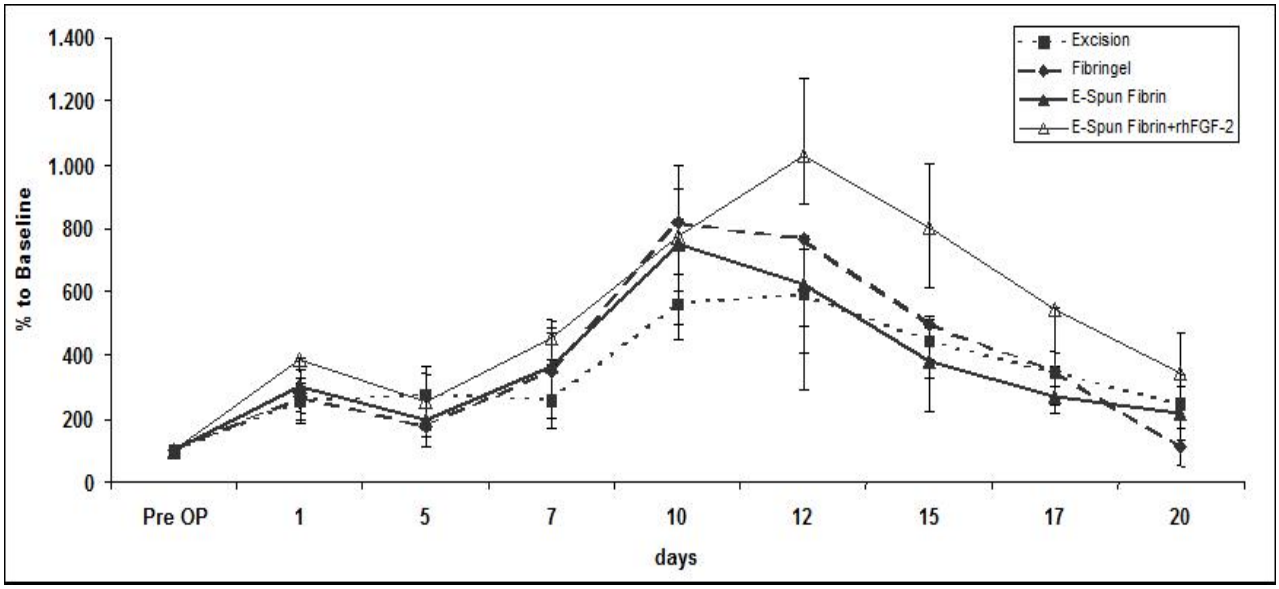

Figure 5.5b. Quantification of luciferase signal (photons/sec) from wound area with Living Image® software. Luciferase activity show a higher signal on days 12 and 15 in he electrospun fibrin containg rhFGF-2 group in comparison to all other groups (n = 6, median ± Q1, Q3).

Planimetric analysis (Figure 5.6a and 5.6b) showed a significant slower wound closure of the fibringel group from day 2 to day 10 post injury in comparison to all other groups. There also is a slight difference in wound closure on days 2 and 5 in the control group to both groups with electrospun fibrin as wound coverage.

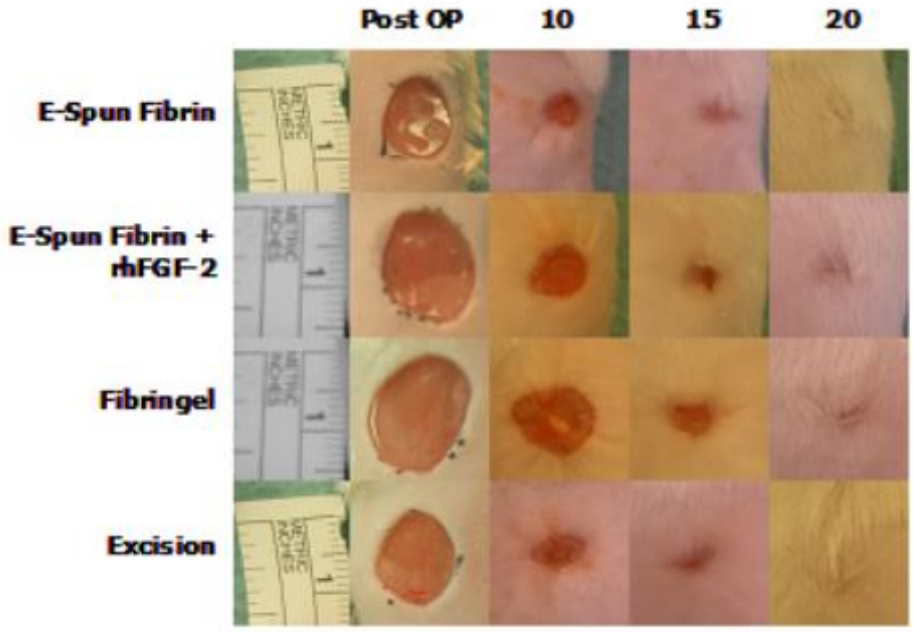

Figure 5.6a. Comparison of wounds at days 10, 15 and 20 to post operative area size. The photographs show the strong contraction until day 10 in the control group and the delayed wound healing of the fibringel group.

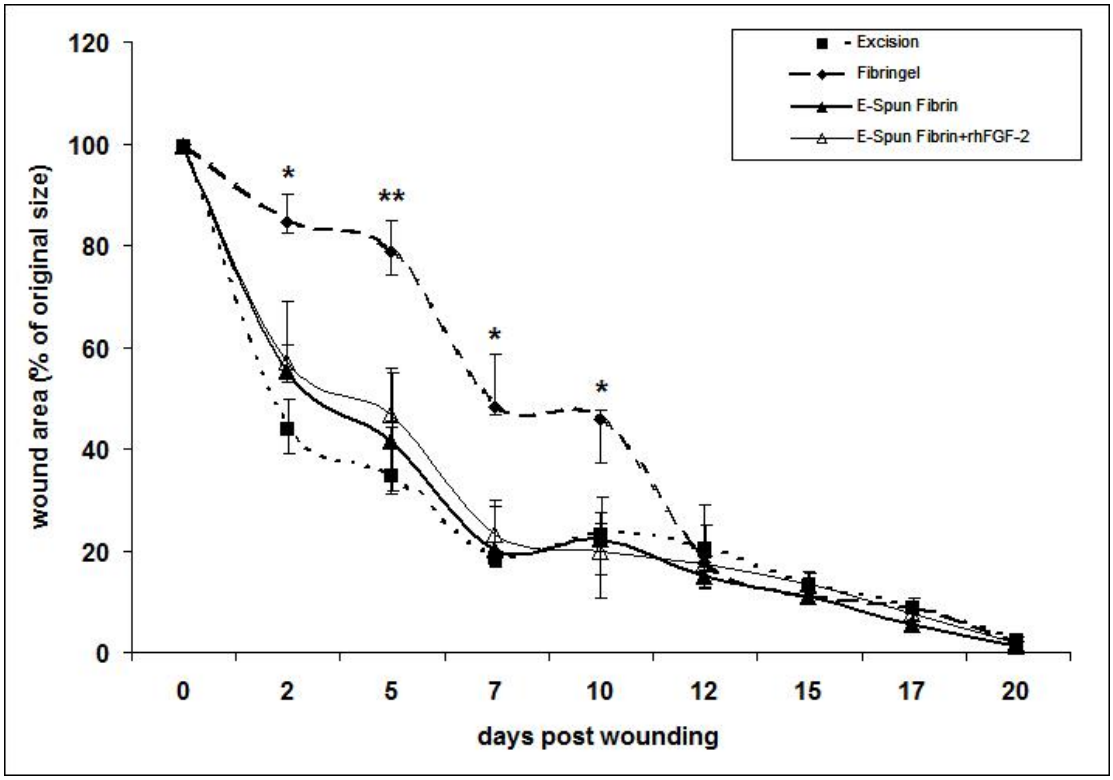

Figure 5.6b. Kinetics of wound closure. Differences in median wound area between fibringel therapy and all other groups were statistically significant at all time points between day 2 and 10 (*$p < 0.05$ on days 2, 7 and 10; **$p < 0.01$ on day 5). Results also show a slight difference in wound closure in both groups of electrospun fibrin wound coverage in comparison to control group. Closure in controls is shown as a reference and represents wound closure only through contraction (n = 6, median ± Q1, Q3).

Discussion

As described by Langer and Vacanti in 1993, tissue engineering is "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function."(38) One part of tissue enginnering has been the design of scaffolds with similarity to native extracellular matrix (ECM) biologically and mechanically. From the results of this study as well as from historical studies fibrin represents an appropriate healing material with biological properties similar to the ECM. With the technique of electrospinning it is possible to create various structures, shapes and sizes of fibrin matrices with a high surface / volume ratio and fibers with small diameters of 0.1 – 1 µm. Another benefit of this method is that almost all scaffolds and structures can be made seamless. This fact will prevent any variation or possible weak areas in the scaffold during development, application and regeneration phase. Preliminary experiments showed that electrospun fibrin alone has a weak structure and is difficult to remove from the collection electrode. Therefore, small amounts of PGLA, a biodegradable and biocompatible copolymer, were added for a better mechanical stability of electrospun fibrin. In addition to PGLA, growth factors i.e. FGF-2 and BMP-2 were also mixed prior into the protein solution for stimulation of cell behavior. Studies have shown that nanometer-sized elements have positive effects on cells.(39) *In vitro* experiments of this study showed good results in cell morphology and cell differentiation on fibrin nanofiber matrices. The influence of BMP-2 in the electrospun fibrin / PGLA led into an osteogenic differentiation of the adipose derived stem cells. Teixeira *et al* also observed that nanogrooved surfaces can induce contact guidance of human corneal epithelial cells, causing them to elongate and align their cytoskeleton along the topological features of the scaffold.(40) The results of this study also represents a tissue engineered scaffold where the cells are well-distributed in the matrix.

Due to the auspicious results from the presumed mechanical properties of the electrospun fibrin / PGLA nanofibers and our *in vitro* experiments the scaffold was used in an *in vivo* dorsal skin excision wound healing model in transgenic VEGFR-2-luc mice. The electrospun matrix could work as an appropriate wound coverage and as well as it could accelerate wound closure and healing. The results demonstrate that the electrospun fibrin is suitable for wound coverage. In comparison to the well-studied and commonly clinically used fibrin glue, the electrospun fibrin has no preparation time, is easier to apply to the skin wound and naturally sticks to the wound. Therefore, it is simple to cover even irregular wounds. The nanofiber network and the high surface area of the electrospun bandage ensure air permeability and absorbance of some wound fluid. Results of the planimetric analysis also showed that this matrix can be seen as a semidry or semiliquid wound bandage that accelerates wound closure but is effective against skin contractions.

With its additionally capacity to function as a drug delivery depot, electrospun fibrin represents a perfect tissue engineerednbiocompatible and biodegradable scaffold for wound dressing with hemostatic qualities.

Acknowledgement

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References

1. Vacanti JP, Vacanti CA. The Challange of Tissue Engineering. In: Lanza R, Langer R, ChickW., editors. Principles of Tissue Engineering. Academic Press, 1997.

2. Greisler HP, Gosselin C, Ren D, Kang SS, Kim DU. Biointeractive polymers and tissue engineered blood vessels. Biomaterials In press.

3. Matthews JA, Wnek GE, Simpson DG, Bowlin GL. Electrospinning of collagen nanofibers. Biomacromolecules In press.

4. Boland ED, Matthews JA, Pawlowski KJ, Simpson DG, Wnek GE, Bowlin GL. Electrospinning collagen and elastin: preliminary vascular tissue engineering. Front Biosci In press.

5. Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol In press.

6. Li WJ, Laurencin CT, Caterson EJ, Tuan RS, Ko FK. Electrospun nanofibrous structure: a novel scaffold for tissue engineering. J Biomed Mater Res In press.

7. Ma PX, Zhang R. Synthetic nano-scale fibrous extracellular matrix. J Biomed Mater Res In press.

8. Huang ZM, Zhang YZ, Kotaki M, Ramakrishna S. A review on polymer nanofibers by electrospinning and their applications in nanocomposites. Composites Science and Technology In press.

9. Pham QP, Sharma U, Mikos AG. Electrospinning of polymeric nanofibers for tissue engineering applications: a review. Tissue Eng In press.

10. Doshi J, Reneker DH. Electrospinning process and applications of electrospun fibers. Journal of Electrostatics In press.

11. Katti DS, Robinson KW, Ko FK, Laurencin CT. Bioresorbable nanofiber-based systems for wound healing and drug delivery: optimization of fabrication parameters. J Biomed Mater Res B Appl Biomater In press.

12. Grafe, T. H. and Graham, K. M. Nanofiber webs from electrospinning. 2003. Donaldson Company Inc.; Minneapolis, MN, USA. 1-3-2003.
Ref Type: Conference Proceeding

13. Wnek GE, Carr ME, Simpson DG, Bowlin GL. Electrospinning of Nanofiber Fibrinogen Structures. Nano Lett In press.

14. Catelas I, Sese N, Wu BM, Dunn JC, Helgerson S, Tawil B. Human Mesenchymal Stem Cell Proliferation and Osteogenic Differentiation in Fibrin Gels in Vitro. Tissue Eng In press.

15. Ho W, Tawil B, Dunn JC, Wu BM. The behavior of human mesenchymal stem cells in 3D fibrin clots: Dependence on fibrinogen concentration and clot structure. Tissue Eng In press.

16. Peretti GM, Randolph MA, Zaporojan V, Bonassar LJ, Xu JW, Fellers JC, Yaremchuk MJ. A biomechanical analysis of an engineered cell-scaffold implant for cartilage repair. Ann Plast Surg In press.

17. Ashammakhi N, Ndreu A, Piras AM, Nikkola L, Sindelar T, Ylikauppila H, Harlin A, Gomes ME, Neves NM, Chiellini E, Chiellini F, Hasirci V, Redl H, Reis RL. Biodegradable nanomats produced by electrospinning: expanding multifunctionality and potential for tissue engineering. J Nanosci Nanotechnol In press.

18. Helgerson S, Seelich T, DiOrio J, Tawil B, Bittner K, Spaethe R. Fibrin. Encyclopedia of Biomaterials and Biomedical Engineering. 2004.

19. Clark RA. Fibrin sealant in wound repair: a systematic survey of the literature. Expert Opin Investig Drugs In press.

20. Martinowitz U, Saltz R. Fibrin sealant. Curr Opin Hematol In press.

21. Mosesson MW. Fibrinogen and fibrin polymerization and functions. Blood Coagul Fibrinolysis In press.

22. Jackson MR. Fibrin sealants in surgical practice: An overview. Am J Surg In press.

23. Jackson MR. New and potential uses of fibrin sealants as an adjunct to surgical hemostasis. Am J Surg In press.

24. Morikawa T. Tissue sealing. Am J Surg In press.

25. Cox S, Cole M, Tawil B. Behavior of human dermal fibroblasts in three-dimensional fibrin clots: dependence on fibrinogen and thrombin concentration. Tissue Eng In press.

26. Schlag G. The Importance of Fibrin in Wound Repair. In: Schlag G, Redl H, editors. Fibrin Sealant in Operative Medicine Otorhinolaryngology. Springer-Verlag, 1986:3-12.

27. MacPhee MJ, Singh MP, Brady R, Akhyani N, Liau G, Lasa C, Hue C, Best A, Drohan W. Fibrin Sealant: A versatile delivery vehicle for drugs and biologics. In: Sierra DH, Saltz R, editors. Surgical Adhesives and Sealants. Lancaste, Basel: Technomic Publishing CO. INC, 2001:109-20.

28. Kenawy e, Bowlin GL, Mansfield K, Layman J, Simpson DG, Sanders EH, Wnek GE. Release of tetracycline hydrochloride from electrospun poly(ethylene-co-vinylacetate), poly(lactic acid), and a blend. J Control Release In press.

29. Morton TJ, Furst W, van Griensven M, Redl H. Controlled release of substances bound to fibrin-anchors or of DNA. Drug Deliv In press.

30. Gospodarowicz D. Biological activities of fibroblast growth factors. Ann N Y Acad Sci In press.

31. Hankemeier S, Keus M, Zeichen J, Jagodzinski M, Barkhausen T, Bosch U, Krettek C, van Griensven M. Modulation of proliferation and differentiation of human bone marrow stromal cells by fibroblast growth factor 2: potential implications for tissue engineering of tendons and ligaments. Tissue Eng In press.

32. Hollinger JO, Schmitt JM, Buck DC, Shannon R, Joh SP, Zegzula HD, Wozney J. Recombinant human bone morphogenetic protein-2 and collagen for bone regeneration. J Biomed Mater Res In press.

33. Noshi T, Yoshikawa T, Dohi Y, Ikeuchi M, Horiuchi K, Ichijima K, Sugimura M, Yonemasu K, Ohgushi H. Recombinant human bone morphogenetic protein-2

potentiates the in vivo osteogenic ability of marrow/hydroxyapatite composites. Artif Organs In press.

34. Karageorgiou V, Meinel L, Hofmann S, Malhotra A, Volloch V, Kaplan D. Bone morphogenetic protein-2 decorated silk fibroin films induce osteogenic differentiation of human bone marrow stromal cells. J Biomed Mater Res A In press.

35. Cheng SL, Lou J, Wright NM, Lai CF, Avioli LV, Riew KD. In vitro and in vivo induction of bone formation using a recombinant adenoviral vector carrying the human BMP-2 gene. Calcif Tissue Int In press.

36. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng In press.

37. Wolbank S, Peterbauer A, Wassermann E, Hennerbichler S, Voglauer R, van Griensven M, Duba HC, Gabriel C, Redl H. Labelling of human adipose-derived stem cells for non-invasive in vivo cell tracking. Cell Tissue Bank In press.

38. Langer R, Vacanti JP. Tissue engineering. Science In press.

39. Flemming RG, Murphy CJ, Abrams GA, Goodman SL, Nealey PF. Effects of synthetic micro- and nano-structured surfaces on cell behavior. Biomaterials In press.

40. Teixeira AI, Abrams GA, Bertics PJ, Murphy CJ, Nealey PF. Epithelial contact guidance on well-defined micro- and nanostructured substrates. J Cell Sci In press.

Chapter VI

Mechanical stimulation of mesenchymal stem cells embedded in 3-dimensional fibrin constructs

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Manuscript submitted

Abstract

The mechanical environment has the ability to alter the differentiation and phenotype of cells. To determine the specificity of this effect, we developed three-dimensional constructs that embedded mesenchymal stem cells (MSCs) within fibrin gel and loaded the constructs simultaneously in compression and extension. The constructs were placed under either static or dynamic loading for 14 days and their morphology as well as levels of proteins and genes related to either tendon/ligament or cartilage phenotype was determined. Histological analysis of the constructs after the 14 days of stretch showed fewer, elongated, and directionally oriented cells and a high density of collagen within the extension zone of both the dynamic and static stretch groups. Since this morphology and the production of large amounts of collagen is not typical of MSCs, the expression of markers of the tendon/ligament lineage were measured to determine whether the MSCs had differentiated down a tendon/ligament phenotype. As expected, the expression of collagen III, collagen Iα 1, and tensin 2 were all increased within the extension region regardless of the dynamics of loading. The compressed sites of the dynamic stimulated samples showed significantly lower expression of collagen III, collagen Iα 1, α -smooth muscle actin and tensin 2. These data indicate that MSCs within fibrin-based gels can be pushed down divergent cell fates solely on the basis of whether they are loaded in tension or compression.

Introduction

The anterior cruciate ligament (ACL) is the primary stabilizer of the knee. It functions to prevent anterior movement of the tibia in relation to the femur and restrict internal rotation of the tibia. Without a functional ACL, the knee rapidly deteriorates since the resulting laxity of the knee alters normal biomechanics.(1) Due to its limited vascularization and poor healing potential, injuries to the ACL need surgical intervention to return to normal function. For this reason, over 100,000 ACL reconstructions are performed annually in the United States alone. Current methods to reconstruct the ACL include: (1) autografts (replacment with the patients own tissue); (2) allografts (replacment with cadaveric tissue); and (3) permanent protheses (replacment with synthetic material). Using autografts means that healthy tissue from the patient is sacrificed and this is associated with donor site morbidity (Mastrokalos et al 2005). Allograft use is limited by the high potential for infectious disease transfer and the potential immunogenic response,(2,3) and permanent or synthetic protheses are not widely used because they fail due to poor abrasion resistance.(4-7)

More recently, biologically based ACL replacements have been developed, first with reconstituted type I collagen fibers (8), later with prestressed collagen sutures seeded with mesenchymal stem cells (MSC)(9) and most recently Hairfield-Stein *et al.* developed self-organized engineered ligament tissue from bone marrow stromal cells without an exogenous extracellular matrix or scaffold.(10,11). Ligament-derived fibroblasts have also been used with varying success.(12,13) Clearly, a number of different methodologies have been developed, using a variety of scaffolds with little focus on the role that the scaffold might play in the development of the tissue.

Biomaterial scaffolds play very important roles in tissue engineering both *in vitro* and *in vivo*. An ideal scaffold provides temporary support and promotes the proper functional determination of the cells within the tissue

prior to being resorbed by the body. It is becoming increasingly clear that beyond the chemical signals provided by the scaffold, cells receive important mechanical cues from the stiffness of the scaffold.(14-17) Simply changing the stiffness of the 2-dimensional (2D) substrate used for culturing MSCs can induce their differentiation into neural, muscular, or adipose cells.(18) In vivo however, cells rarely grow in 2D. In 3D, the mechanical environment is even more complex. Not only does the passive stiffness of the matrix need to be considered, but the active mechanical environment (i.e. tension, compression, torsion, etc.) needs to be considered as well.

The role of the active mechanical environment is particularly important when one considers musculoskeletal tissues. For example, when attempting to engineer cartilage, constructs are regularly placed under compressive loads, while in engineering tendons/ligaments tensile loads are more important.(19,20) However, in most of the studies that have looked at the active mechanical environment, specific chemical agents and growth factors are added to the media to further promote the differentiation of cells towards the desired phenotype. As a result, it is impossible to tell whether it is the mechanical environment, the chemical environment, or both that is required for the differentiation events.

The aim of the current study was to determine the role of mechanical loading alone on the phenotype of MSCs derived from the amniotic membrane. In order to test this aim, we developed a 3D circular construct model and placed these constructs into bioreactors using flat grips. Static or dynamic separation of the grips resulted in compressive loading on the gripped region and uniaxial tensile loading on the ungripped region.(21) Following 14 days of loading, the constructs were collected and histological and expression analyses were performed to determine the phenotype of the cells within the different regions of the graft.

Materials and methods

HAM isolation and cultivation

All experimental procedures and cell isolations were performed with approval of the local Ethical Review Board. Human placentae were collected during caesarean sections with previous consent. Following collection, the amniotic membrane was peeled off the placenta by blunt dissection and washed several times in phosphate buffered saline (PBS). Cells were isolated as described by Moore et al. with some modifications. Briefly, the amniotic membrane was minced and digested with collagenase (1 mg/mL collagenase I (Biochrom AG, Vienna) in endothelial growth medium (EGM-2; PAA Laboratories GmbH, Pasching) and 10% fetal calf serum (FCS; PAA Laboratories GmbH, Pasching) for 2 hours and 37°C. Following digestion, the cell suspensions were filtered through 100 µm strainers, centrifuged, washed, and cultured in the selected medium EGM-2 (Cambrex Bio Science, Verviers, Belgium) at 37°C, 5% carbon dioxide (CO_2), and 95% air humidity to a subconfluent state.

Fibrin-Cell Constructs and stimulation process

Culture plates (35 mm) were pre-coated with Sylgard® (Dow Corning, Wiesbaden, Germany) and allowed to cure for 14 days. On the day of plating, a Sylgard®-mold was pressed into place in the middle of each 35mm plate and the plates were sterilized using ultraviolet-light and 70% ethylene alcohol. Tisseel® VH Fibrin Sealant (Baxter AG, Vienna) was used to prepare 560 µL fibrin gels in the culture plates. The fibrinogen component was reconstituted with an aprotinin solution (3000 KIU/ml) and the thrombin component with a 40 mM calcium chloride solution according to the manufacturer's instruction. Serum-free medium containing fibrinogen and $5x10^5$ cells was mixed with an equal volume of 4 IU/mL thrombin. The final concentration of fibrinogen was 12.5 mg/mL and of thrombin 2 IU/mL. After 2 hours of polymerization, the fibrin gels

were washed twice with serum-free medium, covered with 1.5 mL of medium containing 5% FCS, and put into the incubator at 37°C, 5% carbon dioxide (CO_2), and 95% air humidity for 13 days. Every third day the media was replaced with fresh medium supplemented with 30 IU/mL aprotinin and the fibrin constructs were.

On day 14, the circular fibrin constructs were placed into specially designed grips and connected to uniaxial strain bioreactor (Figure 6.1B). Because of the design of the grips, the gripped portion of the construct underwent compression while the free portion of the constructs underwent uniaxially extension (Figure 6.1C). The constructs were divided into three groups: (1) unloaded; (2) static; and (3) dynamic. The dynamic groups were loaded for 14 days at 0.1Hz. The static control groups were also fixed in the bioreactor and the length of the construct set so that the constructs were under tension, but no dynamic stretch was performed. The unloaded groups were removed from their molds and allowed to float free for the 14-day period. The media was changed every second day during the 14-day loading period. At the end of the 14 days, the constructs were measured and cut into stretched and compressed parts and stored either at -80°C for RNA analysis or in a 4.5% buffered formaldehyde solution for histological analysis.

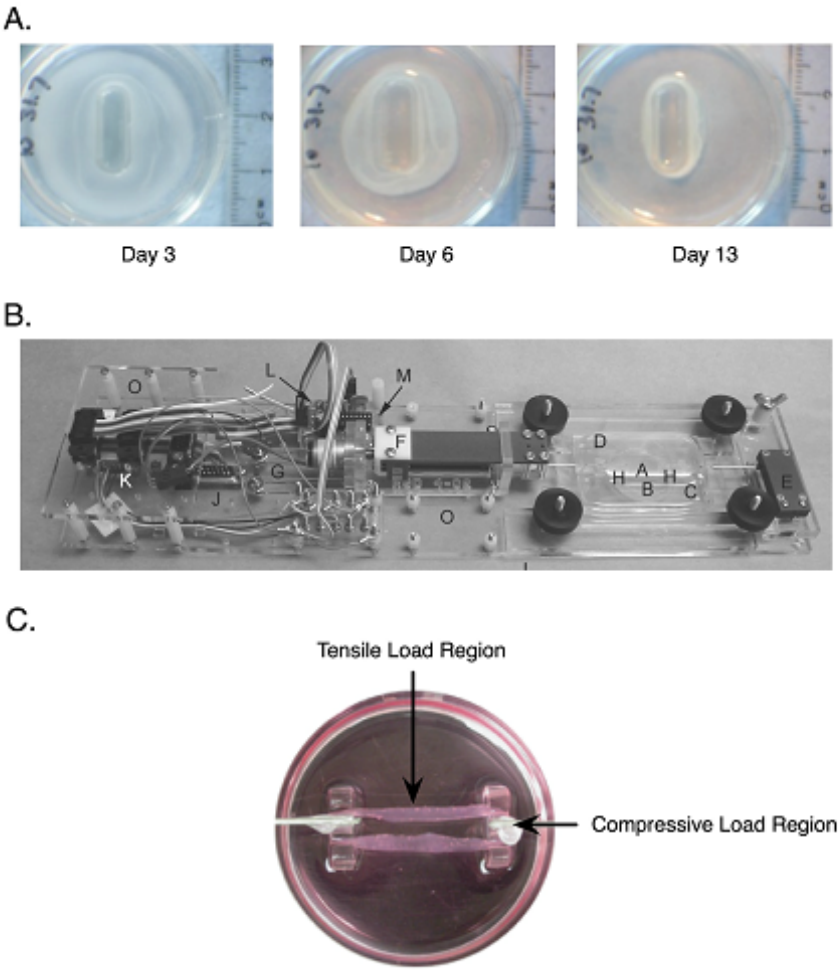

Figure 6.1. (**A**) Formation of fibrin-based 3-dimensional engineered cell construct after 3, 6, and 13 days. The fibrin-cell matrix was forming around the Sylgard™ bar. (**B**) The bioreactor. (**C**) The set up of the mechanical stimulation process. Fibrin-cell constructs were clamped on two hooks in a cell culture dish and covered with medium. The hooks were connected to the stepper motor for stimulation.

RT-PCR

RNA was extracted from cells with trireagent according to manufacturers instructions. Purified RNA was quantified by spectrophotometry, and then aliquoted and stored at -80°C. Prior to reverse transcription, 2 µg of RNA were treated with RNase-free DNase (Promega GmbH, Germany) and the resulting product was transcribed into cDNA using the AMV reverse transcription system (Promega GmbH, Germany). For reverse transcriptase-polymerase chain reaction (RT-PCR) the following primer

sequences (Table 6.1) and conditions were used: 5 min at 94°C, and then 39 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C. The PCR reactions were separated by gel electrophoresis and the product bands were visualized and quantified using ultraviolet light densiometry.

Gene	Primers: sense (s) and antisense (as); ٥' - ٣'		accession#
Col III	s	TGG AGT GTC TGG ACC AAA AG	NM_٠٠٠٠٩٠.٢
	as	ACC ATC TGA TCC AGG GTT TC	
Col XII	s	GAG GGA GTG GAG CTG TTT G	NM_080645.2
	as	GAA CGA TGG GTT CGC TCA G	
TNS2	s	TCA GTC ACC ATG TCA CCT TC	NM_170754.2
	as	GTC CTT GTC CTT CAG CAG G	
COMP	s	CAA GGT GGT AGA CAA GAT CG	NM_000095.2
	as	ACC ACG TAG AAG CTG GAG C	
BMPRIa	s	AAA TGG CGT GGC GAA AAA GTG	NM_004329.2
	as	ACA GCA AGG CCC AGG TCA GC	
CFL1	s	ATG CCC TCT ATG ATG CAA CC	NM_005507
	as	GGA TGG AGG GAG AAG GAA AA	
aSMA	s	CGA CCG AAT GCA GAA GGA GA	NM_001613.1
	as	TTT GCG GTG GAC AAT GGA AG	
bActin	s	ACC TTC TAC AAT GAG CTG CG	NM_001101.2
	as	GGA GTA CTT GCG CTG AGG A	
GAPDH	s	TTA GCA CCC CTG GCC AAG G	NM_002046.3
	as	CTT ACT CCT TGG AGG CCA TG	

Table 6.1. Human specific oligonucleotide primers used for polymerase chain reactions. Col III, collagen III; Col XII, collagen XII; TNS2, tensin 2; COMP, cartilage oligomeric matrix protein; BMPR Ia, bone morphogenic protein receptor Ia; CFL1, cofilin 1; αSMA, alpha smooth muscle actin; β-actin, beta actin; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

Histology

At the time of collection, stretched and control fibrin constructs were fixed in 4.5% buffered formaldehyde solution. Fixed samples were embedded in paraffin, sectioned, and stained with hematoxylin/eosin (H&E) using standard histology protocols. For M.S.B. (martius/scarlet/blue) trichrome staining, samples were deparaffinated, rinsed in alcohol and water, and then treated according to Lendrum *et al*.(22) Finally, samples were rinsed in deionised water, dehydrated, and fixed on object slides with Aquatex (Merck GmbH, Vienna). Evaluation of labeled slides of the fibrin/cell

constructs was done blinded. Samples were analyzed for residual fibrin, cell number and collagen level.

Statistical analysis

The medians and deviations (Q1, Q3) were calculated for all variables tested. Statistical analysis of data was performed by unpaired *t*-test and statistical significance was accepted at $*p < 0.05$.

Results

Morphology

The formation of the fibrin-cell constructs took place within 14 days of plating (Figure 6.1A). The length of the constructs was determined using a digital calliper both following formation and after the 14 days loading period. At the end of the loading period, the dynamic constructs were about 14% longer (pre=20.5±0.0mm and post=24.1±0.3mm) while the length of neither the static nor unloaded constructs had changed over the 14 days in culture.

Histology

Histological examination of the constructs showed evenly spread cells in the constructs of all groups (Figure 6.2). The static and unloaded matrices were continuous, while the dynamic group contained some holes due to the high tensile loads during the experiment (Figure 6.2A). The cell number was significantly higher in the unloaded group compared with the dynamic and static groups (*$p<0.05$, Figure 6.2B). The cells in the dynamic and static stretched areas were orientated within the matrix, while cells in the unloaded group were not. Cells in the compressed regions were grouped and showed no uniform orientation. M.S.B. trichrome staining showed blue-green areas of collagen in the dynamic stretch group (Figure 6.3). Because of the low collagen concentrations in the compressed areas blue areas could not be detected within this region.

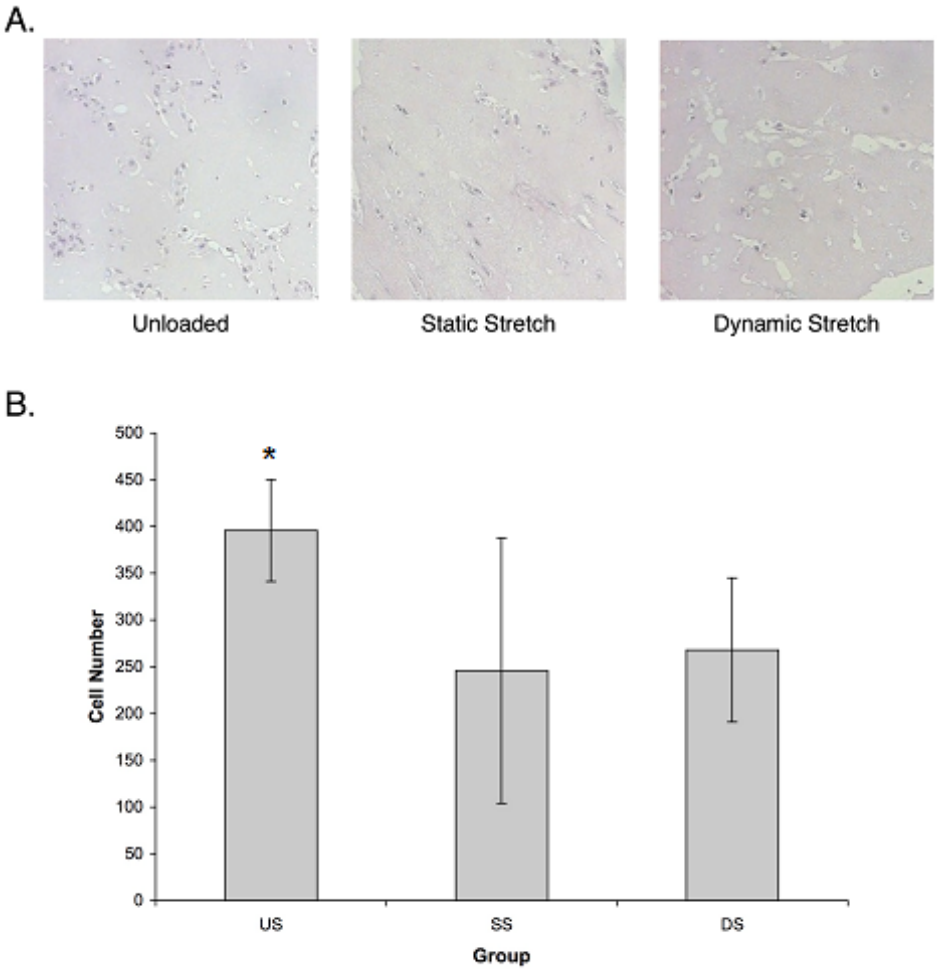

Figure 6.2. Histological and statistical analysis of hämatoxylin and eosin stained samples. (**A**) Most of the dynamic and static stimulated cells were orientated in the direction of mechanical stimulation (10x). (**B**) The cell number was significantly higher in the unloaded group compared with the stretched groups (*$p<0.05$).

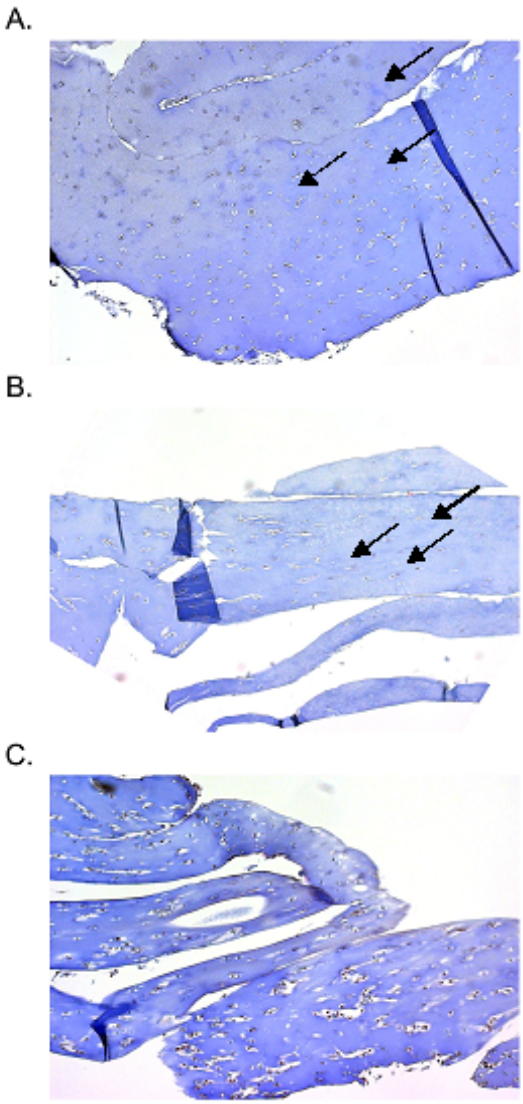

Figure 6.3. Representative slides of M.S.B. trichrome stained (**A**) dynamic, (**B**) static stimulated and (**C**) unstimulated fibrin-cell constructs. Stimulated samples possessed collagen production which is represented in the blue-green colour. Some collagen locations are indicated by black arrows (20x).

Gene Expression

The increased levels of collagen in the stretched groups suggested that the MSCs had differentiated within the fibrin matrix. Therefore, the expression of genes indicative of the tendon/ligament or cartilage fate were determined. The genes analyzed fell into three general groups: those that did not change with tension or compression; those that decreased with either tension or compression; and those that increased under tension. The genes that were unchanged in any group were cofilin, and bone morphogenic protein receptor Ia (Figure 6.4). Surprisingly, the

previously identified tension-dependent genes, cartilage oligomeric matrix protein and collagen XII, were significant higher in the unloaded group (Figure 6.4). Collagen III, and tensin2 were highest in the dynamic stretch group and showed a significantly higher expression in both the dynamic and static stretch samples compared to the corresponding compressed regions (Figure 6.5). Alpha smooth muscle actin was higher in both the dynamic and static groups in the region of extension then in the compression zones (Figure 6.5).

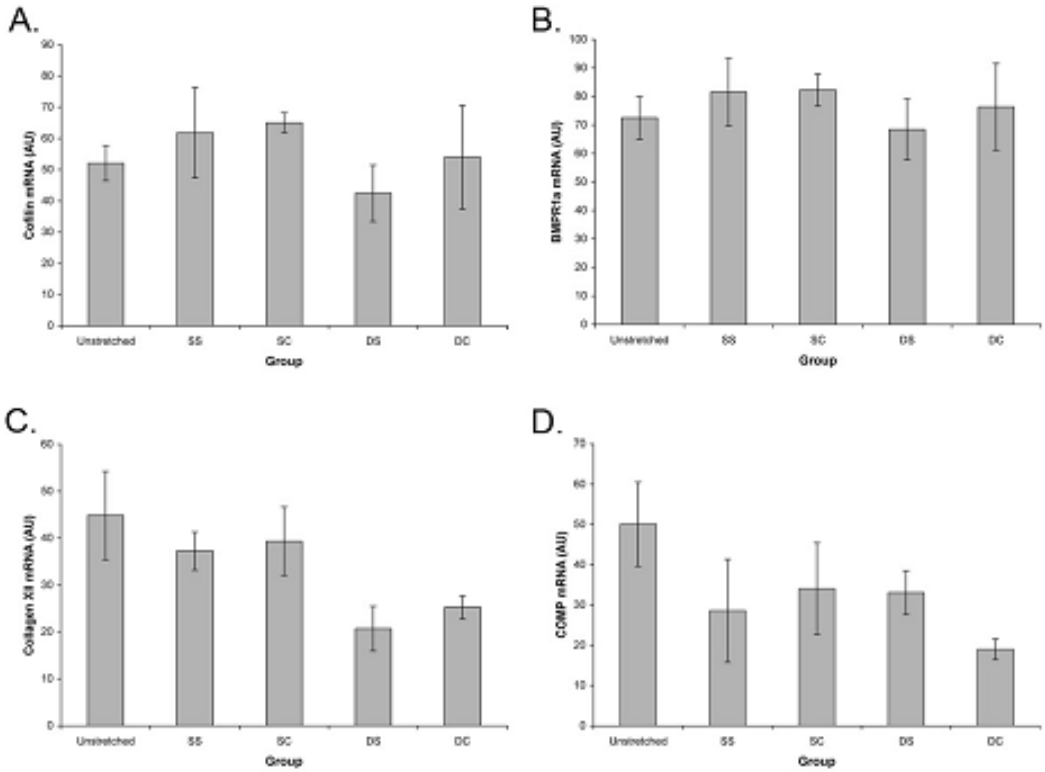

Figure 6.4. Different expression levels of various markers important for ligaments could be observed in amniotic derived mesenchymal stem cells. (n = 6, median ± Q1, Q3). (**A**) cofilin; (**B**) BMPR Ia (bone morphogenic protein receptor Ia); (**C**) collagen XII; (**D**) COMP (cartilage oligomeric matrix protein).

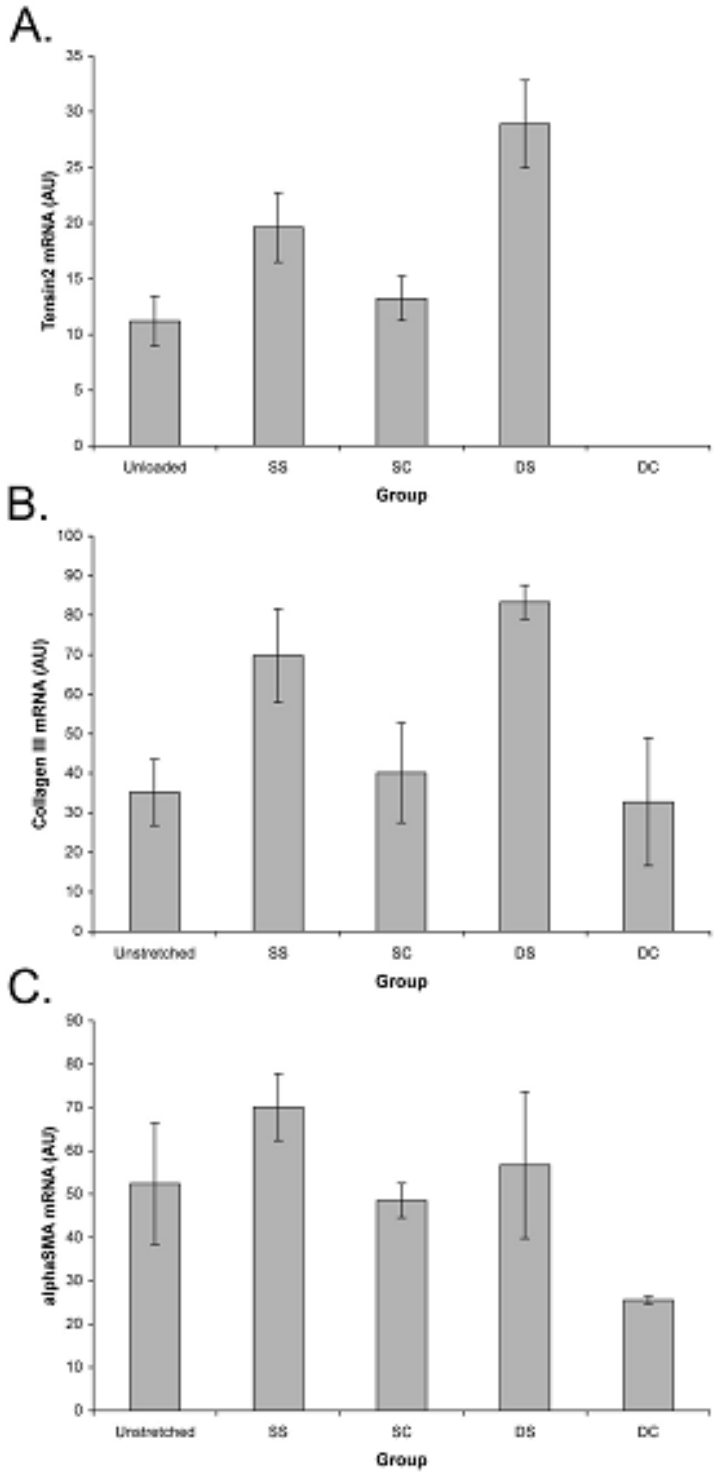

Figure 6.5. Different expression levels of various markers important for ligaments could be observed in amniotic derived mesenchymal stem cells. (n = 6, median ± Q1, Q3). (**A**) tensin2; (**B**) collagen III; (**C**) αSMA (alpha smooth muscle protein).

Discussion

Using a unique circular construct and a flat gripping system, were have shown that mechanical loading on its own provides important cues to MSCs that promote their differentiation into various musculoskeletal tissues. When loaded under dynamic uniaxial tension, MSCs orient along the line of stretch, synthesize a collagen rich matrix, and begin to express markers of developing tendons/ligaments. Under compression or in unloaded gels, cells showed no equal orientation and led to both to low mRNA expression levels for collagen III, alphaSMA, and tensin2.

One of the best studied aspects of mechanical loading is its effect on cell orientation. Both in 2D and 3D, tension causes cells to reorient themselves in a direction that is directly related to the applied load. (23,24) In the current study, the cells in the stretch groups became longer, reoriented, and well-distributed within the fibrin scaffold. Although the fibrin was homogeneous, cells in the unloaded group appeared mostly in clusters, possibly due to the clonal expansion of cells that did not migrate through the fibrin gel. These groups of cells showed no secondary organization and the cells themselves were much smaller than the cells in the stretched groups (Figure 6.3).

Cell number was lower in both dynamic and static stretch group compared to the unloaded group. One possible explanation for there being fewer cells within the constructs following the 14 days of stretch is that the application of mechanical stretch dynamic as well as static inhibited cell growth and promoted cell differentiation. This hypothesis is supported both by mRNA and histological observation. In the regions of extension, there was an increase in the amount of collagen detected by Masson's trichrome indicating that the cells in this region had become more tendon-like (Figure 6.3). Furthermore, collagen III, a collagen that is expressed in developmentally immature tendon, and the focal adhesion molecule Tensin2 were expressed to a greater degree in the tensile region (Figure 5), suggesting that in the loaded area the construct was becoming a developmental tendon.

The dynamic stretch group possessed the lowest concentration of cells. The cells were embedded in the matrix at a greater distance from each other and seemed to reside within holes in the fibrin network. Constructs that underwent static stretch showed a similar cell number but the cell were embedded in a strong fibrin matrix, similar to unloaded cells. The level of collagen XII and cofilin were higher in the static constructs relative to the dynamic groups. Alpha smooth muscle actin tended to be higher in the stretched samples then in the compressed regions. Both collagen XII and COMP, markers associated with the interactions between the fibrils and the surrounding matrix, were highly expressed in cells embedded in unloaded gels.

MSCs embedded in fibrin without any uniaxial tension appeared mostly in clusters and appeared to take on a cartilage phenotype. In comparison, static stretch changed the nature of MSCs dramatically. Cells showed orientation along the direction of tension and expressed markers of developing tendons/ligaments. Although about one third of embeeded cells died during the stretching process amnion-derived mesenchymal stem cells are excellent cells for tissue engineering applications. They have a high potential because of their fetal origin and pluripotency.(25) MSCs derived from amnion also have a high expansion potency allowing the rapid formation of tissues such as the fibrin constructs used here.(24) The cells also appear robust and under the right mechanical cues can differentiate into tendon/ligament or cartilage like cells.

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References

1. Jackson DW, Heinrich JT, Simon TM. Biologic and synthetic implants to replace the anterior cruciate ligament. Arthroscopy In press.

2. Laurencin CT, Ambrosio AM, Borden MD, Cooper JA, Jr. Tissue engineering: orthopedic applications. Annu Rev Biomed Eng In press.

3. Dunn MG, Liesch JB, Tiku ML, Zawadsky JP. Development of fibroblast-seeded ligament analogs for ACL reconstruction. J Biomed Mater Res In press.

4. McPherson GK, Mendenhall HV, Gibbons DF, Plenk H, Rottmann W, Sanford JB, Kennedy JC, Roth JH. Experimental mechanical and histologic evaluation of the Kennedy ligament augmentation device. Clin Orthop Relat Res In press.

5. Paavolainen P, Makisalo S, Skutnabb K, Holmstrom T. Biologic anchorage of cruciate ligament prosthesis. Bone ingrowth and fixation of the Gore-Tex ligament in sheep. Acta Orthop Scand In press.

6. Guidoin MF, Marois Y, Bejui J, Poddevin N, King MW, Guidoin R. Analysis of retrieved polymer fiber based replacements for the ACL. Biomaterials In press.

7. Kock HJ, Sturmer KM, Letsch R, Schmit-Neuerburg KP. Interface and biocompatibility of polyethylene terephthalate knee ligament prostheses. A histological and ultrastructural device retrieval analysis in failed synthetic implants used for surgical repair of anterior cruciate ligaments. Arch Orthop Trauma Surg In press.

8. Dunn MG, Tria AJ, Kato YP, Bechler JR, Ochner RS, Zawadsky JP, Silver FH. Anterior cruciate ligament reconstruction using a composite collagenous prosthesis. A biomechanical and histologic study in rabbits. Am J Sports Med In press.

9. Young RG, Butler DL, Weber W, Caplan AI, Gordon SL, Fink DJ. Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J Orthop Res In press.

10. Hairfield-Stein M, England C, Paek HJ, Gilbraith KB, Dennis R, Boland E, Kosnik P. Development of self-assembled, tissue-engineered ligament from bone marrow stromal cells. Tissue Eng In press.

11. Toyoda T, Matsumoto H, Fujikawa K, Saito S, Inoue K. Tensile load and the metabolism of anterior cruciate ligament cells. Clin Orthop Relat Res In press.

12. Vunjak-Novakovic G, Altman G, Horan R, Kaplan DL. Tissue engineering of ligaments. Annu Rev Biomed Eng In press.

13. Calve S, Dennis RG, Kosnik PE, Baar K, Grosh K, Arruda EM. Engineering of functional tendon. Tissue Eng In press.

14. Byrne DP, Lacroix D, Planell JA, Kelly DJ, Prendergast PJ. Simulation of tissue differentiation in a scaffold as a function of porosity, Young's modulus and dissolution rate: application of mechanobiological models in tissue engineering. Biomaterials In press.

15. Boontheekul T, Hill EE, Kong HJ, Mooney DJ. Regulating myoblast phenotype through controlled gel stiffness and degradation. Tissue Eng In press.

16. Engler AJ, Griffin MA, Sen S, Bonnemann CG, Sweeney HL, Discher DE. Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments. J Cell Biol In press.

17. Engler AJ, Sweeney HL, Discher DE, Schwarzbauer JE. Extracellular matrix elasticity directs stem cell differentiation. J Musculoskelet Neuronal Interact In press.

18. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell In press.

19. Jung Y, Kim SH, Kim SH, Kim YH, Xie J, Matsuda T, Min BG. Cartilaginous tissue formation using a mechano-active scaffold and dynamic compressive stimulation. J Biomater Sci Polym Ed In press.

20. Kapacee Z, Richardson SH, Lu Y, Starborg T, Holmes DF, Baar K, Kadler KE. Tension is required for fibripositor formation. Matrix Biol In press.

21. Birla RK, Huang YC, Dennis RG. Development of a Novel Bioreactor for the Mechanical Loading of Tissue-Engineered Heart Muscle. Tissue Eng In press.

22. LENDRUM AC, FRASER DS, SLIDDERS W, HENDERSON R. Studies on the character and staining of fibrin. J Clin Pathol In press.

23. Sun S, Titushkin I, Cho M. Regulation of mesenchymal stem cell adhesion and orientation in 3D collagen scaffold by electrical stimulus. Bioelectrochemistry In press.

24. Huang YC, Dennis RG, Larkin L, Baar K. Rapid formation of functional muscle in vitro using fibrin gels. J Appl Physiol In press.

25. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells In press.

Chapter VII

Summary

Zusammenfassung

Summary

Tissue engineering approaches urge us to develop and create engineered matrices or scaffolds biomimicking tissue *in vitro*. Tissue engineering implies the presence of reparative/regenerative cells, biodegradable scaffolds, and bioreactors to control the cellular environment. Cells and biomaterial scaffolds can be utilized in many ways. Fibrin represents a useful biodegradable matrix that also binds to regulatory signals such as growth factors. In our studies we tested fibroblast growth factor-2 (FGF-2) peptides, which have a binding affinity to fibrin(ogen) of their biological activity and behavior on isolated human adipose-derived stem cells. FGF-2 peptides had no proliferation effect, but elevated collagen Iα 2 and collagen III mRNA expression within 7 days of incubation compared to FGF-2 addition. Further experiments concerned the controlled release of substances out of fibrin. These agents, not having a fibrin-binding domain, were bound to a fibrin anchor (fibronectin or thrombin), which has a naturally binding domain to fibrin(ogen), and showed continous release from the matrix.

Another approach was the production of fibrin mats using electrospinning. Fibrinogen and thrombin solved in a hexafluoroisopropanol solution were electrospun to nanofibers. Due to the small diameters of the electrospun fibrin nanofiber, they are more attractive for cell attachment. Their similarity in size to native extracellular matrix components and the 3-dimensional structure allows cells to attach to several fibers in a more natural geometry. In addition, seeded cells showed different proliferation patterns on matrices containing growth factors in comparison on nanofibers without additives.

Finally, a novel method for generating three-dimensional mesenchymal stem cell (MSC)-based constructs using fibrin gel casting were used for amniotic MSC, which were mixed within the fibrin gel. Over the next 8 days, cell-mediated tension contracts the gel around two artificial anchors, resulting in small tubular constructs. Following formation, these constructs were connected to a stepper-motor and were uniaxially loaded to 114% of

their resting length at 0.1 Hz over 14 days. Histological analysis of the constructs showed a high density of collagen between the anchors. These data indicate that MSC/fibrin-based gels provide a novel method to engineer three-dimensional functional constructs as tendon or ligament *in vitro*.

Zusammenfassung

Die Methode von Tissue Engineering oder der Gewebezüchtung drängt uns Matrizen oder Stützgerüste , die Gewebearten imitieren *in vitro* zu entwickeln. Tissue Engineering setzt die Präsenz von sich regenerierenden Zellen, biologisch degradierenden Matrizen und Bioreaktoren, die die zelluläre Umwelt regulieren. Zellen und biologische Matrizen können mit unterschiedlichen Methoden verwendet werden.

Fibrin stellt eine gut anwendbare biologisch abbaubare Matrix, an die regulatorische Faktoren wie Wachstumsfaktoren binden, dar. In unseren Studien testeten wir Fibroblasten Wachstumsfaktor 2 (FGF-2) Peptide, welche eine Bindungsaffinität zu Fibrin(ogen) aufweisen, auf ihre biologische Aktivität an isolierten humanen Fettstammzellen. FGF-2 Peptide hatten keine Auswirkung auf die Proliferation der Zellen, aber führten zu einer Erhöhung der Kollagen Ia2 und Kollagen III mRNA Expression innerhalb einer Inkubationszeit von 7 Tagen. Weitere Experimente beschäftigten sich mit dem kontrollierten Freisetzung von Substanzen aus Fibrin. Substanzen, die keine natürliche Bindungsaffinität zu Fibrin(ogen) aufweisen, wurden an sogenannte Fibrin-Anker (Fibronektin oder Trombin), die eine Bindedomäne zu Fibrin(ogen) haben, gebunden. Mit diesem System zeigte sich eine kontinuierliche Freisetzung dieser Substanzen aus der Fibrinmatrix.

Eine weiter Methode der Modifikation von Fibrin war die Produktion von Fibrinmatten durch elektrisches Spinnen. Fibrinogen und Trombin wurden in einer Hexafluoroisopropanol-Lösung gelöst und zu Nanofasern gesponnen. Auf Grund des geringen Durchmessers der Nanofasern bilden sie eine attraktive Matrix für Zellkontakte. Die Ähnlichkeit in der Größe des Netzwerks und der 3-dimensionalen Anordnung der Nanofasern zu extrazellulären Matrixcomponenten erleichtern Zellen den Kontakt und die Anhaftung zur Matrix.

Zuletzt beschäftigten wir uns noch mit einer neuen Methode um 3-dimensionale mesenchymale Stammzell-Konstrukte in Fibrin zu erzeugen. Amniotische mesenchymale Stammzellen wurden mit Fibrin vermischt.

Innerhalb von 8 Tagen wurde das Fibrin durch die zelluläre Spannung zu einem schlauchartigen Konstrukt kontrahiert. Dieses Fibrin-Zell-Konstrukt wurde in einem Bioreaktor über einen Zeitraum von 14 Tagen mit 0.1 Hz auf eine Länge von 114% gedehnt. Histologische Untersuchungen zeigten eine hohe Dichte von Kollagen im gedehnten Bereich. Diese Resultate weisen auf eine neue Methode zur Produktion *in vitro* von funktionellen Konstrukten wie Sehnen oder Bänder hin.

Appendices

Danksagung

Biographie

PhD portfolio summary

List of publications

Danksagung

Diese Dissertation wurde am Ludwig Boltzmann Institut für experimentelle und klinische Traumatologie unter der Leitung von Prof. Dr. Heinz Redl durchgeführt. Mein großer Dank gilt ihm für die Ermöglichung dieser Arbeit und die Möglichkeit, mich in wissenschaftlichen Fragestellungen zu entfalten.

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Biographie

Tatjana J. Morton, geboren als Tatjana Sindelar am 16. Februar 1980 in Wien, Österreich, besuchte das Realgymnasium I Schottenbastei in Wien. Anschließend an die Reifeprüfung inskribierte sie die Studien der Chemie und Physik an der Universität Wien. In ihrem Chemiestudium setzte sie die Schwerpunkte Biochemie und Lebensmittelchemie. Im Mai 2005 schloss sie ihre Diplomarbeit in Chemie am Ludwig Boltzmann Institut für experimentelle und klinische Traumatologie ab. Danach begann sie am selbigen Institut mit dem Studium zum Doktorat im Bereich Tissue Engineering unter der Betreuung von Prof. DDr. Martijn van Griensven. Ihre Dissertation führte sie unter anderem für ein Monat an das Institut für Biomaterialien an der Technische Universität von Tampere, Finnland und für drei Monate an das Institut für Molekulare Physiology an der Universität von Dundee, Großbritannien. Im Juni 2006 heiratete sie ihren Mann Robert Morton und im August 2008 erblickte ihre Tochter Luna Orieta das Licht der Welt. Ihre Dissertation schloss sie im Dezember 2008 ab.

PhD portfolio summary

Name PhD student: Tatjana J. Morton
Institution: Ludwig Boltzmann Institute for Experimental and Clinical Traumatology
University: University of Vienna, Department of Chemistry
PhD period: October 2005 – December 2008
Promotor: Prof. DDr. Martijn van Griensven
Co-promotor: Prof. Dr. Heinz Redl
Co-promotor: Dr. Keith Baar

1. Conferences and presentations

Podium presentations

Mechanical stimulation of human stem cells embedded in a fibrin construct-ligament engineering
Tatjana J. Morton, Keith Baar, Kathrin Reise, Anja Peterbauer, Martijn van Griensven, Heinz Redl
European Tissue Engineering Society (TERMIS)
Porto, P
22.-26.06.2008

Shockwave therapy on human adipose-derived stem cells
Tatjana J. Morton, Joachim Hartinger, Eva Tögel, Sabine Pfeifer, Anja Peterbauer, Wolfgang Schaden, Martijn van Griensven
International Society of Medical Shockwave Treatment (ISMST)
Juan les Pins, Antibes, F
05.-07.06.2008

Electrospun Fibrin Nanofibers for Angiogenesis in vitro and in vivo
Tatjana J. Morton, Lila Nikkola, Nurredin Ashammakhi, Susanne Wolbank, Heinz Redl, Martijn van Griensven
European Tissue Repair Society (ETRS)
Southampton, UK
25.-28.09.2007

Electrospinning of fibrin(ogen)
T.J. Morton, L. Nikkola, N. Ashammakhi, M. van Griensven, H. Redl
Tissue Engineering Today, Not Tomorrow (TETNT)
London, UK
17.11.2006

Electrospinning of fibrinogen
T. Sindelar, L. Nikkola, N. Ashammakhi, M. van Griensven, H. Redl
European Tissue Engineering Society (TERMIS)
Rotterdam, NL
08.-11.10.2006

Fibrin binding conjugates for the use of fibrin as a drug release matrix
T. Sindelar, W. Fürst, M. van Griensven, H. Redl
International Conference on Advances in Biomaterials (ICAB)
Capri, I
11.-16.06.2006

Posterpresentations

Fibrin – An Injectable Smart Hydrogel
T. J.Morton, M. Hofmann, K. Schöbitz, H. Redl, M. van Griensven
European Tissue Engineering Society (TERMIS)
Porto, P
22.-26.06.2008

Electrospun Fibrin Nanofibers for Angiogenesis in vitro and in vivo
Tatjana Morton, Lila Nikkola, Nurredin Ashammakhi, Susanne Wolbank, Anja Peterbauer, Martina Hofmann, Martijn van Griensven, Heinz Redl
SAWC & WHS
Tampa, Florida, USA
28.04.-01.05.2007

Electrospinning of fibrin(ogen) nanofibers
T. Sindelar, L. Nikkola, N. Ashammakhi, M. van Griensven, H. Redl
ESF-EMBO Symposium-"Stem Cells in Tissue Engineering"
Sant Feliu de Guixols, S
28.10.-02.11.2006

Electrospinning of fibrin(ogen) nanofibers
T. Sindelar, L. Nikkola, N. Ashammakhi, M. van Griensven, H. Redl
Biomaterials 2006
Essen, G
05.-08.09.2006

Electrospinning of fibrin(ogen)
T. Sindelar, L. Nikkola, N. Ashammakhi, M. van Griensven, H. Redl
Strategies in Tissue Engineering
Würzburg, G
31.05.-02.06.2006

Electrospinning of fibrinogen
T. Sindelar, L. Nikkola, N. Ashammakhi, M. van Griensven, H. Redl
Expertissues (18 months meeting)
Vienna, A
24.-25.03.2006

2. Study trips

European Network of Excellence `EXPERTISSUES` Mechanical stimulation of cells embedded in fibrin University of Dundee, Scotland, UK	05-08/2007
European Network of Excellence `EXPERTISSUES` Electrospinning of Fibrin(ogen) Nanofibers Tampere University of Technology, Tampere, Finland	10-11/2005

3. Teaching activities

Supervising Bachelor and Master theses

Cloning and expression of recombinant FGF-2	2005-2006
Expression and purification of recombinant FGF-2	2006-2007
Surface modification of fibrin	2006-2007
Influence of growth factors *in vivo* delivered from fibrin	2008
Mechanical stimulation of C2C12 cell line	2008-2009
Influence of fibrin composition on cell behaviour	2008-2009

List of publications

Fibroblast growth factor-2 peptides bind to fibrinogen and fibrin and exert biological activity
Tatjana J. Morton, Burkhard Kloesch, Heinz Redl, and Martijn van Griensven
Submitted

FGF-2 peptide enhance adipose-derived stem cell differentiation into tendon-like tissue
Tatjana J. Morton, Kathrin Lang, Asmita Banerjee, Daniela Dopler, and Martijn van Griensven
Submitted

Electrospun fibrin nanofibers for the use in tissue engineering
Tatjana J. Morton, Lila Nikkola, Susanne Wolbank, Martina Hofmann, Sabine Pfeifer, Christian Grasl, Heinz Redl, and Martijn van Griensven
Submitted

Mechanical stimulation of mesenchymal stem cells embedded in 3-dimensional fibrin constructs
Tatjana J. Morton, Kathrin Reise, Anja Peterbauer, Martijn van Griensven, Heinz Redl, and Keith Baar
Submitted

Low dose BMP-2 in fibrin matrix equivalent to clinical product with high dose BMP-2 in collagen? An experimental study
S.Schützenberger, A.Schultz, T.Hausner, R.Hopf, G.Zanoni, **T.J. Morton**, K.Kropik, M. van Griensven, H.Redl
Submitted

Controlled release of substances bound to fibrin-anchors or of DNA
Morton TJ, Fürst W, van Griensven M, Redl H
Drug Deliv. 2009 Feb;16(2):102-7

Sustained (rh)VEGF(165) release from a sprayed fibrin biomatrix induces angiogenesis, up-regulation of endogenous VEGF-R2, and reduces ischemic flap necrosis
Mittermayr R, **Morton TJ**, Hofmann M, Helgerson S, van Griensven M, Redl H
Wound Repair Regen. 2008 Jul-Aug;16(4):542-50

Fibrin – an injectable smart hydrogel
Hofmann M., **Morton T.J.**, Schöbitz K., Redl H., van Griensven M.
Tissue Engineering, 14 – Part A, 854 – 855, 2008

Local delivery of VEGF165 from fibrin biomatrix significantly reduces tissue necrosis in a dose dependent manner
Mittermayr R., Krammel F., Hartinger J., Simon B., **Morton T.J.**, Hofmann M., van Griensven M., Redl H.
Tissue Engineering, 14 – Part A, 873, 2008

In vivo expression of VEGF-R2 during early hindlimb ischaemia-reperfusion injury
Hofmann M., Mittermayr R., **Morton T.J.**, Redl H., van Griensven M.
European Journal of Trauma and Emergency Surgery, 33, Suppl. II, 6, 2007

Naturally bound and linked growth factors in fibrin matrix to reduce skin flap necrosis
Mittermayr R., Hofmann M., **Morton T.J.**, Helgerson S., van Griensven M., Redl H.
Inflammation Research, 56, Suppl. 2, S237 - S238, 2007

Biodegradable nanomats produced by electrospinning: expanding multifunctionality and potential for tissue engineering
Ashammakhi N, Ndreu A, Piras A, Nikkola L, **Sindelar T**, Ylikauppila H, Harlin A, Chiellini E, Hasirci V, Redl H
J Nanosci Nanotechnol. 2006 Sep-Oct;6(9-10):2693-711. Review