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Original research

Mutant matrix metalloproteinase-9 reduces postoperative peritoneal adhesions in rats

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HIGHLIGHTS

- MMP-9 was low in saline-treated peritoneal adhesions.
- MMP-9 was elevated in Ad-mMMp-9-treated adhesions.
- Severity of peritoneal adhesions decreased in Ad-mMMp-9-treated adhesions.

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ABSTRACT

Introduction: Postoperative peritoneal adhesions continue to be a major source of morbidity and occasional mortality. Studies have shown that matrix metalloproteinase-9 (MMP-9) levels are decreased postoperatively which may limits matrix degradation and participate in the development of peritoneal adhesions. In this proof-of-principle study, we evaluated the effect of gene therapy with catalytically inactive mutant MMP-9 on postoperative peritoneal adhesions in rats.

Methods: Adenovirus encoding mutant MMP-9 (Ad-mMMP-9) or saline was instilled in the peritoneal cavity after cecal and parietal peritoneal injury in rats. Expression of mutant MMP-9 transcript was verified by sequencing. Adenovirus E4 gene expression, adhesion scores, MMP-9, tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1) and transforming growth factor-β1 (TGF-β1) expression were evaluated at sacrifice one week after treatment.

Results: Both mutant MMP-9 transcripts and adenovirus E4 gene were expressed in Ad-mMMP-9 treated adhesions. Adhesions severity decreased significantly ($p = 0.036$) in the Ad-mMMP-9-treated compared with saline-treated adhesions. Expression of MMP-9 mRNA and protein were elevated ($p = 0.001$ *and* $p = 0.029$, respectively) in the Ad-mMMP-9-treated adhesions compared with saline-treated adhesions. While tPA levels were increased ($p = 0.02$) in Ad-mMMP-9 treated adhesions compared with saline-treated adhesions, TGF-β1 and PAI-1 levels were decreased ($p = 0.017$ and $p = 0.042$, respectively). No difference in mortality were found between groups ($p = 0.64$).

Conclusions: Mutant MMP-9 gene therapy effectively transduced peritoneal adhesions resulting in reduction of severity of primary peritoneal adhesions.

1. Introduction

Postoperative peritoneal adhesions are directly responsible for multiple major morbidities including intestinal obstruction [1], female infertility, ectopic gestation [2] and chronic abdominal and pelvic pain [3]. The cost of treating adhesion complications results in a large surgical workload and cost to health care systems [4]. Despite several anti-adhesion products are commercially available,

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none of which has been shown to be consistently effective [5].

Healing following peritoneal injury necessitates degradation of fibrin deposits through the action of the proteolytic enzyme plasmin. The inactive proenzyme, plasminogen, is converted to plasmin by the action of tissue plasminogen activator (t-PA). Experimental and clinical studies have shown that t-PA is the major physiological mediator of plasminogen activation in the peritoneal tissues [6,7] and that both open and laparoscopic surgery decrease peritoneal t-PA [8,9]. We have showed previously that instillation of adenoviral vector encoding human t-PA gene in the peritoneal cavity after de novo or recurrent peritoneal injury in rats results in significant reduction in adhesion formation and reformation [10,11].

Plasmin also activates latent matrix metalloproteinases (MMPs) involved in extracellular matrix (ECM) degradation. It has been demonstrated that fibrin is a critical substrate for MMP9 and that MMP-9 degrades fibrin directly, independent of plasmin, and in a dose-dependent manner [12]. In human peritoneal adhesion studies, the concentration of MMP-9 in peritoneal fluid was found to be significantly lower in women with pelvic adhesions than in women with normal pelvis [13], while its specific substrate collagen type IV was found to be more than two fold elevated in adhesion fibroblasts compared with normal peritoneal fibroblasts [14]. Moreover, elevated serum MMP-9 level was proposed as a marker for peritoneal adhesion [15].

The proteolytic activity of MMPs is regulated in part by their physiological inhibitors, tissue inhibitors of MMPs (TIMPs). Chegini et al. demonstrated that adhesions express elevated levels of TIMP-1 and a lower ratio of MMP-1 to TIMP-1 compared with intact parietal peritoneum in the same patient [16]. It has also been shown that peritoneal fluid levels of TIMP-1 in subjects with extensive adhesions are higher compared with those in subjects without adhesions [17]. Because MMP-9, compared to other MMPs, was found to bind to TIMP-1 with the highest affinity, it has been used to neutralize TIMP-1 [18]. However, it has been suggested that MMP-9 might play a role in cancer invasion [19]. Therefore, wild-type MMP-9 is unsuitable candidate for neutralizing TIMP-1 as an anti-fibrotic approach [20]. A catalytically inactive MMP-9 was developed through a single amino acid substitution resulting in abrogation of the aggressive proteolytic activity without disturbing its tight binding to TIMP-1. This mutant MMP-9 has been successfully used as a specific antagonist of TIMP-1 activity but without the potential for enhancing tumor invasion [18,20].

This study was conducted to test the effect of using mutant MMP-9 gene therapy on reduction of peritoneal adhesion formation.

2. Methods

2.1. Experimental design

Adult male Wistar rats (250–300 g) were used and had free access to water and food ad libitum. Female rats were not used so as to exclude the effect of the cycling female sex hormones on adhesion formation [21]. All animals received humane care in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Faculty of Medicine Minia University Council.

The E1-deleted replication-deficient recombinant human adenovirus type 5 vector expressing mutant murine MMP-9 gene (Ad-mMMP9) under the transcriptional control of the optimized human cytomegalovirus-5 (CMV5) immediate early promoter/enhancer was constructed as described previously [18]. Forty animals, 20 per group, were used for the study. After peritoneal injury, Ad-mMMP9, at a dose of 1×10^9 pfu in 1 mL saline was instilled in the peritoneal cavity in group 1 ($n = 14$) while group 2 ($n = 11$) received 1 mL saline and served as control. All animals were sacrificed one week after installation of the vector, and adhesions were graded as follows; grade 0, no adhesions; grade 1, avascular; easily lysed and failing to bleed; grade 2, vascular; easily lysed but bleeds at time of lysis; grade 3, thick; requires extensive sharp surgical dissection [10]. The severity and incidence of adhesions in each group were calculated as the number of severe adhesions (grade 2 and 3) to the total number of animals and the number of animals with adhesions to the total number of animals, respectively.

2.2. Surgical procedure

Anesthesia was induced by intramuscular injection of ketamine HCl (50 mg/kg) and xylazine (10 mg/kg). After skin preparation and lower midline incision, the cecum was exteriorized and 1-cm^2 of its visceral peritoneum was abraded using a scalpel until punctate bleeding is observed [22]. A segment of the right parietal peritoneum from eight animals measuring 20 mm × 5 mm was excised and served as uninjured healthy peritoneum.

2.3. Preparation of peritoneal homogenate

Blocks of adhesion tissues were snap-frozen in liquid nitrogen and then stored at −70 °C [23]. Tissues were homogenized in 500 uL of PBS containing 1% Triton X-100, 0.1% sodium dodecylsulfate, 0.5% sodium deoxycholate, 0.2% sodium azide, and protease inhibitor mixture (Amersham Biosciences, Piscataway, NJ). Homogenization was carried out for 1 min at 4 °C. The homogenate was centrifuged at 4 °C for 5 min at 10,000 g, and the supernatants were aliquoted and stored at −70 °C.

2.4. Biochemical analysis

Peritoneal homogenates were used for the determination of total protein concentration by the Coomassie binding method [24]. Enzyme linked immunosorbent assay (ELISA) kits were used to measure the concentration of rat MMP-9, transforming growth factor beta 1 (TGF-β1) (R&D Systems, Inc., Minneapolis, MN, USA), tPA (Molecular Innovations, Inc., Novi, MI, USA), plasminogen activator inhibitor 1 (PAI-1) antigen (Zymutest; Hyphen BioMed, Neuville-Sur-Oise, France) according to manufacturers' instructions.

2.5. Expression analysis

Total RNA was purified from homogenized adhesion tissues using RiboZol RNA Extraction reagent (Amresco, Solon, USA) following the manufacturer's instructions. Isolated total RNA (5 μg) was used as template to generate cDNA using OneStep RT-PCR Kit (Qiagen, UK). PCR amplification was performed in a DNA thermal cycler (Progene; Techne Ltd., Duxford, United Kingdom). For PCR detection of the specific adenovirus E4 gene, genomic DNA was isolated from adhesion tissues using a QIAamp Tissue Kit (Qiagen Inc., Valencia, CA, USA). Nucleic acid concentrations were determined by spectrophotometer (Genova Plus, Bibby Scientific, UK). Primers used and amplicon size were as follows; mutant *MMP-9*, sense, GGGGGCAACTCGGCAGGAGA, antisense, GGGGGCGCCTGTAGGGCCA, product size 459; *E4*, sense, TGTGACTGATTGAGCGGTG, antisense, CCCATTTAACACGCCATGCA, size 714; and *18s*, sense, TTGACGGAAGGGCACCACCAG, antisense, GCACCACCACCCACGGAATCG, size 131. Annealing temperature was 56 °C, 55 °C and 54 °C for MMP-9, 18S and E4 respectively. The intensity of the PCR product bands were quantified using gel