Diseases of the Colon & Rectum

Effect of Adenoviral-Mediated Transfer of Transforming Growth Factor-β1 on Colonic Anastomotic Healing

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PURPOSE: Transforming growth factor-β1 plays a central role in colonic repair. We examined the temporal effect of vector-mediated transfer of transforming growth factor-β1 on colonic anastomotic healing. METHODS: Male Sprague-Dawley rats (n = 24) underwent transection of the distal colon and single-layer anastomosis. Proximal to the anastomosis, the colon was again transected and a colostomy was matured proximally. The distal colon was intubated with a silicone catheter, tunneled along subcutaneous tissues, and connected to a swivel apparatus for postoperative luminal infusion. Rats were randomized into four groups (n = 6 each). Two control groups received 10^{10} plaque-forming units of a Type 5 E1-deleted adenovirus carrying the bacterial β-galactosidase gene either immediately following surgery or on postoperative Day 3. The treatment groups received transforming growth factor-β1 with the same viral construct at parallel time points. On postoperative Day 6, anastomotic bursting pressure and site were determined *in situ* with the anastomotic tissue subsequently harvested and analyzed by enzyme-linked immunosorbent assay for β-galactosidase and transforming growth factor-β1. RESULTS: When compared with its corresponding control, the group that received the transforming growth factor-β1 gene on postoperative day 3 had a significantly higher bursting pressure (mmHg; 119 ± 16 *vs.* 160 ± 12, mean ± SD; $P = 0.001$). While the majority of colons (5/6) from the control group burst at the anastomosis, none of the colons in the group that received transforming growth factor-β1 on day 3 burst at the anastomotic site ($P = 0.007$). β-Galactosidase levels (pg/ml) in anastomotic tissue were significantly increased in both control groups when compared with their respective treatment groups (101 ± 43 *vs.* 38 ± 30, $P = 0.01$ when infused the day of surgery and 243 ± 92 *vs.* 50 ± 30, $P = 0.009$ when infused on day 3). Anastomotic levels of transforming growth factor-β1 were also increased in the group receiving the transforming growth factor-β1 gene on day 3 (214 ± 66 *vs.* 135 ± 24, $P = 0.02$). CONCLUSIONS: Gene transfer into the healing colonic anastomosis can be effectively achieved *via* intraluminal administration of adenoviral vectors. Transfer of transforming growth factor-β1 increased the strength of colonic anastomoses when given at Day 3 but not at Day 0, demonstrating its diverse effects in the wound healing sequence. Thus, gene transfer of transforming growth factor-β1 may avoid the need for a diverting stoma in cases of rectal surgery and impaired healing resulting from chemotherapy or radiation. [Key words: Transforming growth factor-β; 1; Adenovirus vectors; Colonic healing; Rat]

Wound healing involves a complex series of events that includes the interplay of collagen, cytokines, and several growth factors. The role of transforming growth factor-beta 1 (TGF-β1) in this process has been well documented by our group in rat models of both normal and impaired colonic healing.[1–3] We have previously demonstrated a temporal relationship in colonic healing between the increasing levels of transcription of TGF-β1 to procollagen I upregulation with maximal mRNA expression of both of these factors on postoperative day 7. These local in-

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creases in TGF-β1 expression are diminished by the administration of 5-fluorouracil (5-FU) with impairment of colonic anastomotic healing. These results underscore a probable role for the direct administration of exogenous TGF-β1 to the wound site *via* a recombinant virus vector.

Replication-deficient adenoviruses have received much attention as efficacious vectors for gene delivery. Unlike retroviruses, recombinant human adenovirus serotype 5 (Ad5) is effectively transferred into a broad spectrum of both replicating and nonreplicating cells across many different species.[4,5] One cell type in which Ad5 vectors have been shown particularly effective is rodent intestinal epithelial cells.[6,7] However, most of these reports on successful transfection of epithelial cells have been performed with small intestine rather than colon.[1,4,6]

The purpose of this study was to investigate the ability to transfer a marker gene *via* an adenoviral vector directly into the rat colon during the early stages of wound healing. With feasibility established, TGF-β1 was similarly transferred and its role and temporal relationship in anastomotic healing and strength were examined.

MATERIALS AND METHODS

Propagation and Purification of Recombinant Adenoviruses

An E1/E3 deleted recombinant type 5 adenovirus with normal fiber structure expressing bovine TGF-β1 was constructed.[8,9] The TGF-β1 sequence was altered *via* two point mutations in its coding region such that the resulting molecule produced was intrinsically active, as opposed to the naturally occurring latent form which is cleaved *in vivo* to its active form. The entire coding region of the TGF-β1 sequence positioned downstream to a human cytomegalovirus promoter was subcloned into pΔE1sp1 and cotransfected with pBHG11 into 293 cells for homologous recombination. For large-scale production, virus was added to the *293 Cell Line* grown in Dulbecco's modified Eagle's medium supplemented with 5 percent fetal calf serum, penicillin, streptomycin, and glutamine. When cytopathic effects were completed, the cells were harvested and viral particles were released by five cycles of freeze and thawing in dry ice and ethanol. Crude viral lysates were purified by means of a previously described method of centrifugation of viral particles against linear sucrose gradients.[10] In brief, crude viral lysates were layered over a 20 percent to 80 percent linear sucrose gradient. After centrifugation at 80,000 × *g* for two hours at 4°C the viral band was collected and layered over a second linear sucrose gradient with centrifugation at 100,000 × *g* for an additional five hours at 4°C. The viral band was again collected; and viral concentration was determined by end-point cytopathic effect assay. A vector expressing the marker gene bacterial β-galactosidase (β–Gal) was similarly constructed.

Animal Preparation and *In Vivo* Gene Transfer

Twenty-four male Sprague-Dawley rats weighing 250 to 275 g (Charles River Laboratories, Bridgeport, NJ) were acclimated to laboratory conditions in individual metabolic cages with wire bottoms to prevent coprophagia. All studies were by approved by Temple University's Institutional Animal Care and Use Committee.

All animals received a preoperative bowel preparation consisting of Vivonex TEN® (Sandoz Nutrition Corporation, Minneapolis, MN) and osmotic purgative polyethylene glycol in a balanced electrolyte solution of GoLYTELY® (Braintree Laboratories Inc., Braintree, MA) for two days. On the third day, all rats were anesthetized with intraperitoneal (IP) ketamine (90 mg/kg) and xylazine (1 mg/kg). A silicone catheter was tunneled from the interscapular space to the anterior abdominal wall in the subcutaneous space and connected to a swivel apparatus for postoperative luminal infusion. The abdomen was entered through a midline laparotomy, and the distal colon was transected 2.5 cm proximal to the pelvic brim. A single-layer, inverting, end-to-end anastomosis was constructed with 7-0 interrupted silk sutures (Ethicon, Inc., Somerville, NJ). Proximal to the anastomosis the colon was again transected 2.0 cm distal to the cecum; a colostomy was matured proximally; and the distal colon was intubated with the above-mentioned silicone catheter. The catheter was then advanced to a point approximately 1 cm proximal to the distal colonic anastomosis (Fig. 1). The catheter was fastened to the colon with a 5-0 silk suture. Postoperatively, rats received water and rat chow *ad libitum*.

Postoperatively, all rats received 10^{10} plaque-forming units of adenovirus vector diluted to 2 ml in Dulbecco's phosphate-buffered saline (D-PBS) at a rate of 0.2 ml/min *via* the intraluminal infusion catheter. After the viral infusion an additional volume (1

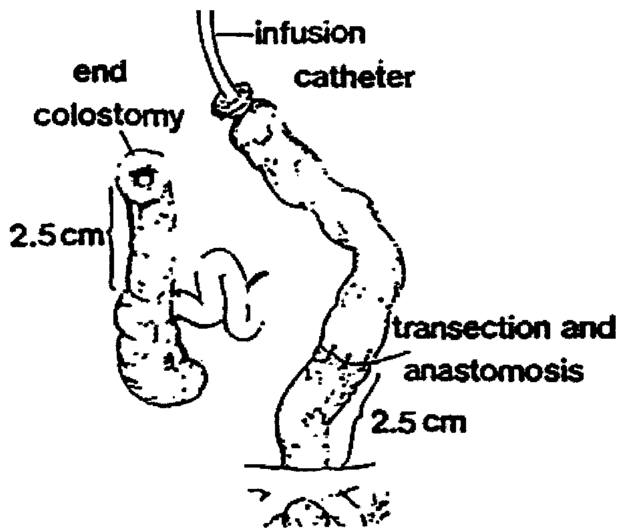

Figure 1. Schematic representation of the proximal colostomy, the infusion catheter, and its relation to the distal transection and anastomosis of the rat colon.

ml) of D-PBS was infused. Rats were randomized to receive either the Type 5 E1-deleted adenovirus carrying the bacterial β-Gal gene (Control) on the day of surgery (AdLacZ0, n = 6) or on postoperative Day (POD) 3 (AdLacZ3, n = 6) or the same viral construct carrying TGF-β1 (Treatment) at the same respective times (AdTGF0, n = 6; AdTGF3, n = 6).

Bursting Pressure and Site

Bursting pressure (BP) and site of burst were determined *in situ* on POD 6. All rats were anesthetized, the abdomen was opened, and anastomotic dehiscence, fistula, and abscess formation were recorded. A ligature was placed across the bowel, 1.25 cm proximal to the anastomosis. After the introduction of a pressure catheter through the anus, a pursestring suture was placed subcutaneously around the catheter and tied to form a watertight seal. The catheter was simultaneously connected to an infusion pump and pressure transducer. The pressure signal, transduced into voltage and displayed simultaneously on a monitor, was entered into a database (Labtech Notebook Software, Wilmington, MA). The Notebook software includes a graphical interface that allows *post hoc* analysis of the pressure curve. Normal saline, dyed blue, was infused through the catheter at 7 cc/min. Time to the first sign of dye in the abdominal cavity was recorded, and a fall in the recorded pressure confirmed the maximum BP. The maximum intraluminal pressure and the area under the pressure curve have been shown to be good indices of the energy applied to the colonic wall to produce anastomotic disruption.[11,12] At the completion of or during the recording of the BP, the site of the burst was identified and bursting energy was calculated. These data allow subsequent analysis and comparisons of tensile strength, and therefore wound healing, at the anastomotic site *vs.* other intact portions of the colon.

Tissue Homogenates and Analysis

Following assessments for BPs and sites, all rats were killed by exsanguination while still under anesthesia. The colon was transected 5 mm proximal and 5 mm distal to the suture line, resulting in a 1-cm strip of anastomotic tissue. Tissue specimens were rinsed in phosphate-buffered saline, immediately frozen in dry ice, and stored at –80°C until time of homogenization.

A 400-mg section of full-thickness colon was homogenized in 2 ml of protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) and centrifuged at 3000 × *g* for 10 minutes at 4°C.

The homogenized samples were quantitatively assayed for β-Gal by use of a commercially available enzyme-linked immunosorbent assay (ELISA) preparation (Boehringer Mannheim). Standards and samples were added in triplicate to microtiter plate modules that contained prebound antibodies to β-Gal, incubated for one hour at room temperature, and rinsed with wash buffer. Each well was then coated with digoxenin-labeled antibody to β-Gal and allowed to incubate for an additional hour before rinsing with wash buffer. Subsequently, the wells were coated with an antibody to digoxenin conjugated to peroxidase and allowed to incubate for another hour. After rinsing with wash buffer, the enhancer solution was added and the color changes in each well were quantified at 405 nm wavelength. Standard curves were generated, and β-Gal concentrations were determined.

For the quantitative assay of TGF-β1, recombinant human TGF-β sRIII/Fc chimera (R&D Systems, Minneapolis, MN) was used as the capture reagent in our ELISA protocol along with a biotinylated TGF-β1 affinity purified polyclonal detection antibody, both cross-reactive with bovine TGF-β1. ELISA plates were coated for 24 hours at 4°C with capture antibody at a concentration of 2 μg/ml of Tris-buffered saline (TBS) containing bovine serum albumin (BSA). The capture antibody was subsequently removed and each well

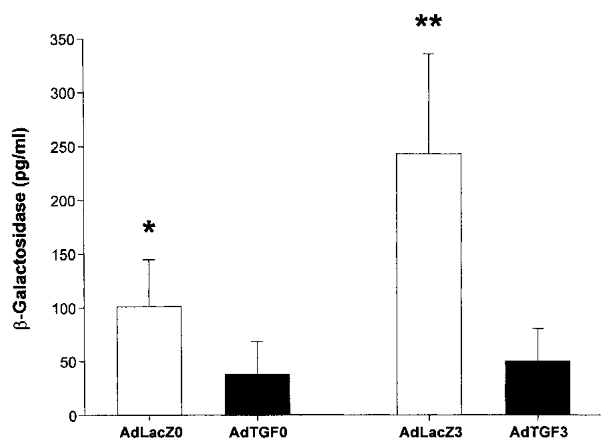

Figure 2. Measured β-galactosidase (pg/ml) levels in the four experimental groups. **P* = 0.01; ***P* = 0.009.

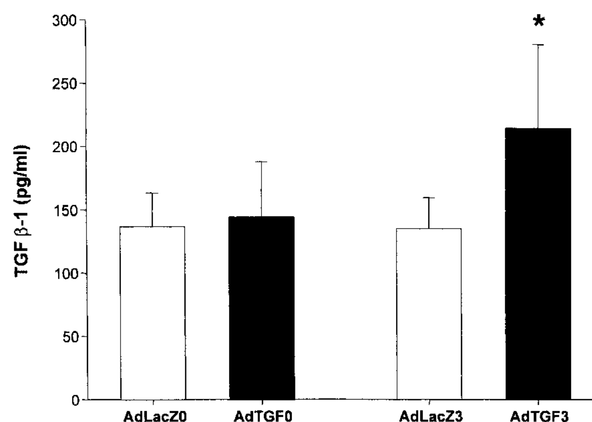

Figure 3. Measured TGF-β1 (pg/ml) levels in the four experimental groups. **P* = 0.02.

blocked for 24 hours with TBS-BSA at 4°C. The ELISA plate was washed with TBS-BSA, and both standards and samples were added to wells in triplicate. After 24 hours the plates were washed and detection antibody was added for three hours. Streptavidin-alkaline phosphatase (1:20,000 dilution) was added to each well for two hours, and each plate was thoroughly washed again. An ELISA amplification system was used to increase the amount of color generated per quantity of immobilized streptavidin alkaline-phosphatase at a wavelength of 450 nm. Standard curves were generated, and TGF-β1 concentrations were determined.

Statistical Analysis

Descriptive statistics were determined on all measured variables by means of Sigma Stat for Windows 2.0 (Jandel Corporation, San Rafel, CA). Comparisons between groups were performed with Student's *t*-test or Fisher's exact test where appropriate.

RESULTS

β-Gal and TGF-β1 Expression in Colonic Tissue Homogenates

β-Gal levels (pg/ml, Fig. 2) from anastomotic tissue harvested on POD 6 were significantly increased in both AdLacZ0 and AdLacZ3 compared with AdTGF0 (101 ± 43 *vs.* 38 ± 30, $P = 0.01$) and AdTGF3 (243 ± 92 *vs.* 50 ± 30, $P = 0.009$), respectively. These results confirmed gene delivery to anastomotic tissue.

TGF-β1 levels in the AdTGF3 group were also higher than those observed for AdLacZ3 (214 ± 66 *vs.* 135 ± 24, $P = 0.02$) (Fig. 3).

Anastomotic Blood Pressure and Bursting Energy

The AdTGF3 group had a significantly higher BP (mmHg, Fig. 4) than its corresponding control AdLacZ3 (160 ± 12 *vs.* 119 ± 16, $P = 0.001$). When the BP of the AdTGF3 group was compared to that of the AdLacZ0, the results approached significance (160 ± 12 *vs.* 136 ± 29, $P = 0.068$). There were no significant differences in the bursting energies within or between groups.

Site of Burst, Adhesions, Anastomotic Dehiscences, and Abscess

Although all animals were observed to have adhesions at the time of sacrifice, no anastomotic dehiscences or abscesses were observed. Each of the AdLacZ0 and AdTGF0 groups demonstrated bursting at the anastomosis in four of six animals. In the AdLacZ3 group, the site of burst was at the anastomosis five of six times. None of the colons (0/6) burst at the anastomotic site in the AdTGF3 group ($P = 0.007$, Fisher's exact test).

DISCUSSION

The role of TGF-β1 in colitis and experimental models of inflammatory bowel disease is well described.[13–17] The role of immunomodulator therapy in

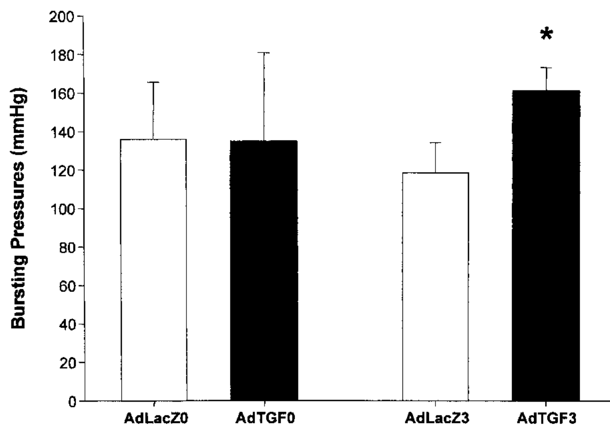

Figure 4. Measured bursting pressure (mmHg) in the four experimental groups. *$P = 0.001$.

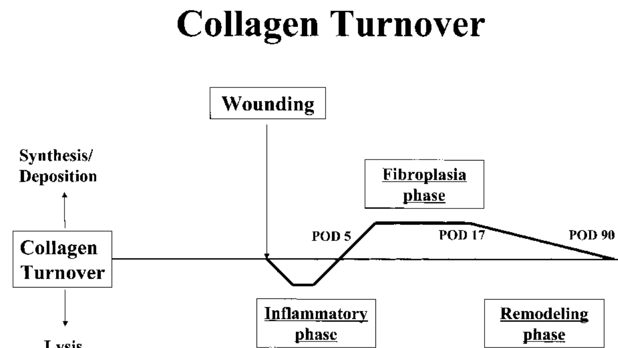

Figure 5. Temporal relationship between collagen deposition and breakdown and the phases of wound healing. POD = postoperative day.

inflammatory bowel disease has proven to be clinically beneficial, but patients need repeated doses secondary to the short half-lives of cytokines.[4,18] Several animal experiments involving vector delivery of immunomodulators, for more meaningful and sustained cytokine delivery, have attempted to address this problem.[19] It is from these experiments that we draw our inspiration. Our purpose was to examine vector-mediated delivery of TGF-β1 as a possible means to improve colonic wound healing.

The healing of a colonic anastomosis proceeds in a stepwise time-dependent fashion (Fig. 5). At the time of transection of the colon, there is an immediate inflammatory response secondary to activation of the clotting cascade, recruitment of platelets, and perpetuation of the inflammatory cascade *via* the elaboration of inflammatory mediators stored in platelet granules. Neutrophils are subsequently recruited into the wound. It is during these first three to five days, termed "the inflammatory phase" of colonic healing, when the collagen matrix undergoes degradation by metalloproteinases. It is in this initial phase that the integrity of the anastomosis depends almost entirely on technical factors and suture materials.

Around the fifth postoperative day there is a crucial switch from collagen degradation to collagen deposition, with TGF-β1 identified in wound healing as being trophic and chemotactic for myofibroblasts. The up-regulation of TGF-β1 is temporally related to the transcription of the procollagen I. The transcription of TGF-β1 increases from the time of wounding through Day 5, and during the crucial switch from the inflammatory phase to the fibroplasia phase reaches its maximal level at Day 7.[2] Any delay or impairment of the fibroplasia phase can result in the potentially catastrophic consequence of anastomotic dehiscence.[3] Indeed, it is at the end of the first postoperative week that anastomotic dehiscences usually occur and become clinically evident.

With this established role of TGF-β1 in the early phases of wound healing, we sought to determine whether exogenous administration of TGF-β1 during early wounding of the colon could positively affect wound healing. Although virtually every cell in the body produces TGF-β1, its release is closely regulated in the wound-healing process. The exogenous administration of such a short-acting peptide growth factor (half-life <90 minutes) would be difficult by direct administration, but adenoviral-mediated delivery of this growth factor could ensure short-term production of TGF-β1 by the transfected colonic epithelium. The choice of the end point of POD 6 approximates the crucial switch from collagen uptake to collagen deposition. Furthermore, it is in this time period that clinically evident anastomotic dehiscences occur. Measurement of BPs permitted the reproducible assessment of wound strength.

The first goal of this study was to demonstrate that the β-galactosidase gene could be successfully delivered to the colon, thus establishing the efficacy of gene transfer *via* viral vector through direct delivery to the colonic mucosa. When compared with the cohorts receiving TGF-β1, the AdLacZ groups were found to have significantly higher levels of β-Gal. The low level of β-Gal detected in the AdTGF groups is most likely due to its production by the endogenous bacteria of the colon itself; the assay used in this study is specific for bacterial β-galactosidase. Immunohistochemical staining or X-Gal staining may have supplied additional information and quantification. A

vector carrying the luciferase gene would also have been another option, but this too can present limitations in quantification.

The second goal of the study was to establish the effect of up-regulating the expression of TGF-β1 *via* exogenous administration of the vector carrying the TGF-β1 gene. The increased levels of TGF-β1 in animals receiving the vector on POD 3 showed that the gene was successfully transferred to the colon. Furthermore, the timing of delivery of TGF-β1 to the colon was shown to be crucial to its effect on the breaking strength of the colonic anastomosis at POD 6. Thus, despite sporadic literature to the contrary, we believe these experiments in combination with previous work by our group support the role of TGF-β1 as a positive stimulant in colonic anastomotic wound healing.[20]

The observation that only the group receiving the vector carrying TGF-β1 on POD 3 had a significant increase in BP is probably related to two factors. First, the natural turnover of colonic epithelia every 24 to 48 hours in combination with the short half-life of TGF-β1 failed to result in demonstrable levels or effect by the sixth postoperative day. Second, maximal expression of vector-delivered genes is usually observed at 24 to 48 hours after delivery. Thus, the timing for our delivery of TGF-β1 on POD 3 may coincide with the shift from collagen uptake to collagen delivery occurring at POD 5.

In animals receiving TGF-β1 at POD 3 there was an unexpected observation that the site of burst was almost exclusively at a site other than the anastomosis. This may reflect the possibility that TGF-β1 accelerated collagen deposition and matrix deposition to the anastomotic site such that the colons of the treatment group resembled a normal colon in strength.

CONCLUSION

Gene transfer into healing colonic anastomosis can be successfully accomplished *via* intraluminal administration of adenoviral vectors. Transfer of TGF-β1 increased the strength of colonic anastomoses when given at Day 3 but not at Day 0, demonstrating its temporal effects in the wound-healing sequence (Fig. 5). Furthermore, despite higher BPs, these colons never ruptured at the anastomotic site. Thus, time-dependent gene transfer of TGF-β1 may avoid the need for a diverting stoma in cases of low rectal surgery and may have an impact on resections of bowel at high risk for anastomotic leakage (e.g., chemotherapy, radiation, diverticulitis, or inflammatory bowel disease).

REFERENCES

1. Buckmire M, Parquet G, Seeburger JL, Fukuchi SG, Rolandelli RH. Effect of bowel preparation and a fiber-free liquid diet on expression of transforming growth factor and procollagen in colonic tissue preoperatively and postoperatively. Dis Colon Rectum 1998;41:1273–80.
2. Buckmire M, Parquet G, Greenway S, Rolandelli RH. Temporal expression of TGF-beta 1, EGF, and PDGF-BB in a model of colonic wound healing. J Surg Res 1998;80:52–7.
3. Fukuchi SG, Seeburger JL, Parquet G, Rolandelli RH. Influence of 5-fluorouracil on colonic healing and expression of transforming growth factor-beta 1. J Surg Res 1999;84:121–6.
4. Wirtz S, Galle PR, Neurath MF. Efficient gene delivery to the inflamed colon by the local administration of recombinant adenoviruses with normal or modified fiber structure. Gut 1999;44:800–8.
5. Trapnell BC, Gorziglia M. Gene therapy using adenoviral vectors. Curr Opin Biotechnol 1994;5:617–25.
6. Cheng DY, Kolls JK, Lei D, Noel RA. In vivo and in vitro gene transfer and expression in rat intestinal epithelial cells by E1-deleted adenoviral vector. Hum Gene Ther 1997;8:755–64.
7. Lozier JN, Yankaskas JR, Ramsey WJ, Chen L, Berschneider H, Morgan RA. Gut epithelial cells as targets for gene therapy of hemophilia. Hum Gene Ther 1997;8:1481–90.
8. Bett AJ, Haddara W, Prevec L, Graham FL. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc Natl Acad Sci USA 1994;91:8802–6.
9. Graham FL, Smiley J, Russel WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 1977;36:59–72.
10. Croyle MA, Anderson DJ, Roessler BJ, Amidon GL. Development of a highly efficient purification process for recombinant adenoviral vectors for oral gene delivery. Pharm Dev Technol 1998;3:365–72.
11. Koruda MJ, Rolandelli RH. Experimental studies on the healing of colonic anastomosis. J Surg Res 1990;48:504–15.
12. Gottrup F. Healing of incisional wounds in stomach and duodenum. Am J Surg 1980;140:296–301.
13. Strober W, Neurath MF. Immunological diseases of the gastrointestinal tract. In: Rich RR, ed. Clinical immunology. St. Louis: Mosby, 1995:1401–28.
14. Elson CO, Sartor RB, Tennyson GS, Riddell RH. Experimental models of inflammatory bowel disease. Gastroenterology 1995;109:1344–67.
15. Strober W, Kelsall BL, Fuss I, Marth T, Ludviksson B,

Ehrhardt R, Neurath M. Reciprocal IFN-γ and TGF-β response regulate the occurrence of mucosal inflammation. Immunol Today 1997;18:61–4.
16. Powrie F. T cells in inflammatory bowel disease: protective in pathogenic roles. Immunity 1995;18:61–4.
17. Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Hapten induced model of colonic inflammation and ulceration in the rat colon. Gastroenterology 1989;96:795–803.
18. Choi PM, Targan SR. Immunomodulator therapy in inflammatory bowel disease. Dig Dis Sci 1994;39:1885–92.
19. Giladi E, Raz E, Karmeli F, Okon E, Rachmilewitz D. Transforming growth factor beta gene therapy ameliorates experimental colitis in rats. Eur J Gastroenterol Hepatol 1995;7:341–7.
20. Fukuda H, Motohiro T, Yamamichi K, Nakane Y, Fujisawa J, Hioki K. Negative effect of transforming growth factor-beta-one on intestinal anastomotic tissue regeneration. Eur Surg Res 2001;33:388–94.