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The Nucleic Acid Scavenger Dendrimer Polyamidoamine Third-Generation Dendrimer Inhibits Fibroblast Activation and Inhibits Granulation Tissue Contraction

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Abstract

Background—Pathologic cutaneous scarring affects over 40 million people worldwide and costs billions of dollars annually. Understanding mechanisms of fibroblast activation and granulation tissue contraction is the first step toward preventing pathologic scarring. The authors hypothesize that nucleic acids increase fibroblast activation and cause granulation tissue contraction and sequestration of nucleic acids by application of a nucleic acid scavenger dendrimer, polyamidoamine third-generation dendrimer, will decrease pathologic scarring.

Methods—In vitro experiments were performed to assess the effect of nucleic acids on pathologic scar-associated fibroblast activity. The effect of nucleic acids on cytokine production (polymerase chain reaction) and migration on mouse fibroblasts was evaluated. Immunofluorescence microscopy was used to determine the effect of nucleic acids on the differentiation of human primary fibroblasts into myofibroblasts. Using a murine model, the effect of polyamidoamine third-generation dendrimer on granulation tissue contraction was evaluated by gross and histologic parameters.

Results—Mouse fibroblasts stimulated with nucleic acids had increased cytokine production (i.e., transforming growth factor- β , monocyte chemotactic protein 1, interleukin-10, tumor necrosis factor- α , and interferon- γ), migration, and differentiation into myofibroblasts. Polyamidoamine third-generation dendrimer blocked cytokine production, migration, and differentiation into myofibroblasts. Using a murine model of granulation tissue contraction, polyamidoamine third-generation dendrimer decreased wound contraction and angiogenesis. Collagen deposition in polyamidoamine third-generation dendrimer-treated tissues was aligned more randomly and whorl-like compared with control tissue.

Conclusions—The data demonstrate that nucleic acid-stimulated fibroblast activation and granulation tissue contraction is blocked by polyamidoamine third-generation dendrimer. Sequestration of pathogen-associated molecular patterns may be an approach for preventing pathologic scarring.

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Pathologic scars such as scar contractures, hypertrophic scars, and keloids limit range of motion and are painful, itchy, and severely disfiguring. Pathologic scarring costs billions of dollars per year, and treatments are marginally effective.¹⁻³ An unmet medical need exists to develop antiscarring therapies; however, pathogenic principles of pathologic scarring are still largely unknown.

Pathologic scarring is caused by overexuberant fibroblast activation, which leads to persistent granulation tissue contraction.⁴ Fibroblast-mediated contraction occurs in a direct and indirect manner. Direct contraction of the granulation tissue occurs as fibroblasts migrate into the granulation bed and within the extracellular matrix. Indirection contraction is attributable to growth factors and cytokines that mediate granulation tissue contraction by activating fibroblasts and other cell types.

Pathologic scars are exacerbated by cutaneous infection and cellular damage. A growing interest has emerged regarding the role of the innate immune system, such as toll-like receptors, in regulating wound healing.^{5,6} Toll-like receptors are a highly conserved family of germline-encoded receptors that recognize structural motifs expressed by bacteria, viruses, and fungi (pathogen-associated molecular patterns) and motifs from intracellular factors from damaged cells (damage-associated molecular patterns). The inappropriate activation of these toll-like receptors can result in a variety of inflammatory, autoimmune, and scarring diseases.^{7,8} Toll-like receptors 1 through 9 are reported to be expressed in skin. Toll-like receptor 9 is expressed 2- to 10-fold higher in pathologic scars, hypertrophic scars, and keloid scars compared with normal tissue.^{7,9-12}

In this article, we present and test the hypothesis that nucleic acid-driven toll-like receptor signaling activates fibroblasts and promotes granulation tissue contraction and accounts for clinical observations. After injury, different types of nucleic acids are released, which in turn activate toll-like receptors. The activation by nucleic acids results in fibroblast activity and resultant granulation tissue contraction associated with pathologic scar contracture. The sequestration of toll-like receptor signaling by a nucleic acid scavenger such polyamidoamine third-generation dendrimer could be a novel method for decreasing scar contracture.

Dendrimers are hyperbranched synthetic macromolecules that are made using controlled sequential processes to give them defined structural and molecular weight characteristics. Polyamidoamine third-generation dendrimer has been shown to bind DNA and RNA and stop activation of toll-like receptors by nucleic acids.¹³ We demonstrate that polyamidoamine third-generation dendrimer acts as a molecular scavenger of nucleic acids and inhibits signaling and downstream activities in fibroblasts associated with pathologic scars. We use a mouse wound granulation tissue contraction model to demonstrate the in vivo efficacy and translational potential of dendrimers to prevent pathologic scarring.

MATERIALS AND METHODS

Animals

To determine whether polyamidoamine third-generation dendrimer reduces granulation tissue contraction, we used a murine model of wound contraction. Wound closure is facilitated by the contraction of underlying granulation tissue. Granulation tissue contraction causes pathologic scarring.⁴ To determine whether polyamidoamine third-generation dendrimer reduces granulation tissue contraction, we used a murine model of wound contraction. Wound closure is facilitated by the contraction of underlying granulation tissue. Granulation tissue contraction causes pathologic scarring.⁴ Excisional wounds were created on the dorsa of mice, and granulation tissue contraction (relative wound area) was quantified by gravitational planimetry. A mouse excisional wound model was approved by the Institutional Animal Care and Use Committee of Duke University Medical Center. Ten- to 12 week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Me.) were anesthetized with inhaled 2% isoflurane and their dorsa were shaved followed by application of a depilatory agent. A full-thickness excisional wound of 4 cm² (2 × 2 cm) was created between the scapular angle of each animal. The wounds were covered with Tegaderm (3M, St. Paul, Minn.). Polyamidoamine third-generation dendrimer (Sigma Aldrich, St. Louis, Mo.) was applied to the wound in a phosphate-buffered saline solution (100 µl per mouse) daily using Tegaderm. Wound size was measured by gravitational planimetry, daily. Wound area was calculated as a percentage of original and remaining wound size as follows: remaining/original × 100. Animals were weighed and assessed for signs of toxicity.

Immunohistologic and Histochemical Staining

On day 14 after wound injury and polyamidoamine third-generation dendrimer administration, the animals were killed using pentobarbital (250 mg/kg intraperitoneal injection). The wounds were excised, including a 5-mm margin of normal skin around the edges of the wound, and fixed in 10% formalin. The samples underwent histologic processing and stained with either hematoxylin and eosin or Masson trichrome stain. The specimens were analyzed for tissue architecture, reepithelialization, inflammation, and collagen deposition. CD31 (Abcam, Cambridge, Mass.) immunostaining was used to quantify angiogenesis, and terminal deoxynucleotidyl transferase dUTP nick end labeling (Roche, Indianapolis, Ind.) staining was used to quantify apoptosis. Formalin-fixed, paraffin-embedded tissue blocks were sectioned at a thickness of 5 to 10 µm. Appropriate positive and negative controls were performed for each antigen assayed. Slides were counterstained with hematoxylin. The sections were incubated with the following: rabbit anti-Ki67 (Thermo Fisher Scientific, Fremont, Calif.) or rabbit anti-mouse CD31 antibody (Abcam) overnight at 4°C. Tissue staining was visualized using the avidin biotinylated enzyme complex system (Vectastain Elite ABC; Vector Laboratories, Burlingame, Calif.) and 3,3'-diaminobenzidine substrate chromogen solution (Dako, Carpinteria, Calif.). The number of Ki67-positive nuclei and CD31-positive vessels were counted in five high-power fields at 40× and 20× magnification, respectively. Epithelial thickness was measured at six different points in the reepithelialized wound per mouse using ImageJ version 1.62 (National Institutes of Health, Bethesda, Md.) and average reported with standard error of the mean.

Cell Culture

3T3 mouse fibroblasts were obtained from the Duke cell culture facility. Cells were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma-Aldrich). Human scar fibroblasts from explants of human skin scar were obtained as whole-tissue surgical specimens from the operating room according to approved institutional review board protocol. In brief, tissues were washed, minced finely, and incubated in collagenase type I with 1% penicillin-streptomycin (at 37°C for 8 hours). The cells were subsequently cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Experiments with primary cell cultures were performed when cells had become 80 to 90 percent confluent between passages 1 and 6.

Migration Assay

To test the role of polyamidoamine third-generation dendrimer on fibroblast cytokine production, we stimulated 3T3 cells with cytosine-phosphate-guanine in the presence of polyamidoamine third-generation dendrimer (0 to 20 µg/ml). As a control, we used a different toll-like receptor 7/9 receptor known inhibitor, IRS954 (2 µM). IRS954 blocks toll-like receptor 7/9 receptor activation by oligonucleotides such as cytosine-phosphate-guanine.

Assays were performed on day 3 following serum starving overnight on day 2. Cells were released by ethylenediaminetetraacetic acid. PET-etched polycarbonate cell migration inserts (8-µm pores; Millipore, Billerica, Mass.) were placed over a 96-well chamber containing desired treatment in Dulbecco's Modified Eagle Medium. Then, 1×10^4 cells in Dulbecco's Modified Eagle Medium were added to the insert. The chambers were placed in an incubator for 10 hours, after which the cell culture of the upper surface of the insert was aspirated to remove nonmigratory cells. Migrated cells were detached, lysed, and stained with CyQuant (Invitrogen, Grand Island, N.Y.). Migration was quantified by measuring fluorescence. Each treatment was performed in triplicate and the experiments were repeated three times.

Quantitative Real-Time Polymerase Chain Reaction

Cell culture assays were performed on day 3 after serum starving overnight on day 2. Following phosphate-buffered saline wash, 3T3s cells were stimulated with cytosine-phosphate-guanine-1668 (InvivoGen, San Diego, Calif.) for 8 hours. Additional treatments included IRS954 (5'-TGCTCCTGGAGGGGTTGT-3'; Integrated DNA Technologies, Coralville, Iowa), polyamidoamine third-generation dendrimer (Sigma-Aldrich), transforming growth factor (TGF)-β (5 ng/ml; Life Technologies, Carlsbad, Calif.), and lipopolysaccharide (*Pseudomonas aeruginosa*; Sigma-Aldrich). On days 7 and 14 after wound injury and polyamidoamine third-generation dendrimer administration, the animals were killed using pentobarbital. The wounds were excised and frozen. In addition, unwounded tissue was collected from the initial operation. RNA from cultured cells and tissue was isolated using a Qiagen RNA extraction kit. cDNA was synthesized using iScript cDNA synthesis kit (BioRad, Hercules, Calif.). Primers used for quantitative real-time polymerase chain reaction analysis were as follows:

TGF- β : forward, CACCTGCACAGCTCACGGCAC; reverse, GGTCCCTTCCTAAAGTCAATGTAC.

Collagen 1: forward, CCTGGTAAAGATGGTGCC; reverse, CACCAGGTTACCTTTTCGCACC.

Interferon- γ : forward, CAAGTGGCATAGATGTGGAAG; reverse, GAGATAATCTGGCTCTGCAG.

Interleukin-6: forward, TGTATGAACAACGATGATGCACTT; reverse, ACTCTGGCTTTGTCTTTCTTGTATCT.

Interleukin-10: forward, CATGGCCCAGAAATCAAGGAGCAT; reverse, CTCTTCACCTGCTCCACTGCCTTGC.

Tumor necrosis factor- α : forward, TTCGAGTGACAAGCCTGTAGCC; reverse, GCCACTCCAGCTGCTCCTCC.

Monocyte chemotactic protein 1: forward, CCCACTCACCTGCTGCTACT; reverse, TCTGGACCCATTCTTCTTG.

Hypoxanthine-guanine phosphoribosyltransferase: forward, GCTGGTGAAAAAGGACCTCT; reverse, CACAGGACTAGAACACCTGC.

Quantitative real-time polymerase chain reaction was performed using SYBR Green reagent in a BioRad iCycler.

Fluorescent Microscopy

Human dermal fibroblasts were plated onto poly-D-lysine-treated coverslips. The next day, growth media was replaced by the desired treatment in Dulbecco's Modified Eagle Medium without fetal bovine serum. After 4 days, human scar fibroblasts were fixed and permeabilized. The coverslips were first incubated for 1 hour at room temperature with primary antibodies: rabbit anti- α -smooth muscle actin (ab5694; Abcam), mouse anti-vinculin (V9264; Sigma Aldrich), and phalloidin (A12379; Invitrogen), followed by three 5-minute washes with phosphate-buffered saline. Human scar fibroblasts were incubated for 1 hour with the secondary antibody Alexa Fluor 568 (Invitrogen) for 1 hour followed by phosphate-buffered saline washing. Next, the coverslips were stained for F-actin with Alexa Fluor 488 phalloidin conjugated antibody (Invitrogen) 1:200 in 1% bovine serum albumin phosphate-buffered saline for 20 minutes at room temperature followed by three phosphate-buffered saline washes. Finally, the nuclei were stained for 1 minute with 4',6-diamidino-2-phenylindole 350 conjugated 1:5000 in 1% bovine serum albumin phosphate-buffered saline at room temperature followed by three phosphate-buffered saline washes. The stained coverslips were then inverted and mounted onto glass slides with glycerol-based mounting media. Fluorescent images were obtained using a fluorescence microscope (Axio Imager; Carl Zeiss, Oberkochen, Germany) equipped with a cooled charge-coupled device digital camera (Retiga1300R; QImaging Corp., Burnaby, British Columbia, Canada). Image acquisition and control were accomplished using MetaMorph imaging software version 6.2 (Molecular Devices, Sunnyvale, Calif.). We counted the number of focal adhesions for five cells per field of view.

Statistical Analysis

All values are presented as mean $\pm$ SD. GraphPad Prism (GraphPad Software, Inc., La Jolla, Calif.) was used for statistical analysis. Differences between groups were compared by means of *t* test, analysis of variance, or Wilcoxon test and are considered to be statistically significant at values of *p* $\leq$ 0.05. We adjusted the *p* value for multiple comparisons using the Bonferroni correction.

RESULTS

Polyamidoamine Third-Generation Dendrimer Inhibits Nucleic Acid–Stimulated Cytokine Production by Fibroblasts

Pathologic scarring is thought to be caused by inflammation and fibroblast activation.¹⁴ Production of cytokines, chemokines, and prostanoids by fibroblasts prolongs fibroblast activation and inflammation by maintaining a proinflammatory environment and promoting recruitment of immune cells.¹⁵ To determine whether nucleic acids increase cytokine production, we stimulated mouse 3T3 fibroblasts with cytosine-phosphate-guanine DNA for 8 hours. Nucleic acid stimulation increased production of TGF- β , monocyte chemotactic protein 1, collagen, interleukin-10, tumor necrosis factor- α , and interferon- γ mRNA in fibroblasts 8 hours after stimulation (Fig. 1). Surprisingly, nucleic acid stimulation did not result in increased interleukin-6 mRNA expression in fibroblasts.

Inhibition of fibroblast activation by nucleic acids could be an effective treatment for pathologic scars. Polyamidoamine third-generation dendrimer has previously been shown to bind extracellular nucleic acids and could be used a potential novel anti–pathologic scar contracture therapeutic. Polyamidoamine third-generation dendrimer significantly decreased nucleic acid–stimulated TGF- β , monocyte chemotactic protein 1, and interleukin-10 cytokine production. In addition, polyamidoamine third-generation dendrimer inhibited interferon- γ and tumor necrosis factor- α production to levels less than the control (Fig. 1). Our data show that IRS954 reduces production of a number of cytokines following cytosine-phosphate-guanine stimulation; however, this stimulation pattern is different from polyamidoamine third-generation dendrimer, suggesting that polyamidoamine third-generation dendrimer and IRS954 function by means of different mechanisms of action.

Cytosine-Phosphate-Guanine DNA Increases Fibroblast Migration

Pathologic scarring results from chemotaxis of fibroblasts to the granulation tissue and migration within the granulation tissue. Fibroblast migration within granulation tissue causes extracellular matrix contraction and results in pathologic scar contracture.¹⁶ The migration of 3T3 mouse fibroblasts was shown to increase with increasing concentrations of cytosine-phosphate-guanine. Migration was significantly increased at concentrations greater than 0.6 μ M and peaked at 5 μ M (Fig. 2, *above* and *center*). The cytosine-phosphate-guanine concentration of 5 μ M was used for subsequent migration assays. To determine whether polyamidoamine third-generation dendrimer inhibits nucleic acid–stimulated migration, increasing concentrations of polyamidoamine third-generation dendrimer were added to the assay. Polyamidoamine third-generation dendrimer inhibited cytosine-phosphate-guanine–stimulated migration in a dose-dependent manner (Fig. 2, *below*). Polyamidoamine third-

generation dendrimer concentration of greater than 10 $\mu\text{g/ml}$ significantly inhibited cytosine-phosphate-guanine DNA-stimulated migration, with maximum inhibition observed at 20 $\mu\text{g/ml}$. Moreover, we used IRS954 as a positive control of inhibiting cytosine-phosphate-guanine-driven migration of 3T3 fibroblasts. As expected, the toll-like receptor 7/9 inhibitor IRS954 inhibited migration (Fig. 2, *below*).

Cytosine-Phosphate-Guanine DNA Stimulates Myofibroblast Differentiation

Myofibroblasts are α -smooth muscle actin-positive fibroblasts that produce extracellular matrix proteins including collagen type I¹⁴ and generate contractile forces that cause granulation tissue contraction.¹⁷ Myofibroblasts have increased expression of F-actin stress fibers and focal adhesions. Persistent differentiation of fibroblasts into myofibroblasts and expression of α -smooth muscle actin is the primary cause of pathologic scarring.

Immunofluorescence staining of untreated human scar fibroblasts showed a sparse actin network predominantly organized around the cell cortex, with few or no intracellular actin filament bundles (Figs. 3 through 5). Cultured human scar fibroblasts showed only minimal staining for vinculin, which was localized to adhesion complexes (Figs. 3 through 5).

Addition of TGF- β resulted in differentiation of fibroblasts into myofibroblasts, as characterized by proliferation, spreading, increased α -smooth muscle actin expression, organization of actin filaments into prominent intracellular bundles, the formation of stress fiber and an increased number of focal adhesions. Similar to TGF- β -treated cells, cytosine-phosphate-guanine-treated human scar fibroblasts spread and had increased proliferation over 4 days compared with the control tissue, as shown by increased confluence. Cytosine-phosphate-guanine increased actin reorganization into stress fibers compared with the untreated human scar fibroblasts, but stress fibers were less than TGF- β -treated human scar fibroblasts and the α -smooth muscle actin staining remained perinuclear. In addition, vinculin staining of cytosine-phosphate-guanine-treated human scar fibroblasts showed an increased number of focal adhesions compared with control tissue, but this was not significant (Figs. 3 through 5).

Both polyamidoamine third-generation dendrimer and IRS954 prevented activation of human scar fibroblasts by cytosine-phosphate-guanine DNA and decreased vinculin expression compared with controls (Figs. 3 through 5). Moreover, polyamidoamine third-generation dendrimer decreased α -smooth muscle actin expression after nucleic acid stimulation as determined by fluorescence microscopy. IRS954 did not decrease fibroblast α -smooth muscle actin expression but increased the intensity of staining of perinuclear α -smooth muscle actin expression after cytosine-phosphate-guanine DNA stimulation.

Polyamidoamine Third-Generation Dendrimer Inhibits Granulation Tissue Contraction and Pathologic Scar-Associated Outcomes in a Murine Model

Polyamidoamine third-generation dendrimer has not been previously applied to open wounds; therefore, we initially determined a maximal tolerated dose using topical polyamidoamine third-generation dendrimer (0 to 400 $\mu\text{g/ml}$ per day) applied for 14 days. All mice survived, but greater than or equal to 20 $\mu\text{g/ml}$ per day adversely affected gross wound healing. Doses between 0 and 2 $\mu\text{g/ml}$ per day were nontoxic; all wounds closed by

day 14 without gross or systemic toxicity (Fig. 6, *above*). Polyamidoamine third-generation dendrimer did not significantly affect relative wound area between days 0 and 5 (Fig. 6, *below*). After day 5, fibroblasts are present and active in the granulation tissue. Polyamidoamine third-generation dendrimer significantly inhibited granulation tissue contraction between days 6 and 10. Based on this observation, further studies were performed with 1 µg/ml per day polyamidoamine third-generation dendrimer applied to the wound and tissue collected on days 7 and 14.

Histologic staining by hematoxylin and eosin showed that on day 7 both inflammation and fibroplasia were present in both groups and all mice had reepithelialized wounds (Fig. 7). On day 14, inflammation had subsided and fibroblasts populated the mature granulation tissue. Both groups had similar epithelial thickness (control, 41.5 ± 10.21 µm; polyamidoamine third-generation dendrimer, 51.0 ± 9.8 µm) on day 14 ($p = 0.05$) (Fig. 7).

In pathologic scars, collagen is arranged along the lines of tension, parallel to the skin surface, compared with random arrangement in normal skin.¹⁸ Using Masson trichrome staining, the collagen deposition of the polyamidoamine third-generation dendrimer-treated and control groups were compared. In both groups, newly formed granulation tissue had remodeled where collagen was deposited along lines of tension that appeared aligned and parallel, demonstrating scarring (Fig. 7). Qualitative comparisons showed that collagen was less aligned and more whirl-like in polyamidoamine third-generation dendrimer-treated tissues compared with controls (Fig. 7).

Vascularity is increased in pathologic scar compared with normal skin.¹⁹ Using CD31 immunostaining to quantify vascularity, day-7 and day-14 tissues were analyzed (Fig. 7). Polyamidoamine third-generation dendrimer significantly decreased vascularization (five vessels per high-power field) compared with controls (seven vessels per high-power field; $*p = 0.05$).

Cell proliferation was also compared between untreated and polyamidoamine third-generation dendrimer-treated mice on day 14 using Ki67 staining (Fig. 7). Polyamidoamine third-generation dendrimer significantly increased proliferation compared with controls.

Using quantitative polymerase chain reaction, we found α -smooth muscle actin mRNA increased in tissue on days 7 and 14 compared with unwounded tissue. However, there was no significant difference between the polyamidoamine third-generation dendrimer-treated group and the control group (Fig. 8).

DISCUSSION

Pathologic scarring is the result of overexuberant fibroblast activation and prolonged granulation tissue contraction. Much effort has been directed to the preclinical development of anti-pathologic scar technologies. Current treatments under development include drugs targeting and modulating ligands, receptors, and signaling pathways such as connective tissue growth factor, angiotensin II, peroxisome proliferator-activated receptor- γ , connexin, and TGF- β 1 or TGF- β 3^{20–23}; and technologies (devices) that target tension in pathologic

scars such as silicone gel sheeting¹ (Neodyne trial). However, technologies do not always successfully translate to the clinic with effective patient outcomes.

Regarding discovery of a potential treatment, whether by rational design or serendipity, there are several obstacles to successful translation of preclinical findings to patient outcomes. The development of a new anti-pathologic scar therapy requires a significant amount of time, capital, and resources. In pathologic scars, hurdles also exist and need to be addressed in the clinical setting before clinical testing can be shown to be effective; these include the paucity of studies demonstrating disease causality to aid and direct preclinical research, and the multifactorial nature in scarring, including symptoms such as pain, redness, itching, contraction, and hypertrophy, which will make a truly quantifiable way of measuring the outcome of a pathologic scar treatment difficult. Strategies to address challenges in discovery and preclinical research, technology transfer, funding, clinical trials, regulatory affairs, and meeting the requirements of the market and will require careful design and management of resources to ensure successful translation of anti-pathologic scar technologies.

We posit that nucleic acids may also have a role in pathologic scars. This is based on observations that cytosine-phosphate-guanine DNA toll-like receptor 9 stimulation increases (1) chemokine/cytokine expression^{24–28}; (2) α -smooth muscle actin expression, actin organization into myofibroblasts,^{24,28,29} and actin remodeling in immune cells³⁰; (3) differentiation of blood monocytes into fibrocytes (epithelial-to-mesenchymal transition)²⁹; (4) collagen expression^{24,28}; and (5) fibroblast invasion, which includes migration.^{31,32} Toll-like receptor 9 expression has also been shown to be increased in both mouse skin following trauma³³ and in human pathologic scars.¹⁰

The rational design of anti-pathologic scar treatments could include reducing inflammation, preventing fibroblast activity, and inhibiting granulation tissue contraction.^{34,35} Using Boyden chambers, we have shown that cytosine-phosphate-guanine DNA increases migration of fibroblasts and that polyamidoamine third-generation dendrimer decreases this stimulated migration. We interpret the effects of polyamidoamine third-generation dendrimer as beneficial because, mathematically, modeling has shown that antiscarring treatments should decrease fibroblast mobility and fibroblast orientation to chemoattractant gradients.³⁶ In addition, we found that cytosine-phosphate-guanine DNA increases myofibroblast formation and activation, and polyamidoamine third-generation dendrimer blocks these effects. In human scar fibroblasts, myofibroblast formation was demonstrated by an increase in stress fiber assembly. However, we found that this assembly did not occur with an increase in α -smooth muscle actin expression. Recently, it has been demonstrated that α -smooth muscle actin is not necessary for myofibroblast formation and function, including wound closure.³⁷ Our data propose a similar finding that myofibroblasts have a mechanism for displaying typical myofibroblast phenotypical and functional characteristics in the absence of an increase in α -smooth muscle actin expression. Cytosine-phosphate-guanine DNA did not significantly increase the number of focal adhesions on fibroblasts as determined by vinculin staining. Cytosine-phosphate-guanine oligonucleotides increased fibroblast expression of known proscarring cytokines: TGF- β and monocyte chemotactic protein 1.³⁸ Polyamidoamine third-generation dendrimer inhibited this increase. The

inappropriate production of such proscarring immunoregulatory molecules by nucleic acid-stimulated fibroblast activity prevents the resolution of the inflammatory response, leading to persistent inflammation.

Previous studies have shown that cytosine-phosphate-guanine, through toll-like receptor 9 activation, stimulates wound contraction.²⁷ Granulation tissue is populated with fibroblasts, which results in contraction of wounds.^{39,40} Fibroblasts promote contraction and resultant pathologic scars.^{4,41} Here, we used a model of wound contraction to demonstrate the efficacy of polyamidoamine third-generation dendrimer in preventing contraction as a potential therapeutic strategy for pathologic scars. Our studies demonstrate that polyamidoamine third-generation dendrimer inhibits contraction significantly. However, polyamidoamine third-generation dendrimer did not inhibit wound healing, as shown by comparing epithelialization between the two groups on day 7. We demonstrated that polyamidoamine third-generation dendrimer did not significantly affect α -smooth muscle actin mRNA expression levels, which suggests that the observed inhibition in contraction by polyamidoamine third-generation dendrimer is α -smooth muscle actin independent.

Pathologic scars such as hypertrophic scars and contractures have increased vascularity, a parameter that is used by clinicians to determine the severity of a scar in scar scales.^{19,42} Immunostimulatory cytosine-phosphate-guanine DNA has been shown to increase cornea angiogenesis²⁵ and vascularization in wound healing studies.²⁷ Our data demonstrate a decrease in the number of vessels present in tissue on day 14 in wounds treated with polyamidoamine third-generation dendrimer. Dendrimers have been shown to decrease angiogenesis in cell-based assays.⁴³ Our data show for the first time that dendrimers decrease angiogenesis in vivo. Decreasing vascularity is an advantageous property of antiscarring treatment; decreasing vascularity improves scar pigmentation.

In this article, we evaluated the feasibility of using dendrimers to treat pathologic scars, in particular, scar contractures. By targeting fibroblast activities within granulation tissue, it would be possible to develop novel anti-pathologic scar treatments. We propose that polyamidoamine third-generation dendrimer acts by inhibiting fibroblast-associated granulation tissue contraction. This study is the first to investigate an antiscarring approach based on nucleic scavenging technology and could have a significant impact on future treatment of patients.

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Fig. 1.

The effect of oligonucleotide (cytosine-phosphate-guanine DNA) signaling on cytokine expression. 3T3 mouse fibroblasts were incubated with cytosine-phosphate-guanine DNA (5 μM, *white*), cytosine-phosphate-guanine DNA and polyamidoamine third-generation dendrimer (20 μg/ml, *gray*), or cytosine-phosphate-guanine DNA and IRS954 (2 μM, *black*). Quantitative real-time polymerase chain reaction was used to determine the response of 3T3 mouse fibroblasts to cytosine-phosphate-guanine DNA by measuring mRNA of cytokines (TGF-β, monocyte chemotactic protein 1, interleukin-10, interleukin-6, tumor necrosis factor-α, interferon-γ, and collagen). The endogenous hypoxanthine-guanine phosphoribosyltransferase was used as a control mRNA. Data are plotted relative to untreated fibroblasts. Data greater than 1 indicate an increase in mRNA. Data points represent mean ± SD. Data are representative of at least three independent experiments using the 3T3 mouse fibroblast cell line. Analysis of variance and post hoc analysis, **p* 0.05. *MCPI*, monocyte chemotactic protein 1; *IL*, interleukin; *IFN*, interferon; *TNF*, tumor necrosis factor.

Fig. 2.

The effect of oligonucleotide (cytosine-phosphate-guanine DNA) signaling on fibroblast migration. Boyden chambers were used to determine 3T3 mouse fibroblast migration in response to cytosine-phosphate-guanine DNA and the toll-like receptor 9 inhibitor IRS954. (Above) Cytosine-phosphate-guanine DNA stimulated fibroblast migration in a concentration-dependent manner (* $p < 0.05$). (Center) Polyamidoamine third-generation dendrimer inhibited cytosine-phosphate-guanine DNA-stimulated fibroblast migration at doses greater than or equal to 10 $\mu\text{g/mL}$. (Below) Cytosine-phosphate-guanine DNA–

stimulated migration was inhibited by polyamidoamine third-generation dendrimer (20 $\mu\text{g/ml}$) and IRS954 (2 μM). Relative migration represents migration relative to unstimulated fibroblasts, data points represent mean $\pm$ SD. Data are representative of at least three independent experiments ($n = 9$). Analysis of variance and post hoc analysis, $*p < 0.05$. *CpG*, cytosine-phosphate-guanine; *FBS*, fetal bovine serum; *PAMAM-G3*, polyamidoamine third-generation dendrimer; *OGN*, oligonucleotide.

Fig. 3.

The effect of oligonucleotide (cytosine-phosphate-guanine DNA) on myofibroblast differentiation. Primary human scar fibroblasts were cultured for 4 days in serum-free media supplemented with cytosine-phosphate-guanine DNA (5.0 μ M), cytosine-phosphate-guanine DNA and IRS954 (2 μ M), cytosine-phosphate-guanine DNA and polyamidoamine third-generation dendrimer (20 μ g/ml), or TGF- β (5 ng/ml). Immunofluorescence staining was used to determine changes in α -smooth muscle actin (α SMA) and stress fiber. DAPI, 4',6-diamidino-2-phenylindole; CpG, cytosine-phosphate-guanine; PAMAM-G3, polyamidoamine third-generation dendrimer.

Fig. 4.

The effect of oligonucleotide (cytosine-phosphate-guanine DNA) on myofibroblast differentiation. Primary human scar fibroblasts were cultured for 4 days in serum-free media supplemented with cytosine-phosphate-guanine DNA (5.0 μ M), cytosine-phosphate-guanine DNA and IRS954 (2 μ M), cytosine-phosphate-guanine DNA and polyamidoamine third-generation dendrimer (20 μ g/ml), or TGF- β (5 ng/ml). Immunofluorescence staining was used to determine changes in vinculin (focal adhesion marker) expression. *DAPI*, 4',6-diamidino-2-phenylindole; *CpG*, cytosine-phosphate-guanine; *PAMAM-G3*, polyamidoamine third-generation dendrimer.

Quantification on Day 14

Fig. 5.

The effect of oligonucleotide (cytosine-phosphate-guanine DNA) on myofibroblast differentiation. The number of focal adhesions per cell was determined by counting 10 cells. Treatment data are plotted as mean $\pm$ SD. Data are representative of at least three independent experiments ($n = 10$). Analysis of variance and post hoc analysis, $*p < 0.05$. CpG, cytosine-phosphate-guanine; PAMAM-G3, polyamidoamine third-generation dendrimer.

Fig. 6.

The effect of polyamidoamine third-generation dendrimer on granulation tissue contraction. Excisional wounds were created on the dorsum of mice and polyamidoamine third-generation dendrimer (*PAMAM-G3*) was applied daily. Granulation tissue contraction was measured by gravitational planimetry. Wound area decreases as a function of granulation bed contraction. Granulation tissue contraction causes pathologic scarring. (*Above*) The gross appearance of wounds in control and polyamidoamine third-generation dendrimer treated mice. There were no signs of adverse effects such as ulceration or infection. Wounds were closed at 14 days after injury in both groups. (*Below*) Wound contraction of control (*circle*) and polyamidoamine third-generation dendrimer (*squares*, 1 μ g/ml per day) mice. Data points represent mean $\pm$ SD. Data are representative of four independent experiments ($n = 10$; $*p < 0.05$).

Fig. 7.

Histologic evaluation of polyamidoamine third-generation dendrimer-treated and control wounds. Tissue samples were collected on days 7 and 14 and stained by hematoxylin and eosin. On day 7, there is inflammation and fibroplasia. On day 14, the inflammatory reaction had subsided and fibroblasts populate the mature granulation tissue. Tissue samples were collected on day 14 for neovascularization (CD31) and proliferation (Ki67). Data are represented as mean ± SEM ($n = 6$; * $p < 0.05$). PAMAM-G3, polyamidoamine third-generation dendrimer; H&E, hematoxylin and eosin; HPF, high-power field.

Fig. 8.

The effect of polyamidoamine third-generation dendrimer (*PAMAM*) on α -smooth muscle actin expression in treated and control wounds. Tissue samples were collected on days 0 (unwounded), 7, and 14 and α -smooth muscle actin mRNA expression was measured by quantitative real-time polymerase chain reaction. Data are represented as mean $\pm$ SD. Data are representative of three independent experiments ($n = 4$).