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Gene Therapy for Tissue Regeneration

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Abstract Tissue repair and regeneration are the normal biological responses of many different tissues in the body to injury. During the healing process, profound changes occur in cell composition and extracellular matrix (ECM) formation. Fibroblasts and equivalent reparative cells migrate to the wounded area and subsequently proliferate. These cells and reparative cells from the surrounding tissue are responsible for the rapid repair which results in tissue regeneration. Growth factors, one of which is transforming growth factor-β (TGF-β), stimulate fibroblasts and smooth muscle cells to proliferate and synthesize ECM proteins. This process of early repair provides a rapid way to restore new tissue and mechanical integrity. This early tissue repair process is normally followed by involution, which requires the production and activation of proteases, tissue maturation and remodeling, reorganization and finally regeneration. Alternately, failure to replace the critical components of the ECM, including elastin and basement membrane, results in abnormal regeneration of the epithelial cell layer. Although remodeling should occur during healing, provisional repair may be followed by excessive synthesis and deposition of collagen, which results in irreversible fibrosis and scarring. This excessive fibrosis which occurs in aberrant healing is at least in part mediated by persistent TGF-β. Because of the central role of collagen in the wound healing process, the pharmacological control of collagen synthesis has been of paramount importance as a possible way to abrogate aberrant healing and prevent irreversible fibrosis. Fibrosis is an abnormal response to tissue injury. J. Cell. Biochem. 88: 418–425, 2003. © 2002 Wiley-Liss, Inc.

Key words: gene therapy; tissue regeneration; complimentary DNA; extracellular matrix; sense phosphorothioate oligodeoxynucleotides; antisense; oligodeoxynucleotide; β-galactosidase (LacZ); plasminogen activator inhibitor (PAI-1); platelet derived growth factor; transforming growth factor-β; vascular endothelial growth factor; fibroblast growth factor

GENE THERAPY FOR REGENERATION

The roles of growth factors in the modulation of tissue repair have been at least in part elucidated. Recent advances in molecular biology and genetics have resulted in techniques for the uptake and expression of growth factor genes in somatic cells. Gene therapy is defined as the transfer of genetic material to achieve the therapeutic effect of tissue repair. The focus of gene therapy is to place therapeutic agents at the injury site at safe levels to avoid toxicity, yet have the desired therapeutic effect. Gene therapy largely involves the transfer of genes encoding growth factors since these agents regulate cell proliferation, cell migration, and the accumulation of extracellular matrix (ECM) components. Several different approaches have been used to transfer growth factor genes into cells and tissues. In the future, tissue specific promoters will aid in localizing the production of factors to certain cell types.

Several different tissue repair models have been used to evaluate the effects of gene therapy. Direct injection of a HVJ-liposome preparation containing the PDGF-B cDNA into injured rat ligament resulted in increased collagen deposition and angiogenesis [Nakamura et al., 1998]. Plasminogen-deficient mice show impaired wound healing and inhibition of arterial neointima formation after arterial injury. Intravenous injection of an adenovirus expressing human plasminogen activator inhibitor (PAI-1) produced an increase in plasma PAI-1 and a suppression of luminal stenosis [Carmeliet et al., 1997].

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Success has also been shown with transforming growth factor-β (TGF-β) and LacZ gene transfer in osteoblasts in vitro and osseous tissue in vivo by using the adenoviral technique of gene transfer [Mehrara et al., 1999]. Direct delivery of the TGF-β transgene to osseous tissue resulted in significant changes in the epiphyseal plate. The adenoviral vector method has been used for the healing of chronic wounds [Liechty et al., 1999]. In another study, collagen matrices were shown to retain both the PDGF-B-encoding adenovirus and transgene products within the delivery site, enabling the transduction and stimulation of infiltrating reparative cells [Doukas et al., 2001]. These studies demonstrated that immobilizing matrices enable the controlled delivery and activity of tissue regenerating genes for injured tissues. In still another study, matrix-mediated delivery of the adenoviral vector encoding PDGF-B in combination with FGF2 targeting overcomes some of the safety and efficacy limitations of current gene therapy strategies and is an attractive approach for tissue repair and regeneration [Chandler et al., 2000]. Adenoviral vectors may also be incorporated successfully into a synthetic calcium phosphate bone mineral substitute to provide effective, sustained local gene delivery [Kirschner et al., 2001]. Ischemic excisional wounds treated with the adenovirus containing the PDGF-B gene healed more rapidly than non-ischemic excisional wounds treated with vehicle. In this model, the adverse effect of adenovirus containing the LacZ gene on wound healing was overcome by the over-expression of PDGF-B. Despite an increased acute inflammatory response after adenovirus injection, no difference in the healing of wounded skin was observed, suggesting that adenovirus-mediated gene transfer for growth factor-mediated acceleration of tissue regeneration is a sound technique [Sylvester et al., 2000]. Administration of adenovirus containing the myogenic determination gene into cardiac repair granulation tissue of healing hearts resulted in the expression of both myogenin and skeletal myosin heavy chains [Murry et al., 1996]. Direct administration of adenovirus containing the focal adhesion kinase gene to tendons resulted in tendon adhesion formation [Lou et al., 1997]. Delayed wound closure in nitric oxide synthetase knockout mice was completely reversed by a single application of an adenovirus containing the human nitric oxide synthetase cDNA [Yamasaki et al., 1998]. Adenoviral-mediated ex vivo gene transfer of human VEGF resulted in enhanced angiogenesis in a rabbit larynotracheal reconstruction model [Samadi et al., 2002]. However, no statistical improvement was observed in graft survival.

The supernatants of cells infected with recombinant vaccina virus containing the human PDGF-B cDNA produced a marked stimulation of 3T3 cell proliferation [Norton et al., 1996]. Human bone morphogenic protein cDNA was cloned into a retroviral plasmid vector and used to transfect periosteal cells [Breitbart et al., 1999]. The cells were then superimposed on matrices and successfully used to repair rabbit cranium.

The success of gene therapy to accelerate normal wound healing depends on gene dosage for the amount of factor at the wound site to be enough to elicit the desired therapeutic effect and not cause adverse effects. For example, transient TGF-β causes normal wound healing, yet persistent TGF-β results in excessive ECM component accumulation and aberrant tissue repair. The continuous production of endogenous TGF-β by the TGF-β autoregulatory pathway in cells may lead to a greater ECM deposition at the wound site.

Local growth factor gene therapy for tissue repair and regeneration offers a rationale approach. Following plasmid transfer, the growth factor is expressed by the cells in the wounded area. Plasmid diffusion from the wounded site will not cause systemic toxicity due to DNA degradation in the blood stream.

The amount of growth factor under regulation in gene therapy should be quantified. Factor levels can be assessed in the plasma only for well vascularized healing tissues. In the future, it is imperative to establish methodology to monitor gene dosage. The amount of the growth factor at the wound site is not only dependent on gene dosage. Degradation of any factor involved in the wound healing process also determines the concentration of the factor. The adverse effect(s) of growth factors during gene therapy also should be addressed. Thus far the majority of studies using gene transfer to accelerate wound healing have used a single treatment. What would the effect on growth factor levels be with repetitive doses of genes transferred by liposomes, viruses, or by DNA particle bombardment? Would these types of treatment result in growth factor toxicity?

SENSE OLIGODEOXYNUCLEOTIDE THERAPY

TGF-β is a pleiotrophic growth factor which is synthesized by many cells in the body. The TGF-βs are a family of potent cytokines with diverse effects on proliferation, differentiation, extracellular matrix proteins, gene expression, and other aspects of cellular phenotype. Evidence in the literature indicates that TGF-β plays an essential role in the production of fibrosis in various tissues by the induction of ECM protein accumulation. TGF-β is secreted by most cultured cells in the inactive form and exists as multiple isoforms, TGF-β 1, 2, and 3 which are structurally closely related to one another. Following either secretion from cells or release from platelets during tissue injury, the inactive polypeptide is converted into the active polypeptide by plasmin. All three isoforms stimulate collagen synthesis in cell culture but are differentially expressed during fibrosis [Coker et al., 1997]. This growth factor is chemotaxic for fibroblasts and stimulates fibroblast proliferation.

The TGF-β activator protein complex is a trans-acting factor which binds to the TGF-β element in the distal promoter of the proα1(I) collagen gene and induces transcription of this gene. Although transient TGF-β results in repair and regeneration of tissues, persistent TGF-β affects fibrosis and ultimately scarring of both skin and internal organs. Scarring of internal organ (e.g., liver and lung) results in a loss of function and ultimately death. Phosphorothioate oligodeoxynucleotide double-stranded oligos containing the TGF-β element would decrease procollagen gene expression, procollagen synthesis, and collagen content during aberrant wound healing. The rationale is that the double-stranded oligo decoys containing the TGF-β element binds the TGF-β activation protein complex [Cutroneo, 2000] preventing the latter from binding to the TGF-β element in the 5′ flanking region of the proα1(I) collagen gene resulting in transcription inhibition (Fig. 1). This article will in part focus on aspects involved in TGF-β induced fibrosis that occur during aberrant tissue regeneration and the use of these double-stranded TGF-β element containing oligo decoys to control excessive collagen synthesis and deposition resulting from persistent TGF-β. In our model of regulation of collagen synthesis, these double-stranded oligo decoys act as promoter competitors, binding to the TGF-β activator protein complex either in the cytoplasm or in the nucleus. The significance of the studies is that these novel antifibrotic will mimic the effect of glucocorticoids on collagen synthesis without the untoward side effects of these steroids. Based on our previous studies on the molecular mechanisms by which dexamethasone decreases collagen synthesis, we have designed phosphorothioate oligodeoxynucleotides which will mimic the effects of dexamethasone at the molecular, cellular and in vivo levels of collagen synthesis.

The consensus TGF-β element (TGCCCACGGCCAG) located in the distal 5′ flanking region of the rat proα1(I) collagen gene has recently been shown to be required for the basal promoter activity of this gene. We have shown that site-directed mutation of the TGF-β element resulted in almost complete abolishment of the basal promoter activity of the fibroblasts transfected with the 3.6 ColCat plasmid which contains a portion of the 5′ flanking region of the rat proα1(I) collagen gene linked to the reporter gene, chloramphenicol acetyltransferase [Meisler et al., 1999], thus indicating the absolute importance of this *cis*-element in the transcription of the proα1(I) collagen gene.

A 27 mer sense single-stranded phosphorothioate oligodeoxynucleotide (ssPT) decreased endogenous TGF-β-induced collagen synthesis and granuloma growth in the sponge-induced granuloma model of aberrant wound healing [Cutroneo and Chiu, 2000] and the TGF-β-induced proα1(I) collagen gene transcription and collagen synthesis in granulation tissue fibroblasts in culture [Meisler et al., 1999]. The 27 mer sense wild type phosphorothioate oligodeoxynucleotide used had the following sequence: 5′-AGCCTAAC<u>TGCCCACGGCCAG</u>CGACGT-3′ where the underlined section is the TGF-β element. After transfection into cells, the ssPT compete with the TGF-β response element for binding to the TGF-β activator protein complex and prevent this activator protein from associating with the TGF-β response element in the natural gene, thereby inhibiting procollagen gene expression, collagen synthesis, and aberrant tissue regeneration. TGF-β reacts with receptors on the cell membrane resulting in signals to the nucleus causing transcription of specific genes, amongst which is that for the proα1(I) collagen gene. This sequence of events involves the TGF-β activator protein complex

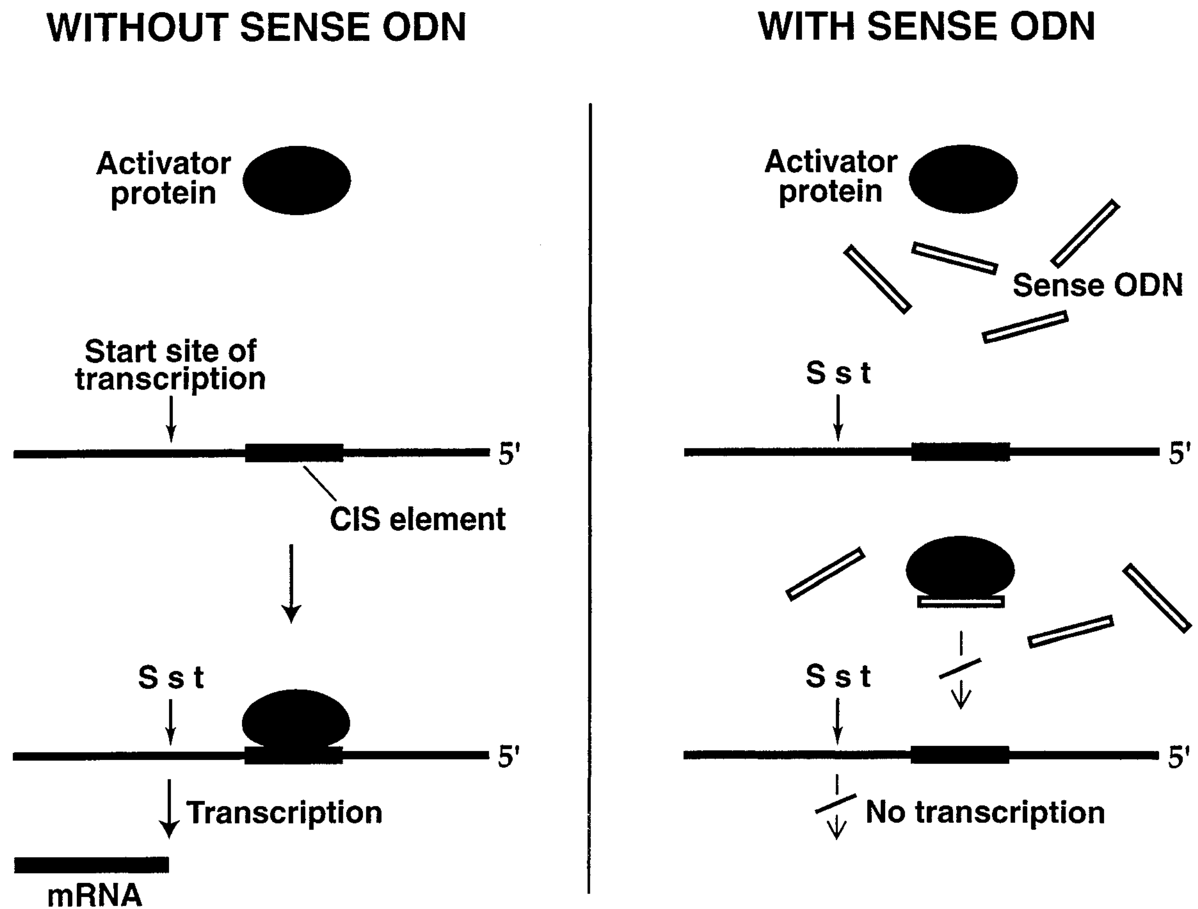

Fig. 1. Schematic representation of how sense oligodeoxynucleotides (ODN) inhibit gene transcription.

as a trans-acting factor, binding to the TGF-β regulatory element in the 5′ flanking region, thereby inducing the transcription of the proα1(I) collagen gene. The ssPTs act as traps for the TGF-β activator proteins in fibroblasts. However, double-stranded oligo decoys containing the TGF-β element (dsPTs) were shown to be more effective than ssPTs in inhibiting the binding of the TGF-β activator protein to ^{32}P-labelled double-stranded oligodeoxynucleotides containing the TGF-β element [Cutroneo and Boros, 2002]. In addition, functional studies using primary fibroblasts isolated from granulomas of schistosomiasis-injured livers indicated that the dsPTs were much more effective than ssPTs in inhibiting collagen synthesis [Cutroneo and Boros, 2002]. Neither ssPTs nor dsPTs inhibited noncollagen protein synthesis, thus ruling out a toxic effect of these decoy oligos in primary fibroblasts isolated from the granulomas of schistosomiasis-injured livers. In collaboration with Dr. Dov L. Boros, we have also demonstrated that direct intrahepatic injection of dsPTs totally abolished collagen synthesis while not effecting noncollagen protein synthesis in schistosomiasis-induced liver granulomas (unpublished data). In other studies after intrahepatic injection of these oligo decoys, gene expression of proα1(I) in liver granulomas as determined by real time RT-PCR was dramatically decreased (unpublished data).

Double-stranded oligodeoxynucleotides provide a therapeutic strategy to regulate gene transcription. This therapy provides precise and effective modification of specific gene expression. One of the principle problems with oligodeoxynucleotide therapy relates to nuclease sensitivity. Unmodified phosphorodiesters [Wickstrom, 1986] have decreased stability within cells which can reduce their potency and shorten the duration of action of these agents. The reduced stability of oligodeoxynucleotides has been largely overcome by the use of backbone modified oligodeoxynucleotides that are

more resistant to nucleases. Phosphorothioates are more resistant to nucleases. Phosphorothioates are more resistant to enzymatic digestion than the corresponding natural oligodeoxynucleotides [Wickstrom, 1992; Letsinger, 1993].These modified oligodeoxynucleotides have a sulfur in place of one of the non-bridging oxygens in the phosphodiester group of DNA. All toxicities associated with phosphorothioate nucleotides were reversible and occurred at doses well above those currently used in clinical trials. In addition, there has been no evidence of genetic toxicity and no changes in reproductive performance, fertility, or fetal development [Henry et al., 1999].

Our studies characterize double-stranded oligo decoys which do not possess many of the untoward side effects of other drug treatments to inhibit collagen synthesis and inhibit wounding fibrosis. In addition, these studies are a direct demonstration of the role of TGF-β in fibrosis and will provide a very important strategy for evaluating the role(s) of TGF-β in development, normal remodeling, and diseases. Furthermore, our studies are the first to establish oligodeoxynucleotides as potential therapeutic agents in tissue regeneration.

ANTISENSE THERAPY

Antisense technology has as its basis the selective impairment of protein synthesis in the cytoplasm through the use of antisense oligodeoxynucleotide (ODN) sequences. A small antisense DNA sequence to the initiating AUG codon and slightly beyond hybridize with the 5′ end of the mRNA, causing translation arrest (Fig. 2). The therapeutic application of antisense has resulted in advances in medicine. However, this potential therapeutic approach is dependent upon the degree of antisense stability, the rate of cellular uptake and accumulation of the antisense molecules, the selection of the proper target gene, the absence of effects

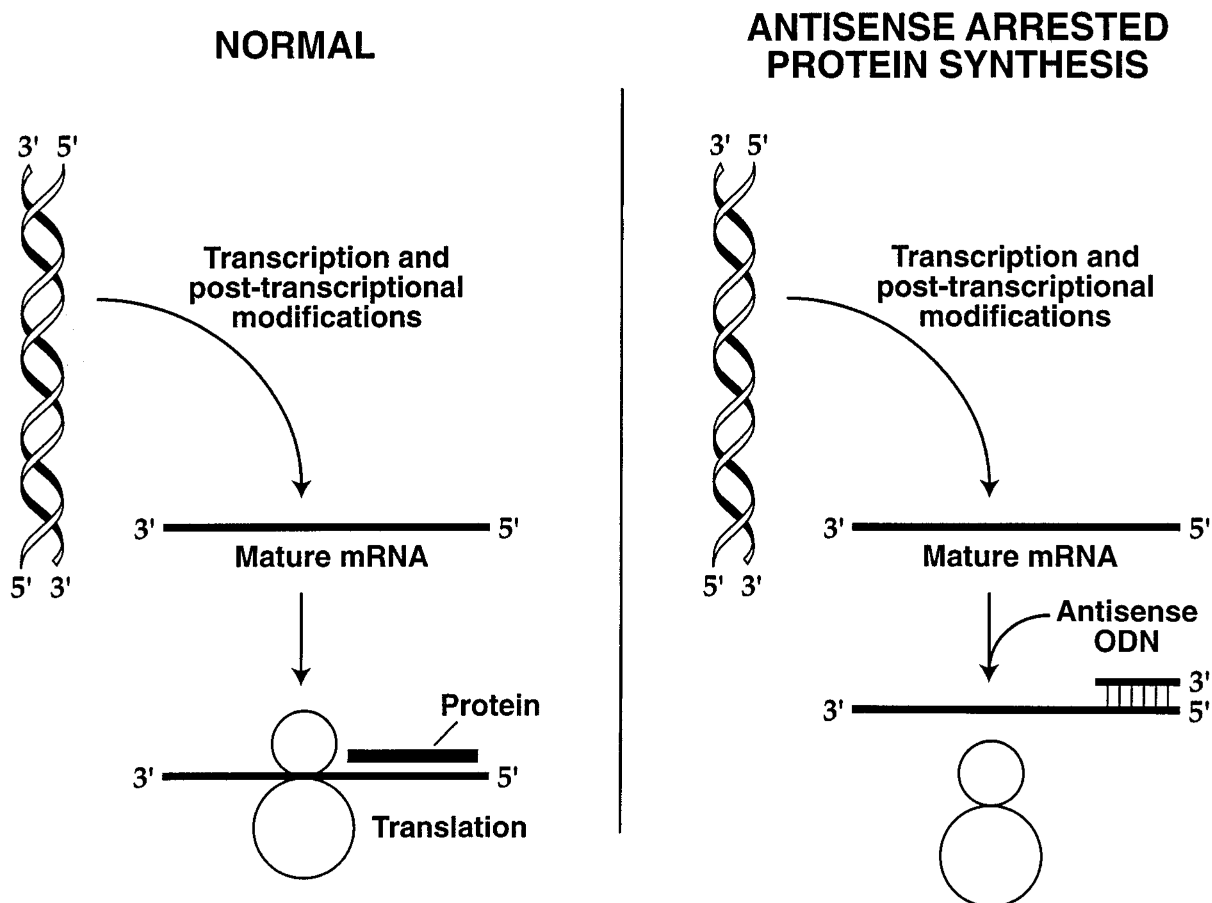

Fig. 2. Schematic representation of how antisense ODN arrest protein synthesis.

on nontargeted genes, minimal toxicity of the antisense ODN, structure and length of the antisense ODN, type and duration of treatment. Antisense ODN may be incorporated into matrices, gels, liposomes, or modified liposomes to target certain tissues or cells for delivery and release over a prolonged period of time. The specificity of antisense is great since targeted mRNA can discriminate between antisense ODN sequences that differ by one or two bases [Wang et al., 1985; Holt et al., 1988; Bennett et al., 1994].

Several model systems have been used to show the effects of antisense ODNs. To determine the most efficient method to treat ligament scars, fluorescent-labeled PTs in HVJ-conjugated liposomes were used to directly treat the scar, underwent systematic direct injection or injection into the femoral artery[Nakamura et al., 1998b]. The intermittent systematic direct injection was the most efficient and was used to deliver antisense ODN for rabbit decorin into ligament scars which resulted in decreased expression of decorin mRNA and protein as compared to sense PT-treated scars. However, in another study while mRNA and decorin protein were decreased by decorin-specific antisense, the mRNA levels for multiple genes were altered which leads one to the conclusion that the observed improvements in scar tissue cannot be directly related to decreasing decorin levels [Hart et al., 2000]. The treatment of human umbilical vein endothelial cells with antisense ODNs targeting endothelial nitric oxide synthetase showed an absolute requirement of this enzyme in endothelin-induced endothelial cell migration [Noir et al., 1997]. Thrombospondin 1 is a glycoprotein involved in tissue repair. Antisense thrombospondin 1 ODN treated wounds contained a marked decrease of thrombospondin positive macrophages with a delay in tissue repair as indicated by a decreased rate of reepithelization and a delay in dermal reorganization [DiPietro et al., 1996]. Protein kinase C alpha has been shown by the use of antisense ODN to be a key player in rabbit corneal tissue regeneration [Chandrasekher et al., 1998]. Protein kinase C alpha antisense ODNs produced a significant inhibition of wound closure in cultured corneas. Topical application of PTs targeted to TGF-β mRNA in skin wounds of mice resulted in a marked reduction of fibrosis associated with decreased expression of the TGF-β gene [Choi et al., 1996]. C-myc antisense treatment has been shown to produce a sustained reduction in neointimal formation and favorable graft remodeling [Mannion et al., 1998]. In addition, an oligomer to c-myc can reduce cell proliferation in regenerating rat liver [Arora et al., 2000]. A single-bolus luminal delivery of antisense PDGFR-B to injured rat carotids reduced intimal hyperplasia, improved the reendothelialization process and led to recovery of endothelium-dependent regulation of vascular tone [Noiseux et al., 2000].

CONCLUSION

Recent advances in the areas of genetics and molecular biology have made it possible to program cells to synthesize growth factors on site to accelerate the tissue repair process. The primary goal for growth factor gene therapy is to have cells express the factors at low abundance yet at an effective and nontoxic dose. Gene therapy for targeted growth factor delivery is used to manipulate normal tissue repair. Various experimental models have been used to evaluate the usefulness and efficacy of this method. The potential major disadvantage of this form of therapy lies with the over-expression of growth factor with a small change in gene dosage.

An oligonucleotide approach to alter the wound repair process is the use of antisense and sense ODNs. Antisense ODNs targeted at specific gene products are complimentary and hybridize to the 5′ end of the mRNA causing translational arrest. Antisense ODNs targeted against specific genes have been used to determine proteins involved in the tissue repair process and to affect normal repair. The disadvantages of antisense therapy are presented in detail in the text.

A novel and more direct approach to inhibit the progression of aberrant tissue repair has been developed. Collagen synthesis is a key player in the fibrotic response. In the future, this sense ODN form of therapy will have numerous applications to regulate aberrant tissue regeneration. This approach may be useful to control the effects of growth factor over-expression if a narrow window exists between the beneficial therapeutic effect and the adverse effect of growth factor gene therapy which may lead to aberrant tissue repair and regeneration.

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