Role of plasminogen activators in peritoneal adhesion formation

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Abstract
Intra-abdominal adhesion formation is a major complication of serosal repair following surgery, ischaemia or infection, leading to conditions such as intestinal obstruction and infertility. It has been proposed that the persistence of fibrin, due to impaired plasminogen activator activity, results in the formation of adhesions between damaged serosal surfaces. This study aimed to assess the role of fibrinolysis in adhesion formation using mice deficient in either of the plasminogen activator proteases, tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). We hypothesize that, following serosal injury, mice with decreased peritoneal fibrinolytic activity will be more susceptible to adhesion formation. Adhesion formation was induced in tPA- and uPA-deficient and wild-type mice following either surgical trauma to the serosa with haemorrhage and acute or chronic intra-peritoneal inflammation. Adhesion formation was assessed from 1 to 4 weeks post-injury. Mice deficient in tPA were more susceptible to adhesion formation following both a surgical insult and a chronic inflammatory episode compared with uPA-deficient and wild-type mice. In addition, the time of maximal adhesion formation varied depending on the nature of the initial insult. It is proposed that the persistence of fibrin due to decreased tPA activity following surgery or chronic inflammation plays a major role in peritoneal adhesion formation.

Key words: fibrin, inflammation, plasmin, post-operative, tissue repair.
Abbreviations used: tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; WT, wild-type; PAI, plasminogen activator inhibitor.
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Introduction
Peritoneal adhesions are defined as fibrous bands of tissue that join together organs that are normally separated and/or the internal body wall. They are a common consequence of serosal repair, occurring in 93–100% of patients following laparotomy, and may lead to serious complications such as intestinal obstruction, pelvic pain and infertility [1–3]. One-third of intestinal obstructions and nearly one-quarter of infertility cases in women are a consequence of adhesions, with removal often resulting in recurrence. The magnitude of the problem was highlighted by a survey performed over a 10-year period suggesting that 5.5% of all hospital re-admissions were directly attributable to adhesions [4]. However, despite their clinical importance, information regarding their formation is sparse, and current prevention is based on careful surgery and the occasional use of physical barriers that are effective in only a proportion of patients [5].

Fibrin persistence and peritoneal adhesion formation
Peritoneal adhesions form when closely apposed visceral and/or parietal peritoneal surfaces are damaged due to surgery, thermal or ischaemic injury, inflammation or a foreign body reaction. The protective surface mesothelial layer is disrupted and a fibrinous exudate ('fibrinous' adhesion) is deposited between the damaged, closely apposed serosal surfaces. These filmy adhesions are often transient and are degraded by proteases of the fibrinolytic system within a few days of injury, leading to restoration of the normal peritoneal surface in association with re-epithelialization by mesothelial cells [6,7]. Alternatively, if there is insufficient peritoneal fibrinolytic activity,
the fibrinous scaffold persists, becomes organized by invading fibroblasts and endothelial cells, and with subsequent collagen deposition forms a permanent fibrous adhesion within 1 week of injury [8,9]. We and others have shown that mature human peritoneal adhesions are well vascularized and innervated bands of fibrous tissue [10,11]. Therefore close apposition of damaged peritoneal surfaces and an imbalance between fibrin deposition and fibrin dissolution are thought to be the key events in adhesion formation [12] (Figure 1).

**Reduced fibrinolysis and peritoneal adhesion formation**

During normal repair, fibrin is degraded principally by the fibrinolytic protease plasmin, which is derived from inactive plasminogen through the action of two physiological plasminogen activators (PAs): tissue-type PA (tPA) and urokinase-type PA (uPA). Inhibition of the fibrinolytic system may occur at the level of plasmin, mainly by α1-antiplasmin, or at the level of the PAs through specific PA inhibitors (PAIs). Although both tPA and uPA can activate plasminogen, they are distinct structural proteins with different tissue-specific expression and biological activities [13]. tPA is thought to be primarily responsible for the removal of fibrin from within the vasculature through its specific affinity for fibrin [14]. Alternatively, uPA binds to a cellular receptor and has traditionally been associated with pericellular proteolysis via degradation of matrix components and activation of latent proteases and growth factors at extravascular sites [15].

The normal peritoneum has inherent fibrinolytic activity that is derived mainly from the surface mesothelial cells and endothelial cells [16]. However, abdominal surgery and/or infection have been shown in animals to dramatically reduce fibrinolytic activity in both peritoneal fluid and tissues [9,17,18]. Gerwin and colleagues [9] reported experimental evidence in dogs that serosal fibrinolytic activity was immediately halved by mechanical abrasion, and that this was associated with adhesion formation. Furthermore, evidence for reduced fibrinolytic activity following surgery, ischaemia and inflammatory disease has also been reported in human subjects [19–22]. Scott-Coombes and colleagues [23] postulated that an early decrease in peritoneal PA activity might be secondary to a decrease in tPA levels, whereas the subsequent decrease in functional fibrinolytic activity is caused by a sharp increase in PAI concentration. However, a direct role for each of the PAs in preventing the formation of peritoneal...
adhesions is unclear. To begin to unravel the role of each PA, we have taken the novel approach of using mice deficient in each of these proteases to address this issue.

**Peritoneal adhesion formation in PA-deficient mice**

Genetic manipulation of the PA system in knockout mice provides a promising experimental approach to study the physiological and pathological roles of each of these proteases following different experimental manipulations. Mice deficient in either PA develop normally, survive to adulthood and are fertile [24]. However, mice lacking tPA have a significantly reduced thrombolytic potential and an increased incidence of endotoxin-induced thrombosis. By comparison, inactivation of the uPA gene results in a more severe phenotype, with fibrin deposition in multiple organs (lungs, liver and intestines) and defective plasmaphenomen of macrophage function, although the mice have a normal plasma clot lysis time. Mice with combined tPA and uPA deficiency have a pronounced phenotype, and suffer from extensive fibrin deposition, delayed wound healing with skin ulceration and occasional spontaneous peritoneal adhesions.

We postulated that, following serosal injury, mice deficient in PA activity would be more susceptible to adhesion formation. Mice deficient in tPA (tPA""-"-) or uPA (uPA""-"-) and wild-type (WT) littermates on a mixed genetic background were established through collaboration with Professor P. Carmeliet (Leuven University, Belgium). The temporal and spatial formation of adhesions was assessed in adult male and female mice following a number of different peritoneal insults. One model involved a standardized trauma to the caecal surface and adjacent abdominal body wall combined with haemorrhage from a damaged mesenteric vessel (surgical model). Our previous studies suggested that 100% adhesion formation occurred only with this amount of trauma plus apposition of injured surfaces with stitches [25], and various combinations of these procedures resulted in fewer animals forming adhesions. Therefore apposition of the damaged surfaces was omitted in order to demonstrate an increase as well as a decrease in the numbers of animals developing adhesions. An alternative model was based on non-lethal inflammatory insults produced either by intraperitoneal injection of 4% Brewer's thioglycollate, providing an acute self-limiting sterile peritonitis resolving over 10–12 days (acute inflammation), or by using heat-inactivated Corynebacterium parvum (100 mg/kg), giving a more chronic peritonitis with hepatosplenomegaly and ascites, persisting for at least 4 weeks (chronic inflammation) [26].

Adhesion formation was assessed in a minimum of four animals from each group (tPA""-"-"-, uPA""-"-"- and WT) at 7 days post-injury for the surgical model, at 5, 7 and 9 days for the acute inflammatory model and at 1–4 weeks for the chronic inflammatory model. By macroscopic examination, each mouse was categorized as positive if it had developed one or more adhesions. In the surgical model, adhesions were divided further into: category 1, if found at the traumatized site (between the caecum and the body wall); category 2, if between one of the injured serosal surfaces and an uninvolved site; and category 3, if between uninvolved serosal surfaces, including the midline incision. In the inflammatory model, the inflammatory cell profile of the peritoneal lavage of each mouse was determined by counting and morphological assessment of cytospin preparations, and confirmed by FACS analysis. All the animals used in this study survived the experimental procedures and suffered no ill effects.

**Adhesion formation following surgery and inflammation**

At 1 week following trauma to the body wall and caecum with haemorrhage, more mice in the tPA""-"-"-"- group had developed adhesions (15/19) than in the uPA""-"-"-"- (10/20) or WT (10/19) groups. In addition, the mean number of adhesions per mouse was highest in the tPA""-"-"-"- group (tPA""-"-"-, 1.63 ± 0.25; uPA""-"-"-, 1.05 ± 0.28; WT, 1.0 ± 0.25). Few mice in any of the groups developed adhesions at the trauma site (category 1), whereas the majority of adhesions were found to involve one injured surface and an uninvolved site, such as caecum to small bowel (category 2), or to be totally independent of the original injury site, e.g. midline incision to pelvic fat body (category 3). Several mice developed multiple adhesions of more than one category (adhesion mass); this observation was more prevalent in tPA""-"-"-"- mice (Figure 2).

An intraperitoneal injection of Brewer's thioglycollate provoked a pronounced inflammatory response in all three groups of mice peaking at 5 days compared with baseline levels before injection. The majority of the mice had developed one or more fine adhesions, generally between lobes of the liver, the omentum, and/or pelvic fat bodies with adjacent organs. By day 7, however,
adhesions were present in only one or two mice in each group (tPA<sup>−/−</sup>, 2/7; uPA<sup>−/−</sup>, 1/7; WT, 1/8). In addition, inflammation was substantially reduced by day 7, apart from in the uPA<sup>−/−</sup> group, in which the number of inflammatory cells remained significantly elevated compared with the other two groups. On day 9 post-injection, adhesions were not found in any of the mice and inflammation had returned to baseline levels in the tPA<sup>−/−</sup> and WT mice, whereas a mild inflammatory response remained in the uPA<sup>−/−</sup> mice.

Following peritoneal injection of heat-inactivated C. parvum, there was no adhesion formation in any of the mice at 1 week, although there was evidence of tabes mesentica, pancreatitis, hepatosplenomegaly and inflammatory nodules on the liver. Inflammation was increased compared with baseline control values, but was similar in all three groups of mice. However, by 2 weeks, more tPA- and uPA-deficient mice had developed adhesions compared with the WT group (tPA<sup>−/−</sup>, 9/12; uPA<sup>−/−</sup>, 7/12; WT, 3/12). In addition, the mean number of adhesions per mouse was significantly elevated in the tPA<sup>−/−</sup> group compared with the WT control group (Figure 3). Adhesions were often found between the omentum and/or pelvic fat bodies and the upper abdominal organs. Inflammation in all groups of mice was substantially increased compared with baseline levels pre-injection. A similar pattern of adhesion formation was apparent at 3 weeks post-injection, with a substantially increased number of mice with adhesions in the two PA-deficient groups (tPA<sup>−/−</sup>, 8/11; uPA<sup>−/−</sup>, 8/12; WT, 4/11); however, the inflammatory cell profile had returned to baseline levels in the tPA<sup>−/−</sup> and WT mice, but remained significantly increased in the uPA<sup>−/−</sup> group. By 4 weeks post-injection, all mice in the three groups had developed adhesions (tPA<sup>−/−</sup>, 4/4; uPA<sup>−/−</sup>, 4/4; WT, 4/4), although further studies with a larger number of mice are warranted to confirm this finding.

Discussion
Our results highlight a number of novel findings with regard to the regulation of adhesion formation, as well as a divergent and specific role for each PA in fibrinolysis following different experimental manipulations. We found that tPA-deficient mice were more susceptible to the formation of permanent adhesions following surgical trauma with haemorrhage, and developed adhesions more readily than uPA-deficient and WT animals following a chronic inflammatory insult. Surgery with haemorrhage is likely to cause a massive extravasation of fibrinogen into the peritoneal cavity. Fibrin would be deposited, as part of the coagulation cascade [27], at injured sites as well as in folds of the viscera and in deep pockets in the peritoneal cavity, where it may be protected from fibrinolytic proteases. Several studies have shown that surgery dramatically diminishes fibrinolytic activity in the peritoneal cavity [17,18], suggesting that the fibrin that is deposited in our surgical model may persist and become organized into fibrous adhesions. Therefore mice deficient in tPA were probably more susceptible to adhesion formation following this procedure due a combination of surgery decreasing fibrinolytic activity and a lack of tPA activity. Carmeliet and colleagues...
[24] have shown that there is no compensatory activity of the other PA in the corresponding PA-deficient mouse, suggesting that tPA is required for the clearance of extensive fibrin deposition and to prevent fibrous adhesion formation, and that the presence of uPA or any other fibrinolytic protease was not sufficient to compensate for the lack of tPA.

Acute inflammation is likely to cause mild peritoneal injury, resulting in fibrinogen leakage from inflamed and damaged blood vessels as well as in the binding of fibrinogen present in peritoneal fluid to areas of damaged serosa. With this model, all mice developed fibrinous adhesions initially; however, these were transient and were degraded within 9 days, suggesting that both groups of PA-deficient mice had sufficient fibrinolytic capacity to degrade any fibrin deposited before it could become organized. Fibrinolytic activity was probably provided by the complementary PA or by another protease with fibrinolytic activity, such as neutrophil elastase and/or a member of the matrix metalloproteinase family. Adhesions were found mainly between lobes of the liver, which is an extremely well vascularized organ with naturally apposed serosal surfaces, and sites protected from fibrinolytic proteases. Omental and pelvic fat body adhesions were also common, although, as these structures are naturally extremely adhesive and may associate with injured serosal surfaces within a few hours, the development of such adhesions may be regulated by a different mechanism [11]. All the adhesions seen with this acute inflammatory model were thin and filmy in nature, and had not become organized into true persisting collagenous structures.

In the chronic inflammatory model, inflammation was maximal at 2 weeks after intraperitoneal injection, and at this time a greater number of both tPA- and uPA-deficient animals had developed adhesions. Chronic inflammation is likely to cause a substantial amount of tissue damage, with increased intravascular fibrin deposition creating areas of ischaemia, partial loss of the protective mesothelial cell layer and extensive fibrin deposition at injured sites. Macrophages are primarily responsible for the clearance of extravascular fibrin deposition, and require uPA for pericellular proteolysis and cell migration. Indeed, Carmeliet and colleagues [24] found that activated macrophages derived from uPA-deficient mice showed impaired fibrin dissolution and migration compared with those from tPA-deficient and WT animals. Secondary effects of uPA may also occur, involving decreased activation of matrix metalloproteases and growth factors. Therefore both PAs are likely to be essential in regulating plasmin-mediated fibrin degradation and preventing fibrous adhesion formation following chronic inflammation. Furthermore, several studies have suggested that peritonitis and ongoing pelvic inflammatory disease cause a decrease in fibrinolytic activity, due mainly to a significant increase in PAI levels [28,29]. Although we did not measure levels of PAIs in the present study, a substantial inhibition of PA activity by PAIs might also explain the increase in adhesion formation in PA-deficient mice and the lack of compensatory activity of the complementary PA in this model.

In summary, we have shown the importance of each PA in preventing permanent peritoneal adhesion formation following different peritoneal insults that are clinically relevant. The contribution of each protease is likely to relate to the site of injury, the physical properties of the fibrinous exudates and the kinetics of induction and inhibition. The use of fibrinolytic agents in adhesion prevention is a logical and promising approach [30]; however, these details need to be addressed before such agents can have a substantial clinical impact.

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