Comparison of Structure, Strength and Cytocompatibility of a Fibrin Matrix Supplemented Either With Tranexamic Acid or Aprotinin

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Abstract: Fibrin sealants are used as hemostats, sealants, tissue adhesives, and as matrix for substances/cells in a number of surgical and tissue engineering procedures. Main characteristics of fibrin are high tensile strength, adhesive strength, biocompatibility, and resorption. A major adverse event would be premature fibrin lysis and recurrent bleeding. This must be prevented by fibrinolysis inhibitors. The most common fibrinolysis inhibitors used are aprotinin and tranexamic acid (t-AMCA). Comparison of commercially available fibrin sealants utilizing aprotinin or t-AMCA revealed a lower sealing efficacy in an *in vivo* lung resection model for a t-AMCA containing product. Therefore, we compared the influence of t-AMCA and aprotinin on structure, mechanical properties, and cytocompatibility of a fibrin matrix. In our experiments, we found that substitution of aprotinin with t-AMCA reduced the tensile strength and formation of fibrin fibers and affected viability of a fibroblast cell-line. In conclusion, t-AMCA negatively affects physical and biological properties of fibrin relevant for clinical application as well as tissue regeneration. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 82B: 109–114, 2007

Keywords: biocompatibility/soft tissue; mechanical properties; wound healing; hemostasis; fibrin

INTRODUCTION

Various surgical tissue adhesives have been developed to serve as hemostats, sealants, and glues. Their characteristics depend very much on the nature of their constituents, which can be fibrin(ogen), collagen, cyanoacrylate, polyethylene glycol, albumin–glutaraldehyde, or gelatin–resorcinol–formaldehyde.[1] In the case of natural products like fibrinogen, the characteristics are also heavily influenced by the isolation procedure and the final formulation of the compounds.

Fibrin sealants, resembling the final step of the coagulation cascade, are concentrated preparations of human fibrinogen and thrombin that form a fibrin clot upon mixing these two components during application to the wound surface. High tensile strength and adhesive strength are mechanical properties characterizing fibrin clots and are necessary for successful sealing.[2] Fibrin has also the potential to promote wound healing by providing a number of growth factors (e.g., FGF-2 and VEGF) and adherence molecules (e.g., fibronectin). These factors are associated with fibrinogen and copurified during the manufacturing process,[3,4] and may attract and/or stimulate cells involved in tissue repair.[5,6]

In contrast to some synthetic tissue adhesives, fibrin clots will be resorbed by the physiological process of fibrinolysis.[7] Primarily, this process is induced by proteolytic cleavage and activation of plasminogen to plasmin. Plasminogen is associated with fibrinogen and copurified by commercially used cryoprecipitation steps. Other fibrinolytic processes include digestion by inflammatory cells via proteolytic enzymes (e.g., elastase).[8,9] Furthermore, fibrinolytic conditions may vary upon different tissues and organ conditions of individual patients. Variations in fibrinolytic status, however, may lead to premature clot lysis followed by recurrent bleeding, leakage, or tissue dehiscence.

Premature clot lysis of fibrin sealants is prevented by supplementation with fibrinolysis inhibitors like aprotinin or tranexamic acid (t-AMCA).[10] These fibrinolysis inhibitors are also used as antifibrinolytic agents in a wide range of hemorrhagic conditions.[11,12] Aprotinin acts as protease inhibitor, for example, against plasmin and is well tolerated. However, repeated administration of aprotinin may cause anaphylactic reactions because of its bovine peptide sequence.[11] t-AMCA blocks binding of plasminogen to

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TABLE I. Sealing Efficacy of Fibrin Sealants in a Partial Lung Resection Model

	T	*Q*
Leakage pressure (mbar)	23.6 ± 3.0	19.0 ± 2.2*
Minute volume at leakage (L)	1.6 ± 0.3	1.1 ± 0.2

T: Tisseel, Q: Quixil; Mean ± SD; $n = 7$; $*p < 0.05$ versus *T*.

fibrinogen/fibrin and does not act as a protease inhibitor.[13,14] Adverse events following systemic application of t-AMCA concern the gastrointestinal tract.[12] However, local application of t-AMCA in CNS induces epileptic seizures via blockade of γ-aminobutyric $acid_A$ receptor-induced chlorine flux.[15,16]

Commercially available fibrin sealants utilizing aprotinin or t-AMCA show an obviously different visual appearance (nontransparent for aprotinin vs. transparent for t-AMCA) and revealed different sealing efficacies in an *in vivo* partial lung resection model (Table I). Thus, we were interested to investigate whether t-AMCA plays a role with regard to these effects on fibrin sealants. In this short-term experiment (~10 min after sealing), resistance to fibrinolysis has no influence. Therefore, we compared the influence of t-AMCA and aprotinin on structure and mechanical properties of fibrin matrix. In addition, the effect of t-AMCA on MRC5 cells, a nonneural human lung fibroblast cell line typically used for assessment of cytotoxicity, was tested and compared to aprotinin. This was done for cytotoxicity by (i) the pure compounds, (ii) having both compounds in the same fibrin matrix, where possible, and (iii) having one fibrin sealant, which contains t-AMCA, in its original state.

MATERIALS AND METHODS

Two-component fibrin sealants, Quixil®/CrossSeal® (Omrix, Israel) and Tisseel VH® (Baxter AG, Austria) were prepared according to the manufacturer's instructions, for direct comparison of sealing efficacy *in vivo*. Sealer protein solutions contained 40–60 mg/mL clottable protein for Quixil/CrossSeal or 75–110 mg/mL clottable protein for Tisseel VH. Homogeneous fibrin clots for all other experiments were prepared by mixing the sealer protein (fibrinogen) with 4 U/mL thrombin. In case of Tisseel, the kit contains a vial with 4 U/mL thrombin, while thrombin from the Quixil/CrossSeal kit was diluted to 4 U/mL with a dilution buffer containing 0.6% human serum albumin, 2% mannitol, 20 m*M* sodium acetate, 77 m*M* NaCl, and 40 m*M* $CaCl_2$, pH = 6.7. Experiments investigating the specific influence of t-AMCA were performed with sealer protein of Tisseel VH (10 % w/v) dissolved either in aqueous solutions of 3000 KIU/mL aprotinin (supplied with Tisseel) or 95 mg/mL t-AMCA. Concentrations of fibrinolysis inhibitors correspond to those used in commercially available products Tisseel VH and Quixil/CrossSeal. Ziehl-Neelson-carbol-fuchsin solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), t-AMCA, and minimum essential medium eagle alpha (MEMα) cell culture medium were obtained from Sigma (MO), fetal calf serum (FCS) was from Biowhittaker (Belgium), and cell culture plates were from Costar (NY). All other chemicals used were purchased from Fluka (Switzerland). MRC5 lung fibroblast cell line was from ATCC and purchased via LGC Promochem (Germany).

Partial Lung Resection Model

Healthy New Zealand white rabbits ($n = 14$, 2.5–3.5 kg, Harlan-Winkelmann, Germany) were anesthetized by subcutaneous injection of 60 mg/kg ketamine hydrochloride and 16 mg/kg xylazin hydrochloride. Anesthesia was maintained with Thiopental® (thiopental sodium) i.v., and alloferin was administered i.v. as muscle relaxant. An arterial catheter (22G) was introduced into the arteria femoralis. A tracheal tube (diam. 3.5/4.0 mm) was introduced into the trachea after tracheotomy. The animals were put on a respirator, tidal volume was adjusted to 10–15 mL/kg, and frequency at 25–30 respirations per min (controlled by peak pressure within a range of 12–15 mbar). Minute volume (liters of gas provided by the respirator per minute) was reduced to half of initial volume after medial thoracotomy was performed. The right medial lobe was exposed and clamped at the base. The apex of the right medial lobe was resected. The injured parenchymal surface was sealed with either one of the commercial fibrin sealants (7 animals per group). Five minutes after sealing, the clamp was removed and the chest was filled with Ringer Solution to submerge the sealed parts. Minute volume was increased slowly (0.01 L after four inspirations) until leakage. Initial pressure (mbar), leakage pressure (indicated by bubbling at the sealed site), and minute volume at leakage were recorded.

The experimental protocol was approved by the local Committee on Animal Experiments of the Viennese government, and care and handling of the animals were undertaken in accordance with the National Institutes of Health guidelines.

Tensile Strength

Solutions of sealer protein (fibrinogen) and thrombin (1 + 1, v/v) were cast into a mold (Figure 1) and incubated in a moist chamber at 37°C for 30 min. Tensile strength was measured and recorded with an universal materials testing machine at 1 cm/min rate of extension (Instron, Type 4301, Darmstadt, Germany).

Scanning Electron Microscopy

Fibrin clots containing t-AMCA or aprotinin were prepared in caps of microtubes (Eppendorf, Germany), incubated for 30 min at 37°C, and fixed in fixing solution (0.1*M* cacodylate buffer (pH 7.4), 2% glucose, 1% glutaraldehyde) for 12 h. Fixed clots were washed three times with cacodylate buffer and dehydrated in a graded series of 40, 60, 80, 96,

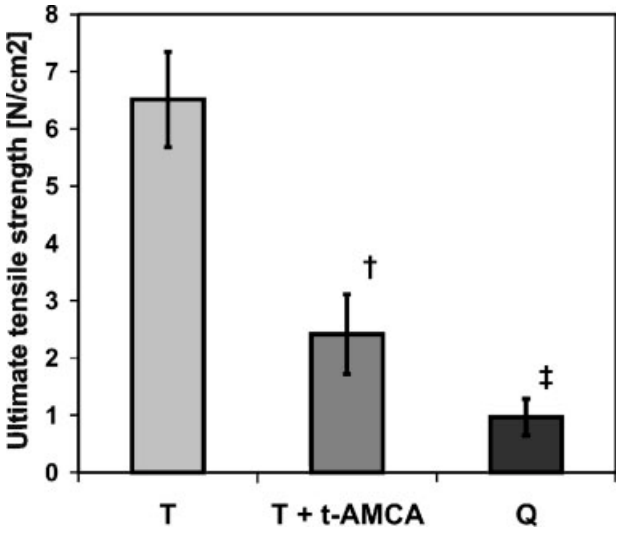

Figure 1. Influence of t-AMCA on ultimate tensile strength of fibrin matrix. t-AMCA containing commercial fibrin sealant (Q) an another commercial fibrin sealant supplemented with t-AMCA (T + t-AMCA) show decreased ultimate tensile strength compared to the respective fibrin sealant supplemented with aprotinin (T). Mean SD; $n = 13$; $^{\dagger}p < 0.01$; $^{\ddagger}p < 0.001$ vs. T.

and 100% ethanol in water. Then samples were frozen in liquid nitrogen, freeze-fractured, and dried by CO_2 critical point drying. Dried samples were stored in a desiccator until coating, using a Au target (10 mA, 10 Pa, 6 × 15 s, Sputter Coater SC502, VG Microtech, UK). Specimens were then transferred to the scanning electron microscope (SEM) operated at 15 kV accelerating voltage (JEOL Scanning Microscope JSM 6310, UK). SEM pictures were made in the secondary electron mode.

FXIII Dependent Crosslinking of Fibrin Monomers

Fibrinogen, containing 95 mg/mL t-AMCA or 3000 KIU/mL aprotinin, and thrombin were mixed 1 + 1 (v/v), and fibrin clots were incubated at 37°C for 30 min to complete FXIII-dependent crosslinking. Then clots were lysed overnight in 5 volumes of clot lysis solution (9*M* urea, 4.5% SDS, and 5% 2-mercaptoethanol), and dissolved fibrin proteins were separated on a homogeneous 5% polyacrylamide/urea electrophoresis-gel. Fibrinogen treated in the same way but mixed with saline instead of thrombin served as reference. After electrophoresis, gels were stained with Coomassie-blue and assessed by densitometry. FXIII-activity was deduced from reduced intensity of bands from α- and γ-subunits of lysed fibrin clots, in comparison to bands from α- and γ-subunits of corresponding fibrinogen samples.

Cytotoxicity Assay

MRC5 human lung fibroblast cells (passage 34–36) were seeded in 24-well tissue culture plates and incubated in modified MEMα medium with 10% FCS, under standard conditions (37°C, 5% CO_2). In experiments with the pure fibrinolysis inhibitors, confluent cells were incubated in medium supplemented with 100 and 50 mg/mL t-AMCA or 3000 and 1500 KIU aprotinin, respectively. These chosen concentrations correspond to the concentration of inhibitor in the sealer component before and after mixing with the thrombin component. After 100 min, viability was assessed via MTT-test. In experiments with sealer components, confluent cells were incubated with sealer protein of Quixil (t-AMCA) or Tisseel (aprotinin). Viability was assessed via MTT-test after 30 min, when visual evaluation of cell morphology revealed that the confluent cell monolayer was disrupted and the cell nucleus diminished clearly.

MTT-Test

Cells were washed once with serum-free medium, overlaid with fresh culture medium supplemented with MTT-solution (650 mg/L), and incubated for 2 h. Afterward the medium was discarded, and the formazan was extracted from the cells with 1 mL of dimethylsulfoxide. Optical density was measured at 540 nm with a Beckman DU-70 UV/Vis Spectrophotometer (CA).

Statistical Methods

Statistical analyses to determine significance between groups was performed using the nonparametric Kruskal–Wallis test and two-sided Mann–Whitney test (partial lung resection model). All calculations were performed with GraphPad® software.

RESULTS

Sealing Efficacy in a Partial Lung Resection Model

Significantly lower leakage pressure was found for partial lung resections sealed with a commercial fibrin sealant containing t-AMCA compared to resections sealed with a fibrin sealant containing aprotinin (Table I).

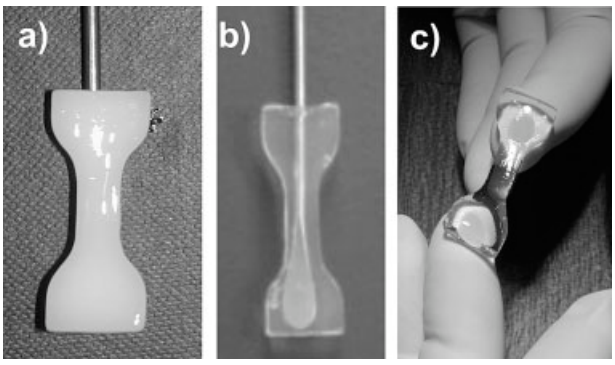

Figure 2. Fibrin clots used as test specimens for measurement of tensile strength. Substitution of aprotinin with t-AMCA changes appearance from non-transparent "coarse clots" (a) to transparent "fine clots" (b). A t-AMCA containing commercial fibrin sealant gives crystal clear clots (c).

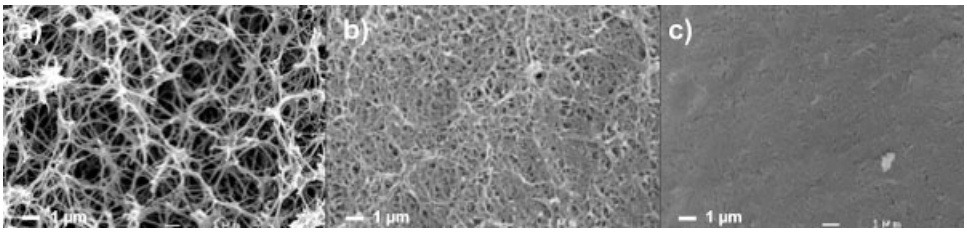

Figure 3. Scanning electron micrographs of fibrin clots formed in the presence of aprotinin (a) or t-AMCA (b, c). Addition of t-AMCA (b) instead of aprotinin (a) causes shortening of fibrin fibers and pore size. For a t-AMCA containing commercial fibrin sealant (c) these effects are even more noticeable. Magnification x5.000.

Tensile Strength

t-AMCA reduced ultimate tensile strength of fibrin, as shown by measurements of fibrin matrix containing either t-AMCA or aprotinin, respectively. Substitution of aprotinin with t-AMCA caused a significant decrease of tensile strength to 40%. A t-AMCA containing commercial fibrin sealant could bear only 15% of the load compared to matrix from an aprotinin containing commercial fibrin sealant (Figure 1).

Three-Dimensional Structure

Substitution of aprotinin with t-AMCA caused obvious changes of fibrin clots from nontransparent ''coarse clots'' to transparent ''fine clots'' (Figure 2). SEM visualized significant changes in three-dimensional structure of fibrin (Figure 3). Fibrin clots formed in the presence of aprotinin are built of long fibers with large diameter. The spatial separation of branching points and the long fibers resemble the fibrin network of natural blood clots. In contrast, fibrin clots formed in the presence of t-AMCA show only small fibers with many but short links. This results in a dense structure and reduced pore size. Clots formed from a t-AMCA containing commercial fibrin sealant (containing the same amount of t-AMCA as used with the other fibrinogen) showed no fibrous network.

FXIII Dependent Crosslinking of Fibrin Monomers

Formation of γ-γ dimers and formation of α-polymers with concomitant reduction in fibrin α-chain due to FXIII-mediated crosslinking could be detected for an aprotinin containing commercial fibrin sealant (Figure 4). No direct effect of t-AMCA on FXIII dependent crosslinking, however, was seen when t-AMCA was used as a substitute for aprotinin in the otherwise aprotinin containing fibrin sealant. In contrast, only slight γ-γ dimer formation and no reduction in α-chains was seen for a t-AMCA containing commercial fibrin sealant.

Cytotoxicity of Fibrinolysis Inhibitors

Viability of MRC5 human lung fibroblasts was decreased by t-AMCA alone in a dose-dependent manner. In contrast, aprotinin did not affect viability (Figure 5). Viability was also decreased to 20% after incubation with the sealer protein of a t-AMCA containing commercial fibrin sealant (Figure 5).

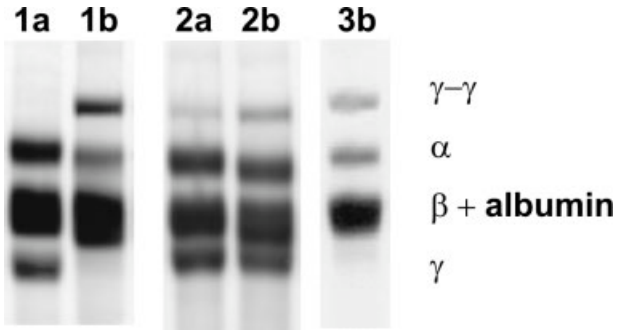

Figure 4. Electrophoretic pattern of fibrinogen (a) and fibrin (b) proteins dissolved in clot lysis buffer. Due to FXIII-dependent crosslinking main part of the γ-band is converted to γ-γ-dimer for an aprotinin containing commercial FS (lane 1a, 1b) while the prevalent part of the γ-chain is not cross-linked for a t-AMCA containing commercial FS (lane 2a, 2b). Neither γ- nor α-chain crosslinking is affected by t-AMCA if used as substitute for aprotinin in the respective FS (3b).

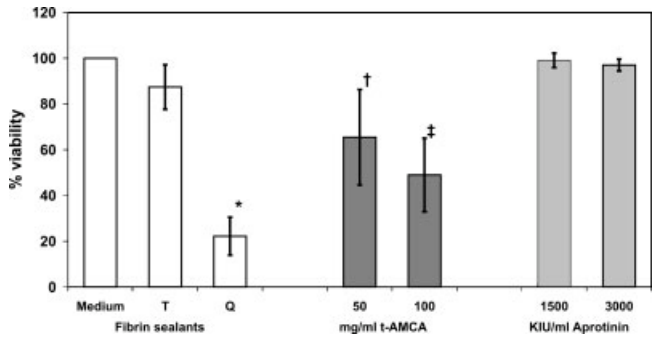

Figure 5. Influence of fibrin sealants and fibrinolysis inhibitors on viability of MRC5 human lung fibroblast cells. A commercial sealant containing t-AMCA (Q) and also t-AMCA by itself reduced viability of cells ($^{*}p < 0.05$, $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.001$ vs. medium). In contrast, an aprotinin containing sealant (T) had only minor effect on cells and aprotinin alone had no effect at all. (Mean ± SD; sealants: 3 assays in duplicate, inhibitors: 6 assays in duplicate).

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DISCUSSION

A fibrin sealant utilizing t-AMCA as fibrinolysis inhibitor showed significantly less sealing efficacy in an *in vivo* partial lung resection model than in a fibrin sealant utilizing aprotinin. Thus, the aim of the present study was to investigate the effect of fibrinolysis inhibitors, t-AMCA and aprotinin, on structure, strength, and cytocompatibility of fibrin matrices. In our *in vitro* studies, we could demonstrate that t-AMCA alters macroscopic appearance as well as microscopic three-dimensional structure, and reduces tensile strength as well as cytocompatibility of fibrin matrices.

Fibrin sealants form strong gels that adhere to the wound area and seal them like a conventional blood clot. A major adverse event in the use of fibrin matrix as sealants would be recurrent bleeding due to leakage of the sealed area. Leakage results generally from bursting of the seal or loosing its adherence to the wound area but not via fluid/gas flow across the fibrin seal. Therefore, mechanical strength is a main parameter for performance of fibrin sealants as surgical sealants or scaffolds.

We could demonstrate that tensile strength is reduced in a fibrin sealant when aprotinin is substituted with t-AMCA. Tensile strength of another commercial fibrin sealant containing the same amount of t-AMCA seems to be further affected by its lower concentration of sealer protein. Reduced tensile strength correlates with a change from physiological coarse to nonphysiological fine clots, which macroscopically become transparent and microscopically are characterized by thinner fibrin fibers, more branching points, and a reduced pore size. These structural changes may be the reason for reduced tensile strength and sealing efficacy. Similar alterations were described for fibrin formed at high ionic strength.[17,18] Thus, t-AMCA may affect fibrin matrices via ionic interaction.

Strength of fibrin matrix depends also on crosslinking of fibrin monomers by FXIII.[19,20] Crosslinking of fibrin monomers, indicated by γ-γ dimer formation and decrease in α monomer, was measured using gel electrophoresis and densitometry. In experiments with t-AMCA supplemented Tisseel, no direct effect on fibrin-crosslinking was detected. However, γ-γ dimer formation was hardly detectable for a commercial fibrin sealant containing t-AMCA. Thus, the strongly reduced crosslinking of fibrin monomers in one sealant may be due to other factors than t-AMCA, such as to the production procedure of its fibrinogen component or other inhibitory ingredients.

Aside the effects of t-AMCA on structure and strength of fibrin matrix and its reported neurotoxicity,[15,16] we were also interested in its cytocompatibility with nonneural cells. Indeed, viability of a lung fibroblast cell line (MRC5) was nearly abolished by diluted fibrinogen components of the t-AMCA-containing fibrin sealant. In separate experiments with fibrinolysis inhibitors, t-AMCA could be identified as one source of cytotoxicity while aprotinin did not affect cell viability. The effects on viability of fibroblasts are rather unexpected, since t-AMCA is used as antifibrinolytic agent in a wide range of hemorrhagic conditions. The concentrations of t-AMCA, however, achieved due to intravenous administration are in the range of 20–500 μg/mL,[21] which is three orders of magnitude less than the concentrations in a commercial fibrin sealant (95 mg/mL). Similarly, reduced proliferation and detachment was already shown earlier for neuronal cells and fibroblasts seeded on polyethyleneimine-coated culture dishes.[22] However, we could not verify that cells recover or readhere after incubation in fresh culture medium without t-AMCA (data not shown). Furthermore, our experiments revealed significant reduction of cellular viability due to incubation with t-AMCA already within 100 min, compared to 3 days in the other report.[22]

CONCLUSIONS

The presented study was intended to compare the influence of the fibrinolysis inhibitors, t-AMCA and aprotinin, on structure, mechanical properties, and cytocompatibility of fibrin matrices. The results of our experiments demonstrate that substitution of aprotinin with t-AMCA affects not only the structure of the fibrin network but also reduces tensile strength (without affecting crosslinking of fibrin monomers) and cytocompatibility of fibrin. Such fibrin t-AMCA interactions seem to be partly responsible for the worse outcome in the *in vivo* lung sealing experiment.

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