

Adenovirus-mediated overexpression of human tissue plasminogen activator prevents peritoneal adhesion formation/reformation in rats

Hussein M. Atta, PhD,^a Ayman Al-Hendy, PhD,^b Mahmoud A. El-Rehany, PhD,^c Mieke Dewerchin, PhD,^d Salama R. Abdel Raheim, PhD,^c Hend Abdel Ghany, PhD,^c and Rasha Fouad, MS,^c *El-Minia, Egypt, Houston, TX, and Leuven, Belgium*

Background. Tissue-plasminogen activator (tPA) demonstrated beneficial effects on peritoneal adhesion formation; however, its short half-life limits its continual fibrinolytic effect. Therefore, we delivered adenovirus encoding tPA to prevent adhesions.

Methods. Rats were subjected to peritoneal injury and assigned to two protocols. In de novo adhesion protocol, adenovirus encoding human tPA gene (Ad-htPA) was instilled after peritoneal injury in group 1 (n = 22), whereas group 2 received phosphate-buffered saline (PBS) (n = 24). In recurrent adhesion protocol, group 1 (n = 15) received the same Ad-htPA dose after adhesiolysis and group 2 (n = 13) received PBS. Adhesion severity was scored 1 week after ad-htPA instillation. Adhesions were analyzed for htPA mRNA by reverse transcription-polymerase chain reaction and levels of htPA, and fibrinolytic inhibitors PAI-1, TIMP-1, and TGF- β 1 were measured using enzyme-linked immunosorbent assay.

Results. htPA mRNA and protein were only expressed in adhesions from treated groups. A reduction in adhesion scores (P < .01) and in fibrinolytic inhibitors (P < .001) occurred in the treatment groups. Also, negative correlation was found (r = -.69, P < .01) between adhesion scores and htPA protein, but a positive correlation was found (r = .90, P < .01) between adhesion score and fibrinolytic inhibitors. No bleeding or wound complications were encountered.

Conclusion. Administration of adenovector encoding htPA is safe and decreased de novo and recurrent peritoneal adhesions. (*Surgery* 2009;146:12-7.)

From the Departments of Surgery^a and Biochemistry,^b Faculty of Medicine, Minia University, El-Minia, Egypt; Department of Obstetrics and Gynecology, University of Texas Medical Branch at Galveston,^c Houston, TX; and Vesalius Research Center,^d Leuven, Belgium

POSTOPERATIVE PERITONEAL ADHESIONS are major source of morbidity, being the most common cause of intestinal obstruction, secondary female infertility, ectopic gestation, and chronic abdominal and pelvic pain.¹ Management of these complications results in tremendous cost to the health care system.² It was reported that the cost of adhesiolysis

only was \$1.18 billion in 1988 and \$1.33 billion in 1994 in the United States.^{3,4}

In an attempt to prevent adhesion formation, many different peritoneal instillates and physical barriers have been used.^{5,6} Although barriers showed some success, this experience is not universally confirmed.⁷ Moreover, the use of barriers has been associated with increased abscess and incisional wound complications.⁸ Furthermore, the surgeon must predict potential sites of adhesion formation in order to determine placement site and to optimize barrier function. One of the instillates that demonstrated consistent success is the fibrinolytic agent, tPA.^{9,10} Many experimental studies have reported reduction in adhesion formation and reformation using intraperitoneal recombinant human tPA (htPA) in a variety of delivery methods and preparations, without impairing the healing of bowel anastomosis and without reduction in wound strength or causing hemorrhagic

Supported by the U.S.–Egypt Science and Technology Joint Fund in cooperation with the University of Texas Medical Branch at Galveston and Minia University under Project BIO9-005-001.

Accepted for publication February 20, 2009.

Reprint requests: Hussein M. Atta, PhD, Department of Surgery, Faculty of Medicine, Minia University, El-Minia 61111, Egypt. E-mail: attahm@ems.org.eg.

0039-6060/\$ - see front matter

© 2009 Mosby, Inc. All rights reserved.

doi:10.1016/j.surg.2009.02.018

complications.^{11,12} Because the action of tPA is localized to fibrin deposits, fibrinolytic activity is limited to this site, which prevents indiscriminate fibrinolysis.¹³ tPA has, however, a short half-life (3–6 min in plasma) and is cleared rapidly from the peritoneum by specific endocytosis. Thus, the limiting problem of tPA therapy is maintaining its fibrinolytic effect within the peritoneal cavity for a sufficient duration of time to prevent adhesions formation.^{10,14} For this reason, recent studies attempted to deliver tPA by a continuous infusion pump,¹⁵ intermittent intraperitoneal injection,¹⁶ or slow-release gel. We hypothesized that the use of adenovirus tPA gene therapy to augment the production of tPA would circumvent this limitation. Therefore, the present investigation examined the effect of locally applied adenovirus encoding htPA gene in increasing the fibrinolytic activity and in preventing adhesion formation and reformation in a rat peritoneal adhesion model.

MATERIALS AND METHODS

Experimental design. Adult male Wistar rats were used and had free access to water and food ad libitum. The E1-deleted replication-deficient recombinant adenoviral vector expressing human tPA gene (Ad-htPA) under the transcriptional control of the cytomegalovirus (CMV) immediate-early promoter/enhancer was constructed as described.¹⁷ Two protocols were evaluated. In protocol I, de novo adhesions, after peritoneal injury, Ad-htPA, at a dose of 5×10^7 pfu was instilled in the peritoneal cavity in group 1 ($n = 22$) while group 2 ($n = 24$) received phosphate-buffered saline (PBS) and served as control. The dose determination was guided by our previous studies to maximize adenovirus transfection of target tissue.¹⁸ In protocol II, recurrent adhesions, rats underwent lysis of adhesions 1 week after peritoneal injury; then they were randomized into 2 groups. Group 1 ($n = 15$) received Ad-htPA intraperitoneally at the same dose as in protocol I, and group 2 ($n = 13$) received PBS. All animals were sacrificed 1 week after installation of the vector, and adhesions were scored according to type (Table I).¹⁶ The incidence and severity of adhesions in each group were calculated as the number of animals with adhesions to the total number of animals and the number of severe adhesions (grade 2 and 3) to the total number of animals, respectively.

Surgical procedure. Anesthesia was induced by intramuscular injection of ketamine HCl (50 mg/kg) and xylazine (10 mg/kg). In protocol I, de

Table I. Adhesion grade

| | |
|---------|---|
| 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 |

novo adhesion model, after skin preparation and lower midline incision, the cecum was exteriorized and 1-cm² visceral peritoneum was abraded using sterile gauze until punctate bleeding is observed.⁷ A punch biopsy was used to excise a portion of the peritoneum and underlying muscles from the right lateral abdominal sidewall.¹⁹ In protocol II, recurrent adhesion model, 1 week after adhesion formation injury, the animals were anesthetized, underwent a second laparotomy, and the adhesions were lysed.

Peritoneal tissue collection and preparation of peritoneal homogenate. Blocks of adhesion tissues (1 cm²) were snap-frozen in liquid nitrogen and then stored at -70°C .²⁰ Tissues were homogenized in 500 μL of PBS containing 1% Triton X-100 (diluted), 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 3 min at 10,000g, and the supernatants were aliquoted and stored at -70°C .

Biochemical analysis. Peritoneal homogenates were used for the determination of total protein according to the method of Lowry et al.²¹ Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the concentration of htPA antigen, rat PAI-1 antigen and activity (Zymutest; Hyphen BioMed, Neuville-Sur-Oise, France), rat TIMP-1, rat TGF- β 1 (R&D Systems, Inc., Minneapolis, MN), and rat fibrinogen (AssayPro, St. Charles, MO). Blood was collected without stasis in acidified citrate tubes to preserve tPA activity and was immediately put on ice and centrifuged (4°C at 10,000g). The supernatant was stored at -70°C and was used to measure tPA using human tPA ELISA kit (Zymutest; Hyphen BioMed, Neuville-Sur-Oise, France).²⁰

Reverse transcription polymerase chain reaction (RT-PCR) analysis of htPA mRNA. Extraction of mRNA from tissue homogenates was performed

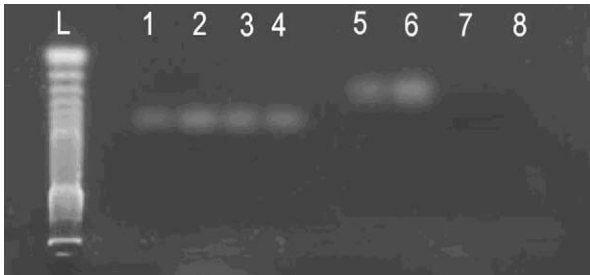


Fig 1. Representative gel showing htPA expression in adhesions from treatment group (lanes 5 and 6) at 300 bp & no expression in control group (lanes 7 and 8). Lanes 1, 2, 3 and 4 show expression of β -actin of the same animals at 550 bp.

using the illustra QuickPrep Micro mRNA Purification kit (GE Healthcare UK Limited, Buckinghamshire, UK). RT-PCR was performed using illustra Ready-to-Go RT-PCR Beads (GE Healthcare UK Limited, Buckinghamshire, UK). PCR reaction was performed in a Progene thermal cycler (Techne LTD, Duxford, UK) using tPA and β -actin primers as described previously.²²

Statistical analysis. The results are expressed as mean \pm standard error of mean (SEM). Statistical analysis was performed with the software Statistical Package for Social Sciences, SPSS 13.0 for Windows (SPSS Inc., Chicago, IL). A P value of less than .05 was considered statistically significant.

RESULTS

Expression of htPA gene in adhesion tissues and in plasma. In both protocols, expression of htPA mRNA was detected in all adhesion tissues obtained from animals treated with adenovirus but none in adhesions obtained from the control groups (Fig 1). htPA protein was detected in adhesions from the treatment groups of protocols I and II (2.9 ± 0.3 ng/mg and 1.4 ± 0.4 ng/mg, respectively) but not in the control groups (Tables II and III, Fig 2). The mean concentration of htPA protein in de novo adhesions was 2-fold greater than that of recurrent adhesions ($P < .05$; independent sample t test). The htPA protein was not detected in plasma using human-specific ELISA kits.

Adhesions scores and complications. A ($P < .01$, Mann-Whitney U test) decrease occurred in severity of de novo adhesions (from 71% to 23%) and recurrent adhesions (from 77% to 27%). The incidence of adhesions decreased ($P < .01$, Mann-Whitney U test) in de novo adhesions but not in recurrent adhesions (Tables II and III). No bleeding complications or abdominal wound dehiscence were encountered, and no difference in mortality was found between groups.

Changes in biochemical regulators of fibrinolytic system. In both protocols, significant reductions occurred in PAI-1 antigen and activity, TIMP-1, TGF- β 1, and fibrinogen in the treatment groups compared with control groups ($P < .001$, independent sample t test). Also, in both protocols, negative correlation was found ($r = -.58$ and $r = -.69$, respectively; $P < .01$; Spearman's rank correlation test) between adhesion score and the htPA protein concentrations but a positive correlation ($r = .90$, $P < .01$; Spearman's rank correlation test) between adhesion score and PAI-1 antigen and activity, TIMP-1, TGF- β 1, and fibrinogen. Interestingly, most studied parameters that promote adhesion formation (ie, PAI-1, TIMP-1, TGF- β 1, and fibrinogen) were increased in the control group of recurrent adhesions compared with the control group of de novo adhesions; but, only PAI-1 antigen and fibrinogen demonstrated statistically significant ($P = .021$ and $P = .018$, independent sample t test) change.

DISCUSSION

We report herein, to the best of our knowledge, the first use of tPA gene therapy to prevent adhesion formation and reformation. In this novel gene therapy study, we showed that adhesion tissues successfully expressed htPA mRNA for 1 week after a standardized peritoneal injury (de novo adhesion protocol) or adhesiolysis (recurrent adhesion protocol). Second, we demonstrated the expression of htPA protein in the adhesion tissues. Third, the measured fibrinolytic inhibitors were markedly decreased in animals treated with the therapeutic viral vector. Fourth, the study showed significant decrease in severity of de novo and recurrent adhesions. Finally, no bleeding complications or abdominal wound dehiscence were encountered.

In both protocols, instillation of adenovirus encoding htPA gene in the peritoneal cavity resulted in significant reduction in severity of adhesion formation (from 71% to 23%) and reformation (from 77% to 27%). The incidence of adhesions, however, decreased significantly in de novo adhesions but not in recurrent adhesions. Also, in both protocols, significant negative correlation was found between adhesion score and the htPA protein concentrations.

Interestingly the concentration of htPA protein in recurrent adhesion tissues was 50% lower than that in de novo adhesion tissues. Because this study was not designed to analyze this finding, we can only speculate that in the recurrent adhesion setting, there is augmented response to tissue injury as manifested by significant increased production of both the fibrin substrate fibrinogen and

Table II. Results of adhesion grades and biochemical analysis of protocol I (de novo adhesion)

| | Control group | Test group | P value |
|------------------------|---------------|-------------|---------|
| Adhesion score | | | |
| Grade 0 | 4 (17%) | 10 (45%) | |
| Grade 1 | 3 (13%) | 7 (32%) | |
| Grade 2 | 11 (46%) | 3 (14%) | |
| Grade 3 | 6 (25%) | 2 (9%) | |
| Total | 24 | 22 | |
| Adhesions, severity | 17/24 (71%) | 5/22 (23%) | <.01* |
| Adhesions, incidence | 20/24 (83%) | 12/22 (55%) | <.01* |
| tPA (ng/mg) | 0 ± 0 | 2.9 ± 0.33 | <.001† |
| PAI-1 activity (ng/mg) | 1.38 ± 0.16 | 0.37 ± 0.12 | <.001† |
| PAI-1 antigen (ng/mg) | 7.43 ± 0.78 | 2.37 ± 0.53 | <.001† |
| TGF-β1 (ng/mg) | 8.27 ± 0.55 | 5.58 ± 0.54 | <.01† |
| Fibrinogen (μg/mg) | 26.7 ± 2.3 | 11.4 ± 1.5 | <.001† |
| TIMP-1 (ng/mg) | 74.2 ± 7.6 | 34.8 ± 5.9 | <.001† |
| Mortality | 1 | 3 | NS* |

*Mann-Whitney *U* test.

†Independent sample *t* test.

Table III. Results of adhesion grades and biochemical analysis of protocol II (recurrent adhesion)

| | Control group | Test group | P value |
|------------------------|---------------|-------------|---------|
| Adhesion score | | | |
| Grade 0 | 1 (8%) | 1 (7%) | |
| Grade 1 | 2 (15%) | 10 (67%) | |
| Grade 2 | 0 | 4 (27%) | |
| Grade 3 | 10 (77%) | 0 | |
| Total | 13 | 15 | |
| Adhesions, severity | 10/13 (77%) | 4/15 (27%) | <.01* |
| Adhesions, incidence | 12/13 (92%) | 14/15 (93%) | NS* |
| tPA (ng/mg) | 0.0 | 1.6 ± 0.41 | <.01† |
| PAI-1 activity (ng/mg) | 1.87 ± 0.21 | 0.67 ± 0.09 | <.001† |
| PAI-1 antigen (ng/mg) | 10.43 ± 0.87 | 5.32 ± 0.52 | <.001† |
| TGF-β1 (ng/mg) | 9.75 ± 0.75 | 6.29 ± 0.49 | <.01† |
| Fibrinogen (μg/mg) | 36.8 ± 3.5 | 20.0 ± 2.5 | <.01† |
| TIMP-1 (ng/mg) | 95.7 ± 11.3 | 38.8 ± 3.9 | <.001† |
| Mortality | 2 | 0 | NS* |

*Mann-Whitney *U* test.

†Independent sample *t* test.

the fibrinolytic inhibitor PAI-1 in the control group of recurrent adhesions compared with their levels in the control group of de novo adhesions. Another possibility is that the adenovirus may be sequestered by the already formed noncellular part of adhesion tissue resulting in decreased numbers of cells transduced with the virus. The defect in both possibilities might be corrected with increasing the dose of adenovirus in the setting of recurrent adhesions.

The ability of expressed htPA to prevent adhesion formation and reformation is supported by the demonstrated decrease in fibrinogen and fibrinolytic inhibitors examined in this study. In

both protocols, significant reductions in PAI-1 antigen and activity, TIMP-1, TGF-β1, and fibrinogen occurred in the treatment groups compared with control groups. Also, significant positive correlation was found between adhesion score and PAI-1 antigen and activity, TIMP-1, TGF-β1, and fibrinogen. These findings are in line with a large body of literature demonstrating that adhesion formation results not only from tPA/PAI imbalance,^{10,20,22,23} but also involves MMP/TIMP imbalance²⁴ and increased TGF-β production.²⁵

This new treatment strategy should be subjected to further evaluation. The efficacy of ad-tPA on

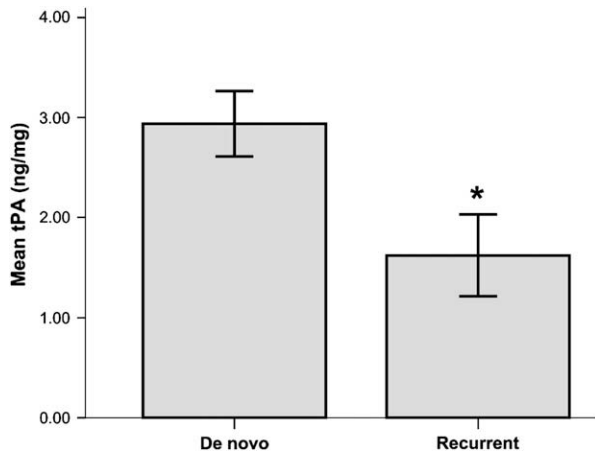


Fig 2. Bar graph representing mean \pm SEM concentration of htPA protein in adhesion tissues obtained from de novo and recurrent adhesions. * $P < .05$; independent sample t test.

adhesion formation/reformation reduction should be determined at longer intervals after treatment. Furthermore, assessment of local and systemic spread of the vector and the effects of such spread such as decreased wound bursting strength and the development of incisional hernia or long-term bleeding complication should be part of future studies.

In conclusion, this investigation demonstrated that instillation of adenoviral vector encoding htPA gene in the peritoneal cavity after de novo or recurrent peritoneal injury in rats results in significant reduction in adhesion formation and reformation without increasing the risk of mortality, bleeding, or wound complications.

REFERENCES

- Liakakos T, Thomakos N, Fine PM, Dervenis C, Young RL. Peritoneal adhesions: etiology, pathophysiology, and clinical significance. Recent advances in prevention and management. *Dig Surg* 2001;18:260-73.
- Ellis H, Moran BJ, Thompson JN, Parker MC, Wilson MS, Menzies D, et al. Adhesion-related hospital readmissions after abdominal and pelvic surgery: a retrospective cohort study. *Lancet* 1999;353:1476-80.
- Ray NF, Denton WG, Thamer M, Henderson SC, Perry S. Abdominal adhesiolysis: inpatient care and expenditures in the United States in 1994. *J Am Coll Surg* 1998;186:1-9.
- Ray NF, Larsen JW Jr, Stillman RJ, Jacobs RJ. Economic impact of hospitalizations for lower abdominal adhesiolysis in the United States in 1988. *Surg Gynecol Obstet* 1993;176:271-6.
- Wallwiener M, Brucker S, Hierlemann H, Brochhausen C, Solomayer E, Wallwiener C. Innovative barriers for peritoneal adhesion prevention: liquid or solid? A rat uterine horn model. *Fertil Steril* 2006;86:1266-76.
- Yeo Y, Ito T, Bellas E, Highley CB, Marini R, Kohane DS. In situ cross-linkable hyaluronan hydrogels containing polymeric nanoparticles for preventing postsurgical adhesions. *Ann Surg* 2007;245:819-24.
- Wallwiener D, Meyer A, Bastert G. Adhesion formation of the parietal and visceral peritoneum: an explanation for the controversy on the use of autologous and alloplastic barriers? *Fertil Steril* 1998;69:132-7.
- Cohen Z, Senagore AJ, Dayton MT, Koruda MJ, Beck DE, Wolff BG, et al. Prevention of postoperative abdominal adhesions by a novel, glycerol/sodium hyaluronate/carboxymethylcellulose-based bioresorbable membrane: a prospective, randomized, evaluator-blinded multicenter study. *Dis Colon Rectum* 2005;48:1130-9.
- Doody KJ, Dunn RC, Buttram VC Jr. Recombinant tissue plasminogen activator reduces adhesion formation in a rabbit uterine horn model. *Fertil Steril* 1989;51:509-12.
- Hellebrekers BW, Trimbos-Kemper TC, Trimbos JB, Emeis JJ, Kooistra T. Use of fibrinolytic agents in the prevention of postoperative adhesion formation. *Fertil Steril* 2000;74:203-12.
- Buckenmaier CC III, Summers MA, Hetz SP. Effect of the antiadhesive treatments, carboxymethylcellulose combined with recombinant tissue plasminogen activator and Septrafilm, on bowel anastomosis in the rat. *Am Surg* 2000;66:1041-5.
- Menzies D, Ellis H. The role of plasminogen activator in adhesion prevention. *Surg Gynecol Obstet* 1991;172:362-6.
- Vipond MN, Whawell SA, Scott-Coombes DM, Thompson JN, Dudley HA. Experimental adhesion prophylaxis with recombinant tissue plasminogen activator. *Ann R Coll Surg Engl* 19194;76:412-5.
- Hellebrekers BW, Trimbos-Kemper TC, Boesten L, Jansen FW, Kolkman W, Trimbos JB, et al. Preoperative predictors of postsurgical adhesion formation and the prevention of adhesions with plasminogen activator (PAPA-study): results of a clinical pilot study. *Fertil Steril* 2009;91:1204-14.
- Orita H, Fukasawa M, Girgis W, diZerega GS. Inhibition of postsurgical adhesions in a standardized rabbit model: intraperitoneal treatment with tissue plasminogen activator. *Int J Fertil* 1991;36:172-7.
- Montz FJ, Fowler JM, Wolff AJ, Lacey SM, Mohler M. The ability of recombinant tissue plasminogen activator to inhibit post-radical pelvic surgery adhesions in the dog model. *Am J Obstet Gynecol* 1991;165:1539-42.
- Carmeliet P, Stassen JM, Van VI, Meidell RS, Collen D, Gerard RD. Adenovirus-mediated transfer of tissue-type plasminogen activator augments thrombolysis in tissue-type plasminogen activator-deficient and plasminogen activator inhibitor-1-overexpressing mice. *Blood* 1997;90:1527-34.
- Al-Hendy A, Magliocco AM, Al-Tweigeri T, Braileanu G, Creltin N, Li H, et al. Ovarian cancer gene therapy: repeated treatment with thymidine kinase in an adenovirus vector and ganciclovir improves survival in a novel immunocompetent murine model. *Am J Obstet Gynecol* 2000;182:553-9.
- Rodgers K, Cohn D, Hotovely A, Pines E, Diamond MP, diZerega G. Evaluation of polyethylene glycol/poly(lactic acid) films in the prevention of adhesions in the rabbit adhesion formation and reformation sidewall models. *Fertil Steril* 1998;69:403-8.
- Holmdahl L, Eriksson E, Eriksson BI, Risberg B. Depression of peritoneal fibrinolysis during operation is a local response to trauma. *Surgery* 1998;123:539-44.
- Lowy OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.
- Saed GM, Diamond MP. Modulation of the expression of tissue plasminogen activator and its inhibitor by hypoxia in human peritoneal and adhesion fibroblasts. *Fertil Steril* 2003;79:164-8.

23. Hellebrekers BW, Emeis JJ, Kooistra T, Trimbos JB, Moore NR, Zwinderman KH, et al. A role for the fibrinolytic system in postsurgical adhesion formation. *Fertil Steril* 2005;83:122-9.
24. Chegini N, Kotseos K, Bennett B, Diamond MP, Holmdahl L, Burns J. Matrix metalloproteinase (MMP-1) and tissue inhibitor of MMP in peritoneal fluids and sera and correlation with peritoneal adhesions. *Fertil Steril* 2001;76:1207-11.
25. Holmdahl L, Kotseos K, Bergstrom M, Falk P, Ivarsson ML, Chegini N. Overproduction of transforming growth factor-beta1 (TGF-beta1) is associated with adhesion formation and peritoneal fibrinolytic impairment. *Surgery* 2001;129:626-32.

ON THE MOVE?

Send us your new address at least six weeks ahead

Don't miss a single issue of the journal! To ensure prompt service when you change your address, please photocopy and complete the form below.

Please send your change of address notification at least six weeks before your move to ensure continued service. We regret we cannot guarantee replacement of issues missed due to late notification.

JOURNAL TITLE:

Fill in the title of the journal here. _____

OLD ADDRESS:

Affix the address label from a recent issue of the journal here.

NEW ADDRESS:

Clearly print your new address here.

Name _____

Address _____

City/State/ZIP _____

COPY AND MAIL THIS FORM TO:

Subscription Customer Services
Elsevier Inc.
11830 Westline Industrial Drive
St. Louis, MO 63146

OR FAX TO:

314-523-5170

OR E-MAIL:

JournalsCustomerService-usa@elsevier.com

OR PHONE:

800-654-2452

Outside the U.S., call
314-453-7041