

## Prevention of peritoneal adhesions: A promising role for gene therapy

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### Abstract

Adhesions are the most frequent complication of abdominopelvic surgery, yet the extent of the problem, and its serious consequences, has not been adequately recognized. Adhesions evolved as a life-saving mechanism to limit the spread of intraperitoneal inflammatory conditions. Three different pathophysiological mechanisms can independently trigger adhesion formation. Mesothelial cell injury and loss during operations, tissue hypoxia and inflammation each promotes adhesion formation separately, and potentiate the effect of each other. Studies have repeatedly demonstrated that interruption of a single pathway does not completely prevent adhesion formation. This review summarizes the pathogenesis of adhesion formation and the results of single gene therapy interventions. It explores the promising role of combinatorial gene therapy and vector modifications for the prevention of adhesion formation in order to stimulate new ideas and encourage rapid advancements in this field.

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**Key words:** Peritoneal adhesions; Tissue plasminogen

### INTRODUCTION

Peritoneal adhesions are abnormal deposits of fibrous tissue that occur in the peritoneal cavity as a result of surgery or peritonitis, or their combination. Adhesions occur in more than 90% of patients following abdominal or pelvic surgery<sup>[1-3]</sup>, and are only moderately reduced after laparoscopic surgical procedures compared to open surgery<sup>[4-8]</sup>. Adhesion reformation occurs postoperatively in 85% of patients, regardless of whether the adhesiolysis is performed *via* laparotomy or laparoscopy<sup>[9]</sup>.

Intraperitoneal adhesions are a major source of morbidity, being the commonest cause of intestinal obstruction<sup>[10,11]</sup>, secondary female infertility, and ectopic gestation<sup>[12,13]</sup>. They may also cause chronic abdominal and pelvic pain<sup>[14,15]</sup>. Small bowel obstruction is the most serious consequence of intra-abdominal adhesions. Retrospective studies have shown that 32%-75% of patients who require abdominal re-operation have adhesion-related intestinal obstruction<sup>[16,17]</sup>.

Adhesions result in a large surgical workload and cost to health care systems. An epidemiological study in the United States showed that 282 000 hospital admissions in 1988 were due to adhesion-related disorders, and the cost

of in-patient adhesiolysis was \$1.18 billion<sup>[18]</sup>. In 1994, 1% of all United States admissions involved adhesiolysis treatment, resulting in \$1.33 billion in health care expenditure<sup>[19]</sup>.

Adhesions and their associated complications are of rising medico-legal interest. Physicians worldwide need to be aware of the increasing burden of medico-legal claims arising from the complications of intra-abdominal adhesions. Successful medico-legal claims include cases of bowel perforation after laparoscopic division of adhesions, delays in the diagnosis of adhesion obstruction of the small bowel, infertility as a result of adhesions, and pain<sup>[20]</sup>.

Currently, there is no effective method for preventing adhesion formation or reformation<sup>[21]</sup>. A better understanding of the pathogenesis of adhesion formation at the cellular and molecular level would undoubtedly help to develop more effective treatment strategies<sup>[3]</sup>.

## PATHOGENESIS

### Vicious triad of trauma, hypoxia, and inflammation

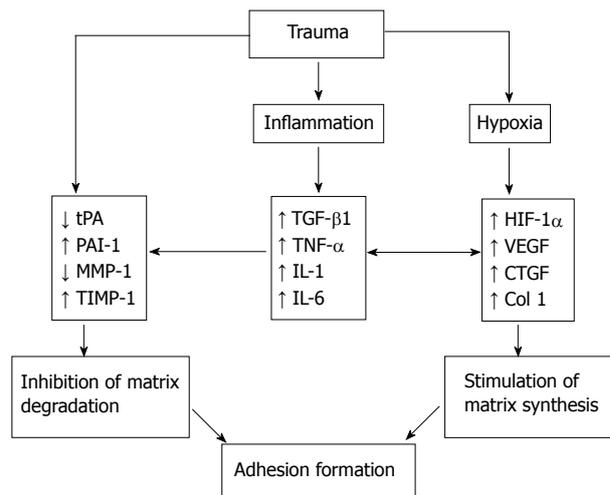
The peritoneum is lined by mesothelial cells loosely attached to the basement membrane, which can readily be detached by the slightest trauma<sup>[22]</sup>. After injury to the peritoneum, a local inflammatory reaction causes increased vascular permeability in blood vessels supplying the damaged area, followed by an exudation of serosanguinous fluid rich in fibrin and inflammatory cells, ultimately leading to the formation of a fibrin matrix. Normally, the plasminogen activator activity (PAA), which resides in the mesothelial cells and submesothelial fibroblasts, degrades the fibrinous mass, resulting in healing of peritoneal surfaces (within three to five days) without adhesions. However, if the level of PAA is diminished, the fibrinous mass persists and the underlying fibroblasts migrate into the fibrinous mass. The fibroblasts then deposit extracellular matrix, including collagen and fibronectin, leading to adhesion formation. Over time, the adhesion may provide the framework for vascular ingrowth, during the process of angiogenesis<sup>[3,23,24]</sup>.

The pathogenesis of adhesions involves three important trauma-induced processes (Figure 1): (1) trauma induces inhibition of the fibrinolytic and extracellular matrix (ECM) degradation systems<sup>[25,26]</sup>; (2) trauma, as well as foreign bodies, incites an inflammatory response with the production of cytokines, mainly transforming growth factor- $\beta$  (TGF- $\beta$ 1), a key regulator of tissue fibrosis<sup>[27-29]</sup>; and (3) trauma also induces tissue hypoxia as a result of interruption of the blood supply to mesothelial cells and submesothelial fibroblasts, leading to increased expression of hypoxia inducible factor-1  $\alpha$  (HIF-1 $\alpha$ )<sup>[30,31]</sup> and vascular endothelial growth factor (VEGF), responsible for collagen formation and angiogenesis<sup>[32]</sup>.

## MOLECULAR CROSSTALK

### Sticky connected pathways

Molecular pathways involved in fibrinolysis inhibition, inflammation, and tissue hypoxia crosstalk and potentiate



**Figure 1** The role of trauma, hypoxia, and inflammation in modulating molecular crosstalk in adhesion formation. tPA: Tissue plasminogen activator; PAI-1: Plasminogen activator inhibitors 1; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitors of MMP; TGF- $\beta$ 1: Transforming growth factor- $\beta$ ; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; IL: Interleukin; HIF-1 $\alpha$ : Hypoxia inducible factor-1 $\alpha$ ; VEGF: Vascular endothelial growth factor; CTGF: Connective tissue growth factor.

the effect of each. The principal molecular aberrations included in this crosstalk are the reduction of tissue plasminogen activator (tPA) and upregulation of TGF- $\beta$ 1 and HIF-1 $\alpha$ .

## INHIBITION OF FIBRINOLYSIS AND MATRIX DEGRADATION

The role of fibrinolysis in adhesion formation/reformation is to breakdown the fibrin clots that are formed during the healing process. The inactive proenzyme, plasminogen, is converted to plasmin by the action of tPA. Plasmin degrades fibrin and thus limits adhesion formation. Experimental and clinical studies have identified the presence of PAA in the mesothelium<sup>[33,34]</sup> and that tPA is the major (95%) physiological mediator of PAA<sup>[35,36]</sup>. Both mechanical and chemical injury reduce peritoneal PAA, with a progressive reduction in PAA in the first hours following a surgical operation, followed by complete loss of fibrinolytic activity up to 72 h after the operation<sup>[37,38]</sup>. Laparoscopic surgery also decreases peritoneal tPA<sup>[39]</sup>. This reduction in PAA is the result of reduced tPA production and increased release of plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2) by mesothelial, endothelial, and inflammatory cells<sup>[3,40]</sup>. Extensive human and animal studies confirmed the central role of altered tPA/PAI-1 balance in adhesion formation and demonstrated that this imbalance is more exaggerated in severe adhesions<sup>[25,26,34,40]</sup>.

Plasmin also activates latent matrix metalloproteinases (MMPs) involved in extracellular matrix (ECM) degradation. The proteolytic activity of MMPs is regulated in part by their physiological inhibitors, tissue inhibitors of MMPs (TIMPs). It has been shown that MMPs and TIMPs are ex-

pressed in the human peritoneum, in adhesion fibroblasts, and in serosal layers of several intraperitoneal organs with and without adhesions<sup>[3,41,42]</sup>. Chegini *et al*<sup>[43]</sup> demonstrated that serosal tissue of intraperitoneal organs obtained during open surgery expresses more tissue inhibitor metalloproteinase-1 (TIMP-1) than matrix metalloproteinase-1 (MMP-1), and that adhesions express elevated levels of TIMP-1 and a lower ratio of MMP-1 to TIMP-1 compared with intact parietal peritoneum. The association between the imbalance of MMP/TIMP production and adhesion formation has been confirmed in another study in women undergoing laparoscopy<sup>[29]</sup>.

## INFLAMMATION AND THE ROLE OF TRANSFORMING GROWTH FACTOR- $\beta$ -1

Several studies have demonstrated that during the acute phase of the inflammatory response, mesothelial cells and peritoneal macrophages produce a variety of cytokines, including TGF- $\beta$ 1, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin-1 (IL-1), and IL-6. These pro-inflammatory cytokines, individually and synergistically, stimulate the production of PAI-1 and reduce the synthesis of tPA by human mesothelial cells (Figure 1)<sup>[3,44-46]</sup>. TGF- $\beta$  not only interacts with the fibrinolytic system and ECM, but also with many other cellular mediators involved in the process of adhesion formation. TGF- $\beta$ 1 overexpression by the peritoneum, as well as increased concentrations of TGF- $\beta$  in the peritoneal fluid, has been associated with increased incidence of adhesion formation in both humans and animals<sup>[3,27,47,48]</sup>.

Several studies demonstrated that increased TGF- $\beta$ 1 is associated with a reduction of tPA and an increase of PAI-1 release<sup>[27,49,50]</sup>, an excess of TGF- $\beta$ 1 leads to an increase in the severity of adhesions formed<sup>[27,51]</sup>, whereas an inhibitory antibody to TGF- $\beta$ 1 decreased adhesion formation<sup>[52]</sup>. TGF- $\beta$ 1 contributes to the synthesis of the ECM by stimulating fibroblastic cell production of collagen and fibronectin<sup>[53,55]</sup>. TGF- $\beta$  also antagonizes ECM resorption by decreasing the activity of MMPs through decreasing MMP-1 and increasing TIMP-1 expression from mesothelial cells<sup>[3,56,57]</sup>. This impairment of MMP activity prevents the ECM deposition that occurs early in wound healing from being adequately remodeled and degraded when necessary, as healing progresses.

## THE ROLE OF HYPOXIA IN ADHESION FORMATION

Several lines of evidence have demonstrated that peritoneal tissue hypoxia plays a key role in adhesion formation<sup>[58-61]</sup>. During laparotomy, tissue injury including trauma, desiccation, and vascular disruption (due to ligatures and other vascular hemostatic methods, including cauterization) reduces oxygen supply to the peritoneum.

Laparoscopic surgery was shown to be less adhesiogenic, not only because it is less traumatic, but also due to elevated peritoneal tissue oxygen tension levels compared to those during laparotomy<sup>[58]</sup>. However, adhesions during laparoscopic surgery increase with duration of pneumoperitoneum and with insufflation pressure. These effects were attributed to desiccation and compression of the capillary flow in the superficial peritoneal layers by the pneumoperitoneum. The addition of oxygen to the insufflation gas decreases adhesion formation<sup>[59]</sup>. Moreover, supplemental perioperative oxygen was found to increase peritoneal tissue oxygen tension and to reduce the severity of adhesions<sup>[61]</sup>.

Hypoxia negatively modulates all pathways involved in adhesion formation. Hypoxia decreases tPA and increases PAI expression in human peritoneal fibroblasts *in vitro*<sup>[62]</sup> and in peritoneal tissues *in vivo*<sup>[61]</sup>, thereby decreasing plasmin, inhibiting lysis of fibrin, and increasing adhesion formation. The *PAI-1* gene contains oxygen responsive promoter sequences, namely hypoxia response element (HRE-1 and HRE-2), to which HIF-1 $\alpha$  binds and induces gene expression<sup>[63]</sup>. Hypoxia was found to increase expression of TIMP-1, but not MMP-1, in both peritoneal and adhesion fibroblasts<sup>[55]</sup>, thus decreasing matrix degradation. Hypoxic conditions in cultured human mesothelial cells and peritoneal fibroblasts also increased the expression of TGF- $\beta$ 1<sup>[55,64,65]</sup>. Hypoxia resulted in increased expression of collagen 1 mRNA in both peritoneal and adhesion fibroblasts<sup>[55,66]</sup>, probably through the production of superoxide<sup>[66]</sup>. Moreover, hypoxia induces proliferation while inhibiting apoptosis in fibroblasts from adhesion, thus favoring adhesion formation<sup>[67]</sup>. Finally, hypoxia increases VEGF production through activation of HIF-1 $\alpha$  in normal and adhesion fibroblasts *in vitro*<sup>[68]</sup> and *in vivo* in human adhesion mesothelial cells<sup>[69]</sup> and in animal adhesion tissues<sup>[30,70]</sup>. VEGF plays a central role in angiogenesis and its role in adhesion blood vessel development has been established<sup>[71]</sup>. Furthermore, TGF- $\beta$ 1 stimulates VEGF and connective tissue growth factor (CTGF) expression<sup>[72]</sup>. CTGF stimulates increased expression of ECM fibronectin, collagen 1, and laminin, while CTGF knockdown inhibits ECM production induced by TGF- $\beta$ 1 in human mesothelial cells<sup>[73]</sup>.

## CURRENT PREVENTION THERAPIES

### Rules of disengagement

Single therapeutic strategies have failed to completely prevent peritoneal adhesions because of the multifactorial nature of adhesion pathogenesis<sup>[74]</sup>. As these multifactorial etiologies act independently and synergistically in adhesion formation, it is imperative to simultaneously address the major molecular aberrations, including reduction of tPA and upregulation of TGF- $\beta$ 1 and HIF-1 $\alpha$ , for any therapeutic strategy to be successful. The current preventive approaches of reducing surgical trauma, use of physical barriers or administration of single pharma-

Table 1 *In vivo* adhesion prevention gene therapy studies

Ref.	Vector/dose	Nucleic acid	Adhesion reduction (%)
Atta <i>et al</i> <sup>[101]</sup>	Adenovirus, 5 × 10 <sup>7</sup> pfu	<i>tPA</i> gene	34
Guo <i>et al</i> <sup>[102]</sup>	Plasmid, 100 µg, sonoporation	<i>Smad7</i> gene	37
Guo <i>et al</i> <sup>[103]</sup>	Adenovirus	<i>Sphingosine kinase-1</i> gene	62
Segura <i>et al</i> <sup>[31]</sup>	Polyethylenimine cationic polymer, 2-4 nmol	siRNA HIF-1α	36 to 52
Liu <i>et al</i> <sup>[104]</sup>	Adenovirus, 1 × 10 <sup>9</sup> pfu	siRNA PAI-1 <i>HGF</i> gene	56

tPA: Tissue plasminogen activator; siRNA: Small interfering RNA; HIF-1α: Hypoxia inducible factor-1α; PAI-1: Plasminogen activator inhibitors 1; HGF: Hepatocyte growth factor.

cological agent or gene therapy have all failed to achieve satisfactory results.

### Surgical precautions

The general surgical precautions aiming at minimizing surgical trauma include meticulous surgical techniques, delicate purposeful tissue handling, achieving optimal hemostasis, minimizing the risk of infection, and avoiding contaminants (e.g., fecal matter) and the use of foreign materials (e.g., talcum powder) when possible<sup>[74,75]</sup>. However, these surgical techniques alone are not effective.

### Physical barriers

Physical barriers work by separating surgically injured tissues during the initial postoperative time period while remesothelization is occurring, a process that is usually expected to take three to five days<sup>[74]</sup>. Currently, three barriers, Interceed® (Johnson and Johnson, Gynecare, Somerville, NJ), Seprafilm® (Genzyme, Cambridge, MA), and ADEPT® (Baxter, Deerfield, IL), are approved by the Food and Drug Administration (FDA) for clinical use in the United States<sup>[74]</sup>. Although barriers have shown some success<sup>[74,76]</sup>, this experience is not universally confirmed<sup>[77]</sup>. In fact, the FDA warns surgeons that when Interceed is used laparoscopically, patients have more adhesions than patients in the control group<sup>[76]</sup>. In the United States, ADEPT is only approved for laparoscopic gynecological surgery, and is contraindicated for patients with infection or allergies to cornstarch, as well as procedures involving laparotomy incision, bowel resection, or appendectomy. If used in these contraindicated procedures, patient may experience dehiscence, cutaneous fistula formation, anastomotic failure, ileus, and/or peritonitis. Thus, application and adoption of this product have been very limited<sup>[76]</sup>. Furthermore, the surgeon must predict the potential sites of adhesion formation in order to determine the placement site and to optimize barrier function<sup>[78]</sup>.

### Molecular therapy

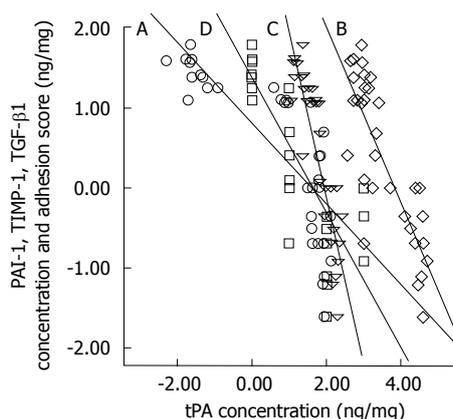
A multitude of pharmacological agents, including recombinant proteins and antibodies, have demonstrated moderate success in reducing adhesion formation in different experimental adhesion models. These agents are applied locally into the peritoneal cavity, and work by correcting aberrant molecular pathways operative dur-

ing adhesion development. One of the most extensively studied pharmacological agents that has demonstrated consistent success is recombinant tPA (reviewed in Ref 92)<sup>[79-92]</sup>. Experimental studies have reported reduction in adhesion formation and reformation using intraperitoneal recombinant human tPA in a variety of delivery methods and preparations, without impairing the healing of bowel anastomosis and without reduction in wound strength or causing hemorrhagic complications<sup>[79,80,92,93]</sup>. The action of tPA is localized to fibrin deposits; therefore, fibrinolytic activity is limited to this site, which prevents indiscriminate fibrinolysis<sup>[90]</sup>. Similar experiences were obtained in studies using neutralizing antibodies for PAI-1<sup>[94]</sup>, TGFβ-1<sup>[45,52,95]</sup>, TNF-α and IL-1<sup>[96]</sup>, IL-6<sup>[97]</sup>, and for VEGF<sup>[98,99]</sup>. However, these agents have short half-lives (few minutes) limiting their fibrinolytic effect for a sufficient duration of time (three to five days) until complete healing of peritoneal surfaces<sup>[92,100]</sup>.

### Gene therapy

Local molecular therapy is inherently limited; therefore, an alternative strategy using gene therapy has been recently employed to correct molecular aberrations induced by surgical trauma in a regulated manner during the period of remesothelialization. Postoperative peritoneal adhesion is an attractive target for gene therapy because of several inherent biological features. The disease is localized to the site of peritoneal trauma and develops over a short period of time, extending for the first few days following surgical trauma. These characteristics lend themselves perfectly to gene therapy using non-integrating vectors. The vector can be applied locally following completion of the operation, and the short duration of gene expression would cover the period of altered molecular aberrations (e.g., depressed tPA, elevated PAI-1, TGF-β1, HIF-1α, *etc.*) following surgery. Nevertheless, gene therapy for peritoneal adhesions is still in its infancy, with very few *in vivo* studies reported in the literature (Table 1).

Using different vectors, the five gene therapy studies reported in the literature were able to express therapeutic nucleic acids (transgenes or small interfering RNA) in the peritoneal tissues after intra-peritoneal administration in a rat adhesion model for at least seven days post-administration<sup>[31,101-104]</sup>. This duration of expression is enough



**Figure 2** Correlation analysis was performed using two-tailed Spearman rank correlation test between tPA with respect to PAI-1 (line A, marker ○), TIMP-1 (line B, marker ◇), TGF-β1 (line C, marker ▽) or adhesion score (line D, marker □). Values of tPA, PAI-1, TIMP-1, and TGF-β1 concentration in adhesion tissues were logarithmically transformed to obtain a normal distribution before statistical analysis. There was significant negative correlations ( $P < 0.01$ ) between human tPA and either of PAI-1 (Spearman rank correlation coefficient,  $r = -0.89$ ), TIMP-1 ( $r = -0.73$ ), TGF-β1 ( $r = -0.87$ ), or adhesion score ( $r = -0.87$ ). tPA: Tissue plasminogen activator; PAI-1: Plasminogen activator inhibitors 1; TIMP: Tissue inhibitors of MMP; TGF-β1: Transforming growth factor-β.

to cover the time required for complete healing of the mesothelial cell layer of the peritoneum. The mechanism of adhesion reduction differed among these studies. Two studies showed that an adenovirus encoding the genes of hepatocyte growth factor (HGF) itself or its downstream signaling molecule sphingosine kinase 1 (SK-1) could achieve adhesion reduction *via* a stimulatory effect on proliferation and migration of mesothelial cells<sup>[103,104]</sup>. The altered tPA/PAI-1 balance occupies a central role in adhesion formation and two studies tackled this molecular imbalance. Atta *et al*<sup>[101]</sup> used an adenovirus vector encoding human tPA, while Segura *et al*<sup>[31]</sup> employed a cationic polymer containing siRNAs to PAI-1 and HIF-1 $\alpha$  to downregulate PAI-1, either directly or through its gene inducer, HIF-1 $\alpha$ . The expression of TGF-β1 was attenuated by overexpressing its downstream Smad2/3 natural inhibitor Smad7 using plasmid vector<sup>[102]</sup>.

The moderate success in adhesion reduction in these gene therapy studies supports the concept that the multifactorial nature of molecular aberration during adhesion formation should be collectively and simultaneously addressed for any therapeutic strategy to be effective. This concept does not contradict, but reinforces, the established hypothesis of the central role of tPA reduction following surgical trauma in adhesion formation. In a recent study of adhesion prevention from our laboratory using an adenovirus vector encoding human tPA, we verified that overexpression of human tPA resulted in abrogation of the elevated fibrogenic molecules PAI-1, TIMP-1, and TGF-β1. The study showed that the reduction of these molecules depends on the concentration of the expressed human tPA protein<sup>[101]</sup>. Further analysis showed that there are significant negative correlations (at 0.01 level, 2-tailed) between human tPA and either of PAI-1 (Spearman's  $r =$

$-0.89$ ), TIMP-1 ( $r = -0.73$ ), TGF-β1 ( $r = -0.87$ ), or adhesion score ( $r = -0.87$ ) (Figure 2).

### Prospects

Gene therapy for the prevention of peritoneal adhesions has not been fully explored. Two potential developments for safe and effective gene therapy studies for adhesion prevention include combinatorial gene therapy and vector modifications.

## COMBINATORIAL GENE THERAPY

The multifactorial nature of adhesion formation proposes that a combinatorial gene therapy would be more efficacious than a single gene therapy approach. For example, this could include overexpression of the fibrinolytic *tPA* gene together with downregulation of fibrogenic genes, such as TGF-β1 and/or HIF-1 $\alpha$ . Overexpression is achieved through delivery of an exogenous gene, while downregulation is accomplished by the delivery of small interfering RNA (siRNA) molecules. Upon delivery, siRNAs complement with specific mRNAs resulting in their degradation, thus enabling the specific silencing of a single gene at the cellular level. As discussed above, single gene overexpression or silencing was moderately successful in reducing experimental adhesions<sup>[31,101]</sup>. The synergistic effects from the combined fibrinolysis stimulation and fibrogenesis inhibition, however, remain to be confirmed.

## SAFE AND EFFICIENT VECTORS

### Viral vectors

Delivery of therapeutic nucleic acid molecules to target tissues is accomplished using either viral vectors or non-viral carrier systems. Replication-deficient recombinant adenovirus vectors have become the most widely used viral vectors for *in vivo* gene transfer<sup>[101]</sup>. Adenovirus vectors have many positive attributes, including their ability to provide efficient *in vivo* gene transfer to both dividing and non-dividing cells, their high *in vivo* stability, and their non-integrating nature into the host genome. These merits make adenoviral vectors suitable for proof-of-principle experimental studies. However, the clinical application of virus-mediated gene delivery *in vivo* is hampered by virus-induced acute inflammation, which could be fatal, high immunogenicity, and low tissue specificity. The broad tropism of adenovirus allows the virus to infect many cell types and is responsible for virus dissemination to distant organs. Various modifications of adenoviral vectors are underway to enhance the targeting of adenoviral vectors towards adhesion fibroblasts, which will provide effective and safe methods for localized treatment of postoperative peritoneal adhesions.

### Nonviral vectors

Given the unresolved safety limitations of viral vectors, significant research efforts have been directed towards

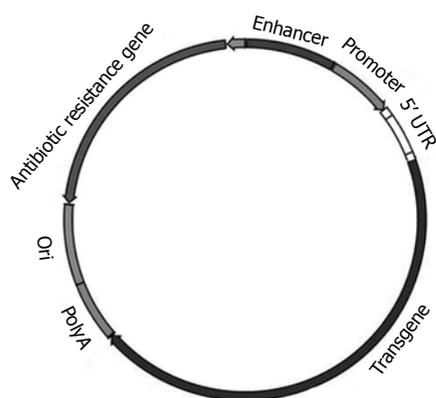


Figure 3 Skeleton of a conventional plasmid. Ori: Origin of replication.

the development of non-viral (plasmid-based) delivery systems. Plasmids are extrachromosomal genetic elements able to replicate autonomously and to be maintained in a host cell. Plasmids, the most basic forms of non-viral gene therapy, carry two main units: a eukaryotic transcription cassette and the bacterial amplification unit (Figure 3). The first bears genetic elements intended for gene expression in mammalian (eukaryotic) cells, such as the enhancer/promoter sequences for gene expression; 5' untranslated region (5' UTR), the gene of interest and polyadenylation (polyA) sequence. The bacterial amplification unit commonly contains an origin for plasmid DNA replication (ori) in bacteria and, generally, an antibiotic resistance selection marker<sup>[105]</sup>. Plasmids do not enter cells efficiently because of their large size, hydrophilic nature (due to negatively charged phosphate groups), and their susceptibility to nuclease-mediated degradation. Plasmid DNA and siRNA are stable for only 0.5 and 2 h, respectively in human serum<sup>[106]</sup>. Plasmids can be delivered to cells either naked (carrier-free) by direct injection, electroporation, ultrasound *etc.*, or complexed with cationic lipids (lipoplexes), cationic polymers (polyplexes), peptides or inorganic nanoparticles<sup>[107]</sup>. Two promising recent modifications of plasmid vectors, minicircles and CpG-depleted vectors, are briefly discussed below. Evaluating the rapid progress in the field of cationic liposomes and polymers is, however, beyond the scope of this brief review, and the reader is referred to excellent recent reviews<sup>[107,108]</sup>.

### Minicircle vectors

Limitations of conventional DNA plasmid vectors are related to their size (> 3 kb). Moreover, bacterial sequences contain immunotoxic cytidine-phosphate-guanosine (CpG) dinucleotides motifs, which are approximately four times more prevalent in bacterial than mammalian DNA. Bacterial CpG dinucleotides have been identified to be major contributors to the low and short-lived transgene expression (transgene silencing) in vertebrates after non-viral gene delivery. These bacterial sequences can also interfere with short hairpin RNA (shRNA, precursor of siRNA) expression<sup>[106,109]</sup>. To overcome these limita-

tions, highly safe and efficient vector systems for gene transfer in eukaryotic cells called minivectors (minicircles) were developed<sup>[110]</sup>. Minicircles are supercoiled minimal expression cassettes, derived from conventional plasmid DNA by site-specific recombination *in vivo* in *Escherichia coli*. As a result, two well-defined circular molecules are generated from the parent conventional plasmid, termed minicircle (mammalian expression cassette) and miniplasmid (bacterial backbone elements). Further purification of the minicircle renders it therapeutically applicable<sup>[105]</sup>. Thus, minicircle DNA lacks the bacterial backbone sequence consisting of an antibiotic resistance gene and an origin of replication. Minicircle DNA is low in immunogenicity due to its lower content of bacterial unmethylated CpG dinucleotides. In addition to their improved safety profile, minicircles have been shown to greatly increase the efficiency of transgene expression in various *in vitro* and *in vivo* studies, compared to the conventional plasmid with the same transgene expression cassette. It has been reported that a minivector incorporating short hairpin RNA efficiently transfected adhesion fibroblasts and was shown to be stable in human serum for > 48 h<sup>[107]</sup>.

### CpG-depleted vectors

Bacterial DNA is rich in unmethylated CpG dinucleotides, in contrast to mammalian DNA, which contains a low frequency of CpG dinucleotide, which are mostly methylated. Recognition of unmethylated CpGs present in the bacterial backbone could trigger an innate immune response following detection in the endosome by toll-like receptor 9 (TLR9)<sup>[109]</sup> and initiate a signaling cascade, leading to the production of proinflammatory cytokines. As plasmids used in *in vivo* gene therapy studies are produced in *Escherichia coli* (*E. coli*), their CpGs are unmethylated and induce immune responses through this host defense mechanism. Recently, plasmids that are completely devoid of CpG dinucleotides have been developed. These plasmids yield high levels of transgene expression both *in vitro* and *in vivo*, and, in contrast to CMV-based plasmids, allow sustained expression *in vivo*<sup>[111,112]</sup>. In these CpG-free plasmids, all elements required for replication and selection of the plasmid in *E. coli* and for gene expression in mammalian cells (e.g., promoter, polyadenylation signal, reporter gene, *etc.*) either naturally lack CpG dinucleotides, were modified to remove all CpGs, or are entirely synthesized.

## CONCLUSION

Gene therapy for the prevention of postoperative peritoneal adhesions is still in its infancy. The potential applications of this strategy have not been fully explored. The recent explosive progress in advanced nonviral gene delivery systems, coupled with the newly developed less immunogenic and more efficient expression plasmids, will undoubtedly accelerate research studies in gene therapy for peritoneal adhesions.

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