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Wound healing and the immune response in swine treated with a hemostatic bandage composed of salmon thrombin and fibrinogen

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Abstract We investigated the inflammatory response in pigs exposed to salmon fibrinogen/thrombin dressings. Animals were exposed to the material in 3 ways: (a) thrombin and fibrinogen were injected intravenously, (b) dual full-thickness skin lesions were surgically created on the dorsal aspect of the swine and treated with the fibrinogen/thrombin bandage and a commercial bandage or (c) a fibrinogen/thrombin bandage was inserted through an abdominal incision into the peritoneal cavity. Blood was collected twice weekly and animals were sacrificed at 7, 10 or 28 days. Animals in the 28-day dermal lesion group were given an injection of salmon fibrinogen/thrombin at the 10 day point to simulate a second bandage application. The immune response manifested itself as induction of germinal centers in mesenteric lymph nodes and in the white pulp of the spleen. Examination of the histology of the skin and organs showed a cellular inflammatory response with granulation tissue and signs of edema that resolved by the 28-day stage. Antibodies reactive to salmon and human thrombin and fibrinogen were detected, but fibrinogen levels and coagulation processes were not affected. In conclusion, animals treated with salmon fibrinogen/thrombin bandages demonstrated a smooth recovery course in terms of both tissue healing and the immune response without adverse effects from the exposure to the fish proteins.

Abbreviations

TNF	Tissue necrosis factor
PDS	Polydioxanone suture
H&E	Hematoxylin and eosin

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1 Introduction

Bleeding from severe wounds is a major cause of preventable death from traumatic injuries on the battlefield [1, 2]. Control of hemorrhage is the initial step in field trauma care and having a widely deployable bandage to staunch blood loss will decrease loss of life. Hemostatic dressings based on coagulation proteins have been shown to be highly effective [3, 4]. However, dressings based on human proteins have the disadvantages of high cost for the raw materials and the possibility of pathogen transmission. Even non-human, mammalian proteins carry the risk of transmission of diseases such as bovine spongiform encephalopathy (Mad Cow disease) [5, 6]. An alternative formulation presented in this report is a dressing composed of salmon fibrinogen and thrombin. These dressings are also effective in stopping bleeding in a swine aorta injury model [7] and have been proposed as an optional material for an active coagulative matrix. A possible drawback to this approach is that it is unknown if exposure to coagulation proteins isolated from highly divergent species such as teleost fish will provoke an immune response that could inhibit the normal host coagulation response.

The possibility of this type of response may not be unexpected because there is a history of adverse reactions to bovine proteins used as hemostatic aids. Transfusion with bovine thrombin caused the production of antibodies against Factor V [8]. This was at first ascribed to impurities in the preparations, however, even highly purified bovine thrombin has been reported to cause an anti-human Factor V antibody associated coagulopathy [9, 10]. These reactions to bovine proteins have not been limited to intravenous applications as the topical use of bovine thrombin has also proven to cause allergic responses [11]. These responses have not been restricted to the exposure to only coagulation proteins. In at least one report, sperm prepared for artificial insemination using bovine serum albumin induced an anaphylactic reaction [12]. Ingestion of cow's milk has also been shown to elicit an immune response and to stimulate lymphocyte proliferation [12].

The goal of this project was to determine if salmon thrombin and fibrinogen would cause an adverse immune and inflammatory response and to examine the cellular basis for that response. We assessed the production of antibodies to the salmon components and determined if the coagulation activity of the swine was altered. We examined the histopathology to characterize the tissue response to salmon dressings in swine after excisional cutaneous surgery that created wounds with separated edges and found a lymphocyte response that included cellular proliferation and cytokine secretion. However, healing occurred normally and there were no signs of adverse immunological reactions to the dressings at the wound site.

2 Methods

2.1 Biochemical and immunological assays

2.1.1 Purification of salmon fibrinogen and thrombin

Salmon proteins were purified from salmon blood as previously described [13]. Briefly, fibrinogen was salt precipitated twice with ammonium sulfate in a method modified from Mosher and Blout [14]. Salmon thrombin was purified from precipitates formed after addition of BaCl to plasma by the method of Michaud et al. [15]. Fibrinogen was used at a concentration of 19.4 mg/cm^2 (2000 mg total in a bandage approximately 10 × 10 cm) and thrombin was used at a concentration of 50 U/cm^2 (5200 U total).

2.1.2 Electrophoresis, Western blotting and ELISA

Immunological reactivity was determined by Western blotting and ELISA. For electrophoresis, proteins were separated on Invitrogen NuPAGE 4–12% Tris-Bis gels and transferred to PVDF. Antibodies were visualized with secondary anti-swine horseradish peroxide-conjugated antibody (HRP-swAB) and treatment with Millipore Chemoluminescence reagent kit.

ELISAs were performed with thrombin or fibrinogen as the substrate. Immunolon B1 plates were coated with 1 μg protein/well, the wells were blocked with and then incubated with porcine serum at 1/10 dilution. Titration curves were performed at dilutions up to 1/5000. Antibody binding to salmon proteins was quantified by incubation with HRP-swAB and Millipore substrate and read at OD450 with a Molecular Devices plate reader.

Cytokine levels for IL1, IL2, IL4, IL6, IL8, IL10, IL12p40, IFNγ and TNFα were assayed by a commercial service, Searchlight Cytokine Custom Multiplex Arrays (Pierce Biotechnology, Inc., Rockford, IL). The Protoarray Human Protein Microarray, a 5000 protein array from Invitrogen Corp., was analyzed to screen the serum from five animals for the presence of anti-human antibodies generated following exposure of the swine to the salmon proteins. This assay would detect antibodies recognizing proteins that are not included in the normal coagulation pathway and, therefore, may not be detected by our standard assays. Serum from blood taken at the time of surgery to implant the vascular access ports (VAP) was compared to serum taken at euthanization of the animals after the exposure to salmon proteins.

2.1.3 Coagulation studies

Coagulation functions (prothrombin time, activated partial thromboplastin time, thrombin time and fibrinogen

concentration) were assayed on a STA4 Compac (America Diagnostic). Complete blood counts were run on a Cell Dyne 1700 (Abbott diagnostics, Abbott Park IL).

2.2 Surgical preparation of animals

Female Yorkshire swine (*Sus scrofa domestica*) (25–28 kg) were prepared for surgery and monitored during the procedure as described previously. A vascular access port (VAP) catheter line (Access Technologies, Skokie, IL) was inserted into the jugular vein using a modified Seldinger technique [16] to permit blood sampling.

Exposure to the salmon proteins was accomplished in several ways. In the first approach, thrombin and fibrinogen were injected intravenously through the vascular access ports. In the second method, paired identical full thickness dermal wounds were surgically created on the right and left dorsal skin surface, paramedial to the spinal column in four pigs. The bandages were applied immediately to the wound site to simulate application of the bandage to an injury. The animals were then monitored for 7 days. A second group of four pigs were subjected to a similar pair of skin lesions and monitored for 28 days. The total number of wounds to evaluate in each time point was eight. For animals in the 7 day group, the right dorsal lesion was bandaged with a dressing composed of lyophilized fibrinogen produced by electrospinning the protein onto a rotating mandrel (Nanomatrix, Inc, Baton Rouge, LA). Bandages were sterilized by gamma irradiation (7 kGy exposure) using a J.L. Shepard Model 109 Cobalt Gamma Irradiator.

The dressings were cut into quarters containing approximately 500 mg fibrinogen and 400 IU thrombin and were applied to a full thickness dermal lesion approximately 2 × 2 cm. The left dorsal lesion was dressed with a commercially available, non-hemostatic bandage. For the 28-day animals, this was reversed and an electrospun fibrinogen/thrombin dressing was applied to the left side. Animals in the 28-day group were injected with thrombin (60 IU) and fibrinogen (200 μg) on day seven to simulate a re-exposure to the dressing. At the end of the time period, the animals in each group were euthanized and the carcass presented for necropsy.

For the third exposure method, a midline abdominal incision was performed and the fibrinogen/thrombin bandage was inserted into the peritoneal cavity. The incision was sutured and the animal was recovered. Animals were maintained for 2 weeks and blood was drawn for analysis of antibody generation.

2.3 Tissue preparation for histological examination

At necropsy, tissue from the salmon fibrinogen/thrombin treated lesions and non-hemostatic bandage treated lesions, the pre-femoral lymph nodes, mesenteric lymph nodes and spleen were harvested for histopathology. Histopathology assessment was performed in a non-blinded fashion using a light microscope. Evaluation parameters on the skin sections included examination of the wound edge and monitoring for signs of inflammation, re-epithelialization, granulation tissue, fibrosis, crust formation and necrosis. Semi-quantitative scoring of the skin samples for superficial and deep inflammation was performed,

2.4 Statistical analysis

Differences between groups were analyzed using a two tailed *t*-test assuming equal variances. Values are expressed as means ± SE. *N* values and *P* values are included with each measurement.

3 Results

3.1 Inflammation and re-epithelialization of the skin lesion in the 7-day group

Paired dermal injuries were produced on the animals and the injury sites were dressed on one side with the salmon fibrinogen dressing and on the other with a non-fibrinogen standard dressing (Fig. 1a–d). After 7 days, the animals were euthanized and taken for necropsy ("7-day group"). In the center of the wound, all four pigs on both the left and right sides, exhibited a leading edge of epithelial cells and superficial wound filling by a coagulum composed of necrotic cellular debris, neutrophils, fibrin, hemorrhage, and edema (Fig. 2a, b). Inflammatory cells, granulation tissue and edema expanded the superficial dermis on both the left and right sides subjacent to this fibrinonecrotic coagulum. Evidence of fibroplasia, characterized by numerous plump, activated fibroblasts with deposition of abundant collagen, extended from the junction of the dermis deep to the panniculus adiposus. This fibroplasia was moderate in severity with a multifocal to diffuse distribution in all four pigs on both the right and left sides.

An attempt at re-epithelialization on the wound edges was evident in all eight wounds at the 7-day time point. Typical findings at these margins included epidermal hyperplasia, acanthosis, spongiosis, deep rete ridges and dermal pegs, parakeratotic hyperkeratosis and projections of regenerative epithelial cells toward the wound center.

To summarize the 7-day group, all wounds were filled with a fibrinonecrotic coagulum. Each wound exhibited superficial granulation tissue in the dermis on both the

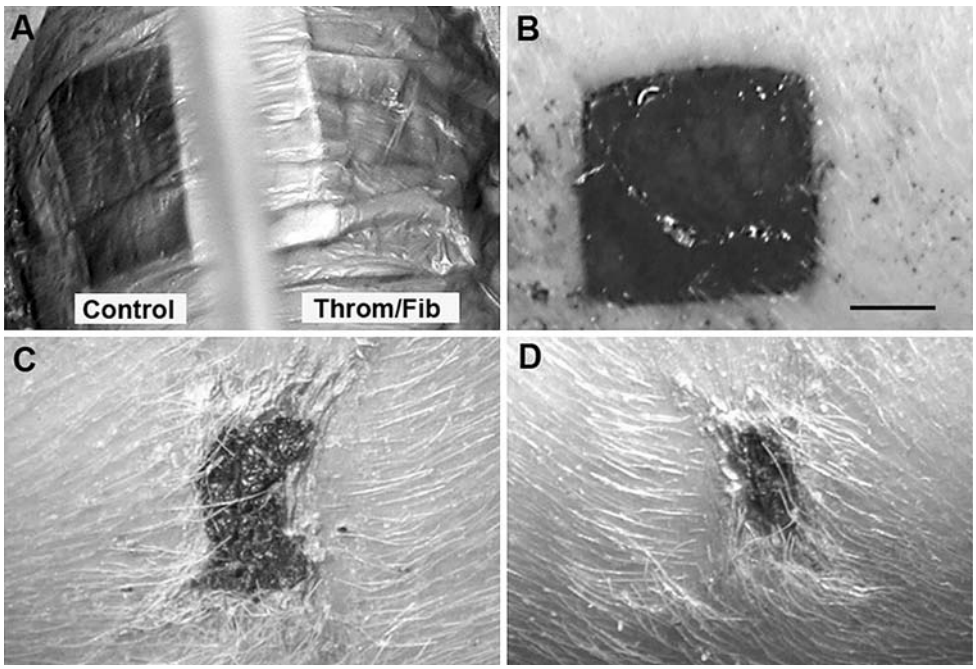

Fig. 1 Gross anatomical view of the dermal lesions. Image **a** shows the control and the thrombin/fibrinogen dressings in place on the dermal wounds following surgery. The control patch is darker due to the more extensive bleeding at the site. Image **b** shows the wound site at 7 days after the bandages have been removed. The lower images show the control site (**c**) and thrombin/fibrinogen-treated site (**d**) after 28 days

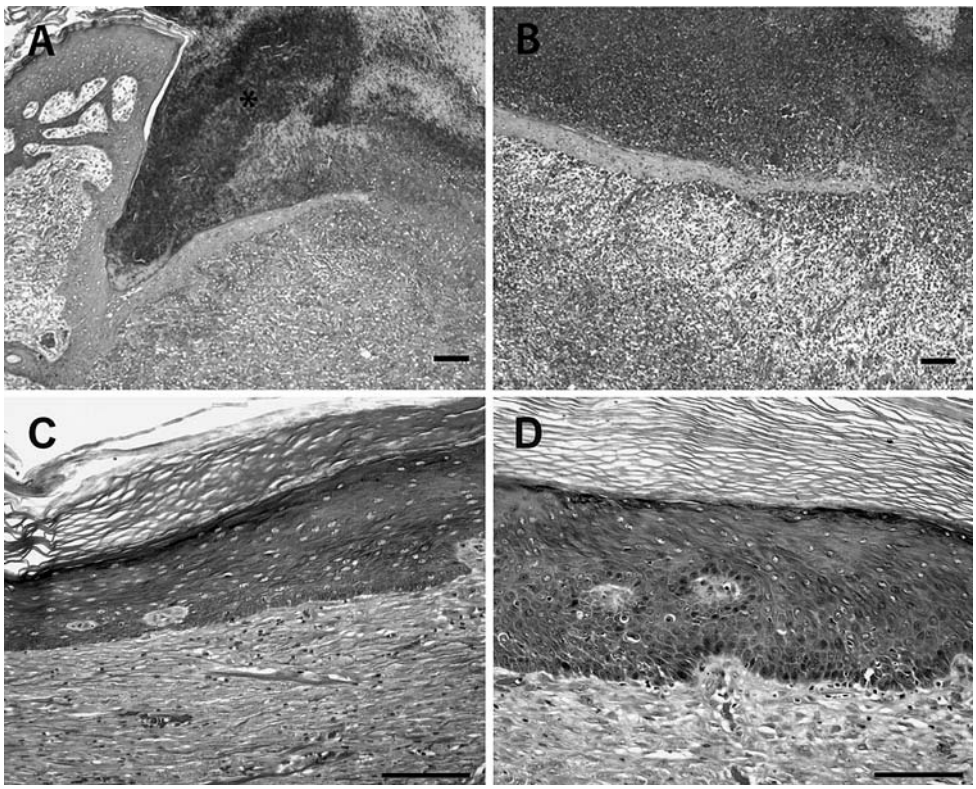

Fig. 2 Dermal healing progresses normally following full-thickness wound. Images from samples taken at 7 days from control (**a**) and salmon bandage-treated (**b**) injuries show a fibrinonecrotic coagulum filling the wound defect (*) and an epithelial cell projection towards wound center in both cases (H&E staining, bars = 100 μm). Samples taken at 28 days from control (**c**) and salmon bandage-treated (**d**) injuries show complete re-epithelialization by a hyperplastic and hyperkeratotic epidermis (H&E staining, bars = 100 μm)

treated and untreated sides. There were numerous neutrophils, fewer macrophages and multifocal hemorrhage. Inflammation variably extended deep into the subcutis and was composed of lymphocytes, plasma cells and macrophages. Re-epithelialization at the margins and moderate fibroplasia was evident in all eight wounds.

3.2 Inflammation and re-epithelialization of the skin lesion in the 28-day group

To fully investigate the healing process, the dermal injuries were repeated in a second set of animals and the course of healing was followed for at least 28 days ("28-day

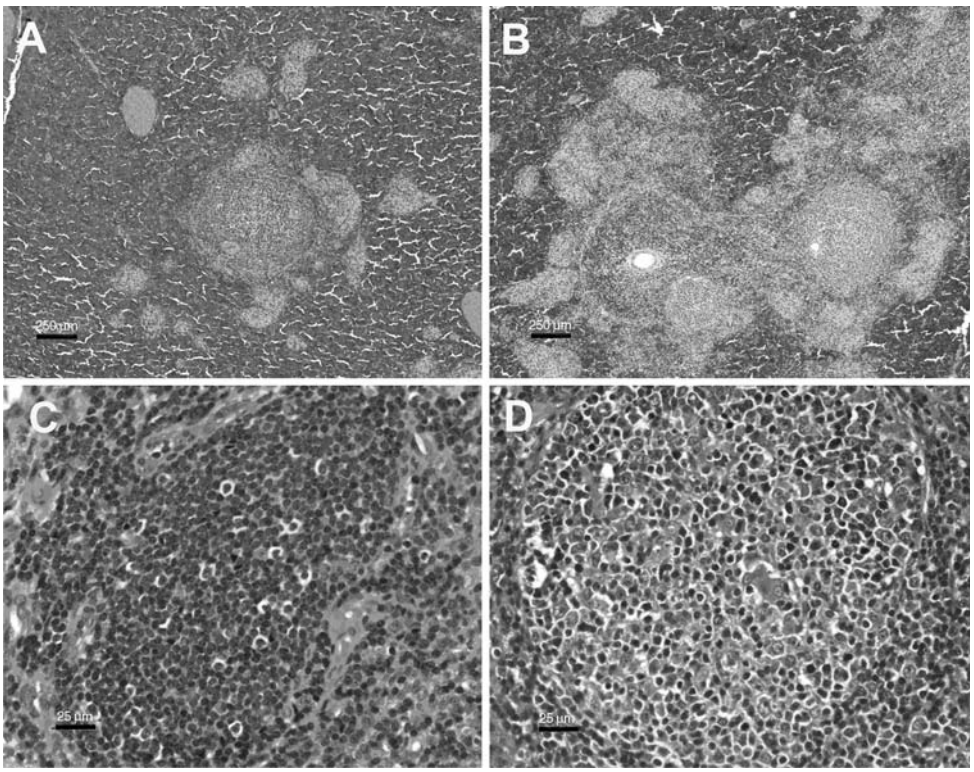

Fig. 3 Immune response in the spleen and lymph nodes is activated following exposure to the salmon fibrinogen/thrombin bandage. Samples taken from the spleen at 7 days (**a**) and 28 days (**b**) post-injury show increased signs of activation at 28 days with increased lymphocyte proliferation in the B-cell areas surrounding the periarterial lymphatic sheaths. Samples were taken at 7 days from the pre-femoral lymph nodes underlying the side treated with the control bandage (**c**) and compared to lymph node samples from the side treated with the salmon bandage (**d**). The node from the salmon bandage treated side shows a larger lymphoid follicle and increased lymphocytolysis suggesting mild activation when compared to control side (H&E staining, bars = 250 µm in (**a**) and (**b**) and bars = 25 µm in (**c**) and (**d**))

group"). In this 28-day group, seven of eight wounds exhibited complete re-epithelialization that was characterized by epidermal hyperplasia and hyperkeratosis (Fig. 2c, d) and, multifocally, there was a superficial clot similar in cellular composition to the 7-day group. In these seven wounds, the superficial inflammation was minimal to mild. One non-hemostatic bandage treated wound in the 28-day group displayed incomplete re-epithelialization and the wound defect was filled by a fibrinonecrotic coagulum along with marked superficial inflammation. The wound edge in this case exhibited similar epithelial cell hyperplastic changes as the 7-day group. All eight wounds exhibited mild amounts of dermal granulation tissue and deep inflammation that was composed of perivascular lymphocytes and macrophages. On the side treated with the fibrinogen/thrombin bandage side, the granulation tissue was contained numerous neutrophils with fewer dermal macrophages, lymphocytes, plasma cells and eosinophils. Neutrophils rarely formed intra-epidermal pustules. Additionally, there was hemorrhage, fibrin and edema with necrosis in this wound.

Deep inflammation in the non-hemostatic bandage treatment varied from minimal to mild in three cases and moderate in one case. This subacute inflammation was predominantly clustered around vessels. In the salmon fibrinogen/thrombin treatment, deep inflammation was minimal in two cases and moderate in two cases; subacute and primarily perivascular.

3.3 Immune organ involvement

The lymph nodes and the spleen were histologically examined for signs of activation, including lymphoid follicle formation and lymphocytolysis. The mesenteric lymph nodes and spleen were found to be similar histologically among the four pigs in each time point. The amount of white pulp (lymphoid tissue containing T and B lymphocytes) contained in the spleen increased slightly from the 7-day samples (Fig. 3a) to the 28-day samples (Fig. 3b).

Although mesenteric lymph nodes showed little difference between the 7-day and 28-day groups, the pre-femoral lymph nodes that drain the ipsilateral area of the skin wound did display differences when examined at the 7-day time point (compare Fig. 3c, d). The node draining the non-hemostatic bandage wound generally exhibited fewer and smaller lymphoid follicles and decreased turnover of lymphoid cells than the pre-femoral nodes that drained the salmon fibrinogen/thrombin wound. By the 28-day time point, lymph nodes from both sides showed equivalent size of germinal centers and amount of lymphocytolysis.

3.4 Systemic changes in the immune status as determined by cytokine levels

To determine if the morphological changes observed in the lymph nodes and the spleen were reflected in the systemic

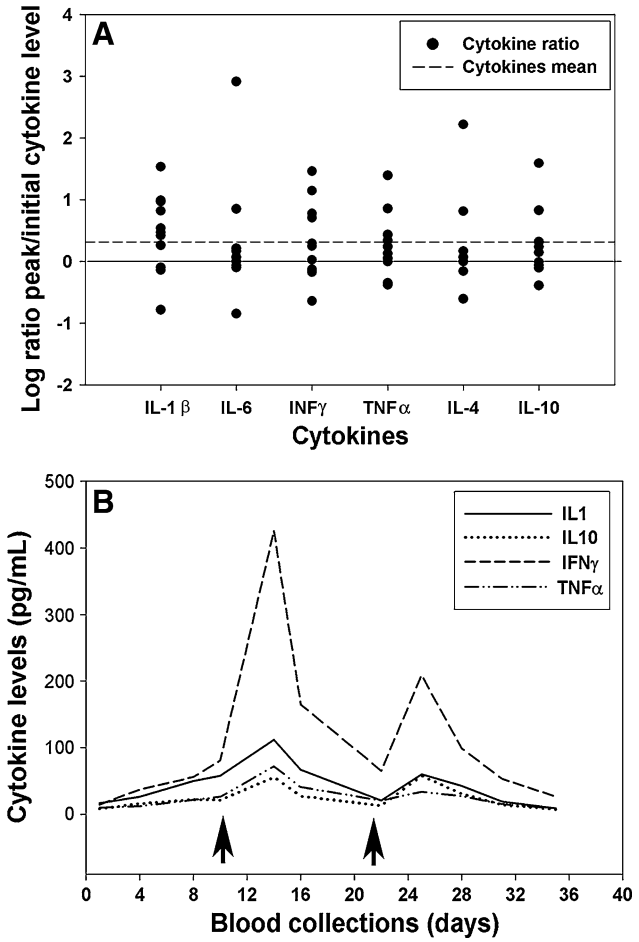

Fig. 4 Changes in cytokine levels in animals exposed to the salmon fibrinogen/thrombin bandage. **a** Levels of IL-1β, IL-6, TNF-α, IFN-γ, IL-4 and IL-10 are shown as the log ratio of the cytokine level determined in blood drawn at the initial surgery to implant the vascular port compared to peak levels following exposure. Changes were seen in both pro-inflammatory responses (IL-1β, IL-6, TNF-α, IFN-γ) and humoral responses (IL-4 and IL-10). Each spot represents one animal. **b** Changes in the cytokines within an individual animal show that initial exposure (first arrow) and the subsequent intravenous infusion of proteins (second arrow) elicited a response that could be detected in samples taken at the next blood draw

circulation of immunomodulatory or inflammatory signaling molecules, levels of a panel of cytokines were measured in the groups of animals exposed to the dermal wound and then intravenously infused 2 weeks later with soluble salmon proteins. Levels of IL-1β, IL-6, TNF-α, IFN-γ, IL-4 and IL-10 are shown in Fig. 4a as the log ratio of the cytokine level determined in blood drawn at the initial surgery to implant the vascular port compared to levels following exposure. Responses varied between individual animals from almost no response to 20- to 30-fold increases. Changes were seen in both pro-inflammatory responses (IL-1β, IL-6, TNF-α, IFN-γ) and humoral responses (IL-4 and IL-10). Changes in the cytokines within an individual animal are shown in Fig. 4b where it can be seen that initial exposure and the subsequent infusion of proteins elicited a response that could be detected in samples taken at the next blood draw.

3.5 Characterization of antibody production in treated animals against thrombin and fibrinogen

Blood drawn from animals ($n = 24$) that had been exposed to salmon thrombin and fibrinogen was analyzed for the generation of antibodies using Western blotting and ELISA. The majority of animals (94%, see Table 1) that were exposed to salmon proteins generated antibodies that recognized salmon fibrinogen (Fig. 5b, c; two different animals) and 66% of the animals developed antibodies that reacted with human fibrinogen. In contrast to the reactions seen against fibrinogen, thrombin antibodies were low or undetectable after one exposure. However, anti-salmon thrombin antibodies were detectable at low titers after a second exposure (Fig. 5e, f; same two animals used in b and c) in some animals (4/24). Antibodies specific for swine thrombin or prothrombin were not detected in these animals. The antibody reactivity varied in that some antibodies only recognized the prothrombin form while others recognized the cleaved thrombin molecule. Isotypes of IgM and IgG were detected for both antigens, but IgA antibodies were not detected by either Western blotting or ELISA. Our second antibodies for both IgM and IgG (anti-swine immunoglobulin) reacted strongly with immunoglobulin contaminants in the fibrinogen and the thrombin preparations. This gave a strong band at a position very similar to one of the fibrinogen polypeptides. This could be demonstrated by using blots in the absence of primary swine serum and in blots using pre-immune serum.

The time course of antibody development was determined by ELISA on sequential blood samples taken from two series of pigs, one set subjected to the skin lesion animals and one set that received the abdominal patch placement. The results were plotted as optical density versus blood collection time, starting with blood collected at the implantation of the VAP until the termination of the experiment. Animals that were in the abdominal patch group had very low responses to human fibrinogen and low responses to both salmon thrombin and human thrombin (Fig. 6). Animals that were exposed to the bandage via the skin lesion modality displayed slightly higher responses compared to animals that were exposed via the abdominal placement of the patch. The blots in general proved to be more sensitive in detecting very low levels of responses to the protein and allowed us to discern between binding to swine immunoglobulins, but the ELISAs enabled us to more easily track the progression of the immune response and antibody production in each animal.

Table 1 Antibody response in swine exposed to salmon fibrinogen/thrombin dressing as accessed by Western blotting

Animal number	Procedure	Salmon FIB	Human FIB	Swine FIB	Salmon THR	Human THR	Swine THR
12171	Skin patch						
12172	Skin patch	+	−	−	−	−	−
12173	Skin patch	+	−	−	−	−	−
12174	Skin patch	+	+	−	−	−	−
13085	Abd patch	+	+	−	−	+	−
13086	Abd patch	+	+	−	−	−	−
13087	Abd patch	**					
13088	Abd patch	+	+	−	+	+	−
14029	Abd patch	+	+	−	NA	NA	NA
14031	Abd patch	+	+	−	−	−	−
14871	Abd patch	+	−	−	−	−	−
14872	Abd patch	+	−	−	−	−	−
14873	Abd patch						
14874	Abd patch	−	−	−	−	−	−
16954	Abd patch	+	−	−	−	−	−
16955	Abd patch	+	+	−	−	−	−
16956	Abd patch	+	+	−	−	−	−
16957	Abd patch	+	+	−	−	−	−
17218	Abd patch	+	+	−	+	−	−
17219	Abd patch	+	+	−	+	+[a]	−
17220	Abd patch	+	+	−	+	+[a]	−
17222	Abd patch	+	+	−	−	+[a]	−

Sera of animals exposed to salmon protein by the dermal protocol and the abdominal were assessed for the production of antibodies as described in Fig. 5. Serum with detectable antibodies (+), undetectable antibodies (−) and serum not assayed (NA) are listed. If antibodies were detectable as binding to any fibrinogen subunit or thrombin or prothrombin, that serum was counted as positive

[a] A band was recognized at the molecular weight of the prothrombin polypeptide

** Died from surgical complications

3.6 Identification of human cross-reactive antibodies in the serum of salmon protein-exposed swine

The Protoarray Human Protein Microarray service from Invitrogen Corp. was utilized to screen the serum from five animals that had been exposed to the salmon proteins for the presence of antibodies reactive against human proteins generated following exposure of the swine to the salmon proteins by either the skin and abdomen placement. These proteins could fall into two categories: (1) proteins that were part of the coagulation cascade and whose function could possibly be inhibited by interfering antibodies or (2) proteins that have no perceived relationship to the coagulation process, but may still react with antibodies induced during exposure. As shown in Table 2, the array had 4 proteins that were part of the coagulation cascade and 5 that were related to transglutaminase activity or were related to coagulation proteins. All 5 of the animals tested had strong initial reactions against the human transglutaminase 2, but none showed an increase following exposure and none of the animals had impaired coagulation responses (see below). Transglutaminase 2 has been implicated to have a role in celiac disease and antibodies against that protein are used as a marker for the syndrome, but it is not involved in the coagulation pathway. These animals also did not evince any digestive difficulties.

A second group of proteins is also presented in Table 2. These are proteins that showed significant increases in antibody responses following exposure of the animals to the salmon proteins, but are not related to the coagulation process. There were 15 proteins that fell into this class with a P-value threshold (initial vs. final value) of <0.05.

3.7 Effect of exposure to salmon thrombin and fibrinogen on coagulation parameters

Coagulation parameters were measured in all animals that were exposed to salmon thrombin and fibrinogen. As shown in Table 3, there were no significant changes in the prothrombin times (PT), the activated partial thromboplastin time (APTT) or the thrombin times in the swine. Coagulation parameters from the pigs remained stable throughout the course of the experiment.

To determine if there was an inhibitory effect on coagulation of human blood that was not detected by measuring the clotting parameters in the swine blood, the blood from swine that had been exposed to the fish proteins was mixed with human blood and the coagulation parameters were measured. As shown in Table 4, mixing swine

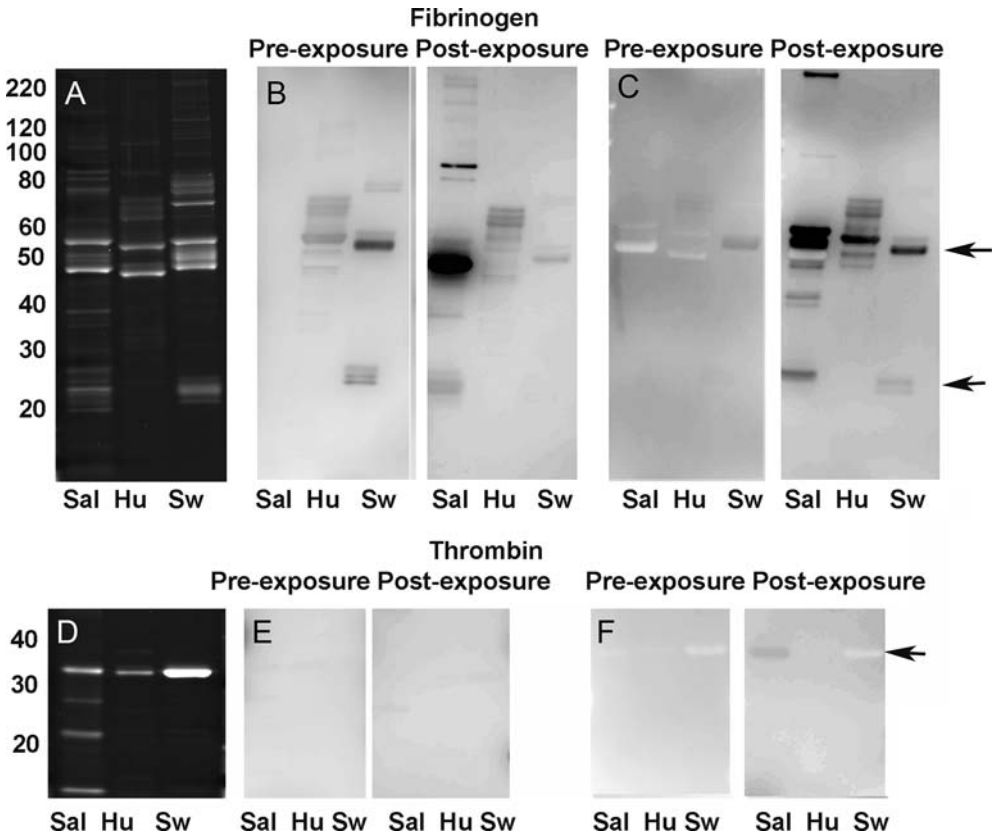

Fig. 5 Qualitative assessment of immunoglobulin production by swine in response to salmon proteins by Western blotting. **a** PAGE of salmon (Sal), human (Hu) and swine (Sw) fibrinogen preparations and corresponding Western blots with serum from two animals (**b** and **c**). Serum from pre-exposure and final euthanasia blood draws are presented in these panels. IgG isotypes were visualized by specific HRP anti-swine IgG second antibodies. Arrows indicate the positions of the IgG heavy and light chains in the swine protein lanes. Molecular weights are show to the left ($\times 10^{-3}$ kDa). **d** PAGE of salmon (Sal), human (Hu) and swine (Sw) thrombin preparations and corresponding Western blots with serum from the same animals shown in (**c** and **d**). In these animals, thrombin was not recognized in (**e**), but there is a faint reaction in the salmon protein lane in (**f**) (arrow). The camera in the detection system detected the heavy swine thrombin protein on the membrane as a white band in (**f**)

blood and human blood did not reveal any cryptic inhibitory effects of the antibodies detected in the swine blood. Values for swine are slightly different from human values and the values, particularly fibrinogen levels, reflect the mixture of the two species.

4 Discussion

4.1 Wound healing in animals treated with salmon fibrinogen/thrombin dressings

Because of the introduction of foreign proteins derived from the salmon blood into a wound site, we were concerned that the wound healing process may be impeded and that coagulopathy may be induced by initiation of an adverse immune response. To investigate these possibilities, we treated full thickness skin wounds with fibrinogen/thrombin dressings, control dressings, and compared the progress of wound healing and the state of activation of the lymph nodes and the spleen.

Excisional cutaneous wounds were surgically created in these eight pigs, bandaged with two different types of dressings, and monitored for two time points of 7 or 28 days following surgery. The 7-day and 28-day time points generally followed the well-established models of cutaneous wound healing by second intention where there are separated edges and no surgical opposition. Cutaneous wounds healed by second intention follow a complex process in closing the defect [17–19]. These types of wounds display a robust, localized inflammatory response, form abundant granulation tissue and have a thin epidermis overlying scar tissue [20, 21]. As expected, the 28-day group exhibited complete re-epithelialization in seven out of eight wounds. Over time, these wounds would likely show some signs of scarring with contracture if allowed to progress for additional weeks and months.

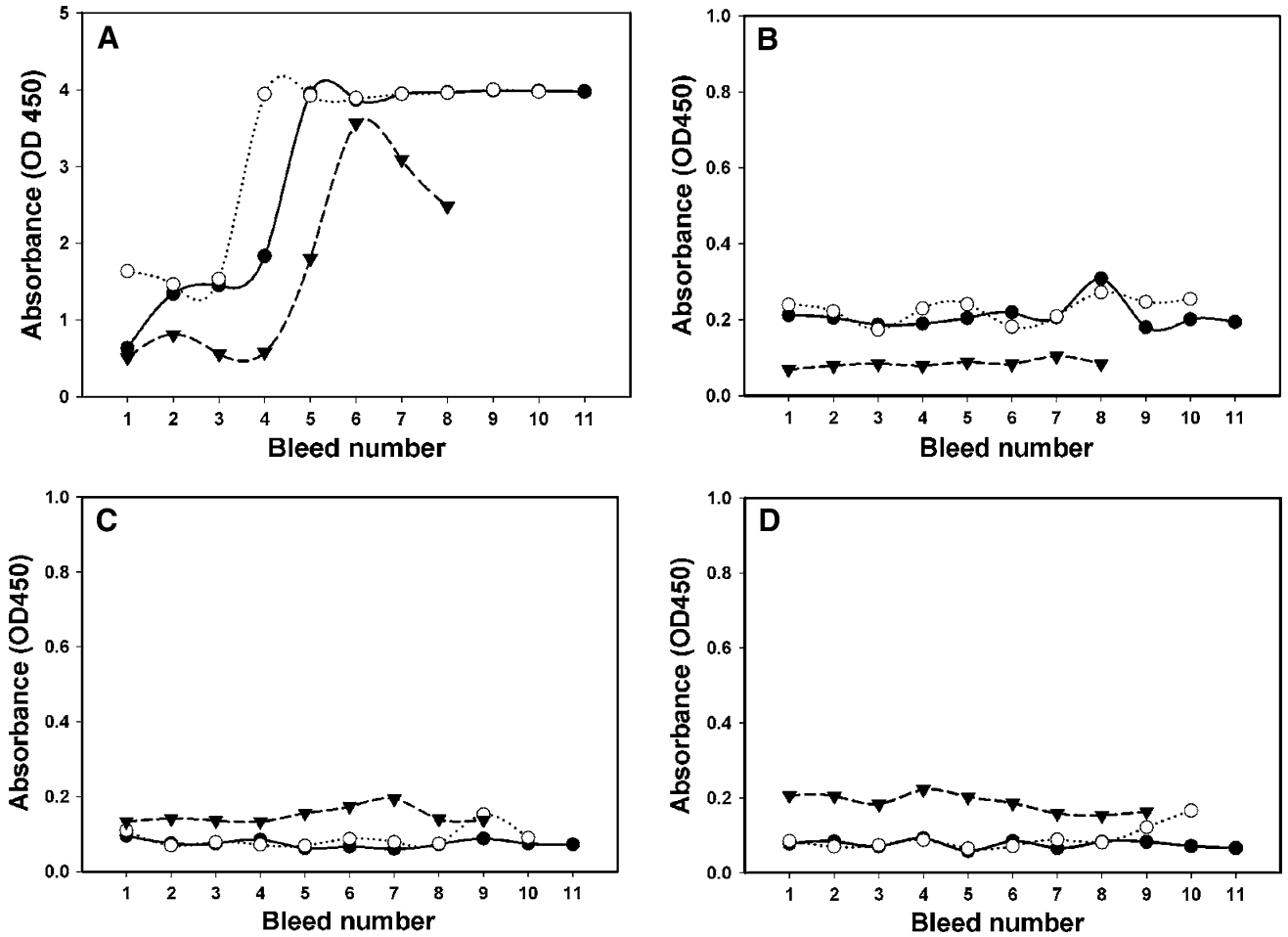

Fig. 6 Time course of antibody development in animals exposed to salmon thrombin/fibrinogen bandages through the abdominal patch protocol. ELISAs were performed using anti-IgG reagents. The following antigens were used as the targets in the ELISAs: **a** salmon fibrinogen, **b** salmon thrombin, **c** human fibrinogen and **d** human thrombin

A notable histopathology difference at the 7-day time point was increased activation of pre-femoral lymph nodes on the salmon fibrinogen/thrombin bandage treated side in contrast to nodes of the non-hemostatic treated side. This was not unexpected as it may reflect a greater degree of immune stimulation on the side exposed to the salmon protein bandages or it may be due to an increased coagulative response at the time of the initial wound, resulting from the fibrin/thrombin bandages.

4.2 The immune response of swine following exposure to salmon fibrinogen/thrombin dressings

Animals exposed to salmon fibrinogen/thrombin were monitored for health complications and blood was drawn to determine the immune response to the salmon proteins. The levels of the cytokines that were measured gave a good representation of the status of the response. The inflammatory cytokines (IL-1, IL-6, TNF-α and INF-γ) often mirrored the surgical manipulations while the humoral signals (IL-4 and IL-10) increased consistently with changes in the antibody titers. Our results show that the animals routinely made immunoglobulins to fibrinogen and, as expected, these were of the IgM and IgG classes. In most animals these antibodies reacted with fibrinogen antigens found in salmon and human fibrinogen. Thrombin, on the other hand, did not induce as universal response as fibrinogen did, with only 6 of the animals generating a thrombin response. Titers of all of the antibodies were low and the Western blots needed to be conducted with a high concentration of the serum to permit any visualization of the thrombin protein bands. There was some diversity in the antigens recognized by the antibodies with some of the antibodies only recognizing prothrombin and not thrombin, even though the prothrombin was a relatively small percentage of the sample.

Measurements of the coagulation parameters were conducted to determine if the antibodies inhibited the coagulation process, but all the measured values remained in the normal range. With the rationale that there may be

Table 2 Screening of human protein-swine antibody interactions with the Invitrogen ProtoArray 5000 protein microarray

Factor	Common name	Related ProtoArray content	Signal level (arbitrary units)	
			Pre	Post
Coagulation or coagulation-related proteins contained on the ProtoArray 5000				
I	Fibrinogen	Fibrinogen-like 1, transcript variant 1	0.5	0.5
II	Prothrombin	Serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1	0.5	0.5
III	Tissue factor, tissue thromboplastin	Coagulation factor III (thromboplastin, tissue factor) (F3)	0.5	0.5
VIII	Antihemophilic factor A (globulin) (AHG)	Coagulation factor VIII, procoagulant component (hemophilia A) (F8)	0.5	0.5
XIII	Protransglutaminase, fibrin stabilizing factor, fibrinoligase			
	Other coagulation related proteins	Multiple coagulation factor deficiency 2 (MCFD2)	0.5	0.5
	Other coagulation related proteins	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	0.5	0.5
	Other coagulation related proteins	Transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase), transcript variant 2	3–5	3–5
	Other coagulation related proteins	Transglutaminase 4 (prostate)	0.1	0.1
	Other coagulation related proteins	Transglutaminase 1 (K polypeptide epidermal type I, protein-glutamine-gamma-glutamyltransferase) (TGM1)	0.2	0.1
	Other coagulation related proteins	CTL2114 TRANSGLUTAMINASE—known Autoantigen	0.2	0.2

	ProtoArray content	Pre	Post
Unrelated proteins contained on the ProtoArray 5000 showing reactivity			
	SUMO/sentrin specific protease, 8	0	4
	WW domain binding protein 2 (WBP2)	1	5
	Cellular retinoic acid binding protein 2	1	5
	RAB3A interacting protein-like 1	0	4
	Nuclear factor I/A	1	5
	Secretogranin III	1	5
	Glycine-*N*-acyltransferase-like 2	0	5
	B-cell CLL/lymphoma 7C	1	5
	Phosphatidylinositol-4-phosphate 5-kinase	0	4
	Mahogunin, ring finger 1	1	5
	Serpin peptidase inhibitor, 6	0	4
	Ataxin 3	0	4
	Casein kinase 1, gamma 2	1	5
	Ribosomal protein S6 kinase	0	4
	NIMA-related kinase 11	1	5

Sera from 6 animals was assayed by Invitrogen for reactivity to a microarray displaying 5000 proteins to determine if antibodies were being generated in the treated swine that may recognize human protein that were not assayed by Western blotting. These proteins were categorized as related or unrelated to the coagulation process. Most antibodies to coagulation proteins did not show significant changes pre- to post-exposure in this assay system. There were 15 non-coagulation proteins showing increased reactivity to antibodies following exposure to the salmon proteins

hidden effects on human coagulation that was not detected in measurements of swine plasma clotting times, we mixed swine and human plasma together. In this experiment as well, we measured normal levels of coagulation. From a gross assessment of the health of the animals as well as the biochemical assays described above, the antibodies did not seem to cause an adverse response.

4.3 Generation of inhibitory coagulation factors in the clinical settings

Acquired coagulopathy is a rare but serious disorder that can arise when a patient generates antibodies that recognize and interfere with the normal function of components of the coagulation pathway. Antibodies have been reported to

Table 3 Hematology and coagulation parameters do not change before and after exposure to the salmon fibrinogen/thrombin dressing

	WBC ($\times 10^9$/l)		RBC ($\times 10^{12}$/l)		HCT (%)		PLT ($\times 10^9$/l)	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Mean ($n = 30$)	19.88	17.30	5.79	5.98	29	30	444	426
SD	3.96	3.10	0.43	0.50	3.23	2.74	89	126
t-Test *P* value		0.03		0.24		0.49		0.62
	PT (s)		APTT (s)		Thrombin time (s)		Fibrinogen (mg/dl)	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Mean ($n = 30$)	13.75	14.66	38.46	40.57	23	23	164	155
SD	1.38	3.63	7.82	10.27	4.59	2.81	40.30	40.63
t-Test *P* value		0.32		0.47		0.83		0.58

Hematological parameters were measured to determine if antibodies developed following exposure to salmon proteins alter normal blood cellular composition or normal coagulation values. Mean, SD and *P* values are shown. None of the parameters was significantly altered following exposure

Table 4 Effects of immunized swine plasma on coagulation of human plasma

Sample	Prothrombin time (s)	APTT (s)	Thrombin time (s)	Fibrinogen concentration (mg/dl)
Swine A alone	13.3	32.2	21.2	137
Human alone	13.0	29.5	16.7	313
Swine A/Human 1:1 ratio	11.9	26.5	22.2	215
Swine B/Human 1:1 ratio	11.8	26.7	21.2	206
Swine A/Human 7:3 ratio	12.2	34.0	23.3	179

Human plasma and swine plasma from two exposed animals were mixed and assayed for coagulation parameters to determine if there may be cryptic factors that could interfere with clotting

recognize and inhibit a range of coagulation proteins. Von Willebrand's disease is the most widely inherited blood disorder [22] and is most commonly due to low expression levels of functional VWF. However, it can also be caused by the inappropriate production of antibodies that can interfere with the von Willebrand polypeptide itself or the protease ADAMTS-13 [23] that processes the polypeptide. Autoantibodies to Factors VIII or IX, although very rare, can lead to acquired hemophilia syndrome [24]. While the underlying cause for these autoantibodies may be due to immune dysfunction in the patient or a triggering health crisis that precipitates the autoimmune response, the use of coagulation agents in surgery as hemostatic agents has also been implicated as the cause of inhibitory antibodies. The use of bovine thrombin as a topical agent in all types of surgical procedures has been widespread with estimates of over a million uses in 2006 [9]. Reports have now documented adverse effects resulting from the antibodies developed against thrombin, prothrombin, factor V and cardiolipin following the use of these hemostatic agents. Many other cases of acquired coagulopathy are associated with surgery without specific reference to whether or not bovine thrombin had been used in those procedures. One approach to combat the autoimmune response has been to attempt to suppress the immune system and the drug rituximab, a monoclonal antibody directed at CD20, has proven to be effective in treating many cases [25, 26]. A disadvantage of this treatment is the increased risk of leukemia and other cancers that occur when the host defense system is impaired. Clearly, any protein-based procoagulative therapeutic agent will need to prove that the risk of autoantibody induction and subsequent acquired coagulopathy is low. Our study has demonstrated that the immune system of pigs, while able to recognize the salmon proteins and generate antibodies, has not mounted a response that leads to coagulopathy and, in the short term, the animals remain healthy. Ongoing studies are now examining the responses of animals over longer periods following exposure. These results suggest that a hemostatic dressing composed of salmon fibrinogen and thrombin could be used in a variety of wounds ranging from external placement on wounds to use internally for surgical hemostasis or treatment of deep trauma. In a separate study, this bandage is being developed for the use with arterial bleeding.

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