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Research Article

Treatment with Spironolactone Reduces Adhesion Formation after Abdominal Surgery and Inhibits Mesothelial-To-Mesenchymal-Transition

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Abstract

Background: Postoperative adhesion formation after abdominal surgery is a common complication and can lead to chronical pain, ileus, emergency surgery and infertility. Previous studies have described an infiltration by myofibroblasts during adhesion formation. Recently the origin of these myofibroblasts has been linked to a mesothelial-to-mesenchymal transition of mesothelial cells. The aim of our study was the investigation of mesothelial-to-mesenchymal transition during adhesion formation in our established ischaemic button mouse model and the inhibition of MMT via spironolactone treatment to prevent adhesion formation.

Methods: Peritoneal adhesion formation was induced by ischaemic buttons on the peritoneal wall in mice. Peritoneal tissue from ischaemic buttons and the unmanipulated peritoneum were analyzed for markers of fibrinolytic activity, collagen production, and cell differentiation by quantitative PCR and immunohistochemistry. One group underwent spironolactone treatment to inhibit adhesion formation. Adhesion formation was measured seven days after surgery.

Results and Conclusion: Gene expressions of fibrinolysis and collagen production markers were significantly increased after surgery compared to unmanipulated peritoneal wall. The significant reduction of Cal+SMA- cells in association with the significantly increase Cal-SMA+ cells display the transition of epithelial to mesenchymal cells during the remodeling processes after surgery. Spironolactone treatment inhibited mesothelial-to-mesenchymal transition and reduced adhesion formation. Our results confirm mesothelial-to-mesenchymal transition during adhesion formation and therefore, spironolactone could be a promising strategy to prevent adhesion formation.

List of Abbreviations: EMT: Epithelial-to-Mesenchymal Transition; Ecad: E-cadherin; SMA: α-Smooth Muscle Actin; Cal: Calretinin; MMT: Mesothelial-to-Mesenchymal Transition; MRA: Mineralocorticoid Receptor Antagonist; IB: Ischaemic Button; tPA: Tissue Plasminogen Activator; PAI: Plasminogen Activator Inhibitor; Col-1/-3: Collagen Type I and III

Introduction

Peritoneal adhesion formation is a common complication after abdominal surgery. Adhesion formation can lead to chronical pain, reduced quality of life, bowel obstruction and is associated with a higher complication rate at subsequent surgeries [1]. It leads not only to a higher morbidity and mortality, but also increases health care costs [2]. An injury of the peritoneum und the subsequent wound healing processes contribute to adhesion formation [3]. Peritoneal adhesion formation involves a local inflammatory reaction, a reduced fibrinolysis activity with an enhanced collagen production [4,5]. Apart from atraumatic and minimal invasive surgery and prevention of infection, approaches to reduce adhesion formation have not reached broad clinical acceptance, especially due to controversially results concerning antiadhesives effects [5-9]. Also, a potential interference of antiadhesives with wound healing can cause reluctance in application of antiadhesive usage [10]. Therefore, the need to understand the pathomechanism during adhesion formation is crucial. It is widely accepted that injury to

the peritoneal surface and the subsequent wound healing processes are causative in adhesion formation. Meanwhile evidence exists that peritoneal adhesions do not exclusively originate from dysregulated collagen production and fibrinolysis, but further involves inflammatory responses [11]. Several cells were identified to infiltrate into adhesive tissue, such as leukocytes, macrophages, mast cells and myofibroblasts [5].

The Epithelial-to-Mesenchymal Transition (EMT) describes the conversion of epithelial cells by losing their cell polarity and cell-cell adhesion and gaining mesenchymal characteristics, such as migratory and invasive properties. EMT is essential for numerous physiological and pathophysiological processes including wound healing [12] and fibrinogenic diseases [13-17]. Several biomarkers for EMT have been identified, such as the loss of the E-cadherin (Ecad) and the upregulation of α-Smooth Muscle Actin (SMA) [18]. Also during adhesion formation, mesothelial cells [3] are losing the epithelial character and transform to myofibroblasts [19]. This transformation has also been termed as Mesothelial-to-Mesenchymal Transition (MMT). Recent studies of biopsies from patients with peritoneal adhesions have revealed an increase of mesenchymal markers as SMA and a reduction of the mesothelial marker Calretinin (Cal) in these tissues. Showing a link between MMT and pathogenesis of postoperative adhesion formation [20]. These findings open new therapeutic strategies in prevention of adhesion formation. Treatment with a Mineralocorticoid Receptor Antagonist (MRA) has shown to reduce EMT in peritoneal mesothelial cells [21] and renal proximal tubular epithelial cells [14] in vivo. To this date, inhibition of MMT during adhesion formation has not been investigated yet. Herein, we investigated MMT during adhesion formation in our established ischaemic button mouse model [22] and inhibition of MMT by spironolactone treatment to prevent adhesion formation.

Material and Methods

Animals

C57BL/6 mice from Janvier (Saint-Berthevin Cedex, France) were enrolled in the study. All experiments were performed with male mice at an age of 8-12 weeks and were acclimatized for at least 5 days under specific pathogen-free conditions including 12/12h light/dark cycle, 21°C and 30% relative humidity in the animal housing facility of the University of Bonn (Germany). No female mice were included as estrogen levels can alter wound healing. The mice were fed (SNIFF, Soest, Germany) and watered ad libitum. Morbidity and mortality were checked once daily during the study. The experiments were performed in accordance with federal law regarding animal protection, ARRIVE guidelines and were approved by the government (AZ.: 84-02.04.2012.A068).

Ischaemic button experiments

Surgery was performed under aseptic conditions. Anesthesia was induced using isoflurane (Abbott, Wiesbaden, Germany) and oxygen as gas carrier (1.5 L/min). Induction of anesthesia was achieved with 3% of isoflurane. The mouse's face was placed into a suitable mask during the surgery (approximately 20 to 30 min.), under 1.5-3% of isoflurane. For analgesia, animals received carprofen 5mg/kg bodyweight subcutaneously. Peritoneal adhesion formation was induced by the construction of four buttons on the peritoneal wall as described before [22,23]. Via a median laparotomy, the peritoneum was lifted with a clamp, and a ligature was applied by first stitching through the base of the button and then ligating the peritoneum. Two buttons were placed on both sides of the peritoneum using a Vicryl® 6/0 suture (Ethicon, Somerville, New Jersey, USA). The abdomen was closed with a double-layered suture of the peritoneum (Vicryl® 5/0) and skin (silk 5/0; Braun, Sempach, Switzerland). Surgery was performed in all groups on day 0. Mice were euthanised on different postoperative days. The ischaemic buttons with adhesions was harvested for further analysis. Unmanipulated peritoneal wall served as control. Eight mice were used per group for ischaemic button surgery and five in the control group.

Spironolactone treatment

To determine the optimal dosage, different concentrations of spironolactone (0.25mg, 0.5mg or 1mg/d) (Aldactone, Riemser Arzneimittel AG, Greifwald, Germany) was applied per gavage for 6 days, beginning three days before surgery. The control group received the vehicle without spironolactone. For further gene expression studies a dosage of 0.25 mg spironolactone was used. As spironolactone leads to hyperkalemia, systemic effect was ensured by measurement of blood potassium levels four hours after spironolactone treatment via puncture of the facial angular veins.

Post-surgery care

Mice were treated with analgesia as indicated below after surgery. They were placed in cages that were placed under an infrared lamp for 2 to 3 hours post-surgery. Afterwards, they were housed again under specific pathogen-free conditions including 12/12h light/dark cycle, 21°C and 30% relative humidity in the animal housing facility of the University of Bonn (Germany).

Animal euthanasia

6 or 24 hours after surgery, animals were sacrificed for further analysis. Euthanasia was performed by cervical dislocation.

Adhesion formation

Adhesion formation after ischaemic button surgery was quantified by counting adhesions on indicated time points. In addition, an adhesion score was used [22]: score 0: no adhesions; score 1: thin, pellucid adhesions; score 2: tensile adhesion; score

3: inseparable and vascularized adhesion; score 4: entire button linked by adhesions.

Immunofluorescence

Cells from the control group, and adhesive tissue were isolated after enzymatic digestion in a solution containing collagenase II (Worthington, Lakewood, New Jersey, USA), Dispase® II (La Roche, Mannheim, Germany), DNase (La Roche), bovine serum albumin and trypsin inhibitor. Cells were centrifuged on to glass slides (cytospin method) and stained with rat anti-mouse F4/80 (1: 200) antibody (BM8; Life Technologies, Darmstadt, Germany) and Arg-1 (1: 200) anti-body (N20; Santa Cruz Biotechnology, Dallas, Texas, USA), followed by secondary donkey anti-rat Alexa 488 (Life Technologies) and donkey anti-goat Cy3 (Dianova, Hamburg, Germany) antibodies. Cells were counted in five randomly chosen areas in each specimen at a magnification of ×200.

Quantitative PCR

Gene expression of (PAI), (tPA), (Col-1), (Col-3), (SMA) and (Ecad) was analysed by PCR. Reagents were from Life Technologies unless specified otherwise. Total RNA was extracted with Trizol® reagent using a tissue homogenizer (Precellys® 24; Peqlab, Erlangen, Germany) followed by DNase I treatment. cDNA was synthesized using a High Capacity cDNA rtkit. Expression of mRNA was quantified in triplicate by reverse transcription–PCR with specific primers for PAI: fwd.: 5´- TTC AGCCCTTGCTTGCCTC -3´, rev.: 5´- ACACTTTTACTCCGAAGTCGGT - 3´,tPA: : fwd.: 5´- TGTCTTTAAGGCAGGGAAGTC - 3´, rev.: 5´- GTCACACCTTTCCCAACATA -3´, Col-1: fw 5′ - ACCTGTGTGTTCCCTACTCA - 3′, rev 5′ - GACTGTTGCCTTCGCCTCTG - 3′; Col-3: fw 5′-AATGGTGGTTTTCAGTTCAGC - 3′, rev 5′ - TGGGGTTTCAGAGAGTTTGGC - 3′ , SMA: fw 5′ - TCAGCGCCTCC GTTCCT - 3′, rev 5′ - TAAAAAAACCACGAGTAACAAATCAA - 3′, Ecad: fw 5′ - GCTGGACCGAGAGAGTTA C - 3′, rev 5′ - GGC ACT TGA CCC TGA TAC G - 3′. GAPDH expression (Glycerinaldehyd-3-phosphat-Dehydrogenase) was analyzed with a pre-developed TaqMan® Assay Reagent. The PCR was performed in Power SYBR® Green or Universal PCR Master Mix by amplification of 10 ng cDNA for 40 cycles (95℃ for 15s, 60℃ for 1min) on an AbiPrism® 7900HT (Life Technologies). Data quantification was performed by the ΔΔCT method and value normalized with respect to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) levels.

Statistical analysis

Continuous data are presented as mean (±SD.). Statistical analysis was performed using one- or two-way ANOVA with Bonferroni's post hoc test with Prism® 5.02 software (GraphPad, La Jolla, California, USA). P <0.05 was considered statistically significant.

Results

Fibrinolysis and collagen production is enhanced during adhesion formation

To evaluate adhesion formation, mice underwent surgery with ischaemic buttons, after different time points adhesion formation was assessed via an adhesion score. Adhesion formation was significantly elevated five (p< 0.01) and seven days (p< 0.001) after surgery compared to day one (Figure 1). As fibrinolysis is an important part of the pathomechanism of adhesion formation, gene expression of tissue Plasminogen Activator (tPA) and Plasminogen Activator Inhibitor (PAI) was analyzed on different time points within the adhesive tissue and compared to untreated peritoneum (CTL). Gene expression of PAI was not significantly altered after surgery (Figure 2A). In contrast, gene expression of tPA was significantly elevated on day five after surgery compared to CTL (p < 0.001). Collagen production was examined by gene expression of collagen Type I (Col-1) and III (Col-3). Gene expression of both Col-1 and Col-3 was significantly elevated on day five after surgery compared to CTL (p < 0.001) (Figure 2C and D).

To explore epithelial-to-mesenchymal transition, gene expression of Smooth Muscle Cell Actin (SMA) and E-cadherin was measured after indicated time points. On day five, gene expression of SMA was significantly elevated within the adhesive tissue compared to control (p < 0.001) (Figure 3A). Whereas in the gene expression of E-Cadherin in the adhesive tissue was not altered in comparison (Figure 3B).
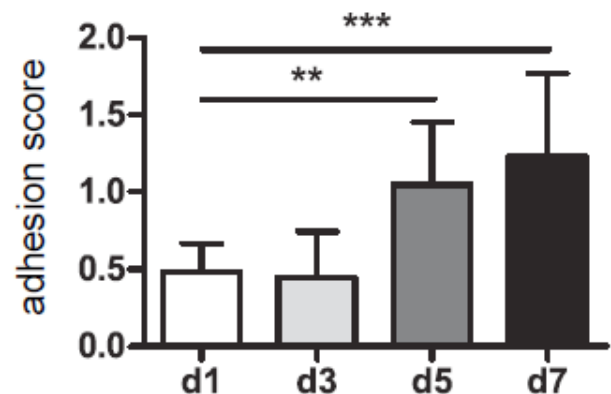
Figure 1: Adhesion formation in wild-type mice was assessed one, three, five and seven days after surgery (score 0: no adhesions; score 1: thin, pellucid adhesions; score 2: tensile adhesion; score 3: inseparable and vascularized adhesion; score 4: entire button linked by adhesions). Data are displayed as mean±SD; n=8 mice/group. Statistical analysis was done by a 1-way ANOVA followed by Bonferroni post hoc test. *p< 0.05; **p< 0.01, ***p<0.001.
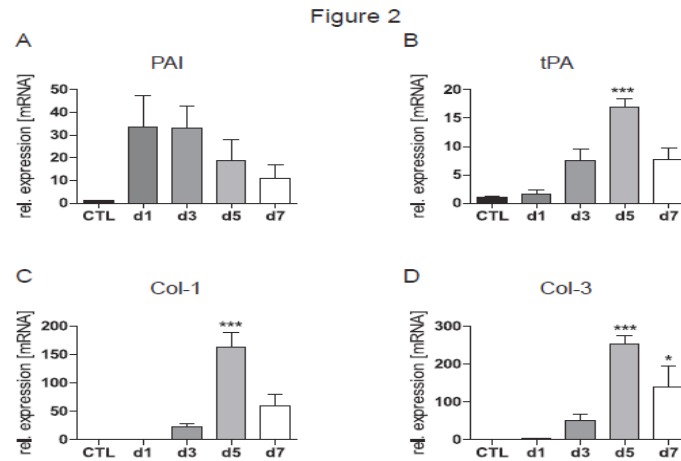
Figure 2: Ischaemic Button (IB) tissue was collected from wild-type mice on days 1, 3, 5 and 7 after surgery. Unmanipulated peritoneal wall served as control tissue (CTL). Gene expression (PAI, tPA, Col-1, Col-3) mRNA levels relative to control were measured after indicated time point. Data are displayed as mean±SD; n=8 mice/group. Statistical analysis was done by a 1-way ANOVA followed by Bonferroni post hoc test. $*p< 0.05$; $**p< 0.01$, $***p<0.001$.
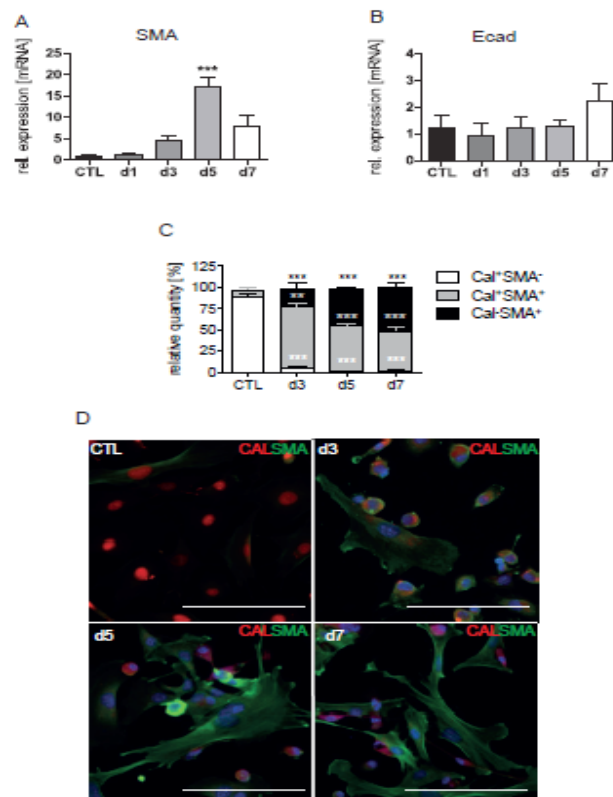
Figure 3: Ischaemic button (IB) tissue was collected from wild-type mice on day 1, 3, 5 and 7 after surgery. Unmanipulated peritoneal wall served as control tissue (CTL). **(A/B)** Gene expression (SMA, Ecad) mRNA levels relative to control were measured after indicated time point. **(C/D)** Cells were isolated and stained after a single-cell cytospin preparation of ischaemic button specimen and stained a mesothelial cell marker, Calretinin (CAL) and a Mesenchymal Cell Marker (SMA). **(C)** Data are displayed as mean ± SD; n = 8 mice/group. Statistical analysis was done by a 2-way ANOVA followed by Bonferroni post hoc test.

*p < 0.05; **p < 0.01, ***p < 0.001. **(D)** Representative immunofluorescence staining after indicated time points, original magnification 400x; scale bar 100 μm).

Epithelial cells transit to mesenchymal cells during adhesion formation

The process of MMT during adhesion formation was verified by immunfluorescence stainings against against Calretinin (Cal), a mesothelial cell marker, and SMA as a mesenchymal cell marker. Relative quantity of Cal$^+$SMA$^-$ cells was significantly reduced after three ($p < 0.001$), five ($p < 0.001$) and seven days ($p < 0.001$), while Cal$^+$SMA$^+$ cells were significantly elevated after surgery compared to CTL (day 3: $p < 0.01$; day 5: $p < 0.001$; day 7: $p < 0.001$). Furthermore, the relative number of Cal$^-$SMA$^+$ cells was significantly increased postsurgery compared to CTL (day 3: $p < 0.001$, day 5: $p < 0.001$, day 7: $p < 0.001$) confirming transition to SMA$^+$ mesenchymal cells (Figure 3C).

Mineralocorticoid receptor antagonist ameliorates adhesion formation

Mineralocorticoid receptor antagonists like spironolactone are known to inhibit EMT. To investigate the effect on adhesion formation, mice were treated with increasing concentrations of spironolactone. Adhesion formation after IB surgery was significantly reduced by treatment with 0.25 mg spironolactone ($p < 0.05$), 0.5 mg spironolactone ($p < 0.05$) and 1mg spironolactone ($p < 0.05$) compared to the control group that received the vehicle, NaCl 0.9 % (Figure 4A). For further analysis, treatment with 0.25 mg spironolactone was applied. As hyperkalemia is a side effect of spironolactone, potassium levels in the blood were measured to ensure systemic effect after spironolactone treatment. Spironolactone treatment significantly elevated potassium levels in the blood ($p < 0.05$) (Figure 4B).
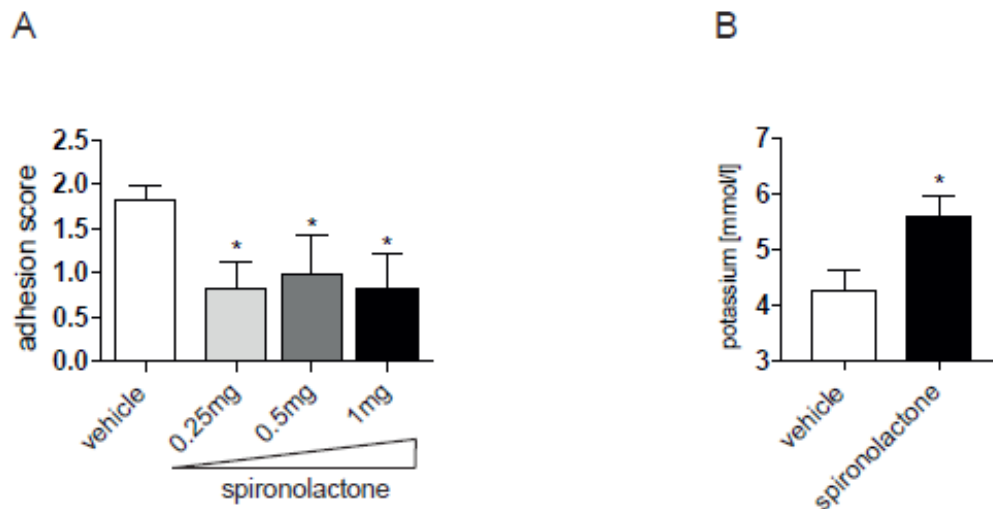
Figure 4: Adhesion formation in wild-type mice after treatment with spironolactone (different dosages) versus vehicle was scored seven days after surgery (score 0: no adhesions; score 1: thin, pellucid adhesions; score 2: tensile adhesion; score 3: inseparable and vascularized adhesion; score 4: entire button linked by adhesions). Potassium levels within the serum was detected four hours after administration of spironolactone versus vehicle. Data are displayed as mean±SD; n=8 mice/group. Statistical analysis was done by **(A)** a 1-way ANOVA followed by Bonferroni post hoc test **(B)** a Student´s t test.

*p < 0.05, **p < 0.01, ***p < 0.001.

Spironolactone treatment alters collagen production and delays MMT

As the fifth day after IB surgery was decisive for gene expression of fibrinolysis marker and collagen production, tissue samples were collected five days after surgery after spironolactone or vehicle treatment. Gene expression of PAI was significantly elevated after spironolactone ($p < 0.001$) or vehicle intake ($p < 0.001$) compared to the associated control tissue (Figure 5A). Also, the measured mRNA levels of tPA and Col-1 were significantly increased after spironolactone ($p < 0.01$ and $p < 0.001$) or vehicle treatment ($p < 0.001$ and $p < 0.001$) (Figure 5B). More interestingly, Col-1 gene expression was significantly lower after spironolactone treatment compared to the vehicle group five days after surgery (Figure 5C). Also, gene expression of Col-3 was significantly elevated in both treated tissues

(each $p < 0.001$), but there was no significant difference between the spironolactone and the vehicle group (Figure 5D).

To explore MMT, gene expression of SMA was measured after spironolactone or vehicle intake. IB surgery induced gene expression of SMA was significantly higher after spironolactone ($p < 0.001$) or vehicle treatment ($p < 0.001$) compared to unmanipulated tissue. Next, cells of adhesive tissue were isolated and stained against Cal and SMA to verify MMT after IB surgery compared to control tissue. Both the treatment with Spironolactone and with NaCl 0.9% led to a significant reduction of Cal^+SMA^- cells compared to CTL after three ($p < 0.001$), five ($p < 0.001$) and seven days ($p < 0.001$). Whereas Cal^+SMA^+ cells were after both treatments significantly elevated (day 3: $p<0.01$; day 5: $p<0.001$; day 7: $p<0.001$). Also the relative number of Cal^-SMA^+ cells was significantly increased on day five ($p < 0.001$) and seven after surgery ($p < 0.001$) compared to the control group (Figure 5F).

Remarkably, the relative number of Cal^+SMA^- cells were significantly lower after spironolactone treatment ($p<0.05$) three days after surgery (Figure 5F), indicating a deceleration of MMT during postoperative adhesion formation.
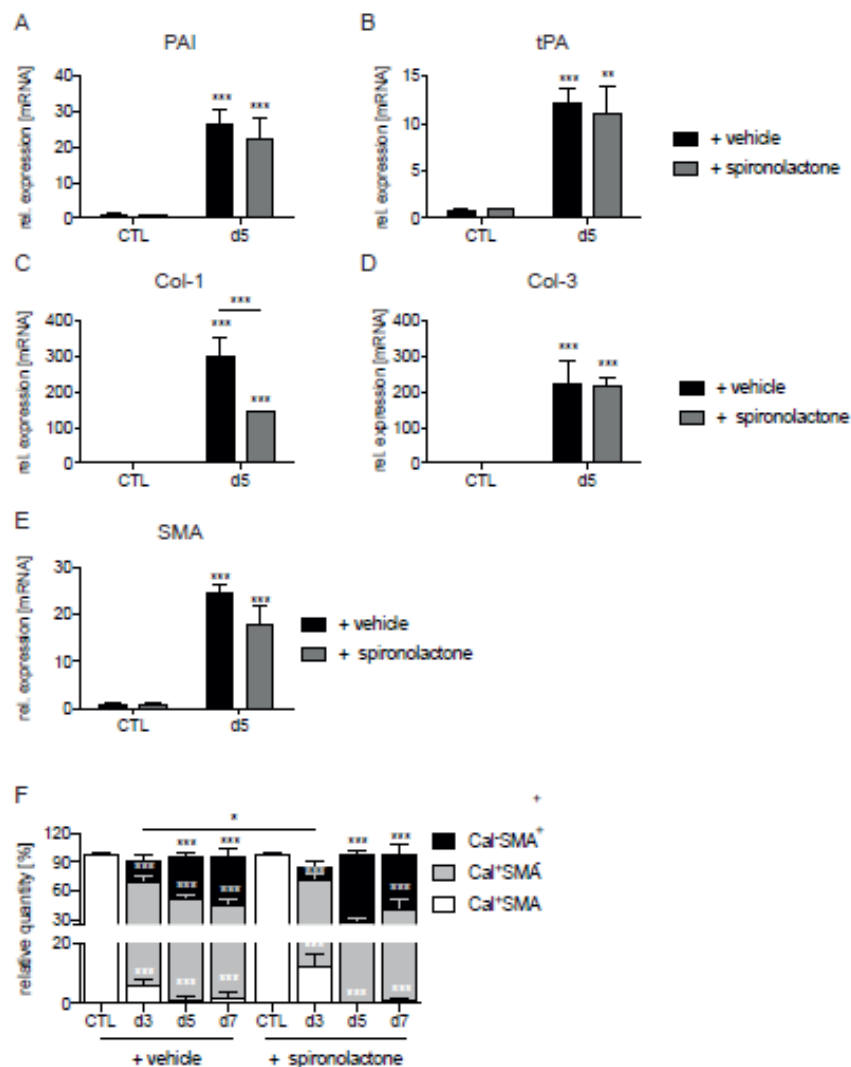
Figure 5: Ischaemic Button (IB) tissue was collected from wild-type mice on day 3, 5 and 7 after spironolactone or vehicle treatment. Unmanipulated peritoneal wall served as Control Tissue (CTL) after spironolactone or vehicle treatment. **(A-E)** Gene expression (PAI, tPA, Col-1, Col-3, SMA) mRNA levels relative to control were measured after 5 days. **(F)** Cells were isolated and stained for a mesothelial cell marker, Calretinin (CAL) and a mesenchymal cell marker (SMA) after a single-cell cytospin preparation.

Data are displayed as mean±SD; n = 8 mice/group. Statistical analysis was done by a 2-way ANOVA followed by Bonferroni post hoc test. $*p < 0.05$; $**p < 0.01$, $***p < 0.001$.

Discussion

After abdominal surgery, injurying of the peritoneum and the subsequent healing processes can lead to peritoneal adhesion formation. Infiltration of myofibroblasts during adhesion formation has been described before [24], recently the origin of these myofibroblasts has been transforming mesothelial cells [20]. This has been also target in recent study, where treatment with an transforming growth factor ß type I receptor kinase inhibitor led to en reduced gene expression of E-Cadherin and an increased SMA within the tissue [25]. To the best of our knowledge, this is the first study to investigate the effect of spironolactone on MMT and adhesion formation after abdominal surgery. Herein, we confirmed MMT by observing a transformation of Cal^+SMA^- mesothelial cells into Cal^+SMA^+ cells and finally transforming into Cal^-SMA^+ myofibroblasts after abdominal surgery. This leads to an enhanced adhesion formation accompanied by an increased collagen production, SMA and an enhanced fibrinolytic activity [26].

Peritoneal mesothelial cells have been shown to express mineralocorticoid receptors. Aldosterone induced MMT by activation of both ERK1/2 or SB20358, an inhibitor of p38 MAPK was shown in human peritoneal mesothelial cells [21]. Also in our study, the inhibition of mineralocorticoid receptors by spironolactone decelerates MMT during adhesion formation. These findings were confirmed by a significantly reduced Col-1 production [27]. Even though number of SMA+cells was reduced after spironolactone treatment, gene expression of SMA was not altered. Also during myocardial fibrosis, spironolactone treatment ameliorated EMT [28]. Actually, MRA is recommended in the treatment by the European society of cardiologists for chronic heart failure and after myocardial infarction to inhibit fibrosis and remodeling. Fibrinolytic activity can be regulated by mesothelial cells, as they can express fibrinolytic enzymes like t-PA or u-PA as well as inhibitors of fibrinolysis by producing PAI-1 [29]. During adhesion formation gene expression of both enzymes have been upregulated in our model. However, deceleration of MMT by spironolactone treatment did not alter fibrinolytic activity during adhesion formation. These findings suggest that fibrinolytic activity during adhesion formation may not depend on mesothelial cells, but other cells like endothelial cells [30].

Also functionally, treatment with spironolactone prevented adhesion formation after abdominal surgery.

Conclusions

In conclusion, MMT is associated with adhesion formation and can be inhibited by spironolactone treatment. Furthermore, adhesion formation was ameliorated by spironolactone treatment and could be a promising strategy to prevent abdominal adhesion formation.

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