Effect of Multiple Gene Transfers of Insulinlike Growth Factor I Complementary DNA Gene Constructs in Rats After Thermal Injury

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**Hypothesis:** Multiple subcutaneous injections of cholesterol-containing cationic liposomes encapsulating the complementary DNA (cDNA) gene for insulinlike growth factor I (IGF-I) increase the rate of transfected skin cells and result in increased IGF-I protein levels in the skin with subsequent improvement in wound healing when compared with a single injection.

**Setting:** Laboratory.

**Intervention:** Twenty-four adult male Sprague-Dawley rats (350-375 g) received a full-thickness scald burn on 60% of their body surface. These rats were randomly divided to receive either 1 injection of liposomes containing 2.2 µg-cytomegalovirus-driven cDNA coding for IGF-I and 0.2 µg of the Lac Z gene cDNA construct, or 2 injections of liposomes containing 2.2 µg cytomegalovirus-driven cDNA coding for IGF-I and 0.2 µg of the Lac Z gene cDNA construct.

**Main Outcome Measures:** Transfection rates and IGF-I protein levels in the skin and physiological responses to the IGF-I gene therapy, evaluated from changes in body weight, protein content in serum and liver, and the rate of burn wound healing.

**Results:** There was a significant decrease in transfection rate and IGF-I protein expression distal from the injection site in animals receiving 1 injection, compared with a consistent increase in rats receiving multiple injections. Multiple injections improved the response to thermal trauma by increasing the extent of the healed burn wound 33 days after thermal injury (single injection, 31% ± 1% vs multiple injections, 38% ± 2%), total serum protein (single injection, 52 ± 0.5 g/L vs multiple injections, 55 ± 0.6 g/L), and total liver protein (single injection, 82.0 ± 0.3 mg/mL vs multiple injections, 91.0 ± 3.8 mg/mL), P<.05.

**Conclusions:** Gene transfer rates can be increased by multiple injections of liposomes encapsulating IGF-I cDNA constructs. Increased transfer results in greater IGF-I protein skin concentrations, accelerated wound healing, and increased serum and liver protein concentrations. The clinical relevance of these findings is that liposomal gene constructs should be applied in well-defined distances to improve gene transfer in the skin, and thus clinical outcome after thermal injury.

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MATERIALS AND METHODS

Twenty-four adult male Sprague-Dawley rats (350-375 g) were placed in wire-bottom cages and housed in a temperature-controlled room with a 12-hour light-dark cycle. The animals were acclimated to their environment for 7 days prior to the start of the study. All rats received equal amounts of a liquid diet (Sustacal; Mead Johnson Nutritionals, Evansville, Ind) and water ad libitum throughout the study. While under general anesthesia and analgesia, each rat received a 60% total body surface area full-thickness scald burn as described by Herndon et al.26 Immediately after the thermal injury, rats were resuscitated with Ringer solution (50 mL/kg of body weight) and then randomly divided into 2 groups to receive weekly subcutaneous injections of liposomes (10 µL in 180 µL of saline) containing 2.2 µg of an IGF-I cDNA construct and 0.2 µg of the reporter gene β-galactosidase, Lac Z gene cDNA construct driven by a CMV promoter (n = 12) at 1 injection site on the edge of the burn wound, or weekly subcutaneous injections of liposomes (10 µL in 180 µL of saline) containing 2.2 µg of CMV-driven IGF-I cDNA construct and 0.2 µg of the reporter gene for β-galactosidase, Lac Z gene cDNA (n = 12), at 2 injection sites on the edge of the burn wound.

The injection scheme is depicted in Figure 1. The IGF-I cDNA construct consisted of a CMV-driven IGF-I cDNA plasmid prepared at the University of Texas Medical Branch Sealy Center for Molecular Science Recombinant DNA Core Facility, Galveston. The IGF-I cDNA was kindly supplied by P. Rotwein, PhD, National Institutes of Health, Bethesda, Md. The liposomes were formulated from 1,2-dimyristoylpropyl-3-dimethyl-hydroxyethyl ammonium bromide and cholesterol suspended in membrane-filtered water (Life Technologies, Rockville, Md). This reagent interacts spontaneously with IGF-I cDNA to form the lipid cDNA complex. Immediately after the thermal injury, each rat received 0.2 mL of the solutions injected at either 1 site, 1 cm from the wound margin, or at 2 sites distal to each other (Figure 1). This was repeated once a week for 4 weeks. Lipoplexes had to be prepared fresh every week prior to injections.

Animals were killed by decapitation 5 days after the last injection (33 days after thermal injury). Blood was collected into serum and plasma separators and spun at 1000g for 15 minutes. Supernatant and pellet were separated and stored at −73°C for analysis. Liver and 3 dorsal skin samples, defined as areas approximately 40 mm × 5 mm, were placed in wire-bottom cages and housed in a temperature-controlled room with a 12-hour light-dark cycle. After 24 hours, liver and 3 dorsal skin samples were collected into serum and plasma separators and spun at 1000g for 15 minutes. Supernatant and pellet were separated and stored at −73°C. Liver and 3 dorsal skin samples at each injection site were frozen in liquid nitrogen, and stored at −73°C for analysis.

Liver and 3 dorsal skin samples were homogenized in 7 mL of 100 mmol/L Tris, pH 7.5, 1 mmol/L EDTA, 150 mmol/L sodium chloride, containing 1× EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, Ind), and lyophilized. Samples were stored at −73°C. Liver and 3 dorsal skin samples were then resuspended in 7 mL of 100 mmol/L Tris, pH 7.5, 1 mmol/L EDTA, 150 mmol/L sodium chloride, containing 1× EDTA-free protease inhibitor cocktail. DNA extraction was performed as described in the kit guidelines (Diagnostic System Laboratories, Webster, Tex).

Protein content in serum was measured using a nephelometer (Behring, Deerfield, Ill). Liver protein concentration was determined by protein assay (Bio Rad, Hercules, Calif) based on the method by Bradford.28 Wound healing was determined as follows. The wound eschar was left intact for the first 28 days and then removed by gentle traction, caution being taken not to disturb or destroy the healing edge along the periphery. After removing the eschar, the animals were placed on a standard surface and the wound area traced onto acetate sheets along the well-demarcated reepithelialized and nonburned interface and the leading edge of the neoeithelium. The areas of these tracings were calculated by computerized planimetry (Sigma Scan and Sigma Plot software; Jandel, San Rafael, Calif).

Body weights were measured at the same time each week. Protein content in serum was measured using a nephelometer (Behring, Deerfield, Ill). Liver protein concentration was determined by protein assay (Bio Rad, Hercules, Calif) based on the method by Bradford.28 Wound healing was determined as follows. The wound eschar was left intact for the first 28 days and then removed by gentle traction, caution being taken not to disturb or destroy the healing edge along the periphery. After removing the eschar, the animals were placed on a standard surface and the wound area traced onto acetate sheets along the well-demarcated reepithelialized and nonburned interface and the leading edge of the neoeithelium. The areas of these tracings were calculated by computerized planimetry (Sigma Scan and Sigma Plot software; Jandel, San Rafael, Calif).

Studies were reviewed and approved for humane animal treatment by the Animal Care and Use Committee of the University of Texas Medical Branch, ensuring that all animals received humane care according to the criteria outlined in the guide for the care and use of laboratory animals published by the National Institutes of Health, Bethesda, Md. Statistical comparisons were made by analysis of variance and t test with the Bonferroni correction. Data are expressed as mean ± SEM. Significance was accepted at P<.05.
subsequent improvements in wound healing rates. We have further shown that increased IGF-I protein expression was limited to a small area around the injection site. This restriction of liposomal migration was most likely due to interactions between positive surface charges on cationic liposomes and contiguous negatively charged outer cell membranes, which limited liposomal migration, and thus, transfection. The purpose of this study was to determine whether multiple injections of IGF-I cDNA constructs via cholesterol-containing cationic liposomes increase efficacy compared with single injections. Efficacy was determined by measuring rates of transfection, IGF-I protein expression, and rates of wound repair.

RESULTS

All rats in each group survived the 60% total body surface area scald burn and drug injections with no evidence of any adverse effects. Total body weight increased almost 2% per week for the first 4 weeks postinjury in animals transfected with single and multiple injections of liposomes–IGF-I cDNA construct. There were no differences between the 2 groups for changes in body weights. Rats receiving multiple injections of the IGF-I cDNA construct had higher serum protein levels (single injection, 82.0 ± 0.3 mg/mL vs multiple injections, 91.0 ± 3.8 mg/mL) compared with single injection–treated animals (P < .05). After the eschar was removed, the percent area of burn wound reepithelialization was significantly larger 33 days after burn in rats receiving the multiple injections of IGF-I cDNA compared with rats receiving the single injection, 38% ± 2% vs 31% ± 2%, respectively (P < .05; Figure 2).

Transfection, determined by chemiluminescent reporter gene assay to detect ß-galactosidase, was increased around the wound perimeter in animals receiving multiple injections of liposome-encapsulated Lac Z gene cDNA and IGF-I cDNA constructs when compared with single injections, P < .05 (Figure 3).

Skin concentrations of IGF-I protein decreased from skin biopsy point 1 to point 3 in rats receiving the single injection of IGF-I cDNA construct along the wound edge (Figure 4, left). Animals receiving multiple injections of the cDNA construct showed consistently elevated IGF-I protein concentrations along the wound edge (Figure 4, right).

Wound healing is of major importance to the survival and clinical outcome of burn patients. Somatic gene therapy is a potentially useful strategy for the delivery of growth factors to accelerate wound healing. Insulin-like growth factor I enhances wound healing through stimulation of collagen formation and mitogenicity of fibroblasts and keratinocytes by binding to their receptors. Despite the advantages of IGF-I therapy after trauma, adverse effects limited the clinical use of IGF-1. We have previously demonstrated that a new form of IGF-I administration, subcutaneous injection of liposomes encapsulating the cDNA coding for IGF-I, increased skin IGF-I concentrations along with improved wound healing without detectable adverse effects. We further showed that liposomal migration, and thus areas of transfection, was restricted to the areas immediately adjacent to the injection site. Therefore, we hypothesized that multiple injections of lipoplexes containing the cDNA for IGF-I enhance transfection with increases in IGF-I concentration around the wound edge, which accelerates the wound healing process.

After the subcutaneous injection of the IGF-I cDNA and the reporter Lac Z gene construct, we determined transfection in dermal cells. The major mechanism by which effective transfection occurs is most likely localized endocytosis. DNA plasmids enter the cell and the cell nucleus and the released cDNA is then taken up by the nucleus. How the cDNA is taken up by the nucleus is currently unknown. The ribosomes then transcribe the IGF-I cDNA into mRNA, which is transported to the rough endoplasmic reticulum, where the messenger RNA is translated into the IGF-I protein. This transient increase in the local expression of IGF-I protein is most likely to cause a concurrent stimulation of insulinlike growth factor binding protein-3 protein synthesis and locally increased levels of the biological active complex IGF-

Figure 1. Schematic of the injection sites. Animals that received 1 injection received the injection only at injection site 1, whereas animals with 2 injections received the injections at injection sites 1 and 2. Skin biopsy specimens 1, 2, and 3 were obtained 33 days after injury for analysis.

Figure 2. Area of wound reepithelization was measured by planimetry. Rats receiving multiple injections of encapsulated insulinlike growth factor complementary DNA constructs had the highest percentage of reepithelization throughout the study period compared with single injections. Insulinlike growth factor complementary DNA multiple injections vs single injections (P < .05). Data are presented as mean ± SEM.
The presence of β-galactosidase protein was detected by chemiluminescent reporter gene assay in skin biopsy specimens 1, 2, and 3. Top, Rats receiving a single injection of the complementary DNA construct demonstrated a significant decrease in β-galactosidase expression along the wound edge. Difference between skin biopsy specimen 1 vs 3, P < .05. Bottom, Rats receiving multiple injections demonstrated consistently elevated levels of β-galactosidase expression. There were no differences between skin biopsy specimen 1, 2, or 3. Data are presented as mean ± SEM.

Figure 3.

In agreement with previous results, we demonstrated in this study that transfection is restricted to a perimeter near the sites of injection. The restricted nature of the expression of IGF-I protein was most likely due to the local character of the interactions between the positive surface charges on cationic liposomes and negatively charged outer cell membranes, which restricted liposomal migration. This restriction of liposomal migration leads to restrained transfection and protein expression. As we demonstrated in this study, liposomal migration, transfection, and protein expression can all be increased by multiple injections. Rats receiving multiple injections of IGF-I cDNA demonstrated high transfection rates around the wound edge, with concurrent increased IGF-I protein expression. Rats receiving 1 injection of IGF-I cDNA demonstrated a gradient of transfection and protein expression, with a high density around the injection site and no detectable transfection as well as protein expression distal from the injection site. This finding is clinically relevant because the liposomes encapsulating the gene should be applied at well-defined distances from the wound to provide optimal transfection and protein expression.

A physiological response to the increased IGF-I skin concentration was accelerated wound healing and improved total protein concentrations in serum and liver. Given that the subcutaneous injection of IGF-I cDNA construct is localized, we suggest that the physiological effects are due to improved wound healing and cell recovery after injury and not from increased circulating levels of IGF-I protein. The advantages of early wound closure, demonstrated in several clinical studies, include a diminished hypermetabolic burn response and a decrease in inflammatory mediators, such as interleukin 1, interleukin 6, interleukin 8, and tumor necrosis factor α. Furthermore, we have shown that IGF-I protein decreases proinflammatory cytokines interleukin 1β and tumor necrosis factor α expression after thermal injury (M.G.J., unpublished data, 1999). Therefore, IGF-I may exert its systemic beneficial effect through the enhancement of re-epithelialization and/or the decrease of the proinflammatory response in the skin, which is one of the major sources of cytokine synthesis and release after burn.

In this study, we showed that multiple subcutaneous injections of the IGF-I cDNA increased the number of transfected cells and protein expression compared with a single injection. The physiological responses to increases in skin IGF-I were an enhancement in wound healing, with subsequently systemic improvements to the hypermetabolic response. From these findings, we conclude that multiple injections of cholesterol-containing cationic liposomes encapsulating an expression plasmid vector for IGF-I cDNA given to rats with a 60% total body...
surface area thermal injury were effective in increasing IGF-1 skin protein concentrations without adverse effects. Thus, treatment with multiple injections of the IGF-1 cDNA construct may be an effective therapeutic approach to improve clinical outcomes after thermal injury.

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